HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN BIOPHARMACEUTICAL ANALYSIS USING COLUMN SWITCHING TECHNIQUES.

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ABSTRACT

High performance liquid chromatographic methods are described for the determination of various drugs in biological fluids, using direct injection and a column switching valve. The methods are based on the enrichment of the drug on a reversed-phase concentration column followed by chromatographic analysis using various mobile phases. Members of three major drug groups were examined, Tricyclic Antidepressants, Antihistamines and Benzodiazepines.

One of the fundamental requirements of a bioassay is the capability to isolate and detect mixtures of polar and non-polar substances simultaneously, as is often the case with a drug and its metabolites. In the determination of amitriptyline and its metabolites, nortriptyline, 10-hydroxynortriptyline and 10-hydroxyamitriptyline, a direct injection/column switching procedure is described which determines all four analytes simultaneously with excellent recovery. A conventional liquid-liquid extraction procedure is also described which failed to isolate the non-polar metabolites. Both methods are fully validated, compared and applied to samples from patients undergoing treatment with amitriptyline.

For the determination of tripelennamine (an antihistamine), in bovine plasma and milk an on-line solid phase extraction technique is described. Bovine plasma proved to be chromatographically cleaner than human plasma but operationally more difficult to handle due to its viscous

nature. The extension of the method for clean-up of milk samples was also investigated. With centrifugation prior to injection this proved possible. The method was fully validated. Setistine, a novel antihistamine was also determined using a solid-phase extraction technique.

In the final section a direct injection/column switching procedure for protein bound drugs is described. It is applied to the determination of diazepam and its metabolites desmethyl diazepam, temazepam and oxazepam, in plasma. The method is fully validated and compared to a classical liquid-liquid extraction scheme.

DECLARATION

I declare that this thesis, submitted in candidature for the award of Ph. D., has been prepared by me and is a result of my own work.

Signed: Anna M. Power

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To my parents

CHAPTER 1: CHROMATOGRAPHY - THEORY AND PRACTICE

CHAPTER 2: DRUG ANALYSIS IN BIOLOGICAL FLUIDS - METHODOLOGY AND CONSIDERATIONS

CHAPTER 3: INTRODUCTION TO COLUMN SWITCHING

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CHROMATOGRAPHY - THEORY AND PRACTICE

CHAPTER I

1.1. INTRODUCTION AND HISTORY OF CHROMATOGRAPHY

The discovery of chromatography is generally credited to Tswett [1,2] who in 1903 described his work on using a chalk column to separate the pigments in green leaves. An English translation of his work is also available [3]. At around the same time D.T. Day was also using chromatography to separate petroleum fractions [4-6] but Tswett was the first to correctly interpret the chromatographic process. He not only separated and isolated the pigments of a green leaf but did this without any chemical change occuring so that they could be used for characterisation purposes. He described the procedure and emphasised its importance in analytical chemistry. The nomenclature used today derives from Tswetts original suggestions. The tube filled with adsorbent is the chromatographic column. The liquid is the chromatographic solvent, wash liquid or developer. The series of zones in the column are called the chromatograph and the washing of the mixture to form the chromatogram is the development. The resolution of the mixture using the chromatographic column is chromatographic adsorption analysis or chromatography.

Despite the work carried out by Tswett, a lull followed for many years and it was not until the early forties that chromatography started to evolve. The foundation for thin layer chromatography (TLC) was laid in 1938 by Izmailov and Scraiber [7] and later refined by Stahl [8,9] in 1958. The remarkable work of Martin and Synge in 1941 [10] not only revolutionised liquid chromatography (LC) but in general set

the stage for the development of gas chromatography (GC) [11]. Between 1952 and the 1960's gas chromatography evolved into a sophisticated analytical technique with much work accredited to Martin and James [12-15], who in 1952 separated fatty acids and later on amines on a stationary phase of silicon oil containing 10% stearic acid. Manufacture of the first commercial gas chromatograph was in 1955.

At first liquid chromatography was performed in large diameter glass columns with long analysis time. In the late 1960's more and more emphasis was placed upon developing liquid chromatography as a complementary technique to gas chromatography. High performance liquid chromatography (HPLC) has evolved from this effort with advances in both instrumentation and column packings occuring rapidly. It is now among the most powerful and versatile separatory techniques ever devised and in the following section I will examine the various modes available today.

The feature distinguishing chromatography from most other physical and chemical methods of separation is that two mutally immiscible phases are brought into contact wherein one phase is stationary and the other mobile. The sample mixture, introduced into the mobile phase, undergoes a series of interactions (partitions) many times between the stationary and mobile phases as it is being carried through the system by the mobile phase. Interactions exploit differences in the physical or chemical properties of the components in the sample. These differences govern the rate

of migration of the individual components under the influence of a mobile phase moving through a column containing the stationary phase.
Separated components emerge in the order of increasing interaction with the stationary phase. The least retarded component elutes first, the most strongly retained material elutes last. Separation is obtained when one component is retarded sufficiently to prevent overlap with the zone of an adjacent solute as sample components elute from the column.

The column is at the heart of chromatography and provides versatility in the types of analyses that can be performed with a single instrument. This versatility, due to the wide choice of materials for the stationary and mobile phases, makes it possible to separate molecules that differ only slightly in their physical and chemical properties but can also be a problem when it comes to select an appropriate chromatographic method.

1.2. CLASSIFICATION OF CHROMATOGRAPHIC METHODS

Chromatographic methods can be divided into two groups according to the mobile phase used. The mobile phase can be a gas (GC) or a liquid (LC).

Chromatographic methods can be classified further according to the nature of the stationary phase. Thus, under the gas chromatography category are GLC and GSC - gas liquid chromatography and gas solid chromatography. Liquid chromatography is divided into two main types: column

chromatography and the planar chromatograpic methods - thin layer chromatography (TLC) and paper chromatography (PC). Column chromatography is subdivided further into five major column types which constitute HPLC. These include LSC (liquid solid chromatography), BPC (bonded phase chromatography), IEC (ion exchange chromatography), and EC (exclusion chromatography).

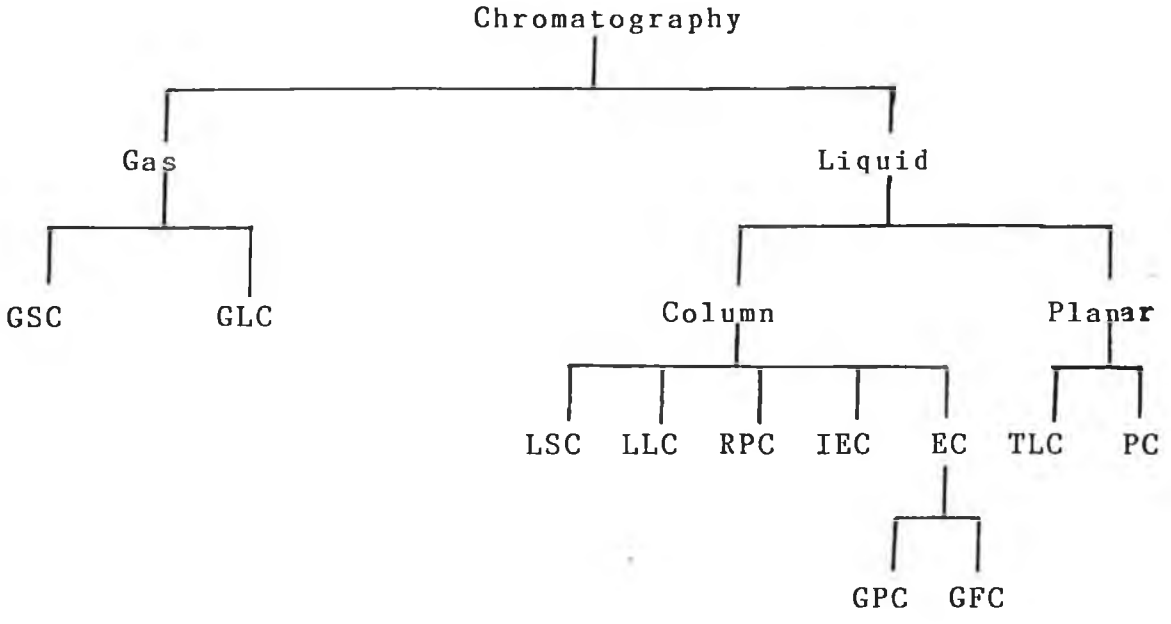


Fig. I - Classification of chromatographic methods.

1.3. THEORY OF CHROMATOGRAPHY

Differential migration of components of a mixture is the basis of separation in chromatography. An understanding of this migration mechanism is therefore essential. This will now be discussed.

When a solute zone migrates through the column, it does so with a fraction R of the velocity of the mobile phase v. At the molecular level, at any given time some of the solute molecules are sorbed while some remain in the mobile phase getting carried along by it. The solute zone therefore constantly moves forward despite the fact that only a fraction of the zone's molecules are mobile at any given time. Due to the stop-go nature of the movement of each molecule in the column, molecules spread randomly in the solute zone. This explanation of zone spreading by random migration is usually referred to as the "random walk model".

One approach to the understanding of zone migration is the kinetic model[16]. It is proposed that when a molecule is in the sorbed state, it remains stationary for an interval of time before it is desorbed again. This time interval (t_d) is called the desorption time. In the next stage the same molecule then desorbs for a period of time (t_a) known as the adsorption time. It is during this time that the molecule is carried forward with a mean flow velocity v. It can be stated therefore, that a molecule spends only a fraction of its time in the mobile phase, this fraction being the period of time during which the molecule is

moving, divided by the sum of the adsorption and desorption times. Since the molecule is part of the solute-zone, the theory can also be applied to the zone.

$$R_f = \frac{t_a}{(t_a + t_d)} \qquad (1)$$

This equation expresses the fraction velocity, R, in terms of the time interval of a single sorption - desorption event indicating the kinetic nature of solute migration through the stationary phase in a column.

The second approach is based on an equilibrium system. In this case, it is considered that an equilibrium is reached in the distribution of the solute between the stationary and mobile phase. It can be considered that in a small region of the column the amount of solute in the mobile phase will equal the local concentration of the solute (C_m) times the volume of mobile phase (V_m) in that region. The amount of solute in the stationary phase will equal the local concentration of the solute (C_s) times the volume of stationary phase (V_s). Therefore

$$R_f = \frac{C_m V_m}{C_m V_m + C_s V_s} \qquad (2)$$

which may also be written as

$$R_f = \frac{V_m}{V_m + (C_s/C_m) V_s} \qquad (3)$$

But since K (distribution constant) = (C_s/C_m) (4)

Then $$R_f = \frac{V_m}{V_m + KV_s} \quad (5)$$

For a given value of V_m and V_s, the value of R_f depends only on the distribution constant. Factors that influence K will also affect the migration of the solute. Temperature has a marked effect on solute migration especially in gas chromatography. The temperature dependance of the distribution constant, is given in equation 6.

$$K = \text{Constant}.e^{-\Delta H^{\circ}/RT} \quad (6)$$

where H° = Enthalpy, R = Gas Constant and T = Absolute Temperature.

An increase in absolute temperature will lead to a decrease in K, since the change in enthalpy is negative for exothermic sorption. This results in an increase in R_f which therefore means a shorter retention time. The temperature dependence of retention can be utilised to improve resolution in chromatography. Another factor affecting R_f is pressure, which has a comparatively smaller influence than temperature. However at very high pressures K can be varied significantly. This has been observed in adsorption and gel permeation chromatography.

The chemical nature of the mobile and stationary phases also influences the value of K. In general polar solutes tend to be sorbed in polar phases and non-polar solutes in non-polar

phases. Thus the migration of solutes can be controlled by changing the polarity of one or both phases.

1.3.1. Zone Spreading

There are three main contributions to zone spreading. These are

(i) multiple paths of a solute through the column packing

(ii) molecular diffusion

(iii) non-equilibrium mass transfer.

1.3.1.1. Multiple paths

Depending upon the bed structure of the column, the velocity of different molecules of the solute moving across the column bed may vary significantly. Some molecules will travel more quickly by going along the more open or low resistance paths near the top of the column. This is called channelling. Others diffuse into spaces between the particles and these get left behind. This is called eddy diffusion. (Fig. 2) Ideally the solvent molecules should proceed through the packed bed by a tortuous path avoiding either one of the above effects. It is the average velocity of the solute that determines its retention time.

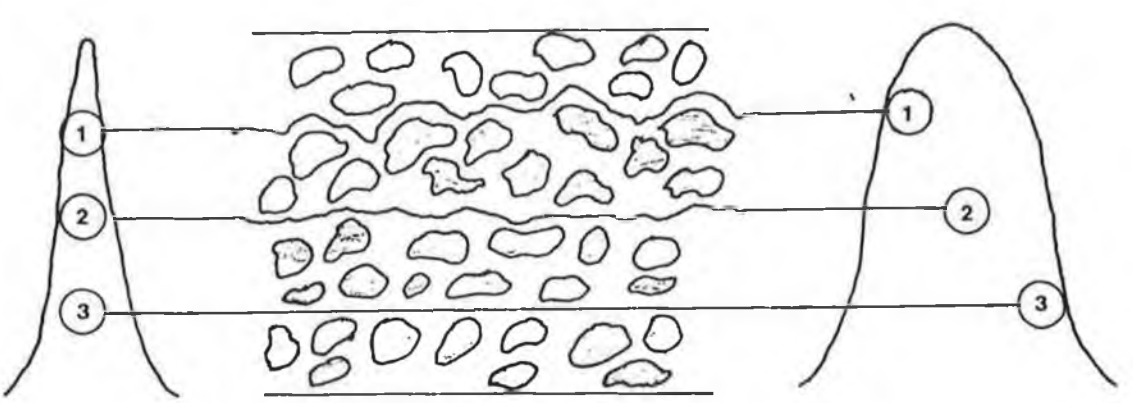
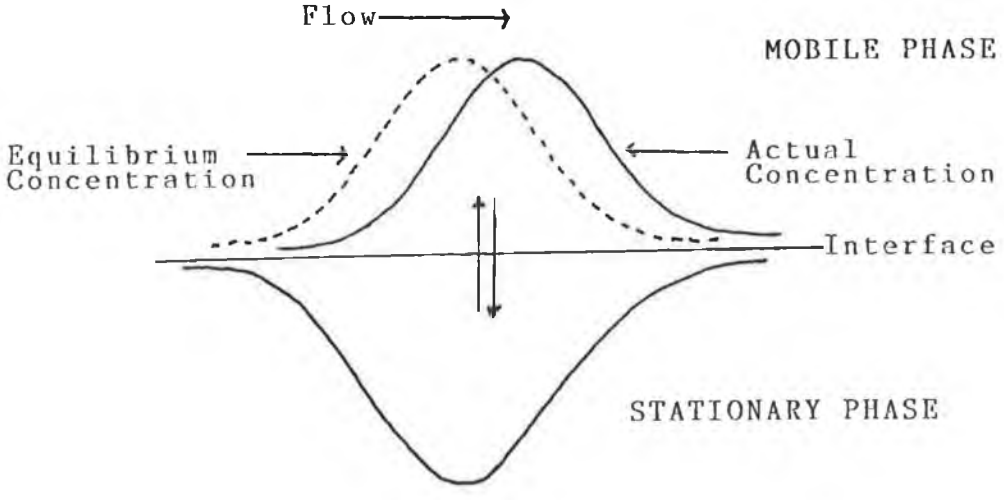


Fig. 2 Schematic diagram showing multiple paths for different molecules moving across a column.

1.3.1.2. Solute Diffusion

Diffusion causes the solute molecules, initially in a narrow band to spread out into the surrounding solvent and broaden the band profile. In principle, longitudinal diffusion may occur in both mobile and stationary phases, but because the rate is minimal in the stationary phase it is usually ignored. This phenomenon is much more important when the mobile phase is a gas. Band broadening by molecular diffusion is rarely a factor important in lowering efficiency in an L C column.

Fig. 3 Illustration of Mass Transfer Effect.



1.3.1.3. Mass Transfer

When the molecule is in the stationary phase, it is retained and falls behind the band centre as the band centre continues to migrate down the column. When the molecule is in the mobile phase it moves with the mobile phase. Its velocity is faster than the band centre since the flow velocity is always greater than the band velocity. This random transfer back and forth between the mobile and stationary phases causes dispersion in the chromatographic peak as some molecules will by chance migrate ahead of the average and others will migrate more slowly than the average (Fig 3). If there were no flow, the band would

have an equilibrium concentration, as shown by the dotted line above the interface and the solid line below the interface. However since there is a flow in the mobile phase, the actual concentration of solute in the mobile phase is always in non-equilibrium with that in the immediately adjacent stationary phase. The dispersion or dilution of the chromatographic peak is minimized by choosing conditions such that the non-equilibrium is reduced and the rate of exchange is maximised.

The undesirable effects of the non-equilibrium processes can be decreased by reducing the flow rate to allow a longer time for the equilibrium to be reached. Another factor is the tightness of the packing. If the channels through which the mobile phase flows are narrow, a greater equilibrium is established since the solute molecules have less distance to diffuse before reaching the stationary phase. This will result in less zone spreading. For the same reason a thinner layer of stationary phase will minimise zone spreading.

1.3.2. Mechanism of Zone Migration

The simplest approach to the mechanism of zone migration is through considering chromatography as a "random walk" process[17]. This model enables us to have a better understanding of the processes occurring during the chromatographic separation.

1.3.2.1. The "Random Walk" model and the van Deemter equation

All of the factors affecting zone spreading occur in a

random fashion. Ordinary diffusion occurs at the molecular level in a random fashion. Eddy diffusion results from the flow of molecules through randomly spaced particles that make up the stationary phase. Non-equilibrium mass transfer is governed by the rate of the kinetic processes that transfer the solute between the stationary and mobile phase. All such kinetic processes are random at the molecular level.

A simplified example of random walk is a drunken man not knowing his way. Each step he takes is independent of the previous one. Each step is the same length but the direction is chosen randomly. If many people were to take a "random walk" from the same point, some would end up on one side of the starting point and some on the other. If a plot of their number at various points against distance was to be drawn, one would obtain a bell-shaped Gaussian curve. Statistically, the extent of their spreading would be the standard deviation, σ . The value of σ obtained from the random walk theory is given by the following equation.

$$\sigma = 1\sqrt{n} \qquad (7)$$

where 1, is the length of each step and n, the number of steps taken. If several random processes are involved, the net result is given by equation 8.

$$\sigma^2 = \sum \sigma_i^2 \qquad (8)$$

In the case of chromatography, three independent factors contribute to the overall result, each of which can be

calculated separately.

1.3.2.2. Calculation of the ordinary diffusion effect

The σ_i^2 value for ordinary diffusion can be related to the coefficient of diffusion, D, in the mobile phase, by the following equation.

$$\sigma_D^2 = 2Dt \qquad (9)$$

where (t) is the time spent in the mobile phase since the beginning of the random process (it is assumed that only a negligible amount of diffusion occurs in the stationary phase).

The equation for distance on the other hand is given by:

$$L = \nu t \qquad (10)$$

where (L) is the distance the zone migrates and ν the velocity of the mobile phase. Substitution of L/ν for (t) in equation (9) will give the final equation for σ_D^2.

$$\sigma_D^2 = 2DL/\nu \qquad (11)$$

This equation represents the total contribution of ordinary diffusion in the overall solute zone spreading.

1.3.2.3. Calculation of the eddy diffusion effect

Eddy diffusion is also a random process due to the solute molecules being carried through an uneven pathway by the

mobile phase. The solute molecules alternate between "fast" and "slow" channels. This is similar to a step forward and a step backward. A channel between two particles will have an approximate length of one diameter, d_p. Depending on whether it is a "fast" or "slow" channel, each step will be in the order of +d or -d with respect to the centre of the solute zone. Thus the equivalent step on the average is approximately, d_p. The number of steps, n, is therefore the number of channels with the length, d_p, present in the displacement distance of the zone, L.

$$L = nd_p \text{ or } n = L/d_p \qquad (12)$$

However, it has to be noted that not all the channels can be classified as "fast" or "slow". All the channels are not equal to d_p, but the assumption taken is approximately correct. In the random walk equation (7) by replacing (1) with d_p and n with L/d_p the final equation representing the eddy diffusion contribution to the solute zone spreading is obtained.

$$\sigma_E = \sqrt{Ld_p} \qquad (13)$$

1.3.2.4. Calculation of the non-equilibrium effect

In this case a similar analysis can be applied. The rate of equilibrium between the mobile and stationary phase can be controlled either by diffusion from one phase to the other or by an adsorption-desorption process. As previously defined, the average time required for desorption of an attached molecule is t_d, and the time taken for adsorption

to take place is t_a. This adsorption-desorption process resembles a random walk process again. A desorbed solute molecule moves faster than the centre of the zone of velocity $R\sqrt{}$ (R<1). In the adsorbed state, it can be considered to be moving backward with respect to the centre of the zone. A resemblance with the random walk can be seen closely here, each step being a phase transfer process depending on a random energetic collision. In order to relate the random walk concept to those processes involved, one has to define the number of steps and the length of each step.

The number of steps, n, is the total number of adsorption and desorption steps (total number of cycles). Since each phase transfer terminates a desorption or adsorption step (or a "lifetime"), n is also equal to the number of phase transfers. Thus, n, is twice the number of adsorption (or desorption) steps. The time needed for a solute zone to move a distance, L, at a velocity $R\sqrt{}$ is simply $L/R\sqrt{}$. The fraction, R, represents the time a molecule spends in the mobile phase while (1-R) represents the time it spends in the stationary phase. The average time spent in the stationary phase would therefore be: $(1-R)L/\sqrt{}R$. To find the number of desorption steps this value is divided by the average time required for a single desorption step, t_d, $(1-R)L/R\sqrt{}t_d$. And from this,

$$n = \frac{2(1-R)L}{R\sqrt{}t_d} \qquad (14)$$

The length of step, 1, is the length of the step the solute

molecule moves back with respect to the centre of the zone. In fact, it is not the molecule that is moving back, but rather the centre of the zone that is moving forward, when the molecule is immobilised during the sorption process. This time is t_d, and the distance moved is $R\sqrt{t_d}$.

Substitution of $R\sqrt{t_d}$ for 1, and $[2(1-R)L]/[R\sqrt{t_d}]$ for n, in the equation (7) results in the following equation:

$$\sigma_C = R\, t_d \sqrt{[2(1-R)L]/R\sqrt{t_d}} \qquad (15)$$

or

$$\sigma_C = \sqrt{2R(1-R)\sqrt{L t_d}} \qquad (16)$$

showing the contribution of non-equilibrium mass transfer to the solute zone spreading. Now it can be seen from the above equation that: (a) an increase in mobile phase velocity will increase the zone spreading, (b) the rapid exchange of solute molecules from stationary phase to mobile phase will decrease the spreading (a smaller t_d), and (c) the spreading due to non-equilibrium mass transfer in directly proportional to $\sqrt{L}$.

The total zone spreading is therefore equal to the sum of the three values of σ, written as:

$$\sigma^2 = L\,(2/D\sqrt{} + d_p + 2R(1-R)\sqrt{t_d}) \qquad (17)$$

A better known quantity representing the solute zone spreading in chromatography is given in "Plate theory". In this theory a chromatographic column is envisaged as being composed of a series of discrete, but contiguous, narrow,

and horizontal layers called theoretical plates, between which the equilibration of solute molecules, between mobile and stationary phases takes place. Movement of the solute and solvent is then viewed as a series of stepwise transfers from one plate to the next.

Based on this theory, it is considered that as the number of plates increases so does the efficiency of a chromatographic column.

The definitions of efficiency are taken from this theory, with efficiency values being expressed as the number of theoretical plates (N) or the height equivalent to a theoretical plate (HETP). From this,

$$N = \frac{L}{HETP} \qquad (18)$$

or

$$N = \frac{L}{H} \qquad (19)$$

where L is equal to the length of the column.

