High Performance

Liquid Chromatographic Separations

in Biopharmaceutical Analysis

by

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of

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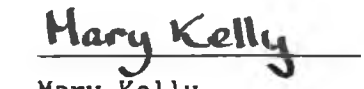
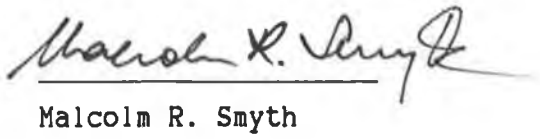
Declaration

I hereby declare that the contents of this thesis, except where otherwise stated, are based entirely on my own work which was carried out at the Dublin City University, Dublin.



Mary Kelly



Malcolm R. Smyth
(Supervisor)

Dedicated to my mother and the memory of my late father

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ABSTRACT

High Performance Liquid Chromatographic Separations in Biopharmaceutical Analysis

Mary Kelly

The application of high performance liquid chromatographic (HPLC) methods to the analysis of drugs in plasma was investigated. A method for the analysis in plasma of the diuretic drug, xipamide was developed from first principles. It was based on reversed-phase chromatography following liquid-liquid extraction of the drug and internal standard into diethyl ether. The method was validated and the coefficient of variation for within-day and between-day replicate analyses were 3.1 and 4.8% respectively.

In chapter 4, reversed-phase micellar chromatography was used to separate the drugs, chlorthalidone and xipamide. A method was developed for the determination of these drugs in plasma based on liquid-liquid extraction into diethyl ether followed by chromatography on a C_8- column using an eluent containing 0.05 M sodium dodecyl sulphate. The method was validated and the coefficient of variation for between-day and within-day replicate analyses were 3.1 and 4.6% respectively. In chapter 5, the retention characteristics of basic drugs on an unmodified silica column using reversed-phase eluents were studied. Drug retention as a function of pH was consistent with an ion exchange mechanism of retention. The analytical method, which used an 80% methanolic eluent, was coupled to a column switching arrangement in order to carry out on-line solid-phase extraction of the drugs from plasma. The mean coefficient of variation for 5 replicate analyses of each drug was less than 8%, even without internal standardisation.

In chapter 6, the retention characteristics of 31 drugs on an alumina column were investigated. Using reversed-phase eluents, retention mechanisms were found to be similar to those on unmodified silica, consisting principally of ion exchange, as shown by drug retention behaviour with changing pH and ionic strength. The analytical method was coupled to a column switching arrangement and on-line solid-phase extraction was carried out.

In chapter 7, the effect of varying the type of column and eluent composition on drug-free plasma profiles was investigated. The study was based on a C_{18}- and a CN- column; methanol and acetonitrile were the organic modifiers used. The plasma profiles were evaluated quantitatively by measuring the number of interfering peaks greater than 2 mm (equivalent to 8×10^{-4} absorbance units) in the area of interest along the chromatogram. Results were subjected to statistical treatment using a 3-factor analysis of variance design. The three factors were the column, type of organic modifier and either the percent organic modifier, pH or ionic strength. Analysis of the data revealed that significant effects were seen with changing eluent composition, particularly with regard to the percent organic modifier and that the observed effects were strongly dependent on the type of organic modifier and the type of column under consideration.

CHAPTER 1

THEORY AND PRINCIPLES OF CHROMATOGRAPHY

1.1. INTRODUCTION

Chromatography is a physical method of separation in which the compounds to be separated are distributed between a mobile phase and a stationary phase. The chromatographic process occurs as a result of repeated sorption onto and desorption from the stationary phase by sample components as they traverse the column bed. Separation between individual components arises fron differences in their distribution coefficients between the two phases.

Chromatography may be fundamentally divided into gas and liquid chromatographic techniques, based on whether the mobile phase is a gas or a liquid. In gas chromatography, the mobile phase is an inert gas and the stationary phase is either an adsorbent or (more usually) a liquid distributed over the surface of a porous inert support. In liquid chromatography, the mobile phase is liquid, and the stationary phase is a solid, an immiscible liquid coated onto a porous support, or a thin film of liquid chemically bonded onto the surface of a sorbent. Liquid chromatography may be operated in planar or column configurations; gas chromatography is only operated in the column configuration.

Liquid column chromatography may be carried out as an open-bed, or closed-bed technique. Gas chromatography can only be carried out as a closed bed technique. In the latter instance pressure gradients are employed to induce movement of the mobile phase down the column. The column is a glass or metal tube of sufficient mechanical strength to withstand operating pressures. In packed column chromatography the sorbent in granular form is packed into a homogeneous bed that totally fills the column. In open tubular chromatography, the sorbent is distributed as a thin film on the internal surface of the column.

Thin-layer and paper chromatography are open-bed planar techniques where separation takes place on a flat surface without the use of pressure gradients and movement of the mobile phase is effected by the action of capillary forces. Thin-layer chromatography employs a layer of stationary phase coated onto a flat surface, and paper chromatography employs absorbent paper. In both cases the stationary phase is a liquid.

The information obtained from a chromatographic experiment is contained in the chromatogram, which is a record of the concentration or mass profile of the sample components as a function of the movement of the mobile phase. As the sample components are successively eluted from the column they are detected using a suitable detection mode, for instance ultraviolet absorbance. Information from the detector is passed to a recording device which displays that data in a trace which constitutes the chromatogram. The kind of information revealed in the chromatogram includes an indication of the sample's complexity based on the number of peaks, concentrations based on peak heights or areas, qualitative identification of individual sample components based on accurate detection of peak position, and an indication of the column's performance over a period of time.

1.2. HISTORY OF CHROMATOGRAPHY

The word "chromatography" was coined by the Russian botanist, Tswett who in 1903, produced coloured bands by separating concentrated plant extract on a column of adsorbent material [1]. The technique then experienced a lull, but was later revived when in the 1930's, Kuhn and Lederer [2], and Reichstein and Van Euw [3], again employed column chromatography in the separation of natural products. The open-bed (thin-layer) version of chromatography was introduced in the 1950s by Kirchner [4], but it was Stahl who popularised the technique some time later [5].

In 1941, Martin and Synge (who were subsequently awarded the Nobel prize) first described the method of liquid-liquid partition chromatography, and in the same work, laid the foundation for gas-liquid chromatography, a technique which enjoyed enormous popularity in the following decades [6]. Martin and Synge also introduced the concept of the theoretical plate [6], which is still a widely used index of chromatographic efficiency. They proposed that column performance was directly proportional to the number of theoretical plates in the system, that higher plate numbers could be achieved by the use of smaller particles at higher operating pressures, and that the mobile phase liquid could be replaced by a gas.

The practical application of gas chromatography was first realised by James and Martin some 10 year later in 1952 [7], and the technique then rapidly evolved into a far more sophisticated form of separation than liquid chromatography, which, excluding the pioneering work by Hamilton on ion exchange [8] and Snyder on adsorption systems [9], remained relatively neglected for a number of decades.

In the early 1960's Giddings showed that the theoretical principles developed to accommodate separation processes in gas chromatography could readily be applied to liquid chromatographic systems [10]. Development of the practical application of liquid chromatography is largely attributed to Horvath, Lipsky and Preiss [11], Huber [12], and Kirkland [13], who developed the method through the embryonic stages during the period 1967-1969. Their new high pressure systems (operating up to 5000 psi) were able to overcome the problem of high liquid mobile phase viscosities relative to gases, and thus the way was paved for efficient and rapid separations in high performance liquid chromatography (HPLC) which were comparable with those then being achieved with gas chromatographic separations.

Advances in HPLC technology then centered around the production of support materials offering improved rates of mass transfer by reducing the distance over which solutes have to diffuse. Short diffusion paths were achieved (a) by coating impervious glass beads with the stationary phase [13,14]; or (b) by producing particles which were totally porous, but of smaller diameter - about 40 m. The latter approach has been widely adopted in the quest for greater plate numbers, and nowadays, columns commonly contain particles between 3 and 10 m in diameter.

The classical solid-liquid adsorption chromatography described above has been largely superceded by bonded phase chromatography, which employs surface-modified silica-based stationary phases. The first bonded phases were produced by Halasz et al. who reacted silica with alcohols [15], and amines [16]. Subsequently, materials of greater hydrolytic stability have been manufactured by reacting silica with alkylsilanes. It is now possible to produce a wide range of bonded supports which can be tailored to suit a variety of analytical needs, and it is the versatility, robustness, and simplicity of practical application which have contributed to the popularity of HPLC in the fields of pharmaceutical, clinical, forensic and environmental analysis.

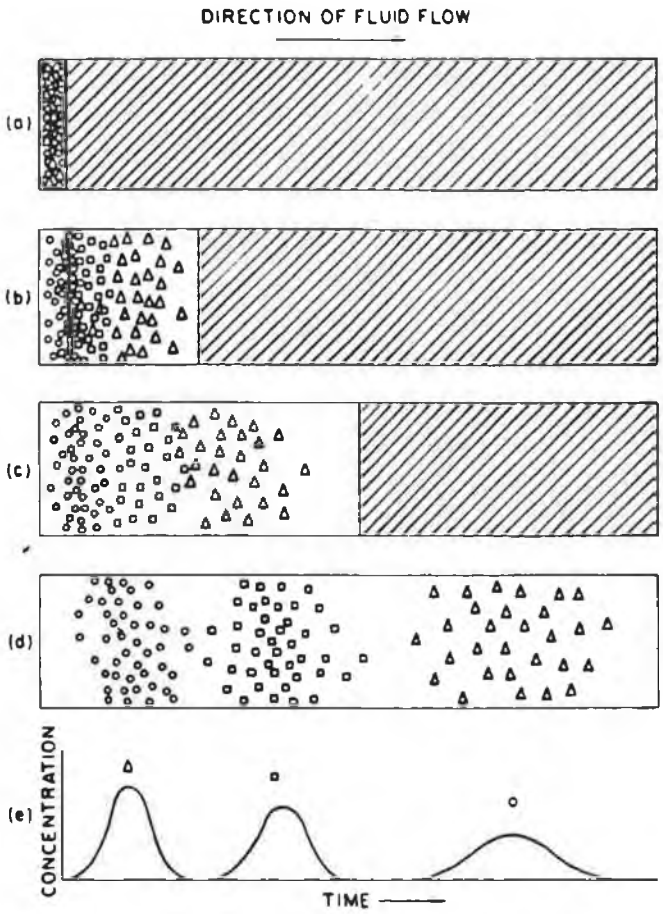
1.3. THEORY

1.3.1. Retention and equilibrium

In HPLC, the migration rate of an individual sample component is determined by the equilibrium distribution of that component between the stationary and flowing phases. Compounds which are distributed mainly in the mobile phase move rapidly down the column, whereas the converse is true for solutes distributed mainly in the stationary phase. These differences in migration rates for individual samples form the basis of chromatographic separation as shown in Figure 1.1. At the beginning of separation following injection, the individual components remain clustered

Figure 1.1.

Schematic illustration of separation by differential migration.



together at the top of the column (Figure 1.1.a). As the sample mixture migrates through the system, the individual components gradually disengage and eventually separate into discreet bands.

A fundamental retention parameter in column chromatography is the retention volume, V_R, defined as the volume of mobile phase required to flow through a column to effect elution of a given compound. The corresponding retention time, t_R, is related to V_R by the expression:

$$t_R = V_R/F \qquad 1.1$$

where F is the volumetric flow rate in ml/min [17].
The measurement of t_R for a 2-component chromatogram is shown in Figure 1.2. The time taken for the elution of an unretained compound is given by t_o, while the point of injection is at t = 0. The elution volume corresponding to t_o is V_M, and may be expressed as:

$$V_M = t_o F \qquad 1.2$$

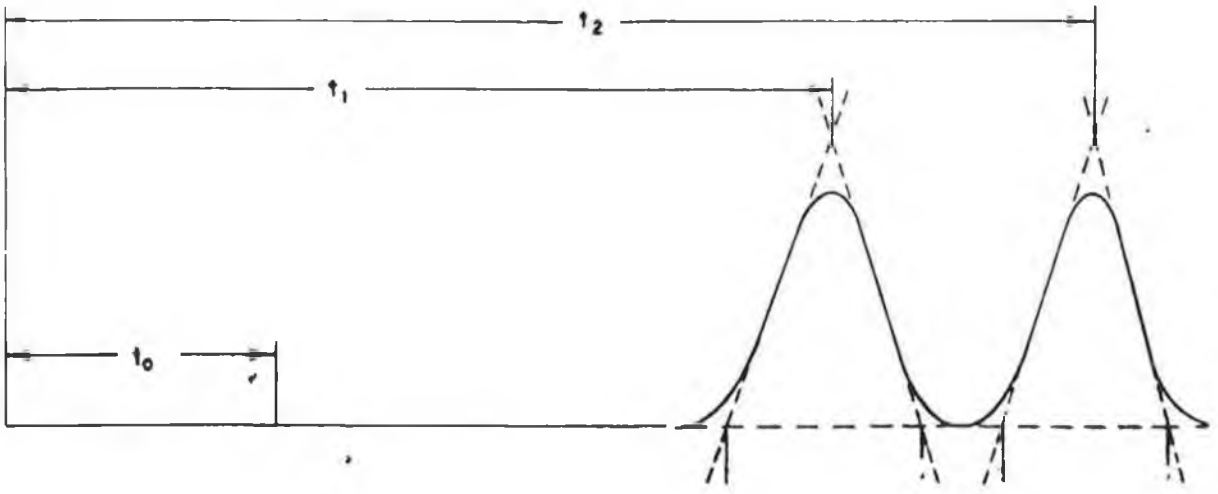
V_M represents the total volume of mobile phase within the column at any given time, and assuming negligible extracolumn volumes, is known as the dead volume. Retention times are measured at the band centre (apex of individual peaks), at which point the distribution of solute molecules in the stationary and mobile phases are considered to be approximately at equilibrium [17].

A useful measure of retention is R, the fraction of solute molecules in the mobile phase, or the probability that that a solute molecule will be found in the mobile phase at any given instant. R may be expressed as:

$$R = n_M/n_M + n_S \qquad 1.3$$

Figure 1.2.

Model chromatogram for the separation of two compounds



TIME (min)---------->

t_o = retention time for an unretained compound

t_1 = retention time of the first compound

t_2 = retention time of the second compound

n_M is the number of moles of solute in the mobile phase, and n_S is the number of moles of solute in the stationary phase. The ratio n_S/n_M is known as the capacity factor k', and substituting this into equation 1.3 , R may be rewritten as:

$$R = 1/(1+k') \qquad 1.4$$

The capacity factor is related to the distribution coefficient (K) for a solute between two phases as follows:

$$k' = n_S/n_M$$

$$k' = (V_S C_S)/(V_M C_M])$$

$$k' = (KV_S)/(V_M) \qquad 1.5$$

where V_S, V_M, and C_S, C_M are the volumes and concentrations of solute in the stationary and mobile phases respectively. Hence:

$$R = 1/(1+K(V_S/V_M)) \qquad 1.6$$

The larger the value of R, the quicker the solute moves through the column. The average migration velocity of a solute (v_S) may be expressed as:

$$v_S = vR \qquad 1.7$$

where v is the mobile phase velocity. But as $v_S = L/t_R$, and $v = L/t_o$, where L is the length of the column, equation 1.7 may be rewritten as:

$$L/t_R = (RL)/t_o \qquad 1.8$$

or:

$$t_R = t_o/R \qquad 1.9$$

Since $t_R = V_R/F$ and $t_o = V_M/F$:

$$R = V_M/V_R \qquad 1.10$$

But as $R = 1/(1 + k')$, V_R may be written as:

$$V_R = V_M + V_M k' \qquad 1.11$$

Since $k = (KV_S)/V_M$, V_R may now be written:

$$V_R = V_M + KV_S \qquad 1.12$$

According to equation 1.12, the retention volume of any given solute depends on the dead volume V_M, and the factor KV_S. The dead volume has no effect on differential migration or separation since it is the same for all sample components, but KV_S does determine differential migration. Hence the net retention volume, V_N may be defined:

$$V_N = V_R - V_M = KV_S \qquad 1.13$$

Since $V_R = t_R F$, and $V_M = t_o F$:

$$V_N/F = t_R - t_o, \text{ or}$$

$$t_N = t_R - t_o \qquad 1.14$$

where t_N is the adjusted retention time. Retention indices are frequently expressed in terms of capacity factor k'. By substituting equation 1.5 into 1.13, one obtains:

$$k' = (V_R - V_M)/V_M \qquad 1.15$$

Substituting Ft_R for V_R, and Ft_o for V_M, one obtains:

$$k' = (t_R - t_o)/t_o \qquad 1.16$$

since the capacity factor for a given solute may be expressed purely in terms of its retention and the time for elution of a non-retained species.

1.3.2. Band broadening

The separation of compounds during migration is accompanied by an increasing dispersion of the initially sharp zones. This dispersion is caused by exchange of compounds between phases, diffusion, and other factors. The extent of band broadening determines chromatographic efficiency: it leads to a distribution of the species in the direction of travel, which can have a detrimental effect on separation. The sample concentration profile which arises is often Gaussian in nature, and the variance σ, of this Gaussian curve is proportional to the migration distance, z.
Chromatographic efficiency is frequently expressed in terms of either the number of theoretical plates, N, or the height equivalent to a theoretical plate, HETP, or H. The plate number, N can be defined from the chromatogram of a single band (Figure 1.3), and may be expressed as:

$$N = (t_R/\sigma_t)^2 \qquad 1.17$$

where σ_t is the standard deviation of the band in time units. N, therefore, is a dimensionless quantity, and its calculation from experimental data is done more conveniently on peak width. The two parameters are related by the equation:

$$\sigma_t = 0.25W_b \qquad 1.18$$

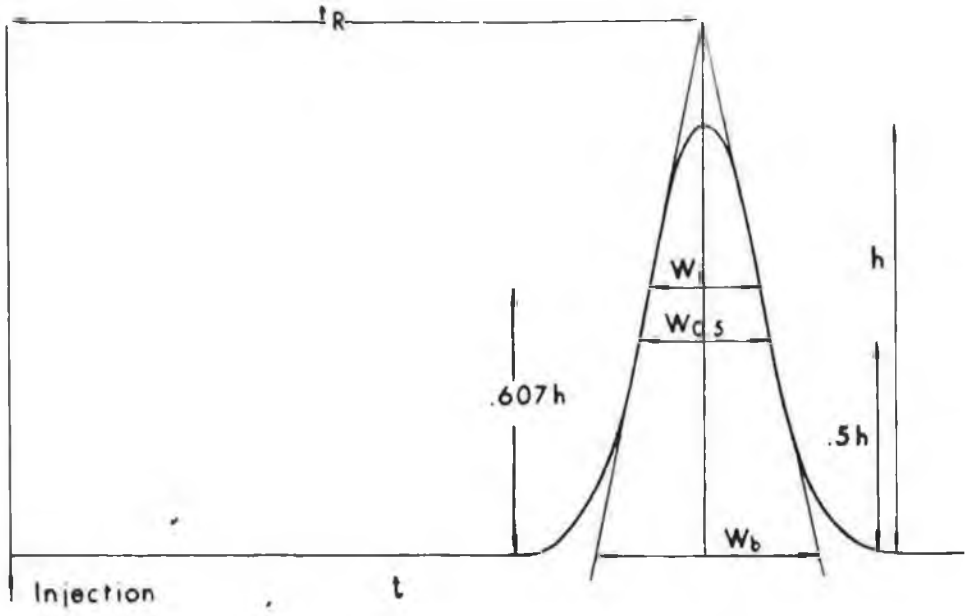
where W_b is the width of the peak at the baseline

Hence: $$N = 16(t_R/W_b)^2 \qquad 1.19$$

The HETP is related to N by:

Figure 1.3.

Evaluation of a chromatographic peak for calculation of column efficiency



TIME (min)---------->

t_R = retention time for the peak

W_b = peak width at baseline

$W_{.5}$ = peak width at half height

W_i = peak width at inflection points

h = peak height

0.5 h = half height

.607h = height at inflection points

t_R = retention time

$$H = L/N \qquad 1.20$$

where L is the length of the column. HETP has the dimensions of length and is usually reported in mm or cm. The terms "plate number" and "plate height" have their origins in the plate model of the chromatographic process as first reported by Martin and Synge [6]. The plate model assumes that the column can be visualised as being divided into discrete units or plates in which complete equilibration of the solute takes place before it progresss to the next plate. It is assumed that distribution between plates is the same for all plates, and independent of solute concentration; that mobile phase flow occurs in a discontinuous manner between plates; and that diffusion of the solute in an axial direction is negligible. Deficiencies with this model arise when the real-life situation deviates from these assumptions, particularly in relation to dependence on solute concentration, particle size, and mobile phase velocity, experimental parameters which vary from one situation to the next. Another useful expression is the reduced plate height, h, which is the HETP divided by the particle diameter, d: i.e. h = H/d.

Where chromatographic efficiency is directly related to the number of theoretical plates, it is inversely proportional to plate height. Hence factors contributing to plate height are considered to promote band broadening, and so reduce efficiency. Band broadening is regarded to be a result of three distinct phenomena:

(i) :longitudinal diffusion;
(ii) :slow mass transfer rates; and
(iii):uneveness of mobile phase flow.

1.3.2.1. Longitudinal diffusion

Molecular diffusion in the longitudinal direction leads to band broadening that is dependent on mobile phase velocity. Its contribution to band broadening increases with the amount of time the solute spends in the column. Its contribution σ^2 to the total band broadening is given by the Einstein equation:

$$\sigma^2 = 2D \times t \qquad 1.21$$

where D is the diffusion coefficient, and t is time.
In general solute molecules spend the time t = L/v in the mobile phase, so equation 1.21 now becomes:

$$\sigma 2 = (2D_M \times L)/v \qquad 1.22$$

D_M is the solute diffusion coefficient in the mobile phase, v is the mobile phase velocity, and L is the length of the column. Since $H = \sigma^2/L$, H_L, the contribution to band broadening from longitudinal diffusion may be written as:

$$H_L = {}_L/L = [2D_M \times \gamma]/v \qquad 1.23$$

where γ is an obstruction or tortuosity factor, which recognises that longitudinal diffusion is hindered by the packing or bed structure; also γ is not totally independent of mobile phase velocity [18]. This arises from the fact that the lowest flow resistance is offered by gaps in the packing structure. Thus at low flow velocities the value of γ is averaged over tightly and loosely packed domains, whereas at high velocities it is weighted in favour of the loosely packed domains where most flow occurs. In practice H_L is a small contribution to overall plate height, H. Only when D_M is large and the mobile phase velocity v is small does it become significant.

1.3.2.2. Mass transfer into the stationary phase

Mass transfer in either the stationary or mobile phases is not instantaneous, and therefore total equilibrium is not established under normal operating conditions. The rate of mass transfer is controlled by diffusion for liquid stationary phases, and by adsorption-desorption kinetics in the case of solid stationary phases. As a result of non-instantaneous mass transfer, the solute concentration profile in the stationary phase is displaced slightly behind the equilibrium position, and the mobile phase profile is slightly ahead of the equilibrium position. Provided the degree of non-equilibrium is small, the plate height contribution arising from non-instantaneous mass transfer may be calculated by means of the one-dimensional random-walk model of Giddings [10]. As solute molecules traverse the column through flow and diffusion processes, they experience many random displacements away from the band centre. Displacements in the direction of flow are regarded as a series of forward steps relative to the continuously moving band centre, whereas dispacements away from the direction of flow are regarded as backward steps. When there is a large number of solute molecules, and hence, steps, there is a normal distribution of molecules around the band centre. Band variance may then be written as :

$$\sigma^2 = l^2 n \qquad 1.24$$

where l is the fixed step length, n the number of steps. These are assumed to be constant and large, respectively.

Solute retention may be regarded as a series of sorption-desorption steps by the solutes into and out of the stationary phase. Assuming simple first order kinetics, the rate of sorption and desorption is proportional to the total number of solute molecules, and the rate constant is independent of solute concentration, a requirement which is normally met at low solute concentrations. The rate constant is equal to the fraction of available molecules which are adsorbed or desorbed within a given unit time.

Hence:

$$K_a = \text{adsorption constant} = 1/t_a;$$

$$K_d = \text{desorption constant} = 1/t_d$$

where t_a and t_d are the mean adsorption and desorption times, respectively. In the random walk model, adsorption represents a step backwards, and desorption represents a step forward. The total number of steps as the band migrates along a distance L, is the sum of all forward and backward steps, or twice the number of adsorption steps since one step follows the next.
The total number of steps may be written:

$$n = 2L/v \times t_a \qquad 1.25$$

The length of each step, l, can be evaluated from the displacement of solute molecules relative to the band centre. The band centre migrates with a velocity of $v/(1 + k')$, so that during one step of duration t_a, the band centre will be displaced by a distance $vt_a(1 + k')$. During this time a molecule in the mobile phase will be migrating with velocity v so that it will have moved a distance equivalent to vt_a before adsorption. The net displacement of the unadsorbed molecule from the band centre during this step is then given as:

$$l = vt_a - (vt_a/(1 + k'))$$

hence

$$l = (vt_a k')/(1 + k') \qquad 1.26$$

Substituting with equation 1.24 gives the variance resulting from slow adsorption-desorption σ_s^2:

$$\sigma_s^2 = (2vt_a L)(k'/(1 + k'))^2 \qquad 1.27$$

Since the plate height contribution from the adsorption-desorption process, H_S is σ_s^2/L, H_S may be

given by:

$$H_S = (2vt_a)(k'/(1 + k'))^2 \qquad 1.28$$

It is more convenient to express H_s in terms of mean desorption time, t_d rather than t_a. Since the fraction of time spent by the solute in the mobile phase is $1/(1 + k')$, the fraction of time spent in the stationary phase is $k'/(1 + k')$. Hence the ratio t_d/t_a equals k', and substituting t_d/k' for t_a in equation 1.28 gives

$$H_S = (2vt_d k')/((1 + k')^2) \qquad 1.29$$

This equation shows that H_s increases with mobile phase velocity, and the time taken for desorption from the stationary phase to occur. This equation pertains to adsorption chromatography, but may be modified to apply to partition chromatography. The mean desorption time t_d is replaced by the mean diffusion time t_D which may be expressed in terms of film thickness, d and solute diffusivity in the stationary phase, D_s. Therefore:

$$H_S = (qvd^2 k')/(D_s(1 + k')^2) \qquad 1.30$$

where the term q is a shape factor included to account for the geometric configuration of the immobilised liquid stationary phase. Where the latter is a thin film on a stationary support, q has a value of 2/3.

1.3.2.3. Mobile phase mass transfer

Solute molecules in the mobile phase are displaced not only by diffusion, but also by flow. In a single flow stream the flow is not uniform. Mobile phase in close proximity to the stationary phase moves very slowly or not at all, whereas mobile phase in the centre of the flow stream moves quickly. Furthermore, small

and large flow inequalities are always present as a result of the irregular packing structure of the stationary phase. The moving mobile phase velocity contribution, H_M, to overall plate height, H, is a function of eddy diffusion effects, H_f, and diffusion effects H_d. H_M is given by:

$$H_M = 1/(1/H_f + 1/H_d) \qquad 1.31$$

As $$H_f = C_1 d_p \qquad 1.32$$

and $$H_d = C_2(d_p^2)/D_M. \qquad 1.33$$

where d_p is the particle diameter, and C_1 and C_2 are functions of the packing structure H_M may be written as:

$$H_M = (1/(C_1 d_p)+D_M/(C_2 v d_p^2))^{-1} \qquad 1.34$$

As mentioned above, mobile phase in close contact with the stationary phase moves very little, and in porous packings the intraparticulate void volume is filled with mobile pahse at rest. Solute molecules must diffuse through this "stagnant" layer to reach the stationary phase; thus an additional term H_{SM} must be added to the overall plate height contribution. H_{SM} is given by the equation:

$$H_{SM} = ((1 - \varphi + k')^2 d_p^2 v)/(30(1-\varphi)(1 + k')^2 D_M \gamma) \qquad 1.35$$

where φ is the fraction of mobile phase inside the particle, and γ is the tortuosity factor inside the particle.

1.3.2.4 Complete plate height equation

To summarise, the overall HETP may be expressed as the sum of the terms from longitudinal diffusion, H_L, stationary phase mass transfer, H_S, the moving mobile phase contribution, H_M, and

the stagnant mobile phase contribution H_{SM}:

$$H = H_L + H_S + H_M + H_{SM} \qquad 1.36$$

The dependence of each individual factor, and the sum of the individual factors on flow velocity is shown in Figure 1.4. The relative magnitudes of the individual plate height contributions vary depending on the type of chromatography and the capacity ratio. The hyperbolic HETP versus mobile phase velocity function is generally described by the Van Deemter equation [19], which may be written:

$$H = A + B/v + Cv \qquad 1.37$$

where A represents the contribution from eddy diffusion, B the contribution from longitudinal diffusion, and C contributions from mass transfer in the mobile and stationary phases to the total column plate height. The form of the Van Deemter equation is shown in Figure 1.5.

The minimum plate height, H_{min}, is observed at an intermediate optimun value of v, v_{opt}. By differentiating the Van Deemter equation with respect to mobile phase velocity, v, and setting the result equal to zero, expressions for v_{opt}, and H_{min} may be obtained:

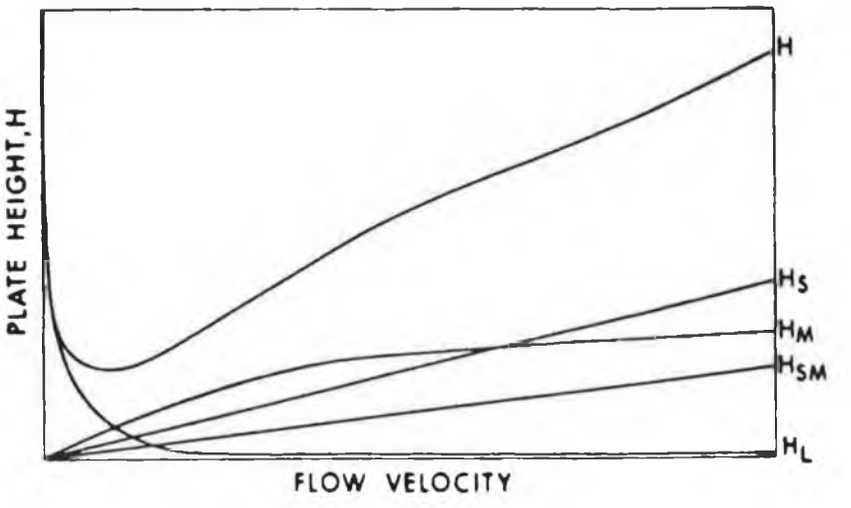
$$H_{min} = \sqrt{A + 2/(B \times C)} \qquad 1.38$$

$$v_{opt} = \sqrt{(B/C)} \qquad 1.39$$

In practice, the flow velocity is kept larger than v_{opt} to provide faster separations so that total band broadening is controlled by the mass transfer term Cv. Provided that the ascending portion of the Van Deemter plot is fairly flat at velocities higher than v_{opt}, then the loss in efficiency will be small and well worth the gain in analysis time.

Figure 1.4.

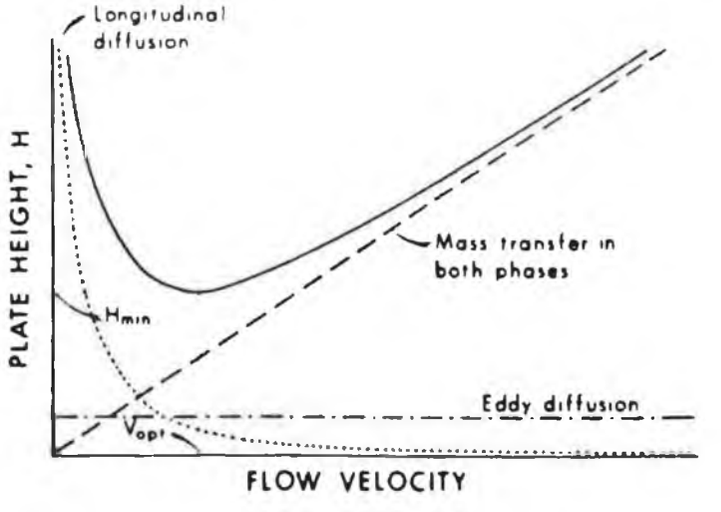
Dependence of overall plate height, H and plate height increments on flow velocity



H_S = Stationary phase contribution; H_M = Mobile phase contribution; H_{SM} = Stagnant mobile phase contribution; H_L = Longitudinal Diffusion.

Figure 1.5.

The Van Deemter plot



1.3.3. Resolution

Resolution in chromatography is determined by two basic factors - the separation of the peak centres, and the size of the peak widths. This is illustrated in Figure 1.6. The degree of separation of two adjacent peaks is commonly defined as the distance between peak centres divided by the average peak width. If retention and and peak width are measured in units of time, the resolution, R_s, may be described as:

$$R_s = 2(t_{R2} - t_{R1})/(W_{b1} + W_{b2}) \qquad 1.40$$

where t_{R1} and t_{R2} are the retention times of the two peaks, and W_{b1}, and W_{b2} are their peak widths, defined by the tangents to the inflection points of a given curve. For closely adjacent bands $W_{b1} = W_{b2}$, and since W_b for a Gaussian curve, is equal to four standard deviations (σ_t), R_s may be written as:

$$R_s = t_{R2} - t_{R1}/4\sigma_t \qquad 1.41$$

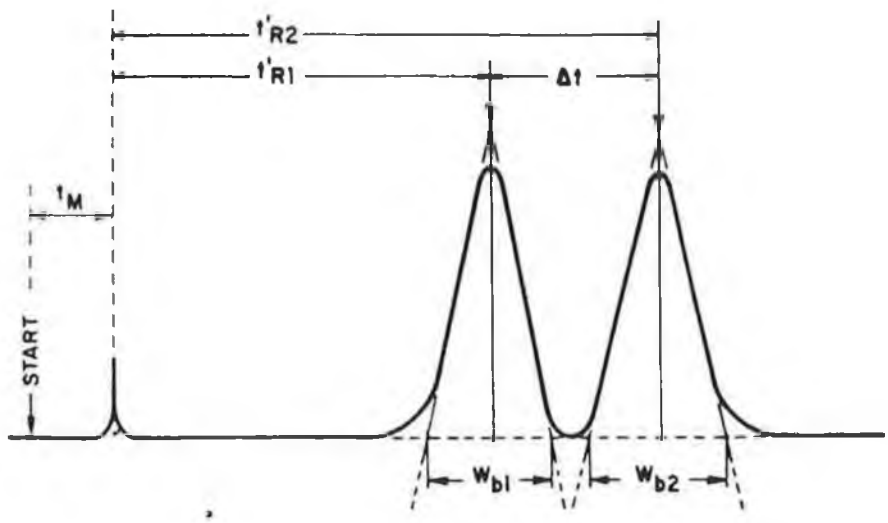
The larger the value of R_s the greater the separation. When $R_s = 1.5$, baseline separation is achieved, but $R_s = 1.0$ is considered to be reasonably good resolution, and the peaks are distinguishable over a wide range of concentrations. At smaller values of R_s, resolution will be determined by the relative concentrations of the two peaks, and where there is a greater than 10-fold concentration differential, the smaller peak may not be recognisable.

Resolution is related to the adjustable variables of chromatography by the equation:

$$R_s = (\sqrt{N}/4)(1 - t_{R1}/t_{R2}) \qquad 1.42$$

Figure 1.6.

Measurement of resolution (Rs) for two closely related peaks



TIME (min)---------->

t_M = retention time of an unretained compound
t_{R1} = retention time of the first compound
t_{R2} = retention time of second compound
W_{b1} = width at baseline for compound 1
W_{b2} = width of baseline for compound 2

$$R_s = 2\sigma t/(W_{b1} + W_{b2})$$

Since:

$$t_{R1}/t_{R2} = (1 + k'_1)/(1 + k'_2),$$

R_s may be written:

$$R_s = (\sqrt{N}/4)((k'_2 - k'_1)/(1 + k'_2)) \qquad 1.43$$

where k'_1 and k'_2 refer to the capacity factors of peaks 1 and 2 respectively. As the separation factor $\alpha = k_2/k_1$, and $k_1 = k_2/\alpha$:

$$R_s = (\sqrt{N}/4)(k'_2/(1 + k'_2))((\alpha-1)/\alpha) \qquad 1.44$$

Resolution is therefore seen to be a function of three separate factors: a column efficiency term, $\sqrt{N}$, a selectivity term which varies with α, and a capacity term which varies with k_2. When α = 1, resolution is not possible in that system, and the larger the value of α , the easier separation becomes. In practice, increasing k' will improve resolution up to a point, beyond which analysis time and band broadening may be unacceptably increased. It is not usual to try to optimise N, k' and α simultaneously; experimental conditions are chosen to give the highest value of N within the constraints of convenience and reasonable separation times.

It is often convenient to combine the plate number and capacity factor terms in equation 1.44 into a single parameter known as effective plate number, N_{eff}, and which is related to N through:

$$N_{eff} = N(k'/(1 + k'))^2 \qquad 1.45$$

The ultimate objective in any separation is to achieve maximum possible resolution in the shortest possible time. The time for separation, which is the retention time for the last eluting

peak, t_1 is related to resolution by the equation:

$$t_i = (16R_s^2)(\alpha/(\alpha-1))^2)((1 + k')^3/k'^2))(H/v) \qquad 1.46$$

Where k' is the capacity factor of the last eluting peak. This equation predicts a 4-fold increase in separation time if it is sought to double R_s, but if α is increased from 1.05 to 1.1, the separation time is decreased 10-fold. Maximum resolution per unit time is obtained when R_s equals 2. This expression also shows that it is desirable to operate at reasonably high flow rates provided the corresponding increase in H does not operate to the detriment of the separation profile. Because H is inversely proportional to particle size, it is desirable to keep the latter as small as possible. This approach is limited by the increase in pressure across the system found with smaller particles.

Because of the inter-relationship between time and resolution, parameters such as plates per second (N/t), or effective plates per second (N_{eff}/t) are more valid criteria in this regard. Classical solid-liquid chromatography before 1965 was characterised by low values (0.01-0.1) of N_{eff}/t, but with modern HPLC techniques it is possible to generate values of 25 or more.

1.4. MODES OF CHROMATOGRAPHY

As described in section 1.1., chromatographic modes may be fundamentally divided into gas and liquid chromatographic techniques, although that distinction has become somewhat blurred with the advent of supercritical fluid chromatography where the eluent is a gas held at a temperature above its critical temperature. The supercritical gas behaves as a liquid in regard to equilibrium properties, and liquid chromatographic technology is frequently used in its operation.

Table 1.1. summarises the classification of chromatographic methods according to the type of mobile and stationary phases they employ. Also shown is the type of interaction which can take place in each of these modes. Sorption includes adsorption and partition, the latter (which is a bulk phase distribution process) is the dominant mechanism in gas-liquid chromatography, liquid-liquid chromatography and supercrititcal fluid liquid chromatography. Adsorption involves interaction of sample components at a surface with fixed sites. Ion exchange occurs where the stationary phase contains fixed charged groups and mobile counter ions with which ionic solutes can compete as they proceed through the column. In exclusion chromatography, which includes both gel filtration and gel permeation, sorption does not occur. Rather sample molecules which are small enough can drift into the pores of the stationary phase. Relative molecular size then determines the distribution of a compound between mobile and stationary phases.

More than one retention mechanism can operate in a given system (for instance, sorption with exclusion, adsorption plus partition). Although such effects are often accidental, it may be useful to deliberately create a dual mechanism of retention to enhance differences in migration of the sample components.

Table 1.1.

Classification of chromatographic modes based on mobile phase

MOBILE PHASE	STATIONARY PHASE	MECHANISM	TECHNIQUE
Gas	Solid	Adsorption	Column
Gas	Liquid	partition	Column
Liquid	Solid	Adsorption	Column, TLC, Paper
Liquid	Solid	Ion exchange	Column
Liquid	Solid	Exclusion	Column
Liquid	Liquid	Partition	Column, TLC, Paper
Supercritical Fluid	Solid	Adsorption	Column
Supercritical Fluid	Liquid	Partition	Column

Liquid chromatographic methods will now be discussed in further detail. Gas chromatography and supercritical chromatography will not be included as it is beyond the scope of this work to permit adequate discussion of these techniques.

1.4.1. Liquid-solid adsorption chromatography

Liquid-solid chromatography (LSC) involves distribution between a liquid mobile phase and a finely divided porous solid adsorbent stationary phase. The adsorbent should have a relatively large surface area, for example 50 to 1000 m^2/g. The equilibrium which governs separation is based on distribution of the sample molecules between the bulk liquid phase and the surface of the adsorbent. In order to achieve this, eluent molecules already adsorbed onto the stationary phase must be displaced, and the system can be thought of as a reaction producing displacement.

1.4.1.1. Theory of liquid-solid chromatography

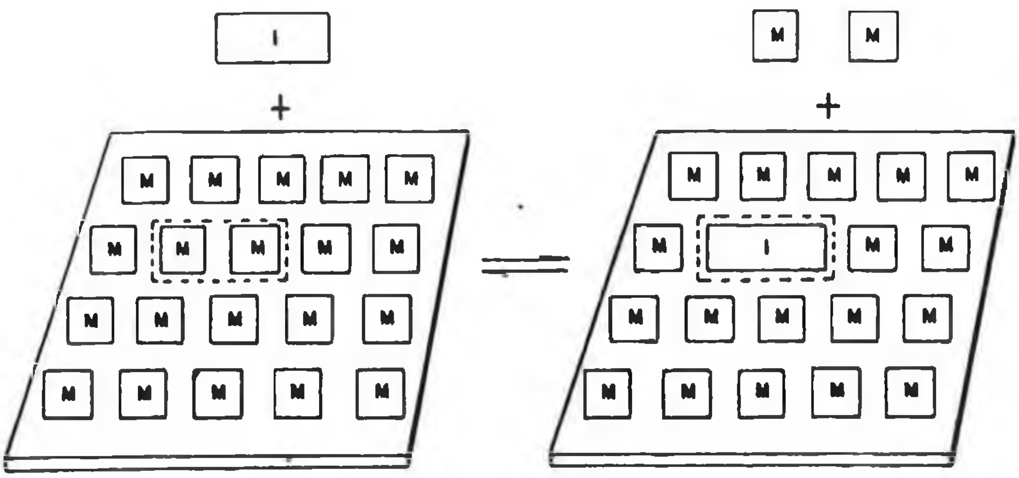
The thermodynamics of adsorption chromatography has been treated in detail by Snyder [9], and a simplified version of his argument is presented here. In almost all practical LSC systems, the surface of the adsorbent is completely covered by a monolayer of adsorbed molecules of solute i, or mobile phase M. The equilibrium for adsorption and desorption of sample molecules can be written as:

$$i_M + nM_a \rightleftharpoons i_a + nM_M \qquad 1.47$$

A solute molecule initially present in the mobile phase [i_M] adsorbs by displacing some number, n, of mobile phase molecules from the adsorbent surface. This then leaves an adsorbed solute molecule [i_a], and some number of desorbed mobile phase molecules [nM_M]. This process is illustrated in Figure 1.7. The magnitude of n is determined by the relative sizes of the solute and mobile phase molecules on the adsorbent surface. In Figure

Figure 1.7.

Hypothetical representation of the equilibrium between solute molecules (i) and the mobile phase molecules (M)



$$i_M + 2M_a \rightleftharpoons i_a + 2M_M$$

1.7., n = 2. The approximate equation relating the sample adsorption coefficient K' to properties of the adsorbent, the solute and the eluent can be derived and expressed as:

$$\log K' = \log V_a + \alpha'(S^o - A_i \epsilon^o), \qquad 1.48$$

where V_a is the quantity of adsorbed (monolayer) mobile phase per unit weight of adsorbent. V_a varies with the type, surface area, and water content of the adsorbent, and to some extent, the nature of M; α' is the adsorbent activity parameter which measures the ability of a unit of adsorbent surface to bind adsorbed molecules; the larger α', the more strongly the adsorbent binds both solute molecules and mobile phase. S^o measures the adsorption energy of i onto a standard adsorbent surface ($\alpha' = 1$), from a standard mobile phase M for which ϵ^o equals 1. The parameter ϵ^o refers to the solvent strength of the mobile phase, and A_i is the molecular area of solute molecule i.

1.4.1.2. The role of the adsorbent

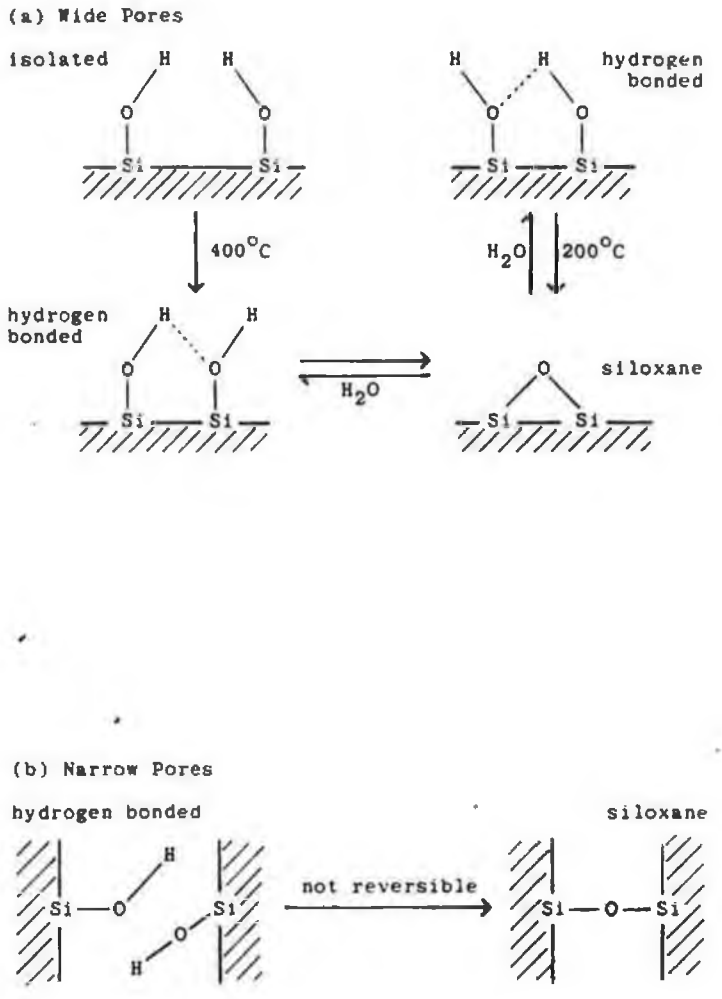
The effect of the adsorbent on separations in LSC is determined by several adsorbent properties; primarily chemical type, surface area, and water content. "Chemical type" refers to the bulk composition of the adsorbent, for example, silica or alumina. Differences in chemical type imply differences in surface functional groups and in the type of interaction between adsorbent and adsorbed molecules. The common adsorbents can be divided into two major groups: polar adsorbents such as silica, alumina and other inorganic gels, and non-polar adsorbents such as charcoal. Microbead particles (diameters 5-10 μm) are now used for high performance LSC applications, with pellicular and macrobead particles (diameters 20-50 μm), being used principally in preparative scale applications. Polar adsorbents interact with solutes via specific forces such as electrostatic attraction and hydrogen bonding. Adsorption energy and chromatographic

retention, and solvent strength tend to increase with the polarity of the adsorbate; such as in the series saturated hydrocarbons < unsaturated and aromatic hydrocarbons < esters < alcohols < acids and bases. Polar compounds are not preferentially adsorbed on the non-polar adsorbents; in this case, dispersion forces are the dominant contribution to adsorption energy, so that higher molecular weight compounds are preferentially retained. The most common polar adsorbents are silica and alumina, and whereas most of the work in the area today employs silica, the classical work of Snyder [9] concentrated largely on alumina. Fortunately the surface structures of the two adsorbents are sufficiently similar to permit single consideration.

The active sites on silica are hydroxyl groups, and in the crystalline form, the groups are isolated, and are not in a position to interact with their nearest neighbours. In amorphous silica, such as that used for chromatography, the arrangement of the surface hydroxyl groups is not as ordered, and interaction between neighbouring moieties is possible [20]. In particular, hydrogen-bonded groups are present, the number of which depends on the pore diameter of the adsorbent. The surface of wide-pore silica is covered mainly with isolated hydroxyl groups, while the surface of narrow-pore silica is covered with hydrogen-bonded hydroxyl groups. If amorphous silica is heat treated, siloxane bridges are formed as shown in Figure 1.8. These functions are weak adsorbent sites, but are thought to partake in hydrophobic interactions [21]. Whether or not the formation of siloxane bridges is reversible is thought to depend on the relative position of the two reacting hydroxyl groups: if the hydroxyl groups are situated on adjacent sites, the reaction can be reversed by heating in the presence of water, but if the they are located on different, but nearby surfaces, such as would be found in narrow-pore silicas, the reaction is irreversible, and permanent loss of surface area occurs. The two types of chromatographically useful active sites (isolated and

Figure 1.8.

Surface processes occurring on heat and hydrothermal treatment of silica



(a): processes occurring in wide pores

(b): processes occurring in narrow pores.

hydrogen-bonded hydroxyl groups) have different adsorption properties; hydrogen-bonded groups are considered to be significantly stronger sites for adsorption, frequently yielding poor chromatographic characteristics. Therefore it is common to cause partial deactivation by the addition of a small amount of water or another polar solvent, thus removing hydrogen-bonded groups from the sphere of activity, and leaving adsorption to occur at a relatively uniform surface of isolated hydroxyl groups.

The surface area of an adsorbent affects V_a in equation B, but has little effect on other parameters. An increase in adsorbent surface area therefore results in a general increase in K' for all solutes, but only slightly affects selectivity. When water is added to an initially dry adsorbent to effect deactivation of the surface, this causes a reduction in surface area, and also in retention. This variation in adsorbent activity with water content leads to a general problem in adsorbent and retention reproducibility: steps must be taken to minimise exposure of the adsorbent to air from which it would attract water molecules, and stringent control must be maintained over the water content in the mobile phase.

1.4.1.3. The role of the mobile phase

The mobile phase in LSC is usually selected to give the desired solute retention times. So-called strong solvents give low retention times whereas weak solvents provide large retention times. Solvent strength is defined quantitatively by the solvent strength parameter ϵ^o, and a series of solvents arranged in order of increasing strength or ϵ^o values is referred to as an eluotropic series. An eluotropic series for selected solvents on silica and alumina, in addition to viscosity and UV cut-off values, is shown in Table 1.2. Relative solvents strengths are roughly the same for all polar adsorbents. A suitable solvent strength for a given separation may be obtained from Table 1.2., or similar type, by trial and error. In this respect, thin layer

Table 1.2.

Properties of some common solvents used in HPLC

Solvent	ε^{o} Silica	ε^{o} Alumina	Viscosity cP. 25°C	UV cut-off nm
Hexane		0.01	0.30	190
Cyclohexane	-0.05	0.04	0.90	200
Carbon tetra-chloride	0.14	0.18	0.90	265
Benzene	0.25	0.32	0.65	278
Diethyl ether	0.38	0.38	0.24	218
Chloroform	0.26	0.40	0.53	245
Dichloromethane		0.42	0.41	233
Tetrahydrofuran		0.45	0.46	212
Acetone	0.47	0.56	0.30	330
Dioxane	0.49	0.56	1.20	215
Ethyl acetate	0.38	0.58	0.43	256
Acetonitrile	0.50	0.65	0.34	190
Dimethylsulfoxide		0.75	2.00	268
1-Propanol		0.82	1.90	205
methanol		0.95	0.54	205
Water		large	0.89	

The solvent strength parameter, ε^{0}, is defined as the adsorption energy per unit area of standard adsorbent.

chromatography (TLC) can prove particularly useful, since the relative migration rates of components in a sample mixture can be determined quickly and easily. Solvent strength is determined by how strongly solvent molecules adsorb on the adsorbent surface. Consequently, for polar adsorbents ϵ^{o}, increases with solvent polarity. On non-polar adsorbents, solvent strength is virtually the reverse of that for polar adsorbents, and hence water is the weakest solvent, while benzene has strong eluting power on surfaces such as charcoal

Fine adjustments in solvent strength are possible with solvent mixtures and a few pure solvents can cover the entire spectrum of solvent strength. Although the primary role of the solvent is control of retention, judicious choice of solvent can produce changes in separation factors. As solvent strength is increased, equation 1.48 predicts that the retention of solutes with larger molecular areas will decrease more rapidly than those of smaller molecules.

1.4.2. Liquid-liquid chromatography

Separation by liquid-liquid partition chromatography depends on the different distribution co-efficients of the components in a sample mixture between a liquid stationary phase and a liquid mobile phase. It was introduced in 1941 by Martin and Synge, who used a liquid-coated porous support to separate amino acid derivatives, and who presented the method in one of the classic papers on liquid chromatogaphy [6]. Two types of partition chromatography may be defined based on whether the stationary phase is coated or chemically bonded onto the surface of the support material. Chromatography carried out on the latter type of material is known as bonded phase chromatography (BPC), and lately, since the decline of the traditional coated phases, has become virtually synonymous with the term "liquid-liquid chromatography".

As regards coated columns, it would appear that the necessity

for pre-saturation of the mobile phase with stationary phase to prevent stripping of the stationary phase, and the incompatibility of these systems with gradient elution is a severe restriction on their use except for special cases. The most important of these is normal-phase ion-pair partition chromatography, which has some application in the separation of ionisable compounds [22].

Bonded stationary phases are prepared by chemically bonding an organic moiety onto the surface of the adsorbent. The advantages of bonded phases are that there is much more freedom in the choice of mobile phase than in liquid-liquid partition chromatography; gradient elution techniques can be used without stripping the stationary phase; and polar and ionic molecules can be efficiently separated. The main disadvantages are that the bonded phase can be cleaved by off by buffer solutions which are too acidic or too basic, as well as by oxidising agents, and that incomplete coverage of the silanol groups can lead to poor chromatographic characterisics for basic drugs. Virtually all currently available bonded materials are based on silica gels, as alumina cannot be modified to produce a mechanically stable surface [23]. A diverse array of silica-bonded phases are now available, and these may be divided into three principal groups:

(i) Hydrophobic chains, especially octadecyl (C_{18}), but also C_8, and C_2 chains.
(ii) Polar goups such as cyano, aminopropyl and ether groups.
(iii) Ionic groups such as sulphonate, amino, and quaternary ammonium groups.

Materials for exclusion chromatography have also been chemically reacted with silane reagents to remove unwanted adsorptive effects [24].

1.4.2.1. Theory of liquid-liquid chromatography

The selection of the correct stationary (s) and mobile (m) phases for a given liquid-liquid chromatographic separation is of critical importance in the design of a working system. The main requirements of the two liquid are as follows:

(i) immiscibility of the two phases;

(ii) retention values in the right range, i.e. $1<k'<20$ approximately.

(iii) sufficiently large separation (α) for samples. The distribution coefficient K_i, for solute i between two immiscible phases s and m is given by the expression

$$K_i = [i]_s / [i]_m . \qquad 1.49$$

where $[i]_m$ and $[i]_s$ are the concentrations of i in the mobile and stationary phases respectively.

since: $$[i]_s = g_s / V_s , \qquad 1.50$$

and: $$[i]_m = g_m / V_m \qquad 1.51$$

where g_s, g_m and V_s, V_m are the weights and volumes of i in the mobile and stationary phases respectively. Hence equation 1.51 may be rewritten as:

$$g_s / g_m = K_i V_s / V_m \qquad 1.52$$

since $K_i V_s / V_m$ equals k' the capacity factor,

$$k' = g_s / g_m . \qquad 1.53$$

The capacity factor is therefore proportional to the weight of the solute in the stationary phase, and indirectly proportional to the weight of the solute in the mobile phase. This argument ignores the effect of the stationary support on the distribution

of solutes between the two phases. So-called support effects can alter K_i values from those observed in the equilibrium distribution of i between two unsupported phases. Support effects include adsorption of the sample onto the support, and ion-exchange interactions between the support and oppositely charged solutes, as is seen between basic compounds and residual silanols on bonded silica.

1.4.2.2. Preparation of Chemically bonded stationary phases

Organic groups are introduced onto the adsorbent by reaction with silanol sites on the silica surface. While there are about five -OH groups per nm^2 of surface on silica gel (corresponding to 8-9 $\mu mol/m^2$) [24], it is stereochemically impossible to react all of them completely even with compounds as small as trimethylsilane. The maximum surface concentrations of various groupings have been measured by a number of workers, and some of their results have been reviewed by Colin et al. [25]. The maximum concentrations of trimethylsilyl-, octadecylsilyl-, and triphenylmethylsilyl- groups are found to be about 4.5, 3.5, and 2.5 $\mu mol/m^2$ respectively, indicating that at best, about half of the available -OH groups can be reacted. The realistic objective is then to obtain maximum possible coverage (up to about 50%), so that remaining silanols are inaccessible to solute molecules, and so do not affect their retention. In practice, however, a proportion of silanol moieties remain unreacted and accessible to solute molecules; interaction with these residual groups is considered to be responsible for poor chromatographic characteristics of basic drugs on bonded silica gel.

The main methods for preparing bonded phases are:

(i) reactions with alcohols and amines,
(ii) reactions involving Grignard and organolithium reagents.
(iii) reaction with organosilanes.

The majority of modern commercially available bonded materials are derived from reactions between organochlorosilanes or alkoxysilanes with the surface silanol groups, and it is only these which will be discussed here. Such reactions can be carried out under a range of conditions, as discussed by Majors [26]. There are two general approaches to the bonding of organosilanes to silica. The first one involves bonding under anhydrous conditions where the reaction is carried under conditions that as far as possible, exclude water form the reaction mixture. Typical reaction conditions might involve heating dry silica under reflux with octadecyltrichlorosilane in toluene [26,27]. In the absence of moisture, no hydrolysis of the Si-Cl bonds in the chlorosilane takes place, and therefore, polymerisation of the silane does not occur. Bonding occurs by elimination of HCl between the organosilane and one or more of the surface silanol groups. After removal of excess silane, the product is hydrolysed to convert unreacted Si-Cl groups to silanol groups as depicted in Figure 1.9.

Residual silanol groups, arising from either hydrolysis of Si-Cl groups as shown, or by non-reaction with the alkylsilane in the first place, may be end-capped, (that is, reacted with trimethylsilane), in order to minimise undesirable adsorptive and/or ionic properties. Some of the more common materials prepared via this scheme include octadecyl-, cyanoalkyl-, and phenyl- groups. Further reaction, for example sulphonation of phenylalkyl supports to form ion-exchange materials, is frequently carried out [28]. The advantage of supports prepared in this way is that the siloxane (Si-O-Si) bonds formed during the reaction are relatively stable to hydrolysis. Bonded phases are generally considered to be stable in the pH range 3-8 [29,30]. Below pH 2-3, the organic groups become cleaved from the support, and above pH 8, the silica itself begins to dissolve, particularly where the eluent contains a high proportion of water.

Figure 1.9.

Reaction of silanol groups to give an alkyl bonded phase using an alkyl trichlorosilane

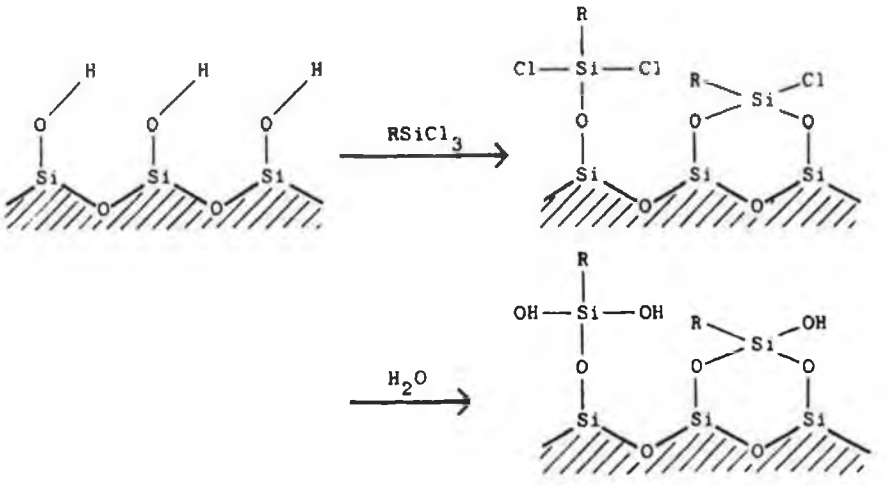
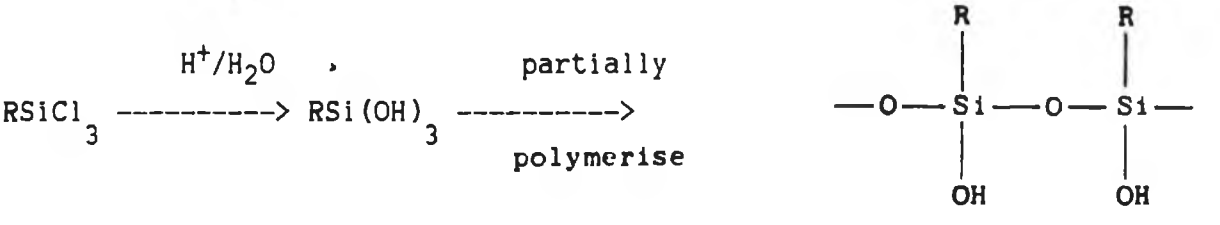
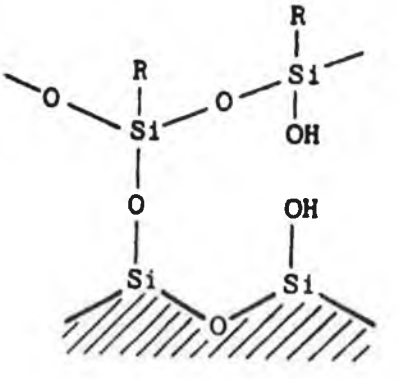


Figure 1.10.

Reaction of silanol groups with a partially polymerised silane to give a polymeric stationary phase



reaction with surface



The second approach involves hydrolysis. As shown in Figure 1.10., the organosilane is first hydrolysed to the silanetriol, which then partially polymerises. The polymer is then bonded to the support by multiple attatchments, again via stable siloxane linkages which confer good hydrolytic stability on these supports [31-33].

1.4.2.3. The range of chemically bonded stationary phases

Although in theory there is no limit to the range of chemically bonded stationary phases which can be prepared, only a fairly small number of bonded phases is required to cope with nearly all the HPLC separations encountered in practice. This is because selectivity may readily be adjusted by changing the composition of the eluent. This contrasts with the situation in GC where where the eluent has fixed properties, and selectivity can only be adjusted by altering the nature of the stationary phase, the support, or operating temperature.

As previously mentioned, bonded phase chromatatography can be operated (a) in the normal phase mode where the eluent is less polar than the stationary phase, (b) in the reversed phase mode where the eluent is more polar than the stationary phase, as well as (c) in an ion-exchange capacity. The latter method will be discussed in section 1.4.3.

1.4.2.3.1. Polar bonded phase chromatography

Polar bonded phases containing diol-, cyano-, diethylamino-, amino- or diamino- functional groups are commercially available. The diol- and diethylamino-bonded phases are used in size exclusion and ion-exchange applications, respectively, which will be dealt with later.

The alkylnitrile-, and alkylamine-bonded phases, when used with a solvent of low polarity, interact with solutes in a manner

similar to that seen in solid-liquid adsorption chromatography; that is, retention of the sample increases with increasing polarity of the solute, and decreasing polarity of the mobile phase. The polar bonded phases are generally less retentive than adsorptive packings, but are less subject to problems such as chemisorption, tailing and catalytic activity associated with unbonded materials. In addition, polar bonded packings are more suitable for gradient elution than adsorbent packings as they respond quicker to changes in mobile phase composition; it is because of these and other advantages that these materials have been proposed as alternatives to microporous adsorbents for separating some sample types [33].

The alkylnitrile-substituted phase is of intermediate polarity and provides good selectivity for the separation of double-bond isomers and ring compounds differing in either the position or number of double bonds [34]. In the alkylamine-substituted phases, the amino function imparts strong hydrogen-bonding properties to the stationary phase, as well as acid or base properties depending on the nature of the solute. Certain problems can arise with the use of these stationary phases: they are readily oxidised so thorough degassing is required, and impurities in the mobile phase containing ketone or aldehyde groups may react chemically with the amine group to form a Schiffs base complex [35].

1.4.2.3.2. Reversed-phase chromatography

Reversed-phase chromatography is so called because it is a reversal of the original mode of solid-liquid chromatography where the stationary phase is more polar than the mobile phase eluent. Reversed phase chromatography has now become the method of choice for the majority of applications, and it is estimated that between 70% and 90% of all HPLC separaions are carried out in the reversed phase mode [35]. The reasons for this include the fact that it is versatile, rugged and simple to use.

The near-universal application of reversed-phase chromatography stems from the fact that practically all organic molecules possess hydrophobic regions in their structure which are capable of interacting with the non-polar stationary phase. Since the mobile phase in reversed phase chromatography is polar and contains water, the method is ideally suited to the separation of polar molecules which are either insoluble in organic solvents, or bind too strongly to solid adsorbents for normal elution; many samples of biological origin fall into this category. In addition, compounds of substantially different polarity may be separated in the same run using gradient elution, within a convenient time frame. Retention in reversed-phase chromatography is a function of solute hydrophobicity whereas selectivity results almost entirely from specific interactions between the solutes and the mobile phase [36].

Generally, the selectivity may be conveniently adjusted by changing the type of organic modifier in the mobile phase. For ionic or ionisable solutes, pH buffers which suppress ionisation, and ion-pairing reagents which form lipophilic complexes, are useful to promote transfer of the solutes into the stationary phase, and so prolong their retention times. These approaches form the basis of ion suppression and ion-pair chromatography, respectively.

Metal-ligand complexes and chiral reagents can be added to the mobile phases to achieve selectivity between optically active isomers, and to this end it is also becoming increasingly popular to bind chiral groups to the column surface itself. Reversed-phase chromatography is gaining attention as a method for separating large biological molecules such as proteins and carbohydrates [37-41], and nowadays, tailor-made packings are commercially available for the separation of a number of large biomolecules.

The mechanisms underlying retention in reversed phase systems have not been fully elucidated [42-45], but the solvophobic theory, discussed briefly below, provides a semi-quantitive explanation of solute retention. To generate a simple view of this theory, it must be assumed that solute retention occurs by adsorption of the solute to the stationary phase without further defining the nature of the stationary phase. The solvophobic theory assumes that mobile phases are highly structured owing to the tendency of water molecules to engage in hydrogen-bonding reactions, and that this structuring is perturbed by the presence of solute molecules. Due to the very high cohesive energy of the mobile phase, solute molecules are forced to partition into the stationary phase. If the solute contains polar groups then the dipolar or hydrogen-bonding interactions between the solute and mobile phase will oppose transfer into the stationary phase. The difference in interactive energies between non-polar and polar solutes with the mobile phase, and differences in hydrophobic solute molecular surface areas are responsible for the functional group selectivity observed in reversed phase chromatography. In practice, solute retention is often complicated by solute interactions, with residual silanols on the column surface, thus both solvophobic and silanophilic binding must be considered.

The solvophobic theory assumes a two-step process of solute solvation, and this is depicted in Figure 1.11. In the first step, a cavity of suitable size is created to accommodate the solute, and δG_C is the energy required to form this cavity. In the second step, the solute enters the cavity and interacts with the sorrounding solvent and the energy required for this process is given by δG_{INT}. The energy required to bring a solute from a hypothetical gas phase into the solvent is the sum of these two terms. The complete equation relating capacity factor and mobile phase composition is as follows

$$\ln k' = \theta + 1/RT[\,\delta A(N\gamma + a) + NA_s\gamma(X^e - 1) + W - \delta Z/\,] + \ln RT/P_oV \qquad 1.54$$

where

k' = solute capacity factor

θ = volume ratio of stationary and mobile phase

R = gas constant

T = temperature

δA = contact area

N = Avogadro's number

γ = Surface tension

W,a = solvent dependent constants arising from Van der Waals contribution to binding energy

V = mole volume of solvent

ϵ = dielectric constant

δZ = contribution of electrostatic interaction to binding

A_s = surface area of solvent molecule

X^e = factor which adjusts macroscopic surface tension to molecular dimensions

P_o = 1 atmosphere

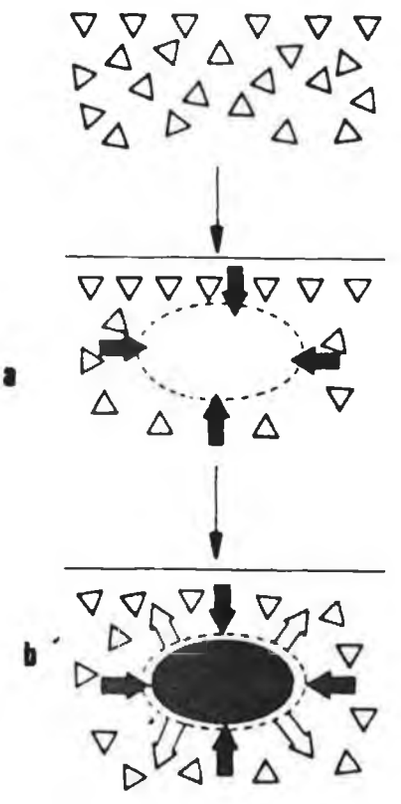
According to this equation the capacity factor decreases as surface tension decreases, and there is an approximately linear relationship between the natural logarithm of the capacity factor and the volume percentage of organic modifier in an aqueous mobile phase. It also predicts increased solute capacity factor for neutral solutes as salts are added to the mobile phase, and a reduction in retention for solute which ionise, in agreement with experimentally observed phenomena.

1.4.2.3.3. Ion suppession and ion pairing

Selectivity for polar and ionic molecules may be manipulated by secondary chemical equilibria. These equilibria are affected by changes in the mobile phase composition; by the addition of buffers and the adjustment of pH (ion suppression), and the addition of ion pair reagents (ion pairing).

Figure 1.11.

Two-step process of solute solvation according to the solvophobic theory



(a) Cavity formation

(b) Reduction in free volume interaction

As mentioned above, retention of solutes in reversed phase systems is adversely affected if these solutes are ionised under prevailing chromatographic conditions. By the addition of a suitable buffer and adjustment of pH it is possible to suppress ionisation by driving the equilibria of the following reactions in favour of the neutral species:

	Low pH		High pH
BASES:	$RNH_3^+ + OH^-$	$\rightleftharpoons$	$RNH_2 + H_2O$
ACIDS:	$RCOOH + H_2O$	$\rightleftharpoons$	$RCOO^- + H_3O^+$

In some cases, however, where the species of interest is strongly acidic ($pK_a < 3.0$), or even moderately basic ($pK_a > 8.0$) adjusting the pH to or beyond these values will compromise the integrity of a bonded silica-based column, as low pH promotes stripping of the bonded phase, and high pH promotes dissolution of the silica packing.

Substances of this type can be separated on ion-exchange columns, but an attractive alternative is the employment of the ion pair chromatographic technique, which is performed on existing high efficiency, small particle normal or reversed-phase packings. Ion pair chromatography has its origins in the use of ion pairing reagents for liquid extraction of ionic compounds in aqueous solution with an immiscible organic solvent. Superficially, it is thought that the basis for ion pair chromatograhy is the formation of a lipophilic complex by association of two ions of opposite charge, that is, the solute and an oppositely charged counter ion which is added to the mobile phase. Ion pair formation occurs in the aqueous phase, and the net result is that the ionic solute is transferred to the stationary phase. Three different models have been proposed to account for ion pair separation mechanisms:

(i) The ion pair model;
(ii) The dynamic ion exchange model;
(iii) The ion interaction model.

The ion pair model assumes that ion pair formation takes place in the mobile phase, and that this is followed by adsorption of the lipophilic complex into the stationary phase. This model describes quite adequately the situation in normal phase and coated partition systems where the stationary phase is behaving as a bulk liquid, but in bonded phase systems, where the stationary phase has a film thickness in the order of one monolayer, the dynamic ion exchange theory presents a more appropriate model. This hypothesis assumes that the ion pair regent is adsorbed onto the stationary phase where it behaves as a liquid ion exchanger, and that separation results from ionic interactions between the ionised solute and the "counterions" adsorbed onto the stationary phase.

The ion interaction model assumes that the the lipophilic ion pairing reagent is dynamically adsorbed onto the stationary phase forming an electrical double layer. Sample retention is assumed to result from coulombic attraction of the solute at the electric double layer due to the surface charge density provided by the charged counterion. This affinity is augmented by the attraction of the non-polar stationary phase for the lipophilic portion of the solute molecule. The net result is that a pair of ions (not necessarily an ion pair) are adsorbed onto the stationary phase.

The general technique of bonded reversed-phase ion pair chromatography is operationally more convenient than normal, or non-bonded phase, ion pairing methods. It lends itself well to gradient elution, and plate efficiencies are in no way inferior to those obtained using simpler chromatographic modes. Early examples of this form of ion pair chromatography were separation of members of the ephedrine class by Schill and co-workers [46] using 0.1M dioctyl hydrogen phosphate dissolved in chloroform as

stationary phase. Not long after this Knox and Laird [47] developed the technique known as "soap chromatography" whereby a reversed-phase bonded silica was used to extract a detergent from an aqueous eluent. They employed cetyltrimethylammonium bromide as the pairing ion, and showed that this was extracted to give what amounted to a monolayer on the surface of the bonded support, a result which was independently confirmed by Kissinger [48]. To select a counterion for a particular separation, the most important consideration is charge compatibilty. The counterion should ideally be univalent, aprotic, soluble in the mobile phase, non-destructive to the chromtographic system, and should not undergo secondary equilibria. The pH of the mobile phase is chosen so that both the counterion and the solute are ionised, and it is usually controlled by adding a buffer salt to the aqueous component. Hence the factors which influence retention and selectivity in ion pair chromatography are pH, the nature and concentration of both the buffer salt and ion pairing reagent, as well as the type and percentage organic modifier in the mobile phase. This confers on these systems an added degree of flexibility which can be useful in the separation of complex mixtures.

Charged surfactants at concentrations below the critical micelle concentration are widely used as mobile phase modifiers in ion pairing chromatography. Because of the amphiphilic nature of the surfactants, there exists some controversy regarding the exact mechanism of separation, but some fundamental considerations have been described by Horvath et al. [49]. Aqueous solutions of surfactants above their CMC, where micelles exist along with monomers, dimers and trimers are used as mobile phases in a technique known as "micellar liquid chromatography. This technique is discussed in detail in chapter 4.

4.3. Ion exchange chromatography

In ion exchange chromatography the chromatographic support contains ions which are capable of being exchanged with ionic solutes in the mobile phase. It has a long history of applications, including the analysis of amino acids [50,51], nucleic acid components [52], carbohydrates [53], and nucleotides [54]. Ion exchangers in HPLC normally use a bonded quaternary ammonium group $[-(CH_2)_nNR_3^+X^-]$ for the separation of anions, and a bonded sulphonic acid group $[C_6H_4SO_3^-H^+]$ for the separation of cations. In classical ion-exchange resins, the acidic or basic groups are bonded to a styrene-divinyl benzene co-polymer. The classical resins have the disadvantages that in HPLC their volumes change in different solvents due to swelling, they are compressible under high pressure, and their mass transfer characteristics are poor. The last defect arises from the fairly high degree of cross-linking required to enable them to withstand pressure. Only the smallest molecules can readily penetrate such resins, and even then, mass transfer is relatively slow because of the highly structured form of the aqueous phase within the pores of the resin.

An advance on the original homogeneous beads was made by Horvath, Preiss and Lipsky in 1967 [11] with the introduction of pellicular ion exchange materials where the resin is coated as a thin film on glass beads. They were followed by the "Zipax" ion exchangers in which a polymer was deposited as a porous layer on glass beads [55]. These materials are more rigid, i.e. are less compressible, and their mass transfer properties are somewhat better than homogeneous particles of the same size, as the solutes have a shorter distance to travel within the resin. The pellicular materials are, however, not as extensively used as the homogeneous bonded materials which are more efficient, and employ fully porous silica particles to which ion exchange groups are chemically bonded [28,56].

1.4.3.1. Theory of ion exchange chromatography

Ion exchange involves the displacement of a counter-ion, present in the eluent, by the solute ion. Considering the following equation, which would apply, for example to an anion exchange material:

$$RN^+Y^- + X^- \rightleftharpoons RN^+X^- + Y^- \qquad 1.55$$

where RN^+ represents an ion exchange site, Y^- is the associated counter ion, which is in the liquid phase. The counter ion can be displaced by solute ion X^- to give the ion pair RN^+X^-. When this displacement reaction is in equilibrium, the retention is controlled by the equilibrium constant K_{IE}, where

$$K_{IE} = [Y^-][RN^+X^-]/[X^-][RN^+Y^-] \qquad 1.56$$

Hence:

$$K_{IE}[RN^+Y^-]/[Y^-] = [RN^+X^-/[X^-] \qquad 1.57$$

$[RN^+X^-]$ represents the amount of solute in the stationary phase and $[X^-]$ the amount of solute in the mobile phase, the term $K_{IE}[RN^+Y^-]/[Y^-]$ equals the capacity factor k'.
Since the concentration of ion exchange sites $[RN^+Y^-]$ is constant and fixed by the structure of the matrix, k' is inversely proportional to the concentration the the counter ion in the eluent, $[Y^-]$. Hence increasing the concentration of counter ions in the in the mobile phase, either by increasing buffer concentration, or by the addition of a neutral salt, increases the number of ions with which the solute must compete for sites on the stationary phase, and this generally produces a decrease in solute retention. Changing the pH of the mobile phase alters the character of the stationary phase and the degree of dissociation the solute, and this facility is usually used to adjust the separation between molecules.

The operating range for a separation can be estimated from the pK_a values of the sample components. For an acid-base equilibrium:

$$HA + H_2O \rightleftharpoons A^- + H_3O^+ \qquad 1.58$$

solute pK_a and mobile phase pH values can be related by the Henderson-Hasselbach equation:

$$pH = pK_a + \log[HA]/[A^-] \qquad 1.59$$

Only ionised solutes are retained through an ion exchange mechanism, and as a general rule of thumb, this equation predicts that optimum buffer pH should be 1 or 2 pH units below the pK_a value of bases, and 1 or 2 units above the pK_a value of acids. When choosing a buffer salt, two criteria must be met. First, the buffer must be able to establish the operating pH for the separation, and second, the exchangeable buffer counterion must provide the required solvent (ionic) strength. Typical buffer concentrations are 0.001-0.05 M.

1.4.3.2. Application and Operation of Ion Exchange Chromatography

Ion exchange columns are generally less efficient than other column types used in HPLC. To improve diffusion and mass transfer these systems are often operated at elevated temperatures. In addition to improving efficiency, this measure also serves to reduce overall capacity factors. Small changes in column temperature frequently result in large alterations in separation selectivity, particularly for structurally dissimilar compound types.

Ion exchange separations are frequently operated at low mobile phase flow rates in order to maximise column efficiency. For most separations, the mobile phase is a wholly aqueous solution, but

water-miscible organic solvents may be incorporated to increase column efficiency and to control solvent strength. This measure is particularly valuable where retention is at least partially controlled by a reversed-phase mechanism, and in these cases, selectivity can be adjusted in the same way as in reversed-phase chromatography.

Interaction can take place between charged silanol groups on an unmodified silica surface and oppositely charged analytes. This interaction, which is thought to proceed largely via an ion exchange mechanism, is widely exploited in the separation of organic bases which show poor chromatographic characteristics on reversed-phase packings. The theory and application of this type of ion exchange chromatography is discussed in detail in chapter 5.

1.4.4. Exclusion chromatography

Size exclusion chromatography is a liquid chromatographic technique which separates molecules according to size. Ideally, no interaction occurs between the sample and support. The support simply acts as a porous matrix containing mobile phase, and separation is effected by the ability of the solute to gain access to the stagnant mobile phase via diffusion through the support pore network. Unlike in other HPLC methods, the mobile phase in size exclusion chromatography is not varied to control resolution; it is chosen to provide good solution properties for the sample, and to be of low viscosity in view of the low diffusion coefficients of macromolecules. All the sample components elute ahead of the column dead volume, V_M, which can be predicted in advance.

Size exclusion chromatography is principally used to separate samples of different molecular size, and is the preferred method for separating compounds of high molecular weight (MW> 2000). It is used to obtain information on molecular weight, and molecular

weight distribution for polymers. Size exclusion columns with pore diameters of 60 Å or 100 Å are now available and can be used to separate molecules with molecular weights below 1000 [57]. In size exclusion chromatography, separation results from the distribution of the sample between the mobile phase inside and outside the pores of the column packing: there is no direct interaction with the stationary phase. The terminology used in exclusion chromatography has a different meaning to most liquid chromatographic techniques.

1.4.4.1. Theory of exclusion chromatography

The void volume relates only to the the interstitial particle volume, and the term "capacity factor" is meaningless in this context. Retention in size exclusion chromatography is better described by the distribution coefficient K_D, which is related to experimental parameters by the equation

$$V_e = V_o + K_D \times V_i \qquad 1.60$$

where V_e = elution volume of the solute
V_o = column void volume (intraparticulate interstitial volume)
K_D = solute distribution coefficient
V_i = internal pore volume.

The solute distribution coefficient represents the ratio of average solute concentration in the pores of the stationary phase support to that outside the support. Thus, the stagnant mobile phase pool contained within the pores of the column packing may be regarded as the stationary phase. Solute concentration within the pores decreases with increasing molecular size. The distribution coefficient is limited to values between 0 and 1, representing the extremes of complete exclusion and complete permeability to the pore volume by the solute. The normal way of

plotting exclusion data is in the form of a semi-logarithmic plot relating molecular weight to V_e, and a typical such curve is presented in Figure 1.12. Such curves have a fairly sharp exclusion limit at the high molecular weight end, a more or less linear portion, and a gradual curve away from the linear towards the region of total permeation. The extent of the linear region generally covers about 1.5 orders of magnitude of molecular weight, and outside this portion, the separation properties of the packing are poor. To separate two components of different molecular size, a column packing for which the two components elute in the middle of the fractionation range should be selected. All other things being equal,the column with the smallest gradient in the fractionation range will provide the highest resolution.

The conceptual idea of a theoretical plate can be used in exclusion chromatography to measure column efficiency and compare performance of packed columns. It is usually measured using small molecules, for example toluene, acetone, or benzyl alcohol, which can explore all the pores of the packing, and thus have K_D values of unity. In size exclusion chromatography the HETP value is due almost entirely to the contributions from stagnant mobile phase dispersion and interparticulate mobile phase mass transfer. Contributions from longitudinal diffusion are insignificant since the large polymeric molecules encountered in this type of chromatography have small diffusion coefficients.

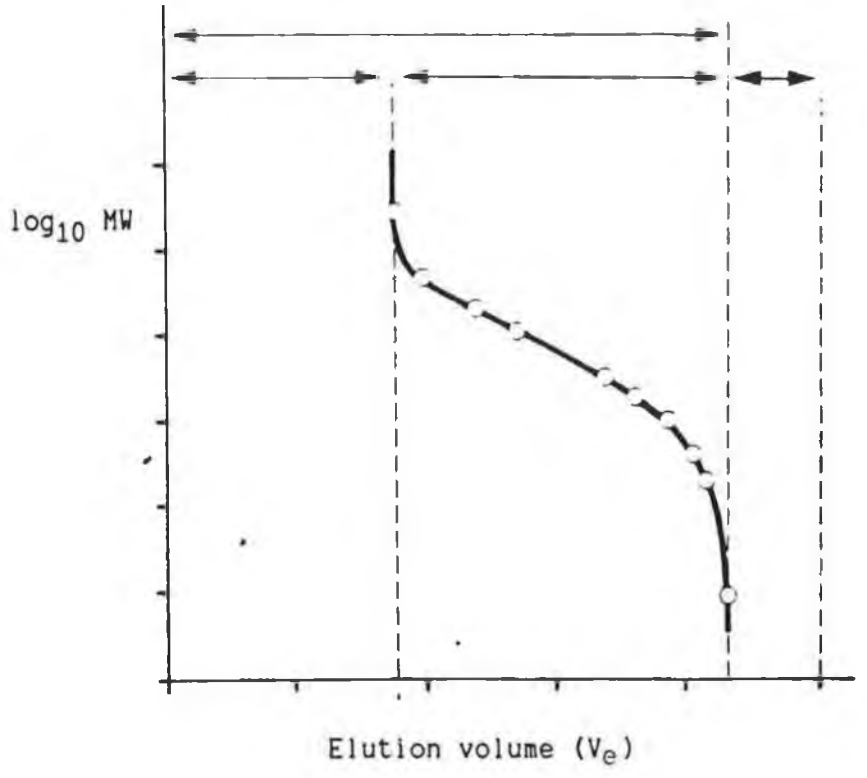
Peak-to-peak resolution in size exclusion chromatography may be defined in terms of the specific resolution factor which relates peak resolution to sample molecular weight [58]. It is assumed that all measurements are made in the linear portion of the molecular weight calibration curve. In this case:

$$R_{sp} = R_s / \log(M_1/M_2) \qquad 1.61$$

where R_{sp} = specific resolution factor

Figure 1.12.

Example of a calibration curve for microparticulate silica gel showing the relationship between molecular weight (MW) and elution volume (V_e)



R_s = chromatographic resolution

M_1/M_2 = molecular weight ratio of two standards.

Assuming a resolution of unity, i.e. $R_s = 1$, then the ratio M_1/M_2 can be defined as the minimum molecular weight ratio R_m:

$$R_{sp} = 1/\log R_m \qquad 1.62$$

R_m is a useful parameter in comparing column performance, and can be used to relate resolution to the basic properties of the chromatographic system:

$$\log R_m = (4mV_e)/(V_i(\sqrt{N})) \qquad 1.63$$

where

m = slope of the linear region of MW calibration curve

V_e = elution volume of standard M_1

V_i = pore volume

N = number of theoretical plates.

This equation indicates that in order to obtain increased resolving power, the column efficiency and the internal pore volume of the packing must be maximised, and the slope of the calibration curve minimised.

1.4.4.2. Application and operation of exclusion chromatography

The principal areas of application for size exclusion chromatography are gel filtration chromatography and gel permeation chromatography. The former technique is used for the separation of water-soluble macromolecules, often of biochemical origin. The following information may be obtained from such experiments:

(i) molecular fractions for characterisation for further use;
(ii) molecular weight determination using calibration standards;
(iii) estimation of molecular association constants.

Gel permeation chromatography is normally used as an analytical technique for separating samples soluble in organic solvents. The following information may be obtained from such experiments:

(i) small molecules may be separated by differences in size;
(ii) calculation of molecular weight averages, or the molecular weight distribution of polymers;
(iii) preparation of molecular weight fractions for further use.

The two predominant types of packing material used in exclusion chromatography are cross-linked polystyrenes and inorganic packings based on silica gel or glass. The silicas normally used for adsorption chromatography can also be used for size exclusion systems. Adsorption processes on these silicas must be curbed, and this can be attained by judicious choice of eluent, or by chemically bonding functional groups in more extreme cases.

Cross-linked polystyrene cannot normally be used with aqueous mobile phases and as a result, current emphasis is shifting towards the use of inorganic packings. The polystyrene materials do, however, have the advantage that they can be used to separate small molecules, and for this reason they are still important.

The choice of packing depends on the size of the molecules to be separated and the compatibility of the packing for the chosen mobile phase. In order to obtain a molecular weight distribution of polymeric substances, a calibration must be carried out, which is preferably done with characterised samples of the same polymer. Detection is usually based on the refractive index of the solutes, as many polymers do not absorb in the UV, and although refractive index detection has low sensitivity, it is usually adequate in exclusion chromatography, as minimal dilution

of the sample occurs. The characterisation of polymers by size exclusion chromatography requires a high degree of control of the mobile phase flow rate [59], as the working volume is usually very small in relation to the molecular weight range of samples undergoing separation. In addition efficiency generally decreases with increasing flow rate, although the quantitative relationship has not been established and may be a function of the nature and type of packing material [60].

Size exclusion chromatography has become a rapid technique for the determination of molecular weight distribution of polymers and can be applied to the separation of a large variety of other chemical compounds. With the advent of chemically and mechanically stable silicas for size exclusion purposes separation on the basis of size will join the ranks of the accepted chromatographic techniques for the analysis of any complex mixture.

1.5. INSTRUMENTATION IN HPLC

The equipment used in modern HPLC is very different from the simple gravity-fed devices which dominated the practice of liquid chromatography for most of this century. Theory and practice have indicated that pressures well above atmospheric pressure are required to operate high efficiency columns, packed with particles of small diameters, at flow rates of a few millilitres per minute. A block diagram of a suitable instrument for HPLC is shown in Figure 1.13. The essential features of this assembly which will be discussed here are the pump, the injector, the column and the detector.

1.5.1. High pressure pumps

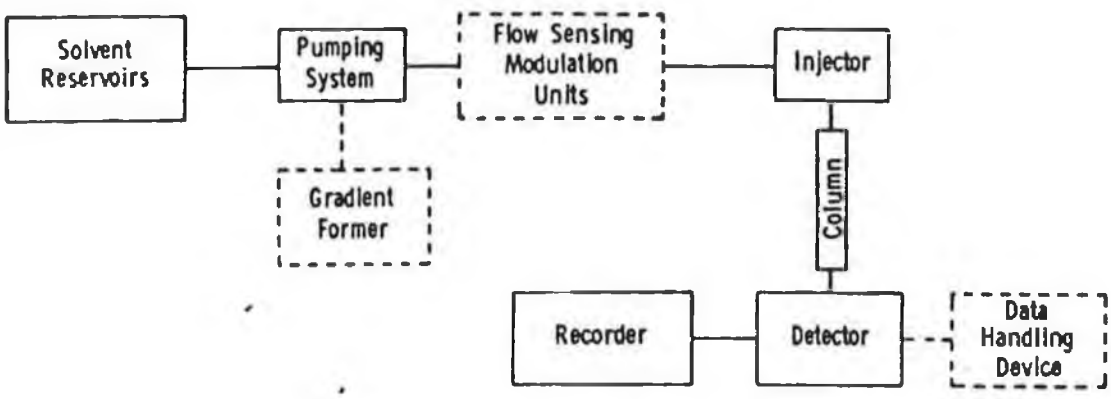
The pump is one of the most important components of the liquid chromatograph since its performance directly affects retention time reproducibility and detector sensitivity. In modern HPLC, the pumping system must meet certain general requirements. For analytical and semi-preparative applications the pump should be able to provide flow rates of 0.5 to 10.0 ml/min at pressures reaching 5000 psi. A high degree of accuracy in pump resetability and flow rate control, with a minimum of pump pulsation and drift are the hallmarks of a good pump. Other practical considerations are resistance to corrosion from a wide range of solvents, serviceability, ease of operation and time required for solvent changeover.

The types of pumps used in HPLC instruments can be divided into two categories: (i): CONSTANT PRESSURE PUMPS, e.g, gas displacement pumps and pneumatic amplifier pumps.

(ii): CONSTANT VOLUME PUMPS, e.g, syringe pumps reciprocating pumps

Figure 1.13.

Block diagram of a typical high pressure chromatograph. The dotted lines refer to optional components



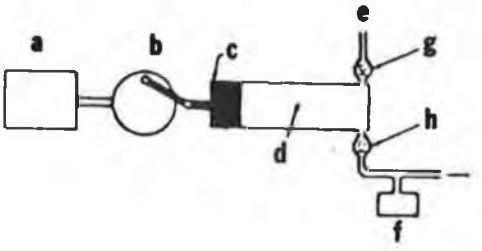
A diagramatic representation of the reciprocating type pump is shown in Figure 1.14. This is the most commonly used model in HPLC, and only it will be discussed here. The cheaper single-piston version uses an eccentric cam to drive a piston in and out of a low volume chamber (30-1000 microlitres). The pistons movement is synchronised with the operation of inlet and outlet check valves, which between them, control the direction of fluid flow during the fill amd pump sequences. The flow output is determined by the length or frequency of piston strokes. The flow output is delivered as a series of pulses which must be damped to prevent interference with detector operation. Pulse dampeners work in a manner analagous to that of a capacitor in an electrical circuit. They store energy during the pressurising stroke, and release it during the refill stroke [61]. Pulse damping has been achieved using a variety of mechanical devices such as syringes, bellows and coiled tubes, frequently in conjunction with a fluid ballast reservoir. A commercially available pulse dampener uses a flattened length of teflon tubing immersed in a degassed compressible liquid. The flexibility of the tubing and the compressibility of the gas thus absorb any pressure fluctuations in the system.

Dual-and triple-headed reciprocating pumps may be operated without a pulse dampener. With a dual-head reciprocating pump, the two pistons are driven by the same motor via a common cam such that they are 180^{o} out of phase. As one chamber is pumping, the other is refilling so that the two profiles overlap, leading to partial cancellation of the peaks and troughs in the total flow output. Likewise, with a triple-head pump the three pistons are 120^{o} out of phase and the degree of pulse reduction is accordingly greater.

Reciprocating pumps deliver a constant flow at a fixed back pressure. At high back pressures some minor flow variability may arise due to compressibility of the mobile phase. Reciprocating pumps can provide continuous solvent delivery, fast solvent

Figure 1.14.

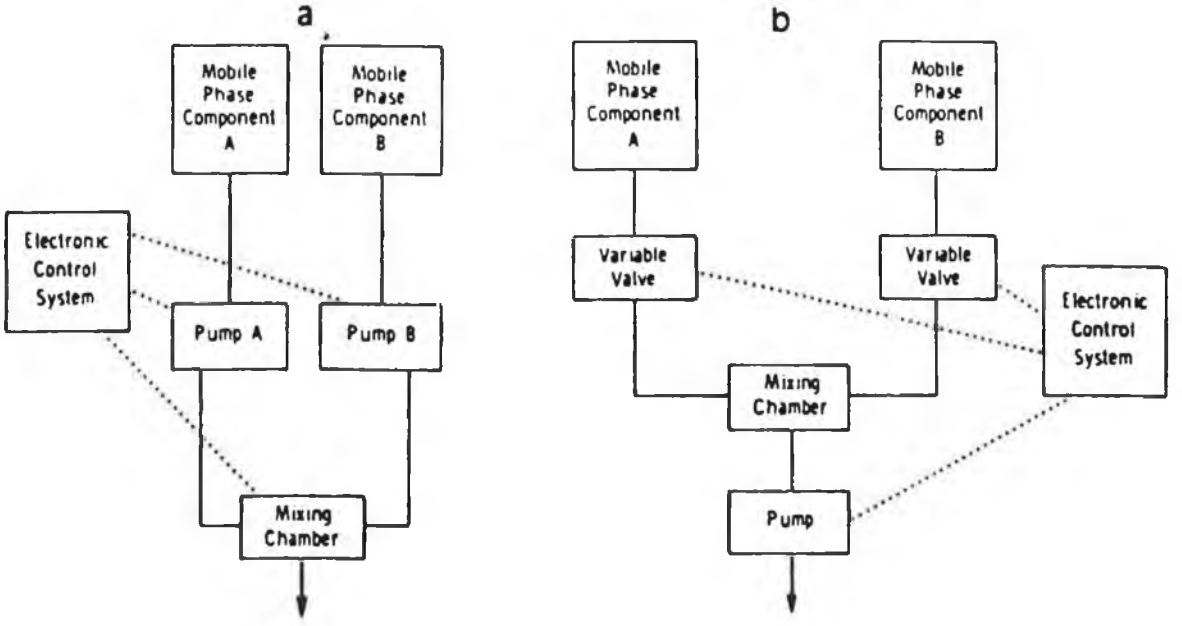
Reciprocating type pump for high pressure liquid chromatography



(a): Motor, (b): Cam, (c): Piston, (d): Chamber, (e): to Solvent resevoir, (f): Pulse Dampner, (g): Inlet check valve, (h): Outlet check valve

Figure 1.15.

Two methods for generating binary solvent gradients



(a): mixing on high pressure side.

(b): mixing on low pressure side.

changeover, gradient elution compatibility, and have low maintenance requirements.

1.5.1.1. Gradient elution

Samples having a wide range of capacity factor are not conveniently separated under isocratic conditions, and gradient elution is frequently employed in these cases. A mobile phase gradient is formed by mixing two or more solvents according to some pre-determined gradient ramp. The most commonly used gradients involve a binary mixture with a linear, concave or convex increase in the percent volume fraction of the stronger solvent. The gradient shape mentioned above can be described by simple mathematical functions [62].

Gradient devices are usually classified according to whether the solvents are mixed in the high pressure side (Figure 1.15.a) or in the low pressure side (Figure 1.15.b) of the pump. Modern instruments use time-proportioning electrovalves, controlled by a microprocessor to control solvent delivery to the pump [63,64]. The low pressure arrangement is little influenced by solvent compressibility effects and can eliminate errors associated with thermodynamic volume changes due to mixing of the solvents. The high pressure arrangement necessitates two pumps which deliver the solvents separately to the mixing chamber. Solvent compressibility and thermodynamic volume changes on mixing can influence the accuracy of the mix ultimately delivered to the column. A significant disadvantage of this arrangement is the need for two pumps.

1.5.2. Injection devices

The ideal sample introduction method should reproducibly and conveniently insert a wide range of sample volumes into the column as a sharp "plug" without adversely affecting the efficiency of the column. These goals are met to varying extents by the following injector types:

(i) Septum
(ii) Septumless
(iii) Stop-flow
(iv) Valves

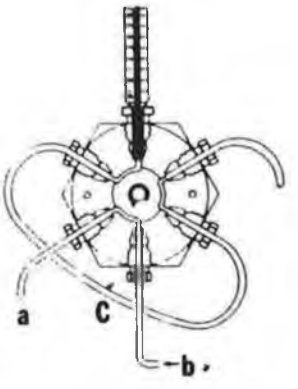
Microvolume sampling valves (Figure 1.16) are the most widely used injection devices in HPLC, and only their operation will be described here. Valve injectors permit reproducible sample introduction without significant flow interruptions. The sample is loaded at atmospheric pressure into a groove in the valve core, or more commonly via an external loop, and the sample is introduced onto the column by short rotation of the valve. The volume of sample injected is varied by changing the external loop, or if a very large loop (up to 2ml) is used, by introducing the volume of sample required using an accurately calibrated syringe. Where the volume injected is determined by the loop size, this method of sample introduction is virtually operator independent and very reproducible with injection errors of less than 2%. Column efficiency is, however, slightly degraded by the method of coupling the valve to the column [65]. This degredation can be minimised if the valve-to-column tubing is short, is of narrow internal diameter(< 0.5 mm), and if low dead-volume fittings are used.

1.5.3. Detectors in HPLC

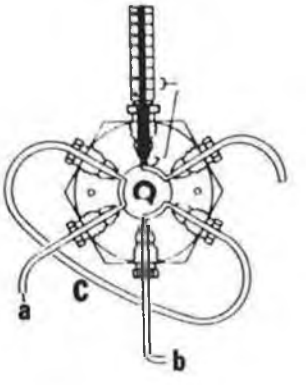
Separations in liquid chromatography occur in a dynamic manner and therefore require detection systems which work on-line to produce an instantaneous record of the column events. There is no equivalent in HPLC to the universal, sensitive flame ionisation detector used in gas chromatography, and a variety of detectors are necessary to cover the wide range of analytical situations to which HPLC is applied. Broadly speaking, the detectors used in liquid chromatography may be classed as bulk property or solute property detectors.

Figure 1.16.

Valve injector for sample introduction



Load position



Inject position

(a): to Column

(b): Mobile phase inlet

(c): Sample loop

Refractive index and conductivity detectors are examples of bulk property detectors. They measure the difference in some physical property of the mobile phase containing solute, as opposed to the solute-free mobile phase. These detectors have fairly universal application, but have poor sensitivity and a limited dynamic range. Bulk property detectors are adversely affected by small changes in mobile phase composition and temperature, which precludes their use in flow programming or gradient elution.

Solute property detectors which include, fluorescence, spectrophotometric and electrochemical detectors, are the most widely used detection schemes in liquid chromatography. They respond to some inherent physical or chemical property of the solute, which is, ideally, independent of the mobile phase. Although this criterion is in reality rarely met, the signal discrimination is usually sufficient to permit flow programming and gradient elution, and to provide high sensitivity with a wide linear response range. These detection schemes will now be discussed in further detail, with more emphasis bing given to ultraviolet absorbance detection which was used throughout the course of this work.

1.5.3.1. Spectrophotometric detection

UV-visible spectrophotometric detectors have been a feature of HPLC instrumentation since its inception and remain the most widely used of the HPLC detectors. Since many organic molecules possess some absorption in the UV region, this detection mode has fairly universal application, though sensitivity is determined by how strongly the solute absorbs in the UV, what its wavelength of maximum absorbance is and the availability of a transparent mobile phase at the chosen operating wavelength. The latter feature is particulary important at the lower wavelengths since many common organic solvents absorb to some extent in the low UV region. The operation of spectrophotometric detectors is based on the measurement of the absorbance of monochromatic

light by the solute in accordance with the Beer-Lambert law:

$$A = \log I_o/I = \varepsilon cl \qquad 1.64$$

where

A = Absorbance

I_o = the intensity of light that would emerge from the cell if it contained a completely transparent liquid

I = the intensity of light folowing absorbance by a solute

ε = molar extinction coefficient

c = concentration in mol/dm^3

Very often the optical absorbing power of a substance in solution is quoted not in terms of its molar extinction co-efficient, ε, but in terms of its A1%(1cm) value. This is the absorbance A of a 1% w/v solution of the solute when measured with an optical path length of 1 cm. The A1%(1cm) value and molar extinction coefficient are related by the equation:

$$A1\%(1cm) = 10\ /MW \qquad 1.65$$

where MW is the molecular weight of the solute. The first commercial flow photometers all used low-pressure mercury arc sources, since such lamps have a long lifetime and give a highly intense and stable output of the resonance radiation at the 254 nm wavelength. This line accounts for about 90% of emmision from the mercury arc source and only the simplest optical filter is required to isolate it. Obviously, these detectors are limited in their application to samples which have at least some absorbance (depending on sensitivity requirements) at 254 nm. These detectors are, however, simple, rugged and inexpensive, and with a highly regulated source and dual beam operation, a very high signal-to-noise ratio can be achieved.

The original mercury lamp was then replaced by a more intense deuterium lamp giving a continuous output from 190-400 nm (or a tungsten filament lamp with an output from 400-700 nm) and a manually adjusted grating which allowed selection of the detection wavelength to match the absorption maximum of the sample. Nowadays, the most generally used detector is the continuously variable multiple wavelength detector which might contain both a deuterium and a tungsten lamp, permitting wavelength selection between 190-700 nm. Wavelengths are selected manually, or in an automated version, the detector may be programmed to change wavelengths during separation. These detectors use high energy sources and stable low noise electronics. Multiple wavelength detectors produce a signal-to-noise ratio which is slightly lower than the fixed wavelength detectors, though in general, deuterium lamps have a sufficiently stable output that the best single-beam instruments have noise levels which are only marginally higher than those of the fixed-wavelength photometers. An example of a multiple wavelength detector is shown in Figure 1.17. Since the light intensity transmitted is proportional to the band width of the radiation and the Beer-Lambert law applies to monochromatic light, a compromise bandwidth of 2-5 nm is usually used.

Careful consideration must be given to the design of the detector cell, as it forms an integral part of both the chromatographic and and the optical systems. The detector cell volume should be as small as possible to reduce extra-column band broadening, a requirement which is becoming more important as advances in column technology are leading towards the use of smaller, more efficient and cost-effective columns. The classical Z-shaped cell which is commonly used is shown in Figure 1.18.a, and a variant on this was the split-flow model introduced by Kirkland [66] which was designed to remove flow-derived disturbances which can produce noise and drift.

Figure 1.17.

Optical diagram of multiple wavelength spectrophotometric detector

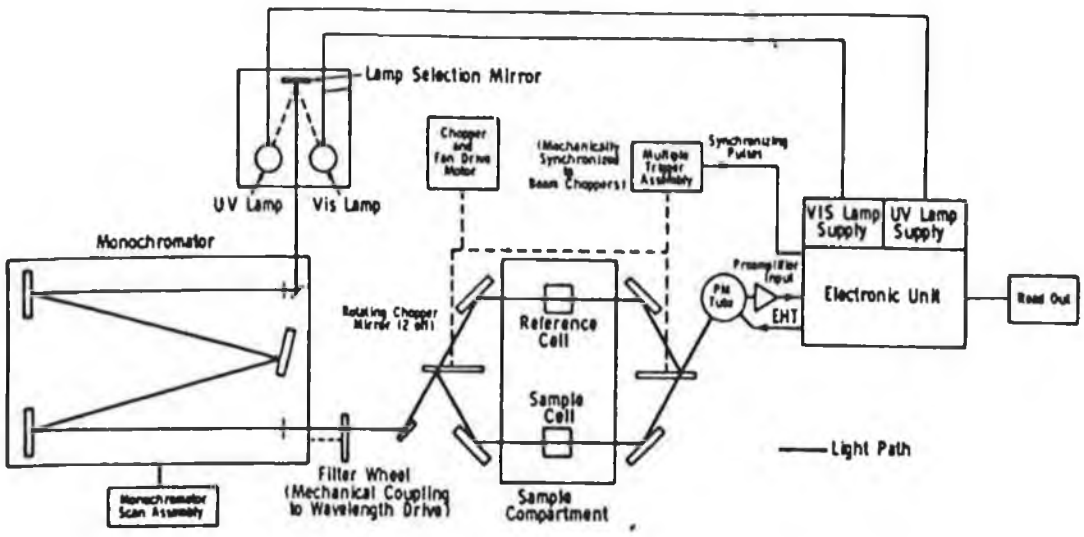
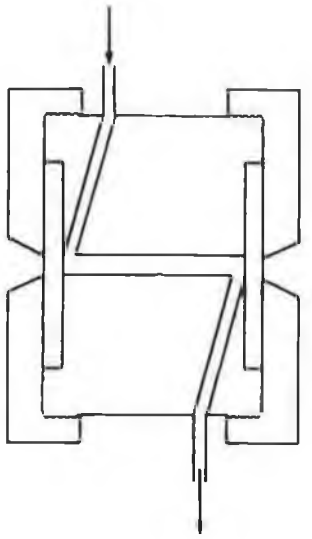
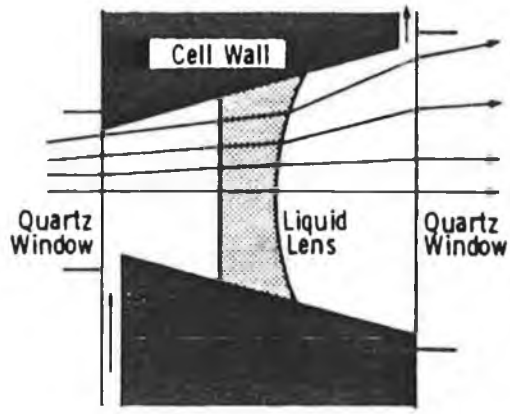


Figure 1.18.

Some types of flow cells used in UV absorbance detection



(a): Z-type cell



(b): Tapered cell

A major source of flow disturbance is non-homogeneous refraction throughout the cell. This arises from temperature gradients, incomplete mixing and turbulence in the mobile phase, which cause some of the incident light to be refracted against the cell walls where it is not detected, and therefore mistakenly taken to represent sample absorption. When a sample of different refractive index enters the cell, a refractive index gradient is created across the bore, and this gives rise to a lens of moving liquid within the cell, which is known as the "liquid lens" effect. These refractive index phenomena are responsible for fluctuating baselines and differential peaks near the solvent front. The tapered cell developed by Little and Fallick [67] and shown in Figure 1.18.b, is designed so that no light is sufficiently refracted to reach the cell wall, and the liquid lens is not created. Flow effects may also be minimised by controlling the temperature of the eluent and the flow-cell, and this may be achieved by housing the column, tubing and the flow cell in a thermostatted enclosure.

With a variable wavelength detector the column flow can be stopped, the solute arrested in the flow cell and scanned manually or automatically to produce an absorbance spectrum in a technique known as stop-flow scanning [68,69]. This exercise can yield information on the optimun wavelength for absorbance by an individual solute, in addition to providing characteristic wavelength ratios for qualitative analysis. Obtaining a single spectrum by stop-flow scanning is quite tedious and time consuming, and stopping the eluent flow requires re-equilibration of the column at the operating flow rate before the next analysis can proceed. A more recent innovation which can provide the same kind of data as stop-flow chromatography, is the photodiode array detector. In this instrument, a beam of white light is passed through the flow cell, and the emergent light is diffracted by a grating so that it falls on an array of diodes, each diode receiving light of a different wavelength. In this way a complete spectrum can be obtained instantaneously for each of

the peaks as they elute off the column. This information is usually stored in a data handling system and it may be recalled and analysed at a later point in time. The diode array spectrophotometer can be used for quantitative analysis even when the peaks are only partially resolved; their sensitivity and noise level are only slightly inferior to those of the more conventional spectrophotometers. The linear photodiode array detector is now commercially available and has, in recent years, become a popular and familiar feature in the analytical laboratory.

1.5.3.2. Fluorescence detection

Many biologically active compounds and pharmaceutical products possess native fluorescence, a fact which has contributed to the popularity of fluorescence detection in the field of biopharmaceutical analysis. In addition, derivatisation to produce fluorescent products is readily achieved pre- or post-column, and the benefit gain in terms of increased sensitivity is frequently worth the compromise in terms of speed of analysis.

When a molecule absorbs a quantum of light, an electron is promoted from the lowest vibrational level of the ground state to higher energy electronic states in accordance with the rules of quantum mechanics. Following this absorption of energy, the molecule relaxes to the lowest vibrational level of the excited electronic state by radiationless routes, from which point a transition occurs to the ground electronic state with energy emitted in the form of light. The wavelength of emitted fluorescence is always longer than that of the absorbed light, and it is therefore possible to irradiate a solute with light which it absorbs strongly (usually its maximum in the UV region) and observe the fluorescent emission through a filter which cuts out the irradiating light, and so creates a dark background. This fundamental property makes fluorescence a very attractive HPLC

detection system, for whereas UV absorbance detection depends on the difference between a full and slightly attenuated light beam, fluorescence measurement starts in principle from a point of zero intensity. Thus, fluorescence detection is 100 fold more sensitive than UV absorption [22].

The selectivity associated with fluorescence detection arises because two wavelengths are used in the measuring process, and from the fact that certain structural features are required for fluorescence to occur. For dilute solutions, the measured fluorescence intensity can be related to sample concentration by the equation

$$I_f = I_o \times \theta_f \times (2.3 \quad \varepsilon cl) \qquad 1.66$$

where

I_f = fluorescence emission intensity
I_o = excitation beam intensity
ε = molar extinction co-efficient
l = path length
c = sample concentration
θ = quantum efficiency
= number of photons emitted / number of photons absorbed

For fluorescence detectors used in liquid chromatography, the above equation is generally linear over a concentration range of 2-3 orders of magnitude. Sensitivity depends on the instrument (I_o and the reduction of scattered and stray light), the sample (quantum efficiency), and the composititon of the mobile phase (solvents, impurities etc).

The optical diagram of a filter fluorimeter is show in Figure 1.19. The measuring optics are arranged at a 90^o angle to the irradiating beam in order to isolate the emmision beam from the excitation beam. The filter is also used for this purpose. As the

Figure 1.19.

Optical diagram of fluorescence detector

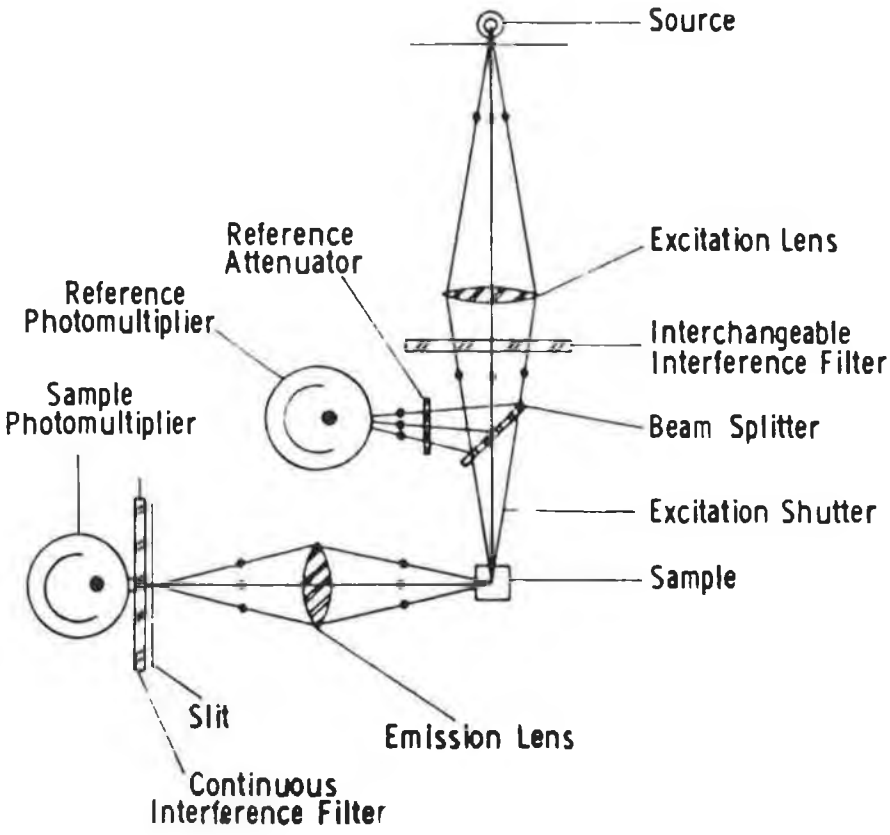
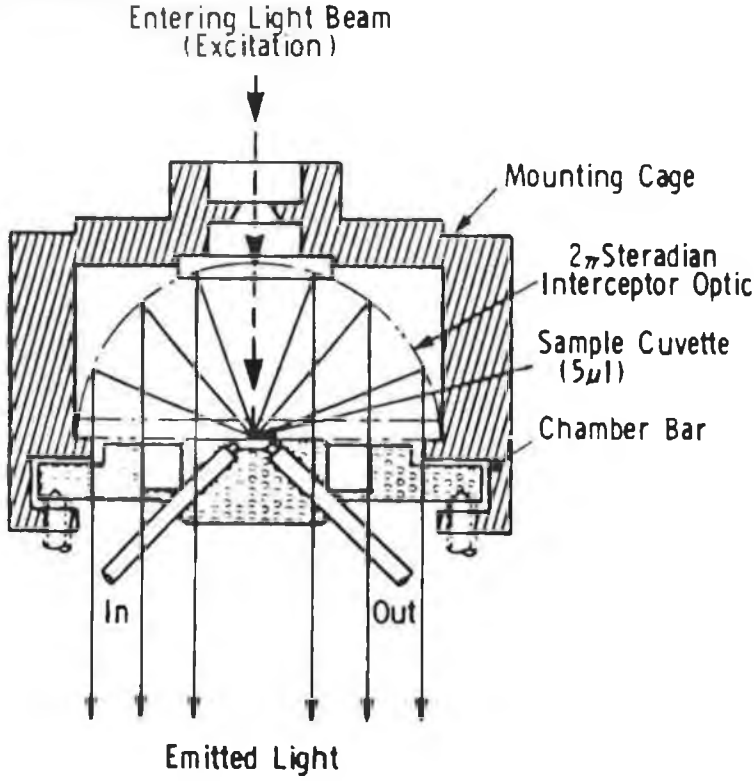


Figure 1.20.

2π sterdian cuvette



signal intensity is directly proportional to the source intensity, high energy line or continuous (tungsten, deuterium, xenon) sources are used. Mercury sources produce a series of intense UV line spectra superimposed on a weak continuum, thus only certain wavelengths are available for excitation, and these may not coincide with the maximum excitation wavelength of the sample. Continuous arc sources are used for continuously variable selection of the operating emission signal. Examples of these arc sources are deuterium (190-400nm), tungsten (400-700nm) and xenon (259-850nm) lamps. As arc sources tend to be unstable, the excitation beam is split between sample and reference beams which are electronically coupled to compensate for fluctuations in source intensity.
Recently lasers have received much attention as excitation sources in fluorimetric detection [70]. Lasers are a more powerful source of incident light, and their inherent spatial coherence and monochromaticity permits all the radiation to be efficiently utilised in a small detection volume. Wavelength isolation is achieved using cut-off of interference filters, or a monochromator diffraction grating. Cut-off filters lack selectivity since they transmit all light above a certain wavelength. Interference filters and monochromators provide narrow bandpasses, though at somewhat lower signal intensity.

Flow fluorimeters have been available since the earliest days of commercial HPLC, but only recently have the problems of flow cell design been adequately resolved. A unique cell which uses a 2π sterdian sample cuvette to maximise the intensity of the emission signal is shown in Figure 1.20. Sample fluorescence is generated in all directions simultaneously and the high light scattering power and low cell volume (3-5 μl) combine to make this design well-suited to modern, high efficiency HPLC columns. The fluorescence signal from a sample may be dramatically affected in both wavelength and emission intensity by the mobile phase composition, and even by the presence of contaminants in the mobile phase. Some of the solvent effects are summarised below.

Under less than ideal conditions, the constancy with which the pump mixes and delivers the mobile phase and the presence of contaminants in the mobile phase may influence detector sensitivity more than fluctuations in the detector operating system. Fluorescence detection can be used with gradient elution, and unless the mobile phase contains as high level of fluorescent impurities, the detector baseline changes very little during the solvent programme.

The types of problems which can develop in fluorescence arise as a result of

(i): too high sample concentration (non-linear responses due to self-absorption by the sample itself or complete absorption of the beam before it reaches the cell centre);
(ii): quenching of the signal from low levels of sample by impurities (especially oxygen) in the mobile phase;
(iii): solute-solvent interactions where a shift in fluorescence spectrum is observed as the dielectric constant of the mobile phase is increased-sensitivity is reduced if the solvent absorbs any of the excitation or emission energy;
(iv): photodecomosition induced by high intensity energy sources which depends on the residence time of the sample in the flow cell; and
(v): fluctuations in the rate of mobile phase delivery by the pump will adversely affect detector sensitivity and reproducibility.

Fluorescence detection is particularly useful when used in conjunction with pre- or post-column derivatisation techniques for the trace analysis of non-fluorescing compounds. Amines, amino acids and phenolic compounds can be detected with high specificity and sensitivity as their fluorescent dansyl derivatives or by post-column reaction with ortho-phthaldialdehyde. The latter reagent produces strongly

fluorescent derivatives with amino acids and biogenic amines in alkaline media which contain a reducing agent. Several anologues of dansyl chloride are also in use; dansyl hydrazine is a selective agent for the analysis of primary amines. Fluorescamine is frequently used for the detection of amines, amino acids and peptides after ion-exchange chromatography.

1.5.3.3. Electrochemical detection

Electrochemical detection is based on the electrolytic properties of solutes, and accordingly they are limited to use with electrically conducting electrolyzable species. A range of therapeutically important species do exhibit these properties and lend themselves well to this detection mode. Examples of compounds which can be conveniently detected in this way are the benzodiazepines, the tricyclic anti-depressants, antineoplastic agents such as cisplatin and mitomycin C, antibiotics such as adriamycin, and cardioactive agents such as tiomolol and pindolol. Selectivity in electrochemistry is achieved by adjusting the applied potential in manner analagous to wavelength selection in UV absorbance detection. This enables one electroactive species to be detected in the presence of another without interference. Electrochemical detection is a useful alternative where the species to be detected possesses no chromophore. Other advantages of electrochemical detectors are their reliability, simple low volume cell design (0.1-5 μl), and high sensitivity; under favourable circumstances, picogramme detection limits may be achieved.

Electrochemical detection requires the use of aqueous or hydro-organic mobile phase which are conducting and contain inorganic salts. These are eluents which are found in reversed-phase and ion-exchange chromatography but which are incompatible with other techniques. Detector operation is critically dependent on the mobile phase: flow rate constancy, pH, temperature, ionic

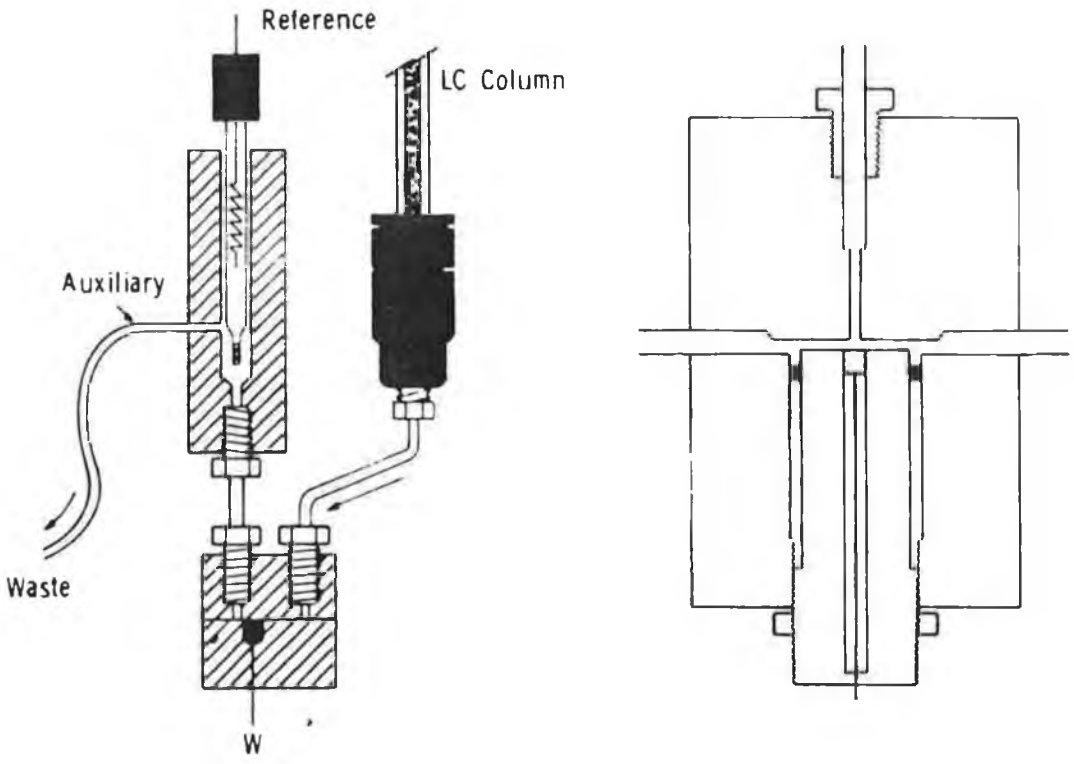
strength and the presence of electroactive impurities, especially dissolved oxygen. It is also related to cell geometry, and sensitivity is adversely affected by contamination of the electrode surface and oscillations in pump output. Since the performance characteristics of the electrochemical detector is profoundly influenced by the composition and flow rate of the mobile phase, solvent programming methods are not normally possible.

Electrochemical detectors include the amperometric, coulometric, and polarographic detectors for the determination of compounds which can be electolytically oxidised or reduced at a working electrode [71-74], and the conductivity detector for the determination of ionic species.

Amperometric detectors are based on thin-layer or tubular electrode cells. The former are more popular as small cell volumes and a variety of electrode materials (such as carbon paste or glassy carbon) can be used [74]. Two examples of thin-layer electrochemical cells are shown in Figure 1.21. The column eluent is introduced either paralled to the electrode embedded in the channel wall as in Figure 1.21.a., or perpendicular to the electrode surface followed by radial dispersion: the so-called wall-jet detector. The wall-jet detector has high sensitivity, can be adjusted to provide various cell volumes and is relatively free from surface adsorption problems. The reference electrode is maintained at a constant potential relative to the working electrode, and the chromatogram is recorded by measuring the detector cell current at a fixed applied potential as the sample is eluted off the column. As long as the mobile phase velocity and composition do not change the background signal will remain constant and be subtracted from the analytical signal. In the amperometric mode, electrolysis is not completed, (typically 1-10%) but if the electrode surface area is increased (for example in the tubular design) almost complete reaction can

Figure 1.21.

Thin-layer electrochemical amperometric detectors



(a): Thin-layer detector (b): Wall-jet detector

occur, and the system is now said to be operating in the coulometric mode. The coulometric detector is insensitive to flow rate and temperature changes, but is more prone to electrode contamination, is more difficult to design, and requires strict potential control over the entire working electrode surface.

Microvolume dropping mercury polarographic detectors can be used in HPLC for the determination of electro-reducible species. Surface contamination is rarely a problem as the electrode surface is continually being renewed. The disadvantages of polarographic detection include high background currents due to current oscillations over the lifetime of the drop, turbulence caused by liquid flow in the region of the drop, and the need for complex cell designs. Recent innovations in cell design and electronic damping of the signal have done much to improve the polarographic detector, though its general use is less widespread than the amperometric detector.

Conductance is a fundamental property of ions in solution and exhibits a simple dependence on ion concentration. The measurement of conductance is thus an obvious choice in the continuous selective monitoring of ions in the column effluent. A typical detector cell consists of a low-volume cavity or tube of insulating material, in which electrodes, made of a noble metal or graphite, are embedded. Generally, a constant alternating voltage is applied to the electrodes and the resistance is measured by a simple Wheatstone bridge arrangement. The cell resistance is related to sample concentration by Ohms law. Conductivity detectors are simple to operate, and in the absence of background electrolyte provide high sensitivity (10^{-8} -10^{-9} g/ml). Conducting impurities in the mobile phase limit absolute sensitivity.

1.5.4. The column in chromatography

1.5.4.1. Column selection

To design a chromatographic method using HPLC, the first decision to be made is the selection of the most appropriate column type. The analyst requires some information about the physico-chemical properties of the sample, in addition to the nature of the information desired for the separation. The decision as to which method to try first could be based on the relative solubility of the sample in polar and non-polar solvents, its molecular weight, or even past experience with different separation methods. An aid to the selection of the first-choice chromatographic method for the various sample types is shown in the flow diagram in Figure 1.22. This selection is based firstly on the molecular weight of the sample, its solubility in polar and non-polar solvents, and on whether or not it is ionic.

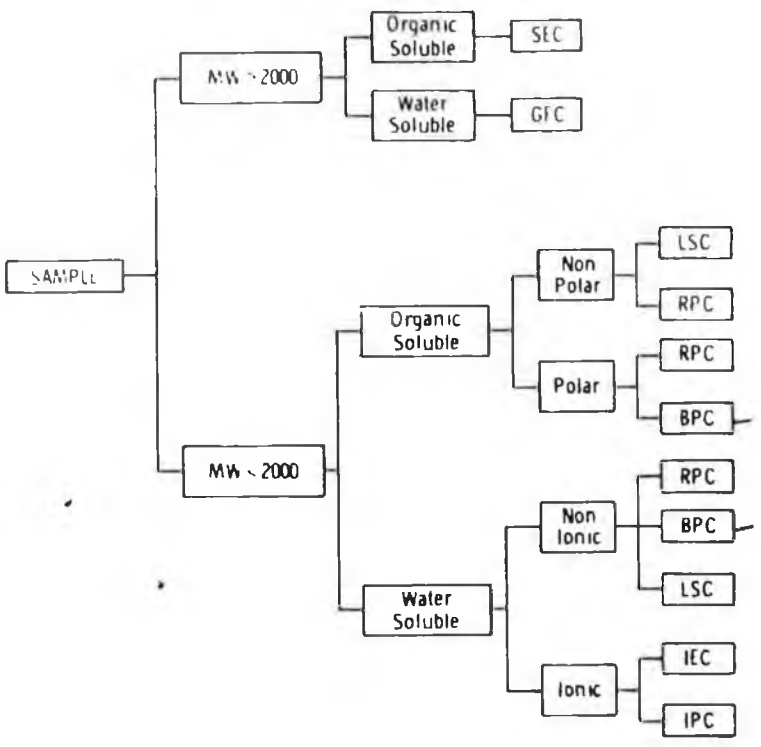
1.5.4.2. Column packings

The high performance packings used in modern liquid chromatography are comprised of small, rigid particles having a narrow particle size distribution. There are basically three different types of packing materials available which are shown in Figure 1.23: superficially porous particles, large totally porous particles, and very small totally porous particles. The latter, also known as microparticulate porous particles are nowadays the most commercially important packing materials.

Rigid porous polymeric beads prepared from polystyrene cross-linked with divinyl benzene were used for ion-exchange and size-exclusion chromatography; these have largely been superceded by more efficient and mechanically stable silica-based packings for many applications. Pellicular packings were originally prepared to replace these porous polymer beads. Known also as porous layer beads, they have diameters of 35-50 μm and consist

Figure 1.22.

Flow diagram of column selection based on sample size



MW = molecular weight; SEC = size exclusion chromatography; GFC = gel filtration chromatography; LSC = liquid solid chromatography; RPC = reversed phase chromatography; BPC = bonded phase chromatography; IEC = ion-exchange chromatography; and IPC = ion pair chromatography.

of a glass bead core to which porous silica or alumina is fused in a layer 1-3 μm thick. They can be used without modification in liquid-solid chromatography, or they may be chemically modified for use in bonded phase chromatography. Porous layer beads have very small pore volumes, providing rapid solute diffusion in and out of the stationary phase. The use of these pellicular type packings for analytical separations declined with the advent of totally porous particles in narrow particle size ranges with diameters of 10 m or less. They do, however, find application as materials for guard and pre-concentration columns and high-quality, modified and unmodified pellicular packings are widely commercially available. Small totally porous silica particles are generally employed as packings for HPLC. They have been described in detail by Unger [75], and Scott [76,77]. Chemically modified silica packings are extensively used for polar and non-polar separations, collectively known as bonded phase chromatography, in addition to both ion-exchange and size exclusion applications. The preparation and application of bonded silica gels is discussed in section 1.4.

1.5.4.3. Types of columns

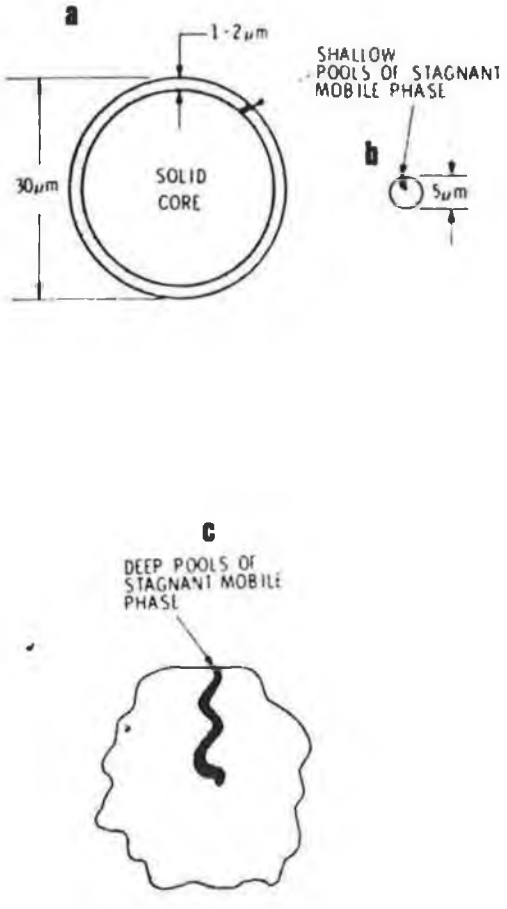
The column is the core of any chromatograph, and it is essential that the design of the chromatograph permits the full potential of the column to be realised in the recorder trace. What this effectively means is that the solute peak should not be significantly broadened by the operations of injection and detection, and by the passage of the solute through any intermediate tubing and connectors.

1.5.4.3.1. Standard analytical columns

The construction material for the column must be able to withstand the pressure at which the column is to be operated and be chemically inert to a wide range of mobile phases. For this reason, seamless polished stainless steel is the preferred

Figure 1.23.

Types of particles used in chromatography



(a): superficially porous particle.

(b): microparticulate porous particle.

(c): totally porous particle.

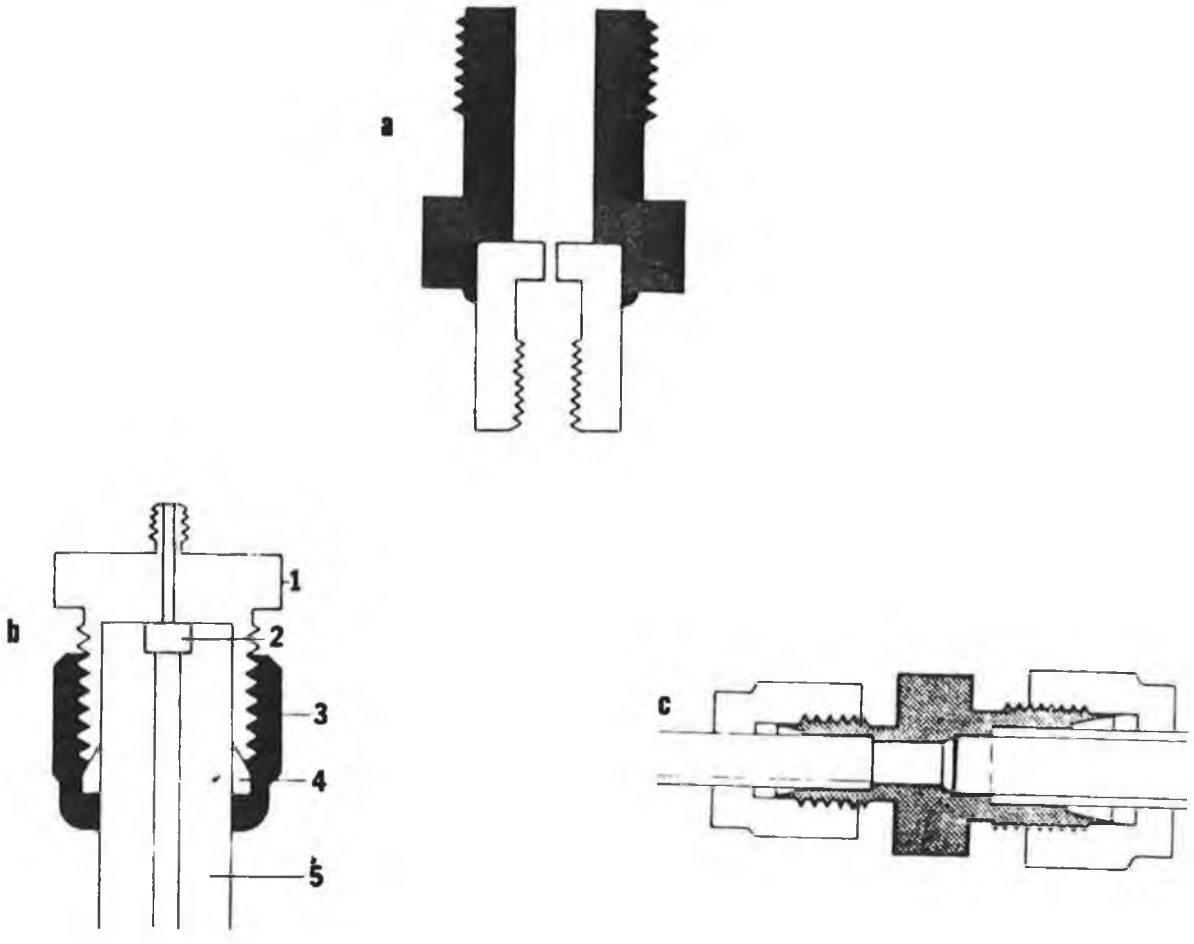
material. It is mechanically strong and is inert to most solvents, with the exception of solutions of halide salts, particularly at low pH [78]. Corrosion occurs when the mobile phase containing halide salts is in contact with the column for prolonged periods. Regular flushing the system with water (for example at the end of each day) will usually prevent serious problems. If corrosion is envisaged as a problem (and this is rarely the case) then glass may be used instead of stainless steel. Heavy-walled glass tubing with an upper operating pressure of 600 p.s.i. is commercially available. Glass-lined stainless steel columns which combine the inertness of glass with the strength of stainless steel, are also available [79].

The most common size for analytical columns packed with 5 to 10μ m diameter materials is 25 to 40 cm in length with an internal diameter of 4.0 to 4.6 mm. With the introduction of column packing having diameters as low as 3 μm, there has been a trend towards the use of shorter columns of 5 to 7.5 cm, with internal diameters of 4.0 to 4.6 mm [80]. Short and narrow bore columns are preferred for trace analysis as they consume less solvent for a separation and provide less peak dilution for a component at the detector. Wider bore columns can separate larger sample sizes without loss in efficiency and are operated at higher flow rates to minimise the effects of extra column band broadening. When longer columns are needed for a particular separation, superior results are obtained by the series coupling of standard length columns using short lengths of stainless steel capillary tubing [81].

Column end fittings and connectors must be designed with a minimum dead volume to eliminate extra column band broadening. Some typical low dead-volume fittings for HPLC are shown in Figure 1.24., and these can be obtained from most chromatographic supply houses. Zero dead-volume fittings are also available and these minimise extra column band broadening by eliminating the space between ends within the fitting. The resolution of solutes

Figure 1.24.

Column end fittings and connectors for HPLC



(a): Low dead volume column end fitting

(b): Standard column terminator

(c): Standatd reducing union

1: stainless steel union

2: porous metal frit

3: stainless steel nut

4: stainless steel ferrules

5: Chromatographic column

produced by a column can easily be lost if the connecting tubing is too long or has too wide a bore. The tubing bore should not exceed 0.25 mm, and although finer tubing can be used, problems such as pressure drop and blockage can arise. The column packing is held in the column by a packing retainer at either end. This is generally either a fine porosity metal frit or a disc of fine stainless steel gauze or cloth. These may be either incorporated into the column ends themselves or made an integral part of the column end fittings. The frits are made from porous stainless steel or PTFE and have an average pore diameter less than the particle size of the packing material (typically 2 μm). The column is pressed home onto the metal frit or screen by the act of tightening the coupling. Because of the problem of tolerance with metal fittings, it is advisable to use the same column and end-coupling together from the first time onwards.

1.5.4.3.2. Radial compression columns

Even in well-packed columns solute dispersion occurs to a greater extent in the region of the column wall than at the core of the column. This phenomenon is known as the "wall effect", and it is due to the differences in packing density of particles close to the wall and those at the column centre. This leads to inhomogeneous flow patterns across the diameter of the column, and results in lower overall column efficiency. Radial compression columns were designed to minimise both column void volumes and the wall effects referred to above. Compared to standard packed columns they have two novel features. A series of distribution plates at the entrance and exit of each column prevents irregular flow patterns from forming in the direction of sample migration. Secondly, the columns are prepared from heavy wall polythene cartridges (packed with normal chromatographic packings) that are uniformly radially compressed by hydraulic pressure in a purpose-built hydraulic press. The column wall is forced to deform and mould to the shape of the internal packing structure. This stabilises the packing structure and reduces the

number of channels available to the mobile phase at the wall-packing interface. Radial compression columns are characterised by high permeability, low operating pressures, and high efficiency over a wide range of flow rates [82]. Compared with a conventional stainless steel column packed with the same material, an increase in efficiency approaching a factor of two might be expected. On an equivalent length basis, the pressure drop across the radial compression column will be greater at a fixed flow rate, due to its higher packing density. Columns are available in 10 cm length with 5 or 8 μm packings. They have been shown to provide good separation for peptides [83], and low molecular weight serum components [84].

1.5.4.3.3. Microcolumns

There were three incentives for reducing the internal diameters of the columns used in liquid chromatography. Analagous to capillary gas chromatography, it was hoped to realise in liquid chromatography the very high column efficiencies for the resolution of complex mixtures. Since these columns operate at flow rates of a few microlitres per minute, they are very economical in solvent consumption. In addition, the low flow rates can open up new possibilities for sensitive detection techniques, provided that detection volumes can be sufficiently miniaturised for this purpose.

Three types of microcolumns are currently in use.

(i) microbore columns are similar to conventional columns except that the bore is reduced to about 1 mm, and the mobile phase flow rate is about 30-40 microlitres per minute.

(ii) Packed microcapillaries have a column bore of 70 microns or less, and are loosely packed with particles having diameters of 5-30 microns. Mobile phase flow rates are of the order of 1 microlitre per minute.

(iii) Open microtubular columns are the equivalent of the capillary column in gas chromatography. Ideally they have column bores of 10-30 microns and contain a liquid phase or an adsorbent either coated on, or chemically attatched to the column wall. Flow rates may be in the sub-microlitre range for operation at maximum efficiency

Columns of very high efficiency and of small cross-sectional areas produce very narrow peaks in the sense that the total volume of mobile phase contained in them may be in the sub-microlitre range. If such peaks pass through a detector connecting tube and cell of significant volume relative to that of the peaks, then the peaks will be broadened. Such broadening is due to both the dispersion resulting from the parabolic velocity profile of the liquid through the tubes and the cell and the logarithmic dilution effect resulting from the finite volume of the cell [85,86]. The operation of microbore columns near their optimum efficiency is possible by modifying liquid chromatographs designed for use with wide-bore packed columns [86-90]. The injection valve should be miniaturised to handle a sample volume of 0.2-0.5 μl. Microbore HPLC is performed at flow rates about 100-fold less than conventional wide bore columns, thus necessitating modification of the gradient former and pump. Since microtubular columns have little flow resistance at the low mobile phase flow rates typically used, an air-tight 50-250 μl syringe controlled by a micro-feeder can be used as a pump [91]. A micro-feeder consists of a small synchronous motor, gears, and a screw thread to advance the syring plunger at a constant rate. The electronics of currently available commercial UV detectors may, however, be inadequate for use with microbore columns, particularly at high flow rates. Though a specially designed UV detector with a total dead volume of 1.0 μl for the connecting parts and 0.1 μl for the detector cell has been described [91]. On-column detection using the microtubular column itself as the detector cell eliminates the problems with dead volumes in connectors, and is now the method of choice in microtubular HPLC

[92]. Other detection devices with low detection cell dispersion volumes have been described. These include electrochemical detectors [93], infrared monitors [94], post-column reaction detectors [95], flame ionisation detectors [96] and fluorescence detectors [97]. A method for generating gradients with microtubular columns has also been discussed [98].

1.5.4.3.4. Guard and pre-columns

A guard column is a short column located immediately in advance of the analytical column. Its function is to protect the latter from damage resulting from particulate matter or contamination by strongly adsorbed substances. It also serves to saturate the mobile phase with packing material and so helps to prevent dissolution of the analytical column. For the latter purpose, an additional short column is often placed betweem the pump and injector, in which case it is referred to as a pre-column. This strategy is normal practice when using unmodified silica in either the adsorption or ion-exchange modes.

The guard column is ideally packed with the same material as the analytical column, and is discarded at intervals dictated by the contamination level of the sample. To maintain an adequate capacity for impurities without introducing excessive peak dispersion, the volume ratio of the guard column to the analytical column should be in the range 1:15 to 1:25. A well designed guard column should not increase peak dispersion by more than 5-10%, a small trade-off for the extended lifetime of the analytical column. The column should be connected to the analytical column using low dead-volume fittings. Ideally the guard column should be slurry-packed with particles of the same diameter as the analytical column, but for practical convenience, they are usually dry-packed with with pellicular packings 20 μm or greater in diameter. The latter type of particle has the advantage of contributing little to the pressure drop across the system. Columns sharing many of the features of guard columns in

terms of packing material and column dimensions are widely used in on- or off-line solid phase sample preparation. The practical application of these columns will be discussed in detail in Chapter 2.

1.5.4.3.5. Semi-preparative columns

There is no clear concensus as to the meaning of the term "preparative chromatography" but depending on the intended use, it may cover a very wide mass range; in the analytical laboratory, preparative chromatography is normally used for the separation of milligramme to gramme quantities. Liquid-solid chromatography remains the dominant technique for separating neutral organic compounds, and a typical semi-preparative column 8 mm x 50 cm would separate around 500 mg of sample. The procedures used frequently represent the operations performed in classical column chromatography; the sample is separated by elution, collecting suitable-sized fractions either manually or with a fraction collector. A single development with 4 to 5 column volumes of mobile phase at a flow rate of 0.5-5.0 ml/min usually suffices for simple separations. A stepwise gradient may be created by changing the polarity of the eluent in incremental steps. At the end of the separation, the individual fractions are collected and subjected to analysis; for instance by TLC and/or HPLC.

As the classical column chromatographic technique is slow and demands the constant attention of the operator several variations have been developed to either automate the methods, improve resolution, or improve sample throughput. All of these goals are realised simultaneously in preparative scale high performance liquid chromatography which is becoming widely used for the separation of anything between 1 mg and 1 Kg of sample material.

1.6 REFERENCES

1. M. Tswett, Proc. Warsaw Soc. Nat. Sci.(Biol.), 14 (1903) 6.
2. R. Kuhn and E. Lederer, Naturwiss., 19 (1931) 306.
3. T. Reichstein and J. Van Euw, Helv. Chim. Acta., 21 (1938) 1197.
4. J. G. Kirchner, Thin-Layer Chromatography (Wiley Interscience, New York, 1967).
5. E. Stahl, Thin layer Chromatography: A Laboratory Handbook, 2nd edition (Academic press, New York, 1969).
6. A. J. P. Martin and R. L. M. Synge, Biochem. J., 50 (1941) 1358.
7. A. T. James and A. J. P. Martin, Biochem. J., 50 (1952) 679.
8. P.B. Hamilton, D. C. Bogue and R. A. Anderson, Anal. Chem., 32 (1960) 1782.
9. L. R. Snyder, Principles of Adsorption Chromatography (Marcel Dekker, New York, 1968)
10. J. C. Giddings, Dynamics of Chromatography, Part I: Principles and Theory. (Marcel Dekker, New York, 1965)
11. C. Horvath, B. A. Preiss and S. R. Lipsky, Anal. Chem., 39 (1967) 1422
12. J. F. K. Huber, J. Chromatogr. Sci., 7 (1969) 85.
13. J. J. Kirkland, J. Chromatogr. Sci., 7 (1969) 7.
14. I. Halasz and C. Horvath, Anal. Chem., 36 (1964) 1179.
15. I. Halasz and I. Sebastian, Agnew. Chem. Int. Rd. Engl., 8 (1969) 453.
16. I. Halasz, O-E Brust and I. Sebastian, J. Chromatogr., 83 (1973) 15.
17. B.L. Karger, L.R. Snyder and C. S. Horvath, An Introduction to Separation Science (Wiley, New York, 1973).
18. R. Thumneum and S. Hawkes, J. Chromatogr. Sci., 14 (981) 576

19. J. J. Van Deemter, F.J. Zuiderweg and A. A. Klinkenberg, Chem. Eng. Sci., 5 (1956) 271.
20. R.K. Iler, Colloid Chemistry of Silica and Silicate (Cornell University Press, New York, 1955)
21. G. B. Cox and R. W. Stout, J. Chromatogr., 384 (1987) 315.,
22. J. H. Knox, High Performance Liquid Chromatography, (Edinburgh University Press, Edinburgh, 1978)
23. C. J. Laurent, H. A. Billiet and L. de Galan, Chromatographia, 17 (1983) 253
24. K. Unger, R. Kern, M. C. Minou and K. F. Krebs, J. Chromatogr., 99 (1974) 435.
25. H. Colin and G. Guiochon, J. Chromatogr., 141 (1977) 289.
26. R. E. Majors and M. J. Hopper, J. Chromatogr. Sci., 12 (1974) 767.
27. J. J. Kirkland, Chromatographia, 8 (1975) 661.
28. G. B. Cox, C. R. Loscombe, M. J. Slucutt, K. Sudgen and J. A. Upfield, J. Chromatogr., 117 (1976) 269
29. A. Wehrli, J. C. Hildenbrand, H. P. Keller, R. Stampfli, and R. W. Frei, J. Chromatogr., 149 (1978) 199-210.
30. P. E. Barker, B. W. Watt and S. R. Holding, J. Chromatogr., 207 (1981) 27.
31. J. J. Kirkland and P. C Yates, U.S. parent 3,772,181 (March 1981).
32. J. J. Kirkland, J. Chromatogr. Sci., 9 (1971) 206.
33. R. E. Majors in C. Horvath (Ed.) High Performance Liquid Chromatography, Advances and Perspectives (Academic press, New York, 1980)
34. R. D. Rasmussen, W. H. Yokoyama, S. G. Blumenthal, D. E. Bergstrom and B. H. Reubner, Anal. Biochem. 101 (1980) 66.
35. C. F. Poole and S. A. Schuette, Contemporary Practice of Chromatography (Elsevier, Amsterdam, 1984).
36. P. Jandera, H. Colin and G. Guichon, Anal. Chem., 54 (1982) 435.
37. F. E. Reigner and K. M Gooding, Anal. Biochem., 103 (1980) 1.

38. M. T. Hearn, Adv. Chromatogr., 20 (1982) 1.
39. F. E. Regnier and J. Fausnaugh, Liq. Chromatogr. and HPLC Magzn., 1 (1983) 402.
40. M. T. Hearn and B. Grego, J. Chromatogr., 266 (1983) 75.
41. M. T. Hearn and B. Grego, J. Chromatogr., 203 (1981) 349.
42. C. F. Simpson (Ed.) Techniques in Liquid chromatography (Wiley, New York, 1982)
43. W. R. Melander and C. Horvath in C. Horvath (Ed.) High Performance Liquid Chromatograaphy, Advances and Perspectives (Academic press, New York, 1980.)
44. C. Horvath, Liq. Chromatogr. and HPLC Magzn., 1 (1983) 552.
45. B. L. Karger, J. R. Gant, A. Hartkopf and P.H. Weiner, J. Chromatogr., 128 (1976) 65.
46. G. Schill, S. Eksborg, P. O. Lagerstrom and R. Modin, J. Chromatogr., 83 (1973) 99.
47. J. H. Knox and G. R. Laird, J. Chromatogr., 122 (1976) 17.
48. P. T. Kissinger, Anal. Chem., 49 (1977) 883.
49. C. Horvath, W. Melander, I. Molnar and P. Molnar, Anal. Chem., 49 (1977) 2295.
50. S. Moore and W. H. Stein, J. Biol. Chem., 45 (1951) 2092.
51. P. B. Hamilton, Anal. Chem., 32 (1960) 1779.
52. W. E. Cohn, Science, 109 (1949) 377.
53. J. X. Khym and L. P. Zill, J. Amer. Chem. Soc., 73 (1951) 2399
54. R. A. Hatwick and P. R. Brown, J. Chromatogr., 112 (1975) 651
55. J. J. Kirkland, J. Chromatogr. Sci., 7 (1969) 361.
56. K. K. Unger and D. Nyamah, Chromatographia, 7 (1974) 63.
57. A. Krishen, J. Chromatogr. Sci., 15 (1977) 434.
58. D. D. Bly, J. Polym. Sci. Part (C), 21 (1968) 13.
59. J. J. Kirkland, J. Chromatogr., 125 (1976) 231.
60. J. H. Dueresken and A. E. Hamielec, J. Appl. Polym. Sci., 12 (1968) 2225.
61. J. G. Nikelly and D. A. Ventura, Anal. Chem., 51 (1979) 1585.

62. L. R. Snyder in C. Horvath (Ed.) High Performance Liquid Chromatography, Advances and Perspectives (Academic press, New York, 1980).
63. F. Erni, R. W. Frei and W. Linder, J. Chromatogr., 125 (1976) 265.
64. P. A. Bristow, Anal. Chem., 48 (1976) 237.
65. N. K. Vadukul and C. R. Loscombe, Chromatographia, 14 (1981) 465.
66. J. J. Kirkland, Anal. Chem., 40 (1968) 391.
67. J. N. Little and G. J. Fallick, J. Chromatogr., 112 (1975) 389.
68. A. M. Krstulovic, R. A. Hartwick, P. R. Brown and K. Lohse, J. Chromatogr., 158 (1976) 365.
69. J. W. Readman, L. Brown and M. M. Rhead, Analyst, 106 (1981) 122.
70. E. S. Yeung and M. J. Sepaniak, Anal. Chem., 52 (1980) 1465A.
71. B. Fleet and C. J. Little, J. Chromatogr. Sci., 12 (1974) 747.
72. D. G. Swartzfager, Anal. Chem., 48 (1976) 2189.
73. P. T. Kissinger, Anal. Chem., 49 (1977) 447A.
74. R. J. Rucki Talanta, 27 (1980) 147.
75. K. K. Unger, Porous Silica (Elsevier, Amsterdam, 1979).
76. R. P. W. Scott, J. Chromatogr. Sci., 18 (1980) 297.
77. R. P. W. Scott, Adv. Chromatogr., 20 (1982) 167.
78. R. E. Majors, J. Assoc. Off. Anal. Chem., 60 (1977) 186.
79. G. Vigh, E. Gemes and I. Inczedy, J. Chromatogr., 147 (1978) 59.
80. R. W. Stout, J. J. De Stefano and L. R. Snyder, J. Chromatogr., 261 (1983) 189.
81. I. Halasz and G. Melander, Anal. Chem., 55 (1983) 1842.
82. J. S. Landy, J. L. Ward and J. C. Dorsey J. Chronatogr. Sci., 21 (1983) 49.
83. W. S. Hancock, J. D. Capara, W. A. Bradley and J. T. Sparrow, J. Chromatogr., 206 (1981) 59.

84. S. P. Assenza and P. R. Brown, J. Liq. Chromatogr., 3 (1980) 41.
85. C. F. Simpson (Ed.) Techniques in Liquid Chromatography (Wiley,New York, 1982).
86. C. E. Reese and R. P. W. Scott, J. Chromatogr. Sci., 18 (1980) 479.
87. R. P. W. Scott ans P. Kucera, J. Chromatogr., 169 (1979) 51.
88. R. P. W. Kucera, P. Kucera and M. Munroe, J. Chromatogr., 186 (1979) 475.
89. T. Tsuji and R. B. Binns, J. Chromatogr., 253 (1982) 227.
90. J. Bowermaster and H. McNair, J. Chromatogr., 279 (1983) 431.
91. D. Ishii, K. Asai, K. Hibi, T. Jonokuchi and M. Nagaya, J. Chromatogr., 144 (1977) 157.
92. F. J. Yang, J. High Resolut. Chromatogr. Chromatogr. Commun., 3 (1980) 589.
93. Y. Hirata, P. T. Lin, M. Novotny and R. M. Wightman, J. Chromatogr., 181 (1980) 287.
94. K. Jinno and C. Fujimoto, Chromatographia, 17 (1983) 259.
95. T. Takeuchi, S. Saito and D. Ishii, J. Chromatogr., 284 (1983) 125.
96. M. Krejci, K. Tasarik, M. Rusek and J. Pajurek, J. Chromatogr., 218 (1981) 167.
97. J. W. Jorgenson and E. J. Guthrie, J. Chromatogr., 255 (1983) 335.
98. T. Takeuchi and D. Ishii, J. Chromatogr., 253 (1982) 41.

CHAPTER 2

BIOPHARMACEUTICAL ANALYSIS

2.1 INTRODUCTION TO THERAPEUTIC DRUG MONITORING

Chemists and biochemists have been routinely analysing biological fluids since the early part of this century when they discovered that a relationship existed between the concentration of various endogenous substances, for example glucose, hormones, enzymes, etc., and the cause of a disease [1]. The analysis of drugs in biological media, however has emerged relatively recently, and it is only in the last twenty years that it has received widespread attention [2]. Growth in the field of biopharmaceutical analysis has paralleled the expansion in the number of new drug compounds introduced onto the market. Assays for proposed new drugs are essential with a view to establishing protocols for new drug registration. It is necessary for a drug company to develop methods capable of determining low concentrations of their products in blood, plasma, urine and other biological matrices in order to establish the pharmacokinetic profile of that product. Pharmacokinetics is the study of absorption, distribution , metabolism and excretion of a drug.

Uncontrollable irregularities in the absorption or distribution of a drug in the body often leads to poor bioavailability, and hence unsatisfactory response of that product. In such cases, for example, where there is a wide variation in individual absorption of a drug, it is necessary to institute a therapeutic monitoring programme for patients receiving that treatment. Therapeutic drug monitoring is also warranted where drugs have a very narrow therapeutic window (range). Examples of such drugs are cytostatic agents and cardioactive drugs (such as digoxin) where serious toxic effects are seen at levels even slightly above their therapeutic dose. In such cases, sub-therapeutic doses could also be fatal due to a depletion of their pharmacological effect. Certain disease states can radically alter the deposition of a drug in the body. Individuals suffering from either renal or hepatic disease may require reduced amounts of drugs as a result of impaired metabolism which enhances both the potency and duration of drug action in the body.

Biopharmaceutical analysis is also useful where there is a question of patient compliance, and has obvious application where it is sought to establish abuse of hedonistic drugs.

2.2. METHODS USED IN BIOPHARMACEUTICAL ANALYSIS

The field of therapeutic drug analysis is largely dominated by chromatographic and immunoassay techniques although the classical spectroscopic methods are applied to some extent. The requirements of an assay are that it is specific, sensitive, reproducible and applicable to a large range of compounds. It should also be relatively simple to develop and execute, and ideally should not involve lengthy and tedious sample preparation stages. The above methods meet these criteria to varying degrees, but the choice of analytical procedure will ultimately depend on the physico-chemical properties of the drug and the required sensitivity and specificity.

2.2.1. Spectroscopic techniques

Absorption and fluorescence spectrophotometry are techniques which do not involve a separation step, and although they are convenient, they lack the selectivity associated with chromatographic and competitive binding assay techniques. Although the use of selective extraction techniques, colorogenic and fluorogenic reactions and differential compensation using the sample matrix can enhance the selectivity of these methods [3], much attention has recently focussed on the development of more sophisticated instrumentation. This includes the use of diode array spectrophotometers, [4-6], or luminescence spectrophotometers which permit the capture of the entire luminescence excitation-emission matrix [7,8], devices which considerably enhance specificity in bioanalytical applications.

2.2.2. Immunoassay techniques

The discovery of competitive binding assays by Yalow and Berson

in 1959 [9], has proved to be a significant development in the fields of both clinical and analytical chemistry. Initially applied only to biopolymers and proteins, these techniques have been extended to the realm of drug analysis by covalently coupling a drug molecule to a protein in order to confer upon it immunogenic properties [10].

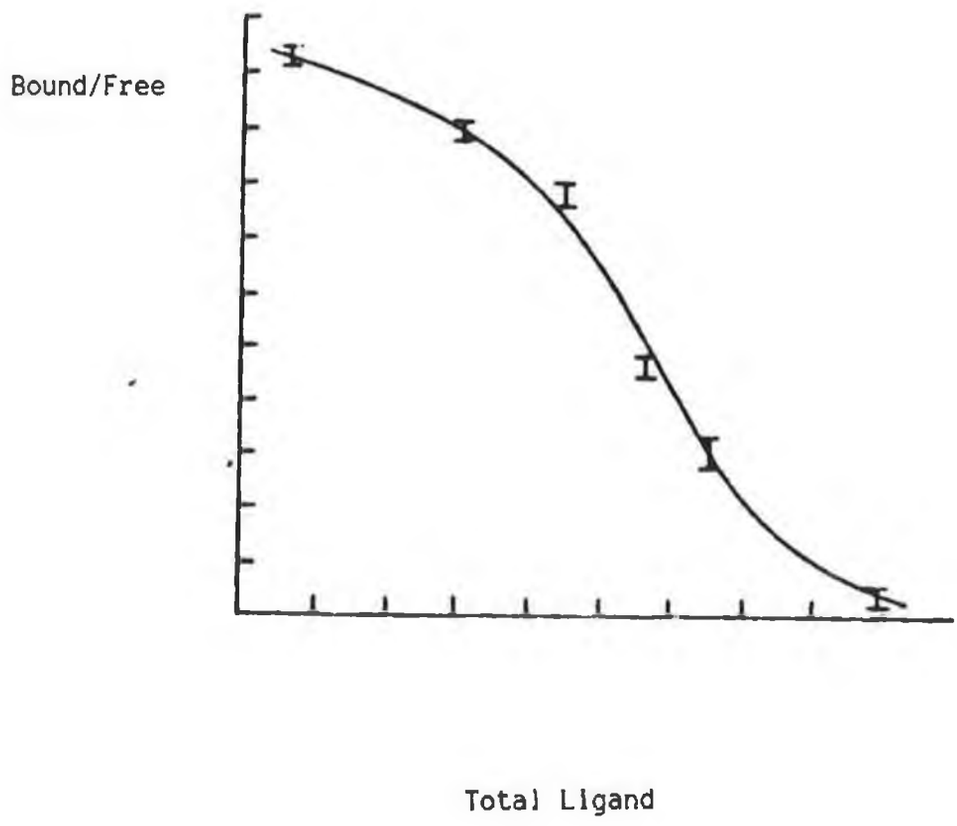
Immunoassays are competitive binding techniques where the drug binds to an antibody specific for that drug. The basic principle of competitive immunoassays is shown as follows:

$$Ab + Ag + Ag^* \rightleftharpoons AbAg + AbAg^*$$

The free antigen (Ag) and the labelled antigen (Ag*) compete for a fixed and limited number of specific binding sites on the antibody (Ab). The antigen in this case is a drug linked to a protein (usually bovine serum albumin). The labelled antigen is a known concentration of labelled drug, and the unlabelled antigen is the drug sample of unknown concentration. Upon admixture of the labelled analyte, the unlabelled sample and the antibodies, the labelled and unlabelled versions of the drug compete with one another for positions on the antibody surface. After an incubation period, the free and antibody-bound antigen are separated from one another and the amount of labelled antigen in one of the fractions is determined. At higher concentrations of drug in the sample (i.e. unlabelled drug-antigen), fewer labelled drug-antigen molecules will be bound to the antibody. If drug-free samples are spiked with known concentrations of unlabelled drug, and the proportion of free and bound labelled drug following reaction is plotted as a function of unlabelled drug concentration, a calibration curve may be constructed from which the concentration of unknown samples can be read. The form of a typical calibration curve is shown in Figure 2.1. In the past, immunoassays have principally relied on radioimmunoassays (RIA), which employ radioisotopes for the production of labelled reagents. Non-isotopic labels such as

Figure 2.1.

Typical form of a plot of the ratio of bound to free ligand versus total ligand in a protein binding assay.



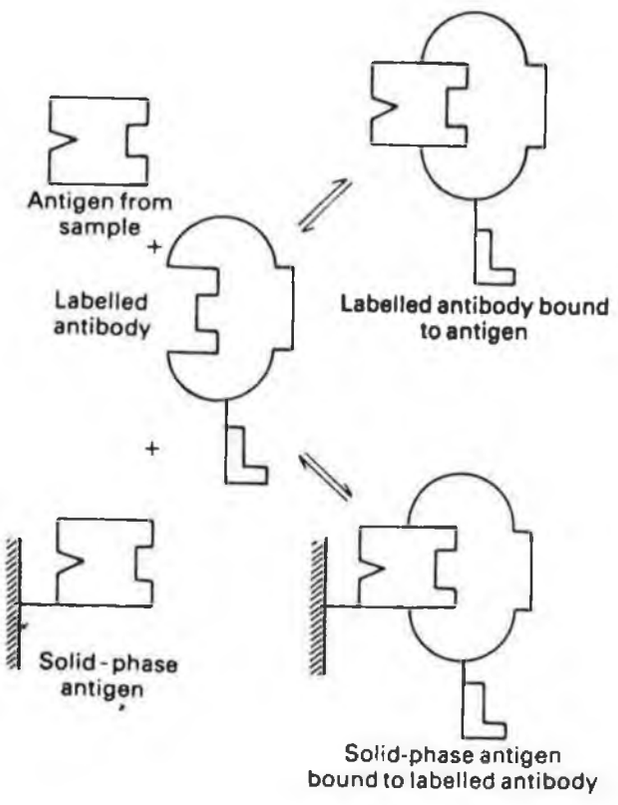
enzymes, fluorophores, particles and cells are gaining broader acceptance because of enhanced user safety, extended reagent shelf life, and ready adaptability to conventional instrumentation [10].

2.2.2.1. Enzyme immunoassays

Enzyme immunoassays (EIA) may be classified as heterogeneous or homogeneous procedures. Heterogeneous procedures originated some 15 years ago in the work of Van Weeman and Schuurs [11], and the principles underlying the various assay protocols are identical to those in which other labels are employed. EIA is analagous to classical radioimmunoassay in that there is competition between labelled and unlabelled antigen for a specific amount of antibody. A widely used derivative of the original heterogenous EIA is the Enzyme Linked Immunosorbent Assay (ELISA) technique where the antibody is labelled with enzyme, reacted with antigen from the sample, and then added to excess solid-phase antigen. The enzyme activity of antibody bound to the solid phase after washing is inversely proportional to the concentration of free antigen originally reacted with the labelled antibody. This procedure is depicted in Figure 2.2. The solid support most frequently used in large scale screening work required in the study of infectious diseases has been the microtitration plate [12]. These plates are cheap and require only small volumes of reagent.

The most widely used homogeneous EIA system is the Enzyme Multiplied Immunoassay Technique (EMIT). Homogeneous immunoassays are so-called because they do not require separation of bound and free ligand prior to measurement of the percentage binding. This technique was developed by Rubenstein et.al. [13], and depends on a change in the specific enzyme activity when an antibody binds to enzyme-labelled antigen. The more unlabelled antigen (drug) present, the less the reduction in enzyme activity, Hence the calibration curve for this technique is the reciprocal of that

Figure 2.2.
Competitive ELISA for antigen.



Enzyme-labelled antibody reacts specifically with with antigen in the sample and is then added to excess solid-phase antigen. After washing, the enzyme label still attatched to the solid phase is measured.

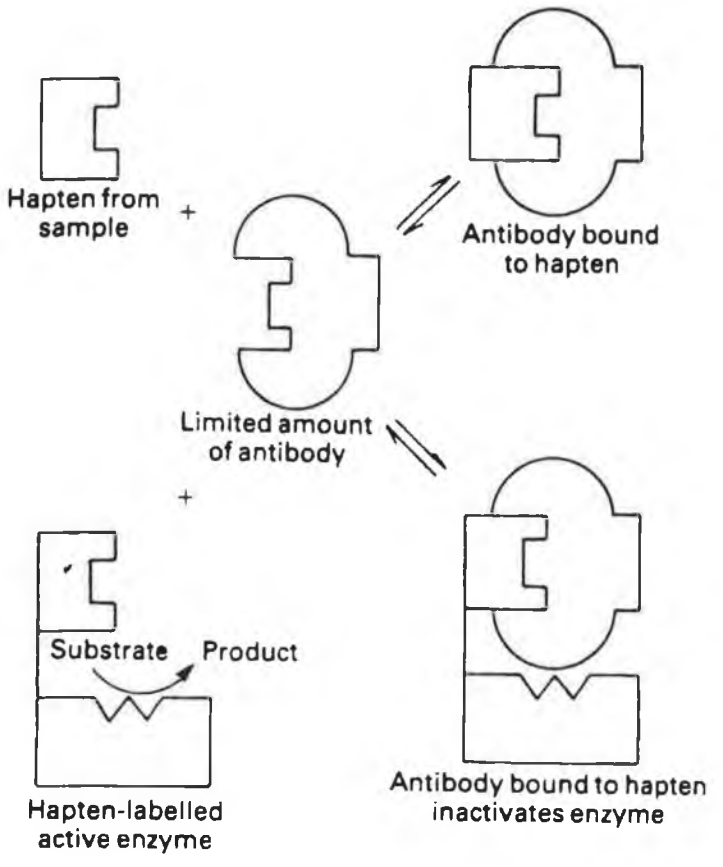
obtained with ELISA. The principle of the EMIT procedure is depicted in Figure 2.3. The earliest EMIT systems employed lysozyme as the enzyme label, though modern drug assays use glucose-6-phosphate dehydrogenase and malate dehydrogenase almost exclusively. The latter enzyme is used in an assay for thyroxine, in a rare instance where binding of the enzyme-labelled antigen actually results in increased enzyme activity. This is because binding of the malate dehydrogenase to thyroxine substantially inhibits enzyme activity, an inhibition which is at least partially overcome by antibody binding [14].

2.2.2.2. Other immunoassays

Substrate labelled fluorescence and fluorescence polarisation immunoassay are other homogeneous immunoassay techniques. In the former case, the label is a fluorophore and the antigen is a fluorophore-drug (F-D) conjugate which does not fluoresce until it is reacted with a fluorescence-inducing enzyme. Only the fraction of F-D which is not bound to the antibody (Ab) will react with the fluorescence-inducing enzyme. Upon mixing of the labelled drug (F-D), the unlabelled drug (D), and the antibody, the labelled and unlabelled versions of the drug compete for the antibody, and as a result, the amount of fluorescence produced is proportional to the amount of unlabelled drug present. In fluorescence polarisation immunoassay the label is again a fluophore and the antigen is a drug-fluophore conjugate. The unlabelled drug, labelled drug and the antibody are irradiated with polarised light. Only the bound species fluoresces with light polarised in the same plane as the incident beam, as the unbound species will loose polarisation through Brownian motion. The more (unlabelled) drug present in a sample or standard, the less bound fluorescent species produced, and the extent of polarisation will decrease.

Figure 2.3.

Principle of the EMIT system of enzyme immunoasssay.



Conjugation of hapten to enzyme does not destroy enzyme activity, but combination with hapten-specific antibody causes marked inhibition of enzyme activity. The measured enzyme activity is dependent on the relative amounts of free hapten and hapten-labelled enzyme.

2.2.2.3. Antibody reagents

An important limitation of immunoassays has been the restricted availability of analytically useful antibodies. Antisera prepared by conventional animal immunisation techniques contain populations of different antibodies varying both in antigenic specificity and affinity [15]. Large numbers of animals must be immunised, and carefully screened to identify animals producing analytically useful antisera. With the advent of monoclonal antibody technology, the problems of lack of uniformity and unavailability of antibodies are more readily solved. Monoclonal antibodies are produced by fusing an antibody-producing spleen cell with a tumour cell [16]. By cloning the progeny of a single hybridoma, the resulting cells, which are clones of the parent, produce antibodies which are identical in chemical structure and homogeneous in binding affinity. Monoclonal antibodies are highly specific since all the binding sites are directed at the same antigenic determinant. Large quantities of monoclonal antibodies can be produced, since hybridomas, by virtue of their tumour origin, will propagate readily.

2.2.2.4. Future trends in immunoassay

More and more drugs are now being analysed on a routine basis using immunoassay techniques, partly because once they have been developed, the ready-to-use kits permit rapid processing often without any sample pre-treatment. Large numbers of samples may conveniently be handled without any sample processing by staff untrained in specialised methodology. As the technique has become more popular, advances in the area have concentrated on the development of more creative detection schemes for the labelled species, particularly with the decline of radioactive labels which are subject to safety, disposal and regulatory problems [10]. The disadvantages of enzyme immunoassay techniques include the fact that plasma constituents may affect enzyme activity,

measurement of enzyme activity can be difficult, lack of selectivity when compared with chromatographic techniques, and development of an assay for a new drug can be time-consuming and costly. However, once a method is available for a drug, the technique has many advantages in terms of ease of operation, versatility, sensitivity, the fact that only a small volume of sample is required, and most significantly, that no sample pre-treatment is required.

2.2.3. Chromatographic techniques in biopharmaceutical analysis

High performance liquid chromatography (HPLC) and gas chromatography (GC) are the most widely used chromatographic techniques for quantitative analysis of therapeutic drugs in biological media.

2.2.3.1. Gas chromatography

Gas chromatography was once the dominant analytical technique for therapeutic drug analysis, but with the development of efficient microparticulate packings and stable bonded phases for liquid chromatography, gas chromatography has somewhat declined in its usage. Part of the reason for this is the fact that few drugs are volatile, and hence must be rendered so using derivatisation processes prior to analysis.

Gas chromatography is carried out using either packed or capillary columns. The large variety of packed columns available for gas chromatography contributed in part to its widespread use as an analytical technique for many years. Another important reason for its popularity is its ability to incorporate the flame ionisation detector (FID) as a detection system. This is regarded as an almost universal detection mode, with the added virtues of being cheap, sensitive (5×10^{-12} g/s), of wide linear range (10^{7}:1), and simple to use. Gas chromatography is also

compatible with other sensitive detectors which are more selective than FID. These include the nitrogen-phosphorous detector (NPD) sensitive to nitrogen and phosphorous, and the electron capture detector (ECD), sensitive to electron-rich compounds, for example organo-halogens.
Although the need to derivatise non-volatile samples is regarded as a drawback of GC, modern ready-to-use reagents have rendered the derivatisation process less cumbersome. For instance, the acidic nitrogen function of the barbiturate and hydantoin moieties are readily converted to their N-methyl derivatives by reaction with trimethylphenylammonium bromide and amines, alcohols, and other groups with replaceable hydrogen may be acylated. Reagents which permit the introduction of different acyl groups, for instance acetyl- or isobutyl- groups are commecially available. The introduction of halogen groups facilitates detection by electron capture, and silylation is useful for the derivatisation of carboxyl groups, phenols and alcohols. Another detection mode which lends itself well to GC is mass spectrometry (MS), and GC-MS has become a widely used tool in trace analysis of drug compounds [17].

Capillary GC offers excellent resolution unparalleled in chromatography [10], though its performance is strongly affected by technical considerations, especially the configuration of the injection device [18], and the quality of the columns used.

2.2.3.2. High performance liquid chromatography

High performance liquid chromatography, unlike GC, is ideally suited to the analysis of compounds which are thermolabile and non-volatile. With recent advances in column technology in terms of the production of microparticulate packings and columns of shorter length and diameters, the efficiency and sensitivity of HPLC has been greatly improved. Liquid chromatography has the further advantages of almost never requiring derivatisation, and of being well suited to simple methods of sample preparation. For

example, in some instances, where plasma drug levels are in the high ng/ml range and above, a simple protein precipitation will suffice as sample pre-treatment. The rapid, efficient technique of column switching for trace enrichment and sample clean-up is readily applicable to liquid chromatographic methods in both the automated and manual versions. In addition, a large number of separations can be carried out in the reversed-phase mode of liquid chromatography.

The importance of reversed-phase materials cannot be over-emphasised; there have been many reports describing the theoretical and practical aspects of the technique [19], and it is estimated that somewhere between 75-90% of all HPLC separations are carried out in the reversed-phase mode [20]. Most drugs possess sufficient hydrophobic character to be retained by the non-polar mechanisms involved in reversed-phase chromatography. In addition, retention and selectivity are easily controlled by altering the type and percentage of organic modifier, by changing the pH, or by the addition of ion pairing reagents for the separation of ionic species. A disadvantage of HPLC is that the routinely used detectors are generally no match for those used in GC. Ultraviolet absorption (UV) is the most widely used detection scheme in HPLC; adequate detection depends on the presence of a chromophore within the molecule and the limit of detection will depend on the molar absorptivity of the drug at the detection wavelength. Where the therapeutic compound absorbs strongly at a reasonably high wavelength, and is present in μg/ml concentrations in the plasma, HPLC is the ideal technique for analysis. Problems can arise where the compound has a weak chromophore and /or where it occurs in the low ng/nl range in the relevant biological matrix: in these cases, where the detection wavelength is in the low UV region, selectivity may be severely compromised and the analyst may have to resort to fluorescence detection with pre- or post-column derivatisation.

2.2.4. Chromatographic versus immunoassay methods in biopharmaceutical analysis

Several studies have been reported in the literature comparing chromatographic methods with immunoassay [21], and abundant data are available to support the fact that both methods are effective in drug determinations [10]. One of the inherent disadvantages of immunoassay is because of its very specificity, an assay developed for a parent drug compound is incapable of providing insight into the metabolite profile of the sample. On the other hand, extraction of the drugs from serum or plasma is not a prerequisite for immunological analysis whereas chromatographic techniques can call for sample clean-up procedures which can be quite elaborate, especially if there is a derivatisation step involved. The need for sample preparation has received much attention in relation to chromatography, and recent innovations resulting from research in this area include the development of an automated centrifugal apparatus for the extraction of drugs from serum prior to GC, GC-MS, or liquid chromatography [10].