Also $$H = \sigma^2/L \qquad (20)$$

or

$$HL = \sigma^2 \qquad (21)$$

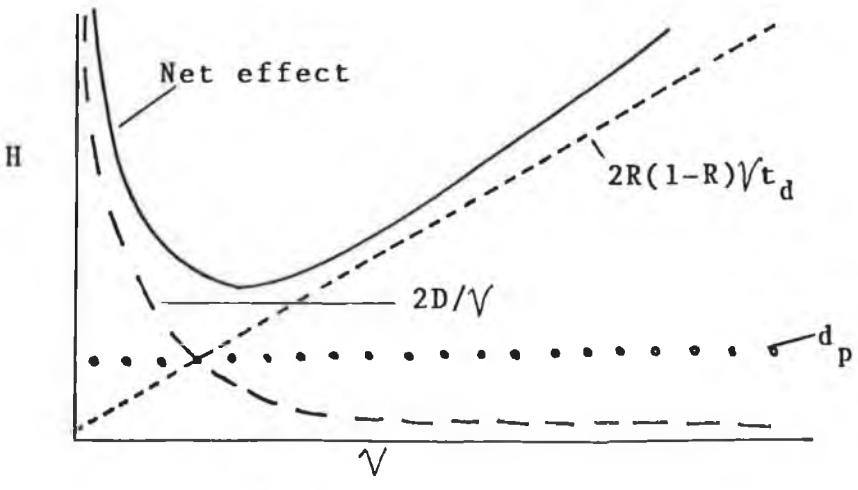
By substituting HL for σ^2 in equation, 17 the final form of the equation is obtained.

$$H = (2D/\sqrt{}) + d_p + 2R\,(1-R)\sqrt{t_d} \qquad (22)$$

This equation is the van Deemter equation [18] and serves as a first approximation to plate height.

The important characteristic of H in this equation is that it is independent of the distance migrated by the solute molecule, in any uniform column. By plotting H, versus $\sqrt{}$ the following graph is obtained.

Fig. 4



van Deemter Plot

A graphical description of the van Deemter plot is shown in fig 4. The plot shows the dependence of H on linear velocity for each of the three spreading sources, and the overall dependence as the sum of the three. At low flow rates, diffusion dominates spreading. At high flow rates, non-equilibrium dominates. There is an optimum velocity at which H is a minimum. In LC the diffusion rates are so low that the minimum is seldom seen.

1.3.3. Optimisation of conditions in Chromatography

Temperature and the nature of the mobile phase are among other variables which affect the value of H. The effect of these variables can be explained by the fact that the efficiency of the separation depends on,

(a) the separation of the zone centres

(b) the compactness of the bands.

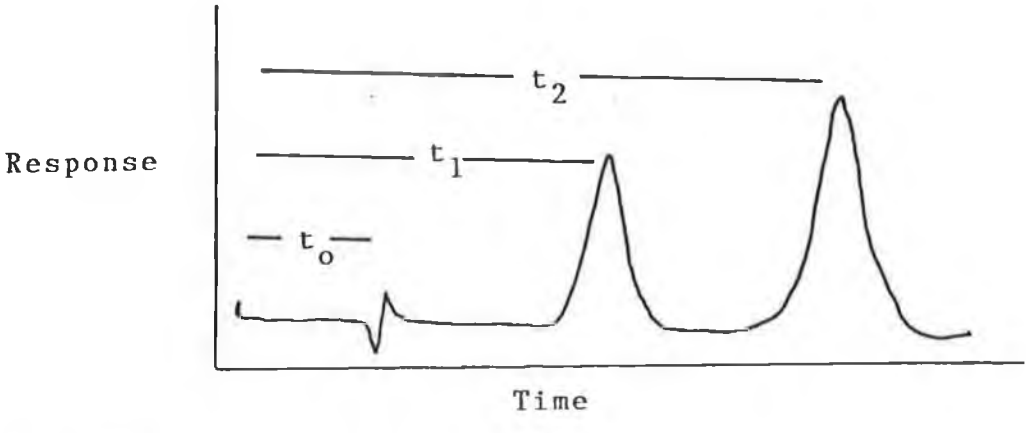
In order to achieve a good separation both these factors must be optimised. Temperature affects both these factors. The velocity of the mobile phase only affects the compactness of the zones.

1.3.4. Resolution

The resolution R_s is defined by the distance between the band centres divided by the average band widths in the same units.

$$R_s = 2 \frac{t_{R_2} - tR_1}{W_1 + W_2} \text{ in time units.}$$

It is an expression of the separation between components, depending on the narrowness of the peaks and the separation between the peak maxima.



$V_o = F \times t_o$

$V_1 = F \times t_1$

$V_2 = F \times t_2$

F = Flow rate (ml/min)
t_o = Retention time of non-retained component
t_1 = Retention time for component 1
t_2 = Retention time for component 2

Purnell[19] has transformed the resolution equation to give

$$R_s = 1/4\ (L/H)[K'/(1+K')]\ (\alpha-1) \qquad (23)$$

where α the separation factor is given by

$$\alpha = (V_2-V_o)/(V_1-V_o) = K'_2/K'_1$$

Resolution may also be expressed as

$$R_s = (t_2-t_1)/[2(\sigma_2+\sigma_1)] \qquad (24)$$

where σ_1 and σ_2 are the standard deviations in units of time. The resolution should be greater than or equal to one for a satisfactory separation of any two compounds. The simultaneous optimisation of variables in the separation process is impossible in practice because of the numerous factors involved. The optimum conditions are best established by relating the individual factors in the resolution equation, (24), to temperature. Therefore t and σ should be expressed in terms of T.

Retention time t, can be expressed as $[L]/[R\sqrt{}]$ with L and $\sqrt{}$ being independent of T. R_f can be related to T by the equilibrium equation.

$$R_f = \frac{V_m}{V_m+KV_s} \qquad (25)$$

and the equation

$$K = \text{Constant}\ .e^{-\Delta H^{\circ}/RT} \qquad (26)$$

The standard deviation in units of time, σ, can be defined as the time required to sweep the zone along a distance

equal to σ. Therefore:

$$l = \frac{\sigma}{R_f} \qquad (27)$$

Of all three contributing factors to σ, only two are influenced by changes in temperatures. Ordinary diffusion depends on temperature through the diffusion coefficient, D. The non-equilibrium term varies with changes in temperature because of its relation with R and T_d. The value of t_d itself is related to rate constant through the Arrhenius equation 6. In this fashion step by step and term by term the temperature dependance of resolution is determined. Then a plot of R_s against T, can be drawn from which the maximum R_s can be obtained. Theoretically the derivative of R_s with respect to T, $[dR_s]/[dT]=0$ should lead to the R_s maximum. An inverse relationship exists between R_s and T with a decrease in temperature improving the resolution.

The more straightforward variables like the column length can easily be related to R_s, since the retention time t, is equal to $[L]/[R\sqrt{}]$, and is therefore proportional to $\sqrt{L}$, thus R_s is proportional to $\sqrt{L}$. This means a longer column will give a better resolution. The major limitation in this case would be maintaining the pressure through a longer column, or in paper chromatography, the evaporation of solvent from the paper strip.

Other variables such as particle size, flow velocity, thickness of the stationary phase layer, and the mobile phase properties can be accounted for in the same fashion.

In the same way, one can establish and calculate the mimimum time required for the separation of a multicomponent mixture, keeping in mind that R_s should not be less than one.

1.4. CHROMATOGRAPHIC MECHANISMS

During a chromatographic separation solute molecules are continually moving back and forth between the stationary and mobile phases. While they are in the mobile phase, they are carried forward with it but remain virtually stationary during the time they spend in the stationary phase. The rate of migration of each solute is therefore determined by the proportion of time it spends in the mobile phase, or in other words by its distribution ratio.

The process whereby a solute is transferred from a mobile to a stationary phase is called sorption. Chromatographic techniques are based on four different sorption mechanisms, namely surface adsorption, partition, ion-exchange and exclusion. The orignal method employed by Tswett involved surface adsorption where the relative polarities of solute and solid stationary phase determine the rate of movement of that solute through a column or across a surface. If a liquid is coated onto the surface of an inert solid support, the sorption process is one of partition, and movement of the solute is determined solely by its relative solubility in the two phases or by its volatility if the mobile phase is gas. Both adsorption and partition may occur simultaneously, and the contribution of each is determined by the system parameters, i.e. the nature of the mobile and stationary phases, solid support and solute. For example, a stationary phase of aluminium oxide is highly polar and normally exhibits strong adsorptive properties. However, these may be modified by the presence of adsorbed

water which introduces a degree of partition into the overall sorption process by acting as a liquid stationary phase. Conversely, paper (cellulose) is relatively non-polar and retains a large amount of water which functions as a partition medium. Nevertheless, residual polar groups in the structure of the paper can lead to adsorptive effects.

The third sorption phenomenon is that of ion-exchange. Here, the stationary phase is a permeable polymeric solid containing fixed charged groups and mobile counter-ions which can exchange with the ions of a solute as the mobile phase carries it through the structure.

The fourth type of mechanism is exclusion although perhaps 'inclusion' would be a better description. Strictly it is not a true sorption process as the separating solutes remain in the mobile phase throughout. Separations occur because of variations in the extent to which the solute molecules can diffuse through an inert but porous stationary phase. This is normally a gel structure which has a small pore size and into which small molecules up to a certain critical size can diffuse. Molecules larger than the critical size are excluded from the gel and move unhindered through the column or layer while smaller ones are retarded to an extent dependent on molecular size.

In each chromatographic technique one of the four mechanisms pre-dominates, but it should be emphasised that two or more may be involved simultaneously. Partition and adsorption frequently occur together and in paper chromatography, for example, ion-exchange and exclusion play minor roles also.

These different modes will now be described in more detail.

1.4.1. Liquid Solid Chromatography (LSC)

Adsorption HPLC, because of its versatility, is often the answer to a separation problem. If the sample is soluble in non-polar or moderately polar solvents such as hexane, methylene chloride, chloroform, or diethyl ether, then adsorption chromatography is a likely choice.

This technique depends on specific interactions of the solute with the surface of a finely divided adsorbent (silica gel, alumina or charcoal).

1.4.1.1. Snyder Model

According to the Snyder model [20], the mobile phase and the solute are in competition for the active sites on the adsorbent. This competition can be represented by,

$$X_m + nS_{ads} \rightleftharpoons X_{ads} + nS_m$$

where n represents the number of mobile phase molecules that must be displaced to accommodate the solute molecule (Fig. 6).

Equilibrium expression

$$K = \frac{(X_{ads})(S_m)^n}{(X_m)(S_{ads})^n}$$

This expression shows that the most important interaction governing the separation by LSC is the adsorbent-solute interaction. As the relative adsorption of the mobile phase increases the adsorption of the solute must decrease leading

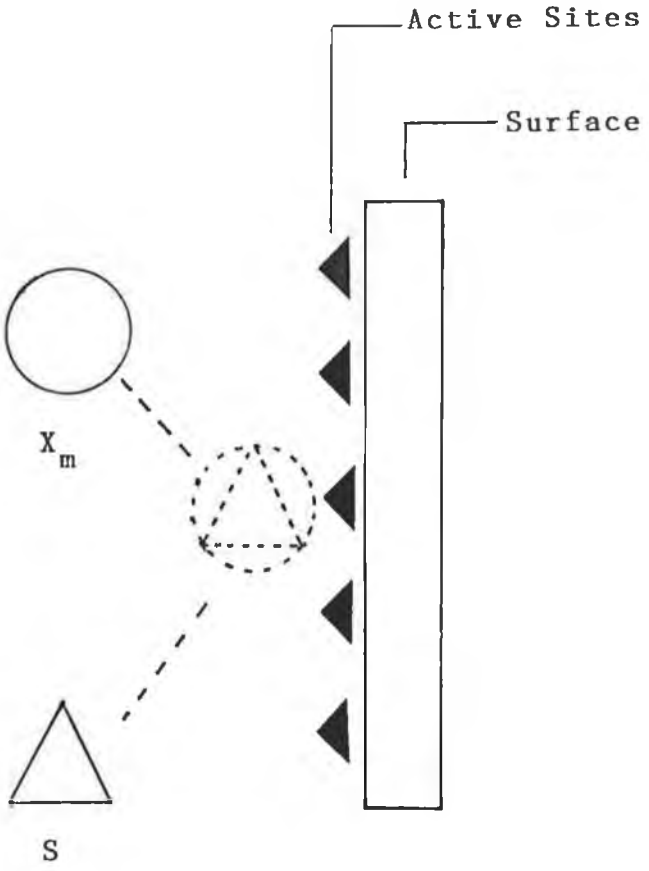


Fig. 6 Schematic diagram of the adsorption process

to shorter retention times. The value for K can be obtained from adsorbtion isotherm data or it is more common to obtain relative K values from TLC data.

1.4.1.2. Packings for L.S.C.

In liquid-solid adsorption chromatography, the mobile phase is a liquid while the stationary phase is either a totally porous particle or a porous layer bead - the pellicular packings, (Fig. 7).

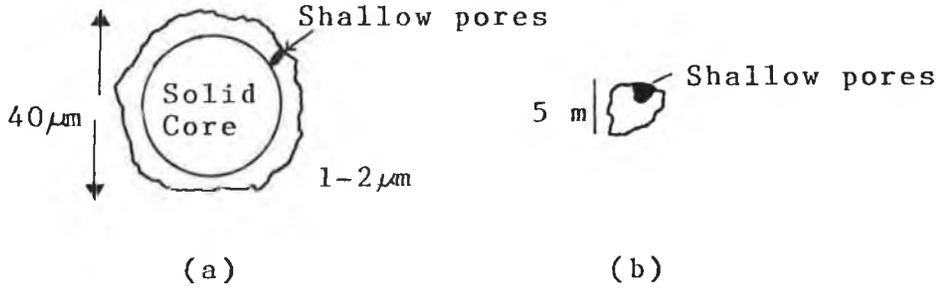


Fig. 7 Stationary phases used in adsorption HPLC: (a) porous layer beads; (b) porous microparticle.

1.4.1.2.1. Porous Layer Beads or Pellicular packing

A pellicular or porous layer bead type of packing consists of a solid, spherical glass bead with an average particle diameter of 30-40 μm, and a thin, porous outer shell. The outer shell, typically I-3 μm thick, may be a silica gel layer, a network of small spherical particles' bonded to the solid core, or a bonded monomeric or polymeric organic phase. Surface areas of the porous layer beads range from 5 to 15 $m^2\ g^{-1}$. Columns are easy to pack with these materials because of the dense core but due to their small surface

areas, porous layer beads suffer from limited sample capacity (approximately 0.1mg g^{-1}). Thus they are less useful for preparative work or for use with the lower sensitivity detectors. However, relative to a porous packing of equivalent diameter, stationary phase mass transfer is greatly improved in a thin porous layer bead and consequently, these packings exhibit good efficiency.

Longer columns are possible because the pressure drop is lower due to the larger particle size of porous layer supports.

1.4.1.2.2. Porous Particles (Microparticles)

The totally porous particle is generally a high surface area, active material such as silica gel or alumina. These are the most common phases in adsorption chromatography. Both silica gel and alumina have a high concentration of surface hydroxyl groups whose number and geometric arrangement determine the activity (retention) of the adsorbent. The slightly acidic silanol groups (Si-OH) in silica gel exist at the surface and extend into the internal channels of the pore structure. These hydroxyl groups interact with polar or unsaturated moieties by hydrogen bonding. During dehydration (activation) a certain proportion of these silanol groups will form siloxane linkages (Si-O-Si) between neighbouring silicon atoms. Siloxane groups are very weak in their adsorptive properties. For silicas, surface areas range from a low of 100m^2 g^{-1} to a high of 860m^2 g^{-1} with the average being 400m^2 g^{-1}.

Aluminas comprise about 20% of the solid adsorbents in use. Aluminas have low surface areas, larger average pore diameters, and higher packing densities than silicas. Aluminas do have some selectivity advantages, particularly for unsaturated hydrocarbons and halogen-containing compounds and are useful for very basic compounds which may adsorb too strongly on the acidic silicas. In addition, alumina possesses greater stability at high pHs where silica tends to dissolve.

In LSC the order of elution is the order of polarity and this has allowed the development of a generalised polarity scale for various classes of compounds. In the case of polyfunctional compounds, the most polar groups will dominate the adsorption characteristics, although all constituent groups contribute to the retention.

Both silica and alumina have surface OH-groups and Lewis acid type interactions. The number and topographical arrangement of these groups determines the activity.

1.4.1.3. Solvent Selection in LSC

The active sites appear to have varying degrees of strength. Very strong adsorption can lead to long rentention times and tailing peaks. To reduce the effect of strong interactions, the adsorbent activity is usually controlled or modified with a fixed water or alcohol content.

For non-polar mobile phases, having the proper water or alcohol content is very important, but as the polarity of

the mobile phase increases the importance of the water content diminishes.

The mobile phase is in competition with the solute for the active sites. This equilibrium is controlled by the mass action law. It is very important to maintain a constant water or modified layer on the adsorber. Only when complete thermodynamic equilibrium exists between the mobile phase and the deactivated adsorbent will retention times be reproducible.

There is a difficulty in saturating non-polar solvents such as hexane with water. Another option is to add a small amount (0.1 to 0.5%) of an alcohol such as isopropanol or methanol. These alcohols serve to modify the adsorbent in much the same way as water and are more soluble in non-polar solvents.

1.4.1.4. Solvent Strength in LSC

The solute and the solvent are in competition for the active sites on the adsorbent, it is easy to deduce that the more strongly the mobile phase interacts with the adsorbent the quicker the solute will elute from the columns. Thus the major factor determining retention in LSC is the relative polarity of the mobile phase and the solute. The solvent strength parameter, ε°, is a quantitative representation of solvent strength or solvent polarity (Table I).

TABLE I Solvent strength Parameter, e^{o} and Physical Properties of Selected solvents.

Solvent	e^{o} (SiO_2)	e^{o}(Al_2O_3)	Viscosity(20°C) mN sec m^{-2}	Refractive Index (20°C)
Pentane	0.00	0.00	0.23	1.358
Hexane		0.00	0.313	1.375
Cyclohexane	-0.05	0.04	0.980	1.426
Carbon disulfide	0.14	0.15	0.363	1.628
Carbon tetrachloride	0.14	0.18	0.965	1.460
1-Chlorobutane		0.26	0.47	1.402
Disopropyl ether		0.28	0.379	1.368
2-Chloropropane		0.29	0.335	1.378
Benzene	0.25	0.32	0.65	1.501
Diethyl ether	0.38	0.38	0.23	1.353
Chloroform	0.26	0.40	0.57	1.443
Methylene dichloride		0.42	0.44	1.425
Methyl isobutyl ketone		0.43		1.394
Tetrahydrofuran		0.45	0.55	1.407
Acetone	0.47	0.56	0.32	1.359
1,4-Dioxane	0.49	0.56	1.54	1.422
Ethyl acetate	0.38	0.58	0.45	1.370
1-Pentanol		0.61	4.1	1.410
Acetonitrile	0.50	0.65	0.375	1.344
1-Propanol		0.82	2.00 (25°)	1.38
Methanol		0.95	0.60	1.329
Water		Large	1.00	1.333

Solvent strength parameter, e^{o} is defined as the adsorption energy per unit area of standard adsorbent.

1.4.2. Partition Chromatography

Two different types of partition chromatography may be distinguished, classical liquid - liquid chromatography (LLC) in which the stationary liquid phase is coated on a support material and bonded phase chromatography (BPC) where there is chemical bonding of groups to the support surface (fig. 8).

In classical liquid - liquid chromatography (LLC) the stationary phase should be a good solvent for the sample but a poor solvent for the mobile phase. Operationally there are many problems. The coated phase may be stripped by strong solvents or gradually removed due to lack of mobile phase presaturation. Under high pressures shearing forces tend to remove the stationary liquid. Another limitation is that column temperatures must be carefully controlled within $0.5^{o}C$. These factors, the inability to use classical LLC with gradients and above all the advent of efficient stable bonded phase packings has almost made the use of these coated phases obsolete.

Partition chromatography can be operated in two different modes (a) normal phase and (b) reverse phase, a division based on the relative polarities of the mobile and stationary phases. Normal phase chromatography uses a polar stationary phase (often hydrophilic) whereas reverse phase employs a non-polar stationary phase (usually hydrophobic) and a polar mobile phase. In the reverse phase mode the compounds that are non-polar selectively interact with the liquid phase and are retained more strongly then the ones

which are polar. Reverse phase chromatography will most likely provide optimum retention and selectivity when compounds have no hydrogen bonding groups or have a predominance of aliphatic or aromatic character. The reverse phase technique in its various forms - regular partition, ion-pair partition and ion-suppression is the most widely used mode in HPLC now that bonded phase supports have provided a suitable stationary phase.

Partition chromatography is potentially more versatile than adsorption LC because of the wide variety of organic stationary phases which can be coated on the packing. However the most popular bonded phase comprises a linear hydrocarbon, most commonly an octadecyl C_{18}, C_8 or C_2 alkyl chain. In normal phase mode, one uses the packings that have polar groups bonded to them. Polar examples of such packings are those with alkylnitrile (CN column) and alkylamine (NH_2 columns) groups bonded to them. The technique is called normal phase since usually the solvents used are those of LSC.

The most common chemical reactions that are employed to bond organic molecules to silica are shown below.

<u>Esters</u> (hydrolytically unstable)

$$\text{silica - OH} + \text{HO-R} \Rightarrow \text{Silica - OR} + H_2O$$

<u>Silicones</u> or <u>Siloxanes</u>

$$\text{Silica - OH} \xrightarrow[H_2O]{RSiCl_3} \text{Silica - O - Si(OH)(OH) - R} + 3\ HCl$$

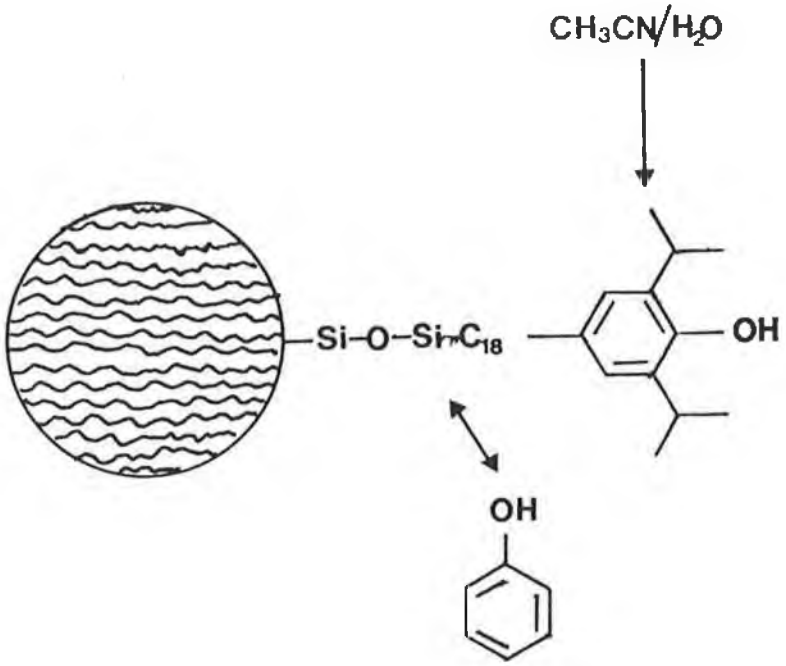


Fig. 8. Interaction on the surface of a bonded phase packing.

In reverse phase liquid chromatographic packings silanol groups on the surface of the silica are reacted with thionyl chloride followed by reaction with a Grignard reagent [16-19]. The R group of this reagent dictates the properties of the stationary phase.

$$SiOH + SOCl_2 \longrightarrow SiCl + SO_2 + HCl$$

$$SiCl + RMgBr \longrightarrow SiR$$

As mentioned the most commonly used hydrocarbons attached to the stationary phase are octyl, octadecyl, and phenyl. Alkylamine may also be attached to the silica surface.

1.4.2.1. Theory

The exact mechanism of bonded phase chromatography is still the subject of much study [21-25]

Briefly when a solute is added to the system, and the system allowed to equilibrate the solute will distribute itself between the two phases according to the equation

$$K = \frac{C_s}{C_m} \qquad (1)$$

where K is the distribution coefficient and C_s and C_m are the concentrations of the solute in the stationary and mobile phases, respectively. The capacity factor k^1 is related to the distribution coefficient by the following equation

$$k^1 = \frac{g_m}{g_s} \qquad (2)$$

gm - grams of solute in mobile phase = (Cm)(Vm) (3)

gs = grams of solute in stationery phase = (Cs)(Vs) (4)

$$K = \frac{C_s}{C_m} = \frac{g_s \times V_m}{g_m \quad V_s} = k^1 \frac{V_m}{V_s} \tag{5}$$

or upon rearrangement

$$k^1 = K \frac{V_s}{V_m} \tag{6}$$

The more soluble the solute is in the stationary phase, the longer it will be retained. In addition retention and resolution can be increased by increasing the amount of stationary phase.

The mechanism that causes separation in bonded phase partition LC is not well understood, however it is clear that columns packed with these materials exhibit separation properties that are similar to adsorption under certain conditions and to liquid - liquid partition under other conditions. This is probably due to the fact that a high initial concentration of silica surface hydroxyls is needed in order to bond the organic phase and many of these hydroxyls remain free for adsorption after even the most careful bonding procedures. In the determination serum theophylline evidence is presented which suggests that an octadecylsilane phase bonded to silica particles acts via mixed retention mechanisms probably due to the action of free silanol groups rather than pure reversed phase partition [26].

Separation due to adsorption on the residual hydroxyls of a bonded-phase packing will be most important for small polar solutes and nonpolar mobile phases. The solute size is important because the highest concentration of residual hydroxyls is likely to be in sterically hindered places where bonding is incompete. The effect of this adsorption mechanism can be tested or eliminated by adding a small amount of polar modifier such as water or acetic acid to the hydrocarbon mobile phase to deactivate the remaining hydroxyls.

1.4.2.2. Normal Bonded Phase

Bonded phases using $Si(CH_2)_n NH_2$ or $Si(CH_2)_n CN$ moieties can be used in normal bonded phase chromatography.

They are very similar to the packings used in LSC but have some advantages

(a) Irreversible retention is less likely due to absence of strong silanol groups
(b) Unnecessary to control the level of water
(c) Equilibration is rapid
(d) Compatable with a wide range of solvents
(e) Difficult to damage
(f) Different selectivities
(g) The alkylamine column can function as a weak anion exchange column.

1.4.2.3. Reverse Phase

Liquid chromatography where the mobile phase is more polar than the stationary phase was termed "reversed phase" (RP) chromatography since early in its use [27, 28]. With the advent of packings with covalently bonded functional moieties on pellicular and microparticulate particals RP became significant in the practice of HPLC. It is now estimated that 60-80% of all HPLC separations are accomplished using RP packings [29, 30]. In a publication by Horvath *et al.* it was considered that RP was primarily a tool for non-polar substances [29] whereas Karch *et al.* believed that the main advantage lies in the separation of polar samples that are not eluted from silica columns [31]. However it is now accepted that RP may be used over a broad polarity range and in diverse applications. In an investigation by Twitchett and Moffat it is claimed that drugs of any lipid solubility, molecular weight chemical structure and acidity/basicity can be chromatographed using RP if an appropriate eluent is chosen [32].

1.4.2.3.1. RP Retention Mechanisms

Although it is generally accepted that in RP systems the most polar solutes are the least retained however the precise mechanism of retention has yet to be agreed upon. Karch *et al.* have suggested that it is due to solute interaction with the nonpolar stationary phase by dispersion forces [31]. In contrast Locke considers the solute interactions with the nonpolar stationary phase to be weak and non selective [24]. Karger *et al.* have emphasised the

importance of understanding water solubility [33]. Starting with water solubility theories which use the idea of cavity formation [34, 35] they discuss hydrophobic effects to explain the experimental observations that reverse phase systems exhibit a marked selectivity for the hydrocarbon structure of solutes. From the hydrophobic effect it can be predicted that an increase in molecular size of solutes in a series of chemical homologues will lead to a longer retention time. An increase in the carbon chain length of the stationary phase will obviously increase the available surface of the non-polar moiety of the stationary phase to that of the solute molecules leading to a higher k^1 value (longer retention time). On the other hand the introduction of polar groups causes a decrease in the retention time.

Horvath et al. also point to the importance of the role of the mobile phase [29]. They have adopted solvophobic theory [36,37] and point out that the most pronounced solvophobic effect is the hydrophobic effect which results from the very high cohesive density of water. Mobile phase surface tension is shown to play a key role. They further point out that, whereas in stationary phases having ionic or hydrogen bonding moieties (normal phase systems) the driving force of retention is predominantly the attraction between the solute and stationary phase, in stationary phases having a hydrocarbon character (RP system) the driving force of retention is the concomitant decrease in the non-polar surface area exposed to the solvent. The hydrophobic "bond" between solute and stationary phase results primarily from the aqueous solvent forcing the molecules to associate rather than from any real attraction.

Snyder has provided a clear description of this effect within a general discussion of polarity and the four bond-breaking or bond making steps involved in the transfer of a solute from mobile to stationary phase [38]. The energetically favourable transfer of non-polar solutes from the aqueous mobile phase to the hydrophobic stationary phase is related to the heat required to form a cavity within the water structure into which the solute is placed. The solute is repelled or "squeezed out" of the water because its interactions with water are weaker than the interactions of water with itself [39]. When the organic constituent of the mobile phase is increased, the surface tension is reduced, understandably this change shortens the retention time.

1.4.2.3.2. Ion Suppression and Ion Pairing

A useful extension of the reversed phase technique is that of ion suppression or ion pairing. Consider a short chain carboxylic acid ($<C_6$) which exists in aqueous solution as follows:-

$$RCOOH \rightleftharpoons RCOO^- + H^+$$

Using a methanol water mobile phase the acid would be poorly retained and elute as a skewed peak. By adjusing the pH on the acid side, the equilibrium will move to the left, suppressing ionization and allowing the acid to be separated by reverse phase techniques. This technique is termed "ion-suppression" and is useful for weak acids and bases.

However strong acids or bases cannot be handled by this technique since many are completely ionized in the pH 2-8 range. However, by forming an ion pair with a counter ion, these strong acids and bases can be made into electrically neutral compounds and therefore be retained on a reverse phase column. The reverse phase ion-pair partition technique is favoured over the normal phase one since a bonded phase is used and the counterion and buffer are added directly to the aqueous mobile phase.

1.4.3. Ion-Exchange Chromatography

Ion-exchange chromatography should be considered when a compound is water soluble and ionic.

Commercial ion-exchange packings consist of porous organic or inorganic gel or resin networks to which various ionic functional groups have been attached. The two most commonly used starting materials are styrene divinyl benzene resin and silica gel. The nature of the ionic functional groups attached to these stationary phases is the most important factor governing retention and selectivity for a given ionic solute. Ion-exchange can be carried out in aqueous or non-aqueous solvents. The mobile phase normally contains a supply of counter ions opposite in charge to the surface ionic group.

Fig. 9.

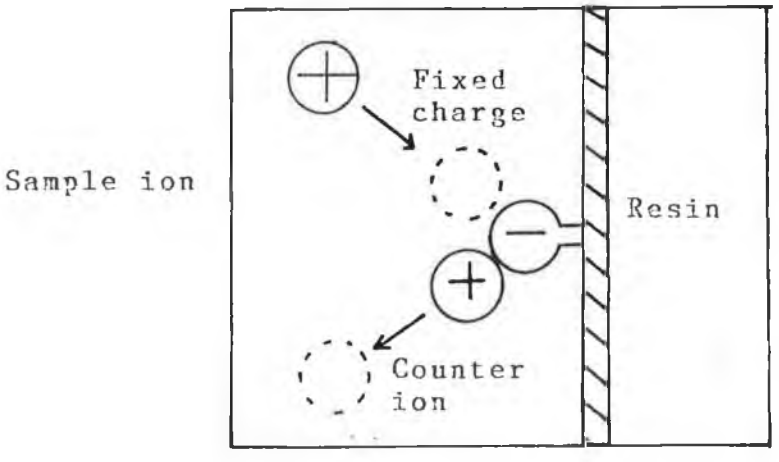


Fig. 9: Schemetic diagram showing ion-exchange.

1.4.3.1. Packings for Ion Exchange Chromatography

Three main types of packing are used, pellicular, phases bonded to silica microparticles and classical crosslinked PS-DVB resins of small particle diameter. Pellicular packings are more widely used in ion exchange than in LSC or BPC. They provide unique selectivities, different to porous resins or ion-exchange silicas, and greater permeability. Due to their larger particle size they display lower pressure drop with the aqueous solutions employed, and show rigidity - neither swelling nor shrinking with changes in pH, temperature or ionic strength. In addition they are easily packed and allow the use of an extended pH range compared to silica but still not as wide as for resins.

1.4.3.2. Ion-exchange equilibria which occur on the column can be represented as follows.

Strong Anion Exchange Packing:

$$R^{\ominus} + M^{\oplus} + X^{\ominus}R_4N - gel \underset{\text{Elution}}{\overset{\text{Retention}}{\rightleftharpoons}} M^{\oplus} + X^{\ominus} + R^{\ominus}R_4N - gel$$

Strong Cation Exchange Packing:

$$R^{\oplus} + X^{\ominus} + M^{\oplus}O_3S - gel \underset{\text{Elution}}{\overset{\text{Retention}}{\rightleftharpoons}} X^{\ominus} + M^{\oplus} + R^{\oplus}SO_3 - gel$$

Weak Anion Exchange Packing:

$$R^{\ominus} + M^{\oplus} + X^{\ominus}H_3N\text{-gel} \underset{\text{Elution}}{\overset{\text{Retention}}{\rightleftharpoons}} M^{\oplus} + X^{\ominus} + R^{\ominus}H_3N\text{-gel}$$

Weak Cation Exchange Packing:

$$R^{\oplus} + X^{\ominus} + M^{\oplus}O_2C\text{-gel} \underset{\text{Elution}}{\overset{\text{Retention}}{\rightleftharpoons}} X^{\ominus} + M^{\oplus} + R^{\oplus}O_2C\text{-gel}$$

The equilibrium expression for a strong anion exchange process may be given by:

$$K = \frac{[X^{\ominus}]\ [-NR_4^{\ominus}R\ ]}{[R^{\ominus}]\ [-NR_4^{\ominus}X\ ]} = k' \frac{V_m}{V_s}$$

The higher the value of K, the distribution coefficient, the more strongly the ionic solute interacts with the ion exchanger. The distribution coefficient will be determined by the nature of the solute and the stationary ionic group and a great number of other variables such as gel pore structure, pH, ionic strength and temperature. In addition, separation characteristics can be influenced by specific interaction between the non-ionic part of the solute and the non-ionic part of the resin or gel. In this latter case, small amounts of polar organic solvents as modifiers to the aqueous mobile phase can have a dramatic effect upon the separation.

Ion-exchange chromatography is probably the most experimentally difficult mode of HPLC because there are more parameters that need to be controlled. The two most important variables that can be adjusted are pH and ionic strength.

The pH should be adjusted so that the solute and stationary ionic groups are in attractive, oppositely charged states. For example with a weak anion exchange packing, select a low enough (acidic) pH so that the amine group is protonated but not so low that the solute is protonated.

1.4.3.3. Solvent Selection

Because of the excellent solvent and ionising properties of water, most ion exchange chromatography is carried out in aqueous buffer salts. It is often useful to choose a buffering salt that has a functional group similar to the sample. For example choose an acetate anion as a buffer to elute organic acids. When developing the chromatographic conditions it is usual to start with a very low concentration of ions in order to maximise the change of retention. A counter-ion usually from a buffer, is one which has the same charge as the solute and occupies the stationary site when the solute is not there. A decrease in the counter-ion concentration forces the equilibrium towards stationary phase retention while an increase in counter-ion concentration forces the solute to elute from the column. In other words increasing the buffer strength in ion-exchange chromatography is analogous to increasing the solvent strength in LSC or LLC.

1.4.4. Exclusion Chromatography

Other names include gel permeation, gel filtration, exclusion, or molecular sieving. It is applicable to a wide range of materials of both high and low molecular weight. It depends upon the physical restriction of the solute flowing into and out of the pores of the column packing. Ideally interactions such as adsorption, partition and ion-exchange should be absent from the ideal steric exclusion system.

The packing contains porous particles of various diameters and only solute molecules which have diameters small enough will diffuse into the pores (Fig. 10). Therefore the component with the largest effective diameter will elute first. As the effective diameter of the solute gets larger, the number of pores that it can fit into and its ability to diffuse into the pores decreases. Thus if it is of such a diameter that it cannot diffuse into any of the pores, it is defined as totally excluded and will be unretained that is, it will elute with the void volume of the column.

1.4.4.1. Types of packing

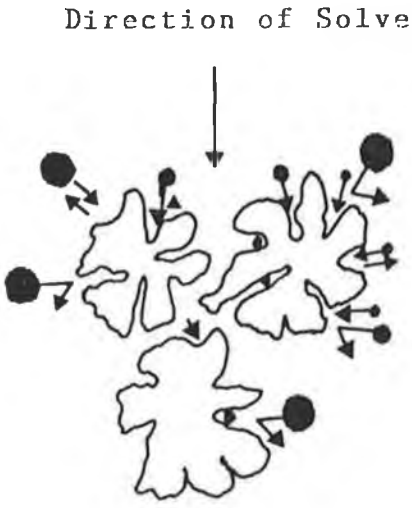
Semi-rigid gels

Rigid packings

Soft gels.

1.4.4.1.1. Semi-rigid gels

An example is Styragel made up of polystyrene divinylbenzene



Large solutes cannot penetrate into the pores of the packing and thus move with the solvent. Small molecules diffuse into the pores and are retained.

Fig. 10: Schematic diagram showing exclusion chromatography.

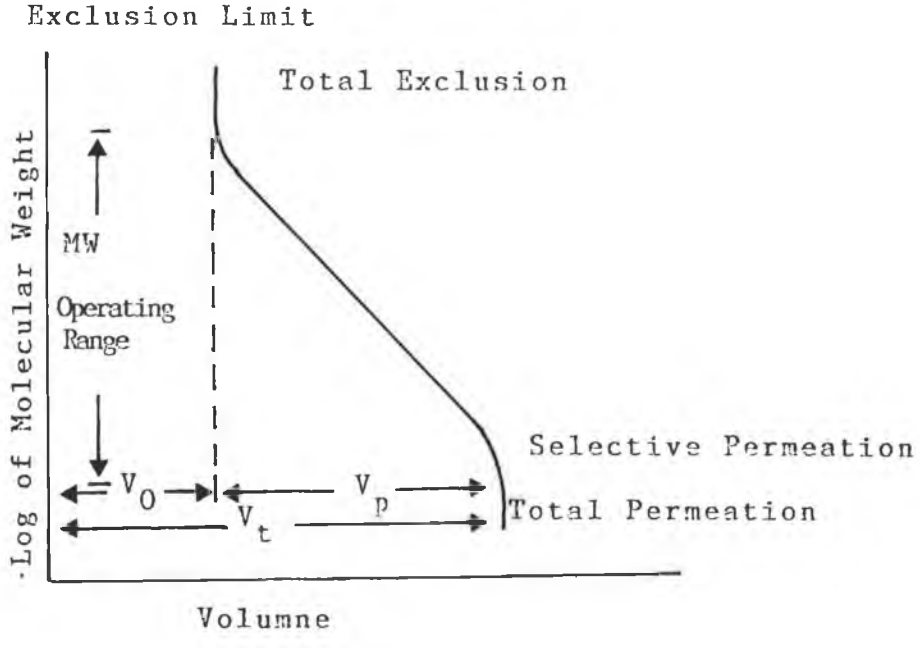


Fig. 11: Typical calibration curve for exclusion chromatography.

cross linked to give a semi-rigid structure. These can separate complex polymer samples such as rubbers and plastics. But the particle size in the region of 37 - 75 μm leads to slow mass transfer which leads to low flow ratio and long analysis times. Smaller particle sizes are now available however with smaller pore sizes and these give increased resolution and allow solutes of lower molecular weight to be separated. One should not change the solvent from that used to prepare the column, this may alter pore size and cause swelling of the packing

1.4.4.1.2. Rigid Packings

These packings are invariably made from glass or silica. They are available in a wide range of pore sizes and in large and small particle diameter. Advantages are that there are no limits on flow rates since higher pressures can be used, columns with high efficiencies are easy to prepare, aqueous and organic solvents may be used, however alkaline solvents with pH > 7.5 should be avoided since they dissolve silica or glass. The major disadvantage is adsorption effects (will alter the calibration curve and lead to tailing).

Adsorption effects can be minimised by treating the beads with Carbowax 400 or polyethyene glycol, or beads may be treated with various silylating reagents to deactivate the polar sites.

1.4.4.1.3. Soft Gels

These materials are typified by cross-linked dextrans, the

most popular being Sephadex. The use of these materials is called gel filtration. The packings swell in the solvents used. They are useful for separating water-soluble substances in the M.W. range 10^2 to 2.5 x 10^7, e.g. proteins and enzymes. They are not widely used in HPLC since they can only withstand pressures of 150 p.s.i.

1.4.4.2. Components of the volume of an exclusion column

1. Void volume - the volume occupied by the flowing mobile phase between the particles.
2. The volume occupied by the solid portion of the packing.
3. The pore volume - (V_p) - the volume occupied by the stagnant mobile phase.

We can define the distribution coefficient K of a particular solute as the ratio of pore volume accessible to the solute (V_A) to the total pore volume V_p.

$$K = \frac{V_A}{V_p}$$

The retention volume is the given by the following equation.

$$V_R = V_o + KV_p$$

The ideal is a small void volume and a high pore volume to achieve maximum possible separation.

Adsorption effects may be noted and these will increase retention time and also lead to tailing of peaks. Packings from porous glass often show adsorption effects probably due to the presence of SiOH groups.

1.4.4.3. Selection of column

This will depend on the size of the solute molecules to be analysed

Soft gels are not compatible with HPLC. The semi-rigid particles are available in high efficiencies and wide pore ranges. They are compatible with organic solvents but not water because it does not wet the surface. Changing of solvent can change the degree of swelling and therefore change elution behaviour.

Each steric-exclusion packing will have its own calibration curve. The distribution of the pores in the packing governs the slope of the calibration curve (fig. 11). If the pore distribution is wide the curve will have a steep slope. The molecular weight operating range will be large but will provide less resolution of species of close molecular sizes. If the pore distribution is narrow, the curve will be flatter, the molecular weight operating range will be smaller but resolution of molecules of closely related size will be increased.

A major disadvantage of rigid particles is adsorption.

Resolution is increased by using a packing with a large pore

volume, a flat calibration curve or a high plate count or by use of longer columns.

1.4.4.4. Selection of mobile phase

In contrast to other modes of liquid chromatography, the solvent in exclusion chromatography is selected to minimise the solute interactions with the support surface. Interactions such as adsorption, ion-exchange and partitioning should be absent in the ideal steric-exclusion system. The solvent chosen must wet the surface of the packing. The solute can then permeate the pores by a totally diffusion type process.

Many of the solvents which are useful in exclusion chromatography are not compatible with U.V. detectors. Toluene, tetrahydrofuran (THF) and halogenated aromatic solvents are widely used because they have excellent solvent characteristics for the heavy molecular weight substances normally analysed by exclusion. Of these solvents, only THF is potentially usable with U.V. detection at 254 nm. It should be noted that exclusion chromatography requires only a single solvent in which to dissolve and run the sample.

1.4.4.5. General Uses of Exclusion Chromatography

Exclusion chromatography may be used in polymer analysis and for sample clean up.

In most polymerisation processes the final product normally contains a range of molecular weights. (Molecular weight

distribution). In routine use exclusion is used to "finger print" a polymer product and gross changes in the polymerisation process can be spotted easily. In sample clean-up, fractions of high molecular weight can be discarded.

Exclusion chromatography may also be used for small molecule separation. Advantages of this approach are:

1. Solvent requirements are minimal, it must dissolve the sample and be compatible with the detector.
2. Gradient elution is not necessary.
3. Retention times can be estimated easily and are short.
4. The technique is easily automated.
5. Because of the mode everything is eluted in a known solvent.
6. The column has high sample capacity.

1.5. INSTRUMENTATION FOR HPLC

The main components of the HPLC system are as follows.

(a) The Solvent delivery system.

(b) The Injection device.

(c) The Column.

(d) The Detector.

1.5.1. Solvent Delivery System

Several features of the solvent delivery system must be considered: precise delivery of solvent over a relatively broad flow range; maximum pressure attainable; compatibility with other components in the HPLC system; compatibility with a wide choice of solvents; and noise level in the detector resulting from any pulsations. There are two main classifications of pumping system: constant pressure and constant displacement. Both types of pumps usually operate by displacing liquid from a chamber by a hydraulic piston.

1.5.1.1. Constant Pressure Pumps

Constant pressure pumps can be used with both large and small hydraulic chamber volumes. The constant pressure

pump is inherently pulse free during its displacement stroke and has additional advantages of being able to refill rapidly and pump liquids at high flow rates. In this type of pump, pressure from an external source (air or N_2) must be supplied to a gas piston of large surface area which is attached to a hydraulic piston of small surface area. Amplification is about 50:1, i.e. a gas pressure of 100 psi will produce an inlet pressure of 5000 psi. The gas pressure is released at the end of the pump stroke, and the pump refills instantly by the action of gas pressure or a spring. A check valve between the pump and column prevents flow reversal during the refill stroke.

The performance of constant pressure pumps depends on the following:

1. Properly functioning seals and check valves.

2. Pressure of the gas supply.

3. Restrictions within the system due to septa particles, particulate matter and column resistance.

4. Solvent viscosity.

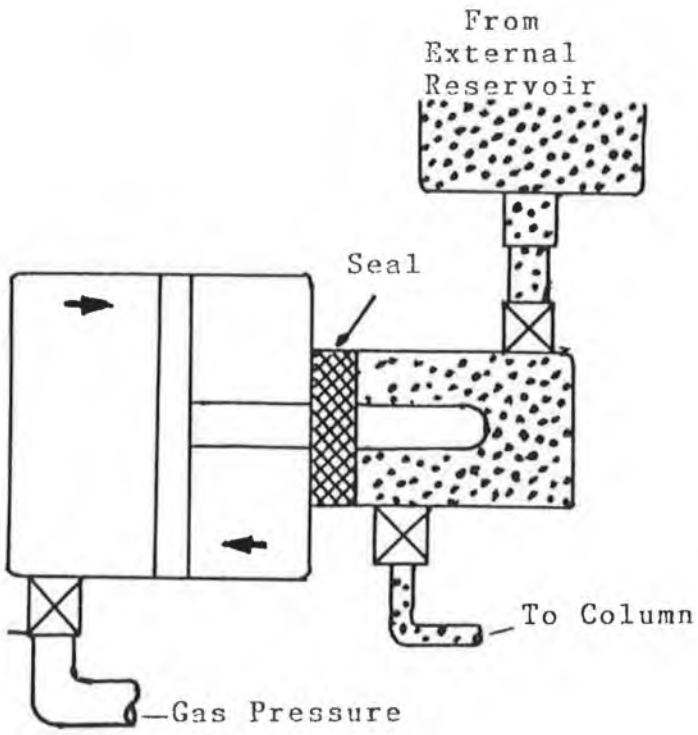


Fig. 12, Constant Pressure Pump.

1.5.1.2. Constant Displacement (volume) Pumps

Constant displacement pumps are divided into two main classes:

a) Single displacement screw drive or syringe pumps

b) Reciprocating (single and dual pistions).