Chromatographic methods enjoy an ease of method development not shared by immunological techniques, for which antibodies must be raised in the developmental stages, resulting in higher costs per sample using the prepared kits. Chromatographic methods are now highly developed and are routine, sensitive and specific. The mass spectrometer combined with GC or HPLC leads to ultimate specific identification for an unknown compound. This is now widely used in conjunction with GC, though this hybrid technology is not as advanced with respect to LC [10]. LC is a serious contender for the low-cost automated clinical analysis of high molecular weight compounds as well as drugs, though thus far, it cannot compete with immunoassay in the determination of whole cells and organisms. Unlike chromatographic methods many immunoassays can be performed simultaneously without the need for elaborate apparatus. This technique is particularly suited to the hospital environment where a large throughput of samples is

carried out by relatively unskilled staff. Chromatographic techniques are ideally suited to the biopharmaceutical laboratory which has a greater need for ease of method development, and requires apparatus which can be tailored to a variety of analytical needs.

Immunoassay and chromatographic techniques have been combined in one case where an HPLC column effected separation of the antibody-antigen complex prior to detection of an activated fluorophore [22], or in another case where the antibodies were immobilised on an HPLC column in order to carry out the immune reaction. This approach has been christened "high performance immunoaffinity chromatography" and is a promising analytical technique [23]. The innovation of combining HPLC with immunoassay may be an indicator of future trends as analysts exploit the power of biochemical assays and apply the knowledge of separation science to further sophistication in the fields of clinical and biopharmaceutical analysis.

2.3. SAMPLE PREPARATION IN HPLC

Before proceeding to discuss the relevance and methodology of sample pre-treatment in biopharmaceutical analysis, it is worth noting that in a few, limited instances it is possible to inject untreated body fluids directly onto the analytical column in liquid chromatography. This approach is not possible in GC. Direct injection is only feasible where the liquid sample, (urine, bile serum) contains a high concentration of analyte. It is important to ensure that the proteins are soluble in the mobile phase to prevent precipitate formation, especially for serum or plasma samples. Micellar chromatography is particularly advantageous in this respect since direct injection of untreated plasma is possible by virtue of the fact that the micelles solubilise blood proteins, thus eliminating the problem of protein precipitation on the analytical column. The application of micellar chromatography is discussed in chapter 4.

When a pre-column and a column switching valve are used, protein and other interferences may be diverted to waste before HPLC separation of the analyte. This approach shall however be treated under the heading of "on-line solid phase extraction". Some workers prefer not to use a pre-column, but rather clean the analytical column by washing with an appropriate solvent at regular intervals, or replace the top 1-2 mm of the column when there are signs of contamination, as shown by an increase in column back pressure or peak tailing. There are obvious limitations to this procedure in terms of limit of detection and column lifetime. Columns have been specially designed for direct injection of serum, for example, internal surface reversed-phase (ISRP) chromatography, a concept which was developed by Hagestam and Pinkerton [24]. These columns totally exclude proteins while retaining smaller organic molecules. Direct injection is a time saving and cost effective exercise,and should be considered whenever detection and interference are not problems; for instance in the determination of theophylline in serum.

In most instances, a biological sample containing a compound of interest requires some kind of sample pretreatment. Such precedures are executed principally to isolate the drug from interfering matrix substances, but these measures also serve to

(i) liberate the drug from protein binding sites
(ii) concentrate the drug for more sensitive analysis
(iii) separate the drug from other drugs or metabolites

The amount of sample preparation required depends on the chemical nature and concentration of the drug and metabolite, the type of sample, and on the nature of the interfering substances. It also depends on instrumental factors such as the sensitivity of the detector to contamination, and in the case of UV absorbance detection, the detection wavelength. The sample preparation step should therefore, be capable of reducing the concentration of endogenous compounds and of concentrating the sample where this is required. Removal of endogenous components is particularly important where these interferents become irreversibly adsorbed onto the packing material of the column, as is the case with lipids, or precipitated in the chromatographic system as is the case with proteins. To achieve this, either the interfering substances may be removed from the sample while the compounds of interest are retained in the original aqueous phase (for example, protein precipitation), or the drugs are selectively removed from the biological specimens (for example, liquid extraction).

2.3.1. Protein precipitation

The preparation of protein-free solutions is especially important for the HPLC analysis of blood and tissue extracts. There are many methods available and the more common ones are described here.

2.3.1.1. Precipitation by addition of organic solvents.

With the addition of a water-miscible organic solvent such as

methanol, ethanol, acetonitrile and acetone to a biological sample such as plasma, the solubility of proteins is lowered and they precipitate out. In addition, drugs are released from protein binding sites. The sample is centrifuged to produce a clear supernatant containing the compound of interest, and an aliquot is injected onto the HPLC column. It is important to use a protein precipitating solvent in which the analyte is highly soluble, otherwise it may adsorb onto, or co-precipitate with the protein. It is sometimes necessary to use a mixture of two solvents to obtain quantitative recovery. For example, methanol with dimethyl sulphoxide has been employed to extract porphyrins from liver tissue [25]. In this case, methanol was added to precipitate the protein, while dimethyl sulphoxide released the otherwise adsorbed porphyrins from the protein.

Diluting the serum up to threefold with the organic solvent will effect removal of 99% of the proteins [26,27]. This degree of protein removal helps to prolong column life, but effectively decreases sensitivity of the method. This drawback can be counteracted to some extent by increasing the volume of injection, though this can adversely affect chromatographic efficiency and peak shape. Alternatively, the supernatant may be evaporated, but this measure also serves to concentrate any remaining interfering compounds.

2.3.1.2. Precipitation by the addition of inorganic salts

Proteins are positively charged in strongly acidic and negatively charged in strongly basic solutions, owing to their zwitterionic nature. Acidic or anionic precipitants such as trichloroacetic acid or perchloric acid form insoluble protein salts with the cationic form of proteins at low pH. These two acids are widely used to extract compounds from tissue and blood samples. A 10-20% (w/v) solution is usually used. After centrifugation, an aliquot of the supernatant may be injected onto the analytical column. The mobile phase should, in this case, contain a high molar

concentration of buffer to protect the column from damage by a strongly acidic solution [28]. More commonly, the acid solution is neutralised with an alkali or removed by extraction into ether before injection. Acidic protein precipitants are obviously unsuitable for compounds which are prone to acid hydrolysis. A combination of an anionic precipitant with an organic solvent can also be useful, where the latter can aid the solubilisation and extraction of the analytes from the proteins.

Proteins may also be precipitated by forming insoluble salts with cationic precipitants such as copper or zinc in alkaline solution [29], for example zinc sulphate in sodium hydroxide or barium hydroxide of copper sulphate in potassium hydroxide. Cationic protein precipitants may also be used in conjunction with organic precipitants. Again, adjustment of pH prior to chromatography is advisable. This method is unsuitable for compounds having a tendency to form metal complexes.

The supernatant liquid contains other constituents besides proteins, and frequently the drug peak can be accompanied and complicated by several other peaks. Thus the likelihood of interfering peaks is about the same as for direct injection, and it may be necessary to carefully manipulate the HPLC conditions to obtain adequate separation of the drug peak. Moreover, small and late-eluting peaks (including late-eluting peaks from previous samples) may co-elute with the drug peak, thereby distorting peak measurements and adversely affecting assay precision. The potential for late-eluting peaks to undermine assay precision has been addressed by Van der Wal and Snyder [30]. The deproteinisation procedure can sometimes give low recoveries for drugs which are strongly protein bound; and as mentioned above, certain precipitants can co-precipitate or degrade drugs or their metabolites. Where dilution of the sample is not of major importance, organic precipitants should be considered as they are less aggressive than the ionic precipitants, with the added advantage of not requiring

neutralisation prior to chromatography. Although methanol is aproximately only half as effective as acetonitrile as a protein precipitant [31], it should be used because of its low cost and low toxicity. The protein precipitation method is particularly useful for highly polar drugs such as antibiotics, and amphoteric drugs like the sulphonamides, which are difficult to extract from plasma with organic solvents. Analyses with good reproducibilities are often possible, even without internal standardisation [32].

2.3.2. Protein removal by Ultrafiltration

A protein-free solution may be obtained by filtration through a size selective semi-permeable membrane under pressure or by centrifugation in a membrane cone. The basic process differs from ordinary filtration only in the size of the particles which are separated, and in the hydrostatic pressure applied as a driving force for the separation process. In ultrafiltration, 1-10 atmospheres of hydrostatic pressure is applied. Ultrafiltration membranes are microporous in their structure. All molecules greater than the largest pore diameter will be retained, and all molecules smaller than the smallest pore completely pass the membrane. All molecules smaller than the largest pores, but greater than the smallest pores will be filtered or retained in accordance with the pore size disribution. Compounds which are protein bound will remain behind with the proteins unless they can be displaced from the binding sites with a competitor before filtration. Hence, ultrafiltration is a useful technique where it is sought to measure the free (and not protein-bound) fraction of a drug, though consideration must be given to the fact that the dynamic nature of the filtration process can disturb protein binding equilibria. The chances of an analyte adsorbing onto the inert membrane must be taken into account, particularly if it is present in trace levels. A major factor limiting the speed and effectiveness of an ultrafiltration process is the build up on the upstream surface of the membrane of a layer of protein

molecules which cannot traverse the membrane. This is called concentration polarisation. Since the layer serves to reduce the rate of ultrafiltration, control of the thickness of the layer is of major importance. If a high shear is not maintained at the membrane surface, this layer will increase in thickness until the flux rate drops to a very low level. Higher shear can be obtained by using either high flow rates across the membrane surface or with rapid stirring. Other problems asssociated with ultrafiltration are the fact that non-ideal membranes may allow a higher filtration rate for water rather than solute molecules, thus resulting in dilution of the drug concentration. The pH and temperature must be carefully controlled throughout the procedure.

2.3.3. Liquid-liquid extraction

Liquid-liquid extraction is a very widely used method for the preparation of biological samples for subsequent analysis, though the more recent on-line solid-phase extraction techniques are steadily gaining in popularity owing to the advantages they can offer in terms of selectivity, ease of operation and compatibility with automation and computerisation. Liquid extraction however, remains an extremely versatile method of sample preparation [33,34]; convenience and ease of use have contributed to the large popularity of the technique. The separations are usually quite clean because the relatively small interfacial area between the two phases helps to avoid effects analagous to the undesirable coprecipitation phenomena which can adversely effect precipitaion separations. In addition, liquid-liquid extraction permits concentration of the analyte.

Liquid-liquid partition is based on the extraction of an analyte depending on its partition between an aqueous and an immiscible organic phase. The partition coefficient (K) of the total analyte concentration is given by:

$$K = [A]_o/[A]_a$$

where $[A]_o$ and $[A]_a$ are the concentrations of analyte in the aqueous and organic phases respectively. Hence, the degree of solvent extraction is dependent on K, and since K is dependent on the type of organic solvent as well as pH and ionic strength of the solution, it is obvious that these must be optimised [35]. It is well known that repeated extraction (two or more times) with small portions of solvent can recover much more analyte than a single batch extraction, but for practical purposes, a biological sample would be extracted no more than twice in most applications.

2.3.3.1. Solvent selection

The solvent is selected to provide maximum extraction efficiency (large K) and minimum contaminants. Polarity is usually the most important factor in the choice of the extraction solvent, and generally as this increases, the range of compounds extracted also increases [34]. Hence, the solvent should be selected with minimum polarity consistent with high recovery of the drug. Drugs of high polarity are difficult to extract and require polar, and hence non-selective solvents. Chin and Fastlich [36] have listed a number of drugs that are extractable in the presence of sodium dihydrogen phosphate with diethyl ether, but not with hexane. Bailey and Kelner have investigated the extraction recoveries of acidic drugs from water and plasma with hexane, diethyl ether, toluene, n-butylchloride and chloroform [37]. As classes of compounds, the barbituates, sulphonamides and diuretics were optimally extracted with dietyl ether from plasma and water. Hexane is advised for the extraction of the tricyclic antidepressants because it gives cleaner blank plasma chroamtograms [37]. Diethyl ether, though it extracts a wide range of drugs introduces many interfering substances from the biological matrix. It will not extract some alkaloids, and chloroform is a more useful solvent in this case; a chloroform-isopropanol mixture is frequently required to extract amphoteric alkaloids such as morphine [38].

It is possible to select a mixture of solvents which, although it does not completely extract the drug, does give cleaner extracts. For example, barbiturates can be extracted from blood at pH 7.5 with a mixture of equal volumes of hexane and ether. The use of a more polar solvent or lower extraction pH substantially increases the co-extraction of interfering material [39]. Hexane mixed wih chloroform or ethyl acetate, and dichloromethane or chloroform mixed with alcohol are other common organic extractants. Van Damme et al. [40] have demonstrated increased recoveries of tricyclic drugs using hexane and dichloromethane if 5% isopropanol is added to the extracting solvent.

As well as having the correct polarity the solvent should be of the highest purity, non-toxic, not highly flammable, and should have a suitable volatility to permit evaporation and concentration of the analyte. It should be redistilled if the preservative or a trace impurity is found to react with the drug or produce an interfering peak on the chromatogram. The most popular solvents for extraction are diethyl ether and chloroform. Although ether is flammable, it has the advantages of being volatile which permits rapid evaporation following phase separation. Phase transfer is facilitated by its low density and the fact that it forms the upper layer when mixed with aqueous solutions. In addition, ether emulsions are easier to break. Solvents such as benzene and carbon tetrachloride should be avoided because of their toxicity; toluene, chloroform or dichloromethane should be used in preference.

It is only the unionised form of a drug which is extracted into the organic solvent, therefore suppression of ionisation is used to increase the hydrophobicity of a compound and promote its partition into the organic phase. Thus, acidic drugs are extracted under acidic conditions, and basic drugs are extracted under basic conditions. The optimum pH for acidic species is 1-2 pH units below their pK_a values, and for basic species it is 1-2 pH units above their pK_a values. The extraction of neutral

drugs is independent of pH; i.e. they are extracted over a wide range of pH, but they remain in the organic phase if a back-extraction into an alkaline or acidic aqueous medium is carried out. Where possible, a higher pH is often desirable to ensure cleaner extracts since many endogenous compounds are acidic and favour extraction at acidic pH.

Another useful method is pre-extraction of the interferents into an organic phase which is then discarded. This is then followed by an extraction tailored to remove the drug, possibly using a different solvent. Urine contains many endogenous compounds, and a preliminary extraction from acidic urine improves the purity of a subsequent basic extract. Sometimes, where K or the amount of drug present is large, the drug may be extracted into a very small volume of organic solvent (100 μl or less). After centrifugation, an aliquot of the organic phase is taken for analysis [41,42]. By eliminating the solvent evaporation step the possibility of drug loss by evaporation, decomposition or adsorption onto glassware is minimised, although this limits the sensitivity of the procedure. And while this approach is quick and simple, it has obvious shortcomings in HPLC where only a limited amount of non-polar solvents can be injected into an aqueous based-eluent stream.

2.3.3.2. Back extraction

By adjusting the pH of a new aqueous phase to that which will reionise the analyte, it can be back-extracted from the organic phase into the new aqueous layer. This should reduce the amount of neutral contaminants which will remain in the organic phase. For back extraction of basic drugs into an acidic aqueous phase, sulphuric and phosphoric acids are preferred to hydrochloric acid because many hydrochlorides are soluble in organic solvents. Either this solution is then used for separation, or more commonly, the pH of the aqueous phase is again adjusted to

suppress ionisation, and the analyte is re-extracted into a fresh aliquot of organic phase. This solvent is then evaporated and the residue reconstituted in the mobile phase. Back extraction is more or less mandatory in GC since extraneous substances in the initial extract tend to contaminate GC detectors, particularly the nitrogen-phosporous and electron capture detectors. This approach is also widely used prior to HPLC analysis as it greatly improves the selectivity of the solvent partition technique, though overall recovery of the drug can be reduced and it adds considerably to analysis time

2.3.3.3. Extraction by ion pair formation

A useful method for dealing with a highly polar ionic drug is to convert it into a neutral ion-pair complex by the addition of an excess of suitable ions of opposite charge, and extract it into an organic solvent. Common ion-pairing agents for acids are tetraalkylammonium salts (for example, tetrabutylammonium hydroxide or chloride), and for bases are alkyl sulphonates (for example, heptane sulphonic acid). Formation of the complex depends on factors such as the pH of the aqueous phase, the type of organic solvent, and the nature and concentration of the counter ion. The ion pair extraction technique is useful for a variety of ionisable compounds, but it offers particular advantages for compounds which are difficult to extract in the uncharged form such as penicillins, amino acids and conjugated metabolites. This method allows extraction of quaternary ammonium compounds such as tubocuarine which are ionised at all pH values. The application of ion-pair extraction has been discussed in detail by Schill [43], and Tomlinson [44].

2.3.3.4. Extraction by salting out

Extraction can be difficult for compounds which are soluble in water at all pH values, for example water-soluble amphoteric and neutral drugs. In some cases, a "salting out" procedure may prove

useful. The addition of buffer salts to an aqueous solution increases its ionic strength and hence its polarity. This tends to decrease affinity of polar compounds for the aqueous phase, and thus shifts the partition equilibrium in favour of extraction. Such an approach has been used by Horning et al. [45] in the screening of plasma and urine for acidic, basic and neutral drugs. By using ammonium carbonate as the salt and ethyl acetate as the extracting solvent, they were able to extract drugs with recoveries better than 80%. An advantage of salting out is that emulsion formation is minimised, as the combination of salt and solvent helps to form better phase boundaries and reduce the water content of the organic phase. It is clearly important that the chosen solvent and the biological fluid should be easy to separate after mixing. Polar solvents which are water-miscible (for example n-propanol) can be forced to form a separate layer using this salting out technique, resulting in a two-phase system with the upper layer consisting mainly of the organic solvent.

2.3.3.5. Solvent extraction following derivatisation

Compounds insufficiently soluble in organic solvents can be extracted following preparation of derivatives. Derivatisation, however, is more commonly used to improve detectability rather than solubility of of the analytes, though in certain cases derivatisation is necessary to release analytes from their protein binding sites. The trans-esterification of plasma bilirubin mono- and dimethyl esters is an example [46].

2.3.3.6. Extraction of metabolites

Metabolites of a drug are usually more polar than the drug itself, and if it is sought to measure the metabolites, then the chosen solvent should be capable of extracting all the compounds of interest so that they can be subsequently separated and

determined chromatographically. However, extraction with solvents of increasing polarity, and/or at different pH values can achieve selective separation of a drug from its major metabolites. Another method for extracting the more polar metabolites is to extract the drug, then add a high concentration of a salt, such as sodium chloride to the aqueous phase which shifts partition in favour of extraction and forces the metabolites into the organic phase. This salting out procedure has been used in the extraction of hydroxy metabolites of barbiturates from urine [34].

Occasionally the parent drug and metabolites are of similar polarity and are co-extracted. Separation from metabolites may be achieved by partitioning between an organic solvent and a buffer of appropriate pH, though absolute specificity is difficult to achieve and co-extraction of metabolites is inevitable. Separation of a parent drug and its metabolites is usually achieved by chromatographic separation, and HPLC is generally the method of choice [47,48]. Martin and Reid have reviewed the isolation of metabolites by solvent extraction and other methods [49].

2.3.3.7. Problems with liquid-liquid extraction

Solvent extraction is relatively time-consuming. It often requires the removal of solvents by evaporation. This lengthy step may also lead to decomposition of unstable compounds, particularly when heating is required. Losses can also occur at the re-dissolution stage [40]. Another factor which must be considered here is the solvent used to reconstitute the residue following evaporation. Van Damme et al. [40] demonstrated the effect on peak shape of the percentage organic solvent in a solution of barbiturates. They found that as the percent acetonitrile in the dissolving solvent was increased, there was a deterioration in peak shape and peak area. Another problem associated with solvent extraction is adsorption of analytes onto glassware which can occur at the extraction or solvent

evaporation stage. Adsorption is much more noticeable when a drug is present at low concentrations. This problem may be partially overcome by silanisation of glassware, by the inclusion of 1-2% ethanol, isopropanol or iso-amyl alcohol into the extracting solvent, or where possible, the use of polypropylene test-tubes. These measures are, however, not always effective, and others may have to be devised [26].

2.3.4. Solid-phase extraction

Solid-phase extraction is a technique which can circumvent many of the problems associated with liquid-liquid extraction. The general approach to solid-phase (liquid-solid) extraction is the adsorption of the drug from the aqueous fluid onto a solid adsorbent. Solid-phase extraction using silica, alumina, celite, talc, charcoal, ion exchange or hydrophobic resins has long been common practice in the clinical laboratory. The development of HPLC, however has lead to new methods in the solid-phase extraction technique. Silica gels bonded with a wide range of functional groups, eg alkyl-, phenyl-, cyano- and diol- moieties provide specific interaction with analytes. Simple procedures involve the addition of the solid to the fluid, agitation and separation by centrifugation. However the most satisfactory approach is to pass the fluid over the adsorbent packed in a short column, referred to in this text as the "pre-column" or the "concentration column".

2.3.4.1. Methods used in solid-phase extraction

The materials used for solid phase extractions fall into two broad categories according to whether they retain all the sample, or only the drugs and related compounds. In the first category, hydrophilic packing materials such as inert particles of diatomaceous earth (kieselguhr) are used to adsorb the sample, including water, over a large surface area. The sample forms a thin aqueous film over the surface of each particle. A small volume of water-immiscible organic solvent such as chloroform is

then passed through the column, and this effectively extracts the drug from the aqueous film of sample. Water and endogenous materials, such as pigments and other polar compounds are retained in the adsorbed phase. This method is essentially one of liquid-liquid extraction, so it can be optimised using the same approaches discussed in that section.

The second, and most currently relevant, method is based on chromatographic principles. Compounds of interest are retained on the adsorbent when samples are passed through. Certain undesirable compounds which are adsorbed at this stage may be removed by washing with a specific solvent. The compounds of interest are then eluted by washing the column with an appropriate buffer or solvent. The elution solvent may be miscible or immiscible with water because (where it is used) little sample water remains on the column. The eluate is processed further or introduced directly onto the the HPLC column

The chromatographic method of solid phase extraction is usually carried out in small columns packed with material similar to that used for analytical separations. These short columns have a number of applications apart from simple extraction purposes.

2.3.4.2. Uses for short or pre-columns

The use of pre-column is multifunctional. For instance, extraction columns are used in biopharmaceutical analysis to isolate drugs from biological matrices which which would cause rapid deteoriation if introduced onto the analytical column. In this respect, they perform the function of sample clean-up. Another function which can be carried out using a pre-column is that of trace enrichment. For compounds with a strong affinity for the sorbent used, adsorption will take place in a small segment of the sorbent bed and large amounts of analytes are retained. This function finds somewhat wider use in the analysis of large volumes of dilute aqueous samples, but the same

principle can be applied to drug analysis: a large volume (up to 1 ml) of a biological sample may be injected onto the pre-column, and if the eluting solvent for the drug is kept small, for example 100 μl, then a 10-fold concentration, or enrichment is obtained. Due to the relatively inert character of many of the sorbent materials, there is a good chance that a compound in the adsorbed state will remain unaltered for a prolonged period of time. Stabilisation of otherwise rather unstable compounds is useful when storage of samples is required. Pre-columns may also be used to carry out derivatisation reactions, and lately they have been employed in immunoassay techniques where the antigen-antibody complexation reaction takes place on an adsorbent within a column.

Solid-phase extraction may be performed off-line or on-line using a switching valve to channel the eluate from the pre-column either to waste or onto the analytical column. These two methods of solid phase extraction will be discussed separately, with most attention being given to the column switching technique which was used during some of this work.

2.3.4.3. Off-line solid-phase extraction.

Extractions of this type are often conducted with home-made columns, for instance, a Pasteur pipette packed with adsorbent. However disposable cartridges of various sizes (up to 20 ml) are available commercially, for example from Waters Associates (Sep-Pak), or Analytichem International (Bond-Elut). These columns have a high sample loading capacity, but are for single use only, since components retained on the column from a previous sample could affect performance in subsequent use. The most popular and useful bonded phases are the reversed phases, commonly, octyl-(C_8-) or octadecyl- (C_{18}-) silica. This is due, at least in part to their versatility; all the methods described for the liquid-liquid partition of an analyte from an aqueous phase into an organic phase are also applicable to

solid-phase extraction with C_8- or C_{18}- bonded silica. Furthermore, some workers [50,51] have shown that C_{18}-bonded phase disposable columns can be regenerated and used again without any loss of performance. Drug retention by ion exchange has been applied to highly polar, ionisable drugs such as gentamycin, which are not extracted by hydrophobic packings [52]. The clean-up of gentamycin has also been carried out using unmodified silica [53]. The mechanism here is also one of ion-exchange involving the positively charged gentamycin and the negatively charged silanol groups. Charcoal and XAD resins are popular for screening of drugs of abuse in urine; XAD-2 has been used as an extraction medium prior to the determination of methaqualone in plasma by HPLC [54].

A more novel concept is the use of internal surface reversed phase packing materials which were developed by Hagestam and Pinkerton [24]. These surfaces present a hydrophilic external surface, while the internal surface (within the pores) retain a more hydrophobic character. When a biological fluid, such as serum or plasma, is injected onto a column packed with this solid phase, the macromolecules will only come into contact with the hydrophilic surface and will elute rapidly, whereas the small analyte molecules will penetrate into the pores where they encounter a more hydrophobic surface upon which they are retained. Large diameter (37-74 μm) particles of this type have been used in a pre-column to retain phenytoin [55]

A typical liquid-solid extraction procedure with reversed-phase cartridge is as follows:

(i) activate the cartridge with methanol;

(ii) equilibrate the cartridge with water or a buffer of controlled molarity and pH, with or without an ion-pairing reagent;

(iii) load the sample onto the cartridge directly, or following centrifugation, dilution with water or buffer, pH adjustment, ion pair formation, derivatisation or complexation;

(iv) wash the cartridge with an appropriate eluent to remove early eluting impurities;

(v) elute the analyte selectively with a solvent mixture optimised for its recovery, leaving late-eluting compounds on the solid phase;

(vi) inject an aliquot of the eluate with or without concentration by evaporation.

The cartridge may be treated with a solution containing a masking agent (for example triethylamine or ammonium acetate) following activation with methanol. This supresses the ion-exchange properties of residual silanol moieties on reversed-phase packings, and so produces more reproducible extractions. Unreacted silanols can have a deleterious effect on reversed-phase extractions in the same way as they adversely affect chromatographic efficiency in reversed-phase HPLC separations.

2.3.4.4. On-line solid-phase extraction

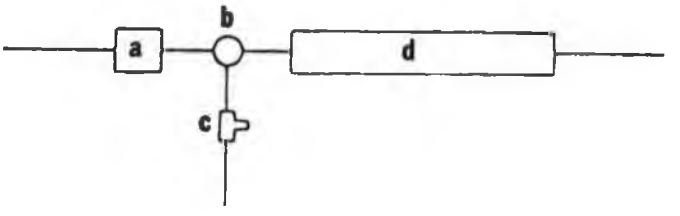
2.3.4.4.1. Single-pump systems

Apart from its protective function, a guard or pre-column is often used to carry out on-line solid-phase extraction. In its simplest form, the pre-column is mounted in place of the injector loop, and the various operations of column conditioning, column preparation, sample loading and sample clean-up are effected by manually injecting the appropriate solvent onto the column while the injector is in the "load" position. By switching the injector valve to the "inject" position, mobile phase eluent

is introduced onto the column whence it desorbs the retained compounds and sweeps them onto the analytical column where they are separated. This method has been applied to the analysis of tricyclic drugs in serum using a C_2- clean-up column [56], and to the analysis of aminopyrine and metabolites in plasma using a C_8- clean-up column [57]. In the latter work, the authors claim a high degree or reproducibility, and recoveries approaching 100% for aminopyrine and all its metabolites. The above method employs only one high pressure pump, and does not call for a switching valve. In this respect, it is less capital cost intensive, though it has obvious drawbacks in terms of ease of operation and the potential for automation. Furthermore, as pressure builds up on the pre-column, it becomes difficult to introduce successive injections of sample and washing solutions, and unless the volume of sample is kept low, it is necessary to change the pre-columnn quite frequently.

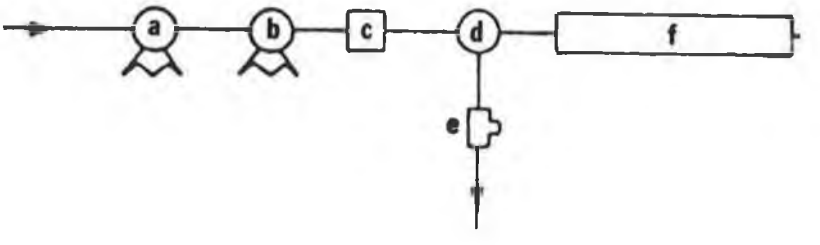
An upgrading in the sophistication of this method was the development by Wahlund [58] of the so-called pre-column venting technique which introduces a three port valve in order to separate the pre-column from the analytical column (Figure 2.4.). The pre-column serves the dual function of acting as a guard column as well as effecting pre-separation of the analytes from the biological fluid matrix. This is achieved due to the fact that the early-eluting matrix components (which are most damaging to the analytical column) are eluted quickly off the pre-column and vented to waste while the compounds of interest are swept onto the analytical column and separated. This approach is well suited to systems where the eluent contains a low percentage of organic modifier; at high concentrations of organic component, proteins present in the biological sample would be precipitated within the tubing or on the columns. A further development on this theme, aimed at reducing the influence of organic modifier is the pre-column venting plug technique [59]. This system is shown in Figure 2.5. and includes a second injection valve with a large loop containing 2 ml of buffer. The biological sample is injected into the centre of this buffer plug which effectively

Figure 2.4.
Pre-column venting system.



(a): pre-column; (b): Three-port valve; (c): Pressure regulator; (d): analytical column.

Figure 2.5.
Pre-column venting plug system.



(a): Plug fluid injector; (b): Sample injector; (c): Pre-column; (d): Three-port valve; (e): Pressure regulator; (f): Analytical column.

serves to isolate it from the hydro-organic mobile phase. An acidic (pH 2) phosphate buffer was found to be the best fluid regarding column stability which was greatly prolonged by this principle. It was applied to the quantitation of lidocaine (lignocaine) and its two main metabolites in plasma [59], producing inter-assay precisions of <2% for therapeutic concentrations of the compounds. The limitations with this type of arrangement are that flow to the analytical column is interrupted during the venting operation, and desorption of the retained compounds can only be done in the forward-flush direction. Forward-flushing of the pre-column can contribute significantly to band broadening.

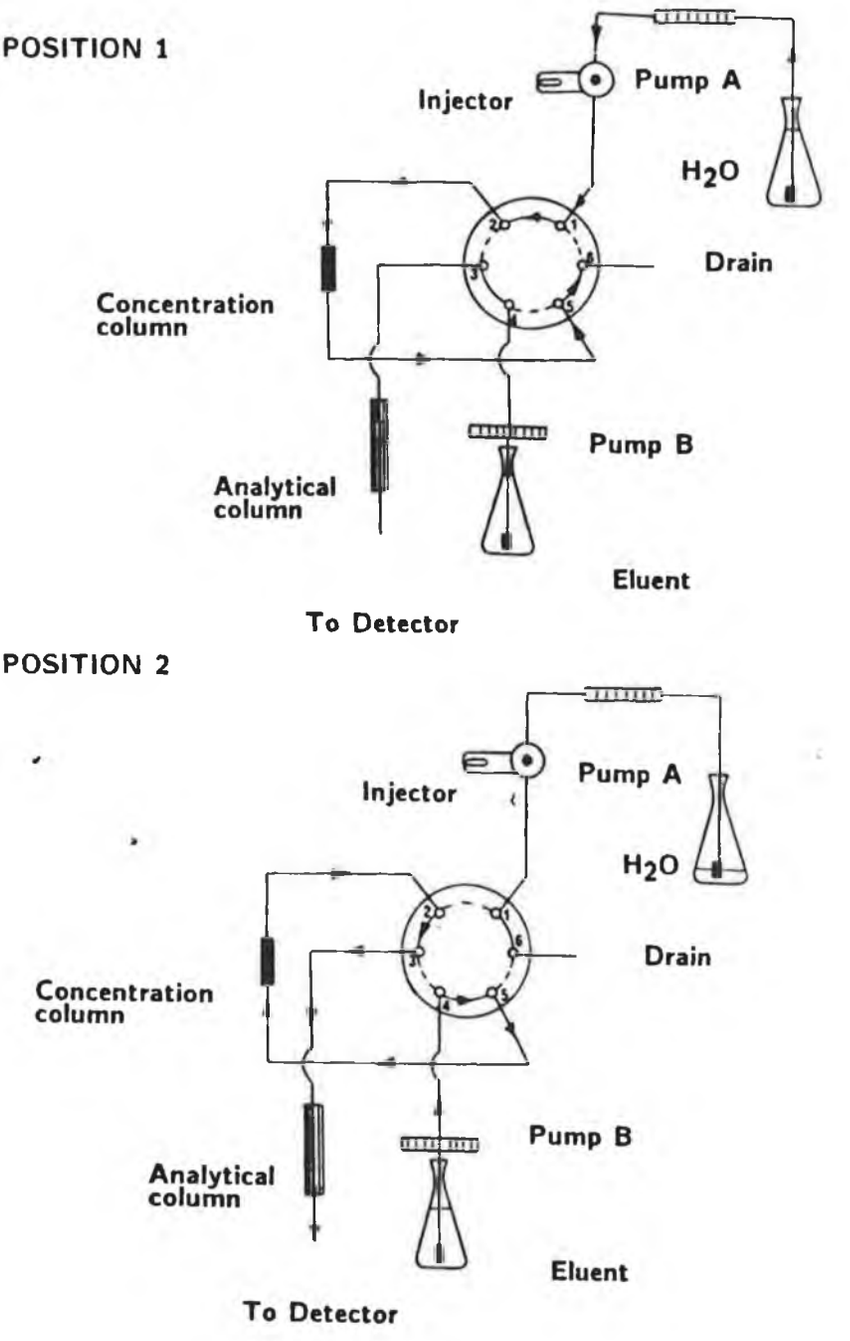
2.3.4.4.2. Multiple-pump systems

A typical scheme for an on-line sample clean-up procedure incorporating two pumps and a six-port switching valve is shown in Figure 2.6. This is the instrument arrangement which was used in all column switching operations in the present work (Chapters 4, 5, 6 and 7).

Pump A is used to deliver the washing solvent, which is usually water or buffer, though a small percentage of organic solvent is sometimes added. The mobile phase eluent is delivered by pump B. When in position 1, washing solution is passed by pump A via the injector and the valve onto the concentration (pre-) column. Meanwhile, mobile phase is being pumped by pump B via the valve onto the analytical column, which is thus maintained in a state of constant equilibration. Injections are made when the valve is in this position. An injected biological sample, for example, is swept onto the pre-column by the washing eluent, whereupon the polar matrix components are eluted to waste and the compounds of interest are selectively enriched on top of a judiciously chosen adsorbent. After a pre-determined wash time, the valve is switched to position 2, which causes the mobile phase to be re-routed onto the concentration column where it desorbs the

Figure 2.6.

column switching arrangement incorporating six-port switching valve.



Injections are made with the valve in position 1. Analytes are desorbed from the concentration (pre-) column when the valve is in position 2. Operation is described in the text.

retained compounds and flushes them onto the analytical column. While this is happening, the washing eluent is passing through the valve and thence to waste.

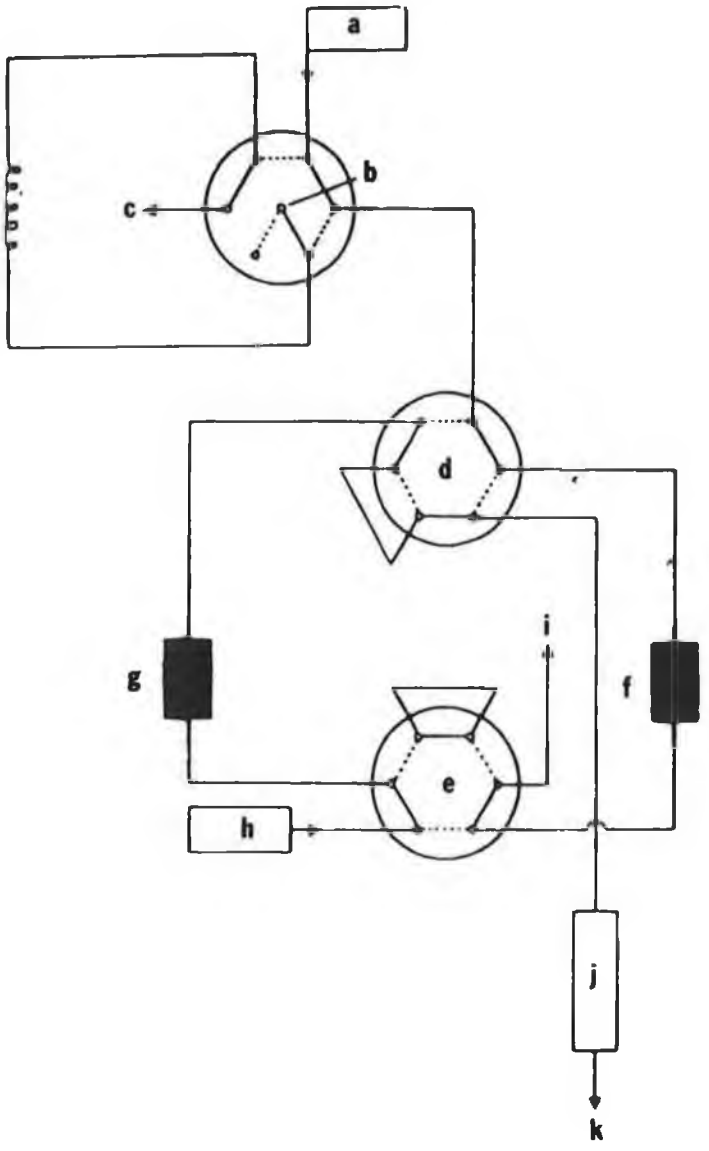
A paper by Roth et al. on "Fully Automated High Performance Liquid Chromatography" reported on a system which contained two pumps, one for the washing solvent, and one for the mobile phase,in conjunction with two switching valves and two pre-columns [60]. The instrument arrangement for an alternating pre-column system is shown in Figure 2.7. The most attractive feature of this arrangement, apart for the the possibility of injecting biological fluid directly without pre-treatment, was the so-called alternating pre-column sample enrichment technique. This involved an efficient and time-saving operation of the analytical column. The sample was injected and loaded onto the first precolumn, and after a purge phase, the analytes were backflushed on to the analytical column. Meanwhile, a new sample was injected onto the second pre-column, and while the first sample was eluted on the analytical column, the second sample was purged and backflushed into the mobile phase solvent stream. While the contents of the second pre-column were being separated on the analytical column the first pre-column was switched back to the solvent stream of pump A, the washing solvent, which removes traces of organic solvent and prepares the pre-column for the next injection.

2.3.4.4.3. Multi-dimensional chromatography

Gradient elution analysis is frequently used for separating sample components with a wide range of capacity factor values. This situation, referred to as the "general elution problem", can also be solved by multi-dimensional chromatographic techniques. Both methods lead to an optimisation of resolution in minimum analysis time. Multidimensional chromatography is where the sample is separated by switching between two or more analytical columns posessing separate, but complementary separation

Figure 2.7.

Alternating pre-column switching system for sample enrichment and backflush.



(a): pump A; (b): injection valve; (c): to waste; (d): switching valve 1; (e): switching valve 2; (f): pre-column 1; (g): pre-column 2; (h): pump 2; (i): to waste; (j): analytical column; (k): to detector. Operation is described in the text.

characteristics. Technically speaking, column switching between a short concentration column for the purpose of sample clean-up (as described above) and the analytical column for separation also constitutes multidimensional chromatography, but for the sake of simplicity the term "multidimensional chromatography" in the present discussion shall be understood to mean the employment of two or more analytical modes subsequent to extraction.
A typical operation would consist of clean-up on a concentration column, followed by transfer of the remaining adsorbed materials onto a second column, which is usually the first analytical column. A zone from this column is then isolated and transferred to the second analytical column, where separation is continued, frequently in a different mode, but often using a mobile phase with greater elution strength on the same type of column. This process where a portion of the effluent from one column is transferred to another is known as "cutting". When the central portion of a chromatogram is isolated for further analysis, the technique is known as "heart-cutting", and it was first described by Deans in 1968 [61].

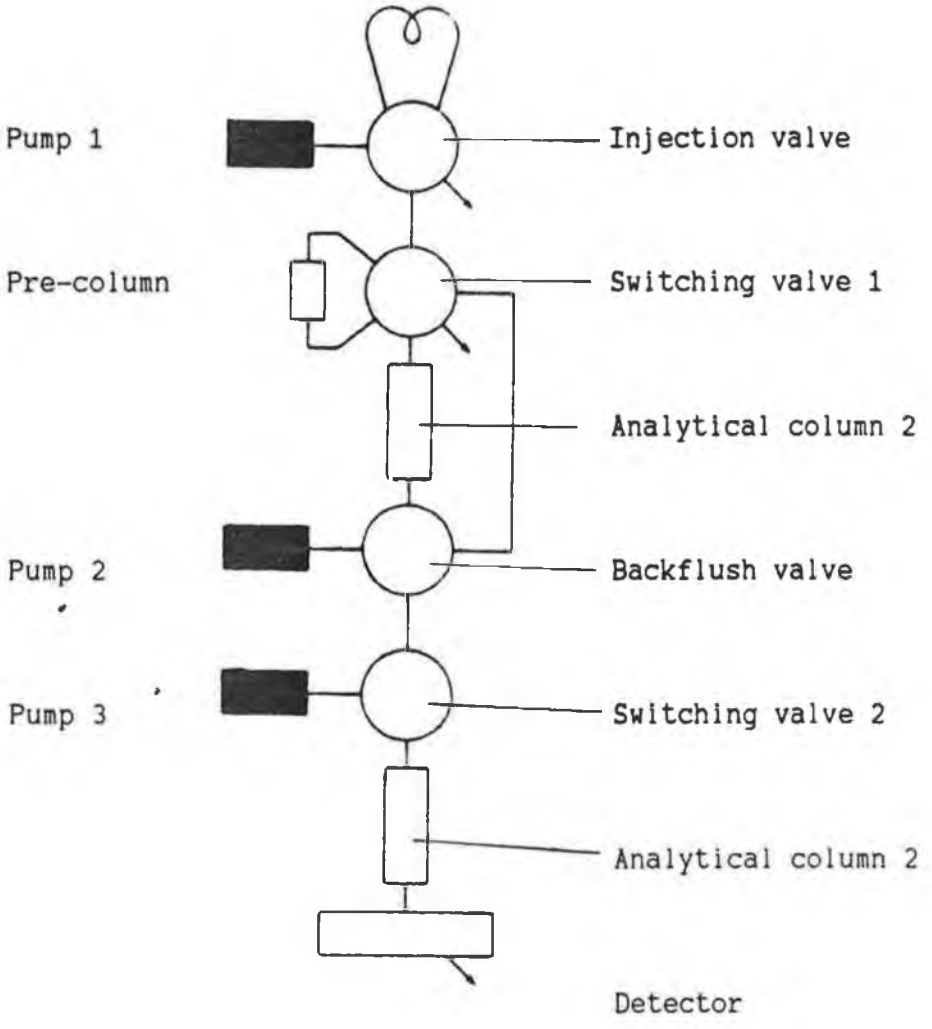
An example of multi-dimensional chromatography is the on-line combination of high performance gel permeation with reverse-phase chromatography which was first described by Johnson and co-workers [62]. Gel permeation was carried out on a cross-linked polystyrene column with tetrahydrofuran as the mobile phase. The problem with this system was that water-soluble samples could not be handled. An advance on this arrangement was described by Erni and Frei [63], in that porous glass particles were used as the exclusion media and thereby permitted the use of complex aqueous samples. With the development of microparticulate (10 μm) aqueous exclusion columns, improved elution profiles are obtained from the first column. The combination of aqueous exclusion chromatography and reversed phase chromatography is ideal from the standpoint that the solvents used in both techniques are compatible; the predominantly aqueous mobile phase used in exclusion chromatography is a weak mobile phase in reversed-phase chromatography, and therefore has little effect on the analytical separation.

Apparatus requirements for multi-dimensional liquid chromatography can be approached at various levels of cost. If a between-column sample valve is used for heart-cutting, two complete chromatographic systems are required. If it is desired to perform heart-cutting using an intermediary column, then three pumps, columns and valves are required. In principle, the heart-cutting operation can be repeated in a variable number of columns, though if extended to many columns, the cost would become prohibitive.

A typical instrument arrangement for multidimensional chromatography incorporating three columns) one clean-up, two analytical) is shown in Figure 2.8. The sample is introduced via the injector and swept onto the pre-column by pump 1 eluent. The analytes are selectively retained while most of the impurities are eluted to waste. After a pre-determined wash period, the flow of pump 2 is directed for a finite period of time onto the pre-column by switching the position of the first switching valve. The stronger pump 2 eluent desorbs retained analytes from the pre-column and sweeps them onto the first analytical column where separation takes place. In what constitutes a heart-cutting operation, the section of interest in the resulting chromatogram is transferred via the second switching valve onto the second analytical column using the mobile phase from pump 2. When transfer is complete, valve 2 is returned to its original position, and mobile phase from pump 3 is introduced onto the second analytical column, where the final separation takes place. While this is occurring, mobile phase from pump 2 is re-routed into a reverse direction. This backflush operation rinses the first analytical column and the pre-column, and slowly moving compounds are backflushed from the top of the columns. After a pre-set time, switching valve 1 is returned to its normal flow direction, and re-conditioning of the pre-column by pump 1 eluent can then take place. The analyte bands are resharpened by the choice of the stationary phases and mobile phases in

Figure 2.8.

Schematic diagram of coupled column system.



Operation is described in the text.

the order of increasing elution power from the pre-column to the second analytical column. Hence, as solvent 2 is a weak solvent for the second analytical column, a large volume of mobile phase 2 can be introduced onto the latter column to concentrate the sample at the head of this column. This is an example of on-column concentration. A detector may be positioned between the first and second analytical columns. This is not an essential component, but is a useful feature when analysing complex chromatograms where it is important to locate precisely the peaks of interest in order to minimise sample loss and contamination. When a detector is not used to control the switching process, a sequential timer can be used to automatically switch the peaks of interest.

This arrangement may be automated by the incorporation of pneumatically actuated valves and a microprocessor control system. Automation improves reliability, sample throughput, analysis time, and minimises sample loss, since analysis is performed in a closed-loop system. Phase exchanges from aqueous mobile phases to organic mobile phases, and vice versa in multidimensional liquid chromatography, may be realised by the incorporation of a purge-and dry sequence. Furthermore, a new chemical dimension may be introduced by the incorporation of a chemical derivatisation step between the first and second analytical columns.

This type of system may also be used for boxcar chromatography which is an advanced column-switching technique placing multiple samples simultaneously onto the analytical column for increased sample throughput [64]. This mode overcomes the objection to liquid chromatography that it is limited in its throughput, because analysis rates as high as 50 samples per hour can be achieved with columns of conventional length [27], and maximal frequency of injections is only limited by the resolution capability of the column. Nazareth and co-workers applied this

arrangement to the analysis of phenobarbitone and primidone in serum [65]. They found no memory effects from the previous sample, achieved 100% transfer between the second and third columns (ie, the first and second analytical columns), and were able to execute up to 40 samples per hour.

2.3.4.5. Some technical considerations for column switching operations

2.3.4.5.1. The valve

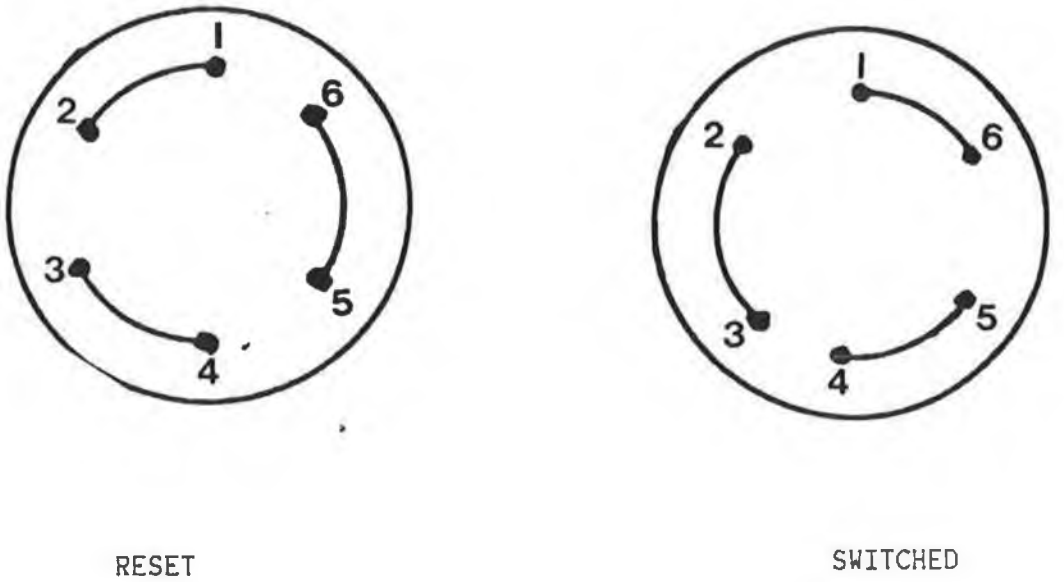
The majority of published methodology uses a six port two-way valve, obtainable from several manufacturers. The essential features of the six-port valve are as follows:

(i) there are two independent flow-paths through the valve, which enables the simultaneous cleaning and equilibration of one column whilst the other is being used; these flow paths are depicted in Figure 2.9.
(ii) the second solvent line can be backflushed.

Column-switching is more readily carried out with a ten-port multifunctional valve which is able to accommodate sample injection and solute switching in the same device for most applications. The more valves that are used, the more flexible the system becomes, particularly for multi-column separations and where it is necessary to monitor the eluent through various stages of the system. Each valve must be capable of high pressure operation without deterioration, and provide low dead-volume sample path so as not to significantly broaden the peaks which pass through it.

The arrangement may be plumbed in such a way that the contents of the concentration column are forward- or back-flushed onto the analytical column. Back-flushing is preferable since it will not exacerbate, and may actually reverse, band broadening which may have taken place during the wash step. It does have the

Figure 2.9.
Flow paths in a six-port switching valve.



In the "reset" position ports 1 and 2, 3 and 4, and 5 and 6 are connected. In the "switched" position ports 2 and 3, 4 and 5, and 6 and 1 are connected.

disadvantage, however, of disturbing the column packing in the concentration column by applying mobile phases alternately in opposite directions.

2.3.4.5.2. Pre-column design

The subject of pre-column design has been discussed in detail by Goewie et al [66]. They recommended that for practical purposes, pre-columns having an inner diameter of 2-4.6 mm and a length of 10 mm be used. This design allows high sampling flow rates with little pressure drop across the system. Within this range, the pre-column does not appear to be critical in the on-line trace enrichment of strongly-retained analytes. Short columns are, however preferable to longer ones. The latter, which must be packed under high pressure may have high efficiency to start with, but this efficiency deteriorates rapidly and good performance is only obtained in the backflush mode. Furthermore, long columns generate quite a substantial pressure drop, posess a large dead volume and can be expensive to buy and repack.

Nazareth et al. [65] investigated the effect of using screens versus porous frits on pre-columns used for extraction purposes. They found that using a 0.2 mm retainer screen backed with a 2 μm filter screen yielded higher eventual plate numbers on the analytical column than if 2 μm porous frits had been used. The importance on the use of screens instead of frits to prevent sample blockage has also been addressed by Roth [67].

2.3.4.5.3. Packing materials

It is generally agreed that the retention of the compounds of interest on the pre- or concentration column should ideally be similar, or even lower, to that on the analytical column in order to prevent band broadening problems during the desorption and actual separation step. However, there seems to be some disagreement as to which particle size range (for example 5-10 μm porous or 37-50 μm pellicular) is the most appropriate for use in

the pre-column. Roth and Beschke [68] investigated the use of commercially available reversed-phase materials of various particle diameters, and concluded that particles in the size range 30-50 μm are best suited for packing concentration columns. Furthermore, Nazareth et al. [65], working with serum and using a pre-column packed with 5 m particles, observed a large increase in back-pressure across both the extraction and analytical columns after less than 10 ml of serum had been injected. This increased pressure drop was accompanied by a reduction in plate number (indicating a deterioration in column efficiency). By switching to particles of diameter 30-70 μm, they found that back pressure and plate count remained satisfactory for longer periods of time. In addition, they found that the use of pellicular as opposed to large-diameter porous packings can significantly reduce recovery of a drug; the recovery of primidone from C_{18} pellicular packing was only 5% whereas 30 μm porous C_{18} material yielded greater than 90% recovery for the same drug [65].

Werkhoven-Goewie et al. [69] assert that HPLC-quality sorbents, i.e, materials having 5-10 μm particle diameter should be used. They based their results on work carried out on column capacity and band broadening of chlorophenols in water at high sampling rates (5 ml/min) over long periods. They found that not only were the column capacities much lower, but band broadening was much greater with the larger particles. While these findings may be worth considering in biopharmaceutical applications, factors such as blockage of small-particle columns by highly viscous biological media almost always demand the use or large-particle diameters. Disadvantages with the use of large particles, the most significant of which is band broadening can be minimised by using a short pre-column, operating in the backflush configuration, and employing low dead volume fittings to reduce band broadening from extra-column sources.

2.3.4.5.4. Washing Solutions

One of the main problems with a biological sample that contains metabolites of differing polarity and lipophilicity, is the choice of an appropriate solvent system so that the various drugs are retained on the pre-column, while the bulk of unwanted plasma constituents are washed through. Roth and Besche [68] studied the effect of using both water and 1% ammonium acetate on the recovery of oxazepam and endogenous interferences from dog urine. Their results show that with water as the washing solution, most of the polar compounds were removed from the C_{18} pre-column, while the drug was retained. When ammonium acetate was used, however, all UV absorbing compounds were retained and the drug peak was obliterated.

Nazareth et al. [65] also varied the composition of the washing solution and found almost identical recovery for a number of barbiturates using either a water or buffer pH 3.5 wash; phenobarbitone was the only compound studied which had zero recovery with water, but almost full recovery with buffer. Nazareth concluded that phenobarbitone (pK_a = 7.3) was ionised in water but unionised in the buffer, and therefore retained on the non-polar pre-column. This approach did not appear to affect the blank serum profile unduly, though the injection volume was only 20 μl. As many interfering compounds of biological origin are acidic, one would expect them to be retained on a non-polar column when the wash solution is buffer of low pH. This was found to be the case in the present work as will be discussed in further detail in chapter 4.

2.3.4.5.5. Breakthrough volumes and sample loading capacity

The breakthrough volume is defined as the volume of sample that can be passed through a sorbent bed before elution of the investigated compound will commence. The larger the breakthrough volume the greater the enrichment factor which can be obtained. The minimum size of the pre-column is a function of the absolute

detection limit of the solute, its concentration in the original sample solution, and its retention, i.e. its capacity ratio k', in the stationary phase system under investigation. These aspects should be considered when conditions are selected so that the solute of interest is efficiently pre-concentrated without the occurrence of breakthrough phenomena. For the pre-concentration of low polarity compounds, only a very small volume of a hydrophobic adsorbent is required to retain large volumes of aqueous samples. The situation becomes more problematic when dealing with medium to high polarity compounds such as the more polar metabolites of low polarity parent compounds.

Pre-concentration based solely on hydrophobicity is often not sufficient, and breakthrough can occur after only a fraction of a millilitre of sample has deposited. In such cases, it becomes necessary to study the breakthrough behaviour of the analytes of interest on a particular adsorbent. What this means in terms of drug analysis, is studying the volume of washing solvent which can be used following injection before the sorbed drugs begin to elute. This can be carried out by measuring peak height on the analytical column after valve switching as a function of wash volume. If an analyte has a very low breakthrough volume on a particular adsorbent, then it is usually necessary to switch to another type of adsorbent, subject to restrictions in regard to the retention of unwanted materials.

Care should be taken to avoid over-loading the pre-column. In biopharmaceutical analysis it is not usually the overloading of drug which is a problem, but saturation of the pre-column with biological interferents. Such occurrences are manifested by a reduction in chromatographic efficiency and an increase in back pressure, and arise more rapidly if the sample is very viscous. Dilution and/or centrifugation can be used to reduce the consistency of the biological sample.

2.3.4.6. Conclusion

The solid-phase extraction technique is becoming a serious challenger to conventional solvent extraction. It is simple to perform and saves in time and labour. There are no losses due to emulsion formation or evaporation, and efficiency and reproducibility are as good, or better than those of liquid-liquid extraction. Solid-phase extractions may be performed on compounds which are difficult to extract at any pH, for example antibiotics, and it has a much wider scope for automation than could be practically realised with liquid-liquid techniques.

2.4. PRACTICAL CONSIDERATIONS IN BIOPHARMACEUTICAL ANALYSIS

2.4.1. Storage of samples

Collection of an uncontaminated sample at the correct time in relation to the dose is vital in any projects involving drug analysis. If insufficient care is taken in collection and handling of biological specimens, data generated by using even the most sophisticated techniques may be invalidated. Considerable time may elapse between the collection of a sample and the analysis, and changes occurring during storage can be important. A common source of error is the adsorption of the drug by the container. Furthermore, some drugs can undergo decomposition, and temperature, pH, salt concentration and light can be involved. Thus, the stability of the drug in a biological fluid should be determined at various temperatures so that the effect of storage on the ultimate analysis can be established. This is especially true if samples arrive irregularly and assays are done on a batch basis. Fresh plasma or serum samples can usually be kept for 6 hours at room temperature, or for 1-2 days at 4°C. For longer term storage, samples should be held at -20°C. For instance, Jonkman *et al.* [70] have shown that theophylline is stable in blood for one day at 25°C, and stable for one month at -20°C. Metabisulphite is sometimes added as a preservative to prevent the oxidation or photo-oxidation of drugs during storage, for example in the photodecomposition of LSD [71].

Changes to the biological material itself can occur as a result of putrefaction during storage, with the appearance of endogenous (breakdown) products which can interfere with the analysis. Bacteria in decaying biological material have been shown to cause the reduction of the nitro group in nitrazepam to an amino group [34].

2.4.2. Sampling

2.4.2.1. Analysis in blood, plasma and serum

The choice must be made as to whether whole blood, plasma or serum is to be analysed. In drug analysis, the most commonly sampled body fluids are plasma and serum because a good correlation between drug concentration and therapeutic effect is usually found [34]. The analyst could, however, be faced with no choice if the samples are haemolysed on arrival at the laboratory. Most drugs are concentrated in the plasma, yet there are a significant number which are concentrated in erythrocytes, such as clorthalidone or cyclosporin. Assays for such drugs will show enormous differences depending on whether analysis is performed on whole blood or on plasma, and if plasma or serum is analysed, the expressions "blood samples" or "blood levels" should not be used to describe the analysis of their concentrations.

On no account should the blood sample be frozen without treatment, because this would cause haemolysis. It is essential to avoid this, since haemolysis prevents subsequent separation of plasma or serum. After collection of blood (5-10 ml), a clot can be allowed to form, and the supernatant collected after centrifugation is serum. Serum contains no fibrinogen, a protein involved in the clotting process, and coagulation is complete in about 30 minutes at room temperature. Alternatively, the blood can be collected in a tube containing an anti-coagulant, and the supernatant which remains is plasma, and it does contain fibrinogen. Since the anticoagulant effect is temporary, collected specimens should be centrifuged quickly to avoid eventual clotting. Plasma is more frequently used than serum in drug analysis since the collected specimen can be centrifuged immediately, whereas the formation of serum is more time-consuming. Moreover, it is relatively easy to centrifuge blood which has been treated with anti-coagulant, as the plasma separates quickly and the maximal volume can be recovered if

required. With the use of plasma, temperature can have a marked effect on the recovery. For example, plasma phenytoin levels increase by 10% when a sample is equilibrated and centrifuged at 4°C rather than 24°C [72]. In addition, the nature of the anticoagulant may dramatically affect results, particulary in relation to the presence of endogenous interfering peaks. Rapaka et al. [73] found that heparin caused the introduction of interference peaks in the chromatographic analysis of frusemide with fluorescence detection. Vacutainer tubes containing EDTA as the potassium salt resulted in no interfering peaks using both fluorescence and ultraviolet absorption detection. Containers with sodium oxalate as anticoagulant caused interference with UV detection, but not with fluorescence detection. Heparin has been shown to interfere with the immunoassay of gentamycin and tobramycin [74].

The effect of plasticizers in the body and stoppers of blood collection tubes on plasma levels of collected specimens is well known [72,75-77]. The presence of the plasticizer tris (butoxyethyl) phosphate in stoppers has been shown to cause redistribution into erythrocytes and distortion of the apparent concentration of the drug in plasma [76]. Drugs which were affected in this way include quinidine, propranolol, lignocaine, alprenolol, some tricyclic antidepressants and some phenothiazines. Other workers established a reduction of the apparent plasma levels of carbamazepine and disopyramide in one brand of a collection tube bearing a stopper containing this plasticizer [77], but no reduction with the same type of collection tube produced without this plasticizer. Collection tubes may also be a source of contaminants which can appear on the chromatogram, making quantitation of the compound of interest difficult or impossible. Some workers found that contaminants from plastic collection tubes caused interference with the analysis of cyclosporin in whole blood and plasma [78]. These interferents were not detected in various kinds of glass tubes. Other workers [79] co-extracted drugs and interferences from plastic extraction tubes, and found that the presence of

interferences originating from the plastic container body increased the coefficient of variation of determinations of 25 commonly administered drugs from 5% for samples collected in glass collection tubes, to >20% for samples collected in plastic tubes.

Many types of contaminants can be encountered in samples; these include, (apart from plasticizers) antioxidants, pesticides, food additives, and vulcanising agents. The problem of these interferents can only be minimised by careful manipulation of the sample. The need for close liasion between the analyst and the person taking the sample is most important, and a typical problem which can arise as a result of lack of communication in this respect, is contamination of a blood sample by a local anaesthetic while a catheter is being inserted.

Frozen samples of plasma or serum should be brought to room temperature and subjected to vortex mixing for 10 seconds to ensure homogeneity prior to analysis.

2.4.2.2. Analysis in other biological media

Urine analysis is useful where a drug or rapidly-formed metabolite is extensively excreted in it. Drug metabolites can be detected in urine for quite some time after they have become undetectable in blood. Analysis of drugs in urine is widely used in bioavailability studies; it is usually done on a single or 24-hour specimen. Both pH and volume are important factors in urine drug analysis and must be recorded immediately on collection. If urine is allowed to stand at room temperature, bacterial action causes the conversion of urea to ammonium carbonate, and thence to ammonia which causes an increase in pH. Urine can be preserved by freezing at $-20^{o}C$ or by the addition of a preservative. Commonly used preservatives are toluene, boric acid or concentrated hydrochloric acid. Freezing is preferable to the latter approach since the preservative can interfere with subsequent drug analysis.

In emergency testing, urine analysis is a useful tool for detecting tricyclic antidepressants as their urine concentration is much higher than that of blood [74]. The determination of drugs of abuse in urine is used in the screening of patients on detoxification programmes. Opiates are measured in urine to check a patients compliance with the dose regimen, and to screen for other drugs of abuse [40].

Less commonly analysed biological media are saliva and cerebrospinal fluid (CSF). Drug concentrations in saliva are sometimes assumed to represent free plasma levels, but this was found to be true only for a limited number of drugs, such as carbamazepine and phenytoin [26]. For other drugs, the correlations are less satisfactory or apparently non-existent [80]. It is not practical to analyse CSF routinely, and such analyses are normally only carried out when damage to the blood-brain barrier is suspected.

2.4.3. Drug conjugates and protein bound drugs

Many drugs and metabolites are present in the urine as glucuronide or sulphate conjugates which are more polar than the unconjugated drug, and not readily extractable into organic solvents.

HPLC offers the possibilty of measuring intact drug conjugates without derivatisation. For instance, paracetamol can be detected in its conjugated form by direct injection of urine with UV detection [81]. Few drugs, however, are present in high enough concentrations to permit this approach, and it is more usual to hydrolyse these conjugates and release the parent drug for extraction. This is done chemically with hydrochloric acid or sodium hydroxide, or enzymatically with enzymes such as glucuronidase or sulphatase. Chemical hydrolysis decreases the yield of labile comopounds, for instance certain benzodiazepines, and compounds sensitive to aggressive reagents. Enzymatic methods, on the other hand, offer mild conditions which are less

likely to cause degradation of the drug in question, though sulphatase can only hydrolyse aryl sulphates [34]. Glucuronidase can be inhibited by high salt concentrations, hence, it is advisable to dilute the sample and to run control experiments to ensure that the enzyme is active. Generally, a urine sample is divided into two fractions, one of which is subjected to hydrolysis. This gives the total (conjugated and unconjugated) concentration following extraction, whereas the unhydrolysed fraction yields the proportion of drug which is non-conjugated. The amount of conjugated drug is obtained by subtraction. This procedure has been carried out in the determination of oxmetidine and its sulphoxide metabolite [82].

A common problem in the analysis of blood levels is protein binding. In pharmacokinetic and clinical studies, differentiation is made between free, protein-bound and total drug levels. Albumin is the most significant binding protein for many drugs, particularly neutral and acidic drugs such as warfarin, phenytoin and valproic acid [83]. Basic drugs such as quinidine, propranolol,and the tricyclic antidepressants bind not only to albumin, but to other proteins such as α-acid glycoprotein and lipoproteins [84-86]. The extent of protein binding is of interest to the clinician, since for many years it was assumed that only the free fraction is active and available to interact with the drug receptor site. Though this would appear to be true for a number of drugs, more recent work [87] has shown that bound fractions of propranolol and lignocaine can be transported into tissues such as the brain. Of great importance, however is how the equilibrium binding is affected by the co-administration of other drugs. The classic example of this occurrence is when the administration of salicylate-based, drugs such as the non-steroidal anti-inflammatory drugs (NSAIDS) causes the displacement of warfarin from its protein-binding sites to which it is extensively bound. This displacement significantly increases the amount of free warfarin circulating in the blood and the concommitant increase in anti-coagulant effect can result

in fatal internal haemmorrhage.

It is usually the total concentration of drug in plasma or serum which is measured rather than the free fraction. Unless specialised techniques which exclude bound drug, such as equilibrium dialysis or ultrafiltration, are employed, the measurement of free drug is difficult since many of the widely-used extraction techniques cause at least partial liberation of drugs from their binding sites. However, providing protein binding of drugs is normal and constant within, and between patients in a particular study, free drug concentration is a reasonably constant fraction of total drug. This relationship does not hold where protein binding is abnormal or affected by drug interaction as outlined above, or where there is individual variation in the extent of binding. One instance of this is phenytoin, and in such cases the free fraction would appear to offer superior correlation with therapeutic effect and toxicity than the total drug concentration [88,89].

Protein precipitation techniques will frequently liberate drugs from their protein binding sites, as will liquid-liquid extraction. Some protein bound-drugs such as propranolol, will bind strongly to reversed-phase packing material used in solid-phase extraction columns, and thus good recoveries will be obtained using this method of drug removal from plasma. In cases where the drug is very strongly-protein bound, the relatively mild extraction techniques outlined above will not release the drug from binding sites, and methods such as acid digestion and enzyme digestion have been employed to obtain a measure of the total drug.

Acid digestion can lead to hydrolysis of the drug, and the search for a more general procedure lead to the use of the proteolytic enzyme, subtilisin Carlsberg. Osselton et al. [90,91] employed this enzyme for the release of antibiotics and benzodiazepines. After incubation with the enzyme, the sample was filtered over a plug of glass wool and analysed in the usual manner. The enzyme

was found to release the drug completely without affecting chemical structures. Enzyme subtilisin Carlsberg has been used to degrade liver tissues at high pH [92]; this has lead to good recovery for drugs, though is unsuitable for substances which degrade under alkaline conditions. Later work on subtilisin Carlsberg revealed that reaction can be carried out at lower pH values with considerable improvement in drug recovery [93].

Werkhoven-Goewie and co-workers [94] demonstrated the application of enzyme hydrolysis using subtilisin followed by automated on-line pre-concentration of the drug secoverine. After enzymic hydrolysis for 15 minutes at 55^{o}C, the drug was liberated from its protein binding sites and 1 ml volumes of the hydrolysate were injected onto short (2-30mm) pre-columns containing cyano-packing material. Up to 50 ml of treated plasma could be injected without appreciable build-up in pre-column back-pressure or loss of performance. As enzymatic hydrolysis produces a large number of small peptides and amino acids, the method may require a selective detection system.

2.4.4. Internal standards

Optimal reliability of analysis is achieved with the use of internal standardisation. The role of an internal standard is to correct for variation in instrument response, injection volume or extraction or derivatisation yield. It is important to add the internal standard at the earliest possible stage in order to achieve maximum compensation for procedural variations. Most internal standards are compounds chemically similar to the drug to be assayed. The internal standard should elute clear of any endogenous interferents, and close to, but clear of the drug. The ratio of the detector response (peak height or area) for the drug and the internal standard is then used in the calibration and assay. In monophasic dilution (such as protein precipitation), the internal standard must only be soluble in the dilution solvent, and its selection then depends

on chromatographic parameters. In liquid-liquid or liquid-solid extraction, the behaviour of the internal standard in the extracting liquid or solid is important. The more its chemical structure resembles that of the drug, the better the control of variation achieved. A molecule having an identical acidity constant and a partition co-efficient similar to the compound of interest would serve as an ideal internal standard. It is essential also that the internal standard and the drug have similar responses in the detection scheme employed. For instance, in the highly sensitive electrochemical detection of imipramine at an oxidation potential of 1000 mV, the use of nortriptyline as an internal standard is precluded because it does not react to this potential [40].

The use of an internal standard belonging to the same chemical class as the drug of interest can present more problems than is commonly assumed. Even when there are close structural similarities, such as among chemical homologues, pronounced differences in physico-chemical behaviour may arise. In addition, while it is desirable that the internal standard belongs to the same chemical group as the drug being monitored, it is inadvisable to use a commonly prescribed drug or metabolite where the patients intake of medication is not closely monitored, especially in toxicology where little or no information can be collected about ingested drugs. The amount of internal standard introduced during the analysis must be selected so that the detector response ratio falls within one range of the calibration curve. An upper limit of 2 is generally chosen as the maximum response ratio [95].

2.4.5. Calibration

Samples of drug-free material (serum, urine) containing known drug concentrations should, where possible, be employed for calibration. This is sometimes not possible in the case of tissue samples such as liver or muscle. Alternative external standardisation based on assaying, or even simply injecting

authentic drug standards ignore the specific peculiarities of the biological matrix; for instance protein-binding would not be accounted for using external standardisation. However, even spiked plasma or serum standards are subject to criticism, since an externally-added drug might not be present in the same physico-chemical state as the in vivo compound [10]. It follows that blank plasma used in the calibration should have been prepared using the same anti-coagulant as employed in taking patient samples: as outlined above, the type of anticoagulant can affect not only the appearance of interfering peaks, but the amount of drug recovered from plasma.

In chromatography, peak-height or peak-area ratios (compound to internal standard) are usually plotted against concentration. The calibration graphs are calculated by linear regression analysis, but in order to avoid inaccuacies near the origin, weighted regression analysis should be performed. This follows because carrying out linear regression of y upon x implies, amongst other constraints that there is no variance in x and a constant variance in y [96]

2.4.6. Evaluation

Several criteria have to be evaluated in order to check the reliability and the overall performance of an assay. Most regulatory agencies, like the Food and Drug Administration, have mandated Good Laboratory Practice regulations for clinical and non-clinical laboratory studies [97,98]. The parameters which are used to evaluate the overall performance of an assay include the drug stability, specificity, limit of detection, accuracy linearity and recovery.

2.4.6.1. Drug Stability

Drugs stored under different conditions of heat, light, humidity and pH should be examined for possible decomposition. If the drug is to be stored for an appreciable length of time, it is

necessary to establish its stability under prolonged storage conditions. The kinds of problems which can arise have been discussed in section 2.4.1., and the extent of this loss must be established before commencing with the assay. If the amount of drug lost is not reproducible then it may be necessary to alter the analytical protocol.

2.4.6.2. Specificity

It is necessary to determine whether endogenous compounds in the biological matrix will interfere with either the drug peak(s) or that of the internal standard. This is readily achieved by taking drug-free plasma (serum, urine, etc) through the sample work-up and analytical process. This procedure should be repeated with each batch during both calibration and assay of unknown samples, in case of a change in column performance or the development of artefacts have caused the appearance of peaks co-eluting with the drug or the internal standard. Drugs which are therapeutically combined with the drug (for instance, anti-hypertensives with diuretics) should be checked for possible interference or cross-reactivity. As caffeine is a common source of interference, it is usual for patients involved in a clinical trial to be given a caffeine-free diet. However, this is not always practicable, and if the samples to be analysed are likely to contain this compound, it may be necessary to exclude caffeine as a potential interferent.

Should any peak interfere with the assay, a systematic search should be undertaken to find the origin of the interference. It may, as mentioned above, arise from the diet, or it may be introduced from the equipment used in the assay. If it proves impossible to actually remove the source of an interfering peak (for instance a vital co-administered drug), it may be possible to eliminate the unwanted peak by selective extraction, more selective detection wavelengths or schemes, or by a revision of the chromatographic parameters.

2.4.6.3. Limit of detection

The limit of detection (LOD) is defined as the lowest concentration of an analyte that the analytical system can reliably detect. In mathematical terms it may be expressed as 3σ above the gross blank signal [99], where σ is the standard deviation of the peak-to-peak noise. Where there is a linear dependence of signal (S) on concentration (C), the following equation may be written:

$$d_{C_x}/C_x = d_{S_x}/S_x$$

where S_x is the signal corresponding to concentration C_x, and d_{S_x} is the signal variability corresponding to concentration variability d_{C_x}.

The limit of detection may vary from day to day as a result of a change in detector response, for instance, but once the LOD has been statisticaly defined, data obtained using this value is not valid and should not be reported.

2.4.6.4. Accuracy and precision

Assessment of accuracy is a fundamental problem because the true value can never be known with absolute certainty. The term "accuracy" denotes the nearness of a measurement to its accepted value and is expressed in terms of error [35]. The accepted error is the difference between the observed and the accepted value. The relative error is expressed as a percentage of the accepted value, and is often used to express the accuracy of a chromatographic assay. It is common to test the accuracy of the method in hand by comparing it with another reliable method. Chromatographic methods are frequently compared with immunoassay techniques, and the correlation between the two is taken as an index of how accurate the method being validated is.

Precision or reproducibility of an assay is defined as the

coefficient of variation (relative standard deviation) of the results at a certain drug concentration. In chromatographic methods of drug analysis, the reproducibility of a method should be determined on the basis of within-day (intra-assay), and between-day (inter-assay) results. In performing both inter-and intra-assays, at least four replicate analyses over the entire concentration range should be carried out. For intra-assay, the mean ratios of drug to internal standard are plotted as a function of concentration to generate a regression curve. The individual ratios are then interpolated as unknowns on this curve to obtain new values of concentration, and these new values are then used to determine precision. For inter-assay, individual regression lines are generated for each set of data, and each set is then interpolated on its own regression line. The new values of concentration thus obtained are then used to determine inter-assay precision.

2.4.6.5. Linearity

The linearity of the assay is defined by linear regression analysis of replicates of spiked biological standards in the anticipated concentration range of the drug to be assayed. Linearity is frequently quoted in terms of the correlation coefficient of the single regression line used for intra-assay calculations. If the signal to concentration function is not linear over the entire concentration range, as is sometimes the case where a wide range of concentrations is to be covered, it may be necessary to split the calibration curve into two portions to permit quantitation of unknown samples. With some techniques, non-linear calibration graphs are frequently obtained. Some workers propose special approaches which extend the linear range artificially [100], but much greater accuracy can be obtained by calculating the actual non-linear calibration graph using weighted polynomial regression analysis [10].

2.4.6.6. Recovery

The extraction recoveries of the drug and internal standard provide useful information and can be calculated either by running isotopically labelled compounds through the procedure, or by assaying drug-supplemented plasma (serum, urine) and comparing the peak heights of the extracted standards versus those of authentic (non-extracted) standards in the same concentrations. By generating a calibration curve of both extracted and non-extracted standards, the ratio of the two slopes can be obtained and used as a second measure of recovery.

2.4.6.7. Quality Control

Routine drug assays should regularly be checked for reliability using internal quality control schemes. This involves the preparation of plasma (serum, urine) pools to which a defined amount of drug (unknown to the analyst) are added. With each determination on patient samples, a control sample is also analysed, and the result is interpreted in terms of established limits of variation. It is recommended that at least 10% of the total samples should consist of quality control standards interspersed amongst the patient samples and calibration standards. If the results of the quality control standards are not within 10% of their anticipated values, then it is normal practice to repeat the batch [101].

Inter-laboratory surveys on a similar basis are currently organised by different institutions, and are often imposed by law. Control samples are distributed to the laboratories participating in such an external quality control programme and the results are statistically evaluated. In order for this type of system to be successful, it is necessary that equipment, materials and operating conditions are identical in each of the participating laboratories. In practice it can be difficult to realise this goal, since there is significant variation between

batches of even the same brand and type of column, and differences in equipment performance can contribute to inter-laboratory variation in results.

2.5. REFERENCES

1. L. J. Kricka, Anal. Proc., 20 (1983) 163.
2. R. E. Hill, Clin. Biochem., 19 (1986) 113.
3. A. F. Fell, Anal. Proc., 19 (1982) 398.
4. I. M. Warner, J. B. Callis, E. Davidson, R. Gouterman and G. D. Christian, Anal. Lett., 8 (1975) 665.
5. D. W. Johnson, J. B. Callis and G. D. Christian, Anal. Chem., 49 (1977) 747A.
6. A. Savitsky and M. J. E. Golay, Anal. Chem., 36 (1964) 1627.
7. J. Steiner, Y. Termonia and J. Deltour, Anal. Chem., 44 (1972) 1906.
8. D. E. Metzler, C. E. Harris, R. L. Reeves, W. H. Lawton and M. S. Maggio, Anal. Chem., 49 (1977) 864A.
9. R. S. Yalow and S. A. Berson, Nature (London), 184 (1959) 1648.
10. A. P. DeLeenheer and H. J. C. F. Nelis, Analyst, 106 (1981) 1025.
11. B. K. Van Weeman and A. H. Schurs, FEBS Lett., 15 (1971) 232.
12. A. Voller, D. Bidwell, G. Huldt, and E. Engvall, Bull. W.H.O,. 51 (1974) 209.
13. K. E. Rubenstein, R. S. Schneider and E. F. Ullman, Biochem. Biophys. Res. Commun., 47 (1972) 846.
14. E. F. Ullman, R. A. Yoshida, J. I. Blakemore, E. Maggio and R. Leute, Biochem. Biophys. Acta., 567 (1979) 66.
15. W. D. Odell and W. D. Daughaday, Principles of Competitive Protein Binding Assay (J. P. Lippincott, New York, 1971).
16. J. A. Miller, Science News, 14 (1978), 444.
17. R. M. Caprioli, J. G. Liehr and W. E. Siefert in G. R. Waller and O. C. Dermer (Eds.) Biochemical Applications of Mass Spectrometry, First Supplementary Volume (Wiley, New York, (1980).
18. G. Verzele, G. Redant, G. Quereshi and P. Sandra, J. Chromatogr., 199 (1980) 105.

19. N. H. C. Cooke and I. Olsen, J. Chromatogr. Sci., 18 (1980) 512.
20. C. F. Poole and S. A. Schuette, Contemporary Practice of Chromatography (Elsevier, Amsterdam, 1984)
21. W. Sadee and G. L. M. Beelen, Drug Level Monitoring (Wiley, New York, 1980).
22. R. N. Zare, W. D. Hunsberg and S. D. Lidofsky, presented at the 2nd Chemical Congress of the North American Continent, Las Vegas, 1980.
23. J. R. Sportsman and G. S. Wilson, Anal. Chem., 52 (1980) 2013.
24. I. H. Hagestam and T. C. Pinkerton, Anal. Chem., 57 (1985) 1757.
25. F. Li, C. K. Lim and T. J. Peters, Biochem. J., 239 (1986) 481.
26. A. C. Metha, Talanta, 33 (1986) 67.
27. R. W. Geise, Clin. Chem., 29 (1983) 1331.
28. C. K. Lim and T. J. Peters, J. Chromatogr., 316 (1984) 397.
29. M. Somogyi, J. Biol. Chem., 160 (1945) 69.
30. S. J. Van der Wal and L. R. Snyder, Clin. Chem., 27 (1981) 1233.
31. J. Blanchard, J. Chromatogr., 226 (1981) 455.
32. P. J. Meffin and J. O. Miners, in J. W. Bridges and L. F. Chausseaud (Eds.) Progress in Drug Metabolism (Wiley, New York, 1980).
33. H. Varley, A. H. Gowenlock and M. Bell, Practical Clinical Biochemistry (Heineman, London, 1976).
34. R. Gill and A. C. Moffat, Anal. Proc., 19 (1982) 170.
35. D. A. Skoog and D. M. West, Fundamentals of Analytical Chemistry (Holt, Reinard and Winton, New York, 1975)
36. D. Chin and E. Fastlich, Clin. Chem., 20 (1974) 1382.
37. D. M. Bailey and M. Kelner, J. Anal. Toxicol., 8 (1984) 26.
38. S. M. Johnson, C. Chan, S. Cheng, J. L. Shimek, G. Nygard and S. K. Wahba, J. Pharm. Sci., 71 (1982) 1027.

39. R. Gill, A. A. T. Lopez and A. C. Moffat, J. Chromatogr., 226 (1981) 117.
40. M. Van Damme, M. Leopold amd A. K. Faouzi, Clin. Toxicol., 23 (1985-86) 589.
41. J. Ramsey and D. B. Campbell, J. Chromatogr., 63 (1971) 303.
42. V. Aggarval, R. Bath, and I. Sunshine, Clin. Chem., 20 (1974) 307.
43. G. Schill, in E. Reid (Ed) Assay of Drugs and Other Trace Compounds in Biological Fluids (North-Holland, Amsterdam, 1976).
44. E. Tomlinson, J. Pharm. Biomed Anal., 1 (1983) 11.
45. M. G. Horning, P. Gregory, J. Nowlin, M. Stafford, K. Lertratanangkoon, C. Butler, W. G. Stillwell and R. M. Hill, Clin. Chem., 20 (1974) 282.
46. F. Li, C. K. Lim and T. J. Peters, J. Chromatogr., 353 (1986) 19.
47. E. Reid and J. P. Leppard (Eds.) Drug Metabolite Isolation and Determination (Plenum press, New York, 1983).
48. G. G. Skellern, Analyst, 106 (1981) 1071.
49. L. E. Martin and E. Reid in J. W. Bridges and L. F. Chasseaud (Eds.) Progress in Drug Metabolism (Wiley, New York, 1981)
50. R. J. Allan, H. T. Goodman amd T. R. Watson, J. Chromatogr., 183 (1980) 311.
51. T. C. Kwong, R. Martinez and J. M. Keller., Clin. Chem. Acta., 126 (1982) 203.
52. J. P. Anhalt, S. D. Brown, Clin. Chem., 24 (1978) 1940.
53. S. K. Maitra, T. T. Yoshikawa and J. L. Hansen, Clin. Chem., 23 (1977) 2275.
54. R. A. Hux, H. Y. Mohammed and F. F. Cantwell, Anal. Chem., 54 (1982) 113.
55. I. H. Hagestam and T. C. Pinkerton, J. Chromatogr., 351 (1986) 239.
56. R. W. Schmid and C. Wolf, Chromatographia, 24 (1987) 713-719.

57. W. Voelter and T. Kronbach, J. Chromatogr., 239 (1982) 475.
58. K. G. Wahlund, J. Chromatogr., 218 (1981) 671.
59. N. Daoud, T. Arvidsson and K. G. Wahlund, J. Chromatogr., 385 (1987) 311.
60. W. Roth, K. Beschke, R. Jauch, A. Zimmer and F. W. Koss, J. Chromatogr., 222 (1981) 13.
61. D. R. Deans, Chromatographia, 1 (1968) 18.
62. E. L. Johnson, R. Gloor and R. E. Majors, J. Chromatogr., 149 (1978) 571.
63. F. Erni and R. W. Frei, J. Chromatogr., 149 (1978) 561.
64. L. R. Snyder, J. W. Dolan and S. T. Van der Wal, J. Chromatogr., 203 (1981) 3.
65. A. Nazareth, L. Jaramillo, B. L. Karger and R. W. Geise, J. Chromatogr., 309 (1984) 357.
66. C. E. Goewie, M. W. F. Nielen, R. W. Frei and U. A. Brinkman, J. Chromatogr., 301 (1984) 325.
67. W. Roth, J. Chromatogr., 278 (1983) 347.
68. W. Roth and K. Beschke, J. Pharm. Biomed. Anal., 2 (1984) 289.
69. C. E. Werkhoven-Goewie, U. A. Brinkman and R. W. Frei, Anal. Chem., 53 (1981) 2072.
70. J. H. G. Jonkman, J. P. Franke, R. Schoenmaker and R. A. De Zeeeuw, Int. J. Pharm., 10 (1982) 177.
71. H. Hellberg, Acta. Chem. Scand., 11 (1957) 219.
72. O. Borga, I. Petters and R. Dalqvist, Acta. Pharm. Suecia, 15 (1978) 549.
73. R. S. Rapaka, J. Roth, T. J. Goehl and V. K. Prasad, Clin. Chem., 27 (1981) 1470.
74. D. J. Krogstad, G. G. Granich, P. R. Murray, M. A. Pfaller and R. Valdes, Clin. Chem., 28 (1982) 1517.
75. O. Borga, K. M. Piafsky and O. G. Nilsen, Clin. Pharmacol. Ther., 22 (1977) 539.
76. V. P. Shah, G. Knapp, J. P. Skelly and B. E. Cabana, Clin. Chem., 28 (1982) 2327.

77. R. Janknegt, M. Lohmann, P. M. Hooymans and F. W. Merkus, Pharm. Weekbl. Sci. Ed., 5 (1983) 287.
78. S. Maguire, F. Kyne and D. UaConaill, Clin. Chem., 33 (1987) 1493.
79. L. J. Dusci and L. P. Hackett, Clin. Toxicol., 14 (1979) 587.
80. R. A. de Zeeuw in E. Reid (Ed.) Trace Organic Sample Handling (Horwood, Chichester, 1981).
81. J. H. Knox and J. Jurand, J. Chromatogr., 149 (1978) 297.
82. G. S. Murkitt, R. M. Lee and R. D. Mc Dowall, Anal. Proc., 21 (1984) 246.
83. M. Wandell and J. Wilcox-Thole, in M. Mungall (Ed.) Applied Clinical Pharmacokinetics (Raven press, New York, (1983).
84. K. M. Piafsky, Clin. Pharmacokinet., 5 (1980) 346.
85. J.J. Valner, J. Pharm. Sci., 66 (1977) 447.
86. M. H. Bickel, J. Pharm. Pharmacol., 27 (1975) 733.
87. W. M. Pardridge, R. Sakiyama, and G. Fierer, J. Clin. Invest., 71 (1983) 900.
88. H. E. Booker and B. Darcey, Epilepsia, 14 (1973) 177.
89. C. J. Kilpatrick, S. Wanwimolruk and L. M. H. Wing, Br. J. Clin. Pharmacol., 17 (1984) 539.
90. M. D. Osselton, M. D. Hammond and P. J. Twitchett, J. Pharm. Pharmacol., 29 (1977) 460.
91. M. D. Osselton, I. C. Shaw and H. M. Stevens, Analyst, 103 (1978) 1160.
92. M. D. Osselton, J. Forensic. Sci. Soc., 17 (1977) 189.
93. M. D. Hammond and A. C. Moffat, J. Forensic Sci. Soc., 22 (1982) 293.
94. C. E. Werkhoven-Goewie, C. De Ruiter, U. A. Brinkman, R. W. Frei, C. J. De Jong, C. J. Little and O. Stahl, J. Chromatogr., 255 (1983) 79.
95. D. J. Greenblatt and R. I. Shader, in A. Richens and V. Marks (Eds.) Therapeutic Drug Monitoring (Churchill-Livingstone, New York, 1981).
96. D. A. Schoeller, Biomed. Mass. Spectrom., 3 (1976) 265.

97. Good Laboratory Practices Regulations for Non-Clinical Laboratory Studies, Federal Register, (43 FR 59986) 43 (1978) No. 247, Part II.
98. Tentative guidelines for Clinical Laboratory Procedure Manuals, U.S. National Committee for Clinical laboratory Standards, Vol. 1 (No. 13) 369-424.
99. Guidelines for Data Acquisition and Data Quality Evaluation in Environmental Chemistry, Anal. Chem., 52 (1980) 2242.
100. B. J. Millard (Ed.) Quantitative Mass Spectrometry (Heyden, London, 1978).
101. D. Dadgar and M. R. Smyth, TrAC., 5 (1986) 115.

CHAPTER 3

THE DETERMINATION OF XIPAMIDE IN HUMAN PLASMA BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

3.1. INTRODUCTION

Xipamide (4-chloro-5-sulphamyl-salicylic acid 2',6' dimethylanilide) belongs to the diuretic group of drugs which increase urinary volume and renal excretion of sodium and chloride ions. Diuretics are usually classified according to their potency or site of action, two characteristics which largely determine the clinical responses of such medications [1]. Xipamide is considered to be a potent diuretic, though it acts on the distal convoluted tubule in the kidneys rather than on the loop of Henle like other potent (so-called "loop") diuretics such as frusemide [2]. Its mode of action is thought to involve interference with sodium ion re-absorption in the distal convoluted tubule, and it causes effective excretion of both sodium and chloride ions for 12-14 hours after oral administration [3]. It significantly decreases the glomerular filtration rate (GFR) and is a potent inhibitor of carbonic anhydrase [4].

Xipamide is used in the treatment of hypertension [5,6], as well as oedematous states due to cardiac, renal and hepatic disease [7-10]. Xipamide may be used alone [5,7,8] or in combined therapy with other drugs,[6,9,10], and it has been tried in cases of oedema with rheumatoid arthritis [11].

It has been reported to closely resemble hydrochlorothiazide in its mechanism of action [4,12], as well as in its kinetic properties [13]. Structurally, xipamide is a benzoic acid derivative bearing a free sulfamoyl moiety [4], and bears a superficial resemblence to both frusemide [14], and chlorthalidone, though the drug is not a phthalimidine or a thiazide [10]. Pharmacologically, xipamide is equipotent with the former drug with respect to its effects on sodium ion and water excretion [10], though they differ in their onset, duration, and sites of action, [2,3,10,15].

After oral administration of a 20 mg dose, maximum plasma levels of 2.0 ± 0.3 g/ml have been shown to occur within one hour [15], and this had fallen to approximately 100 ng/ml after 24 hours. The plasma levels can be described by a two-compartment open model, and the bioavailabliy was calculated to be 73% [15]. Xipamide has a half-life of between 6 and 8 hours and plasma levels are highest between 0.75 and 2 hours after oral administration [13]. Approximately 40% of the given dose is recovered unchanged in the urine [15], and xipamide-o-glucuronide was the only metabolite found in the liver [13]. It is almost completely bound to plasma proteins [13,15]: at a therapeutic concentration of 690 ng/ml it became almost 99% bound to plasma proteins in an in vitro experiment [16].

Xipamide is lipophilic as shown by its solubility and partition coefficient. At physiological pH it forms an anion having an octanol-water partition coefficient of 1 [17]. It has two pK_a values: pH 4.75, when it forms a phenolate anion, and a second at approximately pH 10. At room temperature it is stable in both acidic and basic media. Solutions buffered to between 7.0 and 8.0 are particularly suitable for determination of xipamide by spectrophotometry, though no colour reactions are known which are sensitive enough to permit quantitative determination for pharmacokinetic studies.

To date there has been practically no information published on the determination of xipamide in plasma. Originally, the pharmacokinetics of xipamide were followed using the method of Hempelmann and Dieker [13] which involved injecting patients with ^{35}S-xipamide and following the pharmacokinetics by measurement of the amount of radioactivity in various body fractions over a set time period.

In response to the need for an alternative to the injection of individuals with radiolabelled substances, a thin-layer densito-fluorimetric technique was developed by Sobel and

Mutschler in 1980 [18]. In this method, following adjustment of the plasma or urine pH to 5.0 with citrate buffer, xipamide was extracted into ethylacetate by mixing for one hour. After centrifugation, the organic layer was decanted and evaporated to dryness in a clean test tube. To the residue was added a solution of the fluorescent reagent [4-(7-methoxy-2-oxo-2H-benzopyran-4-ylmethoxy)-benzoyl chloride; BBP-Cl], and derivatisation took place over a three hour period. The derivatised residue was then spotted on a thin layer chromatographic (TLC) plate and the chromatogram was developed using a mixture of toluene and ethyl acetate (1:1). Fluorescence of the derivatised xipamide (Xi-BBP) was measured at 404 nm following excitation at 313 nm. At a spiked plasma level of 20 ng/ml of xipamide, the coefficient of variation was 6.5 % for eight replicate analyses. Recovery was almost 100%.

The authors claimed a limit of detection of 10 ng/ml if a 1 ml sample of plasma was used, but as little as 0.1 ml of plasma may be analysed if necessary. This technique has been adopted by other workers in the analysis of xipamide in plasma [15,19], and while it is clearly an advance on methods involving the introduction of radiolabelled xipamide into the body, it has drawbacks in terms of the need to derivatise the drug, synthesize the fluorescent reagent and the lengthy procedures involved in both the extraction and the actual derivatisation stages.

There have been no methods published for the analysis of xipamide in plasma by HPLC. One method was described where the clearance of xipamide from the body was measured by the direct injection of 10 µl of urine onto a C_8 reversed-phase column [20]. Direct injection of untreated plasma in not a feasible proposition, and in order to determine low levels of the drug, it is necessary to to extract it from the plasma matrix and to incorporate a concentration step into the work-up procedure. The objective of the present study was to develop, from first principles, an HPLC method for the determination of xipamide in plasma, with emphasis on ease of operation without the need for elaborate

instrumentation. The final protocol involved a simple one-step extraction, and using ultraviolet absorbance as the mode of detection, it offers a level of sensitivity suitable for pharmacokinetic studies, without the need to derivatise the drug prior to analysis

3.2. EXPERIMENTAL

3.2.1. Reagents and solvents

Xipamide was received as a gift from the Institute of Clinical Pharmacology, Dublin, Ireland. Mephenesin was obtained from Sigma Chemical Co. Ltd., Dorset, England. HPLC grade methanol, acetonitrile and diethyl ether were obtained from Labscan Analytical Sciences, Dublin, Ireland. Sodium acetate, acetic acid ethyl acetate, hexane, methyl isobutyl ketone and isopropanol (Analytical Grade) were supplied by Riedel de Haen, Hannover, West Germany. Lithium chloride, calcium chloride, sodium sulphate, zinc sulphate, sodium hydroxide and ammonium chloride (AnalaR grade) were obtained from BDH Chemicals Ltd., Dorset, England, and trichloroacetic acid, sodium bicarbonate, sodium carbonate and calcium carbonate (analytical grade) were supplied by May and Baker Chemicals, Dagenham, England.

Deionised water was obtained by passing freshly distilled water through a Millipore water purification system. Dried human plasma from the Blood Transfusion Board, Dublin was dissolved in deionised water used within seven days of reconstitution.

3.2.2. Drug standards

Xipamide (10.00 mg) was dissolved in methanol to yield a stock solution of 100 μg/ml (solution A). A working stock solution of 50 μg/ml was prepared by a 1:1 dilution of solution A in methanol-water (1:1) (solution B). This solution was diluted 1:9 with mobile phase to give a working standard solution of 5 μg/ml.

For calibration, a set of standards were prepared by serial dilution of solution B in methanol-water (1:1). A 1 mg/ml solution of mephenesin (the internal standard) in methanol was prepared. A 100 μg/ml working stock solution was pepared by dilution of the stock solution with methanol-water (1:1). This solution was diluted 1:9 with mobile phase to give a working standard solution of 10 μg/ml.

3.2.3. Plasma standards

Aliquots of drug-free plasma were spiked with 50 μl xipamide and 50 μl mephenesin working stock solutions. Plasma blanks were prepared by adding 100 μl methanol-water (1:1) to drug-free plasma.

3.2.4. Instrumentation and operating conditions

The drugs were separated on a C_8- reversed-phase column, 10 μm, 4.6 x 220mm, supplied by Pierce Chemical Corporation, Rockford, IL, USA. It was protected by a guard column of C_8- Sepralyte (40 μm) packing, supplied by Analytichem International, Harbour City, CA, USA. The mobile phase was delivered by a Waters Model 501 HPLC pump (Waters Associates, Milford, MA, USA).

Injections were made using a Rheodyne (Cotati, CA, USA) Model 7125 6-port injection valve fitted with either a 20 μl or a 50 μl loop. The drugs were detected by Ultra-violet absorption at 220 nm using either a Waters Model 990 photodiode array detector with plotter set a chart speed of 5 mm/min, or a Waters Model 450 variable wavelength detector with a Linseis recorder (Linseis, Selb, West Germany) at a chart speed of 200 mm/hr.

The pH of the solutions was adjusted using a standard glass electrode at ambient temperature. The pH meter was calibrated daily using aqueous standards prepared on a weekly basis.

A stock solution of sodium acetate (1 M) was prepared by

dissolving the appropriate amount of substance in deionised water. The pH was adjusted using the pH meter by the addition of 0.1 M acetic acid. The buffer was diluted with deionised water to give the required ionic strength. Mobile phases were prepared by mixing the aqueous component with either methanol or acetonitrile. The mobile phase was filtered through a 0.45 μm membrane and degassed by ultrasonication prior to use.

3.2.5. Procedures

3.2.5.1. Assay development

When developing the chromatography, 20 μl quantities of the working standard solutions (dissolved in mobile phase) were injected into the system. When investigating various extraction procedures, 0.5 ml aliquots of plasma were spiked with 50 μl of working stock solutions (solution B) of the drug and internal standard or with 100 μl methanol-water (1:1) to generate plasma blanks. The plasma was then treated and subjected to various extraction procedures which are discussed in greater detail in the Discussion section.

3.2.5.2. Calibration and calculation

0.5 ml aliquots of drug-free plasma were spiked with 50 μl xipamide (X) standard solutions (prepared by serial dilution of solution B) so that the the final plasma concentrations of xipamide were 100, 250, 500, 1000, and 2000 ng/ml. The plasma aliquots were also spiked with 50 μl of the working stock solution of mephenesin (IS), so that its eventual concentration in plasma was 10 μg/ml. Plasma blanks were prepared by adding 100 μl methanol-water (1:1) to 0.5 ml aliquots of drug-free plasma. Lithium chloride (200 mg) was added to each test-tube followed by vortex mixing for 10 seconds. After the addition of 3 ml diethyl ether-isopropanol (19:1), the drugs were extracted by vortex mixing for 45 seconds. Following centrifugation at 1000g for 15 minutes, the upper organic layer was transferred to a

clean polypropylene test-tube and evaporated to dryness at 37°C under a gentle stream of air. The residue was dissolved in 100 μl mobile phase and a 50 μl aliquot injected for chromatography.

3.3 RESULTS AND DISCUSSION

3.3.1 Development of chromatography

The structures of xipamide, frusemide, chlorthalidone and mephenesin are shown in Figure 3.1.

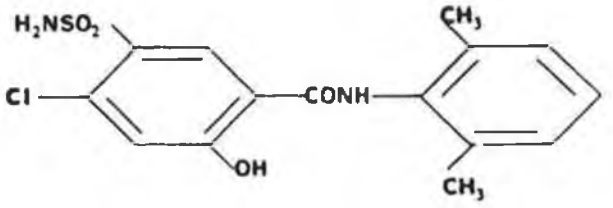
The development of a chromatographic method for xipamide commenced with finding a mobile phase capable of eluting the drug on a C_8- column (a C_8- column had been used for the HPLC determination of xipamide in urine [20]). The chosen starting point was a simple binary mixture of methanol-water (1:1) which caused xipamide to elute with a retention time of 3.5 minutes. Methanol was chosen over acetonitrile because it is a less costly and less toxic solvent for frequent and routine use. It was found that a superior peak shape was obtained for xipamide if a sodiun acetate buffer (0.02 M, pH 7.0) was used as the aqueous component. The use of a buffer also has the advantage of allowing control of pH and thus contributes to reproducible chromatography. Using a 1:1 mixture of buffer and methanol, xipamide eluted with a retention time of 2.8 min. With this retention time, the drug would probably co-elute with endogenous components when it came to the stage of extracting the drug from the plasma matrix. It was therefore sought to increase the retention time by increasing the proportion of the aqueous phase. At a buffer-methanol ratio of 65:35 the retention time of xipamide was increased to 5.8 minutes.

The wavelengh of detection had hitherto been set at 240 nm and it was decided at this point to determine the optimum detection wavelength in order to maximise sensitivity. A three-dimensional plot of xipamide depicting peak height as a function of time and

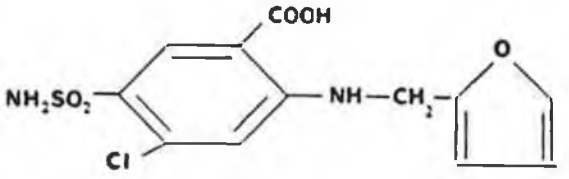
Figure 3.1.

Chemical structures of xipamide, related diuretics, and mephenesin, the internal standard

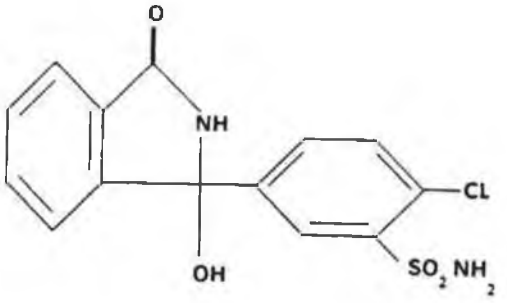
XIPAMIDE (I)



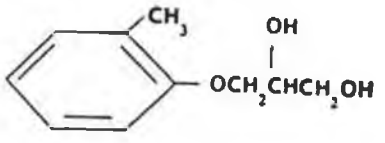
FRUSEMIDE (II)



CHLORTHALIDONE (III)



MEPHENESIN (IV)



wavelength, was generated on the UV photodiode array detector and is presented in Figure 3.2. It shows that xipamide absorbs strongly between 210 and 220 nm with a smaller peak at 230 nm. It was decided to use 220 nm as the operating wavelength since many plasma components absorb strongly in the low UV and would contribute significantly to interfering peaks in the chromatogram.

The next step was to select a suitable internal standard for xipamide. Among the compounds investigated as possible internal standards, mephensein proved to be the most suitable in terms of retention characteristics. The diuretic triamterene eluted with a retention time of 12.0 minutes (Figure 3.3.), but was not selected as an internal standard, partly because it is extremely light-sensitive and decomposes rapidly, but more importantly, because it is frequently co-prescribed with xipamide to counteract the kaluretic effects of the latter [1,20], and as such, would be unsuitable where xipamide was being measured in a combined preparation.

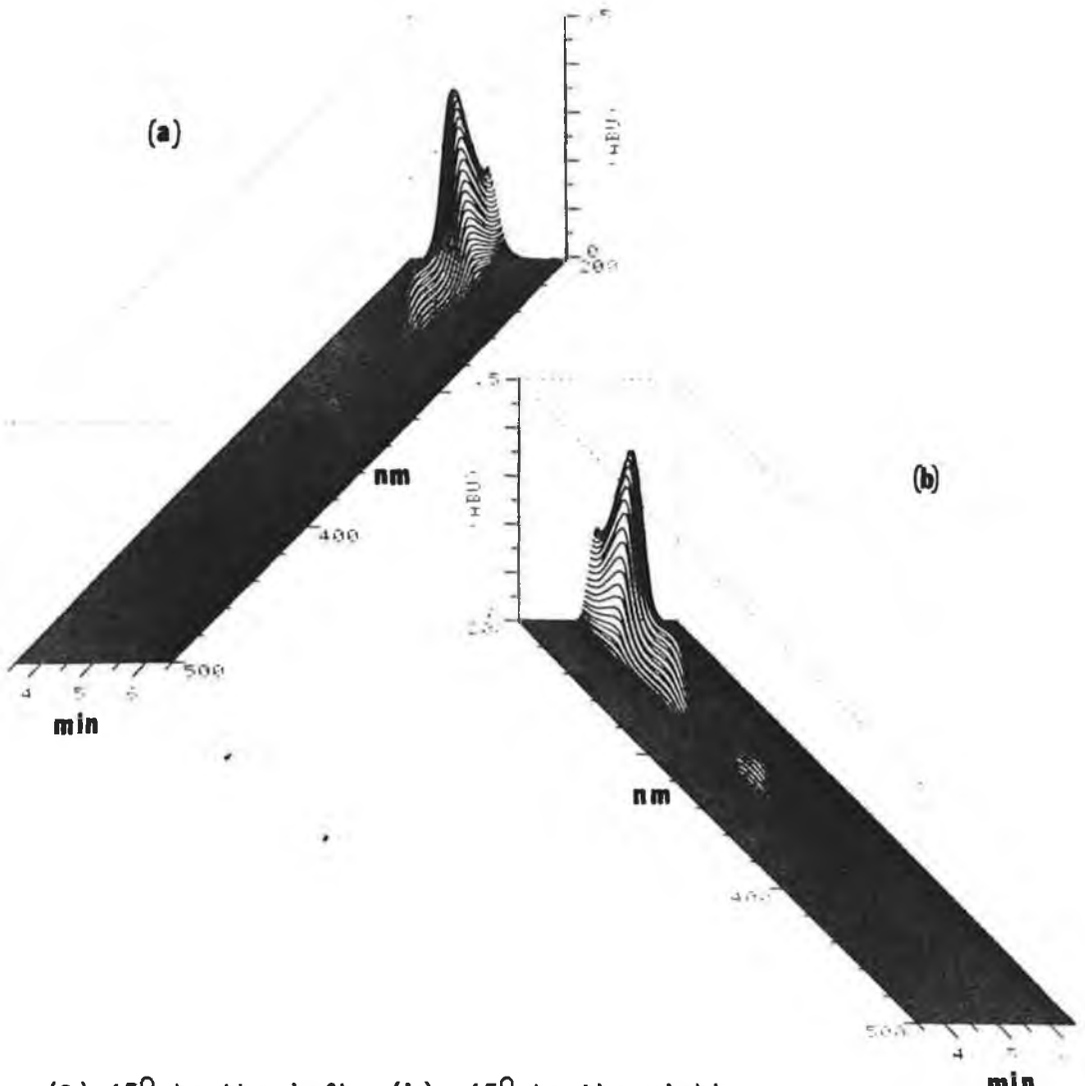
3.3.2 Development of an extraction procedure

Having chosen mephenesin as the internal standard, the next step was to extract the drug from plasma. Reconstituted drug-free plasma aliquots were spiked with the drug and internal standard which were dissolved in methanol water (1:1); plasma blanks were spiked with methanol-water only. As xipamide had previously been extracted from plasma at pH 5.0 into ethyl acetate [18], it was decided to repeat this method as a starting point for the development of an extraction procedure.

Following addition of the drug and internal standard, the pH of the plasma was adjusted to 5 by the addition of an equal volume of 0.1 M sodium acetate buffer, pH 5.0. The drugs were extracted into 3.0 ml of ethyl acetate by vortex mixing for 90 seconds. After centrifugation, the upper organic layer was transferred to

Figure 3.2.

Three-dimensional chromatogram of xipamide showing peak height as a function of time (min) and wavelength (nm)

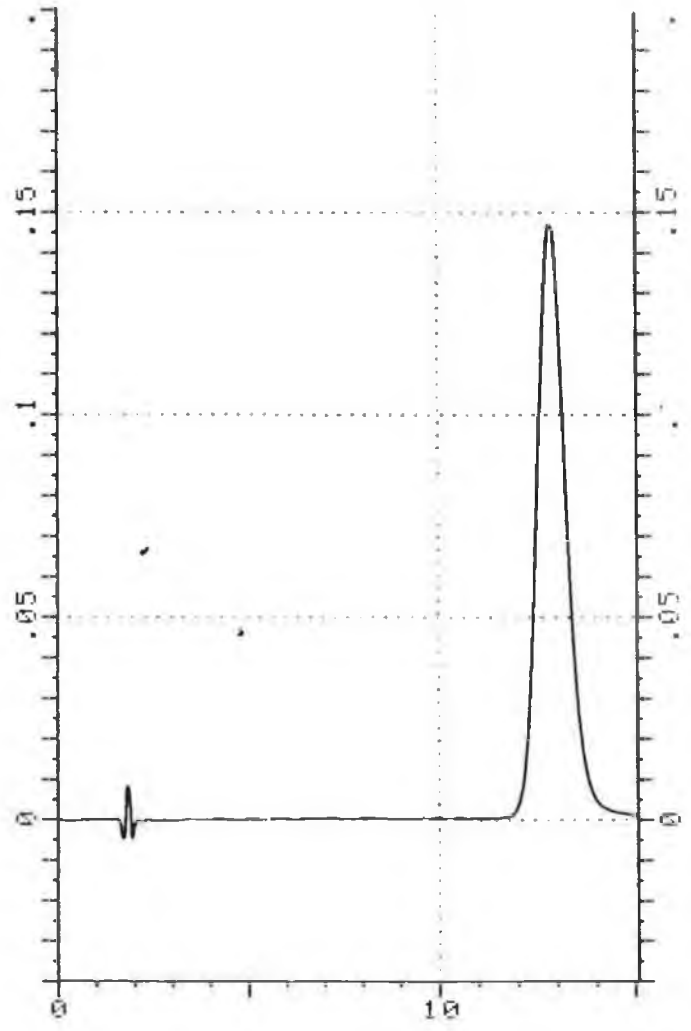


(a) 45° to the left, (b), 45° to the right.

Mobile phase: 0.02 M Sodium acetate buffer, pH 7.0-methanol (65:35).

Figure 3.3.

Chromatogram of authentic standard of triamterene



TIME (min)---------->

Mobile phase: 0.02 M Sodium acetate buffer, pH 7.0-methanol (65:35)

a clean polypropylene test-tube and evaporated to dryness under a gentle stream of air. It was found that many of the endogenous plasma components were co-extracted and produced interfering peaks in the chromatogram.

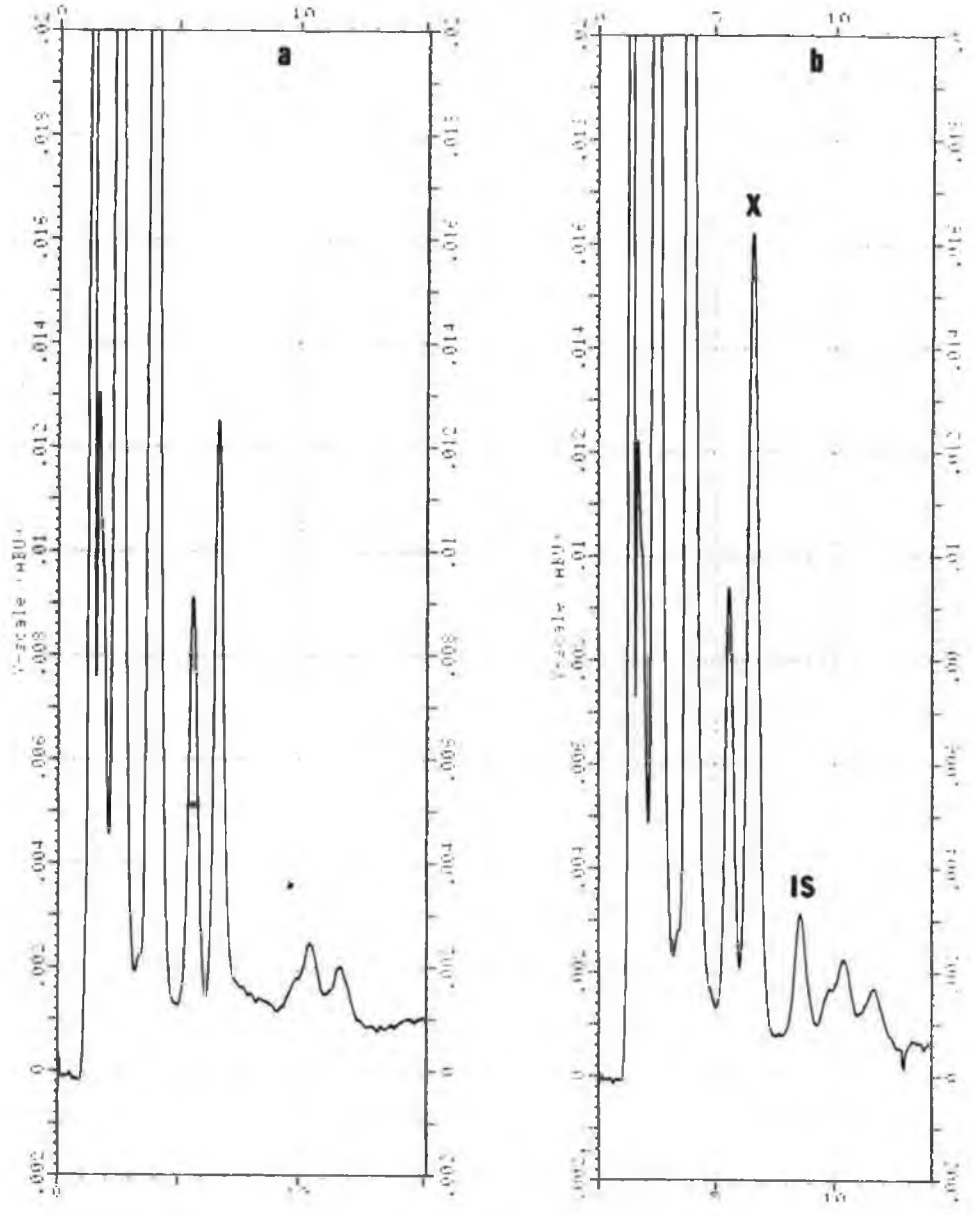
It was then decided to extract the drugs using other solvents in an attempt to achieve more selective extraction. The least polar solvent tried was hexane, and although it gave cleaner blank plasma chromatograms, it failed to extract the drugs. The other solvents investigated were ether, ether-isopropanol (19:1), dichloromethane and methyl isobutyl ketone. None of these solvents gave satisfactorily clean blank plasma chromatograms, though the ether-isopropanol extract was possibly slightly superior to ethyl acetate. Recovery of the drug was poor with methyl isobutyl ketone though the blank extract contained many interfering peaks.

Blank and spiked plasma chromatograms following extraction by some of the solvents are shown in Figure 3.4. Since the ether-isopropanol mix gave slightly better results than ethyl acetate, it was decided to persist with this solvent, because it is easier to evaporate and more convenient to handle in terms of reactivity with equipment. In addition, Bailey and Kelner [21], when investigating the extraction of acidic drugs from water and plasma, found that the diuretics were optimally extracted from diethyl ether, and Van Damme et al. have demonstrated increased recoveries for drugs if 5% isopropanol is included in the extracting solvent [22]. The effect of excluding isopropanol from the extracting solvent was investigated. As may be seen from Figure 3.5., there is no advantage chromatographically in excluding the alcohol, and from a practical point of view, emulsion formation became a significant problem when diethyl ether alone was used to extract the drugs.

The next approach was to change the buffer strength of the mobile phase from 0.02 M to 0.05 M, but this measure caused a deterioration in the shape of the xipamide peak and had no

Figure 3.4.a.

Plasma chromatograms following ethyl acetate extraction



TIME (min)---------->

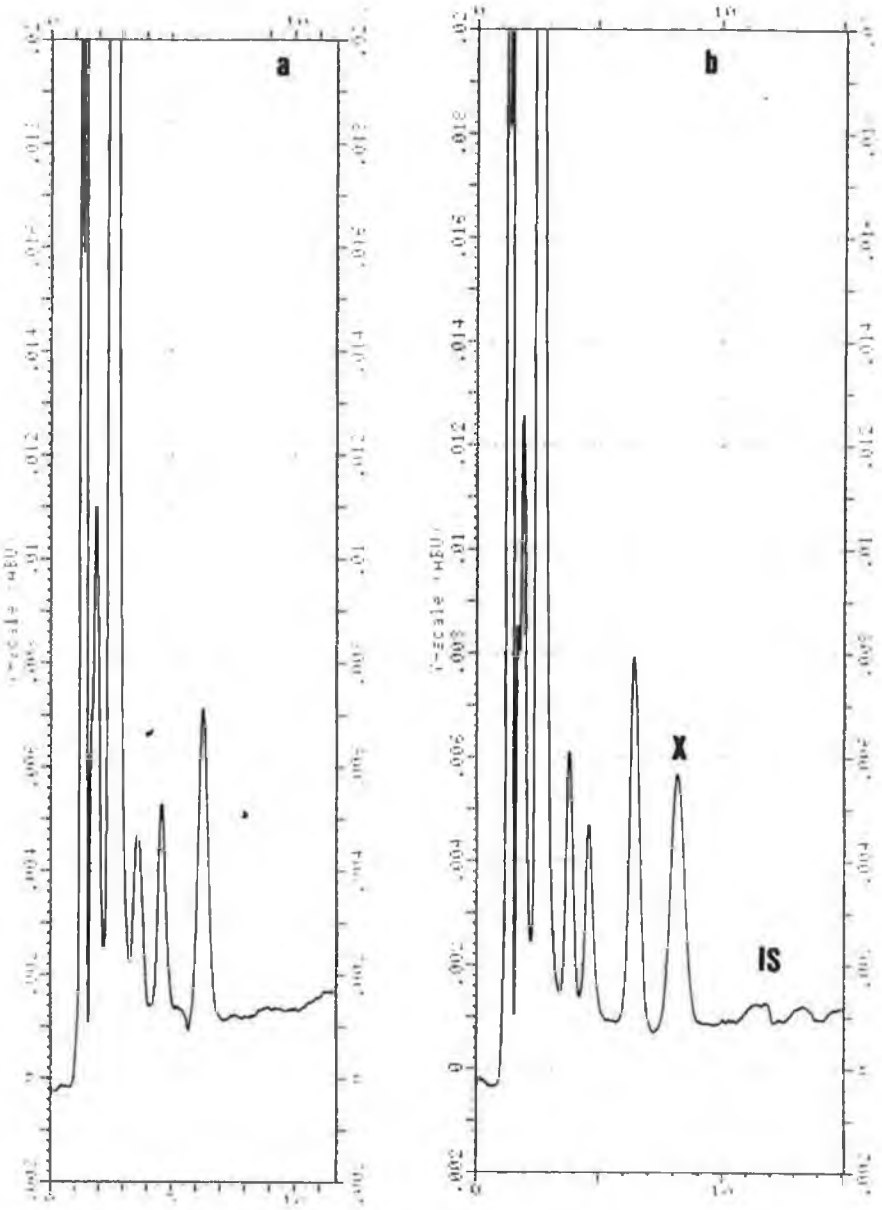
Mobile phase: 0.02 M Sodium acetate buffer, pH 7.0-methanol (65:35)

(a): blank plasma

(b): plasma spiked with xipamide (X) and mephenesin (IS)

Figure 3.4.b.

Plasma chromatograms following extraction into methyl isobutyl ketone



TIME (min)---------->

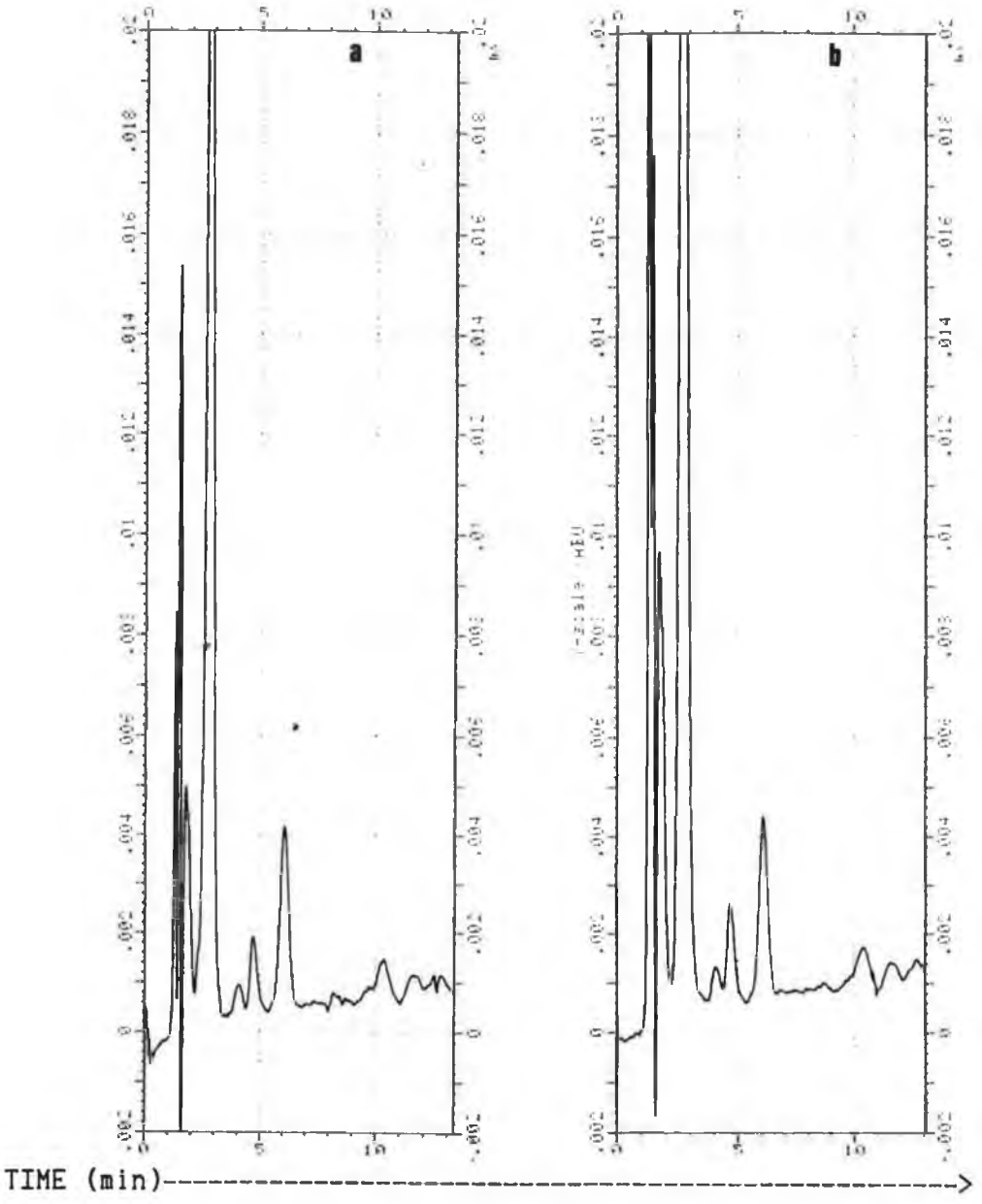
Mobile phase: 0.02 M Sodium acetate buffer, pH 7.0-methanol (65:35)

(a): blank plasma

(b): plasma spiked with xipamide (X) and mephenesin (IS)

Figure 3.5.a.

Chromatograms of blank plasma extracted into diethyl ether

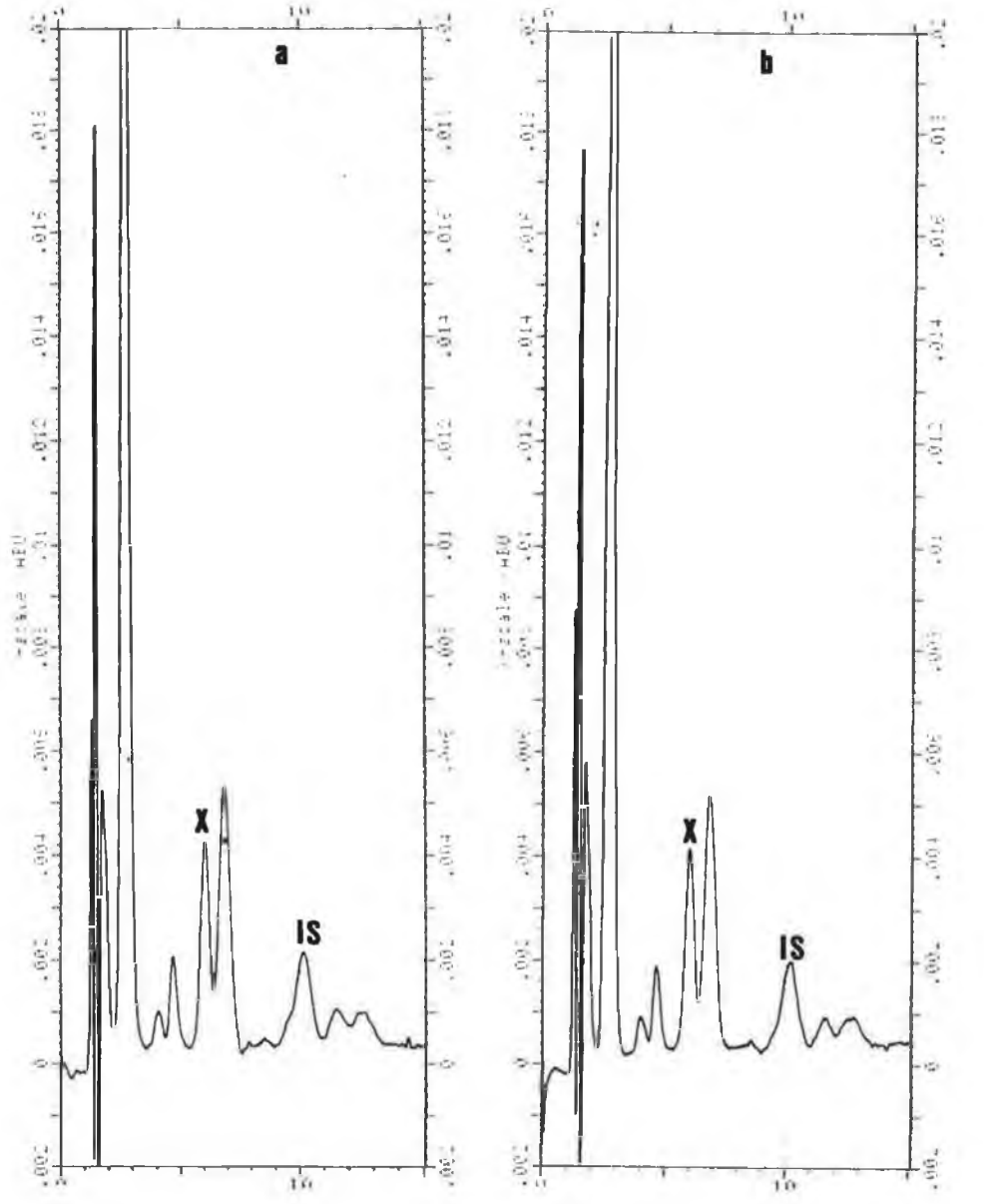


Mobile phase: 0.02 M Sodium acetate buffer, pH 7.0-methanol (65:35)

(a): extraction solvent: diethyl ether alone

(b): extraction solvent: diethyl ether-isopropanol (19:1)

Figure 3.5.b.
Chromatograms of spiked plasma extracted into diethyl ether



Plasma spiked with xipamide (X) and mephenesin (IS)

Mobile phase: 0.02 M Sodium acetate buffer, pH 7.0-methanol (65:35)

(a): extraction solvent: diethyl ether alone
(b): extraction solvent: diethyl ether-isopropanol (19:1)

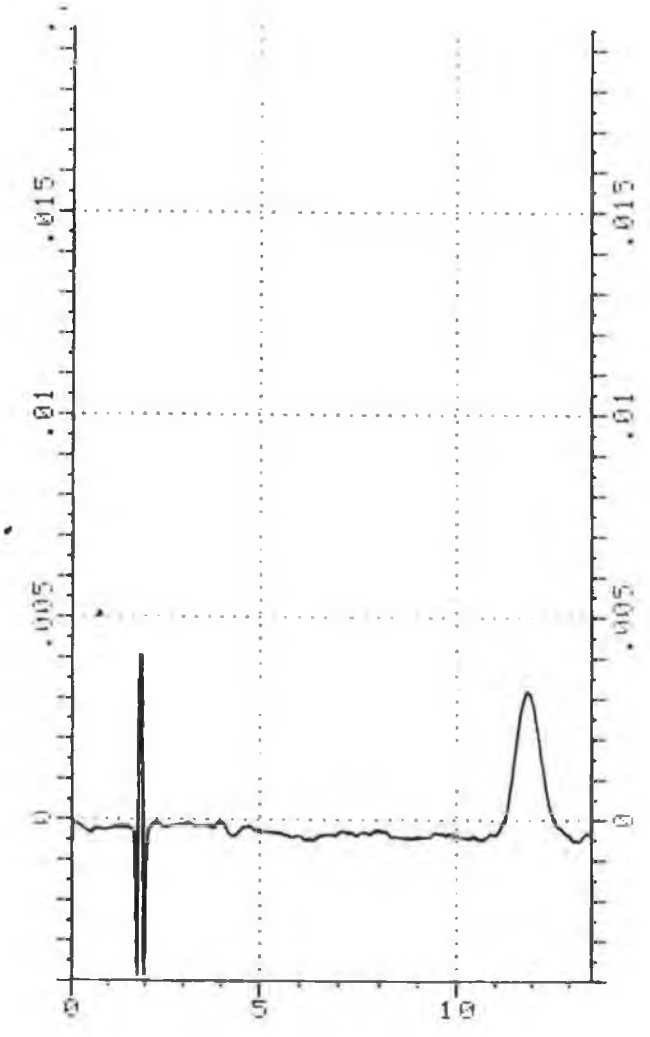
advantage in terms of plasma clean-up. By lowering the pH of the aqueous component in the mobile phase from pH 7.0 to pH 5.0 the retention of xipamide was markedly increased, i.e. from 5.0 minutes to 10.5 minutes (Figure 3.6). This is presumably because xipamide becomes less ionised at the lower pH value, and hence has a greater affinity for the hydrophobic packing in the column. The lower mobile phase pH also served to prolong the retention of plasma components so that they continued to interfere with the xipamide peak. Lowering the extraction pH (by the addition of acetic acid) exacerbated the problem of plasma interferents. This is because many plasma components are acidic in nature and would favour extraction into an organic solvent under more acidic conditions. On the other hand, if the extraction pH was increased, the drug was not recovered as it is ionised at high pH and would not partition into the organic extracting solvent.

Acid, base and organic precipitations were also tried. A base precipitation was carried out by the addition of zinc sulphate (10%) plus sodium hydroxide (0.1 M). This was followed by vortex mixing, centrifugation, and injection of an aliquot (50 μl) of the supernatant. While this measure gave cleaner blank plasma chromatograms, the drug was not recovered. An acid precipitation was carried out by the addition of 10% trichloroacetic acid followed by the same procedure as was used with zinc sulphate. The chromatograms resulting from this procedure contained many interfering peaks, and again recovery was poor. Precipitation with acetonitrile also gave very poor recovery of the drug; these observations may be explained by the fact that xipamide is highly protein bound [13,15,16], and possibly remains associated with the protein fraction following precipitation.

Because methanol and acetonitrile frequently produce differing selectivities on the same column, it was decided to substitute acetonitrile for methanol as the organic component. The order of elution between the two drugs remained the same, but because of the stronger eluting power of acetonitrile, it was necessary to

Figure 3.6.

Effect of lowering mobile phase pH on the retention of xipamide.



TIME (min)---------->

Mobile phase: 0.02 M Sodium acetate buffer, pH 5.0-methanol (65:35)

make some slight modification of the mobile phase to prolong the retention of xipamide on the column. This was achieved by lowering the pH of the aqueous component to 6.7 and by increasing the aqueous-organic ratio from 35:65 to 1:3. Using acetonitrile instead of methanol in the mobile phase also achieved the desired objective of improving the profile of drug-free plasma chromatograms.

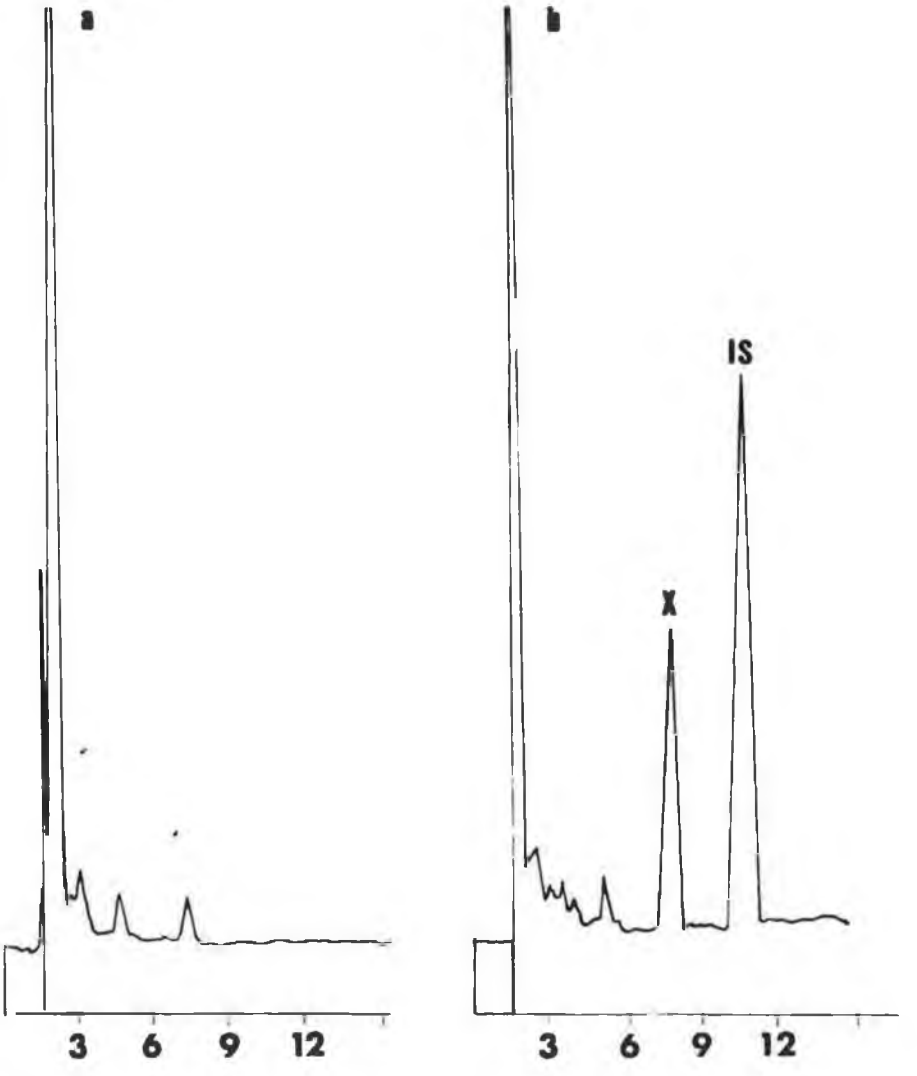
As may be seen from the chromatograms presented in Figure 3.7., there still remain some interfering peaks, so it was decided to investigate a salting out procedure prior to extraction into the organic solvent.*1 As outlined in Chapter 2, the addition of a salt to the biological medium increases the ionic strength of the aqueous phase and shifts the partition equilibrium in favour of extraction. Salting out also helps to minimise emulsion formation by reducing the water content of the organic phase and by imparting better definition to the phase boundaries.

The procedure involved the addition of 300 mg of salt to spiked plasma followed by vortex mixing for 90 seconds. After the addition of diethyl ether-isopropanol (19:1) the drugs were extracted by vortex mixing for a further 90 seconds. A number of different salts were investigated; these were sodium hydrogen carbonate, calcium carbonate, calcium chloride, sodium carbonate, ammonium chloride, zinc sulphate and lithium chloride. The carbonate salts gave clear blank plasma chromatograms, but poor recovery for xipamide.

*1 At this stage the Waters Model 450 detector and Linseis recorder were brought into use. As this detector is less sensitive than the photodiode array, all the peaks appeared to be smaller. In addition the chart speed was set at 200 mm/hr, as the Linseis recorder does not have the facility of the 300 mm/hr chart speed used on the Waters plotter. This gives the appearance of sharper peaks, although obviously column efficiency remains unchanged.

Figure 3.7.

Diethyl ether extractions at pH 5.0 using acetonitrile in the mobile phase



(a): blank plasma

(b): plasma spiked with xipamide, (X) and mephenesin, the internal standard (IS)

Mobile phase: 0.02 M Sodium acetate buffer, pH 6.7-acetonitrile (75:25)

Ammonium chloride, on the other hand, retained interfering peaks in the blank plasma chromatogram, though it yielded better recovery than the carbonate salts (Figure 3.8) Overall, lithium chloride offered the most satisfactory compromise in regard to plasma clean-up and drug recovery. The amount of lithium chloride added was then reduced to 200, 100, and 50 mg in order to determine how little of the substance could be added to the plasma to achieve the desired results. It was found that below 200 mg of lithium chloride, interfering peaks started to re-emerge, so it was decided that this amount would be added when preparing extracts for the validation procedure. The final experimental protocol employed for extraction is described in the experimental section. The mobile phase, as outlined above, was 0.02 M sodium acetate buffer, pH 7.5-acetonitrile (3:1), and the flow rate was increased to 1.5 ml/min. Under the above conditions, the mean retention times for the elution of xipamide and mephenesin were 5.4 and 9.0 minutes, respectively.

3.3.3. Assay validation

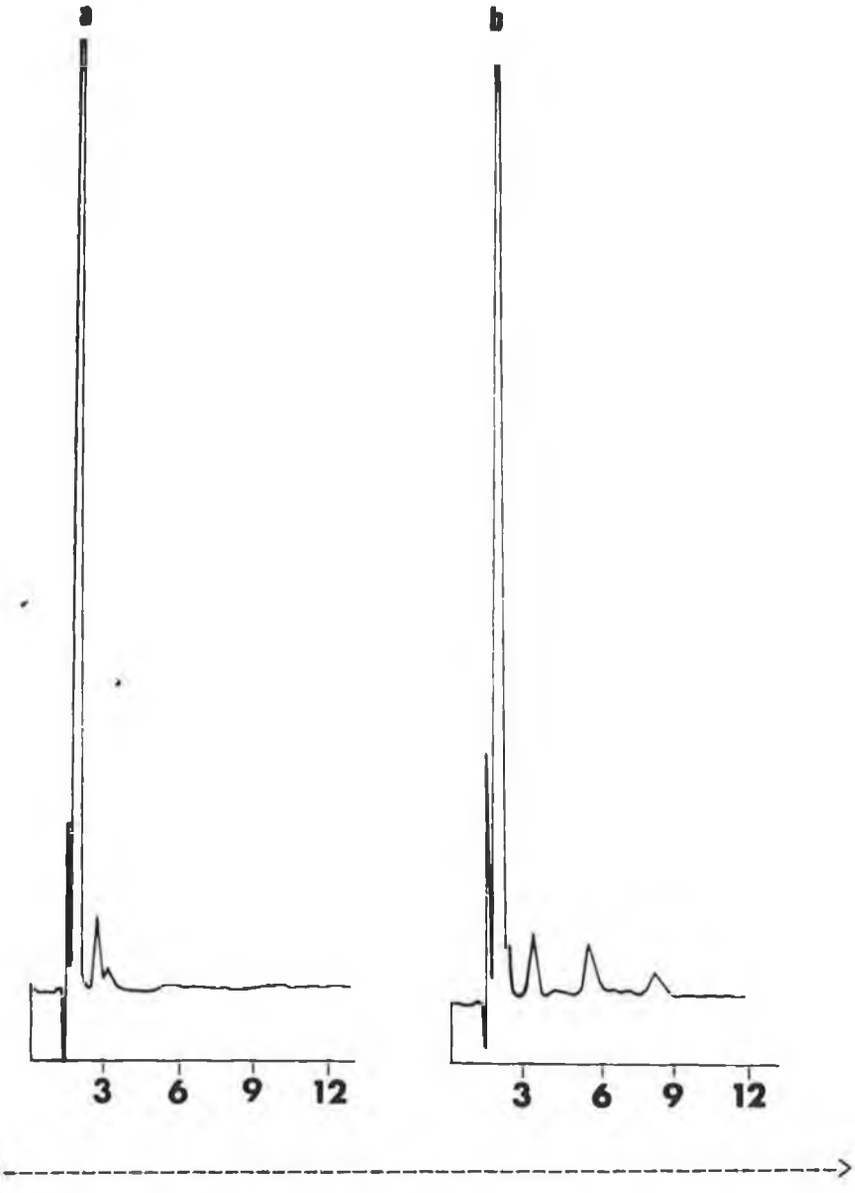
Evaluation of the assay was carried by the construction of a five-point calibration graph covering the concentration range 100-2000 ng/ml of xipamide in plasma. The slope and intercept of the calibration curve were determined through linear regression of the drug to internal standard peak height ratios versus drug concentration. Individual peak height ratios were then interpolated as unknowns on the calibration graph to determine concentration found as compared to concentration added. Chromatograms showing blank and spiked plasma chromatograms from the validation study are presented in Figure 3.9.

3.3.3.1 Limit of detection

The limit of detection was taken to be 50 ng/ml for a signal to noise ratio greater than 3. The limit of quantitation was taken

Figure 3.8.a.

Blank plasma extracts following the addition of salts



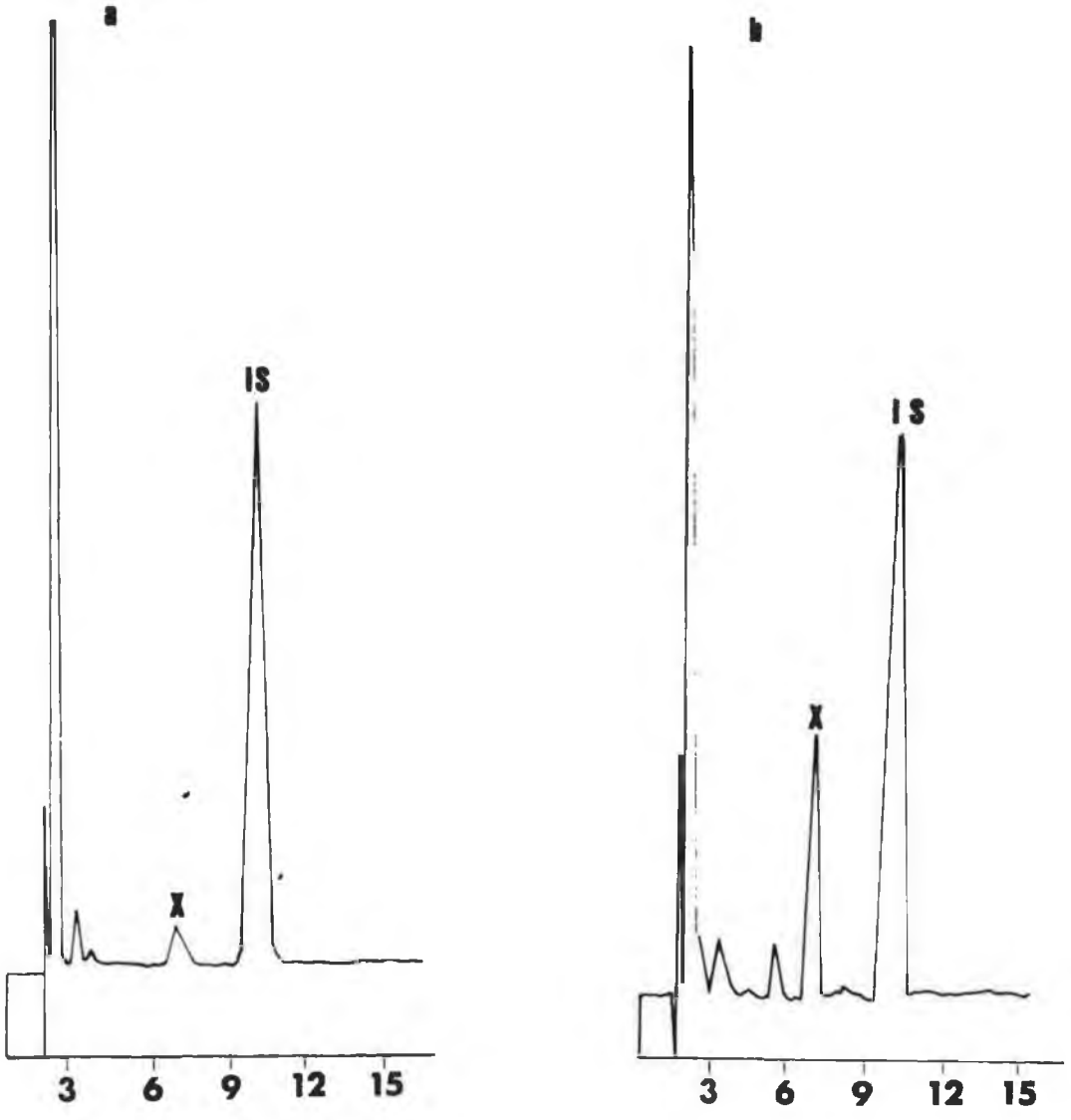
(a): sodium carbonate

(b): ammonium chloride

Mobile phase: 0.02 M Sodium acetate buffer, pH 6.7-acetonitrile (75:25)

Figure 3.8.b.

Spiked plasma extracts following the addition of salts



Plasma spiked with xipamide (X) and mephenesin (IS)

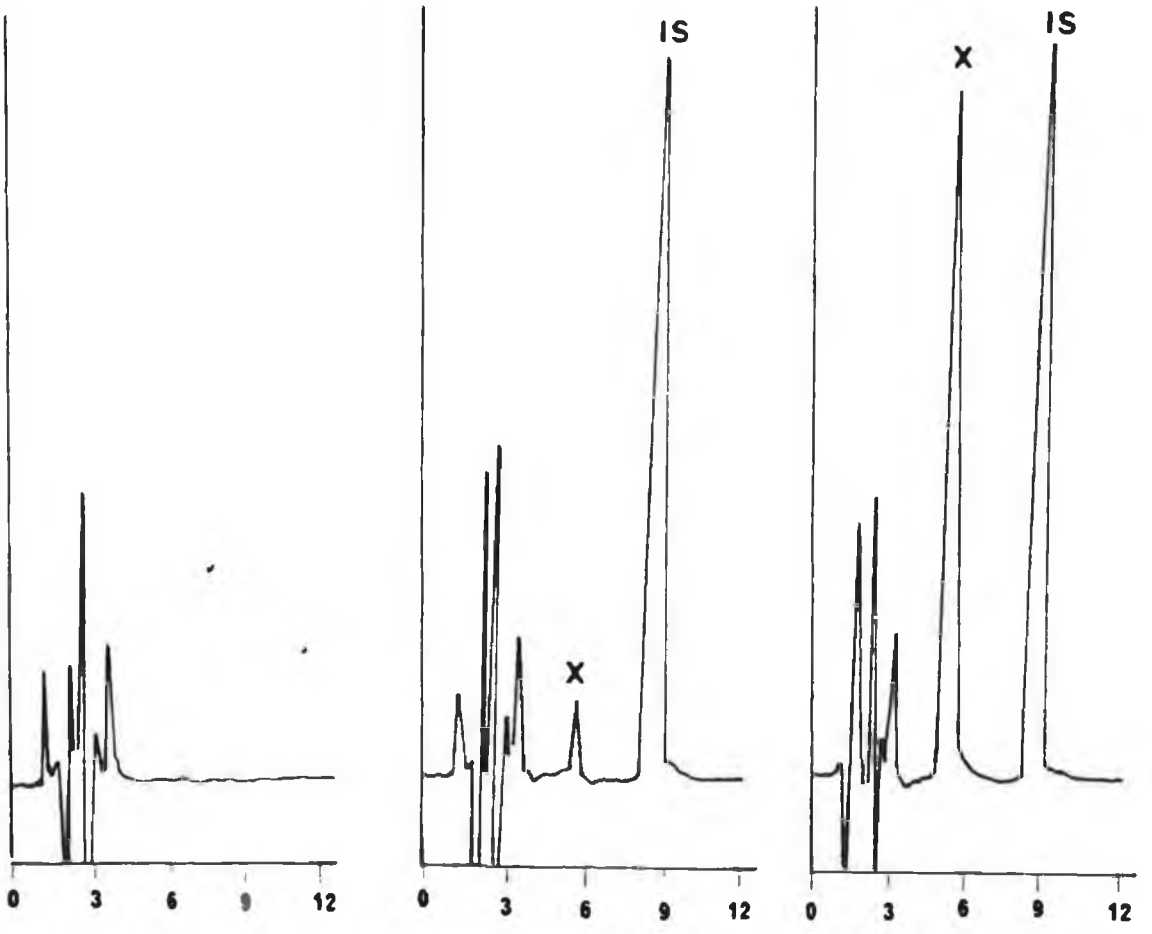
(a): sodium carbonate

(b): ammonium chloride

Mobile phase: 0.02 M Sodium acetate buffer, pH 6.7-acetonitrile (75:25)

Figure 3.9.

Chromatograms of extracted blank and spiked plasma from validation study



(A) Blank human plasma. (B) Plasma spiked to 250ng ml^{-1} xipamide (X) and 10μg ml^{-1} mephenesin (IS). (C) Plasma spiked to 2000ng ml^{-1} xipamide and 10μg ml^{-1} mephenesin

Mobile phase: 0.02 M Sodium acetate buffer, pH 6.7-acetonitrile (75:25)

Detector sensitivity: 0.01 AUFS

to be 100 ng/nl, the level at which quantitative measurement of the peaks was consistently possible. As outlined in the Introduction section, the plasma levels of xipamide 24 hours following an oral administration of a 20 mg dose were found to be approximately 100 ng/ml when the dose was administered on a weekly basis. It is to be expected that where the drug was given on a daily basis in a normal therapeutic regimen, the steady-state plasma levels, even 24 hours after dosing would be somewhat higher than this.

3.3.3.2. Precision

The data in Table 3.1 demonstrate the between-day (inter-assay) and within-day (intra-assay) variation in the method. The inter-assay variability was assessed singly in four replicate runs (on consecutive days) over the concentration range 100-2000 ng/ml. A separate calibration curve based on the individual peak height ratios (drug:internal standard) was constructed for each of the runs; the individual peak height ratios were then interpolated as unknowns on the appropriate regression line to obtain new values of concentration ("drug found"). The mean coefficient of variation over the entire concentration range is taken as an index of the precision and in this case, the inter-assay precision was found to be 4.3%.

For assessment of the intra-assay variability a single regression line was constructed based on the mean (of four) peak height ratios at each concentration level. The individual peak height ratios were then interpolated as unknowns on the regression line to obtain new values of concentration ("drug found"). The means, standard deviations and coefficients of variation of these new values were calculated at each concentration level, where n was equal to four representing the quadruplicate runs. The variability as measured in terms of mean coefficient of variation was 4.1% for the intra assay study.

3.3.3.3. Linearity

A measure of the linearity, as defined by the correlation coefficient (r) of the intra-assay regression line, is presented in Table 3.1. The correlation coefficient of the regression line for the mean intra-assay values was better than 0.999, and as may be seen from the equation of the regression line (also given in Table 3.1), the intercept does not differ significantly from zero.

3.3.3.4. Recovery

Recovery of xipamide from plasma was measured using two methods: (i) by calculating the percentage difference between the peak heights of extracted standards, and those of authentic (unextracted) standards in the same concentrations as reconstituted residues assuming 100% recovery; and (ii) by comparing the slopes of the two regression lines obtained from the extracted and authentic standards used in method (i) with the "extracted" slope as a percentage of the "authentic" slope yielding a measure of the amount recovered. Both authentic and extracted standards were processed and injected in duplicate.

The mean recovery determined by method (i) was found to be 83.8%, and by method (ii) 82.5 %. The mean of these two values yields an overall mean percentage recovery of 83.2%. The results of this experiment are presented in Table 3.2.

3.3.3.5. Selectivity

As may be seen fron the chromatograms presented in Figure 3.9, the drugs are well separated from the plasma peaks and there is no interference present on the blank plasma chromatogram. The drugs chlorthalidone, and frusemide, propranolol and atenolol were tested as possible interferences in the assay. Of these, chlorthalidone and frusemide co-eluted with the xipamide peak.

Precision and Linearity-INTRA-ASSAY

Amount Xipamide Added	Peak Height Ratio	Mean Ratio	Amount Found (ng/ml)
	0.042		102.9
100	0.030	0.041	102.3
	0.043		77.1
	0.041		95.8
	0.113		252.6
250	0.106	0.111	253.6
	0.111		247.7
	0.116		262.5
	0.225		490.4
500	0.223	0.225	506.4
	0.224		527.1
	0.228		507.1
	0.467		1004.1
1000	0.442	0.441	979.6
	0.418		1006.9
	0.438		965.6
			1999.8
2000	0.936	0.897	2008.0
	0.918		1991.9
	0.816		2011.6
	0.917		

Mean Coefficient of Variation = 4.1%

Regression Line: $y = 4.49 \times 10^{-4} x - 8.51 \times 10^{-3}$

Correlation Coefficient (r) = 0.999

TABLE 3.1

Mean Amt. Found (ng/ml)	Standard Deviation (ng/ml)	Coefficient of Variation (%)	Difference between added and found (%)
94.5	12.1	12.8	- 5.5
254.1	6.2	2.4	+ 4.6
507.7	15.0	2.9	+ 1.5
989.1	19.9	2.0	- 1.1
2002.8	8.8	0.4	+ 0.1

Table 3.1. continued
Precision and linearity- Inter-assay

Amount Xipamide added (A) ng/ml	Peak height Ratio	Amount found (F) ng/ml
100	0.045	113.7
250	0.108	251.1
500	0.229	515.1
1000	0.428	949.4
2000	0.919	2020.7
100	0.041	87.7
250	0.111	245.2
500	0.242	524.0
1000	0.456	979.5
2000	0.938	2005.1
100	0.045	106.9
250	0.115	260.3
500	0.228	507.9
1000	0.433	957.2
2000	0.917	2017.8
100	0.041	110.8
250	0.113	261.1
500	0.214	471.9
1000	0.468	1002.1
2000	0.948	2000.6

	Concentration ng/ml				
	100	250	500	1000	2000
Mean Amount Found ng/ml	104.8	254.4	504.7	972.0	2011.0
Standard Deviation	11.7	7.6	22.8	23.8	9.7
Coefficient of variation %	11.2	3.0	4.5	2.4	0.5
Difference between added and found %	+4.8	+1.8	+ 0.9	-2.8	+0.5

Table 3.2.

Results of recovery of xipamide studies

Concentration ng/ml	Xipamide peak height (mm) Authentic standards	Extracted standards	Recovery %
100	4.5	4.0	88.9
250	13.0	11.0	84.6
500	26.0	21.0	80.8
1000	53.5	44.0	82.2
2000	109.0	90.0	82.6

Method (i): mean recovery (± standard deviation) = 83.8 ± 3.1%

Method (ii): mean recovery [slope (extracted)/slope (authentic)] x 100%

Authentic standards: $y = 5.49x \times 10^{-3}x - 0.11$

$r = 0.999$

Extracted standards: $y = 4.53x \times 10^{-3}x - 0.19$

$r = 0.998$

slope(extracted)/slope(authentic) = 4.53/5.49 = 0.825

Mean Recovery = 82.5%

Overall mean percentage recovery = 83.2%

Separation was not attempted as it would be unusual for xipamide (a potent diuretic) to be co-administered with either of these two drugs, which are of moderate potency (chlorthalidone), or equipotent with xipamide (frusemide). If, however, it was desired to separate xipamide from these compounds, this could readily be achieved by minor adjustment of the eluent pH, because, as outlined in the text, the retention of xipamide is strongly influenced by pH under the employed experimental conditions.

3.4. CONCLUSION

A method was developed for the analysis of xipamide in plasma which offers an attractive alternative to previous methods involving densito-fluorimetric analysis of the derivatised drug on TLC plates. Employing a simple effective method of sample clean-up and drug extraction, analyses can be rapidly executed with the minimum of sample handling. Using a volatile extraction solvent, such as diethyl ether helps to minimise the time required for sample preparation, and with a chromatographic run time of less than 10 minutes, a set of samples may be processed within a conveniently short period of time.

3.5. REFERENCES

1. W. P. Leary and A. J. Reyes, S. Afr. Med. J., 59 (1981) 8.
2. W. P. Leary, A. C. Asmal and A. J. Reyes, Curr. Ther. Res., 27 (1980) 166.
3. C. H. Gold and M. Viljoen, Clin. Pharmacol. Ther., 25 (1979) 522.
4. J. W. Strandhoy and A. W. Gomoll, Eur. J. Pharmacol., 54 (1979) 173.
5. R. Dies and N. Heinz, Klinilarzt, 7 (19780 571.
6. R. D. Harding, A. Kalos, J. C. P. Weber and A. S. T. Dixon, Clin. Trials. J., 11 (1974) 45.
7. D. Bonaduce, N. Ferrara, V. Canonico, G. Visciola, M. Posticlione, M. Rengo and M. Condorelli, Curr. Ther. Res., 31 (1092) 293.
8. R. Fischer and A. Lenhartz, Med. Welt., 21 (1970) 270.
9. C. H. Gold and M. Viljoen, S. Afr. Med. J., 54 (1978) 569.
10. K. H. G. Payasena, L. W. H. Havard and J. C. P. Weber, Curr. Md. Res. Opin., 3 (1975) 121.
11. A. Kalos, A. Dixon and J. C. P. Weber, Rheumatol. Rehabil., 13 (1974) 81.
12. F. W. Hempelmann, F. Leuschner and W. Liebenow, Arzneim-Forsch., 25 (1975) 252.
13. F. W. Hempelmann and P. Dieker, Arzneim.- Forsch., 27 (1977) 2143.
14. W. Liebenow and F. Leuschner, Arzneim.- Forsch., 25 (1975) 240.
15. H. Knauf and E. Mutschler, E. J. Clin. Pharmacol., 26 (1984) 513.
16. F. W. Hempelmann, Arzneim.- Forsch., 25 (1975) 258.
17. F. W. Hempelmann, Arzneim.- Forsch., 27 (1977) 2140.
18. M. Sobel and E. Mutschler, J. Chromatogr., 183 (1980) 124.
19. H. Wieholtz, J. Schoelmerich, H. Knauf, W. Gerok, H. Spahn and E. Mutschler, Verh. Dtsch. Ges. Inn. Med., 90 (1984) 1902.
20. W. Diembeck, R. Dies and N. Heinz, Arzneim.-Forsch., 32 (1982) 1482.

21. D. N. Bailey and M. Kelner, J. Anal. Toxicol., 8 (1984) 26.
22. M. Van Damme, M. Leopold and A. K. Faouzi, Clin., Toxicol., 23 (1985-86) 589.

CHAPTER 4

THE DETERMINATION OF CHLORTHALIDONE IN HUMAN PLASMA BY REVERSED-PHASE MICELLAR LIQUID CHROMATOGRAPHY

4.1. INTRODUCTION

Chlorthalidone, 2-chloro-5-(1-hydroxy-3-oxo-1-isoindolinyl) -benzene sulphonamide, is a diuretic-hypertensive drug [1]. It may be given alone or in combined therapy with a hypotensive agent [2]. Pharmacologically, its actions resemble those of the thiazide diuretics although chemically, it differs from the latter in the nature of the heterocyclic ring. It is given in oral doses of between 50 and 200 mg daily: diuresis is initiated in about 2 hours and may last for 48 hours or longer. A number of studies have been carried out on the pharmacokinetics of chlorthalidone [3-10]. The drug is excreted mainly unchanged in the urine [10] and its prolonged duration of action is attributed to the fact that it has a biological half life of 30-80 hours [3-6]. The large inter-and intra-individual variation seen in biological half-life has been attributed to variations in bioavailability (which is relatively poor for chlorthalidone) [5,6], non-linear binding of the drug to red blood cells [7,8], and dose-dependent urinary excretion [9].

The drug appears to have a large volume of distribution (200-400 l [6]) resulting in relatively low peak plasma concentrations of 100-1000 ng/nl depending on the dose [11]. Most of the drug is bound to red blood cells, and this binding is at a maximum after about 8 hours [4-7]. Approximately 50-70% of the drug which is not bound to red blood cells is bound to plasma proteins [2].

A limited number of methods have been published for the determinatiion of chlorthalidone in biological fluids. Pulver et al. used a spectrophotometric method for the determination of chlorthalidone in dogs' urine, but this method is not sensitive enough for human blood level measurements [12]. A spectrophotometric method was also presented by Tweedale and Ogilive [3], permitting the determination of a minimum

concentration of 1 μg/ml. A very sensitive method based on radioactivity measurements was reported by Beizenherz et al. [13], though this technique did not offer the potential to separate drug from metabolites, and the attendant problems with radioactivity-based studies are considerable. Gas chromatographic methods have also been published [14-16]; most of these methods, based on the conversion of chlorthalidone to its tetramethyl derivative, are still quite laborious and may pose considerable methodological problems for a large series of samples. A sensitive and selective GC method was developed by Fleuren and Van Rossum [16], and depending on the volume of sample taken, as little as 10 ng/ml can be determined. An HPLC method for the analysis of chlorthalidone was reported where the drug was extracted from whole blood from acetonitrile followed by HPLC analysis on a cyano- column [17]. The limit of detection was 200 ng/ml, and this method demands the availability of whole blood samples. Chlorthalidone in blood, plasma and urine has been measured by HPLC on a C_{18} column using probenecid as the internal standard [10]. Recovery of chlorthalidone added to whole blood was 47.6%, and from plasma it was 70.0 %. Using UV absorbance detection at a wavelength of 226 nm, the limit of detection was 30 ng/ml.

The aim of the present study was to investigate the use of micellar chromatography for the determination of chlorthalidone in plasma. It was first sought to develop a suitable micellar chromatographic system, and to combine it if possible with an on-line solid-phase extraction system in order to carry out a rapid plasma clean-up procedure. It will be shown that chlorthalidone and the internal standards investigated showed predictable retention behaviour in the micellar chromatographic system, but that on-line solid-phase extraction (followed by micellar chromatogrphy) is difficult due to problems associated with protein binding and modification of the concentration column by surfactant monomers. An alternative liquid-liquid extraction scheme for the separation of the drug from plasma will be presented. The final method is fully validated, and should

provide an analytically useful alternative to conventional reversed-phase methodology.

4.2. MICELLAR LIQUID CHROMATOGRAPHY

Reversed-phase high performance liquid chromatography has become one of the most widely used modes of liquid chromatography, in part because of the impressive selectivity available via mobile phase participation in the equilibrium distribution of solute molecules between the stationary and mobile phases. Retention in reversed phase chromatography is dominated by solute-solvent interactions, with solute-stationary phase interactions playing an important, but secondary role [18]. Thus, the key to selective separations lies in the ability to control solute-solvent interactions by changing the composition of the mobile phase. One way in which this has been achieved is by the addition of surfactants at low concentrations to enhance separation of oppositely charged solute ions, giving rise to a technique known variously as "ion-interaction chromatography", "soap chromatography", or more commonly, "ion pair chromatography".

Surfactant solutions at high concentrations where micelles co-exist with monomers, dimers etc, also form chromatographically useful mobile phases. The chromatographic technique involved is known as micellar liquid chromatography and it can offer an added degree of selectivity to separations because of the possibility of interaction between the solute and the micelle.

4.2.1. Normal micelles

Surfactants exist in a multitude of different forms in which the hydrophobic tail varies in length from 8 to 20 carbon atoms and the hydrophilic portion can be a partially dissociable carboxylate ion, a fully-ionised moiety (such as anionic sulphate, or cationic trimethylammonium ions) plus counterion, or an uncharged species such as polyethylene glycol. They vary not only in their charge type (zwitterionic, positive, negative or

neutral), but in the nature of the hydrophobic portion (single or double hydrocarbon chains, multiple bonds etc.). At low concentrations in aqueous solution, surfactant molecules are dispersed as monomers, and to a lesser extent as dimers and trimers. Above a certain concentration, i.e. the critical micelle concentration (CMC), the monomers aggregate into micelles which are roughly spherical in shape, and consist typically of 60-100 monomers (the aggregation number) [19]. As the surfactant concentration is increased above the CMC, more micellar assemblies are formed with the monomer concentration remaining approximately constant and equal to the CMC.

The alkyl chain length, size of the head group structure, and interactions of the alkyl chains with one another and the solvent, determine the CMC, the micelle size, aggregation number, and structure. Micelles are small enough (3-6 nm in diameters) that the macroscopic solution properties resemble those of a truly homogeneous solution, i.e. they cannot be filtered using conventional methods and they do not cause measurable light scattering error in conventional spectrometry. In aqueous solution, the hydrocarbon moiety is directed inward while the polar head faces the bulk environment. The resulting solution is microscopically heterogeneous, consisting of micellar aggregates approximating to charge-covered oil droplets in a sea of water. Typical surfactants are listed in Table 4.1., along with their respective CMC values. Of these, the surfactants most commonly used in chromatography are sodium dodecyl sulphate and cetyltrimethylammonium bromide.

Much of the understanding of micelle structure and behaviour can be attributed to the pioneering work of Hartley [20]. Later models proposed alignment of the hydrocarbon chain with some water penetration, or alternatively, non-alignment of the tails, with looping of the chains allowing hydration of the sections that would normally be considered completely hydrophobic. The original oil droplet model presents a relatively static picture. Over the years, it has been determined that micelles are

Table 4.1.

Typical surfactants and their CMCs and aggregation numbers

Surfactant	CMC (M)	Aggregation No.
ANIONIC		
Sodium dodecyl sulphate $CH_3(CH_2)_{11}OSO_3^-\ Na^+$	0.008	62
Sodium polyoxyethylene-dodecyl ether $CH_3(CH_2)_{11}-(OCH_2CH_2)_{12}OSO_3^-\ Na^+$	0.030	81
CATIONIC		
Cetylpyridinium chloride $C_{16}H_{33}N^+C_5H_5Cl^-$	0.00012	95
Cetyltrimethylammonium bromide $CH_3(CH_2)_{15}N^+(CH_3)_3\ Br^-$	0.0013	78
NONIONIC		
Polyoxyethylene dodecanol $CH_3(CH_2)_{11}-(OCH_2CH_2)_6OH$	0.00009	400
ZWITTERIONIC		
N-dodecyl-N,N-dimethylammonium-3-propane-1-sulphonic acid $CH_3(CH_2)_{11}N^+(CH_3)_2SO_3^-$	0.003	55

molecular assemblies in dynamic equilibrium with their bulk solvent phase [21]. Two basic processes are occurring; namely exchange with the solvent and other micelles by monomers, dimers, and trimers, occurring over a microsecond to millisecond time scale, and actual replacement of the entire micelle occurring by this process over a time frame of milliseconds to seconds. This dynamic nature of micelles has a profound impact on an analyte residing in or on the micelle, and on analytical measurements centered on the associated analyte.

The effect of micellar dynamics on analytical schemes is based on the ability of micelles to organise reactants on a molecular level [22]; that is they increase the proximity of the reagent and analyte in relation to each other. To take advantage of the micelles organising ability, the species involved must preferentially associate with the micelle over the bulk solvent. In other words, the analyte and associated reagents should exhibit significantly greater rate constants for entrance into the micelle compared to exit constants. A number of factors influence the relative magnitude of the exit and entrance rate constants of analytes in micelles. If the species of interest is highly polar, it may prefer to remain in the aqueous bulk environment. If the analyte is completely anionic, it may be electrostatically repelled by an anionic micelle, or stabilised by a cationic micelle, and vice-versa for a cationic solute. Additional factors which can favourably influence the association between micelle and analyte are pH and ionic strength. Simply by making small pH adjustments, the analyte can be converted to a form which interacts much more strongly with the micellar assemblies.

A useful measure of the strength of the analyte-micelle interaction is the analyte's solubility in water. Generally, in the absence of secondary effects, the less soluble the analyte is in water, the stronger its association with the micellar aggregate. The hydrocarbon nature of the micelle tail serves to greatly increase the aqueous solution solubility of species of

extremely low solubility such as polynuclear aromatics. Low polarity species become solubilised or incorporated into the hydrophobic interior of the micellar assembly. This can greatly ease the solvent requirements for the analysis of highly insoluble organic molecules by permitting the use of non-toxic aqueous solutions.

4.2.2. Reversed micelles

When surfactants are dissolved in apolar solvents, the polar head groups and counterions orient themselves together in an inward fashion in contact with a small water pool where they are hydrated, and the aliphatic tails extend outward in contact with the non-polar solvent. These systems are often more complex and less understood than normal micellar systems. At low water concentrations, the water molecules fill the hydration sphere of the counterion so that solubilisd analytes encounter a more viscous, less polar environment than in ordinary bulk water. At higher water concentrations, there are more free water molecules available, so that the microenvironment is more polar, less viscous, and resembles ordinary bulk water in its properties.

Like normal micelles, these systems are characterised by CMC values and aggregation numbers, though CMC values are ill-defined for reversed micelles, with appreciable pre-CMC association occurring between monomers, dimers, etc. and the analyte. Reversed micelles are also characterised by dynamic monomer-micelle association and analyte-micelle association with both processes occurring on a millisecond time frame. Their usefulness in analytical methodologies resides in their ability to solubilise polar analytes which are excluded from the hydrophobic interior of normal micelles. For example, polar hetrocyclic compounds may be preferentially solubilised into the water pool of normal micelles, thus permitting favourable micelle-enhanced interactions.

These systems have only recently entered the arsenal of the analytical chemist and although not yet fully evaluated or extensively employed in biopharmaceutical analysis, their usefulness as eluents for normal phase chromatography has been demonstrated [23].

4.2.3. General analytical applications of micelles

Generally, surfactant micelles are used in analytical schemes to effect changes in solubility, pK_a, chemical equilibrium, reaction kinetics and spectral distributions or intensities. Acid-base titrations involving water-insoluble carboxylate and amine species using both potentiometric and visual indication systems have been performed using both cationic and anionic charged micelles to solubilise water-insoluble species, a process which can cause a pK_a shift of 0.5 to 3 units [24].

In analyses of selected metals via their coordination with complex anions, surfactant micelles have been used to aid solubilisation of the resulting complex. Charged micelles can also be used to manipulate a molecules excited state acidity constant when the analyte meets certain criteria. One of these criteria is that the molecule must possess a fluorescence or absorption spectrum which distinguishes the ground and excited states of the acid and conjugate base forms. Another requirement is that the analyte possesses some amphiphilic properties to allow solubilisation within or on the micelle.

4.2.4. Micellar solutions in chromatography

The partitioning of solutes between micellar and aqueous phases in liquid chromatography was first treated theoretically by Herries [25], but it was Armstrong and Henry [26] who first effectively demonstrated the usefulness of chromatographic eluents containing micelles above the CMC. They employed as mobile phases, wholly aqueous solutions containing 0.1-0.2M sodium dodecylsulphate (SDS) to separate a range of phenols and

polynuclear aromatic hydrocarbons. They showed that the presence of micelles in the mobile phase provides a hydrophobic site for interaction with the solute, and that surfactant solutions obviate the need for traditional organic modifiers such as acetonitrile and methanol.

If a solute is introduced into a micellar system, it may become solubilised preferentially into or onto the micelle by a process that is dynamic and is characterised by rate constants [27]. In traditional reversed-phase chromatography, selectivity is governed principally by the equilibrium that exists between the concentration of solute in the mobile phase and the amount in the stationary phase. Retention can be controlled by changing the composition of the mobile phase, so shifting the balance of the equilibrium. The situation is more complex in micellar systems: additional interactions of an electrostatic, hydrophobic and steric nature can occur, and these additional interactions impart an added degree of selectivity to the separation of drug and other analytes by HPLC. From a practical point of view, this additional selectivity of separation is what constitutes one of the major differences between hydro-organic and micellar eluents.

Retention of solutes generally decreases with increasing surfactant concentration, an observation first made by Armstrong and Henry [26], but the rate of decrease is strongly solute-dependent, and this frequently produces reversals in orders of retention. These inversions are the result of two competing equilibria: solute-stationary phase interactions, and solute-micelle interaction. These equilibria may be characterised by the partition coefficients, K_{ws} and K_{wm}, defined as the water to stationary phase and water to micelle equilibrium constants respectively. Both of these can affect retention [27], and it is the second partition coefficient, K_{wm}, which imparts uniquness to micellar chromatography. The larger the value of K_{wm} the greater the effect of increasing micelle concentration, which is what accounts for the solute-dependent rate of decrease in retention. The situation is further complicated by the fact

that surfactant monomers adsorb irreversibly onto reversed-phase packings [27-30], and interact with solutes as they progress down the column. Hence, one might expect the value of K_{ws} to change with increasing surfactant concentration. However, the effect of K_{ws} on retention is independent of micelle concentration since, as reported by Hung and Taylor [30], the amount of surfactant adsorbed on the stationary phase remains essentially constant after equilibration once the concentration is above the CMC. K_{wm} and K_{ws} are related to certain column parameters and micelle characteristics and have received detailed mathematical treatment from Armstrong and Nome [27].

An important micellar effect on retention is electrostatic interaction between an ionic surfactant and an ionisable solute in a manner analagous to ion interaction chromatography. The nature of this effect, whether it involves attraction or repulsion, depends on the ionic character of the surfactant and the solute. As previously stated, there is considerable adsorption of surfactant onto a non-polar stationary phase, thereby providing for electrostatic interactions of ionisable solutes with the stationary phase as well as the charged micelle.

When an acidic solute is separated using an anionic surfactant, electrostatic repulsion from both the micelle and surfactant-modified stationary phase can occur. The same holds true for a cation separated using a cationic surfactant. Repulsion from both the stationary phase and the micelle means that the solute would reside exclusively in the bulk macro-environment and would move rapidly down the column. Although a hydrophobic interaction with the stationary phase is still possible, its effect is diminished by the effect of electrostatic repulsion [31].

When a solute is separated using a surfactant of opposite charge, electrostatic attraction between the solute and the surfactant occurs. Considering the micelle first, this attraction would augment the solute-micelle hydrophobic attraction, and decreased

retention would be expected. However, it has been found experimentally that switching from a like-charged to an oppositely-charged surfactant (for example, SDS to DTAB (dodecyltrimethylammonium bromide)) actually increased retention times for a range of phenols [31]. These observations have been accounted for in terms of electrostatic attraction between the charged surface of the stationary phase, supplemented by a solute-surfactant ion pair interacting hydrophobically with the stationary phase. As non-polar compounds are minimally affected by electrostatic effects, they can be separated from other compounds by judicious choice of surfactant and mobile phase pH.

A three-phase equilibrium model relating capacity factor to micellar mobile phase concentration has been developed by Arunyanart and Cline-Love [28], and equations have been developed to allow calculation of the equilibrium constant for the solute between the bulk aqueous phase and the micellar aggregate. They also showed that if the equilibrium constant is available from independent sources, it is possible to predict the chromatographic capacity factor at zero or greater micelle concentrations. This means that spectroscopic methods can be used to predict chromatographic retention, a goal not yet realised for hydro-organic phases. They later extended this work to account for the effect of prototropic equilibria on the association of weak organic acids and bases with micellar aggregates [29]. The equations they developed predicted a sigmoidal dependence of chromatographic capacity factors on mobile phase pH and a parabolic dependence of k' on micelle concentration.

One of the early problems with micellar mobile phases was poor chromatographic efficiency. This problem was first addressed in detail by Dorsey et al. [32]. They proposed that the poor mass transfer characteristics associated with wholly aqueous mobile phases on reversed-phase columns [33] are due to poor wetting of the stationary phase resulting in slow equilibrium across the interface of two highly dissimilar phases. To overcome this problem, they added a small amount of organic modifier to the mobile phase and found propanol to be superior to methanol, ethanol and acetonitrile in

terms of improved efficiency and peak shape. Scott and Simpson investigated the modification of C_{18} phases by organic modifiers, and found that 3% propanol gave more than 90% coverage of the stationary surface, but the figure was only 50% for the same concentration of methanol [34]. This modification promotes faster mass transfer resulting in improved efficiencies. The higher concentrations of methanol which would be required to produce the same improvement as 3% propanol represents an unrealistic situation for micellar chromatography, as added alcohols can loosen the micellar structure and disturb the equilibria which it was originally sought to exploit. Dorsey found little improvement in efficiency or peak asymmetry at concentrations of propanol greater than 3%, though solute retention times are further reduced. For this reason, it may be beneficial to slightly increase the amount of propanol added [32].