Most of todays HPLC systems use reciprocating pumps

1.5.1.2.1. Syringe Pumps

These pumps are pulse-free and relatively accurate. On the other hand, they are limited in flow rate range. Flow must be interrupted during refill, and they are not as convenient as other types for solvent changeover. Solvent delivery is accomplished using a variable speed motor to turn a screw that drives a hydraulic piston. Malfunction of the motor and drive mechanism or leaking seals and valves are the most frequent causes for inadequate solvent delivery. When it becomes necessary to refill the pump, the screw action must be reversed to withdraw the plunger.

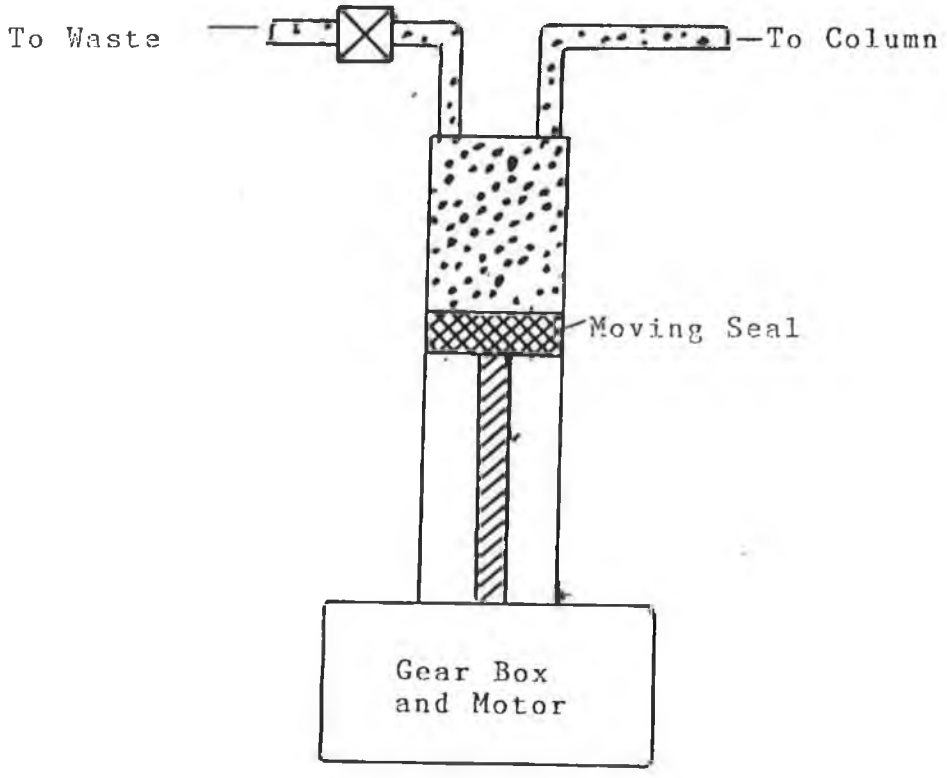


Fig. 13, Screw Driven Syringe Pump

1.5.1.1.2. Constant Volume Reciprocating Pumps

b) Single - Reciprocating Piston Pump:

This pump consists of a small piston attached to a motor via gears and a cam which drives it back and forth in the hydraulic chamber. Pistons are typically fabricated from borosilicate glass, sapphire or Chrom-plated stainless steel with a mirror finish.

Advantages:

- They deliver a truly continuous flow
- Changing from one mobile phase to another is rapid with no waste of solvent (because of small piston volume)

Disadvantage:

- Flow pulsations, thus noise
- The pulsation is normally minimised by using pulse dampers of various designs or the piston is designed with electronic feedback to remove pulsation

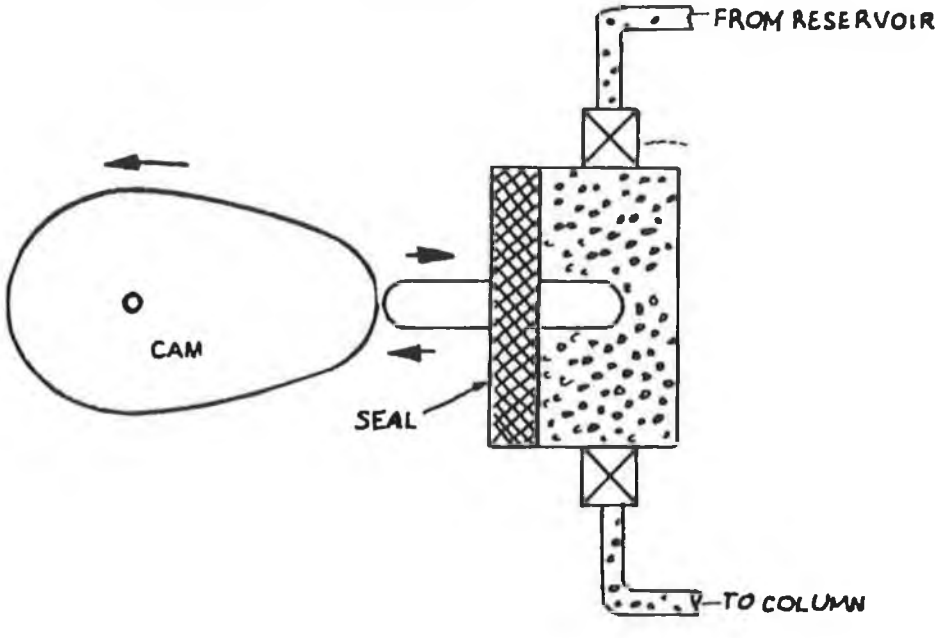


Fig. 14, Single Reciprocating Piston Pump

Dual - Reciprocating Piston Pumps:

This pump features closed-loop flow control to eliminate both flow irregularities and pump pulsations regardless of system back pressure Using a differential pressure transducer that measures the flow-rate by measuring the pressure drop across a restrictor of fixed volume, any fluctuations in flow rate are detected and compensated for by feedback to a pump circuit that rapidly adjusts the pump motor speed. Because the response time of this system is in the order of tenth of seconds and is much faster than the pump noise created by reciprocating pistons, flow pulsations throughout the column and detector are virtually eliminated without the use of a pulse dampner.

The main considerations when choosing a pump are price, performance and convenience.

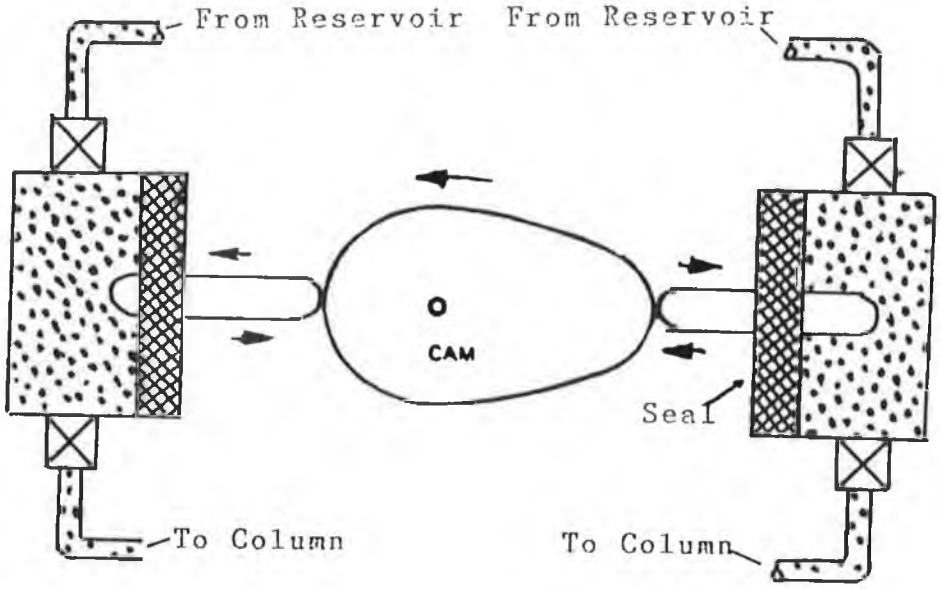


Fig. 15, Dual Reciprocating Piston Pump

1.5.2. Injectors

Typical HPLC injectors include septum, stopped flow, loop valve (either variable or fixed), and autosamplers.

1.5.2.1. Stop-flow

The flow is stopped, the injection made at atmospheric pressure; the system closed and flow reinitiated. This technique can be used because diffusion in liquids is small and thus resolution is generally not affected.

1.5.2.2. Septum

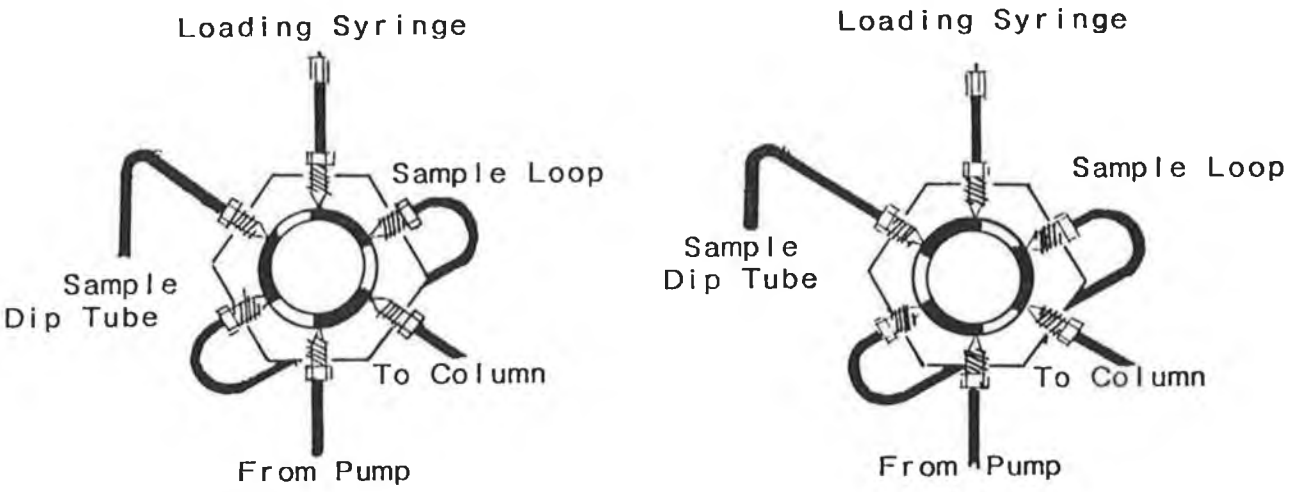
These are on-stream injectors similar to the commonly used gas chromatographic injectors. These injectors are usable up to pressures of about 60-70 atmospheres. They are not compatible with all LC solvents. In addition, small particles tear from the septa and tend to cause plugging problems.

1.5.2.3. Loop-Valve

These are the most popular and most widely used injectors (fig. 16). In the fill position, the sample loop is filled at atmospheric pressure. When the valve is actuated, the sample in the loop is swept onto the column.

1.5.2.4. Auto-injectors

It is possible to automate a loop valve. Here the advantage of on-column injection is sacrificed for automation. The sample is contained in a small vial with a septum cap. The



Load Position

Injection Position

Fig. 16 Loop Valve Injector

injection sequence starts with the needle penetrating the top of the vial. A small pulse of air is added to the top of the vial, which forces the liquid in the vial up the dipper tube and into the sample loop of the loop valve. The amount of air is controlled in such a way that the valve loop is overfilled and flushed with excess sample. A signal to the actuator tells the valve to rotate, placing the sample in the eluent stream. From there it passes directly to the column. The majority of auto injectors for HPLC appear to rely on gas displacement of the sample through the loop valve for filling, but a few utilize suction to fill the loop. Filling by suction has the disadvantage of requiring the use of less volatile solvents for dilution of the sample. Otherwise, the amount of sample taken in may vary due to formation of bubbles in the transfer lines.

1.5.3. The Column

The column is at the heart of the chromatograph and provides the versatility in the types of analyses that can be achieved with a single instrument.

There are three categories of columns, each of which is used for different purposes.

1.5.3.1. Analytical Columns

Analytical columns can be found in different shapes and sizes, they can be stainless steel or a cartridge system made of resistant plastic or glass. Their sizes can vary from 5cm in length up to 30cm with internal diameter of 3.5 - 4.6 mm. Although analytical columns are normally used for trace analysis, a column of say 4mm x 30cm can hold up to 10mg of sample. The packing materials are usually 3µm for 5cm length columns and 5 or 10 µm for longer columns. These columns can provide up to 100,000 theoretical plates per meter.

1.5.3.2. Microbore Columns

Microbore columns are only used for trace analysis. They have internal diameters of 1 or 2mm and can vary in length between 5 to 25cm with 3µm packing materials. To use 1mm I.D. columns small volume detector flow-cells are essential to produce satisfactory results. The advantages of microbore HPLC columns over analytical columns are a massive reduction in solvent usage (up to 95%) and increased sample sensitivity.

1.5.3.3. Semi-Preparative Columns

Semi-preparative columns are primarily used for the purification of components of a mixture for further characterization or study. A typical semi-preparative column 8mm x 50cm can hold up to around 500 mg of sample.

To increase the sample capacity, larger columns, 5 x 50cm, may be used which can hold up to 10g of sample.

In the area of drug analysis, however by far the most popular choice is that of columns containing reverse phase packings. Technical aspects of column selection will be discussed in Chapter 2.

1.5.4. Detectors for Liquid Chromatography

The detector unit, as a component of the HPLC system is employed to continuously monitor the column eluent. Generally the detector signal is then amplified and passes to a potentiometric recorder to obtain a printed output in the form of a chromatogram. Two basic types of detector are encountered (a) a bulk property detector which measures some overall charge in the physical property of the mobile phase e.g. refractive index or (b) a solute property detector which responds to some physical property of the solute which is not exhibited by the mobile phase.

1.5.4.1. Detector Performance

When assessing the performance of a detector the following factors should be considered.

(a) Noise

(b) Response

(c) Linearity of Response

(d) Detection limits

Detector noise may arise from several causes which may be electronic, flow, or pressure effects or due to temperature fluctuations.

(a) Short term noise is considered as the random or periodic signal fluctuations of the order of ten cycles per minute or higher and is usually seen as a fuzzy base line. Short term noise is generally used to define the detection limit.

(ii) Long term noise is of the order of low cycles or fractions of cycles per minute. At normal chart speeds this may be confused with solute peaks in the recorder trace.

(iii) Drift appears as a continuous increase or decrease in signal and its presence often makes the determination of the detection limit difficult.

Response: The response of the detector is defined by the slope of the calibration plot for detector signal against solute concentration (or mass rate). For linear functions we have the following relationship where the response is a fixed value.

(i) Concentration dependent response

$$R_c = k_c \frac{M_1}{M_1 + M_2}$$

R_c = detector signal

k_c = a constant

M_1 = the sample mass rate

M_2 = the mobile phase mass rate w.r.t. time

The units will depend on the physical parameter being measured e.g. refractive index units (R.I.U.) $cm^3\ mg^{-1}$ or optical density units (ODU) for a UV detector.

In HPLC $M_2 >> M_1$ hence e.g. 1 becomes

$$R_c = k_c \frac{M_1}{M_2} \qquad (2)$$

(ii) Mass rate dependent response

$$R_m = k_m\ M_1 \qquad (3)$$

R_m = the detector signal

k_m = a constant

M_1 = the sample mass rate w.r.t. time

The dimensions are usually quoted as coulomb g^{-1}. At the present time none of the well known L.C. detectors function solely as a mass rate device.

The main difference between the two types of response is that the concentration detector response is flow-dependent where as that for the mass detector is not . The equations given above are valid for linear relativity between the detector signal and sample size. In the case of non-linear functions the response is a variable, dependent upon the rate of change of the slope of the calibration curve.

Linearity of Response.

The general response function relating detector signal and sample size can be represented by

$$R = kX^n \qquad (4)$$

X = is a measure of the sample size (concentration or mass rate) and n is known as the response index. For a linear response it is required that n = 1. In practice, detectors are either non-linear or linear over a restricted range. The linear range is defined as that region of response between the detection limit and the point where n deviates from unity by a given value i.e. 5%. Furthermore values of $0.99 < n < 1.01$ provide a satisfactory linear calibration over a range of about 1000 which is acceptable for a wide range of quantification analyses. Equation 4 may be expressed in logaritmic form

$$\text{Log } R = n \log X + \log k \qquad (5)$$

For n = 1 the slope of the plot should be 45°.

Detection Limit

The detection limit is defined as the minimum sample size which will produce a signal equal to twice the short term noise level. The dimensions are g cm^{-3} for a concentration detector and gs^{-1} for a non dependent device.

Other important factors are (1) mobile phase flow rate effects on noise, drift and response (ii) temperature effects on noise, drift and response (iii) pressure effects on noise, drift and response (iv) dead volume in the detector cell and interconnecting tubing (v) time constant and inertial problems of the detector system.

1.5.4.2. The Ultra-Violet Absorption Detector

The ultra violet absorbtion detector is the most widely applied in HPLC, it is relatively insensitive to temperature and flow variations and in the case of some compounds detection at the nanogram level is possible. The detector responds only to substances that absorb U.V. light and a great number of compounds fulfill this requirment due to the presence of C = O, C = S, N = O and N = N groups in their structure.

Both single and double beam instruments are commercially available as well as fixed and variable wavelength

detectors. The fixed wavelength instruments are either single or dual beam arrangements usually containing the following sources: A Tungsten or a Deuterium lamp.

The basic optical layout of a particular design of double beam UV detector is shown in Fig. 17. In this example the source, S, is arranged at the focal length of lens L which is the window of the cells (sample and reference) C_1 and C_2. The element, W, is plane glass representing the other window of the cells. A calibration filter can also be incorporated in the sample side of the detector. A further filter, F, is employed as a block to all other wavelengths other than the one of interest. The resultant UV radiation is converted into electrical signals by the photoconductors D_1 and D_2. The two electrical outputs are generally fed into two log amplifiers, subtracted from each other, amplified and the resultant signal fed into a potentiometric recorder. Some commercial detectors do not employ log amplifiers but depend upon the photodetector response approximating to a log function. Extra care in calibration is essential with this type of device.

There are two cell geometries in common usage; the conventional Z-form and the novel H-form (Fig. 18.) The latter design is claimed to be more stable with respect to

drift caused by flow variations of the mobile phase [40]. In both geometries the flow path has been indicated with arrows. The cells are usually less than 10 µl in volume. A recent innovation is the use of a tapered cell construction which allows better light transmission and offers greater stability and sensitivity.

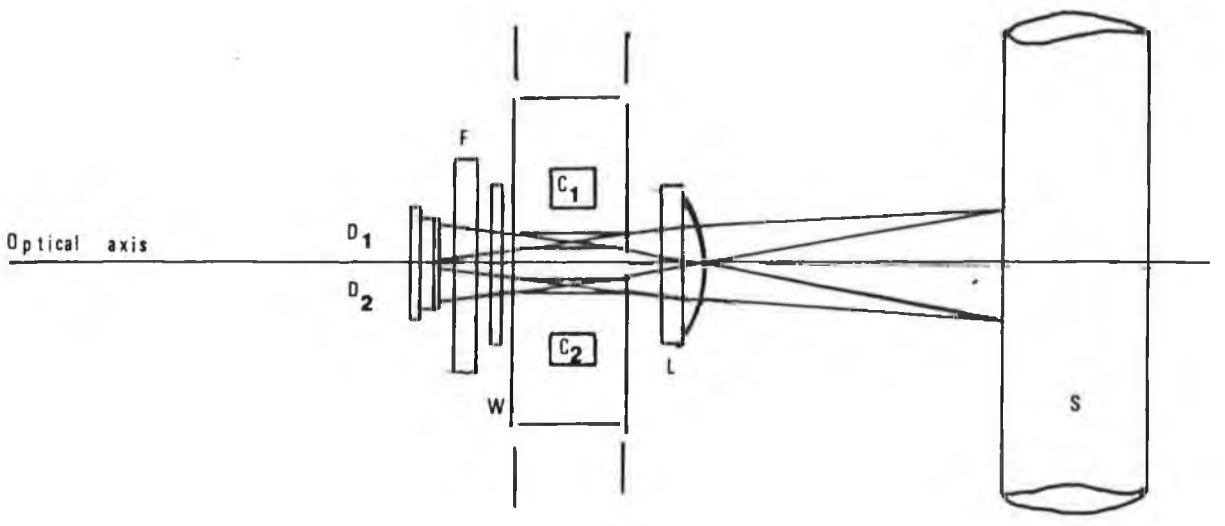
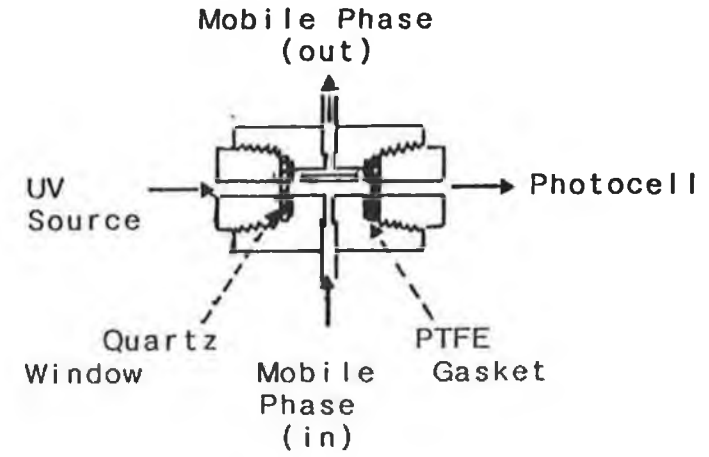
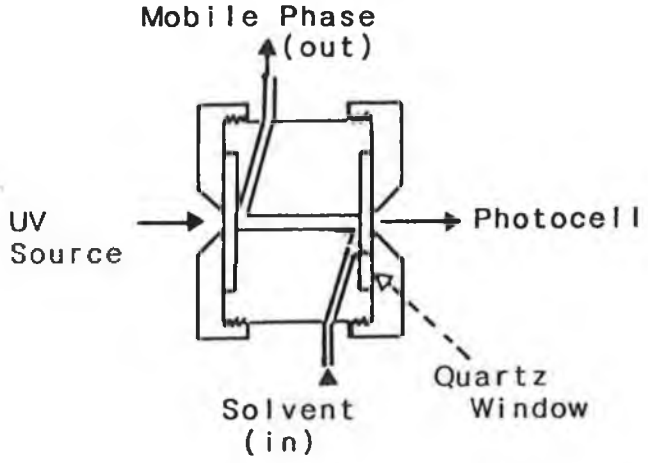


FIG. 17. Diagram of a UV detector



H-TYPE CELL



Z-TYPE CELL

Fir. 18: Different Cell Designs for UV Detector.

Principle of Operation.

The Beer Lambert Law states that the fraction of radiation absorbed by a compound is proportional to the number of absorbing molecules.

$$I = Io \exp(-kcd) \qquad (6)$$

where Io = the intensity of the incident radiation, I is the intensity of the transmitted radiation, d is the radiation path length, c is the concentration of the absorbing sample and k is a constant known as the extinction coefficient, the value of which depends upon the nature of the absorbing molecule and the wavelength of the incident radiation. Equation 6 may be rearranged.

$$\ln \frac{Io}{I} = kcd \qquad (7)$$

By definition we have

$$\text{optical density (OD)} = \log_{10} \frac{Io}{I} \qquad (8)$$

from equations 7 and 8 we get

$$\ln \frac{Io}{I} = 2.303 \log_{10} \frac{Io}{I} = kcd \qquad (9)$$

Now in the detector we have

Io (sample) - sample cell - I_1

Io (reference) - reference cell - I_2

The detector is arranged so that

Io (sample) = Io (reference)

Hence

$$\log_{10} \frac{Io}{I_1} = \begin{matrix}\text{absorbance} \\ \text{of solvent}\end{matrix} + \begin{matrix}\text{absorbance} \\ \text{of sample}\end{matrix} \qquad (11)$$

and $$\text{Log}_{10} \frac{Io}{I_2} = \begin{matrix}\text{absorbance} \\ \text{of solvent}\end{matrix} \qquad (12)$$

From equations 11 and 12 we get

$$\begin{matrix}\text{absorbance} \\ \text{of sample}\end{matrix} = \text{Log}_{10} I_1 - \text{Log}_{10} I_2 \qquad (13)$$

i.e. $\text{Log}_{10} \frac{I_1}{I_2}$ x sample concentration

1.5.4.3. Fluorescence Detectors

Some substances have the property of absorbing light and then emitting it (essentially instantaneously) at a longer wavelength (lower energy). This is called fluorescence. The two main advantages of fluorescence detectors for HPLC are better

detection limits for many compounds and frequently improved selectively. Minimum detection limits for fluorescence detectors can extend below the picogram level (10^{-12} g) for favourable samples.