A second parameter which can be adjusted to improve mass transfer characteristics is temperature [32,35]. Dorsey [32], found that operating at temperatures of up to 40^{o} reduced peak asymmetry, but had little effect on efficiency if the mobile phase contained 10% methanol. On the other hand, Yarmchuck et al. [35], showed a steady increase in column efficiency as the temperature was increased from $25^{o}C$ to $60^{o}C$, although, in this case, the mobile phase contained no organic modifier. Yarmchuck further proposed that efficiency could be improved by keeping the surfactant concentration as low as possible, since an increase in plate height was observed as the concentration was increased from 0.02 M to 0.2 M. In addition, he found that an increase in linear velocity effected an increase in plate height, and that the addition of organic modifiers was of little benefit.

The disadvantages of working at elevated temperatures are considerable, some of the most important being the need to maintain strict temperature control, to preheat the mobile phase above $40^{o}C$ in order to prevent peak distortion from thermal gradients [36] and the fact that the silica-based stationary support dissolves more rapidly at higher temperatures. The approach adopted in the present work was to add a small amount of organic modifier to the micellar

mobile phase; this measure was found to provide satisfactory separation and peak shapes for the compounds under investigation.

Apart from providing unique selectivity in the chromatographic separation process, micellar liquid chromatography enjoys a number of distinct advantages over conventional reversed-phase liquid chromatography. For example, it permits gradient elution without the usual need for re-equilibration after each change in mobile phase composition. This is because, as outlined above, at concentrations above the CMC, any change in surfactant concentration affects only the number of aggregates; the amount of free surfactant in the mobile phase, and that adsorbed onto the stationary phase remains constant irrespective of the number of micelles present. This property of micellar solutions also renders them useful in the coupling of gradient elution to electrochemical detection. With conventional mobile phases, changes in the conductivity, viscosity, and pH of the mobile phase during a gradient programme give steeply sloping baselines owing to changes in residual current [37,38]. Khaledi and Dorsey have demonstrated an improved compatibility of micellar (as opposed to hydro-organic) gradients with electrochemical detectors [39].

Being primarily aqueous-based, surfactant solutions have low compressibility, a factor which makes them well suited to refractive index detection [26]. Micellar solutions have been shown to increase fluorescence quantum efficiencies, and to permit unusual detection schemes, such as room temperature liquid-phase phosphorescence [40,41]. Its usefulness extends beyond the realm of pure organic analysis: both neutral and charged metallo-organic complexes have been separated on the same chromatogram using micellar systems [42], and Mullins and Kirkbright have demonstrated the determination of inorganic anions on a C_{18}- column using micellar systems [43].

4.2.5. Micellar chromatography in biopharmaceutical analysis

In 1985, DeLuccia et al. described the use of micellar mobile phases for direct serum injection (DSI) without prior sample treatment [44].

This type of procedure is possible because of the unique solubilising power of the micelles by which they can form soluble protein-SDS complexes thus preventing protein precipitation on the analytical column. This process also displaces drugs from serum components, freeing protein bound drugs to partition into the stationary phase [44-48]. Granneman and Senello have shown that surfactant monomers will competitively bind proteins thereby releasing protein-bound antibiotics [48]. In the direct serum injection technique described by DeLuccia et al. [44], 20 μl serum samples were introduced onto the column and detection was achieved using UV detection at 254 nm. While this method does permit direct injection, the sensitivity is limited by the low sample volume, the high serum background signal and by the fact that UV detection at 254 nm was employed, which is not necessarily the optimum wavelength for many drugs of therapeutic importance. The limits of detection offered by this scheme would appear to lie in the mid to low microgramme per ml of serum level.

An advance on the procedure of DeLuccia et al. was described by Arunyanart and Cline-Love [45]. In order to enhance chromatographic efficiency, they incorporated 10% propanol into the micellar mobile phase, a measure which did not promote protein precipitation. They also established in this work that approximately 0.02 M SDS was required to solubilise serum proteins. In this case, sensitivity was improved by the use of fluorescence detection, though again, the limit of detection was restricted by the high serum background signal. Using this technique it was possible to determine down to approximately 50 ng/ml propranolol in serum, but the limit of detection for morphine and codeine was greater than 1 μg/ml.

DSI is limited by the fact that there is no pre-concentration of the drug prior to analysis, so large volumes of untreated biological fluid would have to be injected to determine drugs whose therapeutic ranges lie in the low ng/nml range. Injecting large volumes of plasma or serum creates the further problem of blockage of the analytical column and build-up of strongly retained endogenous compounds. Because there is no sample clean-up the biological matrix will produce a large signal eclipsing the peaks of early eluting

compounds. Optimisation could be achieved by judicious selection of a detection scheme or by extracting the drug from the biological fluid.

The use of micellar mobile phases in on-line solid phase extraction processes has been described [47,49,50]. In these instances, the micellar mobile phase constitutes the wash solution. Following injection of the plasma onto the concentration column, the proteins were solubilised and eluted to waste by the surfactant while the drugs were selectively retained by the concentration column. The concentration column was then washed with a 10% methanol-aqueous mixture to remove the SDS and any remaining interferents.

This approach can lead to poor recovery of certain strongly protein bound drugs, and in one instance, increasing the concentration of SDS in the wash phase (with a view to promoting displacement of the drugs from their protein-binding sites) caused severe peak tailing and, in some cases, peak splitting [47]. Another problem with increasing the concentration of SDS in the wash phase is the reduction in drug breakthrough volume, since, as outlined previously, increasing the number of micelles is equivalent to increasing the elution strength of the mobile phase. Furthermore, if a micellar wash phase is to be followed by chromatography with a conventional hydro-organic mobile phase, it is necessary to remove the SDS before coupling the concentration column to the analytical column, otherwise the SDS will interfere with the chromatography, and if the concentration of organic modifier is high, the surfactant will tend to precipitate on the analytical column.

4.3. EXPERIMENTAL

4.3.1. Reagents and solvents

Chlorthalidone and frusemide were purchased from Sigma, Dorset, UK. Xipamide, triamterene, propranolol, atenolol, metoprolol and pindolol were received as a gift from the Institute of Clinical Pharmacology, Dublin, Ireland. Biochemical grade sodium dodecyl sulphate, and AnalaR grade 1-propanol, sodium hydroxide, calcium chloride, sodium carbonate, sodium hydrogen carbonate and lithium chloride were received from BDH, Poole, UK. HPLC grade methanol, acetonitrile and diethyl ether were obtained from Labscan Analytical Sciences, Dublin, Ireland. Hydrochloric acid, zinc sulphate, trichloroacetic acid, salicylic acid, tetramethylammonium bromide, sodium dihydrogen phosphate, disodium hydrogen phosphate and ortho-phosphoric acid (all analytical grade) were obtained form Riedel de Haen, Hannover, West Germany.

Deionised water was obtained by passing freshly distilled water through the Millipore Milli-Q water purification system. Dried human plasma from the Blood Transfusion Board, Dublin, Ireland was dissolved in deionised water and used within seven days of reconstitution.

4.3.2. Instrumentation

The compounds were separated on a C_8- (10 μm) reversed-phase column (220 x 4.6 mm i.d.) which was protected by a C_8-disposable guard column cartridge (15 x 3.2 mm i.d.), both supplied by Pierce, Rockford, IL, USA. The mobile phase was filtered through a 0.45 μm membrane and degassed by sonication prior to use. It was delivered at a flow rate of 1 ml/min (unless otherwise specified) by a Waters (Milford, MA, USA) model 501 HPLC pump. Detection was achieved by ultraviolet absorption using a Shimadzu (Kyoto, Japan) SPD-6A spectrophotometric UV detector with a Linseis (Selb, West Germany) recorder at a chart

speed of 200 mm/hr. Some measurements were made with a Waters Model 900 photodiode array spectrophotometric detector. The drugs were detected at 235 nm.

For direct injection, samples were introduced onto the column using a Rheodyne (Cotati, CA, USA) Model 7125 injection valve fitted with a 20, 100, or 500 μl loop according to the experiment being performed.

For the purpose of column switching a second Waters Model 501 HPLC pump and the concentration column were connected via a Rheodyne six-port, two-position switching valve, the operation of which has been described in Chapter 2, and a 500 μl loop was fitted onto the injection valve. The 10 x 1.5 mm i.d. concentration columns were dry-packed with Corasil (Waters Associates) RP-18 (37-50 μm), Sepralyte (Analytichem International, Harbour City, CA, USA) RP-8 (40 μm) or Supelco (Bellefonte, PA, USA) cyano- (25-45 μm) pellicular packing materials. The washing solvent delivered to the concentration column was filtered degassed deionised water at a flow rate of 1 ml/min.

The pH adjustments were made using a standard pH glass electrode at ambient temperature. The pH meter was calibrated on a daily basis using aqueous standards prepared on a weekly basis.

4.3.3. Standards

Stock solutions of the drugs were prepared by dissolving 10.00 mg of drug in 10.0 ml methanol. Working stock solutions containing 25 μg/ml were prepared by a 1:3 dilution of the concentrated solutions in methanol-water (1:1). These solutions were then diluted with mobile phase for direct injection or added to an aliquot of blank plasma prior to extraction.

4.3.4. Procedures

4.3.4.1. Effect of pH on the retention of chlorthalidone and frusemide

A solution containing 0.08M SDS was made by dissolving the required amount of SDS in 0.01 M disodium hydrogen phosphate. Aliquots of this solution were adjusted to between 4.5 and 7.0 (in increments of 0.5 pH units) by the addition of phosphoric acid. Mobile phases were prepared by mixing the buffered SDS solutions with 1-propanol to produce a 5:95 alcohol-aqueous mixture. Mobile phases were filtered and degassed prior to use.

The 25 μg/ml working stock solutions of chlorthalidone and frusemide were diluted (1:9) in mobile phase and a 20 μl aliquot injected for chromatography. The retention times of the two drugs was measured as a function of eluent pH.

4.3.4.2. Effect of SDS concentration on the retention of chlorthalidone and frusemide.

A solution of 0.1 M SDS in 0.01 M phosphate buffer, pH 4.5 was prepared. This was then diluted with phosphate buffer, pH 4.5 to give solutions containing 0.025, 0.05 and 0.075 M SDS. Mobile phases were prepared by mixing the buffered SDS solutions with 1-propanol to produce a 5:95 organic-aqueous mixture. Mobile phases were filtered and degassed prior to use.

The 25 μg/ml working stock solutions of chlorthalidone and frusemide were diluted (1:9) in mobile phase and a 20 μl aliquot injected for chromatography. The retention times of the two drugs were measured as a function of SDS concentration.

4.3.4.3. Effect of percentage of 1-propanol in the mobile phase on the retention of chlorthalidone and frusemide

A solution containg 0.05M SDS in 0.01M phosphate buffer, pH 4.5, was prepared. Mobile phases were prepared by mixing this solution with 1-propanol to generate either a 5:95 or 3:97 aqueous-alcohol mixture. The 25 μg/ml working stock solutions of chlorthalidone and frusemide were diluted (1:9) in mobile phase and a 20 μl aliquot injected for chromatography. The retention times of the two drugs were measured as a function of percentage organic modifier.

4.3.4.4. On-line solid-phase extraction by column switching

The instrument arrangement used in this section has been previously described in chapter 2. The concentration columns were packed with either RP-18, RP-8, or cyano- packing material. The wash solutions delivered to the concentration column were as follows:

(i) deionised water
(ii) deionised water containing 0.1% phosphoric acid
(iii) deionised water containing 5% methanol
(iv) 0.01 M phosphate buffer, pH 5.0, containing 0.005 M tetramethylammonium bromide

The wash solution was delivered at a flow rate of 1.0 ml/min and for 60-90 seconds, and the drugs were swept from the concentration in a backflush mode in order to minimise band broadening effects. Various pre-treatment operations carried out on the plasma prior to injection will be described in the section 4.4.

4.3.4.5. Extraction procedure

Aliquots of drug-free plasma were spiked with 50 μl of

chlorthalidone and 50 μl of xipamide working stock solutions, or with methanol-water (1:1) to generate plasma blanks. The plasma was then treated either by the addition of an equal volume of 0.067 M potassium dihydrogen phosphate, 0.1 M phosphate buffer of pH 7.4 or 8.0, or by the addition of 200 mg lithium chloride, calcium chloride or sodium bicarbonate. Following vortex mixing for 10 s , 4 ml diethyl ether-isopropanol (19:1) were added, and the drugs were extracted by further vortexing for 60 s. After centrifugation for 15 min at 1000 g, the upper organic layer was transferred to a clean polypropylene tube and evaporated to dryness at 40^{o}C under a gentle stream of air. The residue was reconstituted in 200 μl of mobile phase, and an aliquot of 100 μl was injected for chromatography.

4.3.4.6. Calibration and Calculation

For calibration, a set of standards containing 50-800 μg/ml of chlorthalidone was prepared by serial dilution of the working stock solution in methanol-water (1:1). 0.5 ml aliquots of drug-free plasma were spiked with 50 μl of chlorthalidone (C) and 50 μl xipamide, the internal standard (IS). Plasma blanks were made by adding 100 μl methanol-water (1:1) to 0.5 ml aliquots of drug-free plasma. The plasma pH was adjusted to 7.4 and subjected to the extraction procedure described above. Evaluation of the assay was carried out by the construction of a six-point calibration graph covering the concentration range 50-800 ng/ml of chlorthalidone in plasma. The slope and intercept of the calibration graphs were determined through linear regression of the drug to IS peak height ratios versus drug concentration. Individual peak height ratios were then interpolated on the calibration graphs to determine the values of "concentrations found" compared with "concentrations added".

4.4. RESULTS AND DISCUSSION

4.4.1. Micellar chromatographic characteristics of chlorthalidone and frusemide.

Initially, the micellar chromatographic characteristics of chlorthalidone and frusemide were investigated with the aim of using the latter as an internal standard for the former. The chosen starting point was a mobile phase consisting of 1-propanol-0.08 M SDS in 0.01 M phosphate buffer, pH 7.0 (5:95). This concentration of SDS was selected as it is 10 times the critical micelle concentration (0.008 M [51]), and thus abundant micelles would be available to interact with solutes. Propanol was chosen as the organic modifier because Dorsey et al. found that propanol produces more than double the increase in plate number than methanol when added in the same concentration to solutions of SDS [32]. Under these chromatographic conditions, frusemide and chlorthalidone eluted with retention times of 2.3 and 8.0 minutes, respectively. Whereas this represented an adequate retention for chlorthalidone, frusemide would have to be held longer on the column to permit its separation from the solvent front and its eventual quantitation in plasma. The approach adopted at this point was to alter the mobile phase pH since frusemide is an organic acid (pK_a = 3.6 [52]) and at pH 7.0, would exist principally as the carboxylate anion. Since negatively charged SDS monomers become adsorbed onto the hydrophobic stationary phase [27-30], the like-charged anionic form of frusemide would be electrostatically repulsed by the column surface, and would therefore elute rapidly with the solvent front. This effect is augmented by a similar electrostatic repulsion between the charged drug and the negatively-charged micellar assemblies which induces the drug to move rapidly down the column in the bulk medium. Although hydrophobic interaction with the stationary phase is still possible, its effect will be reduced by the electrostatic repulsion.

The effect of varying the pH between 7.0 and 4.5 on the retention of the chlorthalidone and frusemide is shown in Figure 4.1. As as may be seen, the retention time of chlorthalidone remained virtually unchanged while frusemide was retained longer on the column, consistent with an increased population of the uncharged species as the pH is lowered. Since pH 4.5 yielded satisfactory retention for the two drugs, this value was the chosen pH for following experiments. Chromatograms depicting the retention of chlorthalidone and frusemide are shown in Figure 4.2.

The next variable to be changed was the concentration of SDS in the mobile phase. It has been well established since the inception of micellar liquid chromatography [26] that there is a general decrease in retention as the concentration of surfactant is increased, and as the rate of decrease is solute-dependent, reversals in elution order are frequently encountered. The effect of varying the SDS concentration between 0.1 M and 0.025 M on solute retention is shown in Figure 4.3. This graph shows that the effect of surfactant concentration on chlorthalidone is more pronounced than on frusemide, and at higher SDS concentrations the two lines approach intersection. The smaller decrease in retention observed for frusemide may possibly be explained by the shift to a higher pK_a value induced by the anionic surfactant system, an effect which generally increases with increasing surfactant concentration [53,54]. If the pK_a of frusemide is raised, a greater proportion of molecules will be uncharged thus promoting increased retention, which operates to counteract shorter retention times caused by increased surfactant concentration. For the purpose of the present work, a concentration of 0.05 M SDS was chosen since it gave good separation of the two drugs in a reasonable elution time (8.5 and 11.0 minutes for frusemide and chlorthalidone, respectively).

As Dorsey et al. found little improvement in efficiency (though reductions in retention times were observed) with concentrations of 1-propanol greater than 3% [32], the next step in the present work was to reduce the percentage propanol from 5 to 3%.

Figure 4.1.

Effect of buffer pH on drug retention time

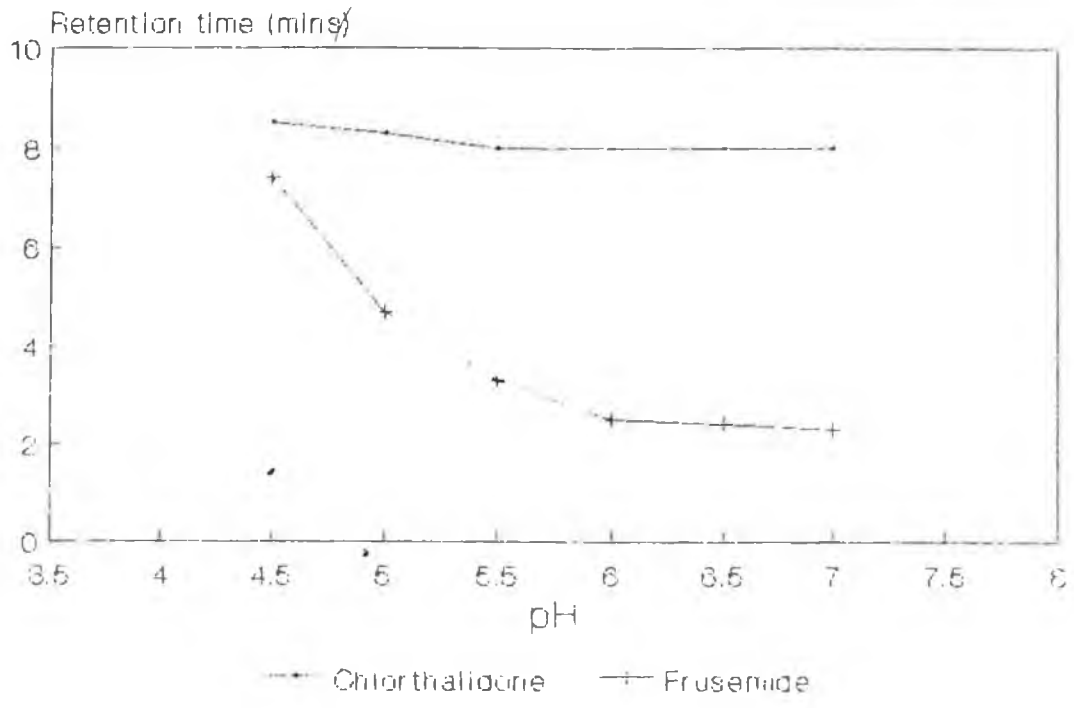
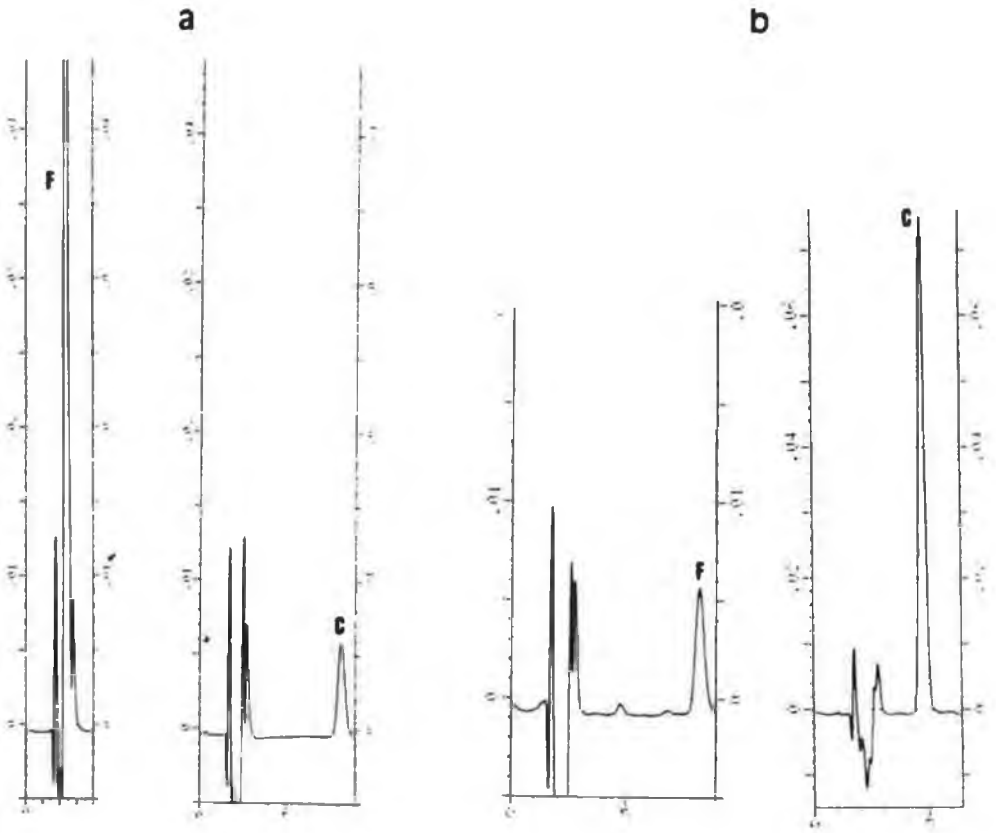


Figure 4.2.
Chromatograms of chlorthalidone and frusemide at pH 7.0 and pH 4.5



TIME(min)---------->

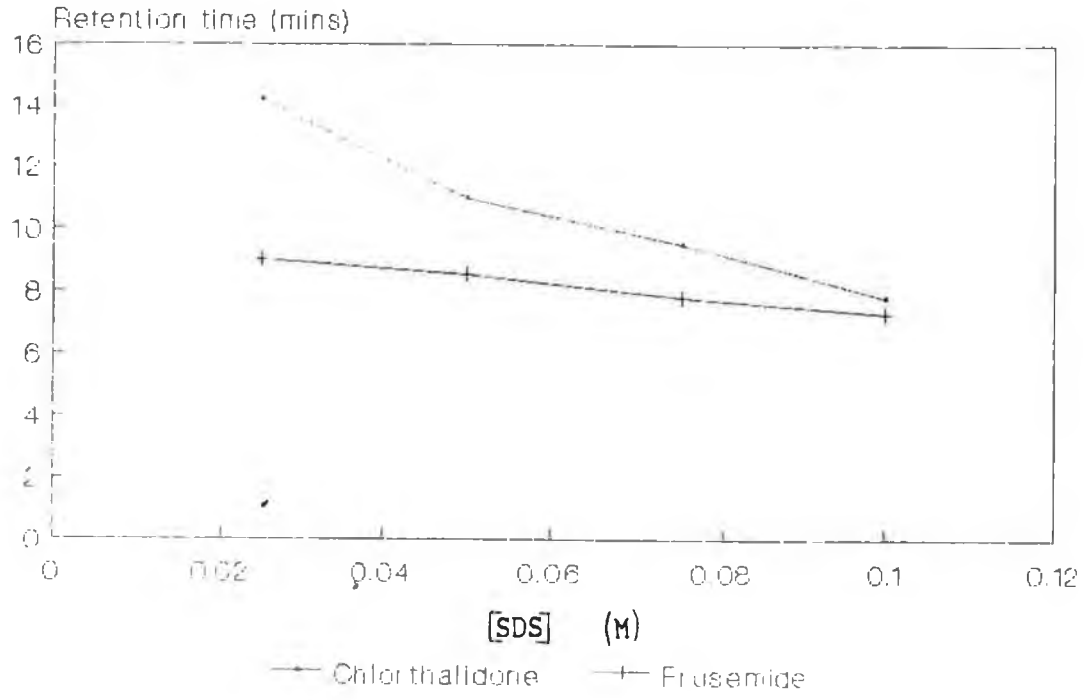
C: chlorthalidone; F: frusemide

(a): mobile phase = 0.08 M SDS in 0.01 M phosphate buffer, pH 7.0-isopropanol (95:5)

(b) mobile phase = 0.08 M SDS in 0.01 M phosphate buffer, pH 4.5-isopropanol (95:5)

Figure 4.3.

Effect of SDS concentration on drug retention time



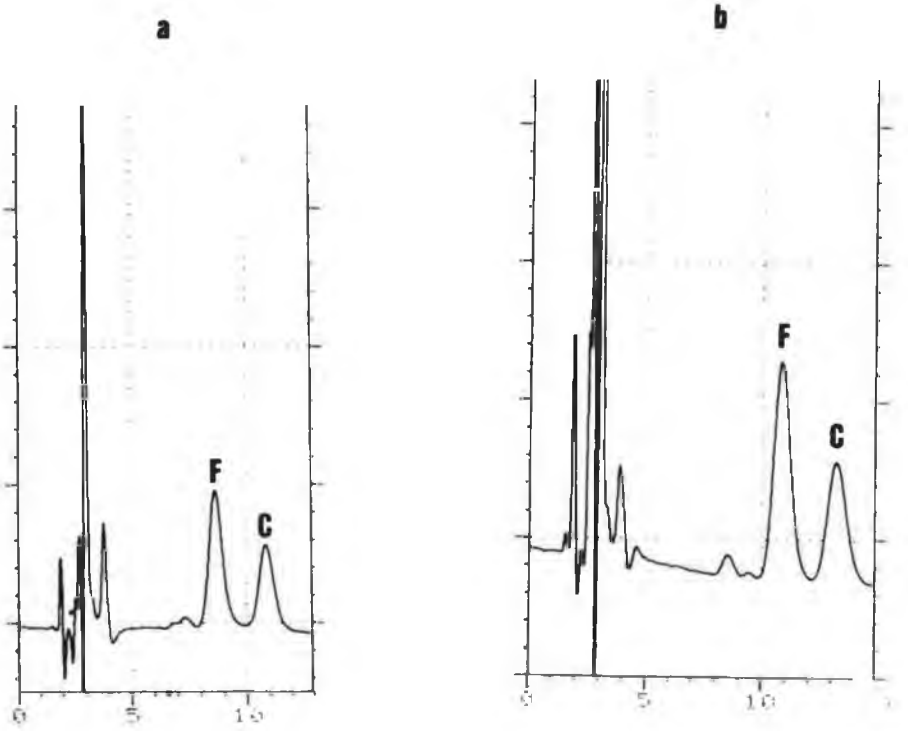
Lowering the percentage of 1-propanol was found to increase the retention times of both compounds, so that chlorthalidone was retained for 13.2 minutes on the column. This finding, coupled with the fact that the higher percentage of propanol helps to reduce the considerable frothing generated when working with micellar solutions, contributed to the decision to continue using 5% 1-propanol in the mobile phase. Higher concentrations of organic modifier were not investigated since it is desirable to keep the concentration low in order to maintain the integrity of the micelle and minimise expense. Chromatograms showing the retention of the drugs with 3% and 5% isopropanol in the mobile phase are presented in Figure 4.4.

4.4.2. Application of on-line solid-phase extraction with column switching to micellar chromatography

The analytical arrangement was coupled to a column-switching assembly as previously described. The general approach was to start with the simplest system possible, and to introduce elaboration only if necessary. The concentration column was packed with RP-18, RP-8 or cyano- packing materials with a wash solution of deionised water at a flow rate of 1 ml/min for 1 min. This wash regimen was maintained throughout the development work, unless otherwise stated. The analytical column was connected to the concentration column for the duration of the chromatographic run, and 250 μl aliquots of plasma or authentic (aqueous) standards in concentrations of 1.0 μg/ml were injected for analysis.

The three types of packing materials were evaluated in terms of drug recovery (as compared to direct injection) and the amount of plasma interferences appearing on the chromatogram. Of the three, the C_8- concentration column proved most promising in terms of recovery of aqueous drug standards: recovery was 55% and 70% for frusemide and chlorthalidone, respectively. However, drug recovery from plasma was 50% or less for chlorthalidone in all

Figure 4.4.
Chromatograms of chlorthalidone and frusemide showing the effect of percentage organic modifier on retention time



TIME(min)---------->

C: chlorthalidone; F: frusemide

(a): mobile phase = 0.05 M SDS in 0.01 M phosphate buffer, pH 4.5–isopropanol (95:5)

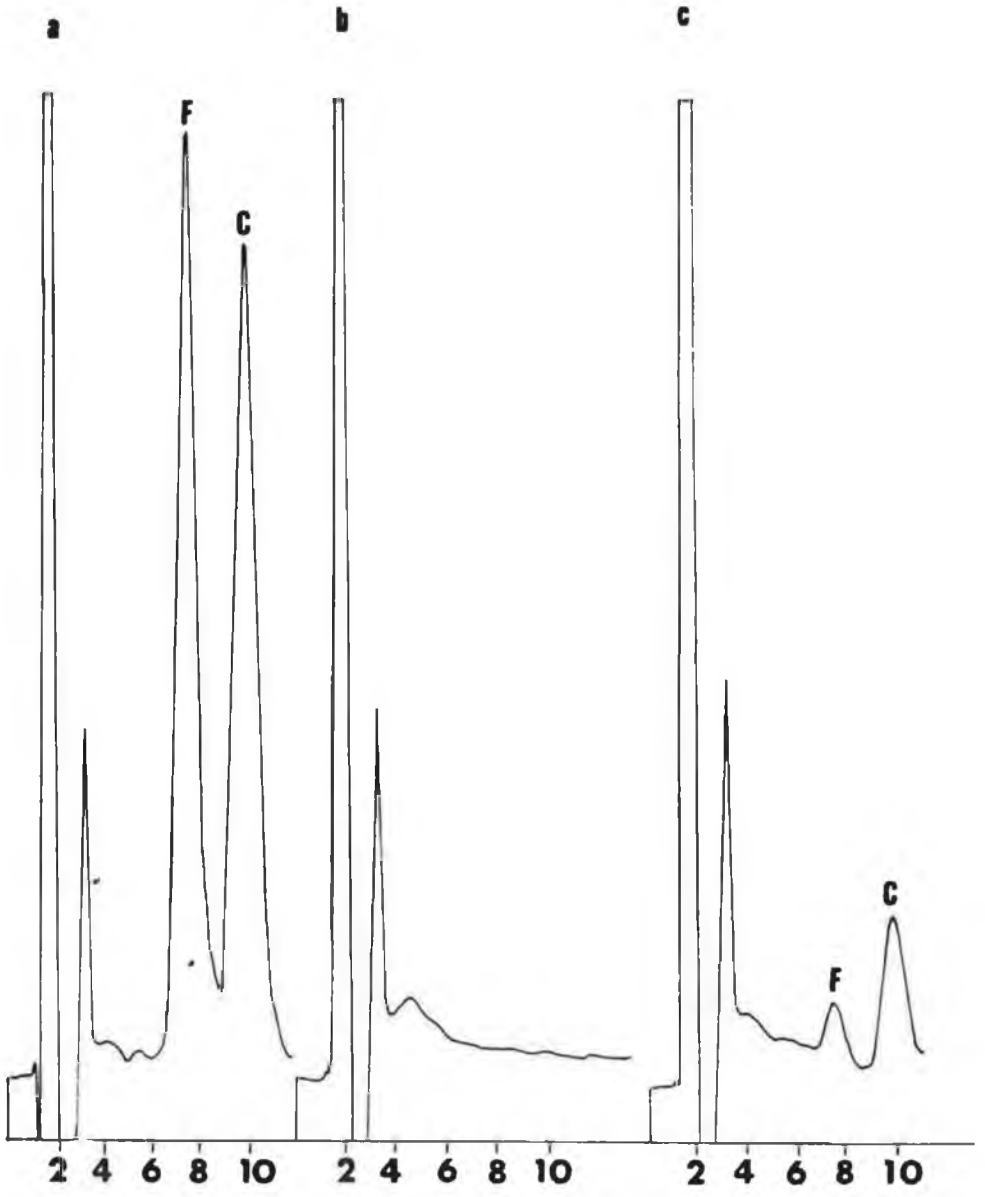
(b): mobile phase = 0.05 M SDS in 0.01 M phosphate buffer, pH 4.5–isopropanol (97:3)

cases and frusemide was not recovered at all from plasma. It was decided to persist with the investigation of the C_8-concentration column with a view to increasing retention of both drugs, (especially frusemide) on this column. It was also sought to increase recovery of both drugs from plasma. As frusemide is an acidic drug, its affinity for the hydrophobic packing material will be enhanced at low pH when its ionisation is suppressed. To this end, 0.1% phosphoric acid was added to the wash solution, a measure which improved recovery of the drug, but which caused the appearance of interfering peaks originating in the plasma. This problem developed because many plasma components are also acidic, and their affinity for reversed-phase packing materials is also enhanced at low pH.

Blank plasma profiles were improved by the addition of 5% methanol to the wash solution but offered no advantage in terms of drug recovery from plasma, and the breakthrough volumes for the pure drug solutions were somewhat reduced. Another wash solution tried was 0.005M tetrabutylammonium bromide in phosphate buffer, pH 5.0. This yielded good recovery (> 70%) for the aqueous standards of both drugs and the blank plasma chromatogram was peak-free. However, the peak heights for both drugs in spiked plasma were reduced to less than 20% of the aqueous standards. Chromatograms showing authentic standards, blank plasma and spiked plasma using TMA-Br in the wash solution are presented in Figure 4.5.

It was decided to attempt treatment of the plasma prior to injection which might enhance drug recovery, particularly frusemide, from plasma, without generating unwanted peaks on the chromatogram. 1 ml samples were treated with an acid, a base and various salts, and 250 μl aliquots were injected. The addition of salicylic acid affected only slight improvement in the amount of drug recovered, and a large interfering peak appeared in the chromatogram. Hydrochloric acid produced a more pronounced increase in drug recovery from plasma, but also produced interfering peaks in the chromatogram (Figure 4.6). It was found

Figure 4.5.
Chromatograms showing drug standards and plasma extracts using TMA-Br as the washing solvent



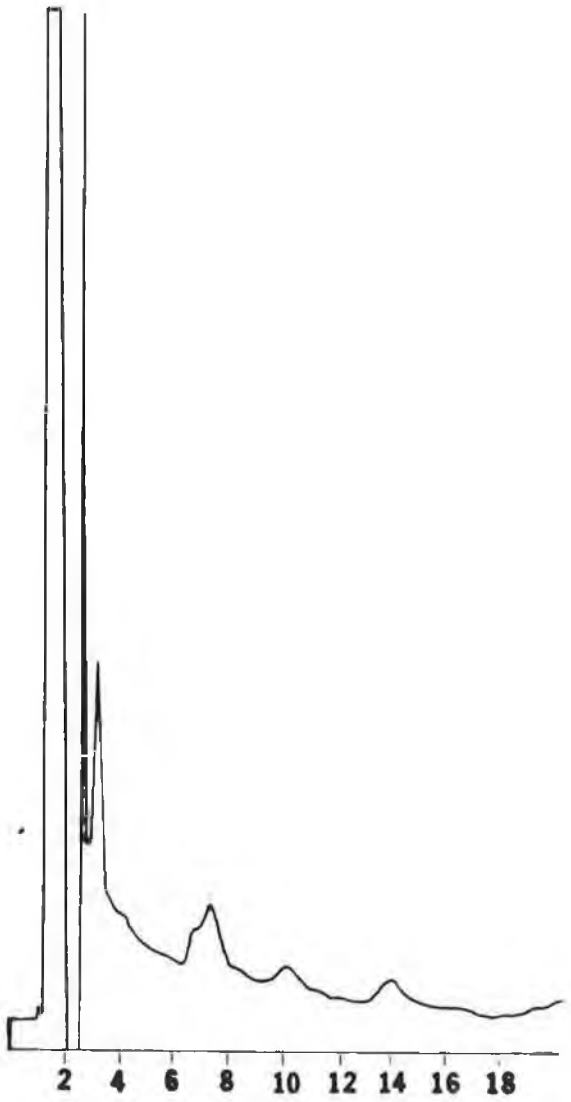
C: chlorthalidone 2.5 μg/ml; F: frusemide 2.5 μg/ml

(a): authentic standards, (b): blank plasma; (c): spiked plasma

Mobile phase : 0.05 M SDS in 0.01 M phosphate buffer, pH 4.5-isopropanol (95:5)

Wash solution: 0.005 M TMA-Br in 0.01 M phosphate buffer, pH 5.0

Figure 4.6.
Effect of pretreating plasma with 20% 1 M HCL prior to injection onto concentration column



TIME(min)---------->

Blank plasma

Mobile phase : 0.05 M SDS in 0.01 M phosphate buffer, pH 4.5-isopropanol (95:5)

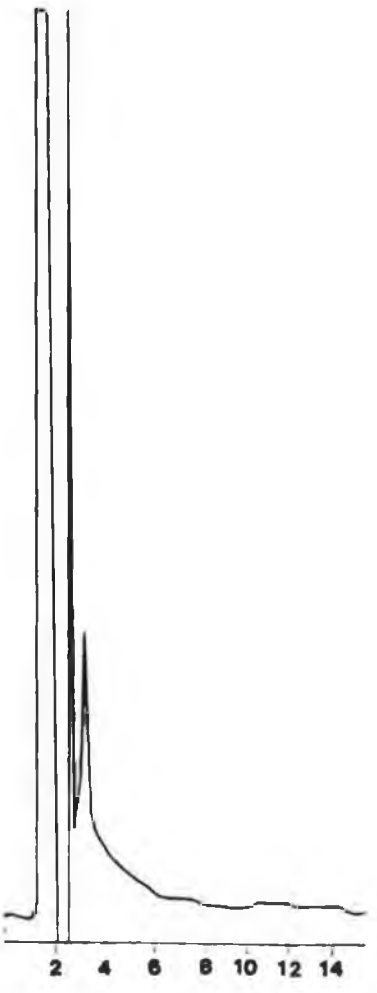
Wash solution: deionised water

that if 10% of a 1 M solution of NaOH was added to the plasma, cleaner blank plasma extracts were obtained, but the drugs were not recovered from spiked plasma (Figure 4.7). Adding salts such as sodium carbonate, sodium bicarbonate and lithium chloride generated peak-free blank plasma chromatograms, but recovery was less than 10% for both frusemide and chlorthalidone.

In addition to the problems of poor recovery and interfering plasma peaks, a pressure drop across the concentration columnm was observed to build up very quickly. It was then found if blank plasma was injected repeatedly using deionised water to wash the concentration column, that with continued injections, progressively more plasma remained on the concentration column, only to be swept onto the analytical column in the next back-flush operation. This process is illustrated in Figure 4.8. It is thought that the concentration column was continually being modified by alternate layers of surfactant and plasma components. It is known that surfactant monomers adsorb onto reversed-phase materials, and it is quite possible that this phenomenon gave the C_8- packing an additional retentive capacity for endogenous plasma constituents, which is absent in unmodified reversed-phase surfaces. Ultimately back-pressure in the system became prohibitive, and as there was a distinct possibility of contaminating the analytical column, it was necessary to perform a pre-treatment step, which would not only encourage drug retention on the concentration column, but would effect removal of contaminants as well.

The approach adopted was to add 10% trichloroacetic acid followed by centrifugation of the precipitated proteins and injection of the clear supernatant. It was hoped that this would release frusemide from protein binding sites. This measure, however, yielded poor recovery for both drugs as well a high background from plasma. A chromatogram depicting a spiked plasma sample after a trichloroacetic acid precipitation and injection onto the concentration column is shown in Figure 4.9. Adding the basic precipitant zinc sulphate plus sodium hydroxide similarly gave

Figure 4.7.
Effect of pretreating plasma with 20% of 1 M NaOH prior to injection onto concentration column



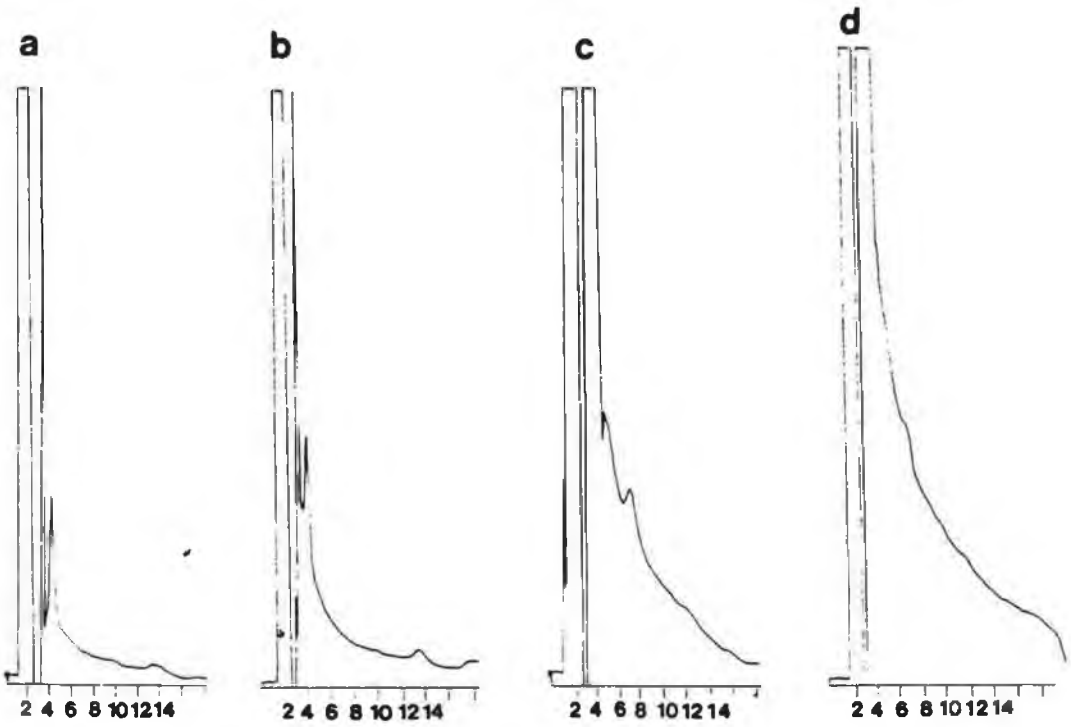
TIME(min)---------->

chlorthalidone 2.5 μg/ml and frusemide 2.5μ g/ml in plasma

Mobile phase : 0.05 M SDS in 0.01 M phosphate buffer, pH 4.5-isopropanol (95:5)

Wash solution: deionised water

Figure 4.8.
Plasma build-up on concentration column with repeated injection of 250μl aliquots.

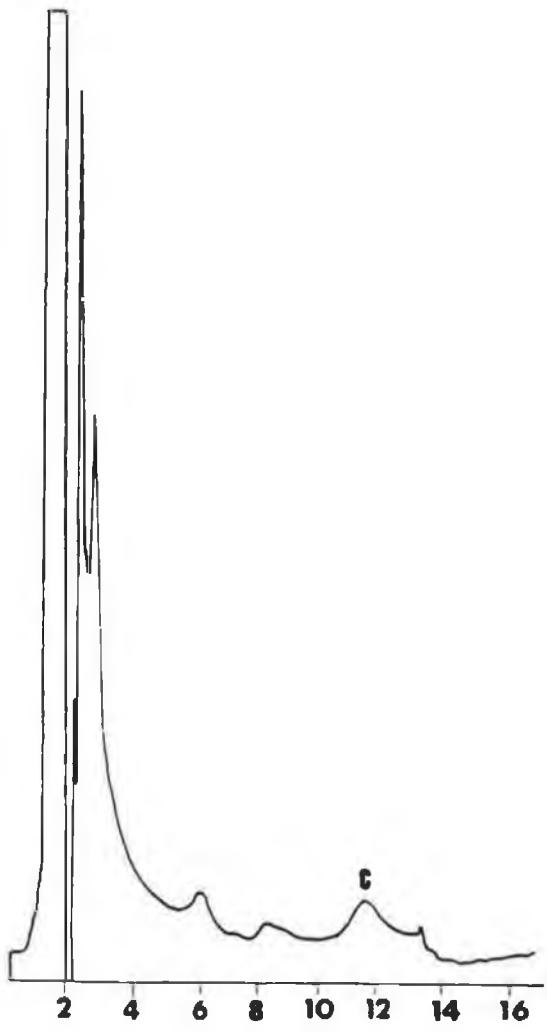


(a-d) : second, fourth, sixth and eight injections of 250 μl aliquots of blank plasma onto concentration column

Mobile phase: 0.05 M SDS in 0.01 M phosphate buffer, pH 4.5-isopropanol (95:5)

Wash solution: deionised water

Figure 4.9.
Spiked plasma chromatogram following precipitation with TCA and injection onto the concentration column



Plasma spiked with 2.5 μg/ml chlorthalidone (C) and 2.5 μg/ml frusemide

Mobile phase : 0.05 M SDS in 0.01 M phosphate buffer, pH 4.5-isopropanol (95:5)

Wash solution: deionised water

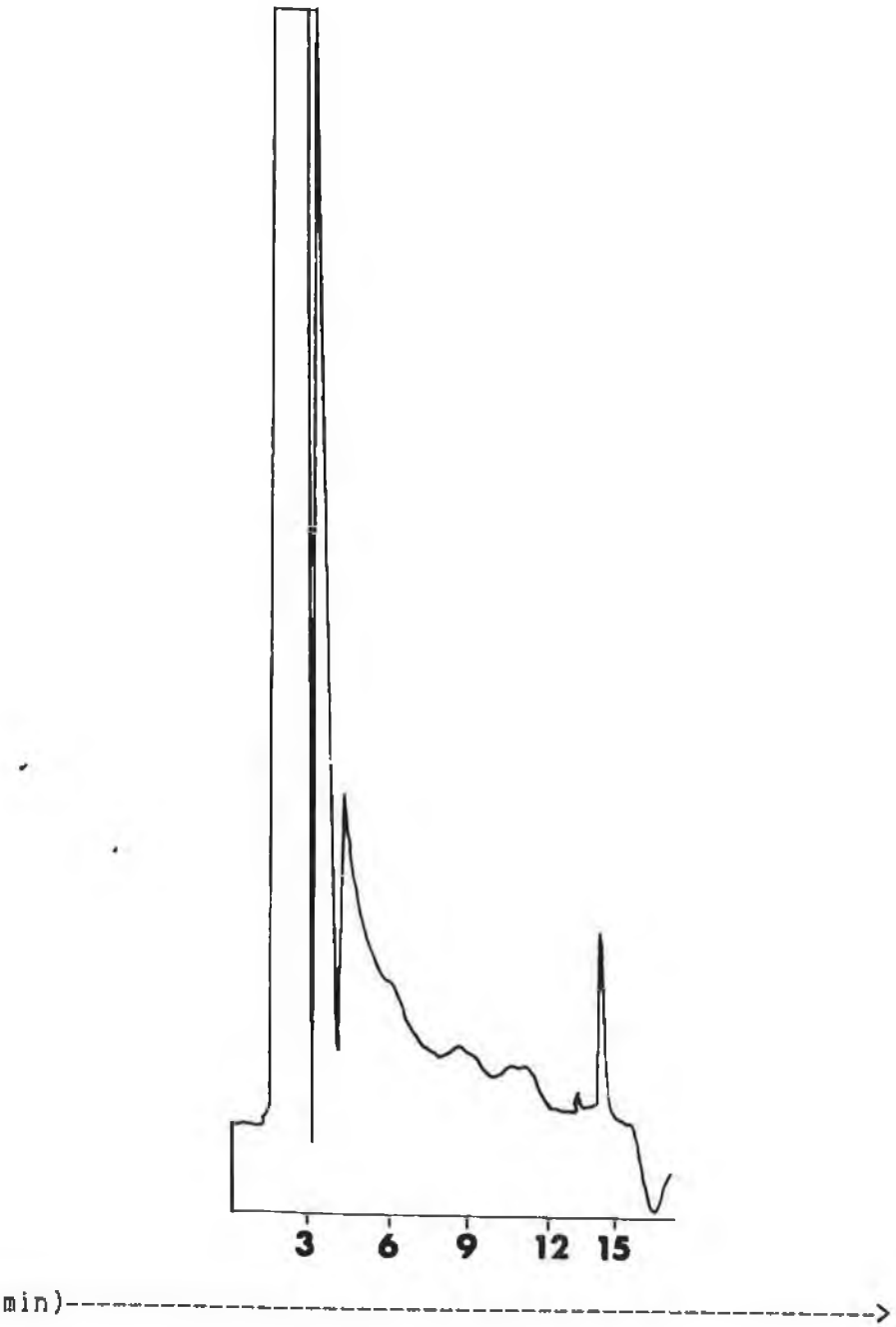
poor recovery though less interferences from the plasma. Repeated injections of up to 500 μl of plasma deproteinised with an equal volume of acetonitrile produced none of the problems associated with the injection of untreated plasma. When drug-spiked protein-precipitated plasma samples were injected, however, recovery of both drugs was unacceptably low owing to the acetonitrile effecting elution of the drugs during the wash phase. The acetonitrile was then removed by partial evaporation, but this resulted in a high plasma background (Figure 4.10) and poor recovery of frusemide, possibly due to the fact that it is highly protein bound.

4.4.3. Liquid-liquid extraction of chlorthalidone and xipamide followed by micellar chromatography.

At this stage it became obvious that a reasonably elaborate plasma clean-up procedure was being employed, i.e., addition of acetonitrile, vortexing, centrifugation, decanting and evaporating the supernatant, followed by column switching, a technique originally devised to obviate the need for all the foregoing steps. It had also, as outlined above, become obvious during the course of this work, that it would be necessary to carry out some kind of pre-treatment to permit repeated injections of plasma onto the concentration column. It was therefore decided to eliminate the column switching step and, if possible, develop a rapid one-step liquid-liquid extraction method which would remove plasma interferents and enrich the drug through pre-concentration. Co-extraction of the two drugs into an organic solvent would have been difficult as frusemide (pK_a = 3.6) would favour low pH for extraction, whereas chlorthalidone is extracted at pH 7.4 [16].

An alternative diuretic was sought to serve as a suitable internal standard for chlorthalidone . Metolazone, which is structurally related to, and elutes with a similar retention time as chlorthalidone, proved not to be a viable alternative owing to its high instability under the experimental conditions employed.

Figure 4.10.
Blank plasma chromatogram following precipitation with acetonitrile, evaporation and injection onto concentration column



Mobile phase : 0.05 M SDS in 0.01 M phosphate buffer, pH 4.5-isopropanol (95:5)

Wash solution: deionised water

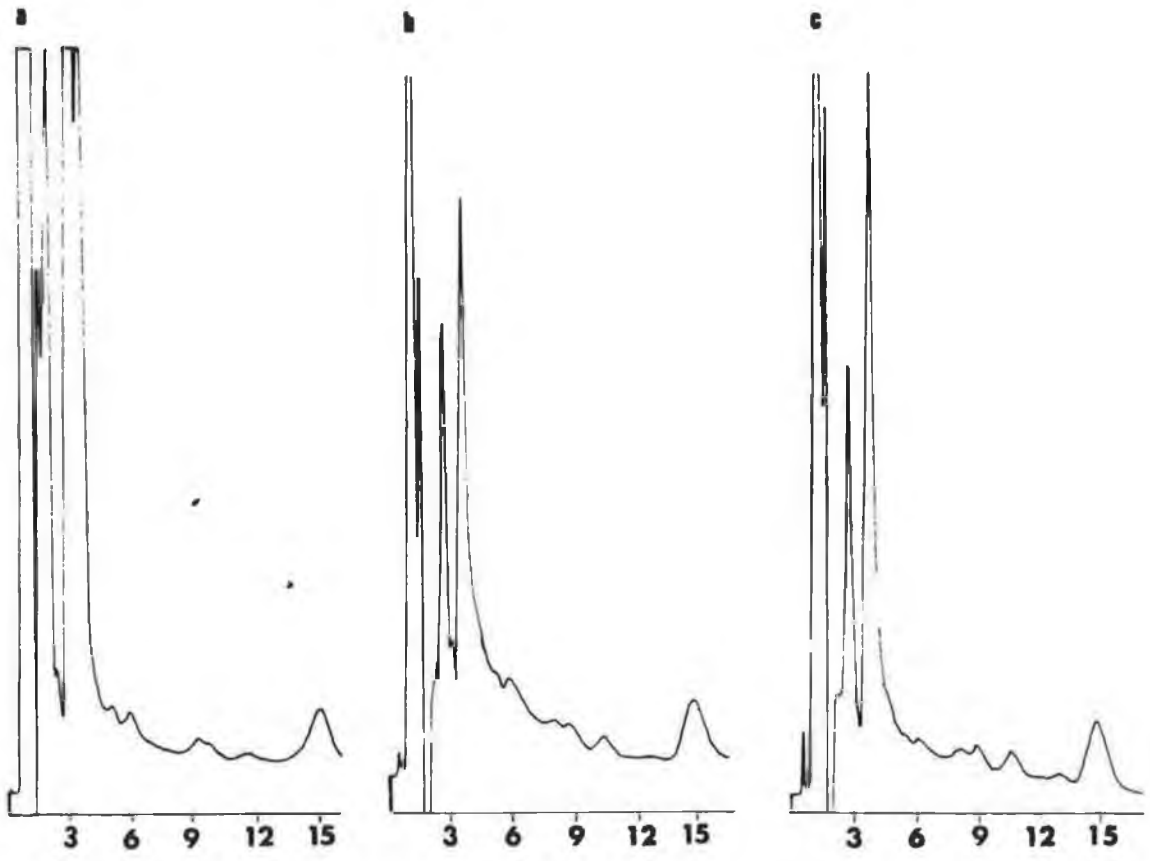
Another diuretic, xipamide, was more stable than metolazone, and was selected as the new internal standard.

In order to reduce the retention time of xipamide from 16.5 minutes, the pH of mobile phase was increased from 4.5 as used above, to pH 5.8. Under these conditions, chlorthalidone and xipamide eluted with mean retention times of 11.5 and 14.5 minutes, respectively. By further increasing eluent pH, it would have been possible to further reduce the retention time of the weakly acidic xipamide (pK_a = 4.75) [55], but this would cause it to merge with the peak for caffeine, which is frequently present in plasma, and would therefore interfere with the assay. The first approach was to add 200 mg lithium chloride to the plasma, followed by extraction of the drugs into diethyl ether-isopropanol (19:1), a method which had worked successfully in the extraction of xipamide into diethyl ether in previous work described in chapter 3.

In this case, although the drugs were extracted, the blank plasma chromatograms contained interfering peaks, and the same result was found for other salts, i.e., calcium chloride and sodium bicarbonate (Figure 4.11). As outlined in the introduction, Fleuren and van Rossum [16] developed a sensitive GLC method which involved the extraction of chlorthalidone from plasma buffered to pH 7.4 and an HPLC method was described by Guelen *et al.* [10] in which an equal volume of 0.067 M potassium dihydrogen phosphate solution was added to plasma prior to extraction into diethyl ether. Both buffers were tried followed by extraction into diethyl ether-isopropanol (19:1). The higher pH buffer yielded cleaner blank plasma chromatograms and comparable recovery for chlorthalidone. Buffering (with sodium phosphate buffer at pH 8.0) was also tried, a measure which did not increase the recovery of chlorthalidone, but caused a marked reduction in the recovery of xipamide.

Finally, an equal volume of 1 M phosphate buffer at pH 7.4 was added to the plasma and the drugs were extracted using the

Figure 4.11.
Blank plasma extractions into diethyl ether-isopropanol following treatment with inorganic salts



Time(min)---------->

(a): $NaHCO_3$; (b): LiCl; (c): $CaCl_2$

Mobile phase : 0.05 M SDS in 0.01 M phosphate buffer, pH 5.8-isopropanol (95:5)

procedure outlined in section 4.3.4.5. The residues following evaporation were reconstituted in 200 l mobile phase and 100 l was injected for chromatography. The mobile phase was 0.05 M SDS in phosphate buffer (0.01 M, pH 5.8) and it was delivered at a flow rate of 1.3 ml/min in order to reduce run times. Under these chromatographic conditions, the mean retention times for the elution of chlorthalidone and xipamide were 8.4 and 12.0 min, respectively, and both peaks were separated from endogenous plasma interferents and an added caffeine peak. Chromatograms showing blank and spiked plasma following this extraction procedure and the described chromatography are depicted in Figure 4.12.

4.4.4. Assay validation

Evaluation of the assay was carried out by the construction of a six-point calibration curve covering the concentration range 50-800 ng/ml chlorthalidone in plasma as outlined in section 4.3.4.6. A number of parameters, which have been discussed in section 2.6.6., were measured in order to check the reliability of the method.

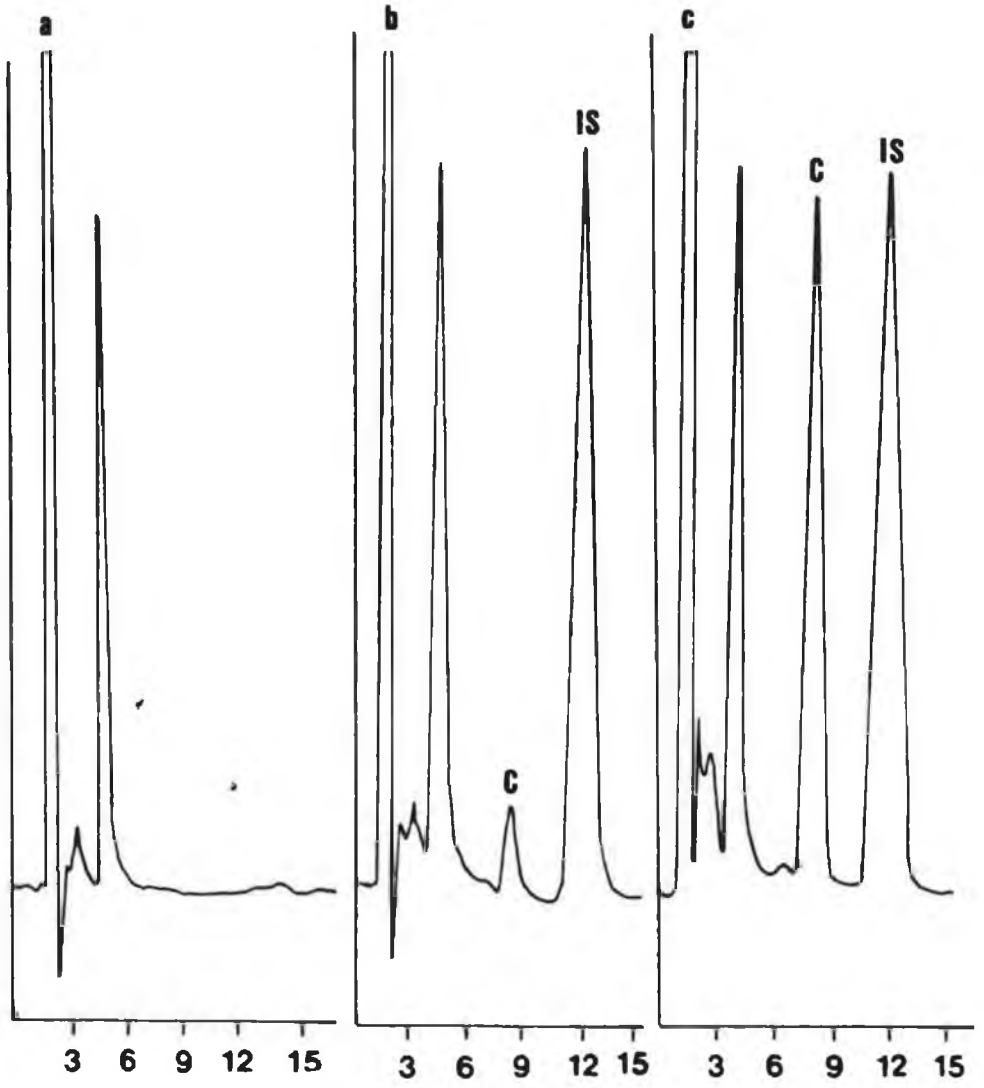
4.4.4.1. Limit of detection

The limit of quantitation of the method was 50 ng/ml: this was the level at which quantitative measurement of the peaks was consistently possible. The chromatograms presented in Figure 4.12. indicate that it should be possible to detect 25 ng/ml or lower, although it is improbable that consistent measurement at this level would be feasible. Although peak plasma levels of chlorthalidone may be as low as 100 ng/nl, they can rise to 1000 ng/ml, depending on the dose.

4.4.4.2. Precision

The data presented in table 4.2 demonstrate the inter- and intra-assay variation of the method. The same methods used in

Figure 4.12.
Sample chromatograms from validation study



(a): drug-free human plasma; (b): plasma containing 100 ng/nl chlorthalidone (C) and 2.5 g/ml xipamide (IS); (c): plasma containing 800 ng/ml chlorthalidone and 2.5 g/ml xipamide

Mobile phase : 0.05 M SDS in 0.01 M phosphate buffer, pH 5.8-isopropanol (95:5)
Detector sensitivity: 0.02 AUFS

Table 4.2

Precision and linearity intra-assay

Amount Chlorthalidone Added (g/ml)	Ratio	Mean Ratio	Amount Found (ng/ml)
	0.054		56.8
50	0.049	0.053	53.0
	0.054		56.8
	0.055		57.7
	0.019		108.5
100	0.112	0.104	112.0
	0.101		100.9
	0.092		92.5
	0.209		202.4
200	0.205	0.200	198.6
	0.194		188.3
	0.190		184.5
	0.434		414.7
400	0.418	0.420	398.7
	0.409		391.2
	0.419		397.8
	0.631		598.8
600	0.634	0.616	602.6
	0.607		576.3
	0.591		561.2
	0.899		850.6
800	0.899	0.588	850.6
	0.812		768.9
	0.823		779.2

Mean % Coefficient of Variation = 4.6%

Regression Line: $y = 1.00 \times 10^{-3} x - 6.45 \times 10^{-3}$

Correlation Coefficient (r) = 0.999

Mean Amt. Found (ng/ml)	Standard Deviation (ng/ml)	Coefficient of Variation %	Difference between added and found (%)
56.1	2.1	3.7	12.2
103.5	8.7	8.4	3.5
193.5	8.4	4.3	3.3
400.6	10.0	2.5	0.2
584.7	19.5	3.3	2.6
812.3	44.4	5.5	1.5

Table 4.2 continued
Precision and linearity Inter-assay

Amount added ng/ml	Peak Height ratio	Amount Found ng/ml
50	0.061	53.7
100	0.128	109.4
200	0.230	194.3
400	0.470	393.8
600	0.697	582.8
800	0.978	816.2
50	0.056	58.5
100	0.106	102.0
200	0.220	196.4
400	0.450	388.9
600	0.695	593.7
800	0.954	810.3
50	0.060	60.5
100	0.111	108.1
200	0.217	198.8
400	0.445	399.7
600	0.600	536.3
800	0.952	846.5
50	0.062	54.9
100	0.126	111.6
200	0.218	193.0
400	0.436	385.9
600	0.667	590.4
800	0.921	814.3

	Concentration ng/ml					
	50	100	200	400	600	800
Mean Amount Found ng/ml	56.9	107.8	195.6	392.1	575.8	821.8
Standard Deviation	3.1	4.1	2.5	6.0	26.6	16.6
Coefficient of variation %	5.4	3.8	1.3	1.5	4.6	2.0
Difference between added and found %	+13.8	+7.8	-2.2	-2.0	-4.0	+2.7

Mean coefficient of variation = 3.1%

chapter 3 for the determination of xipamide were applied here. Inter-assay variation was assessed singly in four replicate runs covering the concentration range 50-800 ng/ml chlorthalidone in plasma. Inter-assay variation was determined in quadruplicate in the same concentration range. The precision of the method (mean coefficient of variation) was determined for chlorthalidone to xipamide peak height ratios after they had been interpolated as unknowns on the regression lines. For inter-assay variation, the interpolations were based on the four regression lines generated from four replicate runs, and the mean coefficient of variation was found to be 3.1%. For intra-assay variation, the interpolations were based on a single regression line from the quadruplicate run, and the mean coefficient of variation was found to be 4.6%.

4.4.4.3. Linearity

Measures of linearity, as defined by the correlation coefficient of the regression line and the percentage difference between added and found concentrations for intra-assay values are presented in Table 4.2. The correlation coefficient (r) of the regression line for the mean intra-assay values was better than 0.999 and, as can be seen from the equation of the regression line (also given in Table 4.2), the intercept does not differ significantly from zero.

4.4.4.4. Recovery

Recovery of chlorthalidone from plasma was measured by two methods:

(i) by calculating the percentage difference between the peak heights of extracted standards and those of authentic (unextracted) standards at each of the six points over the concentration range 50-800 ng/ml chlorthalidone in plasma;

(ii) by comparing the slopes of the two regression lines generated from the extracted and authentic standards used to calculate recovery by method (i), with the extracted slope as a percentage of the authentic slope yielding a measure of the amount recovered. Both authentic and extracted standards were processed and injected in duplicate.

Using these methods, the mean overall percentage recovery of chlorthalidone from plasma was found to be 71.6%. Results of this experiment are presented in Table 4.3.

4.4.4.5. Selectivity

The drugs are separated from any endogenous plasma components as can be seen from the chromatograms presented in Figure 4.12. A number of drugs likely to be co-administered with chlorthalidone were investigated as potential interferents with the method. These included the diuretic triamterene, the beta-blocking agents propranolol, atenolol, pindolol and metoprolol, and the xanthines theophylline and caffeine. None of these drugs interfered with either the chlorthalidone of xipamide peaks, but atenolol was found to elute just after xipamide, showing that it could act as an internal standard for either of these drugs (or indeed, vice-versa). Of greater significance, however, is the fact that chlorthalidone and atenolol are available commercially as a combined preparation, and this method could be applied, possibly without modification, to their co-determination in body fluids. A chromatogram showing the three drugs is presented in Figure 4.13.

4.5. CONCLUSION

Micellar chromatography presents an attractive alternative to conventional hydro-organic mobile phases. The solvents used are cheaper, less hazardous and less toxic than many organic solvents. It offers an added degree of selectivity to separations owing to the unique nature of micellar solutions, and retention may be controlled simply by altering the surfactant

Table 4.3.

Results of recovery of chlorthalidone studies

Concentration ng/ml	Mean peak height (mm) (n = 2) Authentic standards	Extracted standards	Recovery %
50	9.0	5.8	64.4
100	18.0	12.5	69.4
200	35.0	26.5	75.7
400	69.5	50.5	72.7
600	103.0	72.5	70.4
800	148.0	107.5	72.6

Method (i): Mean recovery (± standard deviation) = 70.9 ± 3.8%

Method (ii): Mean recovery = {slope(extracted)/slope(authentic)} x 100%

Authentic standards: $y = 0.018x - 0.125$; $r = 0.999$

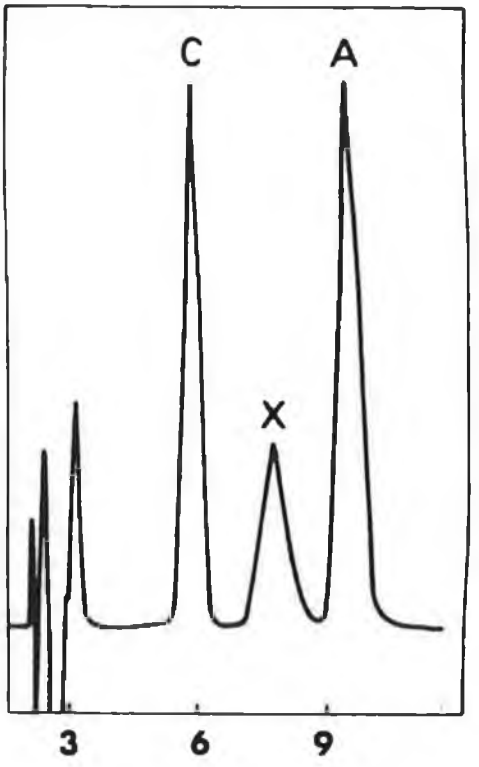
Extracted standards: $y = 0.013x - 0.104$; $r = 0.997$

Slope(extracted)/slope(authentic) = 0.013/0.018 = 0.722

Mean percentage recovery = 72.2%

Overall mean percentage recovery = 71.6%

Figure 4.13.
Sample chromatograms from selectivity study



TIME (min)---------->

Chlorthalidone (C), xipamide (X) and atenolol (A)

Mobile phase : 0.05 M SDS in 0.01 M phosphate buffer, pH 5.8-isopropanol (95:5)

concentration. The principal disadvantage of micellar systems, loss of chromatographic efficiency, can be overcome by the addition of a small amount (3-10%) of propanol, and by operating at elevated temperatures.

The results of this study indicate that micellar chromatography does not lend itself as readily as traditional hydro-organic systems to coupling with an on-line column-switching method of plasma clean-up, and that direct injection onto reversed-phase concentration columns is not a practical proposition for large volumes of untreated body fluids. Where drugs are present in large concentrations, filtered raw serum may be introduced directly onto the analytical column, whereupon the proteinaceous material is solubilised and the compounds are separated free of interferences.

As shown in the present work, the problems associated with direct injection may be circumvented by combining micellar chromatography with a suitable liquid-liquid extaction scheme. Since the attraction of micellar chromatography lies at least in part in its ability to offer added selectivity in the separation of closely related compounds, employment of the technique for this purpose should not present any problems as far as the co-extraction of analytes is concerned.

Statistical evaluation of the inter-and intra-assay results for the chlorthalidone assay reveals that this method can offer levels of reproducibility, linearity and recovery comparable with traditional hydro-organic mobile phases. A limit of detection of 50 ng/ml is adequate for plasma measurements, provided the dose is not very low (i.e. less than 20 mg per day). It should be quite possible, however, to apply this extraction scheme to whole blood samples in order to capitalise on the selective uptake of chlorthalidone into red blood cells, and to follow this with micellar chromatography in order to exploit the unique features of this novel chromatographic mode.

4.6 REFERENCES

1. A. E. Thomson, Int. J. Clin. Pharmacol., 3 (1970) 21.
2. British Pharmaceutical Codex (11th ed.) (Pharmaceutical Press, London, 1979).
3. M. G. Tweedale and R. I. Ogilvie, J. Pharm. Sci., 63 (1974) 1065.
4. H. L. Fleuren and J. M van Rossum, Pharm. Weekbl., 110 (1975) 1262.
5. P. Collste, M. Garle, M. D. Rawlins and F. Sjoqvist, Eur. J. Clin. Pharmacol., 15 (1979) 319.
6. H. L. Fleuren, Th. Thien, C. Verwey-van Wissen and J. van Rossum, Eur. J. Clin. Pharmacol., 15 (1979) 35.
7. W. Dieterle, J. Wagner and J. Faigle, Eur. J. Clin. Pharmacol., 10 (1976) 37.
8. H. L. Fleuren and J. van Rossum, J. Pharmacol. Biopharm., 5 (1977) 359.
9. H. L. Fleuren, C. Verwey-van Wissen and J. Rossum, Clin. Pharmacol. Ther., 25 (1979) 806.
10. P.J. M. Guelen, A. M. Baars and T. B. Vree, J. Chromatogr., 181 (1980) 497.
11. A. Wade, (Ed.) Martindale, The Extra Pharmacopoeia, (27th ed.) (Pharmaceutical Press, London, 1979).
12. R. Pulver, H. Wirz and E. G. Stenger, Schweiz. Med. Wochenschr., 89 (1959) 1130.
13. G. Beizenherz, F. W. Koss, L. Klatt and B. Binder, Arch. Intern. Pharmacodyn., 161 (1966) 76.
14. M. Ervik and K. Gustavii, Anal. Chem., 46 (1974) 39.
15. P. H. Degen and A. Schweizer, J. Chromatogr., 142 (1977) 549.
16. H. L. Fleuren and J. van Rossum, J. Chromatogr., 152 (1978) 41.
17. M. J. Rosenberg, K. K. Lam and T. E. Dorsey, J. Chromatogr., 375 (1986) 438.
18. J. G. Dorsey, M. T. DeEchegaray and J. S. Landy, Anal. Chem., 55 (1983) 924
19. L. J. Cline-Love, J. G. Habarta and J. G. Dorsey, Anal. Chem., 56 (1984) 1132A.

20. G. S. Hartley, Aqueous Solutions of Paraffin Chain Salts (Herman and Cie, Paris, 1936).
21. K. L. Mittal (Ed.), Micellization, Solubilisation and Microemulsions (Plenum Press, New York, 1977).
22. J. H. Fendler and E. H. Fendler, Catalysis in Micellar and Macromolecular systems (Academic Press, New York, 1975).
23. M. A. Hernandez-Torres, J. S. Landy and J. G. Dorsey, Anal. Chem., 58 (1986) 744.
24. E. Pelizzetti and E. Pramauro, Anal. Chim. Acta., 117 (1980) 403.
25. D. G. Herries, W. Bishop and F. M. Richards, J. Phys. Chem., 68 (1964) 1842
26. D. W. Armstrong and S. J. Henry, J. Liq. Chromatogr., 3 (1980) 657.
27. D. W. Armstrong and F. Nome, Anal. Chem., 53 (1981) 1662.
28. M. Arunyanart and J. L. Cline-Love, Anal. Chem., 56 (1984) 1557.
29. M. Arunyanart and J. L. Cline-Love, Anal. Chem., 57 (1985) 2837.
30. C. T. Hung and R. B. Taylor, J. Chromatogr., 209 (1981) 175.
31. P. Yarmchuck, R. Weinberger R. F. Hirsch and L. J. Cline-Love, Anal. Chem., 54 (1982) 2233.
32. J. G. Dorsey, M. T. De Echegaray and J. S. Landy, Anal. Chem., 55 (1983) 924.
33. L. R. Snyder and J. J. Kirkland, Introduction to Modern Liquid Chromatography, 2nd ed. (Wiley-Interscience, New York, 1979)
34. R. P. W. Scott and C. F. Simpson, Faraday Symp. Chem. Soc., 15 (1980) 69.
35. P. Yarmchuck, R. Weinberger R. F. Hirsch and L. J. Cline-Love, J. Chromatogr., 283 (1984) 47.
36. R. J. Perchalski and B. J. Wilder, Anal. Chem., 51 (1979) 774.
37. R. J. Rucki, Talanta, 27 (1980) 147.
38. E. M. Lores, D. W. Bristol and R. F. Moseman, J. Chromatogr. Sci., 16 (1078) 358.

39. M. G. Khaledi and J. G. Dorsey, Anal. Chem., 57 (1985) 2190.
40. D. W. Armstrong. W. L. Hinze, K. H. Bui and H. N. Singh, Anal. Lett., 14 (1981) 1659.
41. R. Weinberger, P. Yarmchuck and L. J. Cline-Love, Anal. Chem., 54 (1982) 1552.
42. C. M. Kirkman, P. C. Zu-Ben, P. C. Uden, W. J. Stratton and D. E. Henderson, J. Chromatogr., 317 (1984) 569.
43. F. G. P. Mullins and G. F. Kirkbright, Analyst, 109 (1984) 1217.
44. F. J. DeLuccia, M. Arunyanart and L. J. Cline-Love, Anal. Chem., 57 (1985) 1564.
45. M. Arunyanart and L. J. Cline-Love, J. Chromatogr., 342 (1985) 293.
46. F. J. DeLuccia, M. Arunyanart, P. Yarmchuck, R. Weinberger and L. J. Cline-Love, LC-GC, 3 (1985) 794.
47. M. A. J. van Opstal, F. A. L. van der Horst, P. Krabbenbourg, J. J. M. Holthuis, W. P. van Bennekam and A. Bult, J. Chromatogr., in Press.
48. G. R. Granneman and L. T. Senello, J. Chromatogr., 229 (1982) 149.
49. M. J. Koeningbauer and M. A, Curtis, J. Chromatogr., 427 (1988) 227.
50. J. V. Posluszny and R. Weinberger, Anal. Chem., 60 (1988) 1953.
51. P. H. Elworthy, K. J. Mysels, Colloid Interface Sci., 21 (1966) 331.
52. R. S. Rapaka, J. Roth, C. T. Viswanathan, T. J. Goehl, V. K. Prasad and B. E. Cabana, J. Chromatogr., 227 (1982) 463.
53. C. A. Bunton, L. S. Romstead and L. Sepulveda, J. Phys. Chem., 84 (1980) 2611.
54. M. S. Fernandez and P. Fromhertz, J. Phys. Chem., 81 (1977) 1755.
55. F. W. Hemplemann, Arzneim.-Forsch., 27 (1977) 2140.

CHAPTER 5

RETENTION CHARACTERISTICS IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF BASIC DRUGS AND PLASMA EXTRACTS ON AN UNMODIFIED SILICA COLUMN USING REVERSED-PHASE ELUENTS

5.1. INTRODUCTION

Reversed-phase chromatography, where the eluent is more polar than the solid support, has become the method of choice for a wide range of analytical applications, particularly in the field of biopharmaceutical analysis. It is now possible to produce a diverse array of bonded phase materials which can be tailored to meet a variety of analytical needs. In spite, however, of the advanced technology in this area, it is possible only to achieve some 50% coverage of the hydroxyl groups on the silica surface [1,2]. These silanol moieties are acidic in nature, and are therefore ionised at neutral or basic pH; indeed some silanol groups are more strongly acidic than others, and these are ionised even under moderately acidic conditions [3]. Since reversed-phase materials are widely used for the chromatography of polar molecules, silanol groups which remain accessible after bonding has taken place are likely to make an important contribution to the chromatography of such compounds [4-6]: in particular, solute molecules containing an amine functionality interact electrostatically with these residual silanol moieties [4-11]. Ionic interactions between the residual acidic sites and oppositely charged protonated bases give rise to badly tailing peaks for basic componds (including drugs of therapeutic and forensic interest) on reversed phase columns [4-6,8,12-15].

Much attention has been paid to circumventing the problem of unreacted silanol groups through ion suppression [16], paired ion techniques [16,17], or by the use of end-capping [18]. Bidlingmeyer *et al.* [12] have proposed that it is not specifically the presence or number of residual silanols which is responsible for the poor chromatographic characteristics, but how accessible they are. He supported this hypothesis by showing no deterioriation in efficiency in going from a fully- to a partially- bonded silica column. He further proposed that on a "fully" bonded column, the remaining silanols may be located in micropores which are blocked by the thicker mobile phase, and as a result of diminished accessibility, mass transfer is reduced,

leading to poor efficiency and peak shapes.

If, instead of vigorously trying to eliminate the surface silanol contribution to retention on bonded columns, an unmodified silica column in conjunction with a reversed-phase eluent is employed, as first reported by Jane in 1975 [19], the amine-silanol interaction is exploited, and efficient separations have been obtained using buffered aqueous methanol-rich mobile phases at high pH in a chromatographic mode which has since been described as "pseudo reversed-phase" [15].

5.1.1. Mechanisms of interaction

The mechanisms involved in these polar interactions on silica with mixed aqueous-organic eluents at high pH have not been fully elucidated. Much work has been devoted to the determination of the factors influencing retention and separation [4,8-10,12-15]. One of the ways silica can interact with solutes is felt by some to involve ion exchange [9,10,12-14,20]. This has been supported by data which show a decrease in retention with an increase in ionic strength [4,9,12-14,20], and an increase in retention for quaternary amines with an increase in pH [10,12-14].

In support of an ion exchange theory, Sugden et al. [8] demonstrated a linear relationship between retention and the reciprocal of the ionic strength on an unmodified silica column for cocaine and a number of other local anaesthetics of varying basicity. Hansen [21] reported that the retention of opiates using eluents containing 50-90% water was governed almost exclusively by ion exchange processes. In a later publication Flanagan and Jane [3] demonstrated a linear relationship between the $\log_{10}$ of the capacity factor and the $\log_{10}$ of the ionic strength at constant pH. They also showed increased retention for basic drugs with increasing pH until their pK_a values were approached, whereupon retention started to decrease concommitant with reduced solute ionisation. The ion exchange theory has also been supported in recent work by Law [20] where he showed

that using alkaline aqueous-organic eluents, retention decreased with increased buffer strength. He also established a linear relationship between the $\log_{10}$ of the retention and drug pK_a, and showed that within any group of homologous compounds, the correlation coefficient for this relationship ranged between 0.959 and 0.996.

The mechanism of chromatography encountered on unmodified silica with mixed aqueous-organic eluents at high pH has been attributed to factors other than ion exchange. Sugden et al. [8] demonstrated reduced retention for the amines as the hydrogen ion concentration was increased, and attributed this phenomenon not to reduced silanol ionisation, but to greater ion-pair formation between the cationic solute and the counter-ion in the mobile phase. Crommen [7], using acidic aqueous eluents of an appropriate pH and ionic strength, also concluded that ion pair formation was the major influence on the retention of the compounds studied. In addition, Flanagan and Jane [3] found some factors which were difficult to reconcile with the simple ion-exchange model of retention. Notably, there was an elution sequence of a series of fully protonated amines and and a quaternary ammonium compound in the order primary, secondary, tertiary, quaternary, meaning that there is increased affinity for the column according to ionic size, which is opposite to that predicted by the ion exchange model. One possible explanation for this is the greater solvation for the less substituted analytes, to give them larger effective ionic radii and hence shorter retention times.

Cox and Stout [15] found a positive intercept when plotting retention against the reciprocal of the competing ion concentration. They took these findings as evidence for retention mechanisms additional to ion exchange, since ion exchange theory [22] predicts that for a pure ion exchange mechanism, the plot of retention against the inverse of competing ion concentration should give a straight line passing through the origin of the plot, with the slope being proportional to the ion exchange

equilibrium constant and the ion exchange capacity of the packing material. Law [20] found a near linear relationship between retention time and the reciprocal of the buffer concentration. The fact that there was a slight deviation was attributed to the operation of more than one retention mechanism. This was also suggested by the fact that the curves did not have a common y-intercept, a phenomenon which is commonly observed with ion exchange media [22].

Bidlingmeyer et al. [12] proposed that the retention of organic amines on silica depended on both electrostatic and adsorption forces. In support of the adsorption theory, they demonstrated increased retention for a series of basic drugs (for example, propranolol, promethazine and chlorpheniramine) as the amount of water in the mobile phase was increased from 30% to 70%. The observed linear relationship of a plot of $\log_{10}$ capacity factor versus solvent composition was typical for reversed-phase systems. They concluded that "reversed-phase" behaviour of silica under these conditions was due to solutes interacting with siloxane bridges, and that the mechanism by which this hydrophobic retention is mediated is similar to the ion interaction model of ion pair chromatography.

Cox and Stout [15] studied the retention of thiamine and morphine under conditions of varying methanol concentration in 0.05 M phosphate buffer pH 4.6. Unlike Bidlingmeyer et al. [12], they found low retention at both very high and very low concentrations of methanol. In agreement with other workers they attributed the U-shape of the curve to hydrophobic interactions at low methanol concentrations and changes in counter-ion solvation at high methanol concentrations [8,23]. In agreement with Bidlingmeyer et al., Cox and Stout also proposed some interaction between analytes and siloxane bridges, but found that it contributed more significantly to the retention of unionised species such as caffeine, than ionised species such as morphine. By carrying out experiments on the retention of solutes both on silica which had been prepared by a standard procedure, and silica which had been

subjected to a hydroxylation procedure analagous to that of Kohler and Kirkland [24], they found that the retention of unionised solutes was reduced on the rehydroxylated silica. As rehydroxylation reduces the population of siloxane bridges on the silica surface by their conversion to silanol groups, they concluded that for unionised species, retention occurs via interaction with siloxane bridges.

On the other hand, Flanagan and Jane [3] found that non-protonated bases (for example, diazepam) were unretained and concluded that there was no evidence to suggest that interaction between unionised silanols and/or siloxane bridges and non-protonated bases was taking place. Law [20] found that retention was influenced by the stereochemistry of the analyte and the degree of substitution at or near the basic centre. Because the effects of certain types of substitution were difficult to rationalise in terms of a simple ion exchange model (for example N-methylation or N-oxidation), he concluded that other factors contribute to retention, but that they did not involve reversed phase or dipole-dipole interactions.

Clearly there are conflicting opinions as to what factors are responsible for the control of retention in this type of chromatography, particularly in relation to the importance of the ion exchange mechanism and the nature of secondary, though obviously still relevant, interactions. Apart from ion exchange, retention has been variously attributed to partitioning of ion pairs [7,8], dipole interactions, Van der Waals forces and hydrogen bonding [25], salting out [8], or adsorption and reversed-phase effects [12]. Most likely, a mixture of some or all of these interactions are taking place and the reason for confusion probably stems from the fact that in a number of the above studies, results have been compounded by the analysis of differing and multifunctioal compounds which are more likely to engage in mixed retention mechanisms [20].

5.1.2. Stability of unmodified silica

It is well known in the literature that liquid chromatographic columns which are based on silica fail rapidly if eluents of high pH are used [26]. Horvath et al. suggested that C_{18}- bonded columns should not be used with eluents above pH 7 [27]. This failure may be as a result of increased solubility of silica in aqueous media as the pH is increased. Sosman [28] has reported that the solubility of silica in water depends on the physical characteristics of silica and that it increases with temperature. For an amorphous form, he reported solubility of about 10 μg/ml at room temperature, and 250 μg/ml at 100^{o}C. Wherli et al. [29] used atomic absorption (AA) spectrometry to measure the silica content of strongly basic solutions (both organic and inorganic) in contact with C_{18}-, C_{8}- and non-bonded silica packing materials. They found that the rate of dissolution was linear with time, and that sodium hydroxide and the strongly basic ammonium hydroxides promote the most rapid dissolution. They also found that the non-bonded silica dissolved at a much faster rate than alkyl-modified silica, and that the octadecyl silica was better protected against attack than octyl silica. Having investigated a range of organic bases, they concluded that triethylamine provided the most suitable alternative in terms of minimal silicate attack, retention and separation properties, as well as availability, handling and good solubility.

Atwood et al. [26] also used an AA spectrometric method to detect silica eluting from the column to measure quantitatively the loss of silica under different analytical conditions. Their findings supported those of Wherli et al. [29] in relation to the dissolution of silica at high pH, at elevated temperature and after prolonged contact with aqueous solutions. They also established that the rate of dissolution decreased with increasing flow rate, and that water flowing through the column at room temperature approaches an equilibrium concentration of silica. Using pure methanol as the mobile phase caused negligible dissolution of the silica packing. By placing a pre-column

between the pump and the injector, the mobile phase was pre-saturated with silica and the dissolution of silica was greatly reduced.

Rabel [30] has shown that dissolution of silicaceous reversed-phase packings can occur at lower pH values than previously thought, and Barker [31] demonstrated appreciable dissolution of silica in water and salt solutions at pH values as low as 5.5. Barker also showed that in the slightly acidic region of pH 3-6, which is frequently used for chromatography, the concentration of salt in solution is an important parameter. In a recent publication which represents a radical departure in opinion on the subject, Law [32] undertook a systematic study to produce quantitative data to demonstrate the stability of silica in the presence of organic-rich eluents. Using a mobile phase of methanol-ammonium acetate buffer pH 9.2, (9:1), he showed that there was no significant loss in efficiency (as measured using five compounds with a range of retention times) when up to 46 l of mobile phase had passed through the column. A dramatic loss in efficiency was observed between 46 and 65 l, but this could easily be restored by repacking the top of the column. This particular study by Law was carried out without the use of a saturation column to represent the worst case situation, though pre-saturation of the mobile phase is recommended for routine use [29,32]. Wheals [25] stated that silica dissolution was not a problem providing ammonium hydroxide was the source of hydroxyl ions and the organic content was high. If, in addition, the mobile phase is pre-saturated with silica, this would further help to alleviate the problem, and these precautions were observed throughout the course of the present work.

5.1.3. Applications of unmodified silica with reversed phase eluents in drug analysis.

Jane [19] developed a method for the separation of basic drugs on unmodified silica with reversed-phase eluents in response to a need for a chromatographic technique which could separate a wide

range of drugs of abuse without gradient elution. Many drugs (which are used for both legitimate and illicit purposes) contain a basic amine functionality: their chromatography on reversed phase materials is characterised by poor efficiency, and gradient elution is usually required. In addition, each of the systems proposed is suitable for only one drug type. The method developed by Jane incorporated an ammonium nitrate buffer and methanol mobile phase, and was capable of separating a variety of narcotic analgesics (morphine, codeine, papaverine), amphetamine-type stimulants and cocaine-type drugs. The retention characteristics of some opium alkaloids was studied in detail by Hansen [33], and in 1983, Law et al. [34] published retention data for 84 basic drugs of forensic interest using a mobile phase of ammonuim nitrate buffer, pH 10.1-methanol (1:9) on a standardised Spherisorb S5W column.

Other applications on unmodified silica include the analysis of analgesics and decongestant drugs in commercial antihistamine preparations [35], the determination of biogenic amines using aqueous acidic mobile phases [36], and the determination of doxorubicin in pharmaceutical preparations using a mobile phase containing acetate buffer, pH 4.5, in isopropanol [37].

These type of separations have also found application in the field of biopharmaceutical analysis. As early as 1979, White [38] demonstrated a method for the analysis of morphine and its major metabolite, morphine-3-glucuronide, in blood which employed electrochemical detection. The mobile phase was ammonium nitrate buffer, pH 10.2-methanol. The method was very sensitive, though the liquid-liquid extraction procedure was rather lengthy and tedious. This type of chromatography has also been used in conjunction with off-line solid-phase extraction. Law [39] demonstrated the separation of beta-blocking drugs in the same run following extraction from plasma on a C_{18}- Bond-elut extraction column. Beta-blockers are particularly well suited to ion exchange on unmodified silica gel since they are difficult to chromatograph together in reversed-phase systems owing to the

wide range of lipophilicities which the group covers. In the same work, Law also demonstrated the chromatography of the tricyclic antidepressants and chlorproguanil and metabolites following solid phase extraction. Other drugs which have been chromatographed in this way using off-line extraction techniques are LSD [40], amiodarone and its metabolite [41,42] and quinidine [43].

Chromatography on unmodified silica using methanol-rich eluents has been applied to an on-line solid-phase extraction technique. In this work, Schmid and Wolf [14] extracted a series of tricyclic antidepressants on a C_2- concentration column. The latter was mounted in place of the injector loop, and with the valve in the "load" position. Clean-up of plasma samples was effected manually by injecting water and a series of weak methanolic solutions onto the concentration column. The drugs were introduced onto the analytical column by the action of switching the valve to the "inject" position, thus bringing the concentration column into the mobile phase stream which desorbed retained analytes and swept them onto the analytical column where they were separated.

Although this type of arrangement has advantages in that it requires only one column and dispenses with the need for a switching valve, its limitations in terms of ease of operation, level of manning and reproducibility are obvious. The objectives of the work presented in this chapter were firstly, to develop an analytical system which could accommodate the separation of a number of basic drugs of therapeutic interest, and secondly , to combine the analytical column with a column switching arrangement in order to facilitate on-line solid-phase extraction of the drugs followed by chromatography by ion exchange on unmodified silica gel. In this arrangement, the concentration column is mounted independent of the injector and a second pump is used to deliver the washing eluent. Switching between the two columns is achieved using a six-port valve and in this way, the concentration column may be re-equilibrated while separation of the previous sample is completed on the analytical column.

5.2. EXPERIMENTAL

5.2.1. Reagents and solvents

The 29 drugs used in this study were received as a gift from the Institute of Clinical Pharmacology, Dublin, Ireland. Ammonium nitrate (reagent grade) was obtained from BDH Chemicals Ltd, Poole, England, and analytical grade ammonia solution (25%) from Riedel de Haen, Seelze, Hannover, West Germany. HPLC grade methanol was supplied by Labscan Analytical Sciences, Dublin, Ireland. Deionised water was obtained by passing freshly distilled water through a Millipore Milli-Q water purification system. Dried human plasma from the Blood Transfusion Board, Dublin was dissolved in Milli-Q water and used within 7 days of reconstitution.

5.2.2. Drug standards

Stock solutions equivalent to 1 mg/ml of the drugs in methanol were prepared. Working stock solutions were prepared in methanol-water (1:1) to concentrations of 1-50 μg/ml, depending on the detector response of the drug. Standard solutions were made to 50-10000 ng/ml. They were prepared in mobile phase for direct injection and in water for column switching. For ultra-violet absorbance measurements, the 1 mg/ml stock solutions were diluted 1:9 in mobile phase.

5.2.3. Plasma standards

Aliquots of drug-free plasma (1 ml) were spiked with working stock solutions (50 μl) to produce the required concentrations. Plasma blanks were prepared by adding 50 μl of methanol-water (1:1) to 1 ml of drug-free plasma. These plasma solutions were then diluted with deionised water (1:1) and 500 μl loopfuls were introduced into the chromatographic system.

5.2.4. Instrumentation and operating conditions

The ultraviolet absorption spectra for the drugs were measured on a Hewlett Packard (Palo Alto, CA, USA) Model 8452A diode array spectrophotometer. For chromatography, the drugs were separated on a LiChrosorb silica 60 (5 μm) column (250 mm x 4.6 mm i.d.), supplied by HPLC Technology, Macclesfield, UK. It was protected by a Waters (Waters Associates, Milford, MA, USA) Guard-Pak module fitted with a silica insert. A pre-column was placed between the pump and the injector. It was packed with Lichrosorb silica (10 μm) (E. Merck, Darmstadt, West Germany) in order to saturate the mobile phase with silica and so prolong analytical column life. The pH of solutions was measured at ambient temperature using a standard glass electrode, which was calibrated daily using aqueous standards prepared on a weekly basis.

Stock solutions of ammonia and ammonium nitrate (both 0.1 M) were prepared by dissolving the appropriate amount of substance in deionised water. These solutions were mixed to produce the required pH (using the pH meter). The resulting solutions were diluted with deionised water to produce the desired ionic strength. Mobile phases were prepared by mixing the aqueous component with the required amount of methanol. The mobile phase was passed through a 0.45 μm filter and degassed by sonication prior to use. The solutions were stored in glass containers which were capped at all times to prevent the absorption of carbon dioxide from the atmosphere, thus raising the hydrogen ion concentration above its initial value.

The mobile phase was delivered by a Waters Model 501 HPLC pump (pump B) at a flow rate of 1.0 ml/min for development work and 1.3 ml/min for evaluation. The drugs were introduced into the system using a Rheodyne (Cotati, CA, USA) Model 7125 6-port injection valve. The valve was fitted with either a 20 μl loop or a 500 μl loop. The drugs were detected by ultra-violet absorption at 254 nm using an Applied Chromatography Systems (Macclesfield,

UK) Model 750/11 fixed wavelength detector, and chromatograms were recorded with a Linseis (Selb, West Germany) recorder at a chart speed of 200 mm/hr.

For the purposes of column switching, a second Waters Model 501 pump (pump A) and the concentration column were connected to the analytical assembly via a Rheodyne Model 7000 6-port switching valve. The 10 x 1.5 mm i.d. concentration columns were dry-packed with either Corasil (Waters Associates) RP-18, (37-50 μm), Sepralyte (Analytichem International, Harbour city, CA, USA) RP-8 (40 μm) or with Supelco (Bellefonte, PA, USA) CN- material (25-40 μm). The washing eluent delivered to the concentration column by pump A was deionised water filtered through a 0.45 μm membrane and degassed under vacuum.

5.2.5. Procedures

5.2.5.1. Effect of pH on drug retention

Ammonium nitrate buffer solutions of pH 6.0, 7.0, 8.0, 9.0, 9.5, and 10.0 were prepared by mixing 0.1 M solutions of ammonium nitrate and ammonium hrdroxide. These mixtures were then diluted (1:3) to produce 0.025 M solutions. Mobile phases containing 1:4 aqueous-methanolic components were made by mixing the aqueous phase with methanol. The retention times of the drugs were investigated as a function of eluent pH.

5.2.5.2. Ultraviolet absorbance spectra

The 1 mg/ml stock solutions of the drugs were diluted (1:9) with mobile phase at both pH 9.5 and at pH 7.0. The absorption spectra of each drug in both mobile phases were measured.

5.2.5.3. Column switching procedure

The operation of the column switching arrangement has been described in chapter 2. The concentration column was packed with

RP-18, RP-8 or cyano- material and the wash solution was delivered at a flow rate of 1 ml/min. The wash time was 1 minute after which the concentration column was connected to the analytical column by actuating the switching valve. The mobile phase delivered to the analytical column was 0.025 M amonium nitrate/ammonium hydroxide, (the pH depended on the pK_a of the drug) plus methanol (1:4). Working stock solutions were diluted in deionised water to generate standards containing 50-10000 ng/ml according to the detector response of the drug at 254 nm. The drugs were introduced in 500 μl loopfuls onto the concentration column and following the wash regimen outlined above, the drug peak heights were investigated as a function of the type of packing in the concentration column.

5.2.5.4. Assay Evaluation

The following procedure was carried out for each of the drugs investigated: drug-free aliquots of plasma (1 ml) were spiked with either 50 μl methanol-water (1:1) or 50 μl working stock solution of the drugs to yield plasma blanks and plasma standards, respectively. The mixtures were vortexed for 10 seconds. The plasma standards were diluted (usually 1:4) with drug-free plasma to generate dilute plasma standards. 500 μl deionised water was then added to the spiked and blank plasma solutions, followed by vortex mixing for 10 seconds. This procedure was repeated four times for the concentrated plasma standards and four times for the dilute plasma standards to generate quintuplicate standards at both concentration levels. 500 μl of each of the standards were injected into the system, and the plasma blanks were injected in duplicate. Aqueous standards of the drugs were injected in duplicate instead of quintuplicate. Having measured the peak heights for each of the compounds, the means, standard deviations and coefficients of variation at both concentration levels were calculated. Drug recoveries from plasma were calculated by comparing the mean peak heights of the quintuplicate plasma standards versus the duplicate aqueous standards at both concentration levels.

5.3 RESULTS AND DISCUSSION

There were 29 drugs investigated in this study. Several therapeutically important classes of compounds were represented: the tricyclic antidepressants, some major tranquillisers such as the phenothiazines, antihistamines, beta-blocking agents such as propranolol, narcotic analgesics, local anaesthetics and and antimalarial drugs. These drugs possess a primary, secondary of tertiary amine functionality, and apart from this shared amine function there are significant structural dissimilarities among some of the drugs which may be seen from the structures of representative compounds shown in Figure 5.1.

5.3.1. Development of chromatography

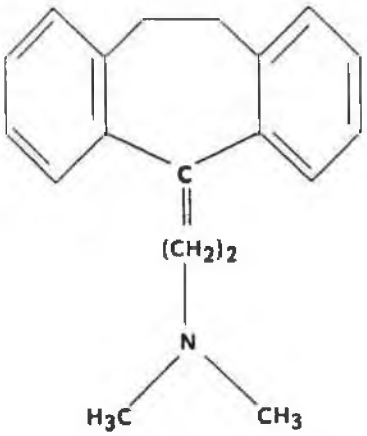
In the interest of reproducible chromatography, it is necessary that the pH and the ionic strength at the silica surface be defined, stable and in equilibrium with those of the eluent [14]. To this end, the aqueous component should contain some kind of buffering salts which do not precipitate in methanol-rich solvents and which preferably do not tend to dissolve the silica packing. Phosphate salts would be unsuitable since they have low solubility in organic solvents, and ammonium salts are preferable to sodium salts as their solutions tend less to dissolve silica [29]. In addition, Sugden et al. found that greater chromatographic efficiency was obtained for the quarternary ammonium compound, amprolinium when the ammonium as opposed to the sodium cation was used in the mobile phase [8]. Referring to the literature on the subject, it became obvious that even within the constraints of these criteria, a number of options were available, ranging from ammonium nitrate as originally used by Jane [19], to ammonium formate [8], or ammonium perchlorate [3].

As pointed out by Law, the ammonium nitrate-methanol eluent is widely used in this type of chromatography on unmodified silica [14,20,34],and since Schmid and Wolf [14] had separated five

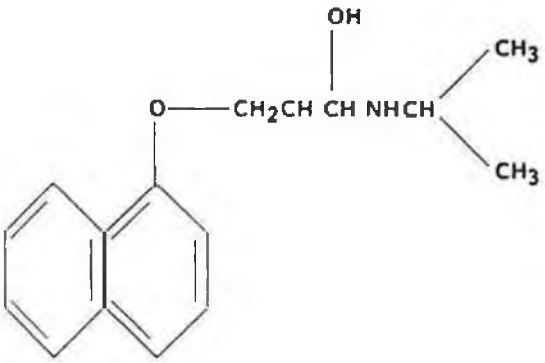
Figure 5.1.

Some representative drug structures

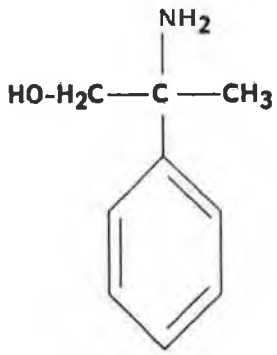
Amitriptyline (I)



Propranolol (II)



Phenylpropanolamine (III)



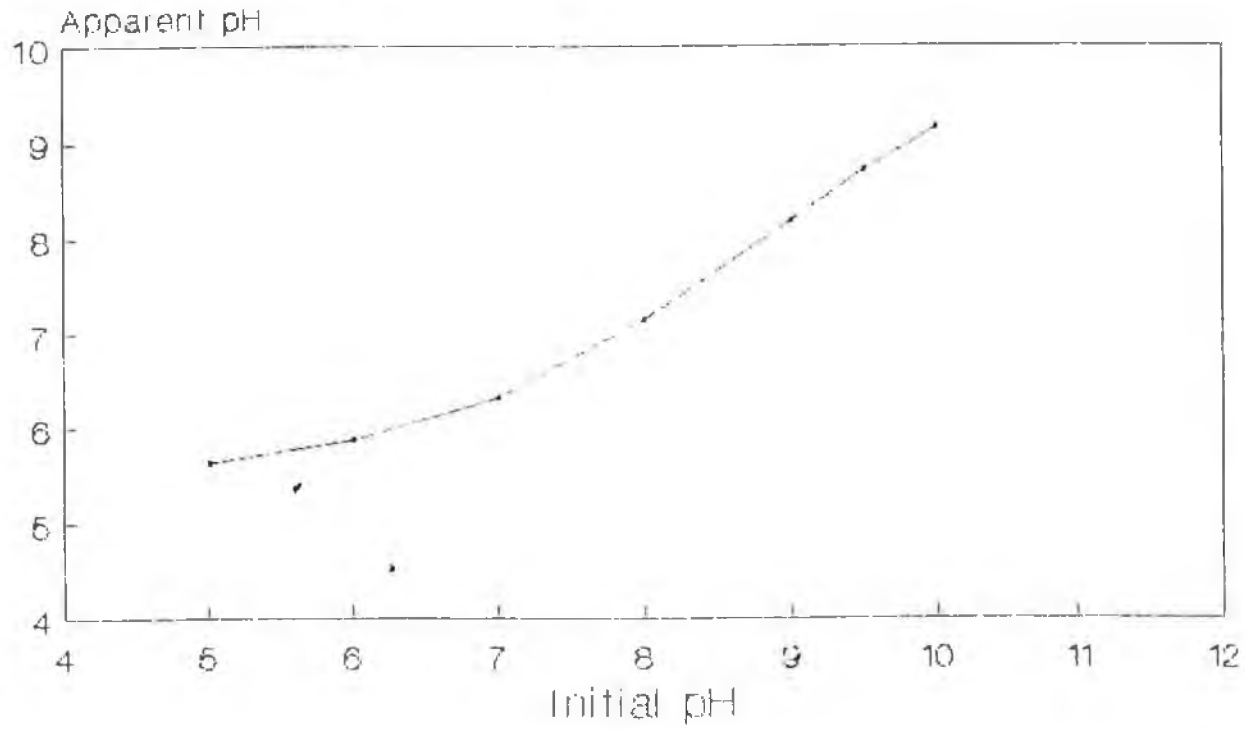
tricyclic drugs using a mobile phase of 0.003 M ammonium nitrate/ammonium hydroxide-methanol (1:4) and as a number of tricyclc drugs were included in the present study, the chosen starting point was an eluent composed of 0.005 M ammonium nitrate/ammonium hydroxide at pH 10.0-methanol (1:4). It was decided not to use the 1:9 ratio of aqueous to organic phase employed by Law and other workers [14,20,34] since this might create problems of protein precipitation when it came to the stage of on-line solid-phase extraction of the drugs from plasma.

As outlined in section 5.2, pH measurements were performed on the aqueous component prior to the addition of the organic phase, and all references to pH are based on the pH of the aqueous component. It is, however, well known that solute pK_a can be reduced in the presence of organic modifier [20]. Also, in the present study, it was observed that the apparent pH of the mobile phase following addition of 80% methanol was lower than the starting pH, and the magnitude of the reduction depended on the original pH in pure aqueous solution (Figure 5.2). The changes in mobile phase pH were not taken into account since meaningful discussion on the subject would warrant measuring solute pK_a values in the mobile phases (the quoted pK_a values were aqueous solution values taken from the literature). The objective of the present study was to observe the trends obtained as the mobile phase pH was altered and to generate suitable mobile phases for the separation of the drugs of interest and plasma components following extraction.

With the above mobile phase, amitriptyline was found to elute with a retention time of 39.3 minutes. If the assumed retention mechanism is one of cation exchange, then increased ionic strength should reduce the retention time. This effect had been widely reported in the literature, and was confirmed here when by increasing the ionic strength to 0.025 M NH_4^+, the retention time of amitriptyline was reduced to 10.5 min. The other tricyclic drugs used were nortriptyline, imipramine, desipramine and protriptyline. As may be seen from the results

FIGURE 5.2.

Effect of the addition of 80% methanol on apparent pH



Mobile phase: 0.025 M NH_4OH/NH_4NO_3-methanol (1:4)

presented in Table 5.1, the drugs (except for desipramine and protriptyline) are separated, though all elute quite late, and there is a large difference between the retention time of the first peak (amitriptyline) and the last peak (desipramine).

Lowering the pH produced different effects on the drugs depending on whether they possess secondary or tertiary amino functionalities; the tertiary amines, amitriptyline and imipramine elute later at the lower pH, while the secondary amines nortriptyline and desipramine elute earlier. These data may be explained by considering the pK_a values of these amines (Table 5.1) and the charged nature of the silanol groups under these conditions. At pH 10.0, due to the acidic nature of silica [3], the silanol moieties are highly dissociated and the column is behaving as a cation exchanger. On the other hand, ionisation of the less basic tertiary amines will be partially suppressed, thus reducing their affinity for the charged silanol groups on the silica packing. As the pH is lowered, their degree of ionisation is increased, though the degree of column ionisation is reduced. However, increased solute ionisation would appear to be the overriding effect since the retention of these amines is prolonged at lower pH values.

The more strongly basic secondary amines are dissociated to a greater extent at pH 10.0 than the tertiary anologues, and with these compounds, the predominant effect observed by lowering pH is a reduction in retention resulting from reduced silanol ionisation. The degree to which the retention of the tertiary amines is increased is less than the degree to which retention of the secondary amines is decreased, possibly because two opposing forces are controlling retention of the tertiary amines at this point, with the increase in drug dissociation overriding the effect of reduced silanol ionisation. Furthermore, it can be noted that even within the secondary amine group, the degree to which the amine is affected by lowering the pH depends on drug pK_a; the higher the pK_a, the greater the reduction in retention time. This observation agrees with the findings of

Table 5.1.
Influence of pH on the separation
of some tricyclic antidepressants

DRUG		RETENTION TIME (min)			
	pK_a	pH10.0	pH9.5	pH9.0	pH8.0
Amitriptyline	9.5	10.5	12.9	13.2	12.0
Nortriptyline	9.8	24.0	20.8	15.9	9.0
Imipramine	9.5	14.0	17.0	16.8	13.5
Desipramine	10.2	32.4	24.1	17.7	9.6
Protriptyline	10.0	29.1	21.0	16.9	8.7

Mobile phase : 0.025 M NH_4OH/NH_4NO_3-methanol (1:4).

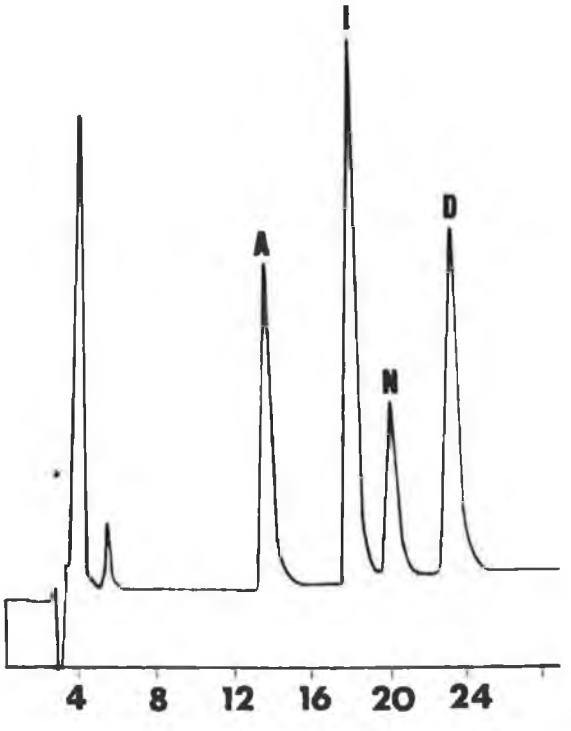
Law when he studied the relationship between retention and pK_a [20]. If the pH is lowered again, there is a further general decrease in retention, which can be explained in terms of a reduction in the charged silanol sites. Other workers [3,12,14] have studied the effect of increasing the pH to 10.0 and greater, and have found that at higher pH values, there is a general fall off in retention as the amines become progressively less ionised. Thus reversals in elution order between secondary and tertiary amines can be achieved, and this can prove an analytically useful tool in the separation of closely related species.

Owing to the high solubility of silica in alkaline media, and in spite of the high methanol content and presaturation of the mobile phase with silica, it is still desirable to use as low a pH as possible. It was found that good separation of the tricyclic drugs was achieved at pH 9.5, and so this was chosen as the operating pH. A chromatogram showing the separation of four tricyclic drugs is presented in Figure 5.3.

The other basic drugs of interest were also tested on this system, and all drugs were tested in mobile phases of pH 6.0 and pH 7.0. As different relative amounts of ammonium nitrate and ammonium hydroxide would be required to generate these different pH values, the nitrate concentration varied from one solution to the next. The cation (NH_4^+) concentration was, however kept constant, and as the proposed retention mechanism is one of cation exchange, it was assumed that a variation in the nitrate concentration would not seriously affect changes resulting from an alteration in pH. The results of this experiment are presented in Table 5.2., and they confirm the trend observed with the tricyclic drugs: i.e. there is a general increase in retention as the pH is raised from 6.0 to 7.0. As the pH is further increased to 9.5, there is a further increase in retention for the more basic amines, while the less basic amines elute earlier.

FIGURE 5.3.

Chromatogram of four tricyclic antidepressants



TIME(min)---------->

A: amitriptyline; I: imipramine; N: nortriptyline
D: desipramine.

Mobile phase: 0.025 M NH_4OH/NH_4NO_3-methanol (1:4)

Table 5.2.
Influence of pH on retention

DRUG		RETENTION TIME (min)		
	pK_a	pH 6.0	pH 7.0	pH 9.5
Acetopromazine	NA	4.5	8.4	15.9
Amitriptyline	9.4	5.7	9.6	12.6
Atenolol	9.6	2.9	6.3	16.5
Chlorpheniramine	8.9	14.6	15.0	19.2
Chlorpromazine	9.3	4.1	11.3	14.6
Clemastine	NA	7.2	7.5	16.2
Codeine	8.2	5.9	9.6	13.4
Desipramine	10.2	4.4	6.8	24.2
Dextromethorphan	8.3	4.1	8.8	28.8
Diltiazem	NA	4.1	5.7	4.8
Fluphenazine	8.1	3.6	5.1	5.1
Imipramine	9.5	6.3	11.0	17.2
Lidocaine	7.9	3.5	5.1	3.6
Mefloquine	NA	3.0	7.8	20.9
Mepivacaine	7.7	3.5	5.1	3.3
Nortriptyline	9.8	4.1	6.3	20.2
Perphenazine	7.8	4.2	5.4	3.9
Phentoloxamine	9.1	6.0	6.0	8.7
Phenylpropanolamine	9.5	2.6	4.8	11.0
Pindolol	9.7	2.6	5.1	11.3
Promethazine	9.1	9.3	9.3	7.5
Propranolol	9.5	2.6	3.2	11.9
Protriptyline	10.0	4.4	6.2	21.1
Quinine	8.5	3.3	7.5	9.0
Setastine	NA	6.0	6.6	12.6
Trimethoprim	7.2	3.0	4.2	3.5
Tripelennamine	9.0	5.9	7.2	13.7
Tyramine	10.2	2.7	5.1	16.8
Verapamil	NA	3.5	4.8	3.6

NA = Not available in standard reference texts

Mobile phase: 0.025 M NH_4OH/NH_4NO_3-methanol (1:4)

One of the main considerations was that the drugs should elute clear of the plasma components following on-line solid-phase extraction. Based on the data presented in Table 5.2., it was clear that some of the drugs, notably the less basic amines, would probably co-elute with the plasma peak at pH 9.5, and that the peaks of many of the more basic compounds would merge at the lower pH values. It was therefore decided to divide the drugs into two groups- one which would be separated using a mobile phase of pH 9.5 (group A) and the other (group B) which would be chromatographed using a mobile phase of pH 7.0. To the latter group was also assigned some of the more strongly basic compounds which had very long retention times at pH 9.5 (for example, dextromethorphan). Some other drugs were included in this group because they could be chromatographed more conveniently at pH 7.0 with other drugs of the same therapeutic class. Table 5.3. lists the drugs in groups A and B.

5.3.2. UV Absorbance measurements

At this stage it was decided to carry our some ultraviolet absorbance measurements to determine whether it was justified to continue using 254 nm as the operating wavelegth for the majority of drugs under study. As a fixed wavelength detector was being employed, the options were limited to 215 nm, 254 nm and 280 nm filters. The absorbance of the drugs was measured in both of the chosen mobile phases using a photodiode array spectrophotometer. The results obtained suggest that there was little difference in the UV maxima between one mobile phase and the other, and most of the drugs had strong absorbance between 230 and 270 nm. Therefore, 254 nm continued to be used as the operating wavelength. The absorption spectra for a number of representative compounds are given in Figures 5.4.a and 5.4.b.

5.3.3. Column switching

Having developed the mobile phases for the drugs, the next stage was to combine the analytical system to a column switching

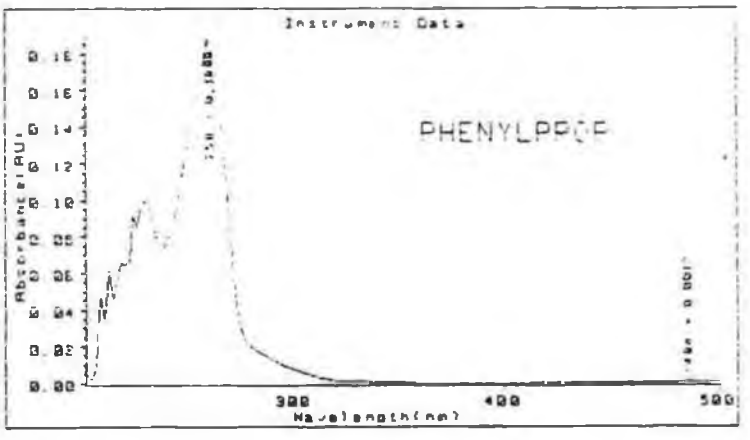
Table 5.3.

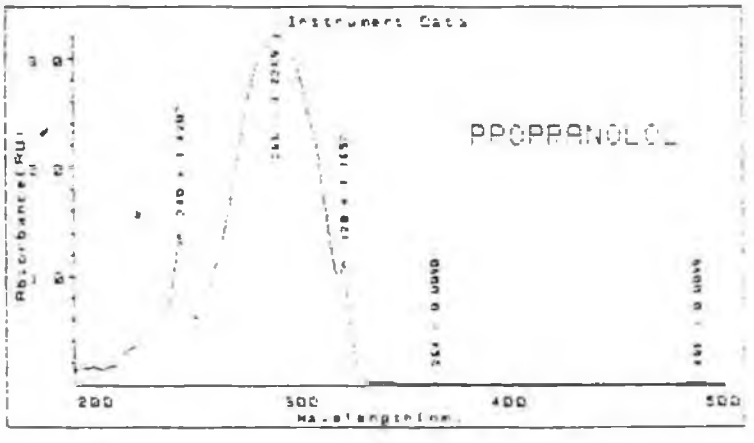
Drug groups

GROUP A	GROUP B
Acetopromazine	Chlorpheniramine
Amitriptyline	Clemastine
Atenolol	Codeine
Chlorpromazine	Dextromethorphan
Desipramine	Diltiazem
Imipramine	Fluphenazine
Mefloquine	Lidocaine
Nortriptyline	Mepivacaine
Phenylpropanolamine	Perphenazine
Pindolol	Phentoloxamine
Promethazine	Protriptyline
Propranolol	Setastine
Quinine	Trimethoprim
Tyramine	Tripelennamine
	Verapamil

FIGURE 5.4.a.

Some ultraviolet absorbance spectra in 0.025 M buffer. pH 9.5-methanol (1:4)





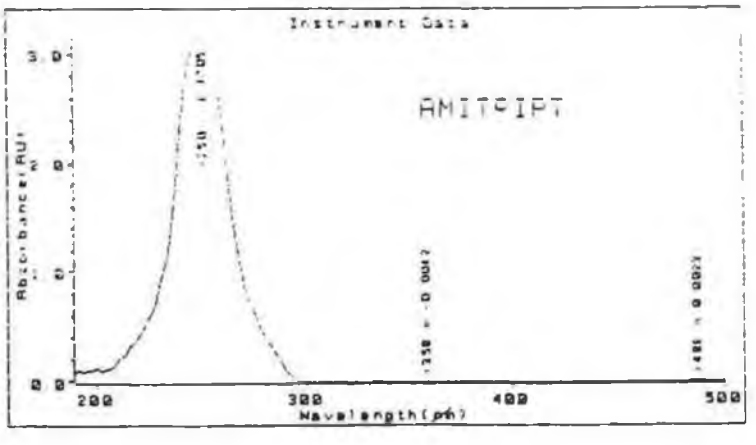
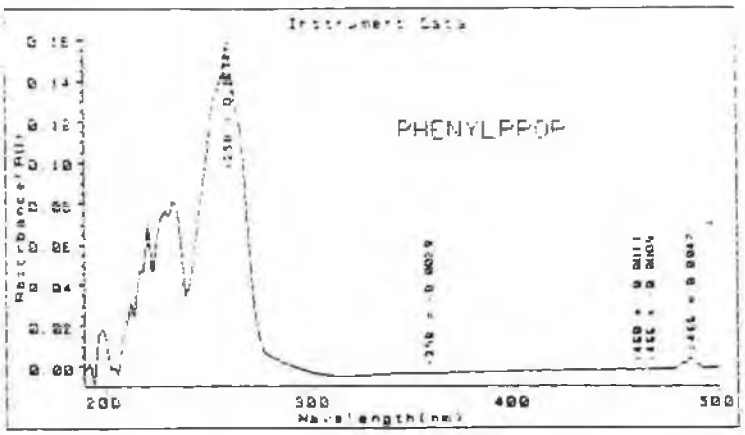
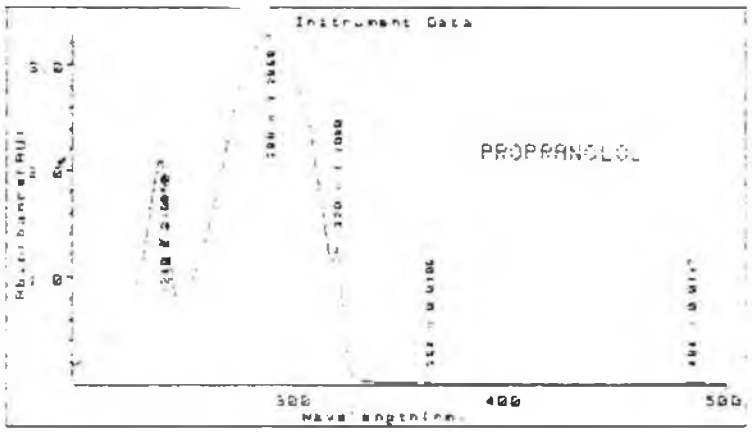
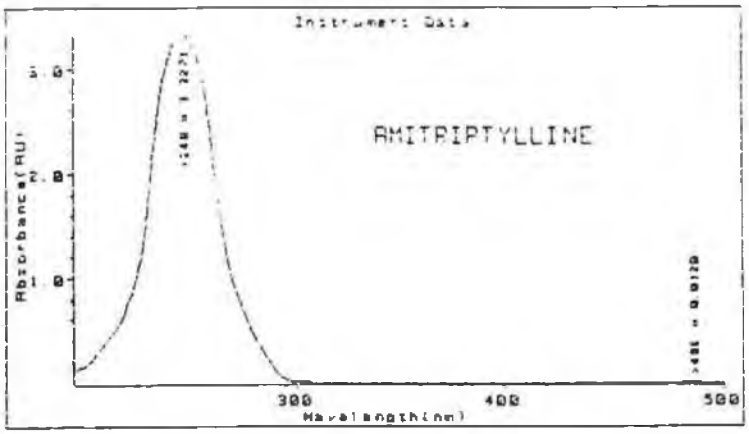


Figure 5.4.b.

Some ultraviolet absorbance spectra in 0.025 M buffer, pH 7.0-methanol (1:4)







arrangement with view to performing on-line solid-phase extraction of the drugs from spiked plasma samples. The operation of the column switching arrangement has been described in chapter 2. It was necessary to determine which type of packing in the concentration column would prove most suitable in terms of drug recovery and sample clean-up. Hence the drugs were first introduced directly onto the analytical column, and then with the second pump and the six-port switching valve in place, they were injected via the concentration column, and back-flushed onto the analytical column. The wash solution delivered by pump A (eluent A) was filtered and degassed, deionised water delivered at a flow rate of 1.0 ml/min, using a wash time of 1 min. Eluent B was 0.025 M ammonium nitrate/ammonium hydroxide (pH 7.0 or 9.5)-methanol (1:4) delivered at a flow rate of 1.0 ml/min. Three types of packing material were investigated; namely RP-18, RP-8 and cyano-. The results of this experiment are presented in Table 5.4., and recovery was determined by comparing the peak heights of recovered versus directly injected samples.

In many cases it can be seen that the apparent recovery is greater than 100%. This is most likely because compounds introduced via the concentration column are concentrated from a large (500 μl) to the smaller volume of the concentration column and associated tubing. The smaller volume of injection produces larger and sharper peaks for the same quantity of drug. In addition, the drugs must be dissolved in water for column switching; if dissolved in the mobile phase, the high methanol content will effect elution of the drugs off the concentration column, which of course, is behaving like a mini-reversed-phase system, and as water is a weaker solvent, it will produce sharper peaks than the mobile phase which must be used for direct injection purposes. This illustrates one advantage of column switching in the analysis of drugs and other compounds, that even where extraction from a biological matrix is not required, large volume dilute solutions may readily be enriched to facilitate their quantitation, and where the compound of interest

Table 5.4.a.

Comparison of recoveries from concentration columns

DRUG GROUP A

DRUG	% RECOVERY VERSUS DIRECT INJECTION		
	C_8	C_{18}	CN
Acetopromazine	70.8	39.6	21.9
Amitriptyline	100.2	90.2	17.6
Atenolol	118.6	39.3	NR
Chlorpromazine	79.7	63.7	23.7
Desipramine	114.4	80.0	6.7
Imipramine	119.7	103.2	7.4
Mefloquine	90.9	36.4	29.5
Nortriptyline	104.7	55.9	17.7
Phenylpropanolamine	113.3	93.3	NR
Pindolol	81.8	72.7	NR
Promethazine	110.0	105.9	7.4
Propranolol	109.5	107.7	NR
Quinine	102.3	26.8	0.9
Tyramine	109.7	NR	NR

NR = Not recovered from concentration column

Mobile phase: 0.025 M NH_4OH/NH_4NO_3, pH 9.5-methanol (1:4)

Wash solution: deionised water at 1.5 ml/min for 1 min.

Table 5.4.b.

Comparison of recoveries from concentration columns

GROUP B

DRUG	% RECOVERY VERSUS DIRECT INJECTION		
	C_8	C_{18}	CN
Chlorpheniramine	114.4	41.7	NR
Clemastine	123.6	116.1	21.2
Codeine	94.4	84.1	NR
Dextromethorphan	117.4	94.6	NR
Diltiazem	120.6	114.6	NR
Fluphenazine	104.8	108.3	124.4
Lidocaine	124.4	70.4	NR
Mepivacaine	119.8	69.8	NR
Perphenazine	95.5	98.3	126.8
Phentoloxamine	121.9	122.6	NR
Protriptyline	140.3	128.4	58.1
Setastine	121.7	115.2	20.8
Trimethoprim	127.0	105.7	8.2
Tripelennamine	123.1	114.2	NR
Verapamil	119.3	137.2	37.7

NR = Not recovered from concentration column

Mobile phase 0.025 M NH_4OH/NH_4NO_3, pH 7.0-methanol(1:4)

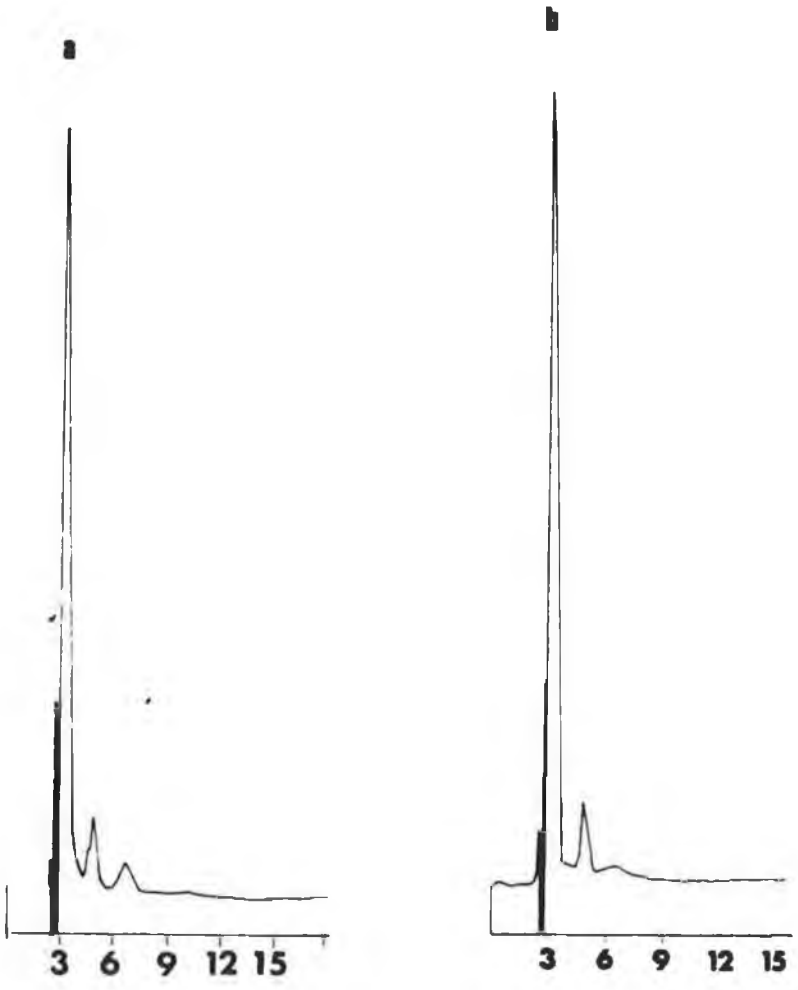
Wash solution: deionised water, 1.5 ml/min for 1 min.

does not occur in a highly contaminated matrix, the volume capacity of these small columns is substantially increased.

Referring to Table 5.4., it may be seen that for the drugs in group A, there is a significant difference between the recoveries from the different column packings. Overall, recovery is optimum from the C_8- concentration column. For the drugs in group B (Table 5.4.b), however, overall recovery (with a few exceptions) is similar from both C_8- and C_{18}- materials. For the majority of drugs in both groups, recovery was poor or non-existent from the cyano- packing material. These trends may be accounted for in terms of polarity differences between the various packing materials: water has stronger eluting power on the more polar cyano- (as opposed to C_{18}- or C_8-) material, thus allowing little or no enrichment, whereas on reversed phase materials, water is a much weaker eluent and the drugs are retained to a much greater extent. The difference in recovery between C_{18}- and C_8- materials may be accounted for in terms of the greater hydrophobicity of the latter, and its inability to retain the more polar analytes.

Hence, while it appears that the C_8- material offers the best match for most drugs, another factor which must be considered is how the various packing materials behave in relation to plasma removal. As cyano- material has little affinity for the drugs (with the notable exceptions of fluphenazine and perphenazine) under the employed experimental conditions, it was omitted from the next stage of the investigation. Blank plasma aliquots were injected onto both concentration column types, and sample chromatograms generated in this experiment are presented in Figures 5.5.a and 5.5.b. As might be expected, the C_{18}- material retained less of the polar plasma components (Figure 5.5.a) than the C_{18}- material. These chromatograms also show that in contrast to the drug compounds, there is little difference between the blank plasma profiles as the pH of the mobile phase is changed. This is presumably because the acidic plasma components would have little affinity for the column under

Figure 5.5.a.
blank plasma chromatograms following solid-phase extraction on a C_{18}- concentration column

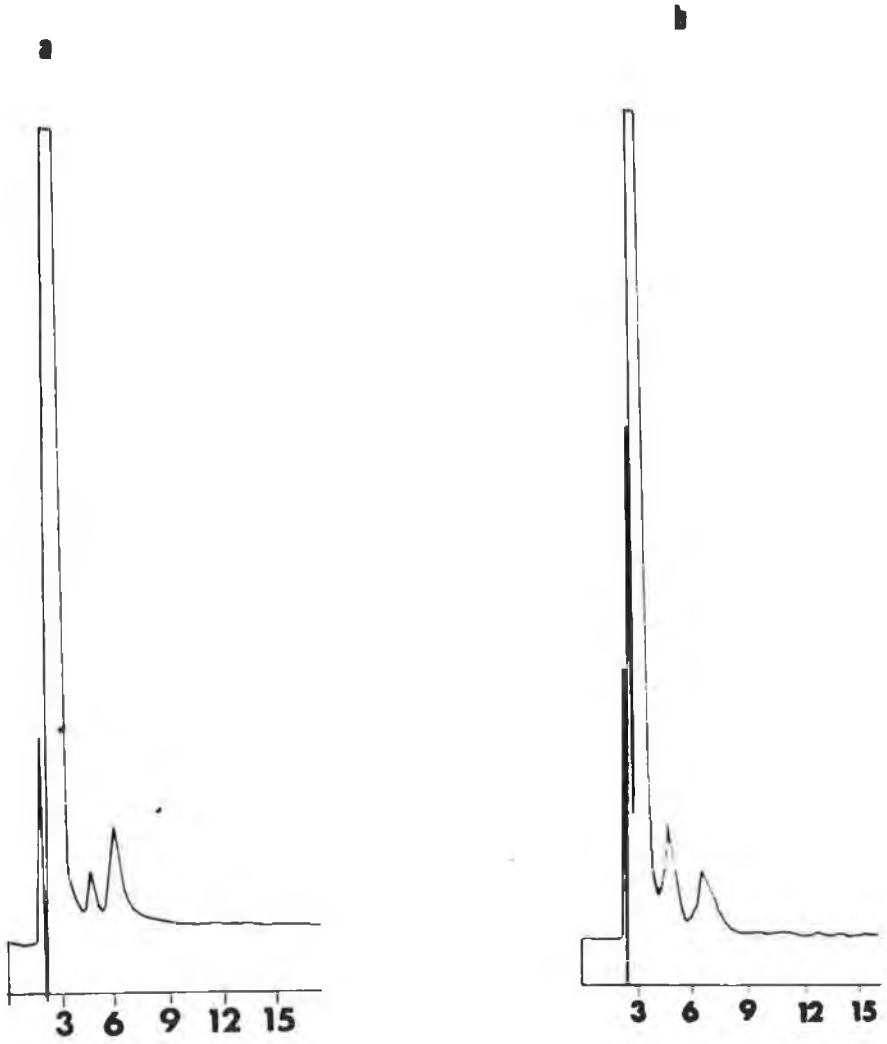


(a): mobile phase 0.025 M NH_4OH/NH_4NO_3, pH 7.0-methanol (1:4)

(b): mobile phase 0.025 M NH_4OH/NH_4NO_3, pH 9.5-methanol (1:4)

Detector sensitivity: 0.04 AUFS

Figure 5.5.b.
Blank plasma chromatograms following solid-phase extraction on a C_8- concentration column



TIME (min)---------->

(a): mobile phase 0.025 M NH_4OH/NH_4NO_3, pH 7.0-methanol (1:4)

(b): mobile phase 0.025 M NH_4OH/NH_4NO_3, pH 9.5-methanol (1:4)

Detector sensitivity: 0.04 AUFS

the employed experimental conditions. These results indicate that it is possible to produce the most appropriate retention times for the compounds of interest by manipulation of the eluent pH with little danger of introducing interfering peaks from the plasma matrix.

For the drugs in group A, the level of plasma removal achieved with C_8- was considered sufficient, as none of the plasma components interfered with the drug peaks. For the drugs in group B, however, it was apparent that some of the drugs would co-elute with the plasma components, irrespective of the type of packing material used in the concentration column.

It was then decided to investigate the effect on group B of changing the pH from 7.0 to pH 5.0 and pH 8.0, in order to obtain better separation between the plasma components and the compounds of interest. The results of this study are presented in Table 5.5, and they reveal that there is a general decrease in retention at the lower pH, possibly due to suppression in the ionisation of the silanol moieties. At pH 8.0 most of the drugs had longer elution times, and though others were hardly affected, it was concluded that this pH would be the most suitable for the compounds in group B.

5.3.4. Analysis in plasma

The drugs in group A were analysed with a mobile phase of pH 9.5, and extraction was performed on a C_8- concentration column. Water had been established as a suitable solvent for the wash phase, and based on previous work in this area [44,45], a wash time of 1 min at 1.0 ml/min was deemed a suitable regimen for the removal of plasma, while retaining the compounds of interest. It has previously been shown [44] that increasing the wash time will not usually remove endogenous compounds or interferents which are retained on the concentration column under the above conditions, but that it will cause band broadening in all retained species, including the peaks of interest. The flow rate of the analytical

Table 5.5.

Effect of pH on the retention of drugs in group B

DRUG	RETENTION TIME (min)		
	pH 5.0	pH 7.0	pH 8.0
Chlorpheniramine	8.4	14.8	18.1
Clemastine	4.2	7.2	8.4
Codeine	7.5	9.6	11.7
Dextromethorphan	5.7	8.9	9.8
Diltiazem	5.4	5.8	6.3
Fluphenazine	5.0	5.0	5.4
Lidocaine	5.1	4.8	4.8
Mepivacaine	5.4	5.1	4.6
Perphenazine	5.1	5.5	5.7
Phentoloxamine	5.7	6.1	9.4
Protriptyline	3.6	6.2	8.9
Setastine	4.5	6.5	6.9
Trimethoprim	4.0	4.2	4.6
Tripelennamine	6.0	7.5	12.2
Verapamil	4.8	4.8	3.6

Mobile phase: 0.025 M NH_4OH/NH_4NO_3-methanol (1:4)

mobile phase was increased from 1.0 ml/min to 1.3 ml/min in order to reduce run times. This measure did not cause merging of the last plasma interferent and the first compound of interest. The drugs in group B were analysed at pH 8.0 using the C_{18}- concentration column, as it had previously been established that most drugs in this category were recovered to similar degrees on both columns and less plasma is retained by the C_{18}- packing material.

It was then sought to measure the reproducibility of the methods in relation to every drug and to determine the percentage recoveries from the plasma matrix. Each drug was injected five times at two concentration levels in spiked plasma (10 injections), and in dupicate as authentic standards. The mean (x), standard deviation (SD), and coefficient of variation (CV) were calculated for the five spiked plasma standards at both concentrations. Recovery of the drugs from plasma was estimated by comparing the peak heights of the quintuplicate extracted standards versus the peak heights of the duplicate authentic standards.

Results of the plasma analyses are presented in Table 5.6 (group A) and Table 5.7 (group B), and some sample chromatograms are shown in Figures 5.6 and 5.7. As may be seen from the results, the coefficient of variation never exceeded 8%, was usually less than 5% and was frequently less than 3%. The low levels of variability, even in the absence of an internal standard, are possible because of the inherent reproducibility of the column switching technique, especially when compared with liquid-liquid extractions which can involve many different steps, and may permit the introduction of artefacts. Recovery from plasma using column switching is generally high. As the results show, recovery is usually in excess of 70%, and is frequently greater than 90%. Some drugs do, however, exhibit particularly small percentage recoveries, especially at low concentrations. The most likely explanation for these observations is that some drugs are protein bound, and would require more vigorous extraction methods such

Table 5.6.
Plasma assays- Drug group A

DRUG	CONC NG/ML	MEAN PKHT* MM	SD MM	CV %	RECOVERY %
Acetopromazine	250	78.0	1.4	1.8	83.4
Acetopromazine	50	14.6	0.5	3.4	78.9
Amitriptyline	500	42.6	1.1	2.6	91.6
Amitriptyline	100	8.4	0.5	5.9	93.3
Atenolol	5000	83.0	0.7	0.8	96.5
Atenolol	1000	16.4	0.5	3.0	96.5
Chlorpromazine	250	72.4	2.3	3.2	53.6
Chlorpromazine	50	13.4	0.9	6.7	58.3
Desipramine	500	46.0	1.4	3.0	93.9
Desipramine	100	11.1	0.2	1.8	89.8
Imipramine	500	71.4	1.1	1.5	97.1
Imipramine	100	15.8	0.8	5.1	101.9
Mefloquine	2000	31.6	0.5	1.6	47.2
Mefloquine	400	4.5	0.4	7.8	36.0
Nortriptyline	500	21.0	0.7	3.4	76.4
Nortriptyline	100	4.8	0.3	6.3	80.1

* : PKHT = Peak height, detector sensitivity = 0.04 AUFS

Mobile phase: 0.025 M NH_4OH/NH_4NO_3, pH 9.5-methanol (1:4)

SD: Standard deviation

CV: Coefficient of variation

Table 5.6. continued

DRUG	CONC NG/ML	MEAN PKHT* MM	SD MM	CV %	RECOVERY %
Phenylpropanolamine	10000	97.6	0.9	0.9	92.9
Phenylpropanolamine	2000	19.4	0.6	3.1	92.4
Pindolol	500	164.0	7.3	4.5	95.1
Pindolol	100	36.2	0.4	1.1	104.9
Promethazine	250	97.2	2.3	2.4	84.5
Promethazine	50	18.0	0.7	3.9	100.4
Propranolol	1250	81.8	0.8	1.1	97.4
Propranolol	250	15.2	0.5	3.3	98.1
Quinine	2000	191.8	1.3	0.7	93.6
Quinine	400	34.4	0.9	2.6	88.3
Tyramine	2000	69.0	0.7	1.0	35.3
Tyramine	400	24.8	0.8	3.2	51.3

*: PKHT = Peak height, detector sensitivity = 0.04 AUFS

Mobile phase: 0.025 M NH_4OH/NH_4NO_3, pH 9.5-methanol (1:4)

SD: Standard deviation

CV: Coefficient of variation

Table 5.7.
Plasma assays-Drug group B

DRUG	CONC NG/ML	MEAN PKHT* MM	SD MM	CV %	RECOVERY %
Chlorpheniramine	2500	125.0	2.9	2.3	96.5
Chlorpheniramine	625	27.6	0.5	1.8	92.0
Clemastine	5000	15.0	0.7	4.7	51.7
Clemastine	2500	5.9	0.2	3.4	40.7
Codeine	5000	95.0	1.7	1.8	109.8
Dextromethorphan	5000	69.6	0.5	0.7	91.6
Dextromethorphan	1250	20.2	0.4	2.0	96.2
Diltiazem	250	124.8	1.8	1.4	76.8
Diltiazem	50	23.0	0.7	3.0	92.0
Fluphenazine	250	89.8	4.8	5.3	86.8
Fluphenazine	100	27.6	2.1	7.6	81.2
Lidocaine	5000	77.6	0.5	0.6	161.6
Lidocaine	200	31.2	0.5	1.6	96.5
Mepivacaine	5000	39.6	0.4	1.2	141.5
Mepivacaine	2000	15.6	0.5	3.2	111.4
Perphenazine	250	82.0	1.2	1.5	75.6
Perphenazine	100	29.8	0.4	1.3	71.0

*: PKHT = Peak height, detector sensitivity = 0.04 AUFS

Mobile phase: 0.025 M NH_4OH/NH_4NO_3, pH 8.0-methanol (1:4)

SD: Standard deviation

CV: Coefficient of variation

Table 5.7. continued

DRUG	CONC NG/ML	MEAN PKHT* MM	SD MM	CV %	RECOVERY %
Phentoloxamine	2500	94.8	2.0	2.1	85.0
Phentoloxamine	625	21.5	0.5	2.3	72.9
Protriptyline	500	90.4	3.8	4.2	76.3
Protriptyline	125	21.8	0.4	1.8	72.7
Setastine	5000	42.6	0.9	2.1	97.9
Setastine	2500	18.8	0.4	2.1	85.5
Trimethoprim	350	109.2	3.9	3.6	53.6
Trimethoprim	80	24.2	0.8	3.3	53.7
Tripelennamine	500	53.0	0.7	1.3	99.1
Tripelennamine	125	12.5	0.5	4.0	89.3
Verapamil	2000	113.8	0.4	0.4	92.9
Verapamil	500	38.8	0.4	1.0	107.5

*: PKHT = Peak height, detector sensitivity = 0.04 AUFS

Mobile phase: 0.025 M NH_4OH/NH_4NO_3, pH 8.0-methanol (1:4)

SD: Standard deviation

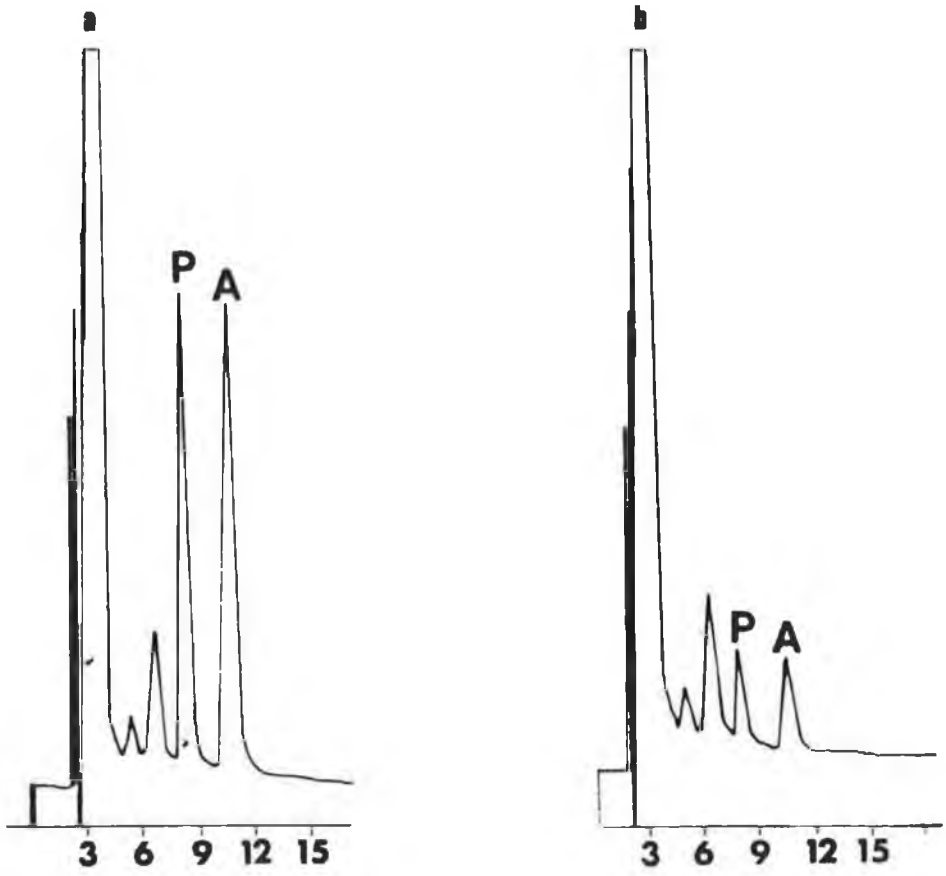
CV: Coefficient of variation

as liquid-liquid extraction or protein precipitation to liberate them from their binding sites.

It is possible, of course, to subject a sample to column switching subsequent to protein precipitation, but acid or base precipitants would first have to be neutralised, and organic precipitants evaporated, as they would effect rapid elution of the drug off the concentration column. In addition, precipitation can cause the loss of some of the sample, and contributes to the elaboration of the system. Another useful measure which can be adopted is to add salicylic acid to the plasma to free drugs from protein binding sites. As salicyate has an extremely high affinity for plasma proteins, it will displace drugs which are already bound, but which have a lower affinity for the binding site. As outlined in chapter 2, the addition of an enzyme, such as subtilisin Carlsberg, can cleave bound drugs from proteins, and this measure has the added advantage of prolonging the lifetime of the concentration column [46]. A more novel approach has been to add a small (to minimise dilution effects) volume of a solution of sodium dodecyl sulphate to the plasma prior to injection onto the concentration column [47]. This measure has been shown to prolong the lifetime of the concentration column, and to promote liberation of drugs from protein binding sites. So far this method has been applied only to relatively high drug concentrations (10 μg/ml) and its usefulness for measurements in the ng/ml region has not yet been demonstrated.

In the case of some of the drugs, recovery was greater at lower concentrations, possibly because extraction efficiency is greater as there is less drug to partition from the liquid into the solid phase. In two instances (lidocaine and mepivacaine), the apparent recovery from plasma was greatly in excess of 100%. In these cases it proved difficult to obtain reproducible recovery of the authentic standards, and it was concluded that the presence of plasma components on the concentration column actually enhances their retention, and so leads to better recovery. In the case of one drug (clemastine), recovery was substantially lower than

Figure 5.6.
Plasma standards extracted on a C_8- concentration column



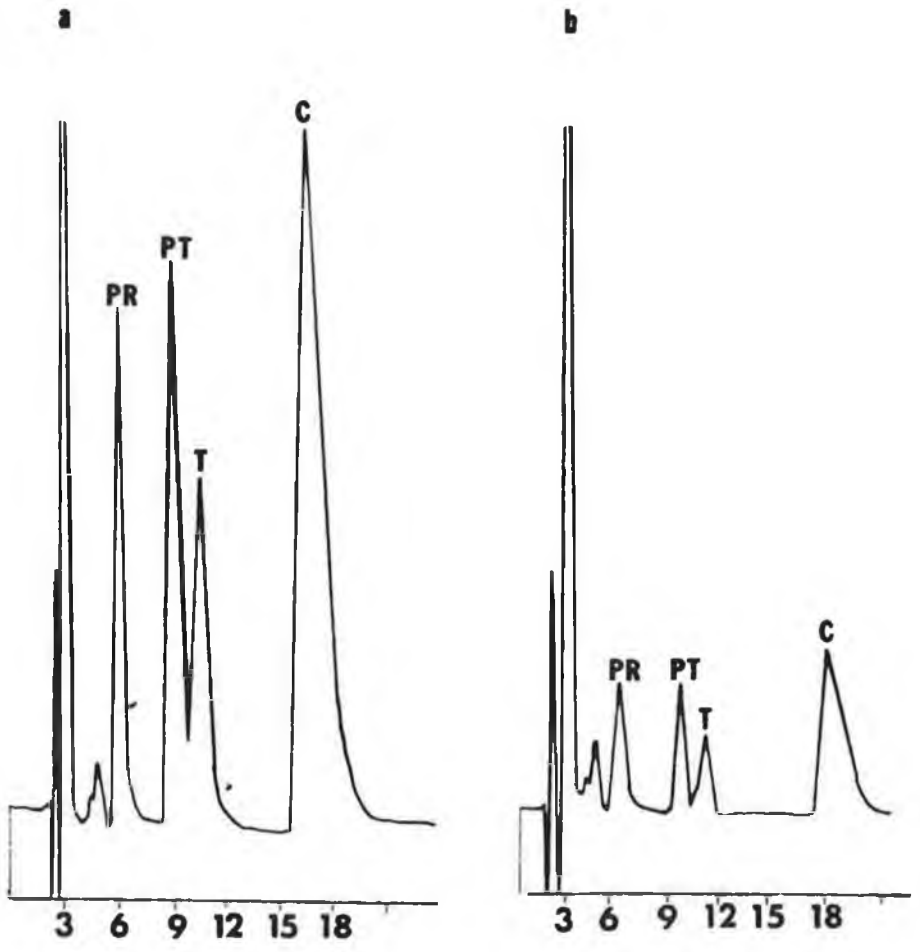
TIME (min)---------->

(a)		(b)
1250 ng/ml	propranolol (P)	250 ng/ml
5000 ng/ml	atenolol (A)	1000 ng/ml

Mobile phase: 0.025 M NH_4OH/NH_4NO_3, pH 9.5-methanol (1:4)

Detector sensitivity: 0.04 AUFS

Figure 5.7.
Plasma standards extracted on a C_{18}- concentration column



TIME (min)---------->

(a)			(b)
500 ng/ml	protriptyline	(PR)	125 ng/ml
2500 ng/ml	phentoloxamine	(PT)	625 ng/ml
500 ng/ml	tripelennamine	(T)	125 ng/ml
2500 ng/ml	chlorpheniramine	(C)	625 ng/ml

Mobile phase: 0.025 M NH_4OH/NH_4NO_3, pH 8.0-methanol (1:4)

Detector sensitivity: 0.04 AUFS

its sister drug (setastine) to which it is closely structurally related. It was found that recovery of this drug depended heavily on the batch of plasma into which it was spiked. This may, in fact, be the case of many drugs, though it was beyond the scope of the this project to test recovery of all drugs from a number of plasma sources. It does, however, illustrate the point that in biopharmaceutical analysis, the condition of the plasma and the factors affecting its composition may play a significant role in determining the amount recovered, and seemingly low levels of a drug may be a result of poor yield from a particular subjects plasma, rather than inefficient bioavailability of that preparation.

5.4. CONCLUSION

It has been shown in this and other studies that a silica column used with a methanol-rich buffered aqueous eluent has wide applicability in the separation of basic compounds of medicinal and forensic interest. In this case, two methods have been developed which can accommodate the analysis of a number of therapeutically important compounds, and these methods have been successfully combined with an on-line column switching assembly to permit rapid extraction of the drugs from plasma. Over the range of drugs studied, the method has been shown to be reproducible, with a coefficient of variation of 3% or less for most compounds, without the need for internal standardisation. The method offers drug recoveries comparable with, and in many cases, superior to conventional liquid-liquid extraction methods. Apart from being less tedious and time-consuming, column switching has the advantage of being less hazardous and expensive than liquid-liquid extraction, as it does not require any organic solvents. The only consumable (apart from filtered water) is the concentration column packing. Obviously, the capacity of the column will depend on how viscous and heavily contaminated the sample is, the size of the concentration column and the nature of the two mobile phases; but it has been shown [44,45] for the type

of concentration columns and packings used in the present study, that a single column can accept up to 7.5 ml of plasma without significant deterioration in column performance, back pressure or peak width.

Ion exchange chromatography on unmodified silica using an organic-rich mobile phase is ideally suited to column switching as the high content of methanol in the mobile phase effects almost total removal of any endogenous components which are retained on the concentration column throughout the wash phase. In this way, the concentration column is regenerated more effectively than if a mobile phase containing low concentrations of organic solvent are used, such as would be common in many reversed-phase separations. In these cases, if there is no separate regeneration step (with methanol) between elution of the drugs from the concentration column and re-equilibration with the wash solution, residual plasma components can build up on and modify the concentration column, necessitating the use of an internal standard and creating problems with regard to contamination and blockage.

A limitation with the use of high concentrations of organic modifier is precipitation of plasma components by the organic solvent, resulting in pressure build-up across the system and the need to change the concentration column frequently. This problem can be minimised by allowing adequate re-equilibration of the concentration column with the aqueous washing solution. Ion exchange chromatography on unmodified silica is particularly suitable for drug screening purposes, e.g. examination of illicit drug preparations [19,34]. It is capable of eluting a wide range of compounds within a relatively small time frame which allows for rapid analysis. However, because of its very suitability in the chromatography of a wide range of basic drugs, it has limited discriminating power, arising from the fact that a large number of compounds are eluting within a relatively short period of time. This means that any identification of unknown substances is tentative and must be confirmed by other tests. This technique

is, therefore, a useful adjunct to other methodologies in the analytical laboratory, and is all the more powerful a tool since it can be combined with column switching to permit plasma analysis, and of course, it can be optimised to develop a specific analytical method for one or more particular compounds.

5.5. REFERENCES

1. P. Roumeltiotis and K. K. Unger, J. Chromatogr., 149 (1978) 211
2. M. Holik and M. Matejkova, J. Chromatogr., 213 (1981) 33.
3. R. J. Flanagan and I. Jane, J. Chromatogr., 323 (1985) 189.
4. W. R. Melander, J. Stoveken and C. Horvath, J. Chromatogr., 199 (1980) 35.
5. A. Nahum and C. Horvath, J. Chromatogr., 203 (1981) 53.
6. K. Bij, C. Horvath, W. R. Melamder and A. Nahum, J. Chromatogr., 203 (1981) 65.
7. J. Chromatogr., 186 (1979) 705.
8. K. Sugden, G. B. Cox and C. R. Loscome, J. Chromatogr., 149 (1978) 337.
9. R. E. Hill, J. Chromatogr., 135 (1977) 419.
10. J. Crommen, B. Fransson and G. Schill, J. Chromatogr., 142 (1977) 283.
11. A. Sokolowski and K. Wahlund, J. Chromatogr., 189 (1980) 299.
12. B. Bidlingmeyer, J. K. Del Rios and J. Korpi, Anal. Chem., 54 (1982) 442.
13. S. G. Weber and W. Tramposch, Anal. Chem., 55 (1983) 771.
14. R. W. Schmid and Ch. Wolf, Chromatographia, 24 (1987) 713.
15. G. B. Cox and R. W. Stout, J. Chromatogr., 384 (1987) 315.
16. B. A. Bidlingmeyer, J. Chromatogr. Sci., 18 (1980) 525.
17. Waters Associates, Bulletin No. N80, 1981.
18. B. A. Bidlingmeyer, S. M. Demming, W. P. Price Jr., B. Sachok and M. Petrusek, J. Chromatogr., 186 (1979) 419.
19. I. Jane, J. Chromatogr., 111 (1975) 225.
20. B. Law, J. Chromatogr., 407 (1987) 1.
21. S. H. Hansen, J. Chromatogr., 212 (1981) 229.
22. J. C. Kraak and C. F. Simpson (Eds.) Techniques in Liquid Chromatography (Wiley, Heyden, 1982).
23. H. Lingeman, H. A. van Munster, J. H. Beynen, W. J. M. Underberg and A. Hulsoff, J. Chromatogr., 352 (1986) 261.
24. J. Kohler and J. J. Kirkland, J. Chromatogr., 385 (1987) 125.
25. B. B. Wheals, J. Chromatogr., 187 (1980) 65.

26. J. G. Atwood, G. J. Schmidt and W. Slavin, J.Chromatogr., 171 (1979) 109.
27. C. Horvath, W. Melander and I. Molnar, Anal. Chem., 49 (1977) 142.
28. R. B. Sosman, The Phases of Silica (Rutgers University Press, New Brunswick, 1965).
29. A. Wehrli, J. C. Holdenbrand, H. P. Keller, R. Stampfli and R. W. Frei, J. Chromatogr., 149 (1978) 199.
30. F. M. Rabel, Int. Lab., 3 (1980) 53.
31. P. E. Barker, B. W. Hatt and S. R. Holding, J. Chromatogr., 206 (1981) 27.
32. B. Law and P. F. Chan, J. Chromatogr., 467 (1989) 267.
33. S. H. Hansen, J. Chromatogr., 212 (1981) 229.
34. B. Law, R. Gill and A. C. Moffat, J. Chromatogr., 301 (1984) 165.
35. H. Richardson and B. A. Bidlingmeyer, J. Pharm. Sci., 73 (1984) 1480.
36. H. Svendsen and T. Greibrokk, J. Chromatogr., 212 (1981) 153.
37. H. G. Barth and A. Conne, J. Cromatogr., 131 (1977) 375.
38. M. W. White, J. Chromatogr., 178 (1979) 229.
39. B. Law, J. Pharm. Biomed. Anal., in Press.
40. J. Christie, M. W. White and J. M. Wiles, J. Chromatogr., 120 (1976) 496.
41. R. J. Flanagan G. C. A. Storey and D. W. Holt, J. Chromatogr., 187 (1980)391.
42. R. J. Flanagan G. C. A. Storey, P. B. Farmer and D. W. Holt, J. Pharm. Pharmacol., 34 (1982) 638.
43. R. G. Achari, J. L. Baldridge, T. R. Koziol and L. Yu. J. Chromatogr. Sci., 16 (1978) 271.
44. D. Dadgar and A. Power, J. Chromatogr., 416 (1987) 99.
45. D. Dadgar and A. Power, J. Chromatogr., 421 (1987) 216.
46. C. E. Werkhoven-Goewie, C. De Ruiter, U. A. Th. Brinkman, R. W. Frei, C. J. De Jong, C. Little and O. Stal, J. Chromatogr., 255 (1983) 79.
47. M. A. J. Van Opstal, F. A. L. Van der Horst, P. Krabbenbourg and J. Holtuis, Personal communication.

CHAPTER 6

RETENTION CHARACTERISTICS IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF BASIC DRUGS AND PLASMA EXTRACTS ON AN ALUMINA COLUMN

6.1. INTRODUCTION

For a number of practical reasons, most separations are carried out on silica (modified or unmodified) rather than on alumina. Silica is less likely to promote unwanted reactions of the sample during separation; it is available in a wide range of chromatographically useful forms; and the literature is replete with applications for silica in both column chromatography and TLC [1]. Furthermore, silica has a higher specific surface area - 500m^2/g for neutral silica as compared to 70 m^2/g for basic alumina. A higher surface area means greater capacity for silica over alumina, and this results in higher capacity ratios and more predictable retention behaviour [2]. Occasionally, however, it is advantageous to consider the use of alumina in place of silica, and the differences in selectivity between the two adsorbents have been reviewed in detail [3].

6.1.1. Structure and properties of alumina

The crystalline alumina generally used in chromatography is known as γ-alumina and is obtained by dehydration of the mineral Bayerite at elevated temperature (200-600^{o}C) [4]. Alumina is hygroscopic and its adsorption properties for chromatographic solutes depend on the amount and the form of water present at its surface [5]. At room temperature, the IR spectrum of hydrated alumina displays two broad bands at 3300 and 1650 cm^{-1} characteristic of water adsorbed in its molecular form. When, for chromatographic use, alumina is subjected to a higher temperature, the above mentioned bands are replaced by bands near 3700 cm^{-1} characteristic of the hydroxyl group [6]. Apparently, the water molecule decomposes into a hydroxyl group, which becomes attatched to the aluminium atom, and a proton which becomes attatched to the oxygen atom. Thus water, through chemisorption gives rise to two hydroxyl groups on the alumina surface. These retain the acidic or basic character of the underlying site and explain the amphoteric properties of alumina as an ion exchanger. It can act as an anion exchanger by

displacing the hydroxyl group chemisorbed to the aluminium atom and it can act as a cation exchanger through the proton chemisorbed to the oxygen atom. Hence, like silica, alumina may be regarded as a typical polar adsorbent with most separations proceeding in the same way on the two oxides. However, whereas silica is only active through its surface hydroxyl groups, alumina posesses two alternative possibilities for interaction with solutes.

Alumina acts as a typical ion-exchanger [7] but since it is amphoteric, its ion exchange properties are strongly pH dependent. The ion exchange properties of alumina have been studied by Laurent et al. [4] who used the adsorption of copper ions to demonstrate cation exchange processes on neutral and acidic alumina. He showed that when neutral alumina is washed with sodium hydroxide, as is common practice in preparing chromatographic alumina, protons which are chemisorbed onto an oxygen atom will be neutralised and replaced by more loosely bound sodium ions. The sodium ions present on alkaline alumina can exchange with other cations, and are responsible for the cation exchange properties of alkaline alumina. In this environment, hydroxyl groups which are chemisorbed onto aluminium ions are too firmly bound to be exchangeable with other anions. When alkaline alumina is washed with hydrochloric acid, the protons desorb the hydroxyl groups, which are then replaced by chloride ions which give rise to the anion exchange properties of acidic alumina. In addition, the protons displace the sodium cations chemisorbed to the oxygen atoms, and it is likely that these processes proceed simultaneously [4].

6.1.2. Ion exchange chromatography on alumina

Ionic solutes on bonded silica columns require a special approach to diminish their interaction with remaining silanol groups, which results in poor chromatographic efficiencies and peak tailing. As outlined in chapter 5, this may be accomplished by exploiting the amine-silanol interaction through the employment

of a buffered aqueous-organic eluent at high pH. In this respect, alumina also offers interesting possibilities for separation. The amphoteric character of alumina permits its use as either a cation or anion exchanger. In acidic media, the cation exchange capacity is small and the anion exchange capacity is large, and vice-versa in basic media. However, the variation with pH is gradual, and either cation or anion exchange properties are seen over fairly broad pH ranges. Alumina has proved to be very stable at extremes of pH. Laurent et al. have demonstrated less than 1% dissolution of the packing material after one months continuous use at pH 13 [4]. This broad pH range is available for chromatographic experiments and it is here that alumina offers an attractive alternative to silica. As a result of its resistance to strongly alkaline environments, basic compounds can be separated in the ion exchange mode in a manner analagous to that on unmodified silica as described in chapter 5, without the problem of loss in efficiency arising from dissolution of the packing material.

It is possible to define a pH where the net charge at the alumina surface is zero. This pH is known as the zero point charge (ZPC) and various methods have been employed to determine its value, including electrosmosis [8], streaming potential measurements [9], electrophoresis [10], and chromatography [11]. Generally, the ZPC of an aqueous suspension does not differ significantly from the solid metal hydroxide. However, the structure of the oxide and the presence of other ions co-precipitated during its preparation exert a large influence on the ZPC [11]. Abrahamson et al. [12] stressed that the determination of the ZPC should be made in the absence of all ions other than hydrogen, hydroxyl groups or those ions inherent in the solid. The specific adsorption of anions tends to increase the negative charge of the surface and shifts the ZPC to a lower pH. The Al-anion-H sites are generally more acidic than the original Al-OH groups.

For example, Clearfield [13] has shown that in the presence of 10^{-5} M H_2PO_4, the ZPC of alumina is shifted from 9.2 (as

originally quoted by Parks [14]) to a value of 6.2. Laurent et al. [11], using chromatographic techniques, demonstrated a similar acidic shift to pH 6.5 (approximately) for the ZPC of alumina in the presence of acetate ions. In the presence of citrate ions, the ZPC was shifted as far down as pH 3.5. Hence, the ZPC moves along the pH scale depending on the nature of the buffer (specifically, the buffer anion) used. Generally speaking, alumina behaves as a cation exchanger at pH values above the ZPC, and as an anion exchanger at pH values below the ZPC. It is usual to select as operating pH, a value which lies between the pK_a of the analyte and the ZPC of the column in the presence of the chosen buffer. In this way, the column and the analyte will be oppositely charged, and hence, available to partake in ionic interactions.

The use of unmodified silica and alumina as stationary supports in liquid chromatography had declined in popularity with the advent of chemically modified hydrophobic silicaceous suports. In particular, the use of alumina became even less widespread, as chemical modification of its surface in a manner analagous to the reaction of silica with alkylsilanes proved not to be a practicable proposition [4].

Many investigators have attempted surface modification of alumina. Knox [15] reported the reaction of alumina with aminosilane, cyanosilane and pyridylsilane. Elemental analysis was then used to show the presence of carbon on the surface. On the basis of this result Novotny [16] caused reaction in situ by passing octadecylchlorosilane through a microcapillary column containing alumina particles. In consecutive reversed-phase chromatographic experiments, the material was observed to have become less polar. The experience of Laurent et al. [4] was somewhat different to these earlier reports. They found that most chemical reagents which have been used successfully with silica showed no reactivity toward alumina. Even trimethylchlorosilane (TMCS), which reacts rapidly with silica, is quite inactive. However, hexamethyldisilazane (HMDS) was found to react with

silica and to generate a non-polar surface. Furthermore, this surface was observed to remain intact up to 200°C, and various experiments revealed that HMDS had replaced water molecules on the alumina and rendered the surface less polar. Unfortunately, this did not mean that modified alumina can be used as a stationary phase in reversed phase liquid chromarography. When contacted with polar solvents, especially water, HMDS was rapidly displaced again. Evidently, however, the silazane was not chemically bonded onto the alumina, but only strongly adsorbed, and hence was rapidly removed on contact with the more strongly attracted water molecule. The Al-O bond is stronger than the Si-O bond. As a result, in silanised alumina the Al-O-Si bond is less stable than the Si-O-Si bond [3].

In the same work, Laurent et al. [4] demonstrated the separation of a range of compounds, including benzoic acid derivatives and basic drugs using an alumina column and a wholly aqueous mobile phase. The factors which influenced retention of the analytes on the column were consistent with both cation and anion exchange processes. It was found, however, that the applicability of the technique was somewhat limited, that it could only be applied to a few components in a sample mixture, and that it was difficult to manipulate the selectivity of the system. In a later work, Laurent et al. [5] demonstrated a more flexible way to influence retention by the introduction of an organic modifier to the aqueous solvent.

They found that while the elution order or a series of primary amines remained intact, there was a retention maximum with increasing additions of organic modifier. They interpreted this maximum in terms of the operation of at least two competing retention mechanisms. This effect was most manifest for acetonitrile and tetrahydrofuran. As the tetramethylammonium hydroxide used as a competing ion would experience enhanced solvation in the presence of an organic modifier [17,18], its competition with the solute ion would decrease, resulting in

longer retention for the latter. They attributed the opposing effect (which became more obvious at higher percentages of organic modifier) to reduced solute ionisation in water-organic mixtures, the effect of which would be to cause a reduction in solute retention. In contrast to reversed-phase chromatography [19], there was no significant difference in eluting power observed between methanol, acetonitrile and tetrahydrofuran: hence solute retention remains approximately constant whether binary or ternary mixtures are employed as long as the total organic modifier content remains the same.

Laurent et al. [17] applied this type of aqueous-organic system to the separation of morphine, codeine and some other alkaloids. Using a buffer of citric acid and TMA at pH 6.0, all components were appreciably ionised in water and selectivity was imparted to the system by the addition of 75% acetonitrile. They found that if a ternary mixture was used consisting of methanol, acetonitrile and the aqueous component, separation, though not radically altered was improved for most of the compounds studied.

Laurent et al. [11] have also demonstrated the applicability of alumina in the separation of proteins using wholly aqueous buffered mobile phases. The retention mechanism in this case is principally one of ion exchange though size exclusion phenomena also play a significant role. The pH dependence of the ion-exchange mechanism is explained on the basis of the isoelectric point of the protein and the ZPC of the alumina, which as stated previously, can be manipulated by judicious choice of buffer anion. They found that for basic proteins, it was profitable to use a buffer such that the ZPC of the alumina surface is well below the isoelectric point of the proteins. This is easily realised by the use of a citrate buffer, which lowers the ZPC to 3.5.

Laurent et al. [20] have also used ion exchange on alumina to separate a wide range of opiates including illicit heroin samples and natural opium originating from a variety of sources.

Quantitation of morphine without the need for internal standardisation was possible. Lingeman et al. [21] have also investigated the retention mechanisms on alumina using a range of basic amines of therapeutic interest. They also made a comparison between unmodified silica and alumina in terms of eluent pH, type and concentration of competing ion and counter ion in addition to the nature and concentration of organic modifier. Lingeman showed that in some cases, silica is preferred over alumina in the analysis of tetracyclines, and that alumina is preferred for the analysis of anthracycline derivatives. The anthracyclines adriamycin and daunorubicin cannot be separated with the silica system, but can be separated on a comparable alumina system [22].

The objective of the present study was to investigate the effect of changing pH, ionic strength, methanol content, and buffer ion on the retention of 31 basic drugs of therapeutic interest. As the on-line column switching technique has lent itself well to to the separation of basic analytes by ion exchange chromatography on unmodified silica gel (chapter 5), it was hoped to attempt similar experiments employing the alumina column in place of the silica column.

6.2 EXPERIMENTAL

6.2.1. Reagents and solvents

The drugs used were received as a gift from the Institute of Clinical Pharmacology, Dublin, Ireland. Potassium dihydrogen phosphate (analytical grade) was obtained from May and Baker, Dagenham, England, and potassium hydroxide and ammonium nitrate (AnalaR grade) from BDH Chemicals Ltd, Poole, England. Orthophosphoric acid, dipotassium hydrogen phosphate and ammonia solution (25%) (all analytical grade) were supplied by Riedel de Haen, Seelze, Hannover, FRG. HPLC-grade methanol was purchased from Labscan Analytical Sciences, Dublin, Ireland. Deionised water was obtained by passing distilled water through a Milli-Q

water purification system. Dried human plasma was obtained from the Blood Transfusion Board, Dublin and dissolved in deionised water. This plasma was then used within seven days of reconstitution.

6.2.2. Drug solutions

Stock solutions equivalent to 1 mg ml^{-1} of the drugs in methanol were prepared. Working stock solutions were prepared in methanol-water (1:1) to concentrations of 1-50 μg/ml according to the detector response of the drug. Standard solutions were made to 50-10000 ng/ml. They were prepared in mobile phase for direct injection and in water for column switching.

6.2.3. Plasma solutions

Aliquots of reconstituted drug-free plasma (1 ml) were spiked with drug solutions (50 μl) to produce the desired concentrations. Plasma blanks were prepared by adding 50 μl of methanol-water (1:1) to 1 ml aliquots of drug-free plasma. These plasma solutions were then diluted (1:1) with water prior to injection of 500 μl loopfuls into the chromatographic system.

6.2.4. Instrumentation and operating conditions

The drugs were separated on a Techsphere alumina 5 μm column (150 mm x 4.6 mm i.d.), supplied by HPLC Technology, Macclesfield, England. The pH of the buffer solutions and the aqueous-organic mixtures were measured at ambient temperature using a standard pH glass electrode. The pH meter was calibrated daily using aqueous standards prepared on a weekly basis.

Stock solutions of ammonia and ammonium nitrate were mixed to produce the required pH (using the pH meter), as were stock solutions of potassium dihydrogen phosphate and dipotassium hydrogen phosphate. Phosphoric acid was added to potassium dihydrogen phosphate to give a solution of pH 3.0, and potassium

hydroxide was added to dipotassium hydrogen phosphate to generate solutions of pH 9.0 and 11.0. All component solutions were 1 M, and the mixtures were diluted with water to give the desired ionic strength. Mobile phases were made by mixing the aqueous component with methanol to produce solutions containing 30-90% organic modifier. The mobile phase was passed through a 0.45μm filter under vacuum and degassed by sonication prior to use. The solutions were stored in glass containers which were capped at all times to prevent the absorption of carbon dioxide from the atmosphere thus raising the pH above the initial value.

The mobile phase was delivered by a Waters (Milford, MA, USA) Model 501 HPLC pump (pump B) at a flow rate of 1.0 ml min^{-1}. The drugs were introduced into the system using a Rheodyne (Cotati, CA, USA) Model 7125 6-port injection valve. The valve was fitted with either a 20 μl or a 500 lμloop. The drugs were detected by UV absorption at 254 nm using a Shimadzu SPD-6A variable wavelength detector with a detector setting of 0.04 AUFS. The resulting chromatograms were recorded with a Linseis (Selb, FRG) recorder at a chart speed of 200 mm hr^{-1}. For direct injection, 20 μl aliquots of the drug solutions in mobile phase were introduced into the chromatographic system. For the purposes of column switching, a second Waters model 501 HPLC pump and the concentration column were connected to the analytical assembly via a Rheodyne Model 7000 six-port switching valve. The 10 x 1.5 mm i.d. concentration column was dry-packed with Corasil (Waters Associates) RP-18 (37-50 μm) packing material. The second pump eluent was deionised water filtered through a 0.45 μm filter and degassed under vacuum. The instrument arrangement and operation of the column switching procedure have been described in chapter 2.

6.2.5. Procedures

6.2.5.1. Effect of pH on retention.

Phosphate buffers of pH 5.0 and pH 7.0 were prepared by mixing 1 M solutions of dipotassium hydrogen phosphate and potassium dihydrogen phosphate. Phosphoric acid (0.1 M) was added to potassium dihydrogen phosphate to give a solution of pH 3.0, and potassium hydroxide (0.1 M) was added to dipotassium hydrogen phosphate to give solutions of pH 9.0 and 11.0. These mixtures were diluted with deionised water to produce 0.04 M solutions. Mobile phases were made by mixing the aqueous phase with methanol (1:4), and the retention times of the drugs were investigated as a function of eluent pH. The pH of the buffer solutions following the addition of methanol was plotted as a function of the initial pH in order to demonstrate the effect of methanol on apparent pH.

6.2.5.2. Effect of ionic strength on retention

Potassium phosphate buffer (1 M, pH 7.0) was diluted to produce solutions of 0.04 M and 0.02 M. Following the preparation of mobile phases containing buffer-methanol (1:4), the retention times of the 31 drugs were measured as a function of ionic strength.

6.2.5.3. Effect of methanol content on retention

A 0.04 M potassium phosphate buffer solution of pH 7.0 was prepared by dilution of a 1 M stock buffer solution. Mobile phases were prepared by adding methanol to produce solutions containing organic-aqueous phase in the ratios 3:7, 5:5, 7:3, 8:2 and 9:1. The retention times of nine representative drugs were measured as a function of the percentage methanol in the mobile phase.

6.2.5.4. Measurement of the zero point charge of alumina in the presence of nitrate ions

Stock solutions containing 1 M ammonium nitrate-ammonium hydroxide at pH 5.5, 6.5, 7.0, 7.5, 8.0, 9.0 and 10.0 were prepared. The retention times of tyramine, which is positively charged over the pH range, and benzoic acid which is negatively charged over this pH range were measured as a function of eluent pH. The zero point charge was determined from the points of inflection of the two curves.

6.2.5.5. Comparison of ammonium nitrate and potassium phosphate buffers

Stock solutions containing 1 M ammonium nitrate and potassium phosphate buffers of pH 7.0 were diluted to produce solutions of 0.04 M. Mobile phases containing aqueous-organic phases in the ratio 1:4 were prepared by the addition of methanol. Drug retention times in the presence of the different buffers were compared.

6.2.5.6. Column switching and plasma experiments

A second pump and concentration column were connected to the analytical column as previously described. The concentration column was packed with RP-18 material and the wash solution was water delivered at a flow rate of 1.5 ml/min for 1 min, after which the concentration column was placed in the mobile phase stream by actuating the switching valve. The mobile phases delivered to the analytical column contained buffer-methanol in the ratio 1:4. The aqueous component was 0.02 M phosphate buffer, pH 5.0; 0.02 M phosphate buffer, pH 8.0; 0.04 M phosphate buffer, pH 8.0; 0.04 M ammonium nitrate buffer, pH 7.0 or pH 9.5. The effect of mobile phase composition on drug-free and spiked plasma profiles following solid-phase extraction was investigated.

6.3. RESULTS AND DISCUSSION

There were thirty one drugs chosen for this study. Several therapeutically important classes of compounds were represented, for instance, the tricyclic antidepressants, major tranquillisers such as the phenothiazines, antihistamines, beta-blocking agents such as propranolol, narcotic analgesics, local anaesthetics and antimalarial drugs. These drugs possess either a primary, secondary or tertiary amine functionality. A very weakly basic drug (nitrazepam) and an acidic drug (frusemide) were also included. Apart from the amine function shared by the other compounds, there were significant structural dissimilarities among some of the drugs, as may be seen from the structures of representative compounds presented in Figure 6.1.