A diagram for a sample fluorimeter is shown in figure 19. Light from a lamp passes through an optical system to form the beam and select the wavelength for excitation of the sample. The light beam is focused on the quartz sample cell. When a fluorescing molecule is present in the cell, light of a wavelength different from that used to excite the molecule is emitted in all directions. The emission optical system collects and filters the light which is then focused onto the detector. Within this general framework there are two different classes of fluorescence detectors for HPLC. The most expensive detectors use monochromators for selection of the excitation and/or emission wavelength. Filter fluorimeters replace the expensive monochromators with filters. Filters generally pass light in a wider band than do the monochromators. This frequently turns out to be an advantage since the detector does not need to be specifically tuned for each compound as it elutes. Generally the light sensitive element is a photomultiplier tube for best detection limits but solid state detectors are used in many less expensive models that are designed to take advantage of the specificity of fluorescence detectors rather than lower detection limits.

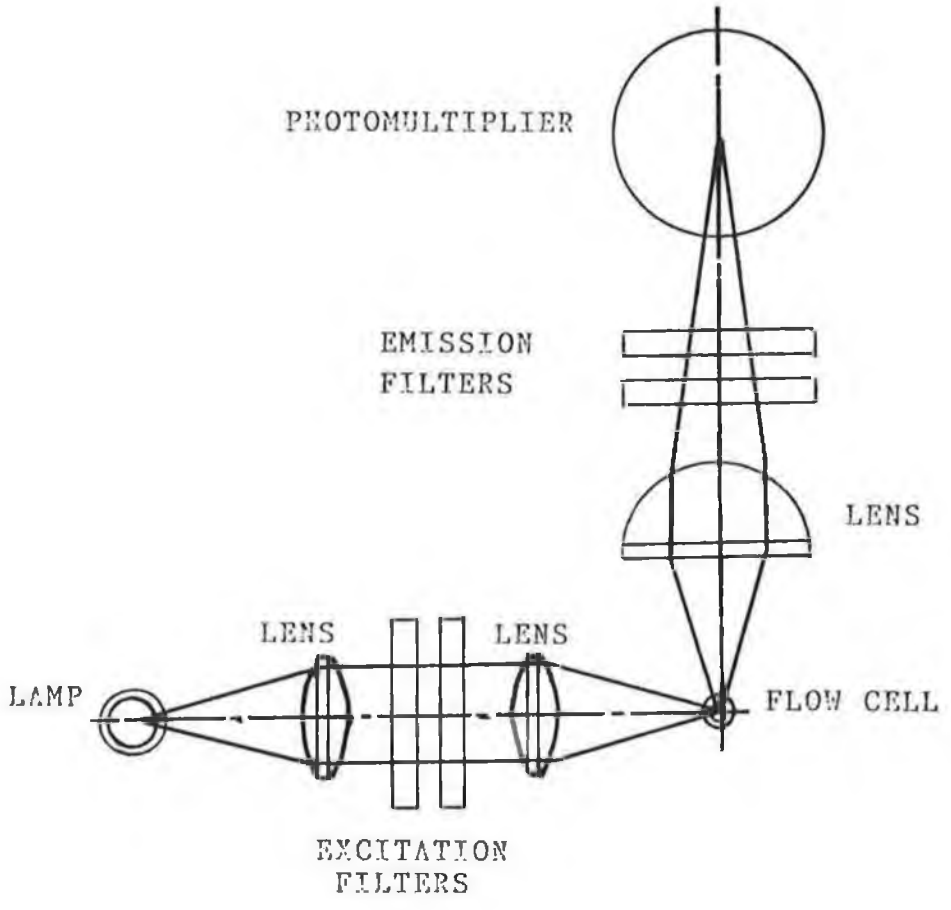


Fig. 19 Optical Diagram of a simple filter fluorimeter for Liquid Chromatography.

Requirements for Fluorescence Detection.

Organic molecules containing at least two conjugated π bonds will contain an absorption at λmax > 200nm characterised by an extinction coefficient $\varepsilon \gtrsim 10^3$ litre/mole. cm. These molecules will be considered "absorbent" in the sense that LC - UV detection at concentration higher than 200 ppb is feasible. Absorbent organic molecules generally have fluorescence quantum yields between 0.0001 and 1.0.

Basic Rules for Predicting Fluorescence in a Sample

(1) Organic molecules which do not absorb strongly above 250 nm will not be fluorescent.

(2) Molecules in which the long-wavelength single absorbtion band (So - S_1) is located at λ > 250nm and is of a (π, π^*) nature will be fluorescent.

(3) The higher the extinction coefficient, ε, for the long-wavelength band and the lower the band energy, the higher will be the fluorescent efficiency.

(4) Substituents which donate electrons to a π system will enhance absorption of light and increase fluorescence. Key examples are OH > OR > NH_2 >SH >SR > CH_3 > CR_3 e.g coumarin is non-fluorescent whereas 7-hydroxy coumarin is highly fluorescent.

5. Functional groups or solvents that introduce a long wavelength (n, π^*) absorbtion band into a molecule will tend to significantly reduce fluorescence. The addition of electron donating functional groups can reverse the effect.

   Most of the solvents used in UV detectors can be used in fluorescence detection, however halogenated solvents such as $CH_2\ Cl_2$ or $CHCl_3$ should be used with care as they tend to quench or diminish fluorescence.

1.5.4.4. Electrochemical Detectors

Electrochemical detectors are instruments which cause the sample to react (be oxidized or reduced) at an electrode surface under controlled potential conditions. The electron current at the electrode is monitored as a function of time to give the signal which is amplified and displayed on a recorder in the conventional manner.

The electrodes are encased in a very low dead volume cell (1µl), which offers good chromatographic efficiency. Quantitative data can be obtained with injected samples in the picomole or nanogram range.

This detection mode is appealing because of its high sensitivity and specificity along with low cost. Unfortunately relatively few drugs are electrochemically active. The main application of the detector in clinical LC analysis is for catecholamines. Different electrochemical techniques include conductiometric (detects ions and has low background current) and polarographic (coulometric) methods.

1.5.4.5. Diode Array Detector

The advent of the microprocessor has led to a new type of detector that offers many advantages to the user. It consists of a lamp that shines via a slit directly through the flow cell, the transmitted light goes through another slit and then to fixed grating, the resulting spectrum being refracted onto a small integrated circuit with a row of photodiodes on it. The diodes are scanned by the microprocessor giving a value corresponding to the intensity of the light over the wavelength range. This makes it possible to monitor all the wavelengths at once, (in practice every 10 ms) and generate either a spectrum at any time or a chromatogram at any wavelength. Combining these two features together can result in a three dimensioned output which may be displayed in real time using high resolution graphics on a data station.

Other detectors besides those discussed above are Evaporative or mass detector, radioactivity, Transport FID / ECD, Infra-red, Atomic absorbtion, photoconductivity, chemiluminescence and optical activity [41].

1.5.5. DERIVATISATION IN LIQUID CHROMATOGRPAHY

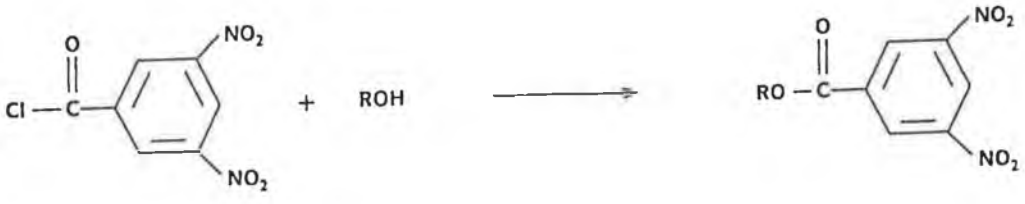
In liquid chromatography derivatisation is used primarily to enhance detection and to a minor extent to reduce the polarity of very polar and ionizable compounds as an alternative to ion-pair chromatography.

The derivatisation step can be carried out either before the injection (pre-column) on-injection or after separation (post column) [42]

Reactions to Enhance Detection

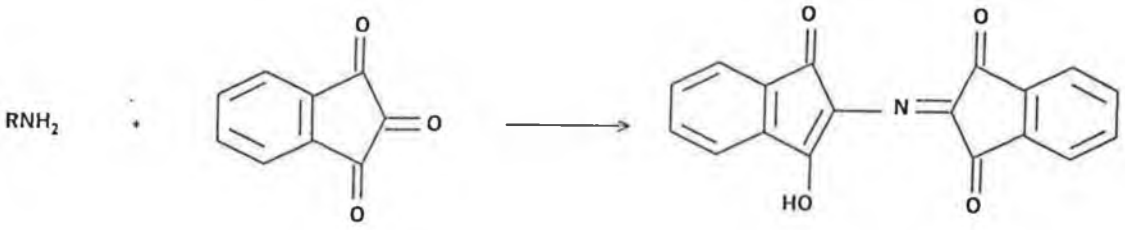
UV - vis Spectroscopy

(a) Precolumn reaction e.g. alcohols to 3,5 dinitrobenzoates.



(b) Postcolumn reaction.

Amines by the Ninhydrin reaction.



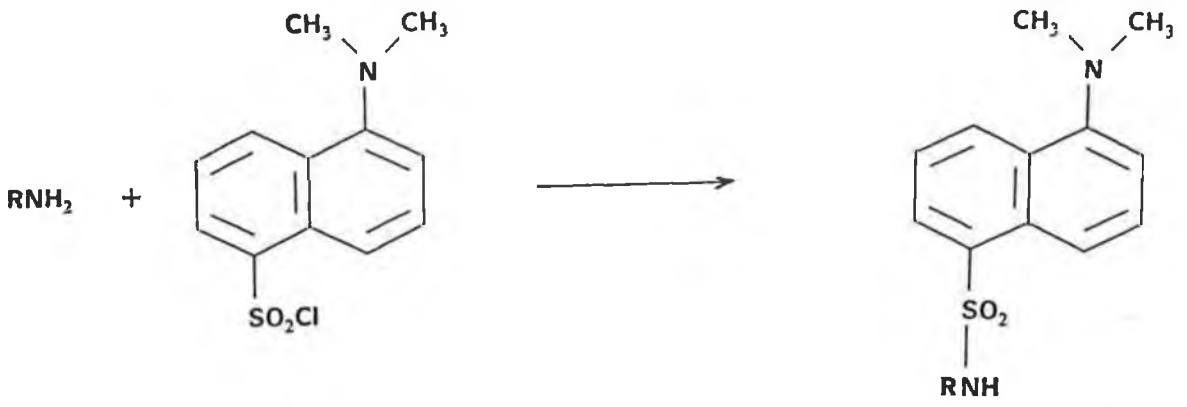
Rulemann purple 570 nm

or 440 nm

Fluroescence Spectroscopy

(b) Pre- column.

Amines and dansyl reagents:

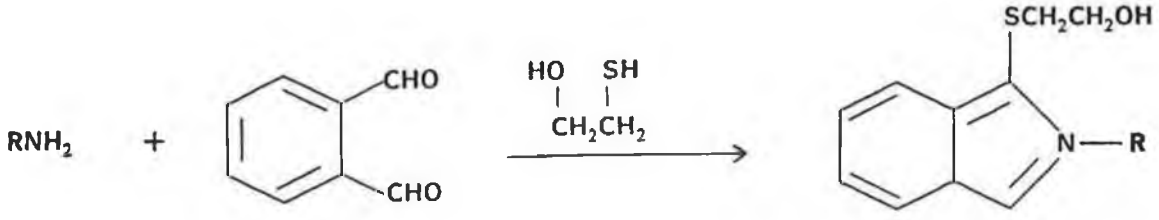


Excitation 350-370 nm

Emission 490-530 nm

Post Column

Amines and O-Phthaldehyde (OPT)



Excitation 340 nm

Emission 455 nm

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DRUG ANALYSIS IN BIOLOGICAL FLUIDS

- METHODOLOGY AND CONSIDERATIONS

CHAPTER 2

2.1. WHY DRUG ANALYSIS ?

With few exceptions drugs are low molecular weight organic compounds of known structure and well defined physico-chemical properties readily available in pure form [1]. Their measurement may be required in areas such as production control and in pharmaceutical preparations but of particular importance is their quantitation in biological samples. The analysis of drugs in biological materials poses a serious challenge to the analytical chemist due to difficulties imposed by the complexity of these media. Drug levels may be monitored for bioavailability studies, in testing the effect of new drug formulations, in cases of drug abuse and overdose and as a guide to optimise individual drug dose regimen (therapeutic drug level monitoring T.D.M.).

Bioavailability studies look at the plasma level time curve and at the pharmacokinetics of the deposition of the drug in man. Pharmacokinetics is the study of the absorption, distribution, metabolism and excretion of the drugs and their corresponding pharmacological, therapeutic or toxic response in animals and man.

Therapeutic drug level monitoring may be required in the following circumstances, (a) where there is a question of patient compliance, (b) where there is a lack of therapeutic effect, (c) where the drug has a narrow therapeutic range, (d) where there is danger of toxicity, (e) where there is a need for medico - legal variation of treatment. In many cases quantitation of metabolites in addition to the parent

drug will be required if these metabolites are pharmacologically active. T.D.M may involve quantitation in the microgram or ppm, nanogram or ppb or picogram or ppt concentration range [2].

2.2. METHODS AVAILABLE FOR DRUG ANALYSIS

Although the chemical structure of the drug will largely dictate the most suitable assay procedure for its determination [2], the magnitude and frequency of the doses administered and the pharmacokinetics of the compound govern the ultimate sensitivity and specificity required of the assay for its quantitation in biological fluids.

The major techniques used in clinical laboratories fall into three general categories

1. Chromatographic methods combined with a variety of detection modes.

2. Spectroscopic analysis (UV, fluorescence and colorimetry)

3. Competitive binding assays.

The requirement of any assay method are sensitivity, specificity, reproducibility and versatility.

2.2.1. Chromatographic Techniques

2.2.1.1. Gas Liquid Chromatography with a variety of detection modes i.e. flame ionization detection (FID), electron-capture (ECD) and nitrogen-phosphorous (NPD), is a widely applied technique. Although analysis of the intact drug moiety (underivatized) is preferred to assure specificity, derivatization is often necessary. Simple derivatization reactions such as alkylation or silylation can be used when needed to yield very sensitive and specific methods. When making a derivative one must take account of the detection system to be used so that during sample preparation contaminants are not introduced into the extract which could be detrimental. NPD is susceptible to interferences by residues of silylating reagents and phosphate plasticizers contained in plastic syringes. The features, applications and limitations of the various GC detectors will be discussed more fully under the different drug groups.

2.2.1.2. Thin Layer Chromatography (TLC) Firstly TLC analysis allows rapid development of chromatographic parameters for use in HPLC analysis. Using a radiolobelled compound, the drug and its metabolites are separated and this allows qualitative identification by radiochromatographic scanning or autoradiography to isolate metabolites from either an *in vitro* 9000g microsomal incubation or from *in vivo* biotransformation studies [3]. The separated compounds can be eluted from the silica gel and analysed by a variety of selective techniques e.g. spectrophotometry/fluorimetry, polarography, GC, GC-MS (Mass

Spectrometry) and NMR. These can be used not only for quantitation but also for structure elucidation purposes.

Quantitative analysis by *in situ* spectrophotometry/fluorimetry has been widely used, especially since the advent of HPTLC using a small sample aliquot (1 µl or less) applied to the chromatoplate followed by rapid development and densitometric analysis [4]. This technique is especially useful in the fluorescence mode and has been applied to the analysis of flurazepam and its metabolite [5]. Some of the advantages of TLC analysis are the ability to analyse the entire sample by either one or two dimensional solvent ascending preparative scale chromogography, rapid development of the separation (HPTLC) and relatively low cost of the separation *per se*. However the spectrodensitometer required for quantitation is expensive.

2.2.1.3. HPLC

HPLC is an attractive approach for a number of reasons. Firstly, sample preparation is often minimal, a simple protein precipitation step followed by centrifugation and injection of the supernatant may be all that is required. Secondly, there is almost never a requirement for sample derivatization prior to the chromatography as there is in GC. Thirdly, it can be used for samples that are thermolabile and unsuitable for GC. Fourthly, most analyses can be performed successfully on reverse phase systems. These systems are ideally suited to therapeutic drug

analysis. Most drugs possess sufficient hydrophobic structure to ensure retention by the stationary phase. However, many also contain hydrophobic groups which will limit this retention and allow the chromatographic process to occur in reasonable time. Mobile phase "strength" can be readily controlled by adjusting the organic solvent to buffer ratio. Variations in pH, ionic strength and the use of ion-pairing agents provide additional versatility.

Detectors for drug analysis using HPLC include ultraviolet and visible absorbance, fluoresence and electrochemical depending on the chemical properties of the drug to be analysed. To date, UV absorbance has found most application. Adequate detection depends upon the presence of the appropriate functional groups within the molecule and in turn the limits of detection will depend upon the value of the molar absorptivity coefficient at the existing wavelength. Many drugs possess conjugated chromophores and UV often allows detection at the low nanogram level for suitable analytes.

2.2.2. Spectroscopic Techniques

Spectrophotometric (UV-VIS) and luminescence emission (fluorescence and phosphorescence) methods have been the classical methods used extensively in drug analysis. They possess adequate sensitivity but lack high specificity since spectral characteristics cannot usually differentiate the parent drug from any metabolites present unless used in conjunction with either differential solvent extraction

techniques using liguid-liquid or solid extraction separations. Most drugs exhibit moderate to strong absorption bands in the near ultraviolet region, exhibiting maxima in the 215-300 nm region. While UV methods are not as selective as chromatographic methods, they are simple and rapid, making them useful for screening purposes.

2.2.3. Immunoassay

All immunoassays are protein binding techniques. The binding of ligand (L) (drug) to a protein antibody (AB) a single specific binding site can be described by the following equation

$$L + Ab = LAb$$

According to the law of mass action

$$K = \frac{[LAb]}{[L]\ [Ab]}$$

Let [Ab], the bound ligand concentration = B; and [L] the free ligand concentration = F, then

$$K = \frac{B}{F} \times \frac{1}{[Ab]}$$

If total antibody = $[Ab_T]$

then $[Ab] = [Ab_T] - [B]$

and

$$K = \frac{B}{F} \times \frac{1}{[Ab_T - B]}$$

or

$$\frac{B}{F} = K\ [Ab_T - B]$$

This equation shows that if the antibody concentration $[AB_T]$ is in excess of the total ligand [F+B] concentration then $[Ab_T] >> B$ and B/F will remain a constant with a value of $K[Ab_T]$. As the concentration of the total ligand approaches the total binding site (antibody) concentration ([Ab]) then

B becomes a significant fraction of $K[Ab_T-B]$ and B/F will decrease. Finally at higher concentration of total ligand, B approaches $[Ab_T]$ and in turn B/F will approach zero. A graph of B/F vs total ligand yields a a sigmoid "standard" curve of protein binding assays (Fig. 1). It is evident that the useful portion of the curve begins when binding is about 50% (B/F = 1). Therefore in any assay the antibody should be diluted to the point where there is 50% binding of the lowest concentration of ligand to be measured.

Furthermore since $B/F \leq K [Ab_T]$, the maximal dilution of the antibody that yields this binding is actually determined by the value of K, the association constant (in fact at 50% binding B/F = 1 and $[Ab_T] = K^{-1}$). In turn since that total ligand concentration is now of the same order of magnitude as $[Ab_T]$ then the minimum concentration of ligand that can be measured in an assay is also determined by K i.e. as K increases (K^{-1} decreases) so does the sensitivity of the assay. A sensitive assay therefore requires a small amount of avid antibody.

2.2.3.1. Methods of Assessing Binding

Binding can be assessed by having present a fixed amount of labelled ligand which competes equally with "unlabelled" ligand for the protein binding sites. This competitive binding arrangement is illustrated in fig. 2 i.e. when no unlabelled ligand is present binding of the labelled ligand is maximal (e.g 50%). However, as unlabelled ligand is added (as standard or sample) it competes with the labelled

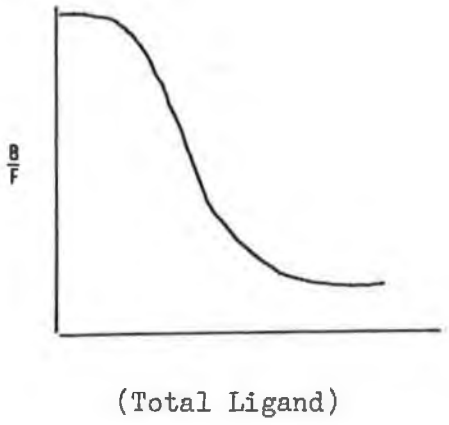

Fig. 1 - Typical plot of bound (B) over free (F) ligand vs total ligand from a protein-binding assay.

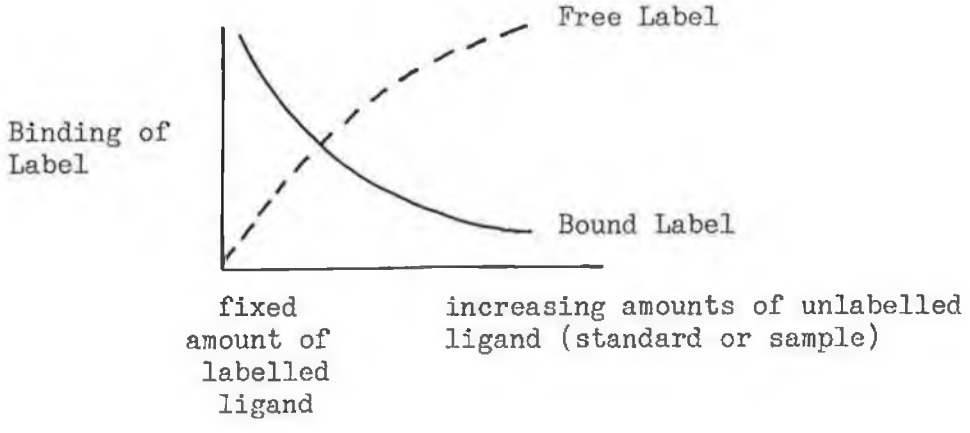

Fig. 2 - The effect of adding increasing amounts of unlabelled ligand to a protein-binding assay.

ligand for a fixed number of binding sites and causes a reduction in the amount of bound label. In conventional radioimmunoassay the fixed ligand is labelled with a radioactive isotope and binding is assessed by measuring the radioactivity associated with the bound or free fraction. These fractions must of course be separated prior to the counting step. The so called homogeneous immunoassays do not require separation of bound and free ligand prior to assessment of the extent of binding because they use labels that are detectable only in one or other of the fractions. Three of these types of assays are particularly prevalent, enzyme multiplied immunoassay in which the label is an enzyme which is active only in the free fractions, fluorescence polarization assays in which the label is a fluorescent molecule and the binding is assessed by measuring the light - polarizing properties of the bound fluorophore - drug species and substrate labelled fluorsecent immunoassay in which the label is again a fluorophore but where binding is assessed by measuring the ability of the unbound fluorphore drug to compete to serve as an enzyme substrate. A good account of the various types of immunoassay is given in a review by de Silva [2].

2.2.3.2 Development of Drug Immunoassays

Drug immunoassays are simple to perform but complex to develop.

1. The drug has to be coupled as a hapten to a carrier protein, usually bovine serum albumin in order to raise

antibodies against them since most drugs themselves are not immunogenic [6].