6.3.1. Effect of pH on retention

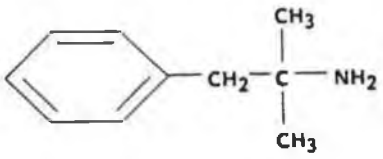
The retentions of the thirty one drugs studied on the alumina column were investigated as a function of pH over the range pH 3.0 to pH 11.0. The mobile phase contained 0.04 M potassium phosphate buffer-methanol (1:4). Additions were not made to compensate for variations in ionic strength from solutions of one pH to the next, as it was assumed that the changes in cation concentration would be small in relation to the changes in solute and column ionisation arising from pH variation.

The results of this study are presented in Table 6.1. From this, it can be seen that the drugs may be roughly divided into three groups; namely (A), those drugs with retention times which increase between pH 3.0 and pH 5.0, followed by a decline in retention at higher pH; (B), those drugs which exhibit decreased retention as the pH is increased; and (C), drugs whose retention times are largely unaffected by pH. These observations may be explained by considering the pK_a of the drugs, the pH of the mobile phase, and the amphoteric nature of the alumina packing. Group(A): Although these compounds are fully protonated at low pH, interaction with the alumina packing is minimal as the

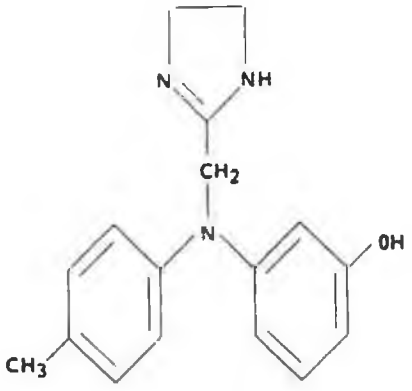
Figure 6.1.

Structures of some drug compounds

Phentermine (I)



Phentoloxamine (II)



Verapamil (III)

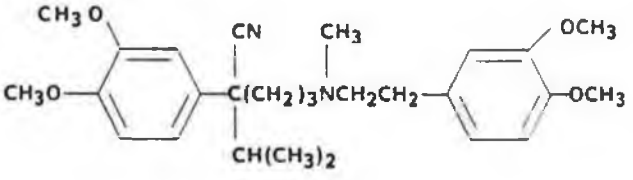


Table 6.1.

Effect of pH on retention

DRUG	pK_a	RETENTION TIME (min)				
		pH3.0	pH5.0	pH7.0	pH9.0	pH11.0
GROUP A						
Amitriptyline	9.5	7.5	9.6	6.0	5.1	4.2
Atenolol	9.6	3.9	5.4	2.7	2.1	2.0
Acetopromazine	NA	6.0	7.6	3.6	3.6	3.0
Chlorpheniramine	8.9	8.7	13.8	7.2	6.6	5.1
Chlorpromazine	9.3	6.2	7.5	3.6	3.0	2.7
Desipramine	10.2	5.4	7.5	7.8	6.6	5.4
Imipramine	9.6	8.1	9.6	6.0	5.1	3.9
Mefloquine	NA	7.5	10.8	5.1	4.5	3.6
Nortriptyline	9.7	5.4	8.7	7.5	6.3	5.4
Phentoloxamine	9.1	6.2	7.8	7.2	3.6	3.0
Propranolol	9.5	3.6	4.5	3.5	3.0	2.7
Pindolol	9.5	3.3	3.6	2.4	2.1	2.1
Promethazine	9.1	7.4	10.5	5.1	3.3	3.0
Protriptyline	10.1	4.8	6.6	6.9	6.3	6.0
Quinine	8.5	4.5	8.2	3.0	2.4	2.1
Tripelennamine	9.0	4.5	5.4	2.7	2.7	2.1
Tyramine	10.2	4.2	9.9	6.6	3.6	3.0

NA = not available in standard reference texts

Mobile phase: 0.04 M phosphate buffer-methanol (1:4)

Table 6.1. continued

DRUG	pK_a	RETENTION TIME (min)				
		pH3.0	pH5.0	pH7.0	pH9.0	pH11.0
GROUP B						
Diltiazem	NA	4.2	3.3	1.8*	1.8*	1.8*
Dextromethorphan	8.3	4.8	4.5	3.0	2.7	2.4
Fluphenazine	8.1	6.1	6.0	2.4	2.4	2.4
Lidocaine	7.9	3.0	2.7	2.4	2.1	2.1
Mepivacaine	7.7	3.0	2.7	2.1	1.8*	1.8*
Perphenazine	7.8	6.9	5.1	3.3	3.0	2.7
Phentermine	10.1	5.4	4.5	3.3	3.0	2.4
Phenylpropanolamine	9.5	7.2	4.8	4.8	2.7	2.4
Trimethoprim	7.2	4.5	2.7	1.8*	1.8*	1.8*
Verapamil	NA	3.0	3.0	2.4	2.4	2.4
GROUP C						
Caffeine	14.0	1.8*	1.8*	1.8*	1.8*	1.8*
Frusemide	3.6	1.8*	1.8*	1.8*	1.8*	1.8*
Nitrazepam	3.2	2.1	2.0	1.8*	1.8*	1.8*
Sulfamethoxazole	5.6	1.8*	1.8*	1.8*	1.8*	1.8*

NA = Not available in standard reference texts

* = drug unretained under these conditions.

Mobile phase: 0.04M phosphate buffer-methanol (1:4)

alumina will also bear a positive charge, thus repelling the like-charged protonated bases. As the pH is increased to 5.0, there was an observed increase in retention consistent with the reduced positive charge on the column. However, as the pH was further increased to 7.0 and above, there was a general decrease in retention. These results may be explained in terms of reduced protonation of the analytes at higher pH, and possibly a shift upwards of the ZPC of alumina, as the changing buffer composition causes a reduction in the negative charge of the column [21]. Furthermore, it is well known that pK_a values of acids and bases vary with the composition of hydro-organic mixtures [23-25], and Laurent et al. [17] have demonstrated an increase in the apparent pH in the presence of organic solvents.

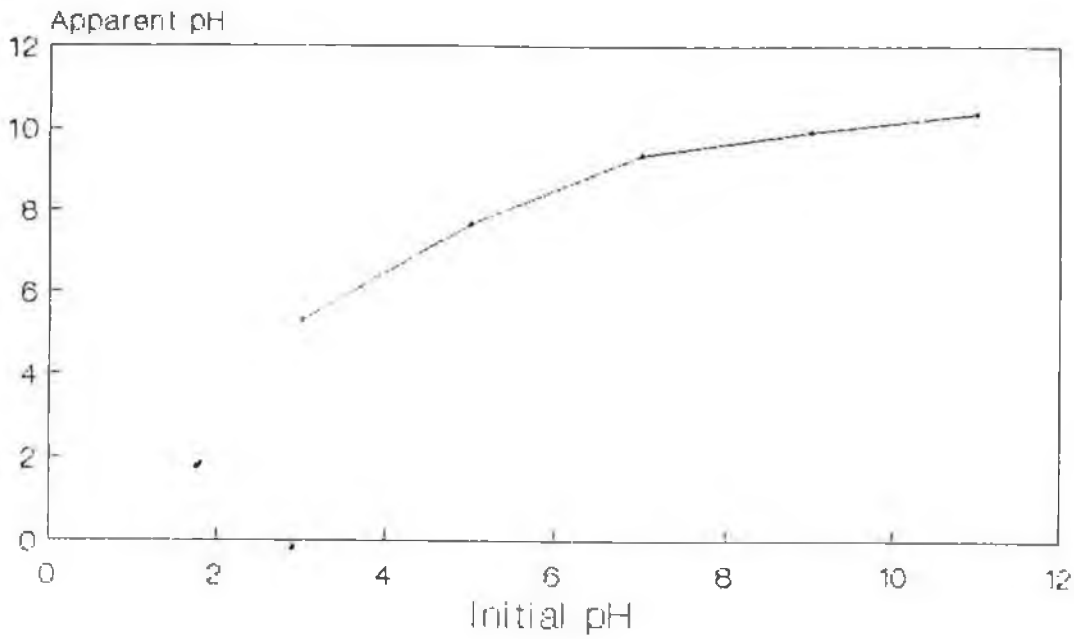
The resulting decrease in retention is exaggerated by a concommitant decrease in solute pK_a. An increase in apparent pH upon addition of 80% methanol to 0.04 M phosphate buffer is illustrated in Figure 6.2, where it can be seen that there is a substantial difference between the initial and apparent pH values of solutions of initial pH 3.0-7.0. This becomes less pronounced for initial pH values of 8.0-10.0, until for a solution of initial pH of 11.0, the addition of methanol has the effect of producing a lower apparent pH value.

Group (B): The drugs in this category exhibit reduced retention times with increasing pH. As they generally have lower pK_a values than those in Group A, it is to expected that these compounds would be less ionised than the more basic amines at any given pH, and that there would be a more pronounced reduction in their retention times under conditions of increasing eluent pH.

Group (C): As expected, solutes which do not ionise, such as caffeine, are unretained by the column in this chromatographic system where the principal mechanisms of solute-column interaction are known to be ionic in nature. Likewise, compounds with low pK_a values, e.g. nitrazepam (pK_a = 3.2), are similarly unretained as they would not be sufficiently ionised to

Figure 6.2.

Effect of the addition of 80% methanol on initial pH of phosphate buffer



Mobile phase: 0.04 M phosphate buffer, pH 7.0-methanol (1:4)

interact with the column at those pH values where alumina behaves as a cation exchanger. The acidic drug, frusemide was also investigated, and was found not to be retained under any pH conditions. Frusemide has a pK_a of 3.6, so that at pH 5.0 the drug should have been ionised. However, this pH is high enough for the column to exhibit cation exchange properties; hence negatively charged solutes would be repelled by the like-charged alumina surface, and therefore be unretained by the column.

6.3.2. Effect of ionic strength on retention

The effect of lowering the ionic strength from 0.04 M to 0.02 M of the potassium phosphate buffer was investigated. The pH of the aqueous component was 7.0 and a 1:4 aqueous to organic ratio in the mobile phase was maintained. The results of this study are presented in Table 6.2, where it can be seen that drug retention increases as the ionic strength is reduced. These observations agree with other workers [4,21] who also found an increase in retention on alumina at lower ionic strengths. These findings are consistent with the ion-exchange theory of retention which explains enhanced solute interaction in terms of decreased competition by the reduced number of competing cations for the charged sites on the column packing.

6.3.3. Effect of methanol content on retention

The effect of varying the methanol-buffer ratio from 9:1 to 3:7 (in a mobile phase where the aqueous component was 0.04 M phosphate buffer, pH 7.0) on the retention of nine of the drugs is shown in Table 6.3. From this it can be seen that as the methanol content is reduced, there is a corresponding increase in drug retention; so much so, that when the methanol content is 50%, or below most of the drugs would appear to be fully retained, not having eluted after 60 minutes or more. These observations differ from those made by Laurent *et al.* [17], and Lingeman *et al.* [21], who found a point of maximum retention as a function of methanol content, which although solute-dependent,

Table 6.2.

Effect of ionic strength on retention

DRUG	RETENTION TIME (min)	
	0.02 M	0.04 M
Amitriptyline	18.3	6.0
Atenolol	3.6	2.7
Acetopromazine	6.3	3.6
Caffeine	1.8*	1.8*
Chlorpheniramine	11.1	7.2
Chlorpromazine	4.5	3.6
Diltiazem	2.1	1.8*
Desipramine	11.7	7.8
Dextromethorphan	3.9	3.0
Fluphenazine	2.7	2.4*
Frusemide	1.8*	1.8*
Imipramine	8.4	6.0
Lidocaine	2.1	2.4
Mefloquine	7.4	5.1
Mepivacaine	2.4	2.1*
Nortriptyline	11.4	7.5
Perphenazine	3.3	3.3
Phentoloxamine	4.5	7.2

* = Drug unretained under these conditions

Mobile phase: phosphate buffer, pH 7.0-methanol (1:4)

Table 6.2. continued

DRUG	RETENTION TIME (min)	
	0.02 M	0.04 M
Phentermine	6.3	3.3
Phenylpropanolamine	4.8	4.8
Propranolol	5.1	3.5
Pindolol	3.0	2.4
Promethazine	6.3	5.1
Protriptyline	10.5	6.9
Nitrazepam	1.8*	1.8*
Quinine	3.0	3.0
Sulphamethoxazole	1.8*	1.8*
Trimethoprim	1.8*	1.8*
Tripelennamine	3.3	2.7
Tyramine	9.0	6.6
Verapamil	2.9	2.4

* = drug unretained under these conditions

Mobile phase: phosphate buffer, pH 7.0-methanol (1:4)

occurred at about buffer-methanol (3:2) for most compounds studied. Laurent et al. [17] attributed low solute retention at high methanol contents to a decrease in solute ionisation, and at low methanol contents to reduced solvation of the competing ions. At any given pH these two mechanisms operate in opposition to one another, as evidenced by the appearance of a retention maximum, with reduced solute ionisation predominating at high methanol contents, and reduced competing ion solvation predominating at low methanol contents.

The observed difference in effect of modifier content between this and previous studies may be accounted for by the nature of the competing ion in the aqueous phase. As Laurent et al. [17] point out, if lithium replaces the tetramethyl ammonium ion as the competing cation, retention times are actually reduced with increasing concentration of acetonitrile. According to the Born model of solvation [26], the lower dielectric constant of organic solvents leads to a positive value for the free energy change when an ion (such as lithium) is transferred from water to an organic solvent. This indicates that the ion is less solvated in the organic solvent. And the less solvated an ion becomes, the greater its ability to compete for sites on the column surface, an effect which causes a reduction in the retention of solute molecules. However, for quaternary alkyl ammonium ions (such as tetramethylammonium hydroxide which was used by Laurent et al. [17]), the free energy change for this transfer was found to be negative, indicating that such ions are actually more solvated in methanol (and especially acetonitrile) than in water, as a result of specific interactions [18,27]. The enhanced solvation of the buffer cation weakens its competition with the solute ions, thus prolonging analyte retention on the column. It is quite possible that in the present study, where the competing ion is the relatively small potassium ion, the degree of buffer ion solvation is reduced as the methanol content is increased, an effect which would act in concert with reduced solute ionisation to minimise the drugs interaction with the stationary phase.

Table 6.3.

Effect of methanol content on retention

DRUG	RETENTION TIME (min) at METHANOL CONTENT (%)				
	30	50	70	80	90
Amitriptyline	-	-	17.1	6.3	3.3
Chlorpromazine	-	-	9.3	3.5	3.0
Dextromethorphan	-	-	4.8	3.0	2.7
Nitrazepam	9.6	3.6	2.1	1.8*	1.8*
Perphenazine	-	-	6.9	3.0	2.4
Pindolol	6.9	3.9	3.0	2.6	2.4
Quinine	-	-	3.9	3.2	2.4
Tripelennamine	-	-	5.4	2.9	2.4
Sulfamethoxazole	1.8*	1.8*	1.8*	1.8*	1.8*

- = Not eluted after 60 minutes

* = drug unretained under these conditions

Mobile phase: 0.04M phosphate buffer, pH 7.0-methanol

The effect on ionic strength of changing the ratio of organic to aqueous phases was not compensated for in this experiment, since it would not appear to be a major contributing factor to the observed shifts in solute retention. If it did significantly affect solute retention, one would expect to observe an increase in retention time at higher methanol contents, as fewer buffer ions are available to compete with analytes for charged sites on the column surface. Adjustment of the ionic strength would be further complicated by accounting for the effect of changing modifier content on the charge of the column, and on the degree of ionisation of the buffer components.

6.3.4. Measurement of the ZPC of alumina in the presence of nitrate ions.

As discussed previously, the nature of the buffer anion determines the ZPC of alumina under a given set of experimental conditions. As the shift in ZPC brought about by the nitrate anion was not available in the literature, it was decided to measure this parameter experimentally. The ZPC was determined in a totally aqueous environment, in spite of the fact that the mobile phase in all other experiments contained methanol in a 4:1 ratio with the aqueous component. This was because previous determinations carried out by other workers [11] were based in aqueous rather than hydro-organic media, and it was sought to compare the results presented here with previous studies.
The chosen pH range was 5.5 to 10.0, and the pH was adjusted using a pH meter.

The anion selected was benzoic acid (pK_a = 4.2) since it is negatively charged over a wide pH range, and besides, it had also been used by Laurent et al. [11] in determining the ZPC in the presence of phosphate, citrate and acetate ions. A number of potential basic test solutes were investigated at various values of mobile phase pH. It was found that some of the larger compounds (for example, desipramine and pindolol) produced broad tailing peaks even at low pH where the cationic character of the

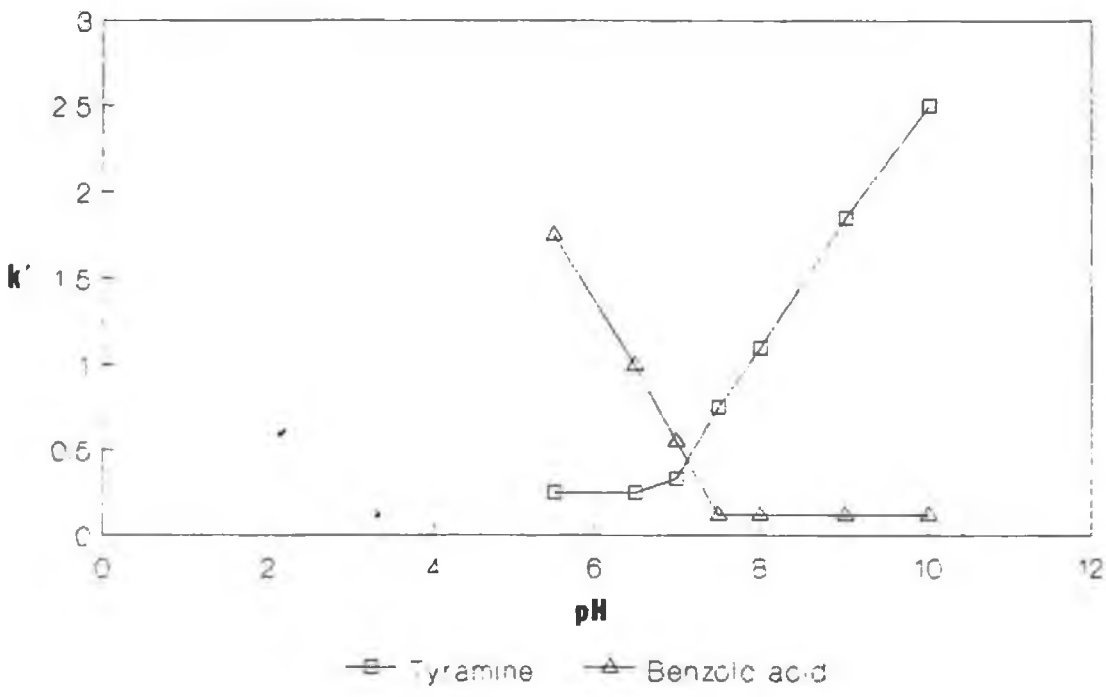
column would be minimal. This observation would indicate that there are possibly factors other than ion exchange processes governing retention on alumina, though these mechanisms have not, as yet been elucidated. The basic compound chosen was tyramine (pK_a = 10.2) since it would be positively charged over a wide pH range. Furthermore, tyramine is a small molecule, and apart from the amino function, is not radically different in structure from benzoic acid. Hence, factors such as molecular size would be less likely to complicate interpretation of the results.

As may be seen from the results presented in Figure 6.3, the negatively charged benzoate ion is strongly retained below a pH valued of approximately 7.0, and unretained at higher pH values, while the opposite is true for the positively charged tyramine. The point at which both compounds are almost completely unretained appears to occur between pH 7.0 and pH 7.6. Hence,the ZPC for alumina in the presence of nitrate ions was taken to be 7.3 ($\pm$ 0.3). Chromatograms depicting the change in elution order between trymine and benzoic acid in the region of the ZPC, in addition to a profile of pindolol are shown in Figure 6.4.

6.3.5. Effect of buffer type on retention

A comparison in terms of drug retention was made between the ammonium nitrate and potassium phosphate buffer systems at constant pH (7.0), buffer-methanol ratio (1:4) and ionic strength (0.04 M). If the difference in drug retention using the phosphate and nitrate buffer systems was considered purely in terms in the difference in ZPC, it would be expected that retention would actually be greater with the phosphate buffer, since it produces a greater acidic shift (ZPC = 6.5) than nitrate (ZPC = 7.3), resulting in greater cationic character for the column at pH 7.0 in the presence of phosphate buffer. However, the results presented in Table 6.4. show that at the stated pH and ionic strength, retention is generally greater in the presence of the ammonium ion than with the potassium ion. These findings agree with other workers [17,21] who found that the larger competing

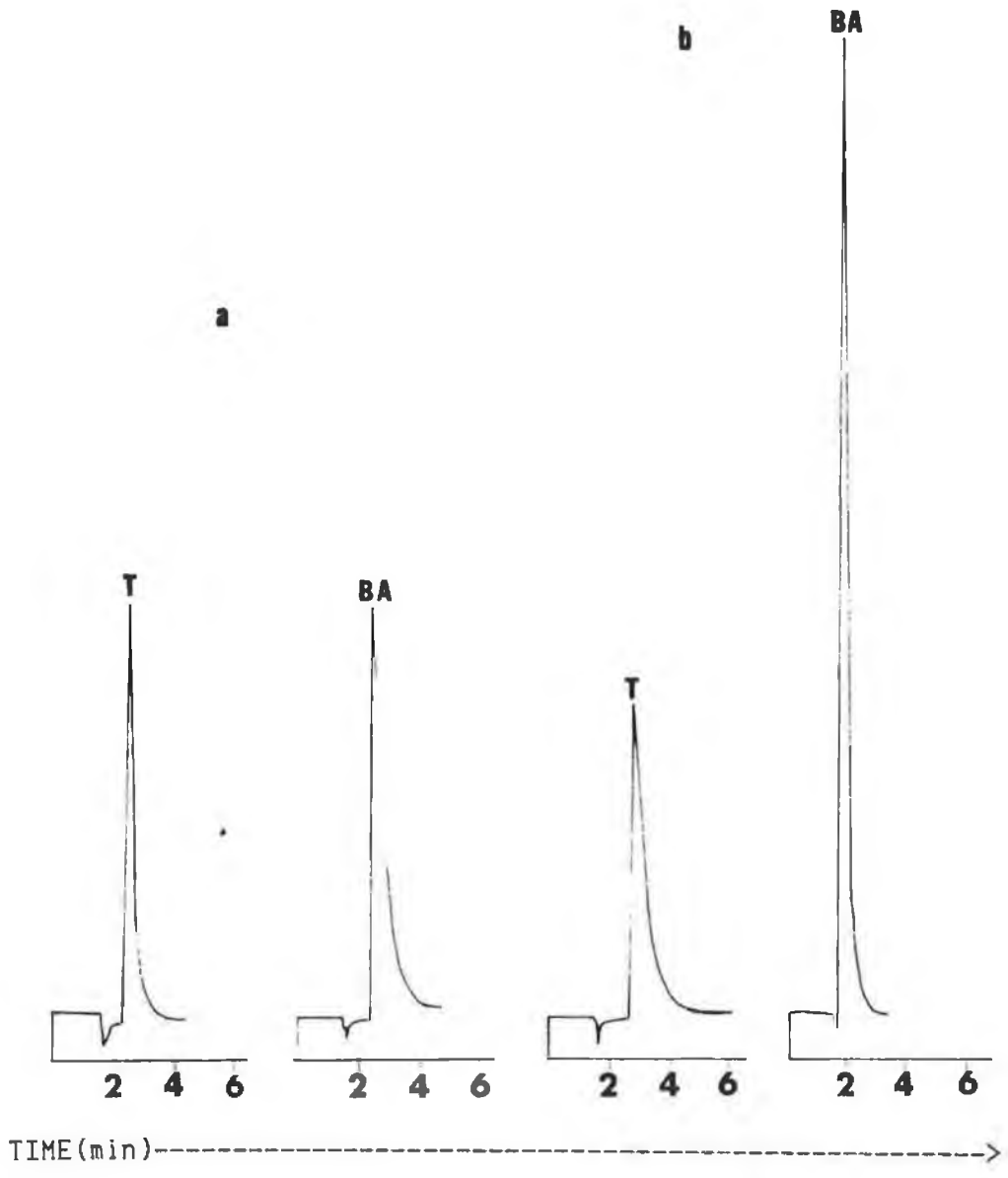
Figure 6.3.
Estimation of the zero point charge of the column using nitrate ions in the mobile phase



Mobile phase: 0.1 M ammonium nitrate/ammonium hydroxide

Figure 6.4.a.

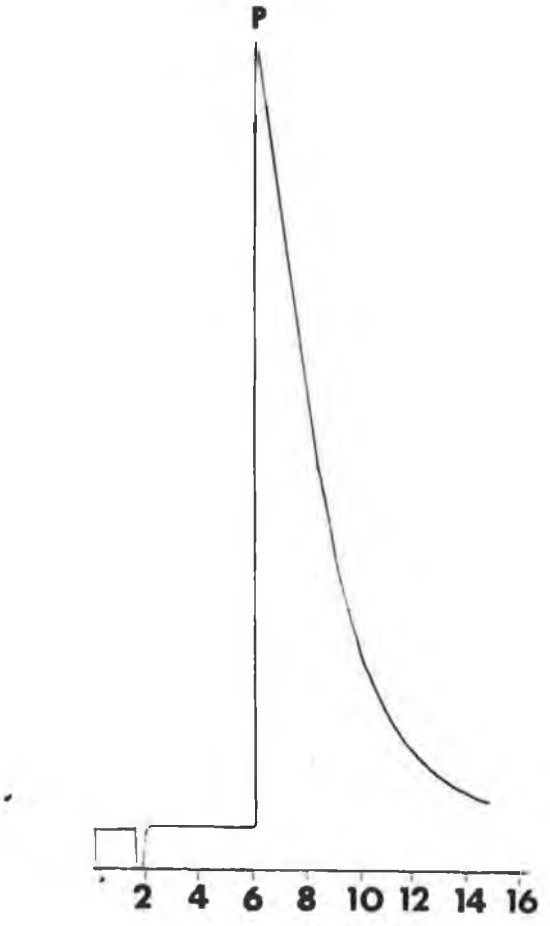
Chromatogram of benzoic acid (BA) and tyramine (T) in 0.1 M ammonium nitrate buffer



(a): mobile phase pH 7.0 (b): mobile phase pH 7.5

Figure 6.4.b.

Chromatogram of pindolol (P)



TIME (min)---------->

Mobile phase: 0.1 M NH_4OH/NH_4NO_3, pH 7.0

Table 6.4.
Effect of buffer type on retention

DRUG	RETENTION TIME (min)	
	POTASSIUM PHOSPHATE	AMMONIUM NITRATE
Acetopromazine	3.6	7.2
Amitriptyline	6.0	8.4
Atenolol	2.7	3.3
Caffeine	1.8*	1.8*
Chlorpheniramine	7.2	11.4
Chlorpromazine	3.6	6.9
Diltiazem	1.8*	3.3
Desipramine	7.8	8.3
Dextromethorphan	3.0	4.2
Fluphenazine	2.4	4.2
Frusemide	1.8*	1.8*
Imipramine	6.0	8.1
Lidocaine	2.4	2.7
Mefloquine	5.1	7.4
Mepivacaine	2.1	3.0
Nortriptyline	7.5	7.6

Mobile phase: 0.04 M buffer, pH 7.0-methanol (1:4)

Table 6.4. continued

DRUG	RETENTION TIME (min)	
	POTASSIUM PHOSPHATE	AMMONIUM NITRATE
Perphenazine	3.3	5.4
Phentoloxamine	7.2	7.5
Phentermine	3.3	3.9
Phenylpropanolamine	4.8	6.3
Propranolol	3.5	3.7
Pindolol	2.4	3.0
Promethazine	5.1	9.6
Protriptyline	6.9	7.0
Nitrazepam	1.8*	1.8*
Quinine	3.0	4.5
Sulphamethoxazole	1.8*	1.8*
Trimethoprim	1.8*	2.5
Tripelennamine	2.7	4.5
Tyramine	6.6	9.0
Verapamil	2.4	3.3

* = drug unretained under these conditions

Mobile phase: 0.04 M buffer, pH 7.0-methanol (1:4)

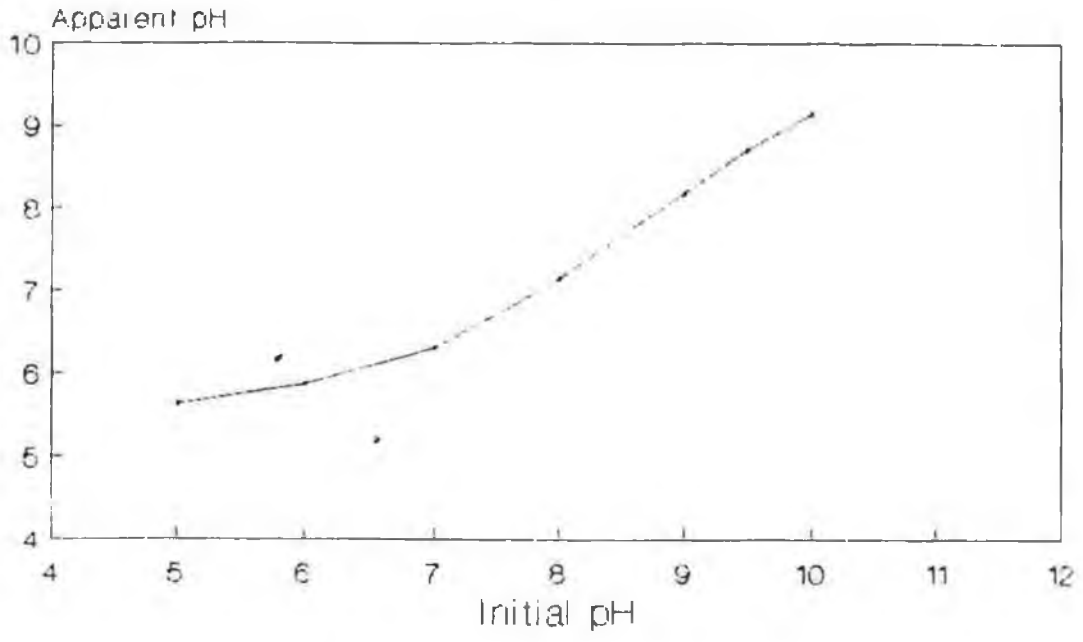
recovery and plasma clean-up. It was found that a C_{18}-concentration column retained less of the endogeous plasma components than the C_8- packing because the former is more hydrophobic and has less affinity for the relatively polar plasma constituents.

In the present study, the concentration column was dry-packed with Corasil C_{18} (25-40 μm) packing material, and the wash solution was filtered deionised water delivered at a flow rate of 1.0 ml/min for 1.5 minutes. Blank and spiked plasma aliquots were prepared as described in the experimental section and 500 μl loopfuls were injected into the chromatographic system.

The objective of this study was to compare drug-free plasma profiles when the pH of the aqueous component was increased from pH 5.0 to pH 8.0, and when the ionic strength was increased from 0.02 M to 0.04 M, in the phosphate buffer system. The nitrate buffers (0.04 M) investigated were pH 7.0 and pH 9.5. In all cases the buffer-methanol ratio was 1:4. The mobile phases studied, along with the resultant chromatograms are presented in Figure 6.6. (phosphate buffer) and Figure 6.7. (nitrate buffer), where it can be seen that a slightly better blank plasma chromatogram is obtained at the higher eluent pH with little difference observed when the ionic strength is doubled. The nitrate buffer system yields a slightly inferior blank plasma chromatogram at pH 7.0 than at pH 9.5, though in all cases, the major peaks continue to elute early.

The effect of pH on the retention of plasma constituents is not as manifest as its effect on the individual drug species. To illustrate this point, a test compound, i.e. fluphenazine, was extracted on-line from spiked plasma prior to chromatography using the phosphate buffer system. As may be seen from the chromatograms presented in Figure 6.8., there is a pronounced shift in the retention time of the drug relative to that of the plasma constituents as the pH is increased from pH 5.0 to pH 8.0.

Figure 6.5.
Effect of the addition of 80% methanol
on the apparent pH of ammonium nitrate buffer



Mobile phase: 0.025 M ammonium nitrate/ammonium hydroxide-methanol (1:4)

recovery and plasma clean-up. It was found that a C_{18}-concentration column retained less of the endogeous plasma components than the C_8- packing because the former is more hydrophobic and has less affinity for the relatively polar plasma constituents.

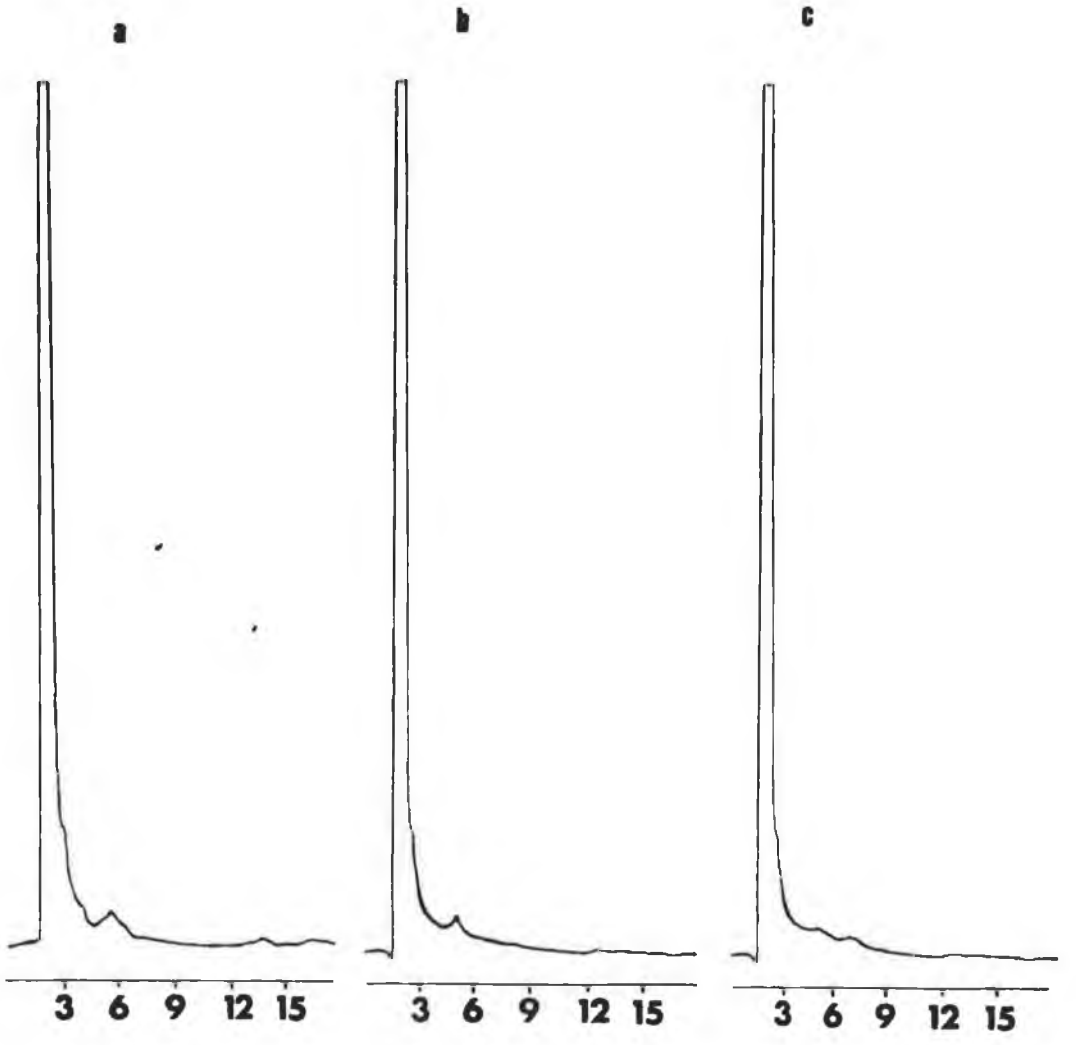
In the present study, the concentration column was dry-packed with Corasil C_{18} (25-40 m) packing material, and the wash solution was filtered deionised water delivered at a flow rate of 1.0 ml/min for 1.5 minutes. Blank and spiked plasma aliquots were prepared as described in the experimental section and 500 l loopfuls were injected into the chromatographic system.

The objective of this study was to compare drug-free plasma profiles when the pH of the aqueous component was increased from pH 5.0 to pH 8.0, and when the ionic strength was increased from 0.02 M to 0.04 M, in the phosphate buffer system. The nitrate buffers (0.04 M) investigated were pH 7.0 and pH 9.5. In all cases the buffer-methanol ratio was 1:4. The mobile phases studied, along with the resultant chromatograms are presented in Figure 6.6. (phosphate buffer) and Figure 6.7. (nitrate buffer), where it can be seen that a slightly better blank plasma chromatogram is obtained at the higher eluent pH with little difference observed when the ionic strength is doubled. The nitrate buffer system yields a slightly inferior blank plasma chromatogram at pH 7.0 than at pH 9.5, though in all cases, the major peaks continue to elute early.

The effect of pH on the retention of plasma constituents is not as manifest as its effect on the individual drug species. To illustrate this point, a test compound, i.e. fluphenazine, was extracted on-line from spiked plasma prior to chromatography using the phosphate buffer system. As may be seen from the chromatograms presented in Figure 6.8., there is a pronounced shift in the retention time of the drug relative to that of the plasma constituents as the pH is increased from pH 5.0 to pH 8.0.

Figure 6.6.

Blank plasma chromatograms: potassium phosphate buffer



TIME(min)---------->

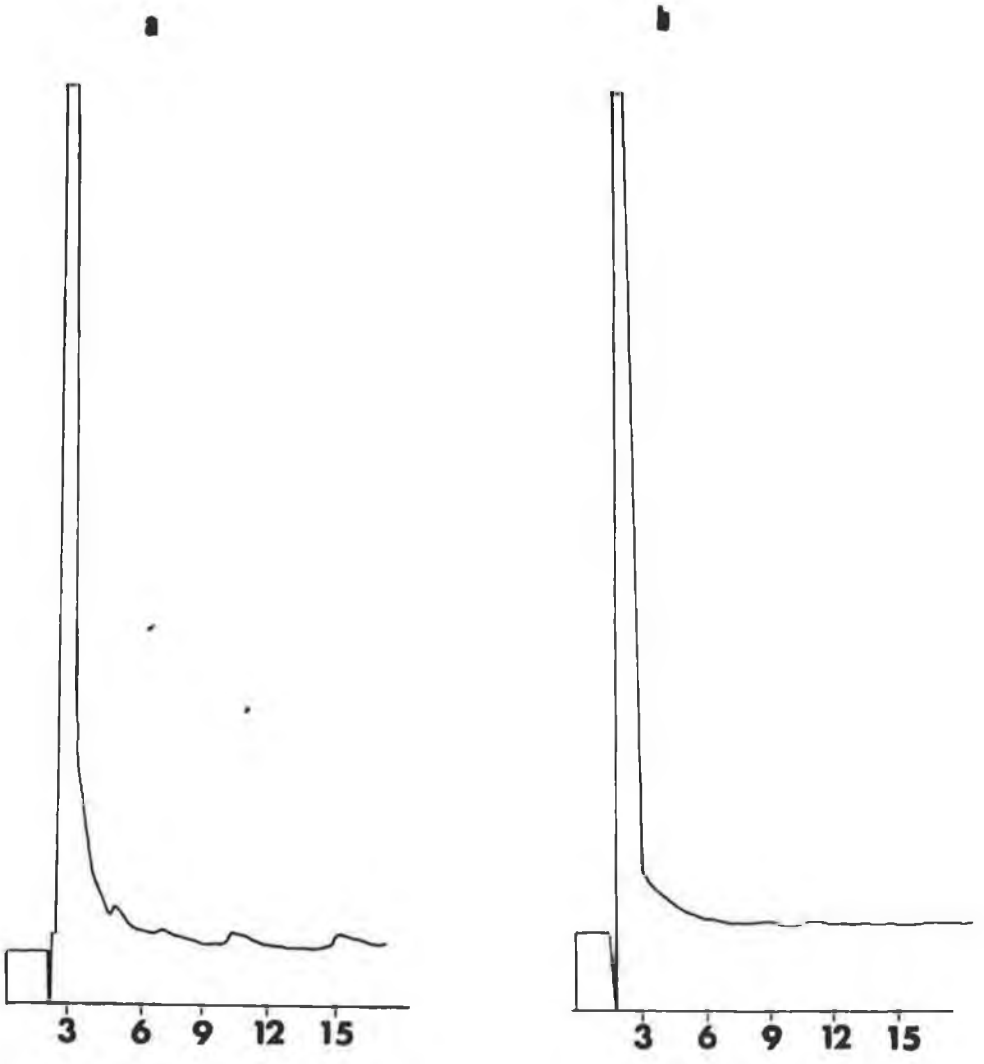
Mobile phases (a): 0.02 M buffer, pH 5.0-methanol (1:4)

(b): 0.02 M buffer, pH 8.0-methanol (1:4)

(c): 0.04 M buffer, pH 8.0-methanol (1:4)

Figure 6.7.

Blank plasma chromatograms: ammonium nitrate buffer



TIME(min)---------->

Mobile phases: (a): 0.04 M buffer, pH 7.0-methanol (1:4)

(b): 0.04 M buffer, pH 9.5-methanol (1:4)

These results demonstrate that it is possible to manipulate the mobile phase to produce the most appropriate retention times for the compounds of interest, with little danger of introducing unacceptable interfering peaks from the plasma matrix. Hence, this type of chromatography would seem to offer an advantage over conventional bonded-phase techniques, in that the plasma constituents are not sufficiently ionised to be retained by the alumina column, and are therefore, rapidly eluted. An important example is caffeine which can interfere with drug analysis in reversed-phase chromatography: because it does not ionise, it will not interact with alumina by the principal mechanism of retention, i.e. ion exchange, and as a result would elute too early to interfere with any of the peaks of interest.

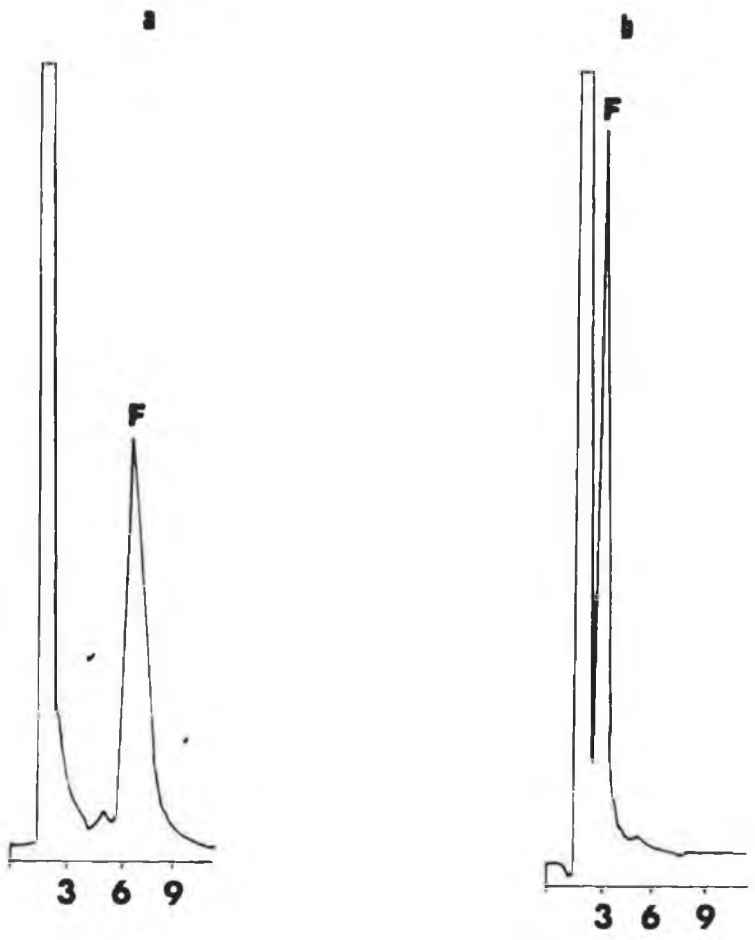
Using a mobile phase containing 0.04 M phosphate buffer, pH 5.0-methanol (1:4), five replicate analyses of plasma containing 200 ng/ml fluphenazine were made. The drug eluted with a mean retention time of 7.0 minutes (standard deviation 0.1 min). The mean drug peak height was 65.4 mm ($\pm$ 2.3 mm) using a detector sensitivity setting of 0.04 AUFS. The coefficient of variation was 3.5%, and 73.1% of the drug was recovered from plasma when compared with authentic aqueous standards injected in the same concentrations as spiked plasma. These results indicate that it should be possible to develop a fully validated bioanalytical method incorporating on-line solid-phase extraction for any drug which can be separated using this chromatographic system.

6.4. CONCLUSION

A preliminary study of the behaviour of drug compounds and plasma extracts on an alumina column has been carried out. Results would indicate that alumina presents an attractive alternative to reversed-phase separations of basic drugs which can present difficulties associated with the presence of unreacted silanol moieties on the silica surface. Alumina has an advantage over silica in that it is more stable at high pH, and thus in the

Figure 6.8.

Spiked plasma chromatograms



TIME (min)---------->

Plasma spiked with fluphenazine (F) 200ng ml^{-1}

(a): Mobile phase: 0.04 M phosphate buffer, pH 5.0-methanol (1:4)

(b): Mobile phase: 0.04 M phosphate buffer, pH 8.0-methanol (1:4)

separation of bases, the cationic character of the column may be maximised without fear of column degeneration. The retention of ionic compounds is strongly influenced by pH, ionic strength, and the percentage methanol in the mobile phase. Any or all of these parameters may be manipulated to produce the desired retention for the compound or compounds of interest. Chromatography on alumina would appear to be well suited to the analysis of drugs in plasma samples as it lends itself to the rapid and convenient technique of solid-phase extraction by column switching.

6.5. REFERENCES

1. L. R. Snyder and J. Kirkland, Introduction to Modern Liquid Chromatography (2nd ed.), (Wiley, New York, 1979).
2. H. Lingeman and W. J. M. Underberg, TrAc, 7 (1988) 346.
3. L. R. Snyder, Principles of Adsorption Chromatography, (Dekker, New York, 1968).
4. C. J. Laurent, H. A. H. Billiet and L. de Galan, Chromatographia, 17 (1983) 253.
5. Z. Suprynowicz, J. Rayss and A. Patrykiejew, Chromatographia, 11 (1978) 742.
6. J. B. Peri and R. B. Hannan, J. Phys. Chem., 64 (1960) 1526.
7. G. M. Schwab, Angew. Chem., 50 (1937) 646.
8. E. J. W. Verwey, Rec. Trav. Chim. Pays-Bas, 6 (1941) 625.
9. Z. D. Koz'mina, M. P. Belova and V. A. Sannikov, Kolloidn Kh., 25 (1963) 169.
10. R. Fricke and I. Leonhart, Naturwissenschaften, 37 (1950) 428.
11. C. J. Laurent, H. A. H. Billiet, L. De Galan, F. A. Buytenhuys and F. P. B. Van Der Maeden, J. Chromatogr., 287 (1984) 45.
12. H. A. Abrahamson, L. S. Moyer and M. H. Gorin, Electrophoresis of Proteins and the Chemistry of Cell Surfaces, (Reinhold, New York, 1942).
13. A. Clearfield, Inorg. Chem., 3 (1964) 146.
14. G. A. Parks, Chem. Rev., 65 (1965) 177.
15. J. H. Knox and A. Pryde, J. Chromatogr., 112 (1975) 171.
16. M. Novonty and Y. Hirata, Anal. Chem., 51 (1979) 1807.
17. C. J. Laurent, H. A. H. Billiet and L. De Galan, Chromatographia, 17 (1983) 394.
18. R. L. Kay, D. F. Evans and S. M. A. Matesich in J. F. Coetzee and C. D. Ritchie (Eds.), Solute Solvent Interaction, Vol. 2 (Dekker, New York, 1976).
19. P. J. Schoenmakers, H. A. H. Billiet, L. de Galan, J. Chromatogr., 205 (1981) 13.

20. C. J. Laurent, H. A. H. Billiet and L. De Galan, J. Chromatogr., 285 (1984) 161.
21. H. Lingeman, H. A. Van Munster, J. H. Beynen, W. J. M. Underberg and A. Hulshoff, J. Chromatogr., 352 (1986) 261.
22. U. K. Underberg-Chitoe, W. J. M. Underberg and H. Lingeman, Anal. Sci., 4 (1988) 91.
23. D. B. Rorabacher, W. J. MacKellar, F. R. Shu and S. M. Bonavita, Anal. Chem., 43 (1971) 562.
24. R. G. Bates, in Determination of pH: Theory and Practice, (Wiley Interscience, New York, 1973).
25. J. L. Van den Venne, J. L. H. Hendrikz and R. S. Deelder, J. Chromatogr., 167 (1978) 1.
26. M. Born, Z. Physik, 1 (1920) 45.
27. M. Scwarc, in Ions and Ion Pairs in Organic Reactions, Vol. 1 (Wiley Interscience, New York, 1972.)
28. C. T. Hung and R. B. Taylor, J. Chromatogr., 202 (1980) 333.
29. E. Papp and Gy. Vigh, J. Chromatogr., 259 (1983) 49.

CHAPTER 7

EVALUATION OF DRUG-FREE PLASMA PROFILES

WITH VARYING ELUENT COMPOSITION

FOLLOWING ON-LINE SOLID-PHASE EXTRACTION

7.1. INTRODUCTION

7.1.1. Characterisation of stationary phases

The most widely used column packings for modern liquid chromatography are those with surface reacted (chemically bonded) organic stationary phases. Bonded-phase columns were originally developed to eliminate the disadvantages of liquid-liquid chromatography (LLC) based on mechanically held supports. They are generally more stable than the latter because the stationary phase is not easily removed or lost during use. The availability of a wide variety of functional groups in bonded-phase packings means that both normal and reversed-phase chromatography can be carried out in a relatively simple straightforward manner.

The most popular reversed-phase columns contain porous silica particles surface bonded with C_{18}- or C_8- alkyl chains. The difference in chromatographic properties between these packings is subtle rather than major, and probably most, if not all, separations can be achieved on either column [1]. Any apparent differences in selectivity between commercial C_{18}- and C_8- phases are probably overshadowed by selectivity differences among columns from different manufacturers [2]. Selectivity differences are less likely between these two packing materials and other, more polar, bonded phases, such as cyano- or phenyl- columns which can also be used in the reversed-phase mode if adequate water is incorporated into the mobile phase. For example, the cyano-bonded phase provides good selectivity for separating tricyclic antidepressants [3-5]. The present study is based on the employment of C_{18}- and CN columns.

Several workers have investigated the mechanisms involved in reversed-phase chromatography [6-8], though application of the proposed mechanisms to real situations is often very difficult. This difficulty can be traced to the fact that, as mentioned above, two columns generically labelled as equivalent, may have vastly different chromatographic characteristics. Description of

a column purely in terms of the alkyl substituent bonded onto the silica support does not adequately describe the chemistry of a packing, and can, in fact provide misleading information regarding the retentivity and selectivity of the resulting chromatographic column. The chromatographic differences in similarly labelled columns are due both to variations in the silica material used as a support and the technique used to form the bonded phase. Since there are no agreed specifications regarding the critical properties of these packings, alternative methods for column classification are essential.

A range of techniques have been used to compare different bonded phases, including pyrolysis-GLC [9], but most methods are based on the reversed-phase separation of a test mixture. The capacity factors (k') are then used for manufacturers' quality control [10-12] or for comparison of different materials [2,13], though many of these tests can produce misleading results [14].

An exhaustive study on retentivity and selectivity involving numerous commercially available packings was undertaken by Goldberg [2]. Characterisation of the packing materials was based both on non-polar hydrocarbons and polar solutes, as the latter are often more revealing in the critical information they yield concerning reversed-phase packings. The expectation that C_{18}- columns are more retentive than C_8- packings, and that the latter are, in turn, more retentive than packing materials bound with shorter alkyl chains, was not realised in a number of cases, even where the test solutes used were the highly non-polar solutes anthracene and naphthalene. Furthermore, there were some instances where one brand of cyano column exhibited stronger retentivity for low- and medium- polarity solutes than other brands of column packed with C_8- material.

More fundamental studies have used nitrobenzene in hexane to test for unreacted silanols [15], or have determined the physical parameters of the column such as porosity [16]. Most of these and the above-mentioned retention studies are based on the

measurement of capacity factors, but there has been a realisation in recent years that accurate determination of the void volume is difficult [17-19], thus generating considerable errors in reported k' values for compounds of short retention times. An alternative method for reporting retentions has been proposed which uses the alkyl aryl ketones to form a retention index scale [20]. Smith used this retention index scale in the determination of differences between eight nominally similar octadecyl columns under varying conditions of eluent composition [14]. Detailed examination of the retention indices showed that the values for each of the six reference standards investigated were more consistent than capacity factor values and that changing the eluent produced systematic changes in index values. Using these indices, Smith was able to demonstrate significant differences in the retention characteristics among the test columns. He also showed that for a given elution strength, the magnitude of these differences depended on the nature of the organic modifier in the mobile phase.

The role of the column in reversed-phase chromatography has been extensively reviewed [21-24], and there is abundant data in the literature on the in-house preparation and evaluation of packing materials which are tailored by the experimentors to suit their individual needs. The range of packing materials which have been produced is extremely large and it is beyond the scope of the present study to discuss them in detail.

7.1.2. Mobile phase considerations

In reversed-phase chromatography, water is usually used as the base solvent with varying proportions of miscible organics added in order to adjust the solvent strength. Methanol is the most commonly used organic solvent since it is relatively cheap and non-toxic. Acetonitrile and tetrahydrofuran are the next most commonly used solvents, in that order. Changing selectivity in reversed-phase chromatography is less easily accomplished than in normal phase separations because water dominates the sample

mobile phase-stationary phase interaction. However, in some cases, significant changes in separation selectivity can be achieved by switching between one organic solvent and another.

Methanol appears not to alter the selectivity of water until very high concentrations are used [25], and although it is less expensive than acetonitrile, it is more polar than the latter and more of it is required to provide equivalent solvent strength, which decreases the cost differential. Acetonitrile also yields a mobile phase with water that has a lower viscosity which makes the column more efficient, and provides a lower pumping pressure. Tetrahydrofuran can offer further contrasting solubility properties (it is less polar than acetonitrile and is nonhydroxylic relative to methanol) and might be considered when attempts to use methanol and acetonitrile are unsuccessful. Studies have shown that ternary aqueous mobile phases (for instance, methanol-dioxane-water) can provide unique selectivity for certain separations [26-28]. Drug separation can be significantly affected by even a small proportion of a second organic modifier, especially if the second solvent is a strong eluent relative to the mobile phase being modified. This is because such a solvent tends to concentrate in, and change the characteristics of the bonded phase [27].

The pH range within which bonded-phase silica-based packings can be used is typically about 2.0-7.0. At lower pH values, the bonded groups are hydrolysed. At higher pH values the silica matrix itself dissolves leading to a release of bonded groups from the surface. However, as discussed in detail in chapter 5, the stability of silica in alkaline media may be considerably enhanced by the incorporation of a pre-column between the pump and the injector, by using a high proportion of organic component and by employing ammonia as the source of hydroxyl ions. Changes in pH can alter the separation selectivity for ionised solutes since charged molecules are preferentially distributed into the more polar phase. Variations in pH are normally not effective in obtaining desired changes in selectivity with solutes which do

not ionise. The aqueous component of the mobile phase frequently contains some kind of buffering salt which principally serves to control eluent pH and so provide for reproducible chromatography. As discussed in chapter 5, changes in ionic strength elicit a profound effect on the solute retention where the mechanisms of retention mainly involve ion exchange. Retention and selectivity in reversed-phase chromatograhy can also be influenced by ionic strength. For instance, the concentration of phosphate in the mobile phase has been shown to significantly influence not only k' values for a series of nonapeptides, but the selectivity of a particular solute pair [29]. In another study, non-linear increases in retention for aromatic acids and bases were reported to occur with increasing salt concentration [30]. Snyder proposed that when the addition of salt increases the solubility of the solute in the mobile phase (salting-in effect), retention is decreased, and that when the addition of a salt decreases the solubility of the solute in the mobile phase (salting-out-effect), retention time is reduced [31].

The role of buffer cations in the retention of aromatic amines in reversed-phase chromatography has been extensively investigated by Papp and Vigh [32,33]. Using a C_{18}- column and an eluent containing 94%, methanol they studied the effect of varying the concentration of a series of commonly used salts on the retention characteristics of a series of aromatic amines. They found that at constant pH, the $\log_{10}$ of the capacity factor decreased linearily with the $\log_{10}$ of the cation concentation, and that the retention-decreasing effect of the cation became stronger in the order of hydrogen, sodium and potassium ions, irrespective of the nature of the amine solute. At constant buffer ion concentration the $\log_{10}$ k' vs pH curves passed through a maximum. They attributed these phenomena to ion exchange between dissociated (residual) silanol groups on the silica packing and oppositely charged protonated bases. Retention of amines would therefore be affected by pH which controls the degree of solute

and silanol ionisation, and by ionic strength which determines the competition between the solute and buffer ions for charged sites on the column. Papp and Vigh [33] and other workers [34,35] further addressed this subject by studying amine retention as a function of pH and salt concentration in the presence of both high and low methanol concentrations. The fact that under certain conditions of eluent composition, amine retention passed through a minimum as a function of modifier concentration was attributed to a dual hydrophobic-silanophilic retention mechanism of retention.

Papp and Vigh [32,33] proposed that for amines on reversed-phase silicas, the silanophilic contribution to retention is large over the entire methanol concentration range, and that it is diminished by the addition of high concentrations of inorganic and organic cations. They further suggested that retention on reversed-phase column of unionised, polar solutes was also dependent on on the type and concentration of the cation in the eluent, and that attention should be paid to these effects in the design and characterisation of a reversed-phase liquid chromatographic system.

7.1.3. Chemometrics in pharmaceutical analysis

Chemometrics is a science where chemistry and pharmaceutical science meet statistics and software development. The primary focus of chemometrics involves the use of mathematical or computational procedures in particular, both to optimise analytical methods in the first place, and to analyse the signals and results obtained. The measurement process may be divided into several steps; first the problem must be specified, then a suitable analytical methodology must be selected and optimised before the chemical determination can be made. Once the raw data has been obtained, it must be treated, using some kind of statistical procedure, followed by interpretation and validation.

The first step, i.e., the decision as to which analytical method should be used, (for instance in chromatography, which chromatographic mode would be most appropriate) has only lately been tackled by chemometrics [36]. Recent breakthoughs in the accessibility of artificial intelligence techniques have made it possible to apply expert system technology to incorporate expertise-related knowledge into computer packages [37-40].

An expert system is an example of an intelligent knowledge-based system which contains encoded expert knowledge and is capable of exploiting this knowledge for problem solving purposes. The information stored in a knowledge base may be derived from a number of sources. Normally, however, these sources fall into two classes, public theoretical knowledge and private experimental knowledge. The public knowledge of a problem domain is that knowledge which is available from books or journal articles. The private knowledge is that accumulated by a human expert and is a combination of the basic theoretical knowledge and a number of empirically derived facts.

Expert systems for method selection in HPLC [38] and for method development [39,40] have been described. Recently, Fell et al. reported on the design of a new expert system for method selection in high performance liquid chromatography [41]. Massart and Buydens [37] have also proposed an expert system for method selection. Its overall strategy is based on certain observations, such as the fact that all drug determinations can readily be carried out on a single stationary phase, the cyano bonded phase, which, as mentioned earlier, is suitable for both normal and reversed-phase separations.

The next step, selection of the optimal mobile phase composition in high performance liquid chromatography is a problem which may be approached in a number of ways. One common approach is the intuitive method wherein the chromatographer makes an initial selection of the mobile phase composition based on the nature of the solutes to be separated, and then refines this selection on a

trial and error basis. This method often fails when complex samples are encountered, or when the nature of the solutes in the samples is unknown. An alternative, and very successful approach is a systematic search over a wide range of solvent compositions with the aid of predicted or extrapolated retention data and a subsequent assessment of the quality of resultant chromatograms by a mathematical optimisation criterion.

Chemometric techniques are widely used in the optimisation of mobile phase composition for HPLC. One method which has found particular use in chromatography is entitled "window diagrams" [42]. The first application of window diagrams to chromatography were for the selection of stationary phases in gas chromatography [43], though, being a general method, it can also be applied to HPLC [44]. Another optimisation strategy is based on the solvent selectivity triangle approach which was conceived by Snyder [45], and further developed by Glajch [46]. This classification has been translated into two solvent selectivity triangles, one for normal phase chromatography and one for reversed-phase chromatography. By plotting the resolution between each set of peak pairs, it is possible to build an overlapping resolution (ORM) map which will define the mobile phase composition which will provide optimum resolution.

A widely used optimisation method is the Sequential Simplex procedure [47,48]. This is a general multifactor optimisation method in which all factors (variables) are varied as the experimental design seeks to progress sequentially towards the optimum region of the selected optimisation criterion. The advantages of using the Simplex procedure are that calculations are relatively simple, the rules of the procedure are precicely defined, and the technique is ideally suited to automated optimisation [48]. The disadvantages of the Simplex optimisation procedure stems from the need to use a chromatographic response function in order to evaluate the qualities of the separations achieved and to guide the procedure towards the optimum. With the advent of microprocessor-controlled chromatographs, Simplex

procedures have been used to guide the automated optimisation of normal-phase separations [49] and a variety of parameters in reversed-phase chromatography [50,51]. Many optimisation methods have been described by Berridge [52] and Schoenmakers [53], and the literature contains a number of reviews on optimisation procedures which employ the Simplex method in the original or highly evolved forms which have been developed since it was first introduced.

Important topics in chemometrics are method development and quality assurance. Much of the validation is to do with accuracy and precision and use well established statistical procedures. Chemometricians are interested in developing methods to do this efficiently.

A different kind of problem is created by the surfeit of information generated by modern chromatographic or spectroscopic methods. Pattern recognition may play a role in digesting this large amount of information. One consequence of automation is that many variables can be determined simultaneously for the same sample. Pattern recognition methods use the available data simultaneously rather than sequentially. The set of measurements which is used to characterise a sample is called a pattern. When only two variables are measured for each sample, the pattern may be represented graphically by a single point with two co-ordinates (x, y). The point may also be defined by a vector, drawn to it from the origin (a "data vector"); the co-ordinate system is known as the "pattern space" [54]. The basis of all pattern recognition methods is that pattern vectors for similar samples lie close together in the pattern space forming clusters. In two dimensions, this clustering can readily be detected by the human eye. However, when more than two variables are measured, graphical representation is no longer possible: if n variables are measured, each sample will be represented by a point in n-dimensional space, and mathematical methods are needed to detect clustering. One such method, known as principal component analysis, allows the pattern vectors to be projected onto a plane

in such as way that as little information as possible is lost. In many cases, only some of the original variables figure significantly in these linear combinations, and thus variables which contribute most to the variation between pattern vectors may be recognised. The remaining variables, which convey little extra information, can be rejected. If too many variables are used, a random and chemically meaningless separation into classes can result, or the "noise" from the superfluous classes may obscure the actual existence of classes.

7.1.4. Introduction to the present study

As outlined in sections 7.1.1. and 7.1.2., much effort has been devoted to the characterisation of columns and mobile phases in terms of their ability to separate various types of solutes which are both polar and non-polar, ionic and non-ionic in nature. Much data has been presented regarding the efficacy of various extraction schemes designed to effect removal of drugs from plasma, serum or urine, but this information is usually generated only in relation to the separation of the compounds of interest to the experimentor presenting the results. To date there has not been a systematic study undertaken on the characterisation of stationary phases purely in regard to the separation of extracted plasma components. The closest approximation to a comprehensive study in this area was an investigation by Blanchard [55] into the relative merits of different methods of protein precipitation which would liberate drugs from binding sites and render the plasma suitable for direct injection onto a chromatographic column.

The objective of the present study was to investigate drug-free plasma profiles on two different analytical columns (C_{18}- and CN-) under a variety of mobile phase compositions. The variables in the eluent were the type (methanol or acetonitrile) and percentage of organic modifier, and the pH and ionic strength of the aqueous component. The plasma which was used was pooled at the outset so that this would not provide a source of variation

during the course of the study, and it was extracted using the on-line column switching technique in order to permit rapid analysis and to minimise the formation of artefacts. The plasma profiles were evaluated in terms of the number of interfering peaks in the area of analytical interest along the chromatogram, and these data were incorporated in a factorial design; the factors in each case being the column, the type of organic modifier and either the percent organic modifier, pH or the ionic strength as the third factor. It was also sought to determine the type of pattern (if any) which was obtained when one or more of the operational variables were altered, and so construct a kind of "mini-library" which would be useful when optimising an analytical method for the analysis of drugs in plasma or serum.

7.2. EXPERIMENTAL

7.2.1. Reagents and solvents

Methanol and acetonitrile (HPLC grade) were obtained from Labscan Analytical Sciences, Dublin, Ireland. AnalaR grade sodium acetate and analytical grade acetic acid were supplied by BDH Chemicals, Poole, UK, and Riedel de Haen, Hannover, West Germany, respectively. The drugs and a number of batches of frozen plasma were received as a gift from the Institute of Clinical Pharmacology, Dublin Ireland. Deionised water was obtained by passing freshly distilled water through the Millipore Milli-Q water purification system.

7.2.2. Drug and plasma solutions

Stock solutions equivalent to 1 mg/ml of the drugs in methanol were prepared. Working stock solutions were prepared in methanol-water (1:1), and these were diluted with mobile phase immediately prior to injection. Small pools of mixed batches of frozen drug-free plasma were thawed and then pooled to produce a single batch. The plasma was divided into 10 ml aliquots and placed into deep freeze storage. When required for use, one of these fractions was thawed, vortexed, and diluted 1:1 with deionised water to generate "blank" plasma solutions.

7.2.3. Instrumentation and operating conditions

Drug-free plasma and authentic drug standards were separated on a Spherisorb (Phase Separations, Clywd, UK) C_{18}- (10 μm) and a Spherisorb CN- (10 μm) column (both 250 mm x 4.6 mm i.d.). They were protected by a Chrompak (Middelburg, The Netherlands) module containing a 10 x 1.5 mm stainless steel column dry-packed with Corasil (Waters Associates, Milford, MA, USA) C_{18}- (37-50 μm) or Supelco (Bellefonte, PA, USA) CN- (24-40 μm) material.

A stock solution of 1 M sodium acetate was prepared by dissolving the appropriate amount of substance in deionised water. The pH of this solution was then adjusted by the addition of 0.1 M acetic acid. The desired ionic strength was obtained by dilution with deionised water. Mobile phases were made by mixing the aqueous component with the required amount of methanol or acetonitrile. The mobile phase was passed through a 0.45 μm filter, and degassed by sonication prior to use.

The mobile phase was delivered by a Waters Model 501 HPLC pump (pump B) at a flow rate of 1.0 ml/min. The plasma was introduced into the system using a Rheodyne (Cotati, CA, USA) Model 7125 six-port injection valve. The injector was fitted with a 20 μl loop to permit direct injection of authentic standards of selected drug compounds.

For the purposes of column switching, the injector was fitted with a 500 μl loop and a second pump (pump A) and the concentration column were connected to the analytical assembly via a Rheodyne Model 7000 six-port switching valve. The 10 x 1.5 mm i.d. concentration columns were packed with either Corasil C_{18}- or Sepralyte (Analytichem International, Harbour City, CA, USA) C_8- material. The washing eluent delivered by pump A to the concentration column was deionised water which had been filtered through a 0.45μ m membrane and degassed by sonication. It was delivered at a flow rate of 1.0 ml/min for 90 seconds. The pH of the aqueous component of the mobile phase was adjusted using a standard glass electrode which was calibrated daily using aqueous standards pepared on a weekly basis. Detection was by ultraviolet absorption at 254 nm using a Shimadzu (Kyoto, Japan) Model SPD 6A spectrophotometric detector with a sensitivity setting of 0.01 AUFS. The resultant chromatograms were recorded by a Linseis (Selb, West Germany) recorder at a chart speed of 200 mm/min.

7.2.4. Procedures

The effect of various parameters (including mobile phase composition and type of anticoagulant) on blank plasma profiles were studied. Blank plasma chromatograms were generated on two different analytical columns (C_{18}- and CN-) following on-line solid-phase extraction using the column switching technique described in chapter 2. 500 μl quantities of plasma wereinjected onto the concentration column, and except where a comparison was made between C_{18}- and C_8- materials in the concentration colums, all solid-phase extractions were carried out on Corasil C_{18}- packing.

A fresh concentration column was used for each different mobile phase on both columns, and a new 10 ml aliquot of frozen plasma was thawed and prepared in each case. The blank plasma was injected in quadruplicate for each set of experimental parameters, and the second, third and fourth injections were used in the analyses. The plasma profiles were assessed in terms of the number of peaks greater than 2 mm occurring in the chromatogram in the time frame 5 minutes to 20 minutes after injection. This is the principal area of interest in terms of drug analysis. The results obtained were subjected to statistical analysis as described in section 7.3.

7.2.4.1. Effect of type and percentage organic modifier on blank plasma profiles on both columns

The pH of the sodium acetate stock solution was adjusted to pH 6.0 by the addition of acetic acid. It was diluted to 0.025 M by the addition of deionised water. Mobile phases were made by mixing the aqueous component with methanol in the ratio 3:7, 4:6, 5:5, 6:4, 7:3 or 8:2 aqueous to organic phase. This procedure was repeated for acetonitrile and the 12 eluents were run on both the C_{18}- and the CN- columns.

7.2.4.2. Effect of pH on blank plasma profiles on both columns

Aliquots of the 1 M stock solution of sodium acetate were adjusted to pH 7.0, 6.0, 5.0, 4.0 and 3.0 by the addition of acetic acid. These were then diluted to 0.025 M with deionised water. Each of the resulting solutions were added to methanol to give mixtures containing aqueous to organic phases in a ratio 1:1. This procedure was repeated for acetonitrile and the 10 eluents were run on both columns.

7.2.4.3. Effect of ionic strength on blank plasma profiles on both columns

The pH of the 1 M stock solution of sodium acetate was adjusted to 6.0 by the addition of acetic acid. This was then diluted with deionised water to generate solutions of 0.1, 0.025, 0.01 and 0.005 M. Each of these solutions was added to methanol to give mixtures containing aqueous to organic phases in a ratio 1:1. This procedure was repeated for acetonitrile and the 8 eluents were run on both columns.

7.2.4.4. Concentration column reproducibility on a between-day and within-day basis

Reproducibility was tested on the C_{18}- analytical column only. The mobile phase was 0.025 M acetate buffer, pH 6.0-acetonitrile (1:1). Injections were made on three different C_{18}- concentration columns on three successive days (nine concentration columns in total). Column-to-columm reproducibility was measured on a within- and between-day basis.

7.2.4.5. Effect of type of anticoagulant on plasma profiles

Blood from a healthy volunteer was taken into evacuated glass tubes containing lithium heparin, fluoride oxalate, sodium

citrate (two brands) or disodium edetate as anticoagulants. The blood was centrifuged immediately and the maximal volume of plasma recovered. Serum from the same volunteer was obtained by taking blood into a tube with no anticoagulant, and after allowing 30 min for the clotting process to occur, serum was separated by centrifugation. The plasma and serum samples were frozen until required for use, whereupon they were treated in the same manner as the frozen plasma in foregoing experiments. They were separated on the C_{18}- column with a mobile phase of 0.025 M acetate buffer, pH 6.0-acetonitrile (1:1), and compared in the same way as blank plasmas in the previous experiments.

7.2.4.6. Comparison of C_{18}- and C_8 concentration columns in terms of plasma removal

The concentration column was packed with Corasil C_{18}- material as has been used above, or with Sepralyte C_8- material. The wash solution was as before, and the analytical mobile phase was 0.025 M acetate buffer-acetonitrile (1:1). Analysis was performed on the C_{18} column. The two column were compared in terms of the amount of plasma recovered.

7.2.4.7. Comparison of the C_{18}- and CN- columns in terms of drug retention

The working stock solutions of drugs were diluted with mobile phase (0.025 M acetate buffer, pH 6.0-acetonitrile (1:1)), and 20 µl aliquots introduced by direct injection onto both analytical columns. Drug retention on both columns was measured and compared.

7.3. RESULTS AND DISCUSSION

7.3.1. Selection of variables

The principal objective of this study was to examine drug-free plasma on two columns under a range of varying eluent compositions. Having selected the biological matrix which would be tested, the next step was to decide what columns to use. As outlined in the Introduction to this chapter, it would be quite possible to select two generically equivalent columns from different manufacturers and obtain completely different results from them. Such an exercise would, however, be of little value in this study since it was sought to characterise the differences in retention behaviour between different types, rather than different brands of columns.

Due to time and other constraints, the project was limited to the investigation of two columns. A C_{18}- column was chosen because it is the most widely used stationary phase in biopharmaceutical, and many other types of separations. C_8- was rejected as the second column since, as mentioned by Geise [1], the difference in chromatographic properties between C_{18}- and C_8- bonded materials is subtle rather than major. Chromatographic modes such as normal phase were not considered, since it was sought to use the same mobile phases on both columns, and besides, normal phase chromatography does not lend itself to the intended extraction procedure of on-line solid-phase extraction. Hence, there remained such possibilities as C_2-, phenyl-or cyano-bonded columns. The cyano column was chosen since it can be used in both the normal and reversed-phase modes and has been claimed by Massart [37] as an almost universal stationary support. In addition, many separations of basic drugs have been successfully achieved on a cyano column in this laboratory. In view of the differences in columns from one manufacturer to another, both the columns were Spherisorb, (10 m)supplied by Phase Separations.

Methanol and acetonitrile were chosen as the organic solvents since they are the most commonly used organic modifiers and differ in their solvent strength and slightly in their selectivities. The chosen percentage range was 30 to 80% which reflects practically the complete range which would normally be used on both columns. Higher percentages of organic solvent can be used on the CN- column since water has stronger eluting power on this surface than on C_{18}- materials. Using a higher percentage of oganic modifier has advantages in terms of helping to prevent column contamination by strongly adsorbed contaminants, and of effecting almost complete rengeneration of the concentration column during the desorption phase of the on-line extraction procedure.

The aqueous phase of the eluent was buffered with sodium acetate since this buffer is widely used in biopharmaceutical applications. As outlined in section 7.1., the pH of the mobile phase affects drug retention and selectivity to a greater or lesser extent depending on whether the analyte is ionic or not, its pK_a and its polarity. The pH of the aqueous component in the mobile phase was, therefore, varied between pH 3.0 and pH 7.0, which represents the range where many separations are carried out. The effect of ionic strength of the aqueous component was studied since it can also affect retention, especially on C_{18}- columns where separations involving basic compounds can proceed through ionic interactions with residual charged silanol groups on the column surface. The ionic strength was varied between 0.005 M and 0.1 M, although the latter concentration is probably outside the upper limit of ionic strengths commonly encountered in reversed-phase applications. This broad range was selected in order to demonstrate the existence of an effect which tends to be less manifest on retention than other factors such as the type and percentage organic modifier.

As it would have been a major undertaking to study all possible combinations of pH, ionic strength and percentages of organic modifier in the ranges listed above, it was decided to set some

variables while examining others. In addition, evaluation would have been difficult in the more elaborate study, as a high order multifactor design would have been required, and these schemes are complicated in their interpretation, especially if many-way interactions were present. Hence, for the organic modifier study, the pH and ionic strength of the aqueous component were set at pH 6.0 and 0.025 M respectively, since these values were deemed to be typical of the kind of conditions used in separations for biopharmaceutical analysis. As discussed in chapter 5, the type and concentration of organic modifier does affect the apparent pH, in addition to the degree of solute and column ionisation, but for the purposes of the present work it was assumed that these influences were small in comparison to the actual effect of changing the ratio of aqueous to organic components. Likewise, when studying the effect of pH, the ionic strength was nominally held at 0.025 M, though it is recognised that small changes in ionic strength occur with a shift in pH. For the experiment on the effect of ionic strength, the pH of the aqueous component was set at 6.0, and in each case mobile phases were made containing acetonitrile-buffer (1:1) and methanol-buffer (1:1).

The on-line column switching technique of extraction was employed in the interests of analytical expediency and to minimise the formation of artefacts which, as discussed in chapter 2, can pose considerable problems with liquid-liquid extractions. It must be recognised, however, that because the analytical mobile phase is actually involved in the extraction process itself, the chromatographic profile will be a function not only of the eluent interaction on the analytical column, but of how efficiently it removes adsorbed plasma components from the concentration column following the wash cycle. Therefore, one would expect that the "optimal" mobile phase following solid-phase extraction to differ from the "optimal" mobile phase following off-line extraction where the extraction efficiency is independent of the mobile phase composition. This consideration is dealt with in further detail later. The operation of, and instrument arrangement for the column switching assembly are described in chapter 2.

The commonly-used C_{18}- Corasil packing was used for most experiments in this study. It was replaced with C_8- packing only to compare the two materials in terms of plasma removal. Since the flow rate of the washing solvent, as well as the duration of delivery is (for any particular application) dependent on a number of factors, including the nature of the solute, the type of packing material in the concentration column, and the solutes breakthrough volume on that column, it was necessary to choose a rather arbitrary wash regimen, which, in this case was 1 ml/min for 90 seconds. Shorter wash cycles will tend to produce less band broadening, but it was found here that more plasma was removed with a 90 second rather than a shorter wash time, and although this difference was slight, the longer wash cycle was preferred in the interest of preventing contamination of the analytical columns. Water was chosen as the wash solvent since it is widely used and presents the least elaborate option, although for particular applications, the addition of a buffering salt or a small amount of organic modifier may be appropriate.

Distilled water was passed twice through the water purification system, as minor impurities may become concentrated on the concentration column and will generate spurious peaks on the chromatogram following the desorption step. In order to verify that interfering peaks observed during the analyses did actually originate in the plasma, the switching valve was actuated without having made an injection (i.e. with the injector in the "load") position, and any adsorbed contaminants originating in the water would, at that stage be removed and detected. In order to eliminate the possibility that interfering peaks were derived from the syringe or the injection loop, injections of purified water were made periodically, and subjected to the same wash regimen as the plasma solutions.

In the same way that the wash regimen may be optimised to suit a particular analyte, it is possible to vary the duration of the

desorption stage with the valve in the "switched" position, and, by association, the length of time for which the concentration column is re-equilibrated with water following switching of the valve back to the "reset" position. The length of time for which the concentration column is placed in the stream of the analytical mobile phase will depend on how strongly adsorbed the analyte is and on the solvent strength of the mobile phase. Generally, if the mobile phase contains high proportion of organic modifier, it will desorb the drug rapidly from the concentration column, but it will also remove any remaining plasma components which might interfere with the drug peak. However, this latter feature also means that a strong eluent is more effective at regenerating the concentration column than eluents containing low concentrations of organic solvents. In fact, if a very weak eluent is used, it might be necessary to incorporate a third (regeneration) step into the cycle to avoid having to change the concentration column very frequently. The disadvantage of using strong eluents is that they are more like to cause precipitation of residual plasma components when contacted with the concentration column, so their use normally demands fairly thorough re-equilibration of the concentration column with water before the next injection of raw plasma is made.

Since mobile phases of varying solvent strengths were to be used in this study, a single desorption-re-equilibration cycle could not be identified as being superior to all others. It was decided, therefore, that following the 90 second wash phase, the concentration column would be introduced into the eluent stream for a period of 20 minutes, and that it would be re-equilibrated with water for a further 10 minutes before making the next injection. It was hoped that this would allow adequate time for desorption using the weaker eluents and adequate time for re-equilibration of the concentration column when the stronger eluents were employed.

As described in the Experimental section, small pools of mixed batches of frozen drug-free plasma were thawed, pooled, divided

into 10 ml aliquots and refrozen until required for use. Having re-thawed the plasma it was mixed (1:1) with purified water in order to reduce its viscosity, and 500 l loopfuls were injected onto the concentration column. Because the loop contained exactly 500 l, it was assumed that the injection volume would not prove to be a source of variation. Four replicate injections using each of the mobile phases were made. Analysis was performed on the second, third and fourth injections only. This was because the chromatogram of the first injection differed markedly from successive injections in that it generally contained more and larger plasma peaks. This observation may be accounted for by the fact that the retentive properties of the concentration column are considerably reduced following even one injection of plasma. A similar effect is also seen with the second and successive injections, but to a much lesser extent. It is quite possible that the first plasma injection causes masking of exposed silanol groups on the column surface, thus dramatically reducing its retentivity, and that successive injections cause slight modification of the column surface through adsorption of plasma components. This surface modification would appear to diminish the retentivity of the column, since as more injections are made, the plasma peaks continue to diminish.

This suggestion is supported by the fact that the above effect is less manifest when an organic-rich eluent is used, which indicates that the latter removes more plasma components from the concentration column than a weaker eluent, and thus minimises the extent of surface modification amounting to effective regeneration of the concentration column. Clearly, a large number of replicate injections would not offer significant advantage over a smaller number in this case, since plasma profiles within a given set of experimental parameters depends on how many injections have preceeded it. While it is recognised that this time effect is important, it was beyond the scope of the present work to take account of its contribution to the observed results, and measuring three replicate injections was settled upon as a suitable compromise, where any time dependence or serial

correlation of responses was likely to be zero.

From the foregoing discussion it is clear that a new concentration column would be necessary for each mobile phase on both columns. The (concentration) column-to-column variability on both a between-day and within-day basis was considered in case a large variation between columns was serving to complicate interpretation of the data. The results of this exercise are discussed later.

Having defined the variables and selected the extraction procedure, the next step was to decide how the blank plasma chromatograms would be evaluated. Obviously the criterion was that there would be no interfering plasma peaks in the area of chromatographic interest. In regard to the latter, the first 5 minutes on the chromatogram were eliminated since they normally contain the large early-eluting plasma peak. Measurements would be made over a time frame of 15 minutes (from 5 to 20 minutes) since analysts would normally endeavour to execute a chromatographic run in 20 minutes or less.

It is a simple matter to assess the chromatograms qualitatively: it is quite obvious which chromatograms are "better" than others based on the absence of small or large interfering peaks, how quickly the baseline returns to the horizontal and the magnitude of the principal early-eluting plasma peak. The latter is not a major consideration since, as outlined above, the chromatography is usually developed such that the drugs have a longer retention time than the principal plasma peak. How quickly the baseline returns to the horizontal can be relevant if a drug peak is masked by the curving portion of the chromatogram. It is not uncommon to see the drug peak appearing to reside on the curved portion of the plasma peak, when what is actually being observed is the top of a peak, the remainder of which is under the tailing plasma peak. Obviously, this tailing of a large plasma peak will have serious implications for the limit of detection of a bioanalytical method.

It is, however, the interfering peaks which appear later on in the chromatogram which can present the most difficult problems in relation to drug analysis in biological fluids. As mentioned above, chromatograms may be qualitatively compared, but if it is desired to quantitatively characterise a large series of chromatograms such as was the case in the present study, some kind of response variable must be sought. If an electronic data processor (integrator) had been used, it would have been possible to measure the areas under the individual interfering peaks in the chromatogram, and to sum these if so desired. However, a problem with using an integrator is that a type of baseline must be defined from which the integrator can make measurements. Hence if there is a large tailing portion to the plasma peak and a horizontal baseline has been set, a very large peak area will be computed, and that chromatogram would be classified (possibly erroneously) as being inferior to another. Conversely, if a sloping baseline had been set, and the chromatogram attained the horizontal very quickly, that chromatogram might be wrongly judged to be superior to another.

For a particular analysis, the merits of using an integrator over a chart recorder cannot be disputed, since (among other factors) the type of baseline appropriate to that method is much easier to define. In the present case, however, as a large number of experimental conditions were to be investigated, and both curved and flat baselines were to be expected, it was decided that the rapidity with which the baseline returned to the horizontal position would not form part of the analytical evaluation, and measurement would be made of peaks superimposed on the baseline, irrespective of whether it was curved or flat.

As the peaks were usually quite small and of indeterminate shape, it would have been a difficult task to measure the area under each one manually using the chromatograms generated on the X-t recorder. Another possibility was to measure peak heights and to use the total peak height of all interfering peaks in each

chromatogram as a response factor. Again this would have been an arduous task to execute manually, and it would not account for the number and location of the peaks contributing to the overall peak height. What appeared to be a practical alternative was to measure the number of interfering peaks in the area of interest and to use this as a response variable in evaluating the the chromatogram. In assessing the data, it became obvious that a large number of very small deviations around the baseline appeared in most chromatograms; though clearly they originated in the plasma, they could not be defined as "peaks" per se, being too small and ill-defined to qualify for that definition. And since these small deviations would not, in most cases, interfere with drug quantitation, it was decided that only peaks or deviations greater than 2 mm (which represents approximately 8 x 10^{-4} absorbance units) would be counted for the purposes of chromatogram evaluation.

Having gathered and counted the data, the next step was to perform the appropriate statistical analyses in order to determine whether real effects in chromatogram quality were being observed as the experimental parameters were varied. Since data were available as a series of means (of three replicate injections), it was decided to use an analysis of variance test (ANOVA) to establish whether the difference betweeen the means was too great to be explained by random error. The statistical analysis was carried out by computer using the "Statgaphics" package. A three-way factor design was used to assess the effects of type of organic modifier, column and either percent organic modifier, pH or ionic strength on the number of interfering peaks. The significance of the three possible two-factor interaction terms within each experiment was also examined in order to determine how strongly the effect seen by changing one factor was dependent on the other two. The three-factor interaction terms were not computed, since the two-factor interactions are easier to examine visually, and hence, the kinds of trends which merit more sophisticated analysis may be readily identified. A two-way ANOVA was performed on the data generated

in the reproducibility study as there were, in this case, only two variables (concentration column and day). The results of the other experiments, comparison of different anticoagulants, concentration column packings, and comparison of the two columns in relation to retention of pure drug solutions, were evaluated qualitatively and will be discussed later.

7.3.2. Effect of percentage organic modifier on the number of interfering peaks

A block design of the number of peaks obtained in the replicate analyses at each percentage level of both organic modifiers on the two columns is presented in Table 7.1. Also shown is the ANOVA table for the three-factor design in which the sources of variation and their corresponding F-ratios are listed. The calculated F-ratios were compared with critical values in statistical tables of the F-distribution in order to determine whether any variations seen in the results were due to systematic differences or random error. Where the empirically derived F-ratio was greater than the value in the tables, a systematic effect was considered to exist. For the appropriate degrees of freedom the F-ratios were compared at two levels of significance, i.e. 5% and 1% levels. So where a null hypothesis was rejected at the 5% level, there was a 95% chance that the variation in results was due to a systematic effect rather than random error, and this is represented in the table by a single asterisk. Where there was a 99% chance of the results being due to a systematic effect is indicated by two asterisks. Where the calculated F-ratio was smaller than the tabulated value at the 5% level of significance, the null hypothesis was accepted and the variation ascribed to random error. Such cases are indicated in Table 7.1. by NS (not significant)

Interaction terms were included in order to determine whether the variation in one factor was dependent on another factor, e.g., if the variation in the mean values as the percent organic modifier was changed was dependent on the type of column or the type of

Table 7.1.
Effect of percentage organic modifier on the number of interfering peaks.