2. If the drug molecule does not have an appropriate group for direct conjugation reaction a chemical derivative may have to be prepared prior to the conjugation step.

3. After formation of the immunogenic species an animal (usually a rabbit or a goat) is immunized and the appropriate antibodies isolated.

4. The drug must be labelled with the appropriate label.

Finally when all the reagents are available the assay procedure itself must be optimized in terms of variables such as antibody dilution pH, temperature and time of incubation [7].

The advantages of immunoassay are that for determination of drug concentration in biological fluids they are (1) very sensitive (ii) only a small volume of sample is needed (iii) no purification (iv) chemically they are gentle (v) they can be applied to all classes of compound (vi) easy to perform and can be automated.

2.3 HPLC VS IMMUNOASSAY

Two techniques dominate the field of biopharmaceutical analysis (a) Chromatography especially HPLC and (b) Immunoassay.

Chromatographic analysis requires that the drug be physically separated from the original matrix. The chromatographic process is actually part of the isolation procedure and is largely responsible for assay specificity. The subsequent quantification is outside the matrix. Immunoassay on the other hand, isolates the drug within the matrix and it is the quality of the antibody that determines specificity. Subsequent quantitation is often in the presence of the original matrix.

Currently immunoassays are used much more than LC for therapeutic drug monitoring because they are technically less demanding, avoid sample pre-treatment and have been able to offer a much higher throughput due to batching of samples and can be automated more quickly. Some further advances in this technique may arise from the potential application of monoclonal antibodies to drug analysis [8].

On the other hand LC because it is a powerful separation technique can be used to quantify closely related drugs and their metabolites such as tricyclic antidepressants. This capability is probably beyond the specificity available from antibodies. Related to this LC allows simultaneous determination of several drugs in a single run whereas separate immunoassays have been required for each drug and metabolite. LC is potentially more accurate because it is a more direct method. The potential for inaccuracy with immunoassays is illustrated by the tendency for antibodies

recognising digoxin to cross react with digoxin metabolites [9]. The final advantage of LC is that one can set up new drug assays more quickly.

2.4. THE SIGNIFICANCE OF FREE DRUG MEASUREMENT

Drug molecules circulate in blood in two forms, those in association with blood components such as proteins, red cells or platelets and those which are unbound and are dissolved in plasma water [10, 11, 12]. The extent of drug binding to plasma proteins varies widely among different types of drugs. The effects of changes in the extent of protein binding on free drug fraction and on drug metabolism are significant only for highly bound drug (greater than 80% bound) since even a relatively small change in the degree of binding has a dramatic effect on free fraction.

Albumin is quantitatively the most important drug binding protein for many drugs, particularly for the neutral and anionic (acidic) drugs such as warfarin, phenytoin and valproic acid [13]. In recent years, cationic (basic) drugs such as propranolol, quinidine, and the tricyclic antidepressants have been shown to bind not only to albumin but also to other blood proteins such as α-acid glycoprotein (AAG) and lipoproteins [14-16].

A widely held view on the pharmacological effect of protein binding is that only the free form of the drug can cross the capilliary membrane and cell membranes. It is therefore assumed that the free fraction <u>in vitro</u> is equivalent to the

pharmacologically active fraction *in vivo* and free drug levels will correlate better with drug effects and clinical conditions of the patients. It should be appreciated that the free drug hypothesis is unproven. The basic assumption that only unbound drugs can cross cell membranes has been questioned lately when it has been shown that globulin bound lidocaine and propranolol were transported into tissues such as the brain [17]. The equilibrium measurement of plasma free drug *in vitro* may underestimate the amount of exchangeable plasma drug *in vivo* that is available for transport into tissues.

2.4.1. Methods for Free Drug Measurement

In reality for routine therapeutic drug monitoring, the total concentration of the drug in serum is usually measured rather than the free fraction - a custom that probably reflects technical limitations rather than clinical utility. The two most common methods used to measure free drug are equilibrium dialysis and ultrafiltration.

2.4.1.1. Equilibrium dialysis

Equilibrium dialysis suffers from certain limitations, detailed reviews on the subject are available [18, 19, 20]. Briefly the technique is based on the principle that unbound drug will equilibrate across a semi-permeable membrane which is permeable to small drug molecules but not to drug binding proteins. The method is performed by placing serum or plasma in a chamber separated from buffer in another chamber by the membrane. At equilibrium the drug concentrated in the buffer compartment (dialysate) is considered to

represent the unbound drug concentration which will be the same as the free drug concentration in the protein compartment (retentate). Experimental conditions such as temperature and buffer concentration can give irreproducable results. A number of papers which critically address the problems have appeared [19, 21-27]. Briefly the problems are (a) that new equilibria are set up during dialysis so that the bound and unbound fractions at equilibrium are different from those of the original sample (b) significant Donnan effect (c) osmotic dilution (d) non specific adsorption of free drug and protein molecules to the surface of the dialysis apparatus (d) lack of attainment of equilibrium and (e) protein leakage into the dialysate.

2.4.1.2. Ultrafiltration

Ultrafiltration is the other commonly used technique. A pressure gradient generated by centrifugation is used to force plasma water and small molecules through a semi permeable membrane. Ultrafiltration is an attractive alternative to equilibrium dialysis because of the ease and speed with which it can be accomplished. Speed is an important consideration in studying the binding of drugs to albumin since binding of some drugs may be deceased by fatty acids [28, 29] which are released through hydrolysis of plasma triglycerides when stored for long periods and in dialysis of long duration. Problems associated with ultrafiltration are:-

(a) disturbance of protein binding equilibria during filtration

(b) non-ideal membranes which allow a higher filtration rate for water than for the drug resulting in dilution of the drug concentration.

(c) temperature and pH must be carefully controlled.

(d) adsorption loss to the membrane

(e) the Donnan effect.

In principle therefore, T.D.M. based on the measurement of free drug should be more meaningful than that of total drug because free drug reflects drug concentration at receptor sites. Unfortunately the technology to date for free drug measurement is complex compared to the routine assays for total drug, hence clinical correlation of drug effects with plasma levels has been based on total drug. As long as protein binding of drugs is normal and constant (within and between patients) free drug is a fairly constant fraction of the total drug. As such measurement of total drug is an adequate estimation of free drug making free drug measurement unnecessary.

This relationship between free and total drug does not hold however where protein binding is variable or abnormal which may be due to abnormal protein concentration, drug interaction or displacement and concentration dependent binding. For example with some commonly monitored drugs such as phenytoin the extent of binding varies greatly among individuals and the free fractions seem to correlate more closely with therapeutic efficacy and toxicity than does the total concentration [30, 31].

2.5. CHOICE OF SAMPLE FOR DRUG ANALYSIS

In drug analysis the most commonly sampled body fluid is plasma or serum because a good correlation between drug concentration and therapeutic effect is usually found.

Whole blood can be broken down into its general components as follows: plasma which contains the serum and fibrinogen and the cellular elements which contain the erythrocytes, leucocytes and platelets. Plasma is the liquid portion of circulatory blood. The cells are separated from the blood by centrifugation of whole blood. If blood is allowed to clot, the fibrinogen is removed from the plasma leaving serum. The majority of clinical analyses are performed on whole blood, plasma or serum and most of these use serum.

Urine is useful when a drug or a rapidly formed metabolite is extensively excreted in it. Drugs can usually be detected in urine for some time after they have become undetectable in blood. Urine analysis for drugs is used in connection with urinary excretion and bioavailability studies. Saliva and cerebrospinal fluid (C.S.F.) are also analysed for drugs, but less frequently. Drug concentrations in saliva are sometimes assumed to represent free plasma levels, but this is found to be true only for a few drugs (e.g. carbamezepine and phenytoin). For others the correlations are less satisfactory or apparently non existant [32-34]. It is not practical to analyse C.S.F. samples routinely but occasionally C.S.F. levels may be required if damage to the blood brain barrier is suspected.

2.6 COLLECTION AND STORAGE OF SAMPLES

Collection of an uncontaminated specimen at the correct time in relation to dose is vital in any projects involving drug analysis. If insufficient care is taken in the collection and handling of biological specimens the data generated will be invalidated. If plasma or serum is analysed, the expressions "blood samples" or "blood levels" should not be used in description of the analytical procedures unless whole blood samples are to be analysed for some reason (for example drugs with a high affinity for red cells such as chlorthalidone or cyclosporin). Specimen collection tubes containing anti-coagulants (for blood) or preservatives (for urine) are available. The choice of anticoagulant may affect assay results as may some plasticizers released from blood collection tubes. The selection of glass or plastic tubes for blood collection and subsequent separation of plasma or serum, and its effect on the drug in an in vivo sample with respect to adsorption losses, contamination by plasticizers, displacement of protein bound drug by plasticizers and their effects on the plasma to haematocrit ratio warrant investigation. Drug concentration data can be adversely biased by these phenomena, hence also pharmacokinetic implications. For example, significant decreases is plasma Tricyclic Antidepressant concentrations have been attributed to sample contact with the plasticizer tris - (2 - butoxy ethyl) phosphate (TBEP) which may be present in the rubber stopper of collection tubes. TBEP free stoppers are now available [35]. Serum separator gel contained in serum separator blood collection tubes has also been demonstrated

to decrease the resultant serum concentrations of lidocaine, phenobarbital and phenytoin [36].

On no account should a blood sample be frozen without treatment since this would result in haemolysis, preventing the subsequent separation of plasma or serum. After collection of blood (5-10 ml) a clot can be allowed to form and the supernatant liquid (serum) collected after centrifugation. Coagulation is complete in about 30 minutes at room temperature. Alternatively the blood can be collected in tubes containing an anticoagulant (e.g. heparin, E.D.T.A.) and the supernatant liquid (plasma) collected after centrifugation. Since the anticoagulant effect is temporary, collected specimens must be centrifuged quickly to prevent eventual clotting. Plasma is more frequently used than serum in drug analysis since although the results from both fluids are identical plasma is preferred since the anticoagulated blood can be centriguged immediately, whereas serum cannot be sampled until coagulation is complete. Secondly it is relatively easy to centriguge blood that has been treated with anticoagulant, since the plasma separates quickly and the maximal volume can be recovered if required.

Drug may be lost to the container or be degraded during storage. Thus the stability of a drug in a biological fluid at various storage temperatures should be studied. Fresh plasma or serum samples can usually be kept for 6 hours at room temperature, 1-2 days in a refrigerator at 4^{o} or frozen at -20^{o} for long term storage. The Council of the British Pharmaceutical Society has issued guidelines for a pharmacy

based pharmacokinetic service which includes instructions on sampling and storage of blood [37].

Urine drug analysis is done either on a single or 24 hr. specimen. Both pH and volume are important factors in urine analysis. If urine is allowed to stand at room temperature bacterial action causes the decomposition of urea into ammonium carbonate and then to ammonia, with a resulting change in pH. Urine may be preserved by freezing or by addition of preservative (toluene, boric acid or concentrated HCl). Frozen samples of plasma/serum or urine should be brought to room temperature and subjected to vortex mixing for 10 seconds to ensure homogeneity before analysis.

2.7. DIRECT INJECTION

In a few cases, direct injection of the biological fluid into the chromatograph is possible. For example in the case of theophylline which is therapeutically present in relatively high concentration (mg/l) and possesses good UV absorption characteristics, a small volume of dilulted serum can be injected directly onto the chromatograph, thus minimizing the amount of non-eluting contaminants placed on the column with each injection. In this case the mobile phase and the column packing release the drug from protein binding sites as the sample enters the column. Direct injection has also been applied to analysis of salicylate and naproxin in serum [38].

Urine can also be directly injected onto the chromatograph following centrifugation to remove particulate matter and suitable dilution preferably with the mobile phase. For example, antibiotics [39] and other drugs [40] can be determined in urine by HPLC with on-column injection.

When using direct injection the analytical column should always be protected by a guard column to prevent irreversible adsorption and blockage. Biological samples contain materials such as lipids and proteins which deposit on the chromatographic column and lower its performance. Direct injection is limited in its application.

Normally to improve both the sensitivity and selectivity of an assay some form of sample clean-up prior to injection will be necessary. These will now be considered.

2.8. SAMPLE PRETREATMENT

Some form of pretreatment of the biological sample prior to analysis is normally required. This is necessary for the following reasons (a) to release the drug from the protein binding sites, (b) to isolate the free drug, (c) to remove interferents which may collect in the column and lower its performance and (d) to concentrate the drug for more sensitive analysis. Various types of sample pretreatment will now be discussed.

2.8.1. Protein Precipitation

In this method, one volume of plasma or serum is mixed with a certain volume of precipitant e.g. trichloroacetic acid, perchloric acid, methanol or acetonitrile. The volume of precipitant used will depend on how effective it is [41]. These solvents precipitate the proteins and release the drug from protein binding sites. After vortex mixing and centrifugation an aliquot of the clear supernatant is injected onto the HPLC column. Care must be taken that the solvent front does not interfere with the peaks of interest as some precipitants absorb in the UV. This method of sample preparation is simple, rapid and accurate. The protein precipitation technique protects the column from deposition of proteins. Up to 99% of protein may be removed by the addition of sufficient precipitating agent. The major drawback here is that dilution of the sample occurs which decreases sensitivity. This drawback may be counteracted by using a larger injection volume. Another drawback is that the procedure sometimes gives low recoveries of drugs that are strongly protein bound since they may co-precipitate or degrade during precipitation. When a new method is being developed, several precipitating agents in various proportions should be investigated to test their efficiency in removing protein and producing a relatively interference free supernatant with good recovery of drug. Although methanol is slightly less effective as a protein precipitant than other organic solvents [41] it should be used whenever possible because of its relatively low cost and low toxicity.

The protein precipitation method is particularly useful for highly polar (e.g. antibiotics) or amphoteric (e.g. sulphonamides) drugs which are difficult to extract from plasma with organic solvents. Analyses with good reproducibilities are often possible, without the use of internal standards [42].

It should be noted however that the drug peak may often be accompanied by other peaks, because the likelihood of interfering peaks is probably about the same as with direct injection because most if not all of the drugs and analogous physiological solutes will be included in the supernatant of these samples. Moreover late eluting peaks from previous samples may co-elute with the drug peak, thereby distorting peak measurements and adversely affecting precision. The potential for late eluting peaks to degrade assay precision in T.D.M. has been pointed out [43]. Protein precipitation did not prove useful for any of the work described in the following chapters as it did not give adequate sensitivity or clean chromatograms.

2.8.2. Solvent Extraction

Liquid-liquid extraction is by far the most popular method used for the clean up of biological fluids mainly due to its versatility [44-47]. It provides chromatographically clean extracts and allows for concentration of the sample by evaporation thus enhancing sensitivity. The size of the sample chosen will depend on the sensitivity required of the assay. Generally however 1ml of plasma or serum is used and

extracted into 5ml or more of organic solvent by vortex mixing followed by centrifugation. The addition of a large amount of extractant minimizes emulsion formation. Glassware used in extractions must be scrupulously clean and free from any residual detergents.

Polarity is usually the most important factor in the choice of extracting solvent and generally as this increases the range of components extracted also increases. The solvent should be selected with minimum polarity consistant with high recovery of the drug. Drugs of high polarity are difficult to extract and require strongly polar and hence non-selective solvents. Polar solvents that are miscible with water (e.g. n-propanol) can be forced to form a separate layer by saturating the aqueous phase with an inorganic salt, a technique useful for very polar drugs [48]. Salts can be important in other ways, as they help to form better phase boundaries (i.e. avoid emulsions) and can also reduce the water content of the organic phase. Further the addition of salts can be used to increase the selectivity of an extraction by aiding the transfer of a drug from the aqueous to the organic phase.

A solvent used for extraction should be of the highest purity, non-toxic, not highly flammable and have a suitable volatility. The most popular solvents are diethyl ether and chloroform. Although ether is flammable, it is reasonably selective, easy to evaporate and its low density favours phase transfer after extraction. Moreover ether emulsions are easy to break.

Modification to the pH of the aqueous phase is also most important in the development of an extraction scheme. It is the unionised form of the drug that is extracted into the organic solvent. Thus acidic drugs are extracted under acidic conditions and basic drugs under basic conditions in order to suppress their ionisation. The optimum pH for extraction of acidic drugs usually lies 1-2 pH units below the pKa value and for basic drugs 1-2 pH units above the pKa value. Amphoteric drugs also show an optimium pH for extraction, the extraction of neutral drugs is independent of pH, they are extractable over a wide pH range. However consideration of pH is important even when assaying for neutral drugs, since a higher pH is often desirable to ensure cleaner extracts since many endogenous compounds are acidic and hence not extracted from alkali. Extraction can be problematic when the drug is water soluble at all pH values (water soluble amphoteric and neutral drugs, zwitterions) since pH adjustment will not help in this situation. In some cases a salting out procedure can shift the partition equilibrium in favour of extraction. Horning et al. [49] used this approach in the screening of plasma and urine for acidic, basic and neutral drugs and their metabolites. By using ammonium carbonate as the salt and ethyl acetate as the solvent they were able to extract the drugs with recoveries better than 80%.

Gas chromatographic analysis often requires a back extraction to prevent extraneous substances in the first extract from contaminating the detector especially NPD and ECD detectors. The drug present in the first extract

is back extracted into an aqueous phase of appropriate pH and the pH of this aqueous phase is then readjusted to the pH of the first extraction and the drug re-extracted into organic solvent.

Metabolites of a drug are usually more polar than the drug itself and if they are to be quantified the solvent chosen for the extraction should be capable of extracting these. However, if desired,extraction with solvents of increasing polarity and/or at different pH values can achieve selective separation of a drug from its major metabolites. Another approach to extracting the more polar metabolites is to extract the drug, then add a high concentration of salt such as sodium chloride to the aqueous phase and re-extract. This forces the desired compounds into the organic phase. Absolute specificity by selective extraction alone is difficult to achieve and thus separation must usually include a chromatographic step. HPLC is usually the method of choice since the drug and its metabolites can usually be analysed without conversion into derivatives or use of extensive clean-up procedures.

2.8.3. Hydrolysis of Conjugates

Many drugs and metabolites are present in urine as conjugates such as glucuronides or sulphates which are very polar and not extractable into organic solvents. It is often necessary to hydrolyse these conjugates so as to release the parent molecules for extraction. This is done chemically with hydrochloric acid or sodium hydroxide or enzymatically with enzymes such as beta-glucuronidase. Chemical hydrolysis decreases the yield of drugs that are

heat labile (e.g. certain benzodiazepines) and sensitive to aggressive reagents. Enzymatic methods on the other hand are slow but mild. After hydrolysis the products can be extracted following pH adjustment or back extracted if necessary. Generally a urine sample is divided into two sub-samples, one hydrolysed to give the total concentration of the drug and the other extracted to give the free concentraction of the drug. The concentration of the bound form is obtained by subtraction.

2.8.4. Ion Pair Extraction

A useful approach to dealing with a highly polar ionic drug is to convert it into a neutral ion-association complex by addition of an excess of suitable ions of opposite charge and extract it into an organic solvent such as chloroform. The counter ions may be inorganic or polar organic. Formation of the complex depends on factors such as pH of the aqueous phase, polarity of the extracting phase and the nature and concentration of the counter ion. The ion-association complex technique can be used for all kinds of ionizable drugs but it offers a particular advantage for compounds that are difficult to extract in uncharged form e.g. quaternary ammonium compounds [50].

2.8.5. Liquid - Solid Extraction

Sample pretreatment by solid phase extraction involves applying the sample to a small column filled with adsorbent material, which binds the drug before elution. Among the adsorbent materials that have been used are Celite, alumina, silica, chemically bounded silica, Florisil and non-ionic and ion-exchange resins. Disposable columns of various sizes and covering the wide range of adsorbents are available commercially (e.g. from Waters Association or Analytichem International).

These materials fall into two broad categories. In the case of the first group, a hydrophilic packing material (e.g. inert particles of diatomaceous earth) is used to adsorb the whole sample so that an aqueous film is formed on the surface of each particle. A small volume of a 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 other polar endogenous materials are retained by the matrix. This type of extraction is essentially a liquid-liquid extraction so that drugs can be selectively eluted by approaches similar to those used in liquid-liquid extraction.

The second type of liquid-solid extraction works on chromatographic principles. Compounds of interest are retained on the absorbent surface when the sample is passed through. If undesirable compounds are adsorbed at this stage they may be removed by washing with a specific solvent or buffer. The drug of interest is then eluted with a

specific solvent and processed by HPLC. The key to obtaining high recoveries of the purified compound of interest is to identify the bonded silica phase that is most selective for that compound. The selectivity will be determined by the affinity the sorbent has for the isolate versus the interference in the matrix. To a great extent, one can predict the best type of sorbent by considering the following [51].

(1) The solubility of the isolate in non polar and polar solvents.
(2) Whether the isolate has the potential to become charged, thus becoming a candidate for ion-exchange.
(3) Whether the isolate has the ability to form a covalent bond (reversibly) with a sorbent such as phenylboronic acid bonded silica.
(4) To what extent the undesired compounds in the sample matrix have the potential to compete with the isolate for the sorbent binding sites [52].

The liquid-solid extraction technique is becoming a serious challenger to conventional solvent extraction (liquid-liquid partition). It is simple and time and labour saving (though prepacked disposable columns are by no means cheap). There are no losses due to emulsion formation and the extraction results in cleaner samples which extend the lifetime of columns. The efficiency and reproducibility are as good as or better than those of liquid - liquid extraction and they can be applied to a wide range of compounds.

2.9. SOME TECHNICAL CONSIDERATIONS

2.9.1. Columns

For general clinical and biochemical L.C. analysis and particularly for T.M.D., reversed phase columns are the ones of choice [53]. Advantages of these columns are (a) resistance to contamination and water based mobile phases, (b) ease of equilibration and (c) broad applicability. The most popular reversed phase columns are C_{18} and C_8. The differences in chromatographic properties between both these columns are subtle but at higher flow rates chromatographic efficiency decreases less with C_8 than with C_{18}. More polar bonded phases such as cyano and phenyl can be also used in reverse phase mode by incorporating adequate water in the mobile phase.

Other important factors influencing separation efficiency are column length and particle size of the packing. The most widely used columns are those 15cm in length packed with particles 5μm in diameter. Gaining in popularity is "high speed liquid chromatography" which uses short columns 3 to 8 cm long, internal diameter 4.6mm and packed with 3μm particles. These are operated at a high flow rate (2 to 5 ml/min), using a small volume flow cell (2.4μl) and a detector system with a fast response time. Analysis time is significantly shortened and there is less solvent consumption but since a higher than usual backpressure is used, wear and tear on equipment can be great. Another

strategy is the use of short columns with 3μm packing in order to increase the internal diameter to 6.2mm and use a flow rate that is only moderately higher, perhaps twice the conventional [54]. In this case the chromatogram is about the same in overall appearance as that obtained with a conventional column but is obtained in a third of the time. A problem associated with the use of both these types of columns is that they can easily become clogged with particular matter from plasma because of the small particle size. Secondly clogging is more likely in the lower porosity filters and frits (0.5μm vs 2.0μm conventional) used in these columns.

Most analytical columns have an internal diameter ranging from 3 to 6 mm. These dimensions provide a good balance among (a) ease of packing with minor contribution from wall effects [55, 56] (b) compatibility of the sample and peak volumes on these columns with detectors in routine use, (c) limited elution volume of costly mobile phase, (d) limited amount of costly packing and (e) moderate pressure drop.

The stability of a column can be greatly improved by the use of a guard column before the analytical column which protects it from contamination and clogging. Modern columns can withstand more mechanical stress but more work is required to investigate the possibility of regenerating columns contaminated by impure substances, such as the usefulness of a strong solvent injection between samples [57]. A major factor affecting the stability of columns is the pH of the mobile phase. The pH range within

which bonded-phase silica-based packings can be used is typically 2 to 7. At lower pH the bonded groups are hydrolyzed, and at higher pH, the silica matrix itself dissolves, leading to the release of bonded groups from the surface. The stability at higher pH may be extended by the use of a silica-packed pre-column between the pump and the sample valve, presaturating the mobile phase with silica before it enters the analytical column [58]. The use of amine vs alkali metal buffers also improves the column stability at higher pH values [59]. Mobile phases with a high pH can be used in normal phase L.C. with silica particles because the high organic content of the mobile phase minimizes the solubility of the silica. An example of this will be seen in Chapter 4, in the determination of the tricyclic antidepressants.

2.9.1.1. Column to Column Reproducibility

It is important that the same chromatographic conditions for a drug analysis give similar results with each new column. This is often not the case and modification to certain parameters is often required in order to achieve separation on a new column particularly for drugs that are susceptible to active sites on the column. It is important to develop mobile phase conditions not only to achieve separation but also to minimize column to column differences. Probably the most severe test of column to column reproducibility is the relative retention of acidic and basic substances analysed simultaneously. Although many drugs are netural, avoiding major problems in column to column reproducibility when

analysed by LC, some of the potential interferences may be acidic or basic and so their retention may vary from column to column giving rise to interference in the chromatogram.

2.9.2. Mobile Phase

A major advantage of LC over GC is the opportunity to influence the separation of drugs by changing the composition of the mobile phase, giving extra flexibility and control.

Reverse phase chromatographic systems are the most popular for drug analysis. The two most commonly used organic solvents in the reverse phase mode are methanol and acetonitrile, acetonitrile is the less polar of the two providing an equivalent solvent strength to methanol at a lower concentration. Acetonitrile also yields a mobile phase with water which is less viscous than with a water-methanol mixture, this makes the column more efficient and allows a lower pumping pressure. Selectivity differences between the two solvents arise because acetonitrile is a hydrogen bond acceptor while methanol is a hydrogen bond donor. Another solvent which may be considered is tetrahydrofuran because it offers further contrasting solubility properties (less polar, weaker dipole moment than acetonitrile, nonhydroxylic relative to methanol).

Another possibility is the use of a ternary solvent system i.e. a combination of two organic solvents and water. Drug separation can be significantly affected by even a small proportion of a second organic solvent, especially if this solvent is a strong eluent relative to the mobile phase being modified [60]. This is because such a solvent tends

to concentrate in and change the characteristics of the bonded phase. An example of this phenomenon is the observation that the separation of TCA's on an RP column is markedly improved by adding 5ml/l of methylene chloride to the mobile phase.

Temperature of the mobile phase can also affect chromatographic behaviour. The primary effect of increasing the temperature is to increase the solubility of drugs in the mobile phase solvent thus diminishing their chromatographic retention. A higher temperature also improves column efficiency, largely because the mobile phase is less viscous. A disadvantage of higher temperature is decreased column stability.

2.10. DEVELOPMENT AND VALIDATION OF A CHROMATOGRAPHIC ASSAY

The most logical approach in the development stage of an assay involves opposite chronology to the final protocol. Although isolation precedes measurement, it can be more convenient to develop the analytical technique first. This is followed by selection of an internal standard and development of the isolation procedure [61].

Samples of drug free material (serum, urine) spiked with known drug concentrations should always be employed for calibration. Alternative "external" approaches on assaying or even just injecting (in the case of chromatography) pure standard solutions are unacceptable because they ignore the specific peculiarities of the biological matrix. However, even drug supplemented samples are subject to criticism, as

since externally added drug might indeed not be present in the same physico-chemical state as an *in vivo* delivered drug.

Most regulatory agencies, like the Food and Drug Administration, have mandated Good Laboratory Practice (GLP) regulations for clinical and non-clinical laboratory studies [62, 63]. Based on these regulations, the fundamental criteria required for validation of chromatographic methods to check their reliability and overall performance are the evaluation of drug stability, specificity, limit of detection, accuracy, precision, linearity and recovery. Each of these individual criteria will now be discussed.

2.10.1. Drug Stability

Drugs stored under different conditions of heat, light, humidity and pH (stock solutions and spiked biological samples) should be examined for possible decomposition. The stability under prolonged storage conditions for possible adsorption onto glass or other materials, such as plastics, must be determined. In certain instances, plasticisers from plastic syringes and containers and certain polymers from Vacutainer stoppers have been known to interfere with drug measurement. The stability of the drug must also be checked under the conditions of analysis, since any losses or decomposition due to experimental manipulations can significantly affect the overall recovery, reproducibility and precision of the assay. The method chosen should be sufficiently sensitive to detect low levels of decomposition products and sufficiently precise to observe small changes in the assay of the drug.

2.10.2. Specificity

The biological sample should be examined for the presence of endogenous components and/or other drugs which might interfere with the drug or its metabolites in the assay system. Should any peak interfere with the assay, a systematic search should be undertaken to find the origin of the interference. For example, an interfering peak eluting at the same retention time as phentermine was found during its GC analysis in plasma [64]. This was subsequently found to be due to the use of plastic pipette tips. However, selective extraction procedures, use of specific detectors or a change in chromatographic parameters should normally eliminate interferences of this kind.

2.10.3. Limit of Detection

The limit of detection (LOD) is defined as the lowest concentration of an analyte that the analytical process can reliably detect. In mathematical terms, the most popular quantitative approach seems to be that which defines LOD as 3S (S = standard deviation of the peak to peak noise) above the gross blank signal, taking into consideration that for the case of a linear dependence of R_x upon C_x

$$\frac{S_{Cx}}{C_x} = \frac{S_{Rx}}{R_x}$$

Where R_x is the response corresponding to concentration C_x and S_{Rx} is the response variability corresponding to

concentraction variability S_{cx}. It should be noted that the limit of detection need not be a constant, because of e.g. day-to-day variation in detector response. The detection limit must be statistically defined, below which no data should be reported.

2.10.4. Accuracy and Precision

The term accuracy denotes the nearness of a measurement to its accepted value [65] and is expressed in terms of error. The absolute error is the difference between the observed and 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.

In real terms, accuracy should be tested by comparison of results to another reliable method. Correlation of the two methods against each other by regression analysis ($r > .98$) is the most acceptable form of validation.

The term precision is used to describe the reproducibility of the method [65]. The precision of a measurement is given by the percentage coefficient of variation of replicate experiments performed under identical conditions.

In chromatographic methods of drug analysis, the precision and reproducibility of the method should be described both in terms of intra-assay (within-day) and inter-assay (between-day) results. In performing intra and inter-assays at least four replicate analyses over the entire

concentration range of calibration should be performed. For intra-assay, the mean values of the peak height or peak area ratios should be obtained and a linear regression analysis performed by plotting the mean values of ratios versus the accepted value (the accepted value is based on the amount of test drug added to the drug-free biological fluid). Then the concentration based on each individual value, expressed either as a peak height or peak area ratio is calculated from the generated linear regression curve and precision defined based on these results. For inter-assay, a linear regression analysis is performed for each day of calibration and then four or more of these calibration curves are used for statistical calculations and for expressing reproducibility. Many of these calculations will be shown in following chapters.

2.10.5. Linearity

The linearity of the assay should be defined by the linear regression analysis of replicates of spiked biological standards in the expected concentration range of the "unknown". In practice the linear regression curve obtained in intra-assay may be used to demonstrate the measure of linearity of the curve.

2.10.6. Recovery

The overall recovery of a given drug from spiked biological standards is calculated from the limit of detection to the upper end of the linear calibration curve by two different methods [64]. The first method compares the peak heights/area of a series of spiked biological samples which

have been taken through the entire analytical procedure with the peak heights/area of a series of authentic standards. The second method compares the slope (determined by linear regression analysis) of a processed standard curve to that of the reference standards.

2.10.7. Quality Control

The performance and reliability of routine drug assays should be checked with each batch of samples. Quality control standards are prepared by accurately spiking drug-free biological fluids with known amounts of the test drug by a person other than the analyst. At least 10% of these standards should be subjected to quality control and the results of these should be within $\pm$10% of their accepted value, or the batch repeated [63].

If a method is modified it should be statistically revalidated even if one assumes that it had been correctly validated in the first place. A method might have to be modified in the following cases.

If the initial method had been developed on a used column and then is repeated on a "new" column, the two columns may behave differently for any of the following reasons

1. The lack of readily available identical columns, e.g. not all C_{18} columns behave similarly.
2. If the chromatographer decides to use a column which may perform differently, he or she may be forced to change other chromatographic conditions.

3. The chromatographer may have different requirements from the method in terms of sensitivity, for example and may be forced to change the extraction technique say.

All the above points necessitate revalidation.

The above strategy for the development and validation of chromatographic methods of analysis of drugs in biological fluids forms the foundation for all the analytical work in the following sections. These studies demonstrate how the strategy has been applied in its entirity to the determination of levels of (1) Tricyclic antidepressant drugs, (2) antihistamines and (3) benzodiazepines.

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INTRODUCTION TO COLUMN SWITCHING

CHAPTER 3

3.1. INTRODUCTION TO COLUMN SWITCHING

As has been mentioned in the previous section, HPLC has become a widely applied analytical technique, particularly in the determination of pharmaceutically active compounds. Coupled with this growth there has been a rapid development in the quality of chromatographic supports and equipment available. However, particularly in the case of biological samples, extensive sample clean-up is often required prior to analysis and this has been a limiting factor in the past. HPLC coupled with column-switching offers the analyst a powerful tool, allowing him to combine the separative powers of HPLC with simple sample clean-up procedures.

Sample clean-up of biological samples is critical for the following reasons (1).

(a) Complicated sample clean-up is time consuming and expensive.
(b) There is a risk of loss of interesting compounds.
(c) Sensitive compounds may decompose during the sample treatment.

The goal of all sample preparation system therefore must be to cope with these problems. The following points have to be considered if the method is to be used for routine determinations.

(1) The total analysis time should be short.
(2) To facilitate the operation, the sample preparation should be on-line to the HPLC separation system.
(3) The sample preparation should be suitable for automation.

(4) The reproducibility should be good enough to use external standardisation, which eliminates complicated searches for adequate internal standards and the problem of interferences with the internal standard.

(5) The sample preparation should be mild so that sensitive compounds can be handled.

(6) To minimize the problem of interferences no loss of the separation power of the chromatographic systems is acceptable, on the contary an improvement in selectivity is desirable.

(7) To reduce the time for the development and optimisation of a given analytical problem the sample preparation should be generally applied in such a way that the experimental parameters can be changed on a rational basis with high flexibility.

In the following section, valve switching and in particular column-switching will be presented as on on-line method for sample preparation which allows the analyst to attain the goals set out above. The section includes a thorough review of important research in this area to date and highlights the versatility, efficiency and never-ending possibility of employing this technique, in particular for sample clean-up and trace analysis in biological fluids.

3.2. VALVE SWITCHING

In general, valve switching is a method of rerouting chromatographic eluents by means of valves. When combined with the automatic switching of pumps, samplers, detectors

and other apparatus, valve switching allows automation of those areas of chromatography that can be time consuming and require large numbers of man hours [2].

The type of valve commonly used in valve switching is the Rheodyne 6-port, 2 way valve (model 7000) which operates at high pressure (fig. 1). This valve has two internal pathways connecting ports 1 and 2, 3 and 4 and 5 and 6 in the nonswitched or RESET position and in the SWITCHED position parts 2 and 3, 4 and 5 and 6 and 1 are connected. Depending on the valve position, different external connections to the valve are selected.

NONSWITCHED POSITION SWITCHED POSITION

Fig. 1: Rheodyne 6-port switching valve.

Valve switching finds application in the following areas.

1, Sample clean-up
2, Trace enrichment
3. Method development
4. Sample identification
5. Boxcar chromatography
6. Multi-column chromatography
7. Incremental gradient elution [3]

Of these, sample clean-up and trace enrichment are major areas of interest.

Valve switching may be used to carry out any of the following:-

1. Column switching
2. Column selection
3. Solvent switching
4. Solvent selection
5. Zone cutting
6. Auxillary pump on/off
7. Trap solute in detector
8. Recycle
9. Fraction collection
10. Sample injection
11. Detector selection

3.3. COLUMN SWITCHING

Here an eluent can be re-routed from one column to another, by using valves.

The procedure is very important and is used to

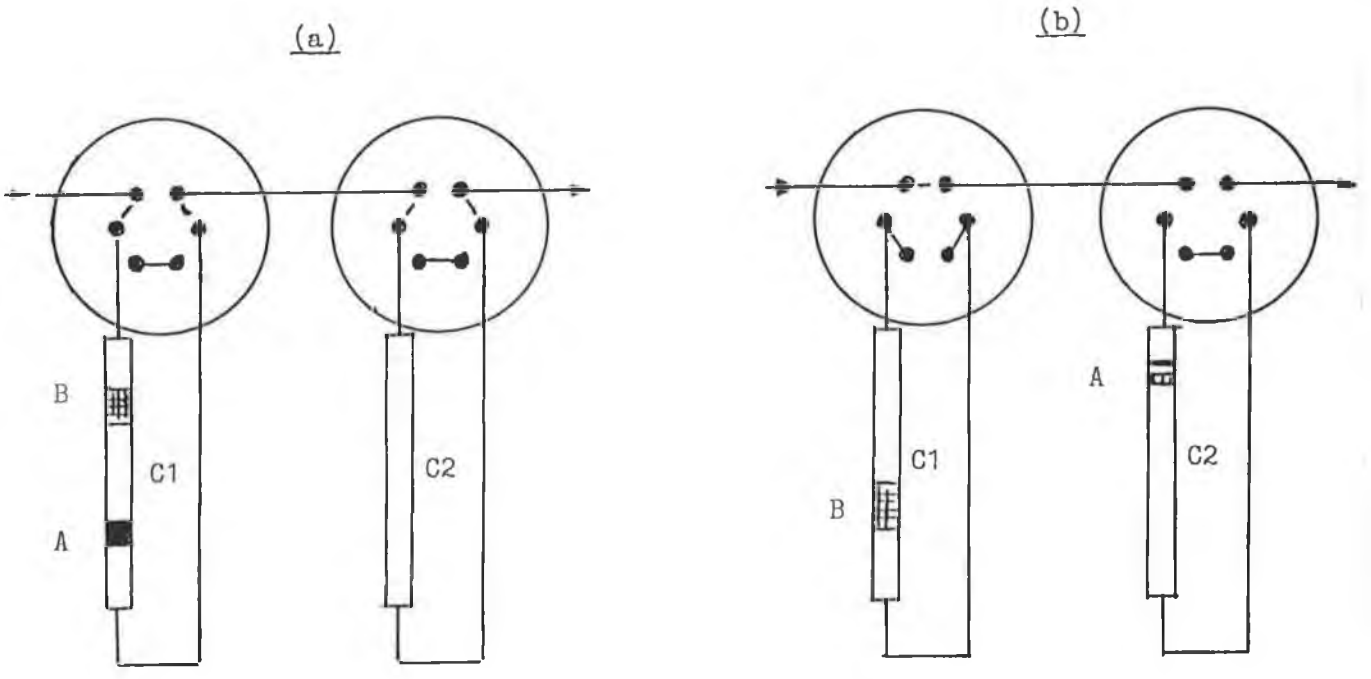
(a) carry out zone transfer and zone cutting
(b) optimize analysis time
(c) change the selectivity of the system
(d) increase column length.

Zone transfer is illustrated in Fig. 2 and represents the basic principles of column switching where a zone is allowed to elute from one column to another. To minimize extra column band broadening, the tubing connecting the column to the valve should be made from the finest bore capillary tubing.

Zone cutting is an extension of this principle and probably the most useful and versatile of all the column switching techniques[3].

Figure 2 shows how a front cut can be taken from column C_1 and transferred to column C_2. Once the cut has been made, C_1 can be isolated from the system. If further zones are required, C_1 can be used to store the remainder of the chromatogram until elution from C_2 is complete.

Further zone cuts can be made in the same way as the first. This cutting technique overcomes the problem of the



zones A & B eluted from
Column C1

zone A transferred from
Column C1-C2

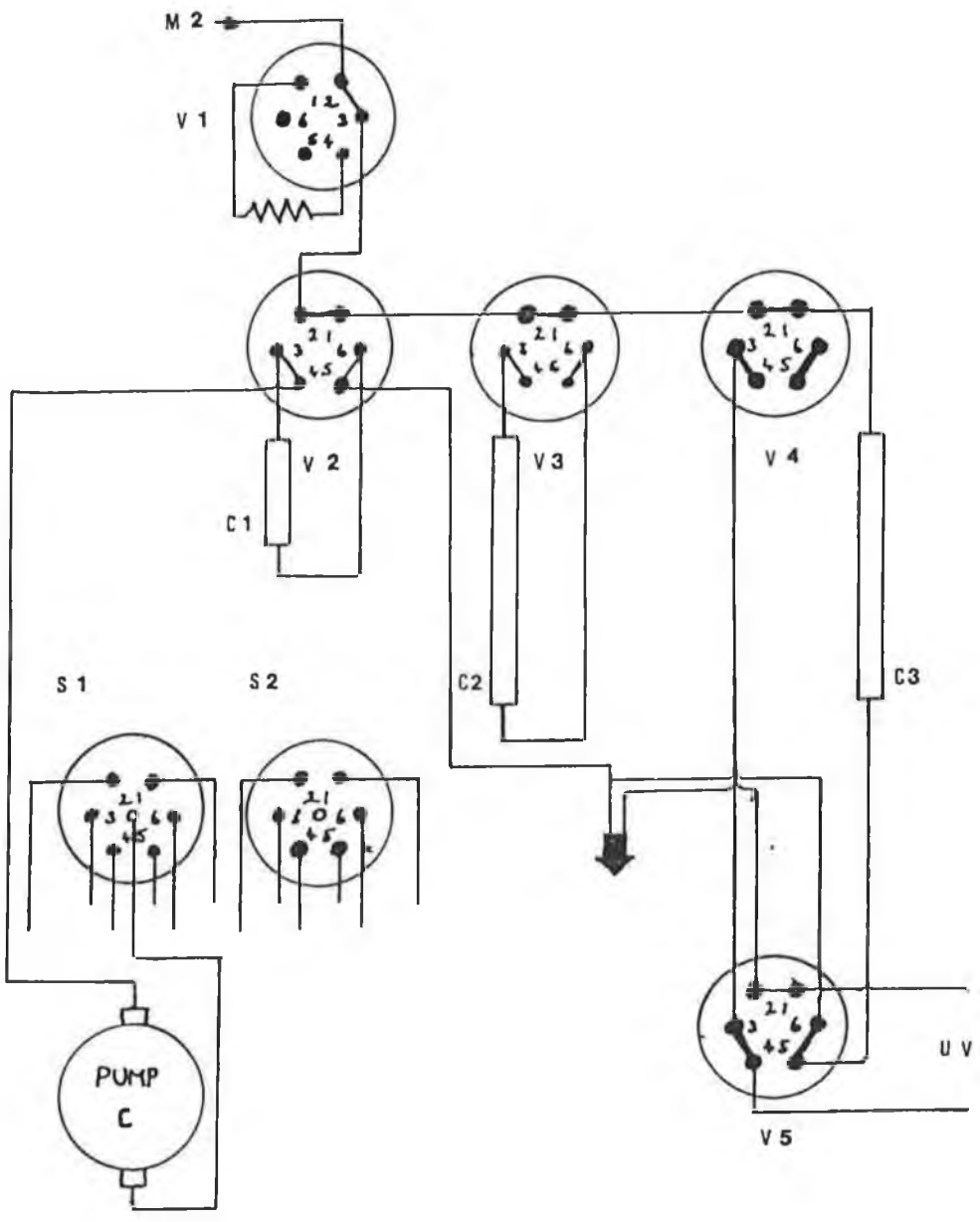
Fig. 2: Description of zone cutting by column switching.

concentration of interferences on the pre-column.

In front-cutting only the first part of the chromatogram, including the interesting peaks are analysed by C_2. After the elution of the last peak of interest from C_1 the column is bypassed by switching valve 1 and can be cleaned by the flow of a good eluting solvent using back-flush or forward flush mode. Using this front-cut the analysis time can be significantly reduced because late-eluting background peaks are not analysed on the second column [1].

Very often the first part of the chormatogram contains a large amount of polar substances. The tailing of peaks due to these substances often interfere with the compounds of interest. To eliminate these polar interferences, an end-cut technique is used, bypassing the column C_2 during the elution of the first part of the chromatogram. By combining front cut and end-cut only the relevant part of the chromatogram (heart-cut) is analysed by the second column. This minimises the interference of fast-eluting and late eluting peaks and also reduces the ballast load on the main separation column which could change the separation characteristics. The valve system shown in figure 2 is a simple but versatile one, however much more sophisticated systems are is use (Fig. 3), by other workers [2].

Fig. 3: Kontron MCS 670 Tracer configuration suitable for sample cleanup using zone cutting methods and automatic solvent change for regeneration of pre-columns.



3.4. APPLICATION OF COLUMN SWITCHING

3.4.1. Sample Clean-up

One of the most important applications of column switching is in sample clean-up. The fundamental concept of column switching which relates to sample clean-up is "zone-cutting" which has been described above. C_1 is the pre-column where clean-up takes place and once the zone cut has been made, C_1 is then cleaned and re-equilibrated. The system has been applied to the determination of organic acids in urine, vitamin C in blackcurrant syrup and vitamin C in rose hip syrup [3]. The chromatograms with and without sample clean-up are show in Fig. 4. The pre-column used for the analysis was the Brownlee S5 ODS pre-column. However a wide variety of column types may be used such as size-exclusion, ion-pair, ion-exchange, adsorption and reversed-phase.

The valve switching system described above has also been applied to the determination of estriol in gelatin capsules, where the complex fatty matrix in which the estriol is dissolved is cleaned up by valve switching. By using a back flushing mode the difficulty of washing the fatty matrix through the column is overcome [4].

3.4.2. Trace Enrichment

Trace enrichment allows on-line concentration of samples and can be of particular use when dealing with samples from

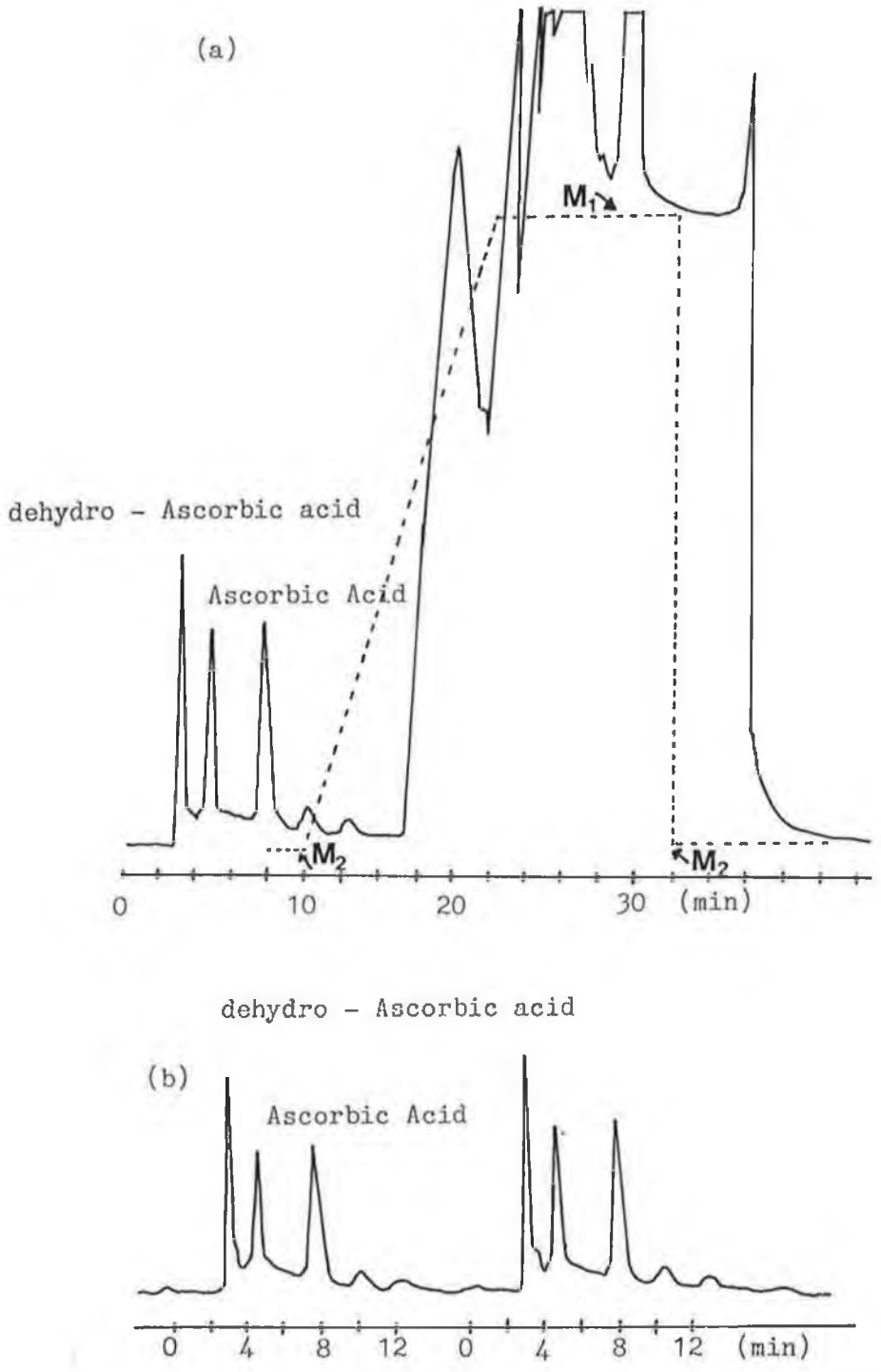


Fig 4. Chromatogram showing the separation of blackcurrant syrup without (a) and with (b) sample cleanup.

environmental media such as sea water or mineral water. A large volume of the sample is passed through a concentration column (10-100 ml) under chromatographic conditions where the sample does not elute and is adsorbed onto the column. A reversed-phase column and water are typically used. After concentration, the precolumn is switched in line with a different solvent, so that it is rapidly eluted onto another column where separation can take place. Unfortunately trace enrichment also concentrates other material in the sample and this may cause interference and lack of sensitivity. One way to overcome this problem is to combine trace enrichment with automatic sample clean-up.