Number of interfering peaks(3 measurements per cell)

	Methanol %						Acetonitrile %					
	30	40	50	60	70	80	30	40	50	60	70	80
Column												
C18	3,3,3	4,3,3	4,5,6	6,5,5	7,6,6	6,7,6	4,3,5	4,6,4	6,5,5	6,6,5	7,7,6	5,5,5
CN	4,4,4	4,4,3	4,3,4	3,3,3	3,2,2	3,1,2	8,8,9	4,5,5	3,2,3	1,0,0	0,0,0	0,0,0

Mobile phase: 0.025 M sodium acetate buffer, pH 6.0 plus percentage and type organic modifier as described in table

Three-way Analysis of Variance of above data

Source of Variation	Sum of Squares (SSQ)	Degrees of Freedom(DOF)	Mean Square (MSQ)	F Ratio
Main effects				
A	13.8	5	2.8	3.6**
B	82.3	1	82.3	106.8**
C	0.1	1	0.1	0.2 NS
2-factor interaction				
A-B	153.7	5	30.7	39.9**
A-C	35.8	5	7.2	9.3**
B-C	2.3	1	2.3	3.0 NS
Residual	40.9	53	0.8	-
Total	328.9	71	-	-

Key: A: Percentage organic modifier
B: Column (C_{18} or CN)
C: Type of organic modifier (MeoH of ACN)

**: significant at 5% and 1% levels
NS: not significant

organic modifier being studied. As may be seen from Table 7.1., there was, if one considers the F-ratios, a substantial difference between the various percent levels over the means for both columns and types of organic modifiers. The results also show that the observed effect with changing organic modifier concentration was strongly dependent on both the type of column and the type of organic modifier employed, indicating a strong interaction between the percent organic modifier and these factors. The magnitude of these interactions are indicated by the large values of F; 39.9 for the interaction between percent organic modifier and the column; and 9.30 for the interaction between the percent and type of organic modifier.

The fact that the F-ratio for the column-type of organic modifier interaction was below the tabluated F-value indicates that at this level of significance, the variation in results between the two columns was not dependent on the type of organic modifier, though this does not necessarily deny the existence of an interaction. There was a very large difference (F = 106.9) between the two types of columns with respect to the means of both types of organic modifier and the percentages in which they were used. Overall, there was no significant difference between the two types of organic modifiers if the means of the columns and percentages of organic modifiers were taken. This is indicated by the very small F-ratio for the type of organic modifier term.

The above effects may be demonstrated graphically by constructing plots of the relevant mean values. Figure 7.1.a shows a plot of peak number at each level of percent organic modifier versus the percent organic modifier. In this case, the means of 12 values (i.e. two columns, two types of organic modifiers, three replicates at each level) were plotted. This plot shows that there was a decrease in the number of interfering peaks as the amount of organic modifier in the mobile phase was increased, though there were minor increases at the 50% and 70% levels superimposed on the overall simple trend. In order to examine this effect on the two different columns, the means of the

Figure 7.1.a.
Plot of peak number versus percent organic modifier- mean of types of column and organic modifier

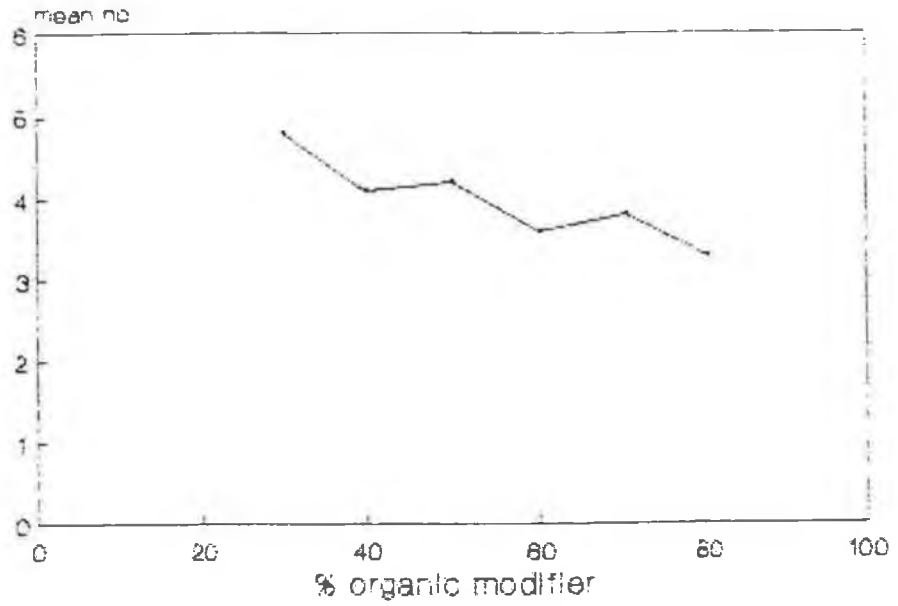
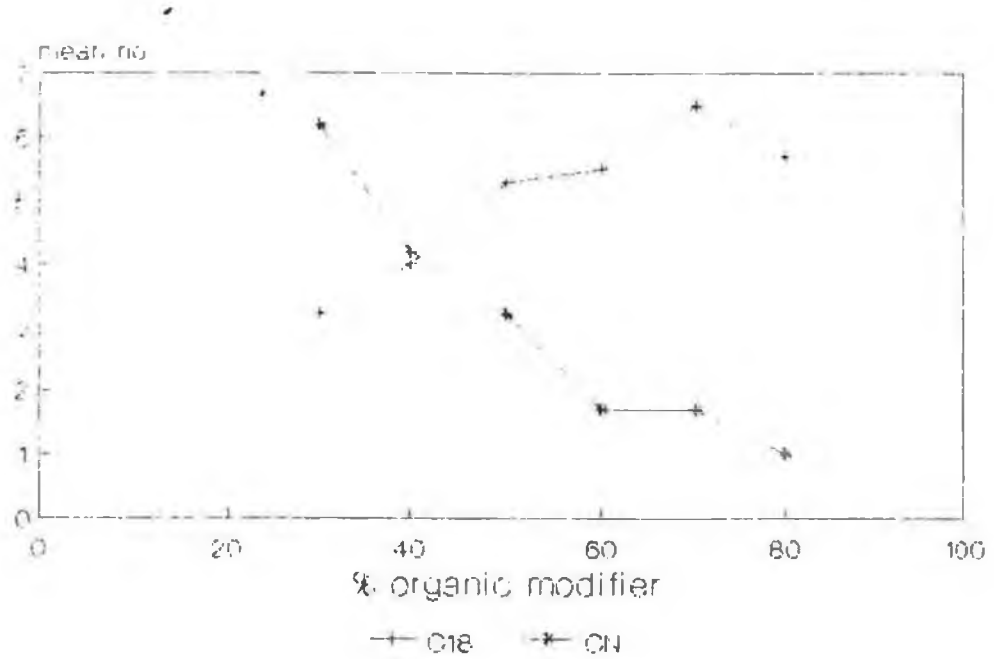


Figure 7.1.b.
Plot of peak number versus percent organic modifier- breakdown on column type



Mobile phase: 0.025 M acetate buffer, pH 6.0-organic modifier, percentages as per graphs

column-percent organic modifier interaction terms were plotted as a function of the percent organic modifier (Figure 7.1.b). Like the previous values used in Figure 7.1.a, these values may be obtained from the computer programme used to caclulate the F-ratios for the 3-factor design. Alternatively, they may readily be calculated from the raw data by summing the appropriate cells, i.e., column 1, percent level 1, and column 2, percent level 1 etc. The plot in Figure 7.1.b shows that the effect of increasing organic modifier produced virtually the opposite effects on the C_{18}- and the CN- columns. The fact that the two lines are non-parallel, i.e. that the pattern is different for the two columns, suggests the presence of an interaction between these two factors. These findings must be considered in terms of the elution strength of the various eluents not just on the analytical columns, but also on the concentration column following the wash cycle.

Considering first the C_{18}- column: using low levels of organic modifier, one would expect the plasma components swept off the concentration column to have long retention times, (leading to the presence of many interfering peaks) due to the weak eluting power of the mobile phase on this highly non-polar surface phase. The fact that the least number of interfering peaks were observed on this column at the lowest level of percentage organic modifier (i.e. 30%) may be explained by the fact that the mobile phase also has weak eluting power on the concentration column and therefore only a portion of the plasma components remaining after the the wash cycle were desorbed. Conversely, with a high proportion of organic modifier, more plasma was removed which produced a greater number of peaks in the chromatogram. As the percentage organic modifier was increased from 70 to 80%, the number of peaks began to decrease. This is because rapid elution of the plasma components from the analytical column outweighs the adverse effects of greater plasma desorption from the concentration column with increasing eluent strength.

The opposite effect was seen with the CN- column. The number of

peaks decreased with increasing eluent strength in this case. As the trend in extraction efficiency remained unchanged, the differences between the two analytical columns may be accounted for purely in terms of the greater eluting power of the mobile phase on the CN- phase. As pointed out earlier, water is a stronger eluent on the more polar CN- phase than the C_{18}- phase, though clearly, the addition of the organic solvent enhances the solvent strength in relation to both supports. Even though more plasma was removed as more methanol or acetonitrile was added to the eluent, the overriding effect was decreased retention time for the interfering peaks, which caused them not to appear in the area of interest in the chromatogram.

The means for the percent-type organic modifier interaction were also plotted as a function of percentage organic modifier. The resultant graph is presented in Figure 7.1.c, and it shows that (if the two columns were taken together) there is a slight increase in the number of peaks as the percent methanol was increased, but that it reached a plateau at about 50%. On the other hand, there was a decrease in the number of peaks as the percent acetonitrile is increased. These observations may be explained used the same general arguments as applied above. That is, increasing amounts of both modifiers removed more desorbed plasma from the concentration column, but that with acetonitrile, this tendency was outweighed by its enhanced eluting power on the analytical columns, whereas in the case of methanol, the opposing effects on the number of interfering peaks became almost equal at about the 50% level.

If it is desired to separate the effect of percent organic modifier between the two columns and the two organic modifiers, the means of each cell (i.e., the three replicate analyses) may be plotted as a function of percent organic modifier (Figure 7.1.d). This graph again reflects the increase in the number of peaks on the C_{18}- column, and the corresponding decrease on the CN column. However, it further depicts how the decrease in peak number in going from 70 to 80% organic modifier on the C_{18}-

Figure 7.1.c.
Plot of peak number versus percent organic modifier- breakdown on types of organic modifier

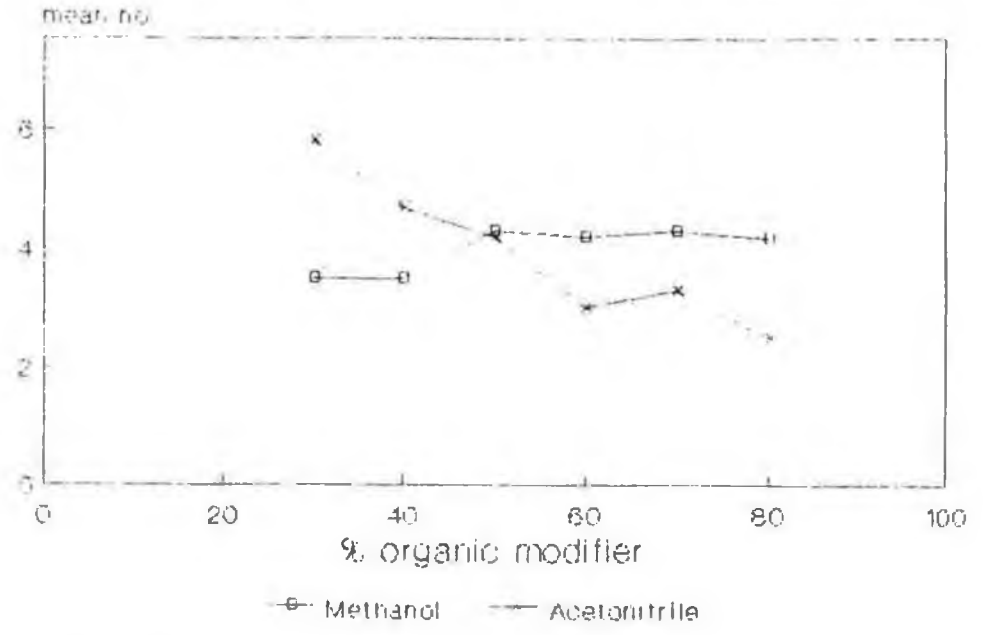
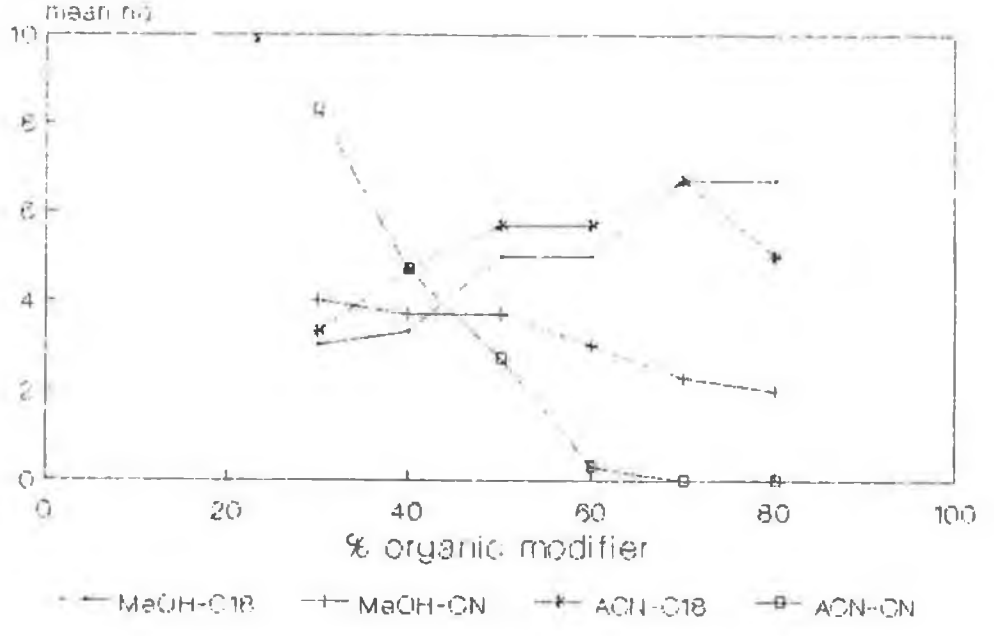


Figure 7.1.d.
Plot of peak number versus percent organic modifier- breakdown on type of column and organic modifier



Mobile phase: 0.025 M acetate buffer, pH 6.0- organic modifier, ratio as per graphs

column was due entirely to the contribution from acetonitrile since the number of peaks remain constant between 70 and 80% methanol on this column. In addition, the decrease in peak number on the CN column is much more profound with increasing concentrations of acetonitrile rather than methanol.

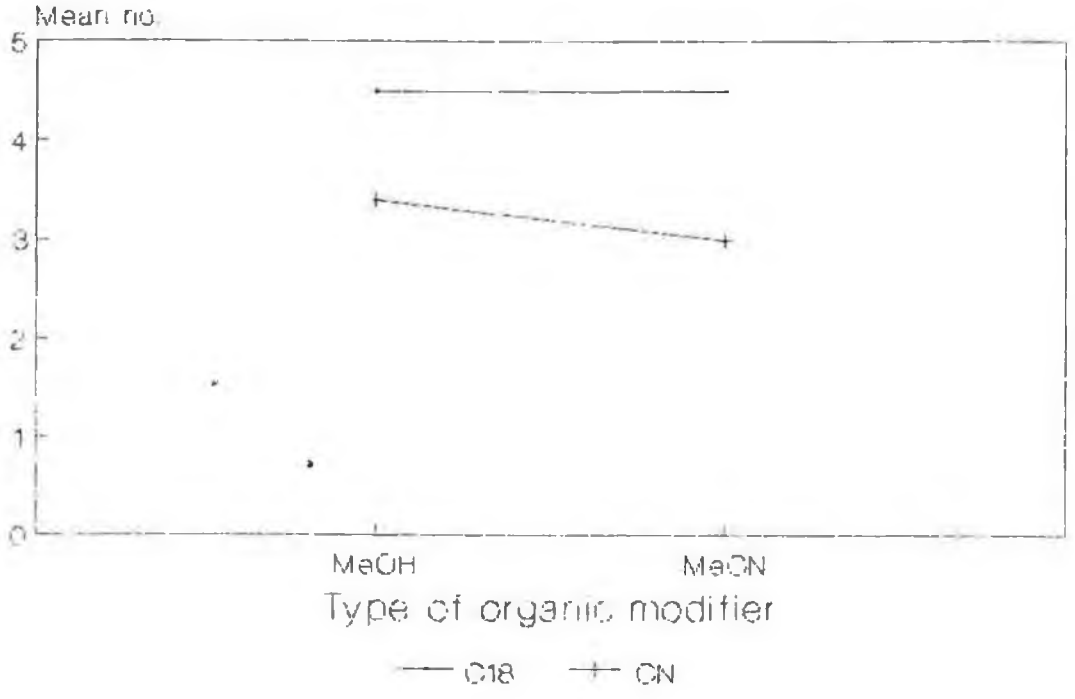
The graph in Figure 7.1.e shows that the interaction between the type of column and the type of organic modifier under the employed experimental conditions is very small. The F-ratio for this interaction is slightly below the 5% level of significance, suggesting that the difference between the two columns was independent of the type of organic modifier and that the difference between the type of organic modifier is independent of the types of columns using these experimental parameters. It is recognised, however, that the column-type of organic modifier interaction may become significant if the experimental conditions were to be slightly varied.

The salient points which have emerged from this experiment are that the ratio of water to acetonitrile seems to have a more pronounced effect on retention characteristics than the ratio of aqueous to methanolic phases, particularly on the CN column; the C_{18}- column provides better chromatograms at the lower levels of organic modifier, with the converse being true at higher percentages of organic modifier; and over the means of both columns, increasing acetonitrile concentration tends to reduce the number of interfering peaks, whereas there are more interfering peaks with increasing methanol concentration.

The above points are illustrated chromatographically in Figure 7.2. The chromatograms also serve to demonstrate that one set of parameters might be superior to another in one respect, but inferior in another respect. For example, in terms of the criterion used in this study, 70% methanol on the C_{18}- column offers a better proposition than 30% acetonitrile on the C_{18}- column. However, by studying the chromatograms themselves, it can be seen that although the peaks are more numerous in the former

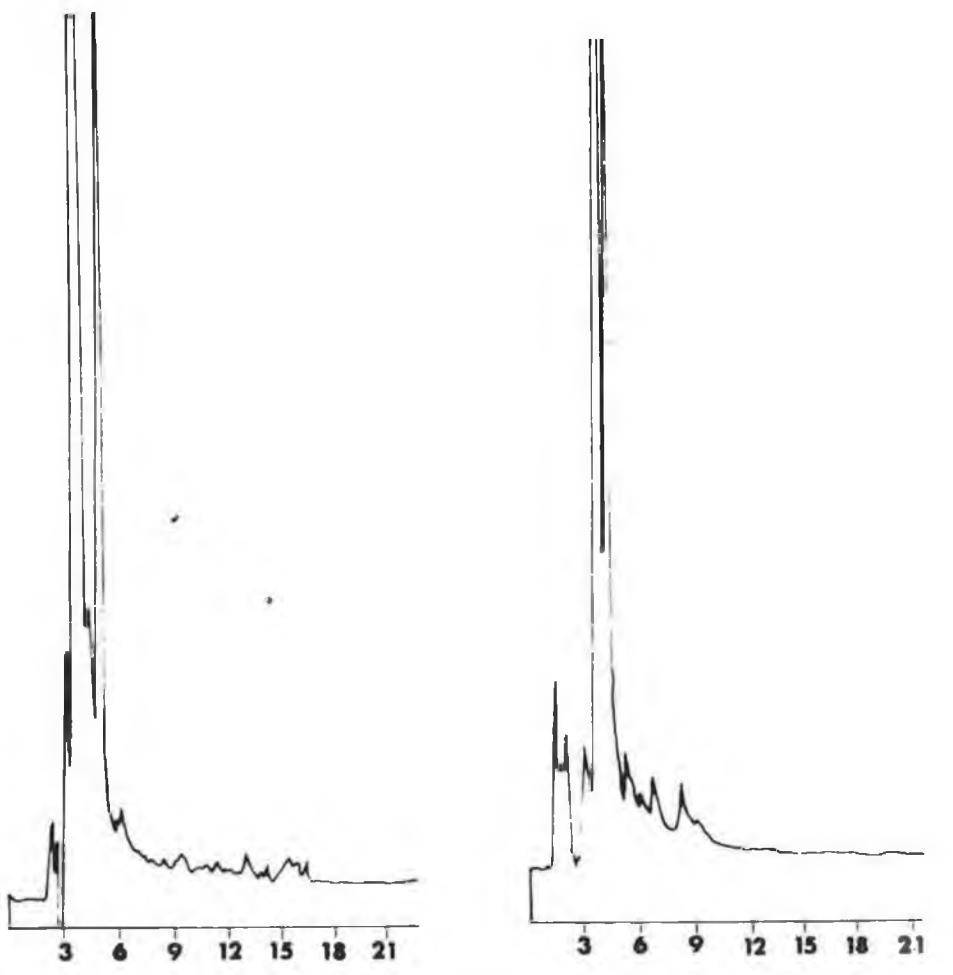
Figure 7.1.e.

Plot of peak number versus type of column and organic number- mean of percent organic modifier



Mobile phase: 0.025 M acetate buffer, pH 6.0- organic modifier

Figure 7.2.
Chromatograms showing effect of organic modifier



Mobile phase: 0.025 M acetate buffer, pH 6.0 plus:

a: 70% methanol on the C_{18}- column

b: 30% acetonitrile on the C_{18}- column

case, they are larger in the latter case and more likely to operate to the detriment of a quantitative determination. This is an example of one of the limitations of the chosen response variable: the size of the peaks are not accounted for in evaluating the various mobile phases, and this can sometimes lead to a misinterpretation as to what constitutes an inferior or a superior chromatogram.

7.3.3. Effect of pH on the number of interfering peaks

The pH of the aqueous component was varied in 1-unit increments over the range pH 3.0 to pH 7.0. The ionic strength was set at 0.025 M and a constant organic-to-buffer ratio of 1:1 (with both methanol and acetonitrile) was employed. This yielded 20 cells of three replicate injections, which are presented in a block design in Table 7.2. The three-way ANOVA table for the determination of the significance of the results is also shown in Table 7.2. The results show that over the means of both columns and types of organic modifiers, there was a systematic effect observed with changing pH. Similarly, there was a systematic effect seen between columns and between types of organic modifiers over the means of the remaining two factors. As evidenced by the very large F-ratio, there was a strong interaction between the pH and the type of column, and an even larger interaction between the column and type of organic modifier; the pH-column interaction was marginally below the 5% level of significance, though again, it was non-negligible.

The graph in Figure 7.3.a. was generated by plotting the mean peak number at each pH level versus pH, discounting (for the moment) the effect of column and type of organic modifier. This graph shows that there was a general decrease in the number of interfering peaks with increasing pH. This effect may be explained by the fact that many endogenous plasma components are acidic in nature and therefore, in more basic eluents, they would become more ionised and tend less to partition into the stationary phase. Hence, they would elute rapidly off the column

Table 7.2.
Effect of pH on the number of interfering peaks.

	Number of interfering peaks(3 measurements per cell)									
	50% Methanol					50% Acetonitrile				
	pH 3	pH 4	pH 5	pH 6	pH 7	pH 3	pH 4	pH 5	pH 6	pH 7
Column										
C18	2,1,1	1,1,1	2,2,2	2,2,2	3,2,2	7,6,7	6,7,5	5,5,4	3,5,4	4,4,3
CN	4,4,3	3,3,3	3,1,2	3,3,3	2,3,2	4,4,3	3,2,3	3,3,1	2,2,3	1,1,0

Mobile phase: 0.025 M sodium acetate buffer, plus 50% organic modifier. pH as described in table.

Three-way Analysis of Variance of above data

Source of Variation	Sum of Squares (SSQ)	Degrees of Freedom(DOF)	Mean Square (MSQ)	F Ratio
Main efects				
A	16.4	4	4.1	8.4**
B	11.3	1	11.3	23.1**
C	29.4	1	29.4	60.0**
2-factor interaction				
A-B	4.9	4	1.2	2.4 NS
A-C	16.4	4	4.1	8.4**
B-C	56.1	1	56.1	114.5**
Residual	21.4	44	0.5	-
Total	155.9	59	-	-

Key: A: pH
B: Column (C_{18} or CN)
C: Type of organic modifier (MeoH of ACN)

**: significant at 5% and 1% levels
NS: not significant

and not appear in the area of chromatographic interest. If this graph is broken down between the the two types of organic modifier (Figure 7.3.b), the resulting plots show that increasing pH caused a marked reduction in the number of peaks if acetonitrile was used in the mobile phase, and a less-defined effect in the presence of methanol which showed a slight decrease between pH 3.0 and 4.0, and between pH 6.0 and 7.0, with a slight increase between pH 5.0 and 6.0.

If, on the other hand, Figure 7.3.a is broken down between the two columns (Figure 7.3.c), it may be seen that the trend was for fewer peaks to appear at the higher pH values. A breakdown on the types of organic modifiers and columns (Figure 7.3.d) reveals that with acetonitrile on both columns there is a marked decrease in the number of peaks with increasing pH. The fact that at most pH levels, there was a greater number of peaks seen with acetonitrile may be accounted for by the fact that it removed more plasma from the concentration column than methanol, and thus more peaks appeared on the chromatogram at the 1:1 buffer-to-organic ratio employed in this experiment. It is quite possible that the more dramatic effect seen with acetonitrile was due to the fact that in this case, there was a greater number of peaks present upon which changing pH could exert an influence. The situation is less clear with methanol. On the CN- column, the general tendency was for a decrease in peak number to occur, with a small increase between pH 5.0 and pH 6.0. On the C_{18}- column, there was a greater number of peaks in the chromatogram at higher pH values. There was, however, a small increase in peak number and from a chromatographic point of view, would not represent a serious deterioration in chromatogram quality. Studying the chromatograms presented in Figure 7.4. explains this observation. The general appearance of the chromatograms in the presence of methanol remained almost constant between pH 5.0 and pH 7.0, whereas there was a noticeable difference between quality of the chromatograms with acetonitrile. This effect was most pronounced on the CN column. Thus, studying the chromatograms serves to illustrate the value of a qualitative assessment in certain

Figure 7.3.a.
Plot of peak number versus pH- mean of columns and types of organic modifier

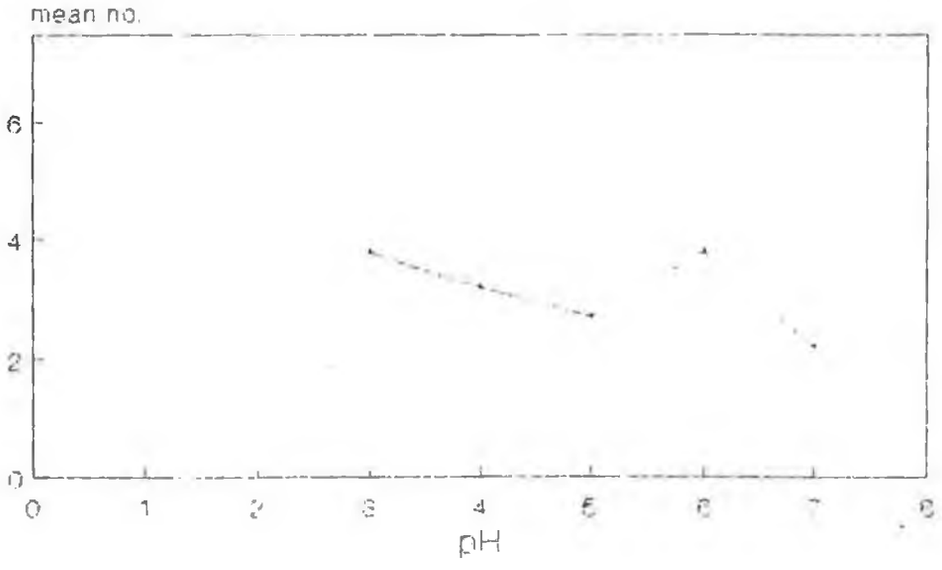
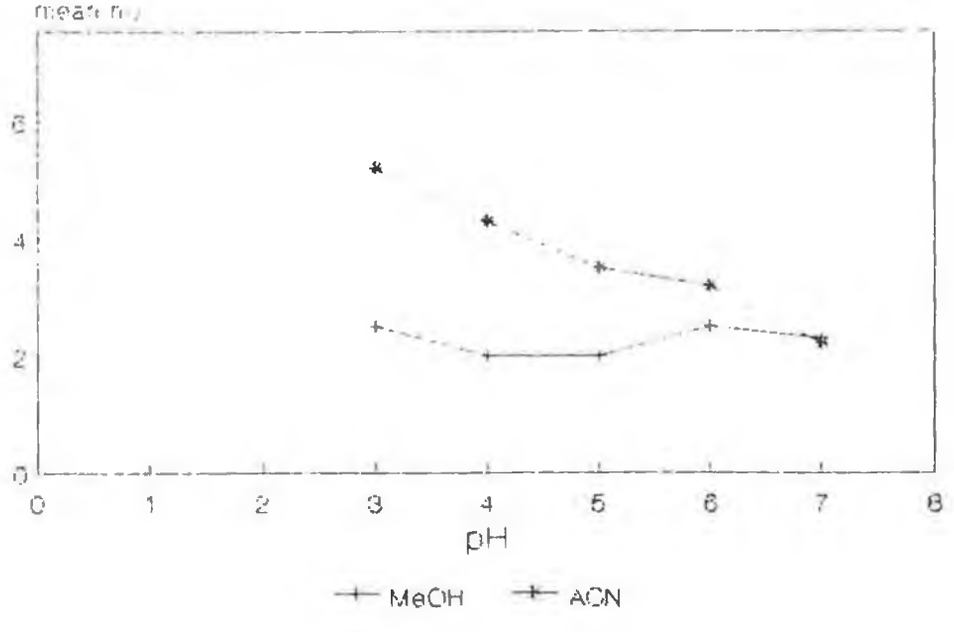


Figure 7.3.b.
Plot of peak number versus pH- breakdown on type of organic modifier



Mobile phase: 0.025 M acetate buffer-organic modifier (1:1), pH as per graphs

Figure 7.3.c.
Plot of peak number versus pH- breakdown on type of column

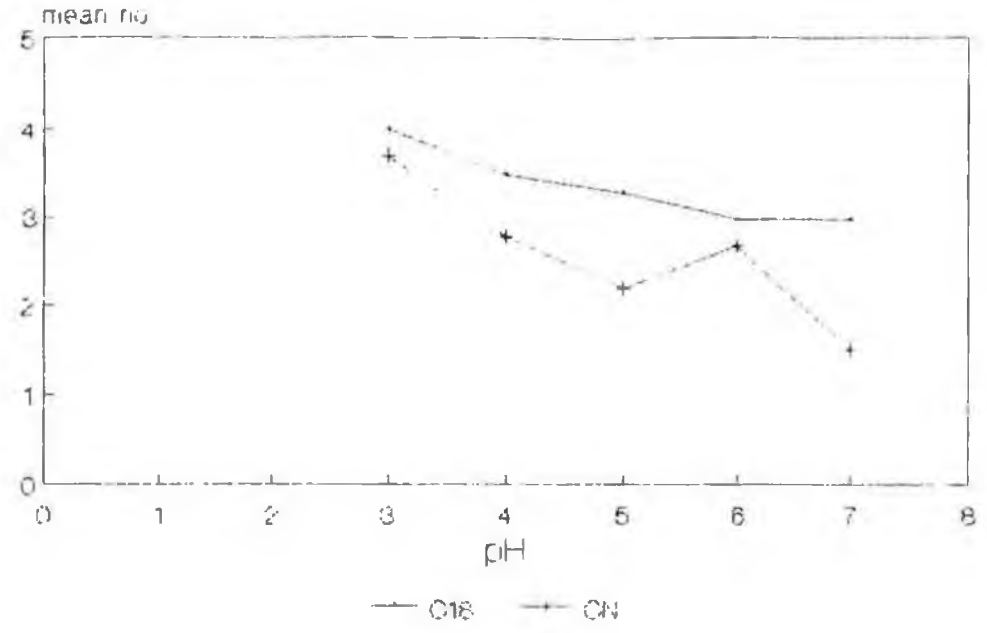
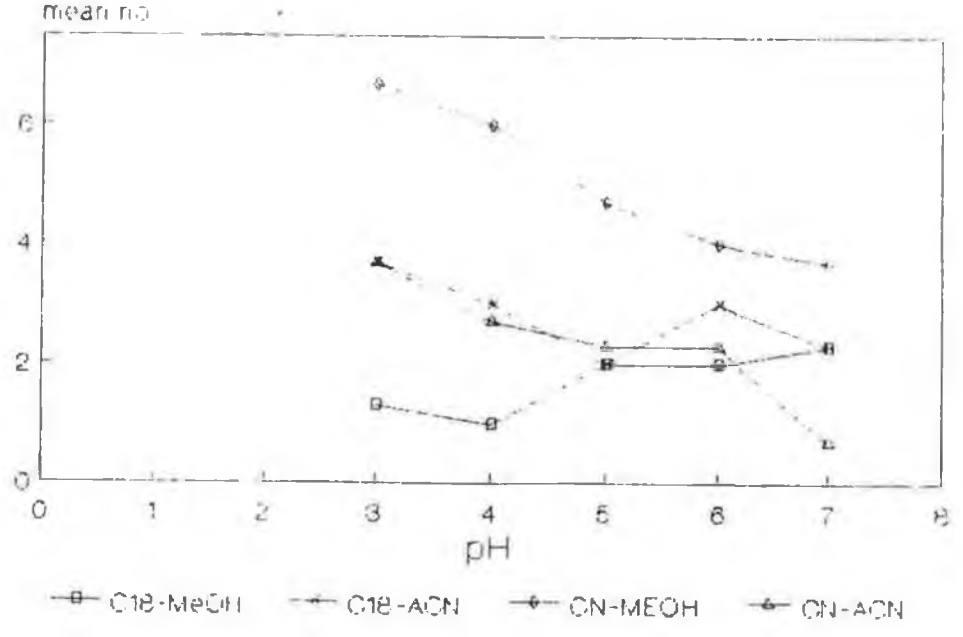


Figure 7.3.d.
Plot of peak number versus pH- breakdown on type of column and organic modifier



Mobile phase: 0.025 M acetate buffer-organic modifier (1:1), pH as per graphs

instances. Another example is that whereas there may be a quite a number of peaks present on the columns with acetonitrile, the large peak at about 7.5 minutes seen on the C_{18}- column with methanol could present significant problems in the development of a biopharmaceutical assay using this particular combination of column and mobile phase.

It may be seen from Table 7.2. that there is a strong interaction between the column and type of organic modifier terms. The graph shown in Figure 7.3.e further illustrates the inter-dependence of type of column and type of organic modifier on the changing peak number.

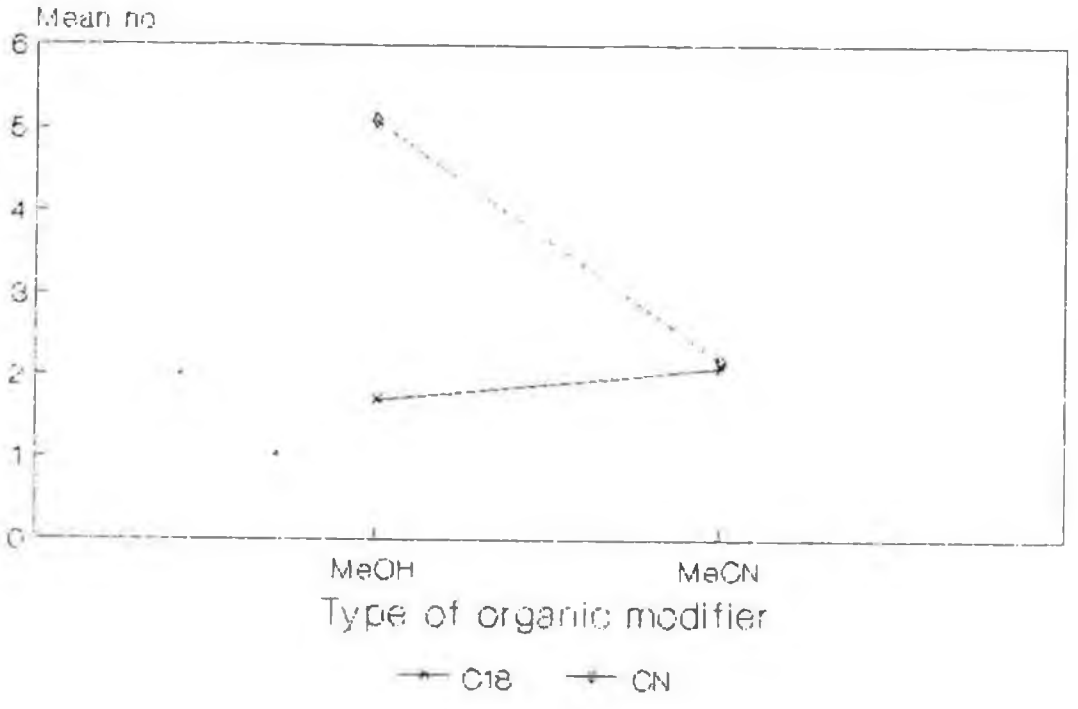
7.3.4. Effect of ionic strength on the number of interfering peaks

The ionic strength of the aqueous component was varied between 0.005 M and 0.1 M acetate buffer at a constant pH of 6.0. Mobile phases containing either methanol-buffer (1:1) or acetonitrile-buffer (1:1) were prepared. A block diagram showing the results for the 16 cells is presented in Table 7.3. Also shown is the three-way ANOVA table used to determine the significance of the observed results.

From these data it may be seen that there was a statistically significant effect with varying ionic strength at both the 5% and 1% levels of significance. Over the range of ionic strengths studied, there was a significant difference between the columns and between the organic modifiers as indicated by the large values of F (35.2 and 75.2 respectively). There were also significant interactions between the ionic strength and the columns and the ionic strength and the types of organic modifier, though in the former case this interaction was only significant at the 5% level. By plotting the number of peaks (averaged over both columns and organic modifiers) as a function of ionic strength (Figure 7.5.a), it may be seen that there was a general decrease in peak number with increasing ionic strengh. Breaking

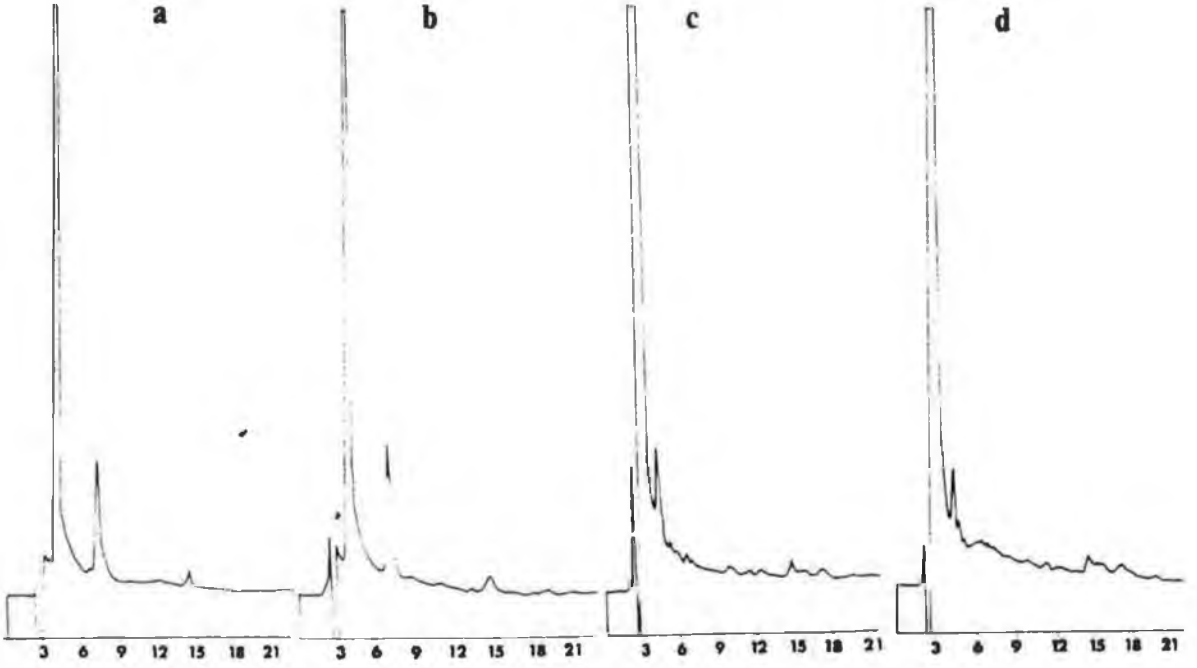
Figure 7.3.e.

Plot of peak number versus type of column and organic number- mean of all pH levels



Mobile phase: 0.025 M acetate buffer, pH 6.0- organic modifier

Figure 7.4
Chromatograms showing effect of pH on C18- column



Time (min)---------->

Mobile phase (aqueous-organic phase, 1:1):

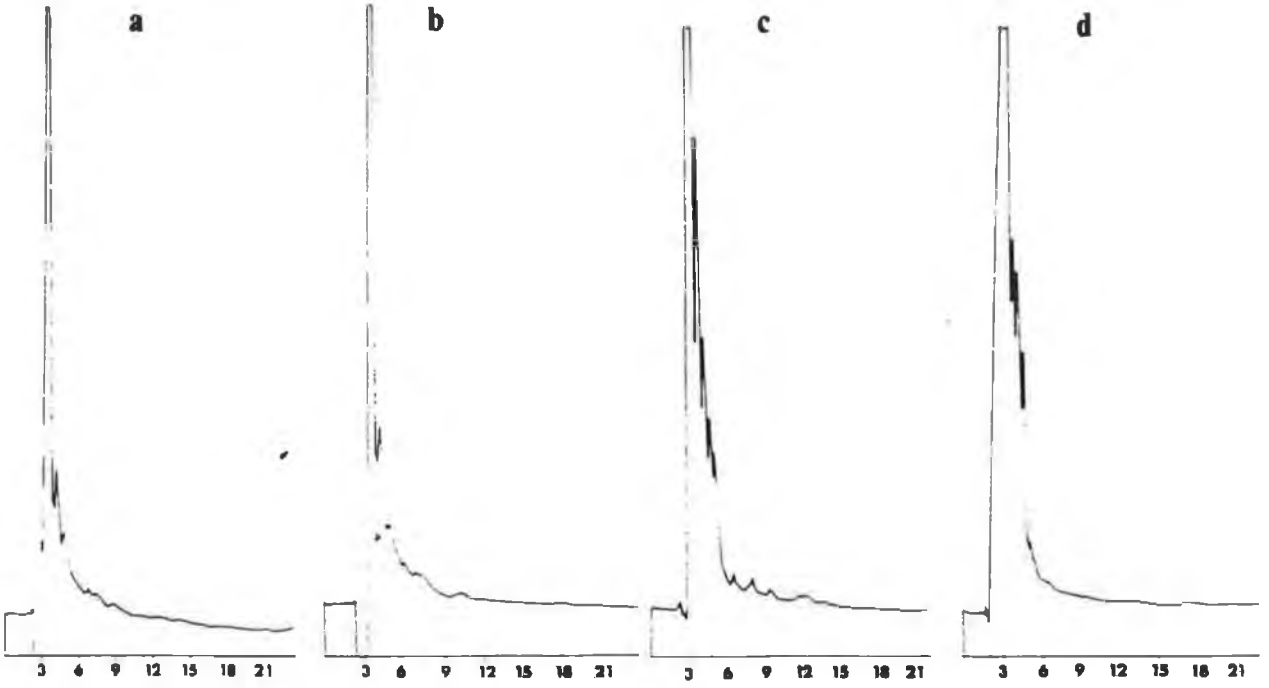
a: acetate buffer, pH 5.0-methanol (1:1).

b: acetate buffer, pH 7.0-methanol (1:1).

c: acetate buffer, pH 5.0-acetonitrile (1:1).

d: acetate buffer, pH 7.0-acetonitrile (1:1).

Figure 7.4 continued
Chromatograms showing effect of pH on CN- column



Mobile phase (aqueous-organic phase, 1:1):

a: acetate buffer, pH 5.0-methanol (1:1).

b: acetate buffer, pH 7.0-methanol (1:1).

c: acetate buffer, pH 5.0-acetonitrile (1:1).

d: acetate buffer, pH 7.0-acetonitrile (1:1).

Table 7.3.
Effect of ionic strength on the number of interfering peaks.

	Number of interfering peaks(3 measurements per cell)							
	50% Methanol				50% Acetonitrile			
	IONIC STRENGTH (M)							
	0.005	0.01	0.025	0.1	0.005	0.01	0.025	0.1
Column								
C18	3,3,3	3,2,4	2,2,2	1,2,2	5,7,6	6,6,5	5,5,5	7,6,6
CN	5,5,3	4,3,4	3,3,3	2,1,1	4,4,3	3,4,2	3,2,3	3,3,2

Mobile phase: sodium acetate buffer, pH 6.0, plus 50% organic modifier. Ionic strength as described in table.

Three-way Analysis of Variance of above data

Source of Variation	Sum of Squares (SSQ)	Degrees of Freedom(DOF)	Mean Square (MS)	F Ratio
Main effects				
A	13.4	3	4.5	11.2**
B	14.1	1	14.1	35.2**
C	30.1	1	30.1	75.2**
2-factor interaction				
A-B	5.1	3	1.7	4.2*
A-C	6.4	3	2.1	5.2**
B-C	36.7	1	36.7	91.8**
Residual	14.1	35	0.4	-
Total	119.9	47	-	-

Key: A: ionic strength
B: Column (C_{18} or CN)
C: Type of organic modifier (MeoH of ACN)

**: significant at 5% and 1% levels
* : significant ot 5% level only
NS: not significant

this graph down between the two columns (Figure 7.5.b) shows that whereas peak number decreased across the range on the CN- column, there was a decrease between 0.005 M and 0.025 M on the C_{18}- column, followed by an increase between 0.025 M and 0.1 M. Splitting Figure 7.5.a. between the two types of organic modifiers (Figure 7.5.c) shows that an almost identical pattern is seen between methanol and acetonitrile mobile phases, except that the effect reaches a higher level of significance because the effect is slightly more pronounced. By plotting the mean number of peaks in each cell (Figure 7.5.d), it becomes apparent that except for acetonitrile on the C_{18}- column, the changing trend was for the number of peaks to decrease with increasing ionic strength. The effect appeared to be most pronounced for methanol on the CN- column. As shown by the large value of F (91.8), there was a strong interaction between the change in peak number between columns and type of organic modifier, with the same applying in the reverse when comparing the effects of the types of organic modifiers. This is also depicted graphically in Figure 7.5.e.

As discussed in the Introduction section, the effect of ionic strength in reversed-phase chromatography is more difficult to define than in ion exchange chromatography. In the latter case the effect of ionic strength is readily explained in terms of the number of ions available to compete with solute molecules for sites on the column surface. With reversed-phase chromatography, the same argument has been put forward to explain the behaviour of strongly basic amines in reversed-phase systems, i.e. that ionised bases interact with oppositely-charged residual silanol groups on the reversed-phase column, and therefore their retention times are strongly affected by ionic strength [32,33]. It is known that species which are uncharged under the employed experimental conditions are also affected by ionic strength [31,32,33], though the nature of these effects have not been fully clarified. Snyder [31] proposed that when the addition of a salt to the mobile phase increases the solubility of a solute in the mobile phase, retention decreases, and that when it decreases

Figure 7.5.a.
Plot of peak number verses ionic strength- mean of types of column and organic modifier

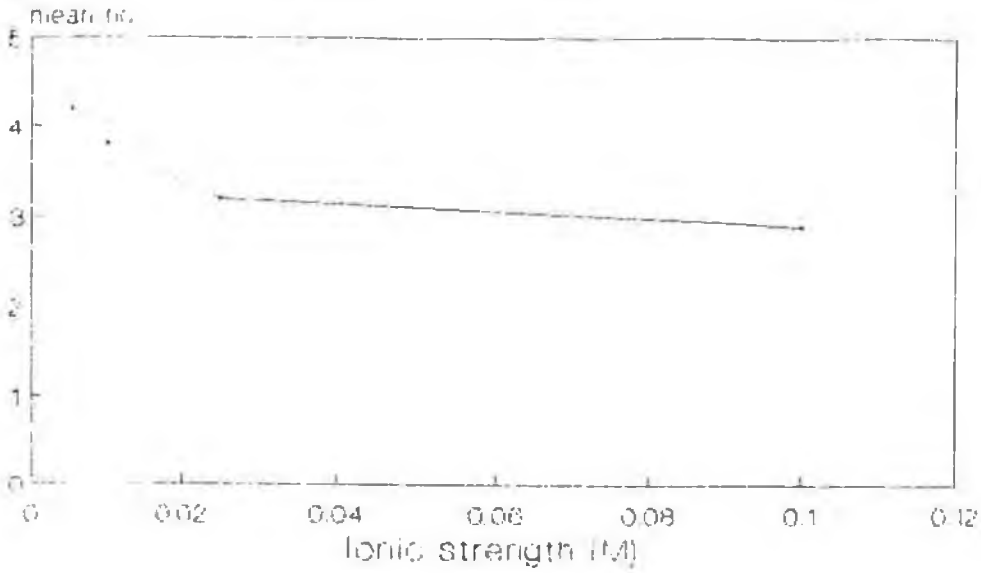
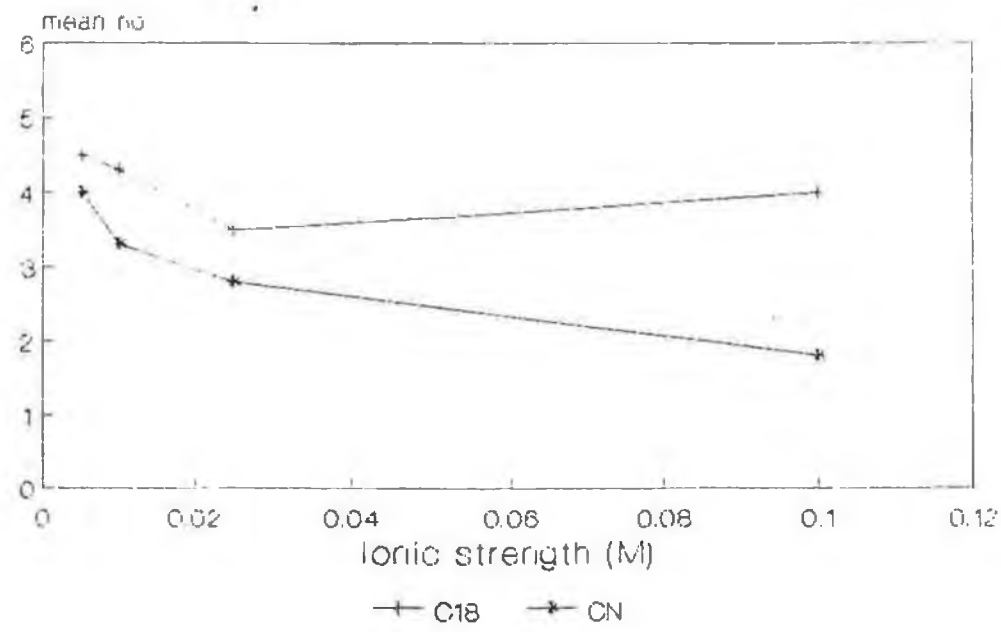


Figure 7.5.b.
Plot of peak number versus ionic strength- breakdown on column types



Mobile phase: acetate buffer, pH 6.0-organic modifier (1:1), ionic strength as per graphs

Figure 7.5.c.
Plot of peak number verses ionic strength- breakdown on types of organic modifier

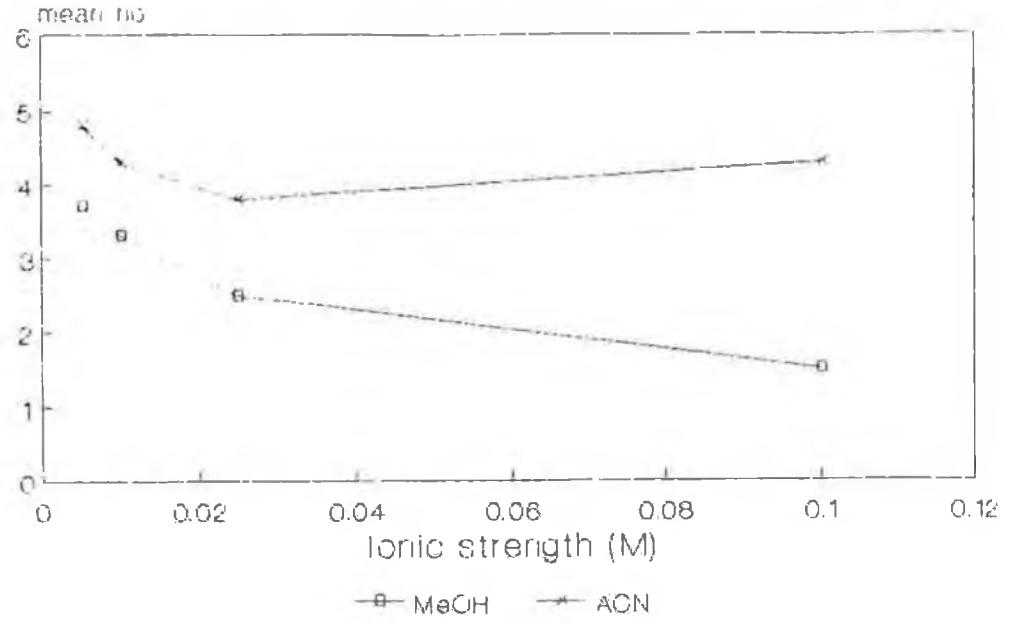
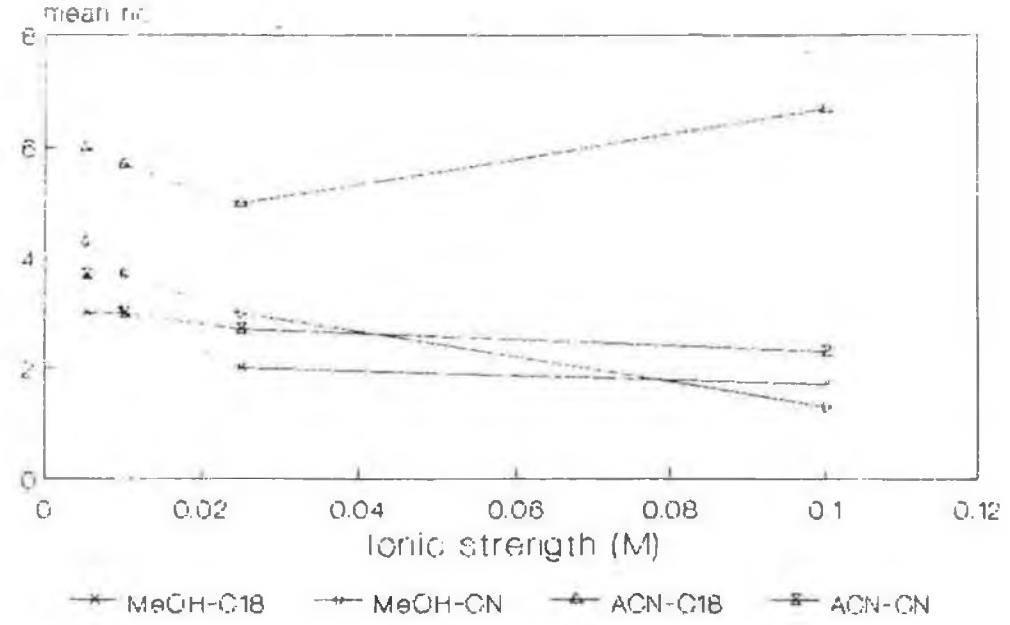


Figure 7.5.d.
Plot of peak number versus ionic strength- breakdown on types of column and organic modifier



Mobile phase: acetate buffer, pH 6.0-organic modifier (1:1), ionic strength as per graphs

the solubility of the solute in the mobile phase, retention is increased. Hence, the results in the present study might possibly be explained by a "salting-in" effect which lead to decreased retention of the peaks, thus causing them to elute near the solvent front and be undetectable in the area of interest along the chromatogram. This is, however, a tentative speculation, since the natures of the interferences have not been identified, and it is also possible, if they possessed a basic function, that decreased retention is due to reduced competition at the charged silanol sites.

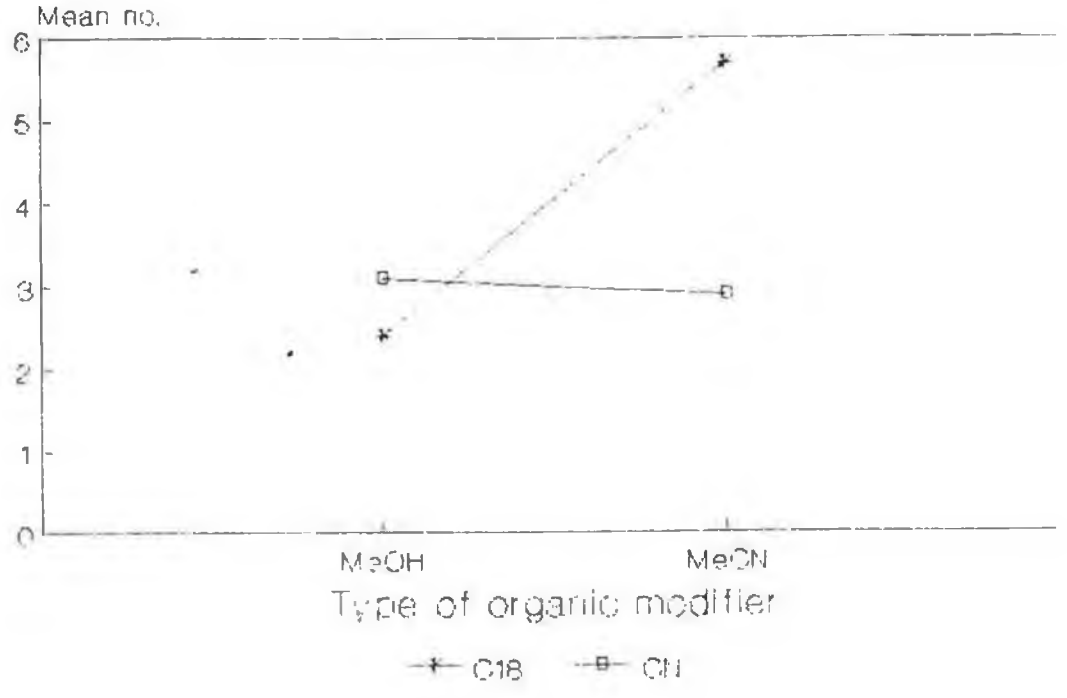
Referring to the chromatograms presented in Figure 7.6., it can be seen that as with the experiment on varying pH, the appearance of the chromatograms with a given column-organic modifier combination did not vary greatly between the higher and lower ionic strengths. This figure indicates that particularly on the C_{18}- column, the differences were slight, although the appearance of slightly more minor peaks at the lower salt concentrations, gave, under the statistical analysis applied here, the impression of a marked change in chromatogram quality. The salient point to be taken from this experiment was that if it is desired to increase the ionic strength in order to improve the chromatographic characteristics of particular analytes, this measure is unlikely to adversely affect the blank plasma profile as shown by statistical and qualitative analysis.

7.3.5. Effect of anticoagulant on the number of interfering peaks

Interference by materials in blood collection tubes is well documented in the literature [56-62]. The kinds of problems which can arise include a redistribution of drugs into erythrocytes brought about by a certain plasticizer in the stopper, or the presence of interfering peaks in the chromatogram originating in the body of plastic collection tubes [59]. Some workers found that heparin caused the introduction of an interfering peak in the chromatographic analysis of frusemide with fluorescence

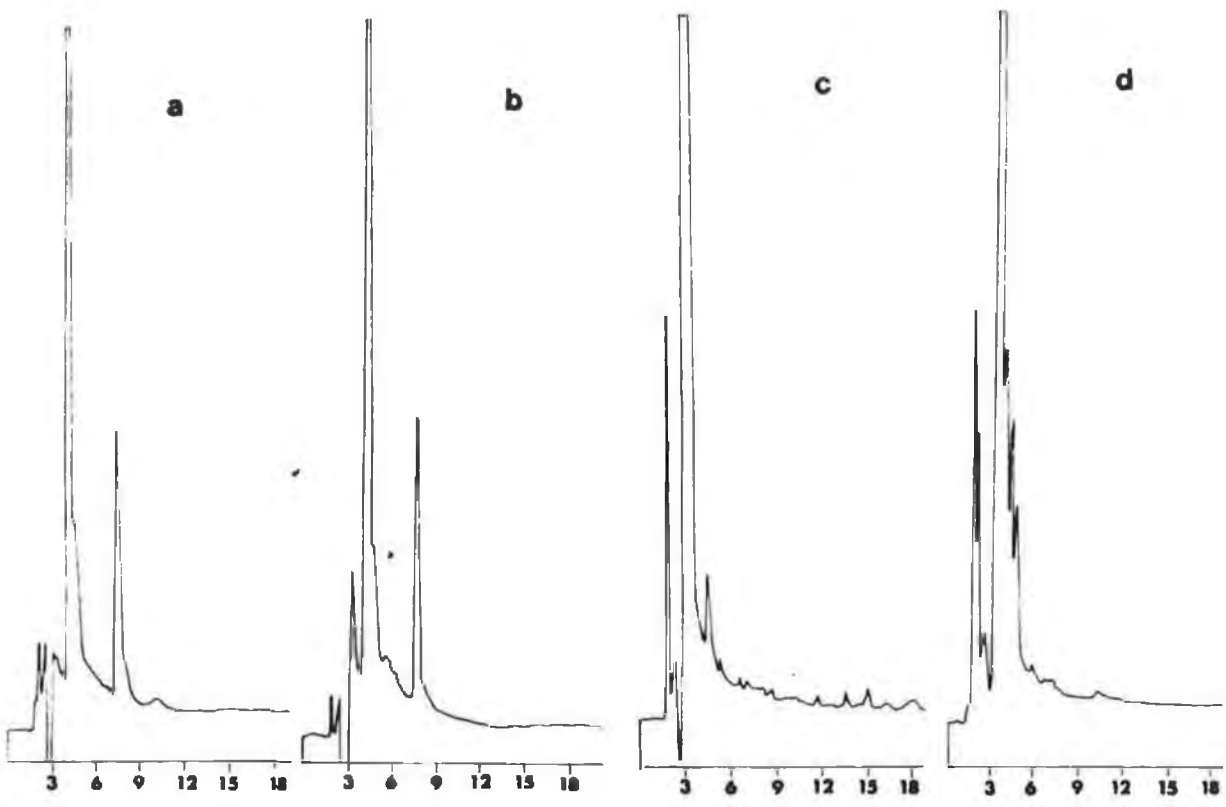
Figure 7.5.e.

Plot of number of peaks versus types of column and organic modifier- mean of ionic strengths



Mobile phase: acetate buffer, pH 6.0-organic modifier (1:1)

Figure 7.6.
Chromatograms showing effect of ionic strength on the C18 column



Mobile phase:

a: 0.005 M buffer, pH 6.0-methanol (1:1)

b: 0.1 M buffer, pH 6.0-methanol (1:1)

c: 0.005 M buffer, pH 6.0-acetonitrile (1:1)

d: 0.1 M buffer, pH 6.0-acetonitrile (1:1)

detection [56]. Vacutainer tubes containing EDTA (as the potassium salt) resulted in no interference using both fluorescence and ultraviolet absorbance detection.

Since the reports were usually a result of a problem arising during an assay for a particular drug, it was decided to investigate the effect of various anticoagulants on blank plasma profiles using the on-line solid phase extraction technique, and to observe whether differences in anticoagulants were detectable under constant mobile phase conditions.

Blood from a healthy volunteer was drawn into evacuated tubes containing various anticoagulants, in order to separate the plasma fraction, and into an empty tube in order to collect serum. As the blood from which this plasma was derived had a different origin than the pooled plasma used in previous experiments, comparisons were not made between the two. In addition, since the plasma used in this section was not screened, the possibility that some of the peaks may be due to xenobiotic products rather than endogenous plasma components must be recognised.

The plasma and serum samples were subjected to the same treatment in terms of preparation and extraction as the pooled plasma in previous experiments. The mobile phase was 0.025 M sodium acetate buffer, pH 6.0-acetonitrile (1:1). Acetonitrile was chosen since it generates more peaks in the chromatogram under these experimental conditions, and differences between anticoagulants would be more obvious. The types of anticoagulants investigated and the results obtained from three replicate injections of each plasma type are presented in Table 7.4. These data show that there were considerable differences between the numbers of peaks depending on the type of anticoagulant. The least number of peaks were obtained with lithium heparin and the greatest number with sodium citrate. The chromatograms presented in Figure 7.7. demonstrate more explicitly the differences between some of the anticoagulants, since in some cases the peaks may have been more

Table 7.4

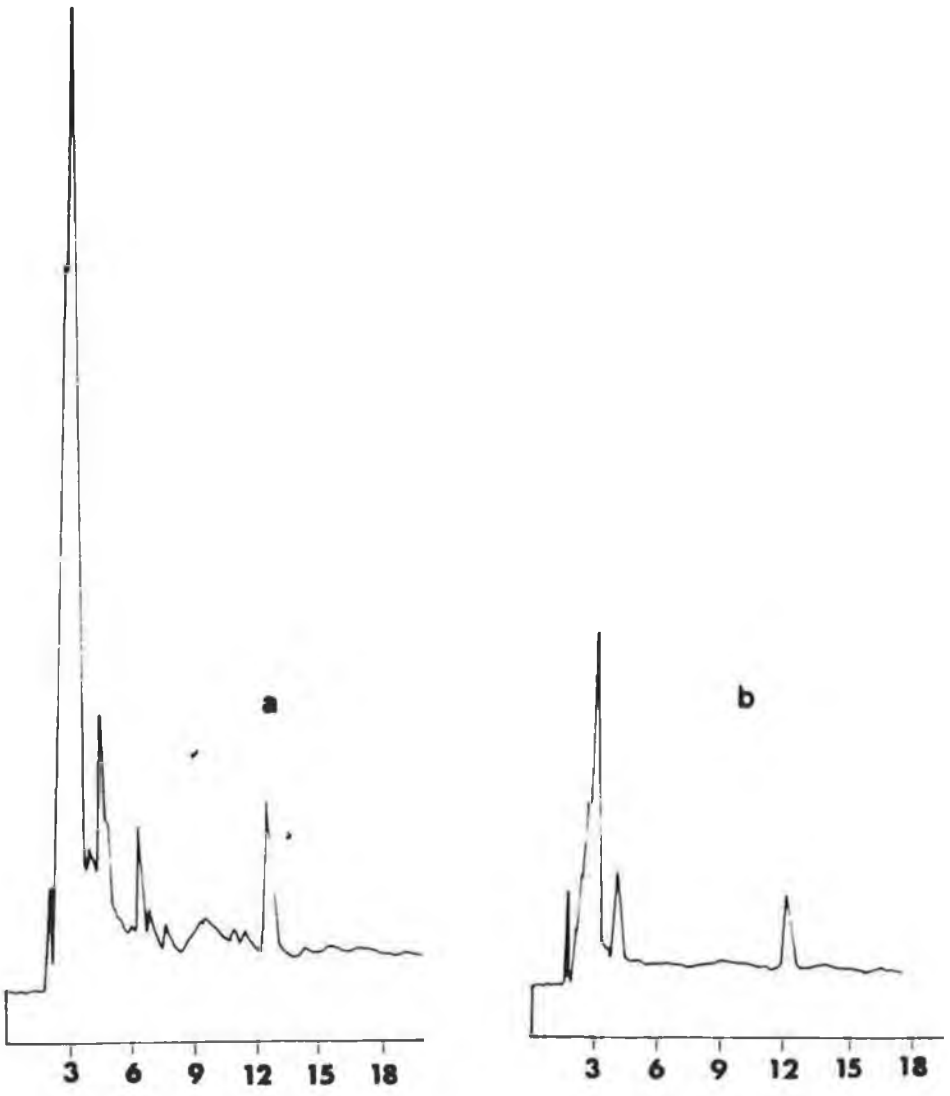
Effect of type of anticoagulant on blank plasma profiles

Type of anticoagulant	Number of interfering peaks		
	Injection number		
	1	2	3
No anticoagulant *	5	5	6
Lithium heparin	2	2	2
Fluoride oxalate	5	5	4
Sodium Citrate brand A	6	6	7
Sodium citrate brand B	7	7	7
Disodium edetate	5	6	5

* Serum

Mobile phase: 0.025 M sodium acetate, pH 6.0-acetonitrile (1:1)

Figure 7.7.
Chromatograms showing effect of anitcoagulant on peak number



Time (min)---------->

a: sodium citrate brand B

b: lithium heparin

Mobile phase: 0.025 M acetate buffer, pH 6.0-acetonitrile (1:1)

Column: C18

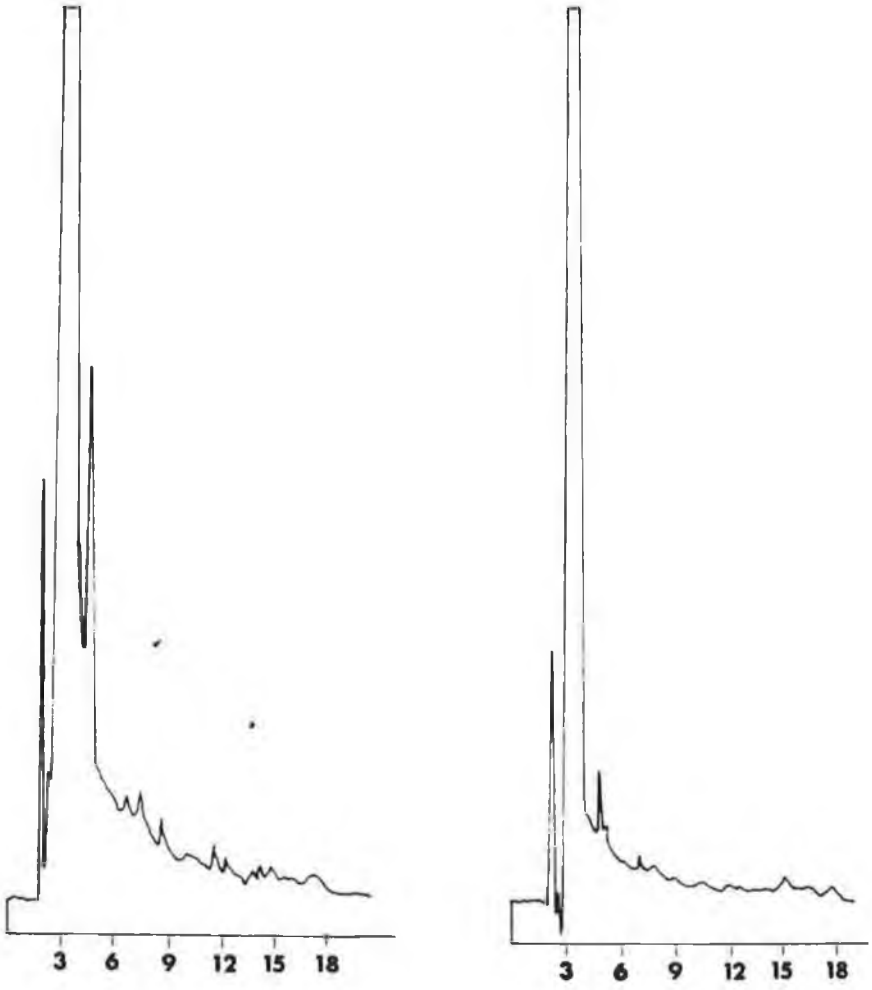
numerous but of smaller size, and vice versa. Although this was not an exhaustive study on the effect of anticoagulants, it does serve to illustrate that this element cannot be ignored when designing an analytical method for a drug in plasma or serum. Clearly, the type of anticoagulant should be defined when developing an analytical method and care should be taken to use the same one for method development and for the analysis of real samples, unless it can be established that the type of anticoagulant has no effect on the method being used.

7.3.6. Effect of type of packing in the concentration column

Using a mobile phase of 0.025 M acetate buffer, pH 6.0-acetonitrile (1:1) on the C_{18}- analytical column and the same wash regimen as used previously, the packing material in the concentration column was replaced with C_8- material in order to demonstrate the difference in the amount of plasma recovered from the two concentration columns.

The results obtained are illustrated by the chromatograms presented in Figure 7.8. This figure shows that in agreement with previous findings in Chapters 5 and 6, more plasma was retained by the C_8- column. The type of packing material used in the concentration column is usually dictated by the physico-chemical properties of the solute to be analysed, but where there is a choice, it would appear that C_{18}- material presents the more attractive alternative. The observed differences might be explained by the fact that the less hydrophobic C_8- material has greater retentivity for the relatively polar plasma constituents than the C_{18}- material. However, as outlined in the Introduction to this chapter, the chromatographic differences between the two materials are subtle, and frequently, variations between different brands are responsible for observed differences between and within generic types of columns.

Figure 7.8.
Chromatograms showing effect of type of packing in concentration column on amount of plasma recovered



: Sepralyte C_8- concentration column

: Corasil C_{18}- concentration column

Mobile phase: 0.025 M acetate buffer, pH 6.0-acetonitrile (1:1)

Column: C18

7.3.7. Concentration column reproducibility

In order to establish that the differences between concentration columns themselves were not contributing significantly to the trends observed in the foregoing experiments, reproducibility among different concentration columns was measured both on a within-day basis and a between-day bases. The wash regimen was 1.0 ml/min of deionised water for 90 seconds, and the analytical mobile phase was 0.025 M acetate buffer, pH 6.0-acetonitrile (1:1) on the C_{18}- column. Three replicate injections were analysed on each of three Corasil C_{18}- concentration columns on each of three consecutive days (9 columns, 27 injections). Having counted the number of peaks greater than two mm on the chromatograms, the results were analysed by carrying out a two-way ANOVA in order to identify significant differences among the columns within days and between days.

A block diagram of the number of peaks obtained on each column is presented in Table 7.5., in addition to the results of the ANOVA analysis. These findings show that the differences among the columns, on either a within- or between-day basis, were not statistically significant, though as indicated by the higher F-ratio, the between-day variation was, as expected, greater than the between-column variation. The results further demonstrate that the day-column interaction was not significant indicating that the variation in peak number between columns is not dependent on the day the analysis was carried out. Based on these results, it can be concluded that variation among concentration columns did not contribute significantly to the systematic effects seen in previous experiments, although it must be recognised that even relatively small variations among columns may have contributed slightly to the trends observed within a given set of experimental parameters.

7.3.8. Drug retention on the two columns

Finally, in order to demonstrate differences between the two

Table 7.5.
Reproducibility study of concentration columns

Number of interfering peaks(3 measurements per cell)

	Concentration column		
	1	2	3
Day 1	4,4,4	4,3,4	4,5,4
Day 2	4,5,4	5,4,4	4,4,4
Day 3	5,4,4	4,5,4	4,4,5

Mobile phase: 0.025 M sodium acetate buffer, pH 6.0-acetonitrile (1:1). Analytical column: C_{18}.

Two-way Analysis of Variance of above data

Source of Variation	Sum of Squares (SSQ)	Degrees of Freedom(DOF)	Mean Square (MSQ)	F Ratio
Main effects				
A	0.5	2	0.3	1.0 NS
B	0.1	2	0.1	0.1 NS
2-factor interaction				
A-B	0.8	4	0.2	0.8 NS
Residual	4.7	18	0.3	-
Total	6.1	26	-	-

Key: A: Day
B: Concentration column number

NS: not significant

columns in terms of drug retention chatacteristics, a selection of drugs of varying polarity and pK_a values were introduced in 20 μl aliquots by direct injection onto both analytical columns. The mobile phase was 0.025 M acetate buffer, pH 6.0-acetonitrile (1:1). The results of this experiment are presented in Table 7.6 in which drug retention times on both columns are tabulated. For many of the drugs, retention times were lower on the CN- column than on the C_{18}- column, as this mobile phase has stronger eluting power on the more polar column. It would seem, however, that the difference in many cases was not very large, and with some drugs (chloramphenicol and diazepam, for instance) there was no difference in their retention times on the two columns.

Selectivity differences between the two columns is illustrated by the fact that the three benzodiazepine drugs (diazepam, nitrazepam and chlordiazepoxide) had virtually the same retention times on the CN- column, but were retained to varying degrees on the C_{18}- column. On the other hand, the tricyclic drugs, desipramine and imipramine, in addition to quinine and piroxicam, had extremely long retention times on the C_{18}- column, but eluted with more acceptable retention times on the CN surface. The poor chromatographic characteristics of the tricyclic drugs on the C_{18} material is due to ionic interaction with residual silanol moieties, as previously discussed.

The fact that the two columns have similar retention properties for certain drug compounds, and highly dissimilar retention properties for plasma components, means that changing from one column to another may achieve separation between the compounds of interest and endogenous interferents, without the need for radical change in mobile phase composition.

7.4. CONCLUSION

The difference in selectivity between a C_{18}- and a CN- column for extracted plasma components has been demonstrated. It has been shown that the percentage organic modifier exerts a profound

Table 7.6.

Drug retention times on the C_{18}- and CN-columns

Drug	Retention time (min)	
	C_{18}	CN
Chloramphenicol	2.7	2.7
Chlordiazepoxide	4.8	3.0
Chlorthalidone	2.4	2.7
Chlorpheniramine	1.8	2.4
Desipramine	NE	18.0
Diazepam	5.4	3.0
Frusemide	1.8	1.8
Imipramine	NE	21.9
Nitrazepam	3.9	2.7
Piroxicam	NE	1.8
Quinine	NE	24.9
Sulphamerazine	3.3	2.7
Theophylline	3.3	3.0
Trimethoprim	5.4	4.5
Verapamil	10.2	12.3

NE = Not eluted after 60 minutes

Mobile phase: 0.025 M sodium acetate, pH 6.0-acetonitrile (1:1)

effect on blank plasma profiles and that this effect is strongly dependent on the column, and the the type and percent organic modifier employed. Based on the chosen response variable, best results were obtained on the cyano-column at high percentages of acetonitrile, and on the C_{18}- column with low percentages of methanol. These findings have been accounted for in terms of the different polarities of the columns, the relative solvent strengths of methanol and acetonitrile and the on-line method of plasma extraction.

The number of interfering peaks was also affected by eluent pH and ionic strength, though not to the same extent as by percentage organic modifier. The observed effects were also dependent on the type of column and organic modifier. Using the number of peaks criterion, the differences with changing pH and ionic strength seemed greater than was shown by the general apearance of the chromatogram, which suggests that an alternative response variable may be more appropriate when evaluating the influence of these factors.

It has been shown that more plasma components are recovered from the C_8- concentration column than from the C_{18}- concentration column. This may be due to the more polar packing having a greater affinity for the polar plasma constituents, although between-brand variability may be the principal contributing factor to the observed differences. Evidence suggests that the type of anticoagulant affects the quality of the blank plasma profiles, although the pattern found in this study may change depending on the column, mobile phase, the detection mode and origin of the plasma. Selectivity differences between the two columns as regards retention of a number of drug compounds has also been demonstrated. Under the employed experimental conditions, some of the drugs exhibit similar retention indices on both columns, a feature which may be exploited in the resolution of difficult separations of drugs and plasma.

The 3-factor analysis of variance design using the peak number response variable served to demonstrate the significance of the effects resulting from changing mobile phase composition, particularly in relation to percentage organic modifier. It also highlighted some very significant two-factor interactions within each set of experiments, and the significance of more than two of the three possible interaction terms in each case is strong presumptive evidence that a significant higher order interaction is present. The significance of this 3-factor interaction was not tested as the 2-factor interactions are easier to visualise and interpret. It is, however, recognised that since this term was not extracted from the residual term, the results for both main effects and interactions were conservative, and that this interaction may account for some findings, particularly in relation to the pH and ionic strength experiments. The incorporation of a 2-factor analysis of variance design tested the variation among concentration columns on a within-day and a between-day basis. The non-significance of the results was taken as evidence that reproducibility among columns sufficient to identify trends resulting from changing column and mobile phase compostition.

The on-line extraction technique proved useful for the evaluation of plasma extracts in this study. Because of the speed and convenience of the method, a large number of samples could be analysed; because of the inherent reproducibility of the method, effects resulting from changing mobile phase composition were easy to identify. However, as the amount of plasma extracted is related to the analytical mobile phase, an interesting exercise would be to perform a similar investigation based on off-line solid-phase extraction or the more traditional liquid-liquid extraction. On account of the large variety of possible off-line solid- and liquid-phase extraction schemes, there is considerable potential for further research in this area of biopharmaceutical analysis.

7.5. REFERENCES

1. R. W. Geise, Clin. Chem., 29 (1983) 1331.
2. A. P. Goldberg, Anal. Chem., 54 (1982) 342.
3. The Supelco Reporter (Supelco, Bellafonte, PA, USA) 1 (1982) 8.
4. A. Power and D. Dadgar, Anal. Proc., 23 (1986) 416.
5. D. Dadgar and A. Power, J. Chromatogr., 416 (1987) 99.
6. C. Horvath and W. J. Melander, J. Chromatogr. Sci., 15 (1977) 393.
7. W. Melander, D. E. Campbell and C. Horvath, J. Chromatogr., 158 (1976) 215.
8. R. P. Scott and C. F. Simpson, J. Chromatogr., 197 (1980) 11.
9. L. Hansson and L. Trojer, J. Chromatogr., 207 (1981) 1.
10. J. G. Atwood and R. Goldstein, J. Chromatogr. Sci., 18 (1980) 650.
11. J. L. DiCesare and M. W. Dong, Chromatogr. Newsl., 10 (1982) 12.
12. R. W. Stout, Techscan, 2 (1982) 14.
13. C. Gonnet, C. Bory and G. Lachatre, Chromatographia, 16 (1982) 242.
14. R. M. Smith, Anal. Chem., 56 (1984) 256.
15. J. N. Done, J. Chromatogr., 125 (1976) 43.
16. J. C. Chen and S. G. Weber, J. Chromatogr., 248 (1982) 434.
17. R. M. McCormick and B. L. Karger, Anal. Chem., 52 (1980) 2249.
18. M. J. Wells and C. R. Clark, Anal. Chem., 53 (1981) 1341.
19. A. M. Krstulovic, H. Colin and G. Guiochon, Anal. Chem., 54 (1982) 2438.
20. R. M. Smith, J. Chromatogr., 236 (1982) 313.
21. I. Halasz, Anal. Chem., 52 (1980) 1393A.
22. H. F. Walton, Anal. Chem., 50 (1978) 36 (R).
23. B. L. Karger and R. W. Greise, Anal. Chem., 50 (1978) 1048A.
24. H. F. Walton, Anal. Chem., 52 (1980) 15 (R).

25. B. L. Karger, J. R. Grant, A. Hartkopf and P. H. Weiner, J. Chromatogr., 165 (1979) 3.
26. S. R. Bakalyar, R. McIlwrick and E. Roggendorf, J. Chromatogr., 142 (1977) 353.
27. N. Tanaka, H. Goodell and B. L. Karger, J. Chromatogr., 158 (1978) 233.
28. S. van der Wal and J. F. Huber, J. Chromatogr., 149 (1978) 431.
29. K. Krummen and R. W. Frei, J. Chromatogr., 132 (1977) 27.
30. C. Horvath, W. Melander and I. Molnar, J. Chromatogr., 125 (1976) 129.
31. L. R. Snyder and J. J. Kirkland, Introduction to Modern Liquid Chromatography (Wiley Interscience, New York, 1979).
32. E. Papp and Gy. Vigh, J. Chromatogr., 282 (1983) 59.
33. E. Papp and Gy. Vigh, J. Chromatogr., 259 (1983) 49.
34. R. Gill, S. P. Alexander and A. C. Moffat, J. Chromatogr., 247 (1982) 39.
35. R. J. Flanagan, C. G. Storey, R. K. Bhamra and I. Jane, J. Chromatogr., 247 (1982) 15.
36. M. A. van Lancker, H. J. Nelis and A. P. de Leenheer, J. Chromatogr., 254 (1983) 45.
37. D. L. Massart and L. Buydens, J. Pharm. Biomed. Anal., 6 (1988) 535.
38. R. Bach, J. Karnicky and S. Abott in (T. H. Pierce and B. A. Hohne, Eds.) Artificial Intelligence Applications in Chemistry, (ACS, Washington, 1986).
39. T. P. Bridge, M. H. Williams and A. F. Fell, Anal. Proc., (1988) 43.
40. T. P. Bridge, M. H. Williams and A. F. Fell, Chem. Brit., 23 (1987) 1085.
41. F. Fell, T. M. Bridge and M. H. Williams, J. Pharm. Biomed. Anal., 6 (1988) 555.
42. R. J. Laub, Int. Lab., 5 (1981) 16.
43. R. J. Laub and and J. H. Purnell, J. Chromartogr., 112 (1975) 71.
44. J. C. Berridge, Anal. Proc., 22 (1985) 323.
45. L. R. Snyder, J. Chromatogr., 16 (1978) 223.

46. J. L. Glajch, J. J. Kirkland, K. M. Squire and J. M. Minor, J. Chromatogr., 57-59 (1980) 199.
47. S. M. Demming and S. L. Morgan, Anal. Chem., 45 (1973) 278A.
48. W. Spendley, G. R. Hext and F. R. Himsworth, Technometrics, 4 (1962) 441.
49. J. C. Berridge, Chromatographia, 16 (1982) 172.
50. J. C. Berridge, J. Chromatogr., 244 (1982) 1.
51. J. C. Berridge, Analyst, 109 (1984) 326.
52. J. C. Berridge in Techniques for the Automated Optimisation of HPLC Separations, (Wiley Interscience, New York, 1985).
53. P. Schoenmakers in Optimisation of Chromatographic Selectivity, (Elsevier, Amsterdam, 1987).
54. J. C. Millar and J. N. Millar, Statistics for Analytical Chemistry, (Ellis Horwood, Chichester, 1988).
55. J. Blanchard, J. Chromatogr., 226 (1981) 455.
56. R. S. Rapaka, J. Roth, T. J. Goehl and V. K. Prasad, Clin. Chem., 27 (1981) 1470.
57. D. J. Krogstad, G. G. Granich, P. R. Murray, M. A. Pfaller and R. Valdes, Clin. Chem., 28 (1982) 1517.
58. O. Borga, I. Petters and R. Dalqvist, Acta. Pharm. Suecia, 15 (1978) 549.
59. O. Borga, K. M. Piafsky and O. G. Nilsen, Clin. Pharmacol. Ther., 22 (1977) 539.
60. V. P. Shah, G. Knapp, J. P. Skelly and B. E. Cabana, Clin. Chem., 28 (1982) 2327.
61. R. Janknegt, M. Lohmann, P. M. Hooymans and F. W. Merkus, Pharm. Weekbl. Sci. Ed., 5 (1983) 287.
62. S. Maguire, F. Kyne and D. UaConaill, Clin. Chem., 33 (1987) 1493.

8. PUBLICATIONS

1. Drug Interactions in Community Pharmacy.
   Mary T. Kelly, Irish Pharmacy Journal, Oct 1984

2. HPLC Determination of Xipamide in Human Plasma.
   Darioush Dadgar and Mary T. Kelly, Analyst, 113 (1988) 329.

3. Determination of Chlorthalidone in Human Plasma by Reversed-Phase Micellar Liquid Chromatography.
   Darioush Dadgar and Mary T. Kelly, Analyst, 113 (1988) 1223.

4. Retention Characteristics in HPLC of Basic Drugs and Plasma on an Alumina Column.
   Mary T. Kelly, Malcolm R. Smith and Darioush Dadgar
   J. Chromatogr., 473 (1989) 53.

5. Recent Developments in High Performance Liquid Chromatography in Biopharmaceutical Analysis.
   Mary T. Kelly, Malcolm R. Smyth and Darioush Dadgar
   Anal. Proc. in Press.

6. Analysis of Basic Drugs by High Performance Liquid Chromatography using On-Line Solid-Phase Extraction and an Unmodified Silica Column.
   Mary T. Kelly, Malcolm R. Smyth and Darioush Dadgar
   Analyst, 114 (1989) in Press.

7. A Comparison of Silica and Alumina Columns for High Performance Liquid Chromatographic Separations of Basic Drug and Plasma Constituents following On-Line Solid-Phase Extraction.
   Mary T. Kelly and Malcolm R. Smyth, J. Pharm. Biomed. Anal.,in Press.