3.5. SAMPLE CLEAN-UP IN BIOLOGICAL FLUIDS

The use of column switching has been applied to the determination of drugs in biological matrices with much success. A pre-column is used for direct purification and enrichment of sample, thus circumventing "classical" purification via extraction. A drug and its metabolites are often separable by reversed-phase HPLC however, proteins (e.g. from plasma) and inorganic compounds have to be removed by time consuming work up procedures which are the most inaccurate steps owing to the often unknown partition coefficients for all tested compounds. If these by-products are not removed, the lifetime of the column will be dramatically reduced and contamination of the analytical

column will result in a loss in efficiency. In biological samples interesting compounds are often present at low concentrations and may be accompanied by excessive amounts of high-molecular weight material (e.g. proteins) or salts.

Of growing importance therefore in clinical applications is the principle of adsorption of the aqueous plasma matrix on a special pre-column followed by:

1. Water wash to remove proteins
2. Solvent change and backflush of drugs onto the analytical column
3. Isolation of the precolumn for clean-up and re-equilibration. The need here is to remove strongly adsorbed species such as lipids and fats [6].

One of the first designs of major importance in the use of valve switching and pre-columns for the clean up of biological fluids was that described by Roth et al [7]. It allows direct injection of plasma followed by "alternating pre-column sample enrichment". It uses an on-line enrichment technique on a small pre-column followed by pre-column backflushing. Many of the succeeding studies were based on this work with some modification. It will therefore be described briefly here (fig. 5).

Samples of the body fluid are pipetted into the sample holders of an automatic sampling device. Volumes of between 10 and 2000µl of plasma, urine or saliva, preferentially between 10 and 150µl can be automatically injected.

HPLC - switching technique with alternating pre-columns

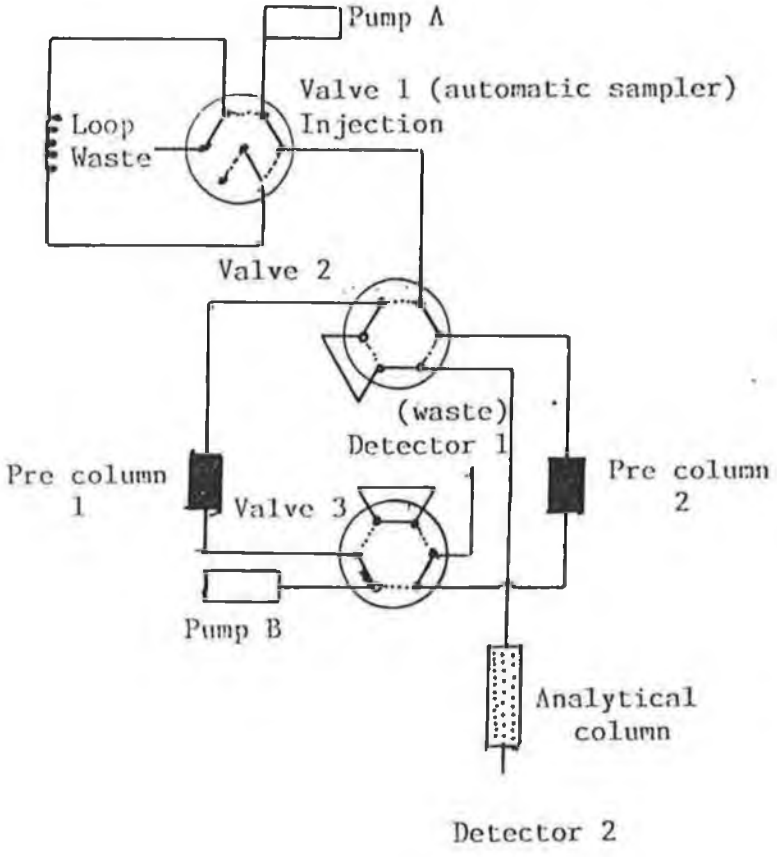


Fig. 5 : System for alternating pre column sample enrichment.

It should be checked that the samples contain no solid particles which might block the injection needle. Pre-column 1 (PC1) onto which the first sample is injected, has been conditioned with the purge phase (water or buffer solution delivered by pump A (Fig. 5). After the injection to PC1 (typical dimensions 23mm x 4.6mm ID) the pre-column is washed for a further 5 minutes with water or buffer.

The adsorption material for the pre-column is reversed - phase or ion-exchange material with a particle size of about 20-50um. Here the substances to be detected are selectively adsorbed on C_{18} Corasil (37-50µm) and thus enriched. At the same time all accompanying water-soluble co-products are eliminated with the purge phase (pump A). A second pre-column (PC2) has been added to save time. While PC1 is reconditioned, PC2 is eluted in the backflush mode onto the analytical column and vice versa (= alternating pre-column sample enrichment). Simultaneously to the application/ injection step, the autosampler (WISP) activates an electronic controller (time relay) which controls the purge phase period, after which it switches the pneumatic valves and starts the printer/plotter integrator. This switching process causes two subsequent steps; pre-column 1 (PC1) where the injected drug has been adsorbed is switched to the solvent stream of pump B which delivers the eluent coctail necessary for separation and chromatography in the backflush mode form PC1 to the analytical column. Parallel to this process PC2 is switched to the eluent steam of pump A (purge phase) which removes the rest of the organic solvent from the pre-column. The whole working cycle thus

consists of an equilibration phase, an adsorption phase and a purge phase on the pre-column and a chromatographic phase on the analytical column.

The characteristic features of the device are that there is no sample pretreatment, only one pipetting step per sample and no further standard compound is required. Peak width is independent of the injected volume, fully automated chromatography with high precision is achieved with overnight runs and low cost.

3.6. CONSIDERATIONS IN SETTING UP A SYSTEM FOR BIOPHARMACEUTICAL ANALYSIS

The above basically describes how the system works, but in order to optimise the recovery and selectivity of an assay major consideration must be given to the various parameters listed below.

(a) Size and design of the pre-column
(b) Pre-column packing material
(c) Eluent for the wash phase
(d) Frits and tubing
(e) Backflush vs forward flush mode
(f) Sample pretreatment
(g) Lifetime of the pre-column
(h) Protein binding
(i) Memory effects

To help in a discussion of these various conditions a table has been drawn up which reviews many of the studies which have been carried out in biological fluids. (Table I.) Reference to this table will prove useful during the discussion.

3.6.1. Size and Design of the Pre-column

Of major importance is the construction and geometry of the pre-column into which the adsorbent will be loaded. The design of the pre-column can be quite diverse ranging in length from 2mm to 3cm and an inner diameter anywhere from 1mm to 4.6mm (for details see table I). It is best to use as short a pre-column as possible to save on expensive adsorbent, enable easy and manual packing and to reduce backpressure [8].

In a study by Werkhoven-Goewie et al. [9], different size pre-columns were examined for their effects on band broadening ranging from (1.45mm x 4.6mm) to (41mm x 1.1mm) to (30mm x 4.6mm). Under the conditions of the study the on-line coupling of the pre-column to the analytical column has a negligible effect on band broadening and the system performance is hardly dependent on the pre-column design used. For more polar compounds the design of the pre-column may be more critical with shorter columns being preferred. Other workers [10] when comparing a pre-column of dimensions 40 x 4.6mm with cartridges 5, 10 and 20mm long obtained full recovery of the analyte with all four types but found the number and amount of interfering peaks was reduced

considerably with shorter columns, the 5mm cartridge giving the best result.

For the determination of Clobazam and its active metabolite desmethyl clobazam a micro pre-column was designed [11]. A hole 4.5 x 1mm I.D is drilled within the axis of a valve and filled with suitable packing material. This when used in conjunction with a narrow bore column allowed a limit of detection of 2.5ng/ml for each drug in plasma after direct injection of the biological fluid, with little contribution to band broadening.

So in general best results are obtained with a small pre-column leading to fewer contributions to band broadening. The pre-column serves as a preconcentration and clean-up column and as a guard column to prevent contamination of the analytical column.

3.6.2. Pre-column Packing Material

The pre-column packing chosen should display a high affinity for the analyte during the sampling step, a low affinity during the desorption stage and have high loadability. The most widely used packings are commercially available alkyl modified silica (C_{18}, C_8 and C_2), pyromodified carbon black, ion-exchange resins, XAD-2 and styrene divinyl benzene co-polymer based materials (PRP). The PRP based packings have a higher affinity for chloro and nitro-substituted aromatics than does C_{18} in fact PRP displays 10-20 times greater affinity towards aromatics than does C_{18} packing [12].

In a study by Roth et al [12], the results for various packings reveal that different RP materials possess different affinities for the injected compounds. The packing in the pre-column should be chosen in such a way that the retention on them is lower than on the analytical column and ideally zero. This can be achieved by the selection of a packing material with different surface characteristics or using pellicular packings. For the analysis of Clovoxamine and Fluvoxamine in plasma an RP-8 analytical column with methanol -0.01M phosphate buffer pH7 (62:38 v/v) as a mobile phase was used [13]. However the use of RP-8 as a packing material in the pre-column was found to cause intolerable band broadening because of a too long retention time. With the use of RP-2 as the packing, the retention time of clovoxamine and Fluvoxamine are much less and consequently there is less band broadening caused by the pre-column. Plate numbers for the analytical and the pre-column were typically 4000 and 200 respectively and using the equation for variance below.

$$\sigma^2 \text{ total} = \left(\frac{tR^2}{N}\right)_p + \left(\frac{tR^2}{N}\right)_a$$

one can calculate that for the RP-8 pre-column σ-total = 1.9 σ anal and for the 32um RP-2 pre-column σ total = 1.1 anal. The behavior of a much more expensive 10µm RP-2 is similar to that of a 32µm RP-2. Also the observed total standard deviation is often higher than the theoretical one because connections between the valve and the columns can

contribute to band broadening [14-16]. A possible disadvantage of RP-2 with respect to the more apolar RP-8 is the smaller recovery of the pre-concentrated step. However in the determination of clovoxamine and fluvoxamine this was not found to be a problem. Zech et al. [17] have also found that RP-2 is to be preferred to RP-18 as a packing in the pre-concentration column as it allows faster transfer of compounds from the column due to weaker retention. They found that the combination of C_2 and C_{18} material (pre-column and analytical column) results in a more complete removal of interfering matrix from the pre-column after injection of serum and a faster transfer of the compounds under investigation to the analytical column.

Particle size exerts a profound effect on pre-column performance. Although it is known that larger size particles cause greater band dispersion [18] most workers favour particles with size 25-50 μm as pre-column packing in order to prevent clogging of the pre-column with particles from plasma. From an inspection of table 1 it can be seen that all of the studies which employ a direct injection of plasma use particles in the range 25-50 μm with Corasil C_{18} (37-50μm) and Lichroprep RP C_{18} (25-40μm) being the ones of choice. In studies where a smaller particle size packing is used in the pre-column some form of sample pre-treatment of plasma prior to injection is required.

Although these pellicular type packings give adequate recovery for medium polarity compounds, porous particles may be needed for more polar drugs [19]. In a study by Juergens

et al. it was found that Nucleosil 30 C_{18} (30μm) gave better recovery and longer lifetimes than pellicular packings [20].

A packing which shows great potential is XAD-2 which was used in the determination of Metaqualine [21]. It has several properties which render it ideal for sample preparation and clean-up. It has strong adsorbent properties [22, 23] and is inert at all pH's. Most plasma proteins have their isoelectric point below 7 [24] and thus have a large negative charge at pH 9.3 which contributes to higher water solubility. Similarly at pH 9.3 many small molecules in plasma are negatively charged and therefore should be unretained on XAD-2. However despite these advantages, the use of XAD-2 is likely to be limited due to its influence on band broadening.

A more recent approach is the use of pre-columns packed with immunosorbents. When a plasma sample containing phenytoin [25] was injected onto a pre-column containing a immunosorbant specific for the drug, a dissociation and partition of phenytoin from plasma proteins to a complete association with on-column insolubilized antibodies results. Desorption onto the analytical column then follows.

Whatever the choice of packing it should be such that the drug is completely retained while interfering substances in plasma are completely removed during the wash step.

3.6.3. Choice of Eluent

The eluent must be chosen in such a way that the drug and its metabolites are retained while the bulk of unwanted constituents from plasma are flushed through. Almost all workers employ a purely aqueous wash solvent i.e. either distilled water or an aqueous buffer. We found in our laboratory that pure water as wash solvent gave the cleanest chromatograms as there is no possible interference from buffer salts when they come into contact with plasma constituents. Some workers may add a small amount of some organic modifier e.g. 4% acetonitrile to the wash phase but this may result in lower recovery for a drug [26].

Of the utmost importance however is that the mobile phase for flushing be pure otherwise impurities will be concentrated and later eluted onto the analytical column where they will cause interference.

Care must also be taken that breakthrough does not occur, this is a function of the sample capacity of the concentration column which depends on the composition of the sample and the eluting strength of the solvent.

3.6.4. Frits and Tubing

It is recommended [27] that the steel capillaries from the injector to the pre-column should be of wider bore than those from the pre-column to the analytical column. This is especially important for viscous plasma samples.

Zech and Huber point to the importance of using sieves (although with an adequate filter function) instead of frits in order to avoid column blocking [17]. Sieves of the pre-column should not be too fine, sieves of at least 18μm pore size are recommended [27]. Also to guarantee a homogenous distribution of the administered plasma to the top of the guard column, crossed grooves should be fitted onto the surface of the end fitting which crosses the top of the pre-column [27]. Other wokers have also found screens to be better than frits [19]. We found in our laboratory that if frits are used they need to be changed frequently.

3.6.5. Back flush/Forward flush

On inspection of table 1 outlining the various studies, it will be seen that the backflush mode for the desorbtion of the drugs from the pre-column to the analytical column is by far the favoured mode. Backflushing prevents contamination of the pre-column and minimizes band broadening. It can be shown by the use of a strong fluorescent substance that the analytes are concentrated in a small region at the top of the pre-column so that their removal by backflushing minimizes their dispersion [7]. In the "elute through" mode these drugs initially in a narrow band at the top of the pre-column must pass through say the full 2cm of packing experiencing all the band dispersion effects of the column [21].

In the case of the few studies [28, 17, 29, 20] where forward flushing is employed, it will be noted that the

pre-columns are very small, less than 1cm in length so that the overall efficiency does not deteriorate as we might expect in straight flush mode. Forward flushing does prevent the build up of contaminants at the head of the analytical column.

However in general back flushing is to be favoured.

3.6.6. Sample Pre-treatment

In many cases the biological fluid is directly injected onto the pre-column with no sample preparation whatsoever [10, 12, 20, 21, 25, 27, 28, 30 -40]. If this is the case care should be taken that there are no particles present which might clog up the column. This offers obvious advantages in terms of speed of analysis and sample throughput. Some workers favour centrifugation of plasma prior to injection [13, 17, 36, 41, 42], followed by injection of the supernatant onto the pre-column.

In order to prevent clogging of the pre-column and reduce the viscosity of samples, plasma may be diluted with water [43] or aqueous buffer [44, 45] prior to injection. This procedure will increase the limit of detection and may not be possible in the case of drugs which are present in the very low nanogram range. Plasma may be deproteinized prior to injection [29, 42, 46, 47] with an organic solvent such as acetonitrile which causes precipitation of plasma proteins which are then separated by centrifugation and the supernatant injected. We have found in our laboratory that

this procedure usually results in low recoveries for the drugs since acetonitrile acts as a strong eluent and the drugs do not become trapped on the pre-column [36, 37].

A problem which may arise and which will certainly result in low recovery for a drug during preconcentration is that of protein binding. Werkhoven-Goewie et al. [48, 49] have shown that incubation with the proteolytic enzyme subtilisin A releases strongly bound drugs from proteins and in addition prevents clogging of the pre-column. Proteinase K may also be used but since subtilisin A is milder towards labile drugs it is the one of choice. The use of proteinase K may however be favoured in the case of thermolabile drugs as its optimum incubation temperature is $25^{0}C$ compared to $55^{0}C$ for subtilisin A. The effect of the pre-column loading with (a) untreated and (b) hydrolysed serum and a backflush wash step with water on the pressure drop over the pre-column has been studied.

The pressure drop is expressed as the relative pressure drop Prel., where

$$P_{rel} = \frac{P - P_o}{P_{max} - P_o} \times 100\%$$

P = pressure measured

P_o = the initial pressure drop over the pre-column

P_{max} = the pressure reached upon complete clogging of the system.

A pre-column was almost completely clogged (P_{rel} = 90%) after injection of 7-10ml of 2 month old untreated serum,

however for 1 month old hydrolysed serum P_{rel} = 10% after 8 subsequent 1ml injections. Upon introduction of backflush wash steps, the pressure could be kept consistently low (Prel = 0-10%) with all samples. It was also observed that dilution of the blood samples in an equal volume of enzyme solution had a favourable effect on the pressure profile due to the decreased viscosity of samples. A disadvantage of using subtilisin A is that some of the peptides formed by its action may interfere with the detection of the analytes of interest.

In general direct injection will provide best recovery, with faster analysis time and rapid sample throughput, the disadvantage is that the system will be more prone to clogging and build up of back pressure.

3.6.7. Protein Binding

It is essential that the analyte of interest has a greater affinity for the sites on the surface of the packing than for the proteins in plasma. Otherwise recovery of the drug from the pre-columns will be low due to plasma protein binding. In order to assess the extent of binding, the peak heights obtained for a series of recovered authentic standards are compared with the peak heights obtained for a series of recovered plasma standards from the pre-column. If there is evidence of binding, the drug must be released from the proteins prior to quantitation. As described above this may be achieved using an enzyme such as subtilisin A. In my work on the benzodiazepines I describe a simple

novel procedure for the release of diazepam from plasma proteins. It involves the addition of a drug which preferentially binds to plasma proteins releasing the diazepam for measurement. This is an approach which may become useful.

3.6.8. Pre-column Lifetime

Although the lifetime of the pre-column will depend on a number of factors such as the particle size and type of the packing, the porosity of the frits, sample pretreatment and the age of the plasma in general each pre-column will be able to handle between 10-20mls of plasma with the average being 15mls. The lifetime of the column depends on the total amount of plasma injected rather than the size of each injection. A pre-column therefore that can handle 400 x 50 µl injection will only handle 80 x 250 µl injections. With larger injection volumes, the re-conditioning of the pre-column between injections is particularly important. Eventually the surface of the adsorbent in the pre-column will become modified with matrix elements from plasma resulting in a loss of efficiency and a decrease in recovery.

3.6.9. Memory Effects

Memory effects from the pre-column ("substance bleeding") may be easily checked by injecting blank solutions between approximately every 10 injections. No memory effects should be observed.

The switching procedure employed for clean-up of biological fluids in our laboratory will be described in Section 4.2.1.5.

TABLE 1

Name of Substance	Biological Medium	Sample Pre-treatment	Valve System	Size & Type of Pre-Column	Mobile Phase	Analytical Column	Backward/ Forward Flush	L.O.D.	Purge Time	Detection Mode	Internal External	Ref
Triamcinolone	Urine 1ml sample	Direct injection	Automated 3 valves 1 low pressure 2 high pressure	2 pre-columns both 40 x 4.6mm (a) PRP (20-30um) (b) ODS silica	A variety of mobile phases	Shandon ODS - Hypersil	Backflush	5ng/ml		U.V. at 254nm	Ext.	32
Methotrexate	Plasma	Deproteinization	2 valves	4.6 x 3mm filled with RP - 8 Merck (10um)	A: Water B: Sodium phosphate containing sodium chloride methanol 4.1	Partisil SAX (10um) 250x4.6mm	Backflush	9 ppb in plasma	20ml	U.V. at 306nm	Ext.	46
Clovoxamine Fluvoxamine	Plasma	Centrifugation of 200ul 5-10 samples/ column	2 valves	5cm x 4.6mm RP - 2 (32um)	A: Water B: Methanol - buffer to pH 7 62:38	Lichrosorb RP - 8 (7 um) 150x4.6mm	Backflush	3ng/ml in plasma	5 mins	Derivatization + fluorescence Xex = 380 nm Xex = 470 nm	Ext.	13

TABLE 1 (Contd.)

Name of Substance	Biological Medium	Sample Pre-treatment	Valve System	Size & Type of Pre-Column	Mobile Phase	Analytical Column	Backward/ Forward Flush	L.O.D.	Purge Time	Detection Mode	Internal External Standard	Ref
Pentoxifyll-ine + Hydroxy-metabolite	Plasma	Direct injection 500ul	2 valves automated	40 x 4.6mm and cartridges 5, 10, 20mm filled with lichroprep RP - 2 + RP - 18 Best: 5mm with RP - 2	A: 5mM/l Na_2HPO_4 pH 7.1 B: acetonitrile Phosphate pH 6.7 23:77	Nucleosil Phenyl (7um) 40 x 4.6mm	Backflush	50ng/ml	4.5min	U.V. at 273nm	Ext.	10
Metoprolol + Hydroxy - metabolite	Plasma	Diluted + Centrifugation (0.5 or 1 ml)	2 valves air actuated	25cm x 4.7mm Lichroprep RP - 2 25-40um or 10cm x 4.7 mm ID nucleosil 5CN (5um)	A: Water B: acetonitrile /sodium acetate 75:25	Lichrosorb RP - 8 (5um) 25cmx4.7mm	Backflush	10ng/ml		Fluorescence Xex = 225nm Xend = 320nm	Int.	43
Lonazolac + P-hydroxyl-Metabolite	Serum	Direct injection 200 ul	1 valve	30 x 4 mm Lichroprep RP - 8 (30um)	A: Water B: Gradient Acetonitrile Water pH 2.7	Lichrosorb RP - 8 (5 um) 125 x 4mm	Backflush	10 ng/ml	4 min	U.V at 282 nm or Fluorescence Xex = 282 Xem = 389	Ext.	30

TABLE 1 (Contd.)

Name of Substance	Biological Medium	Sample Pre-treatment	Valve System	Size & Type of Pre-Column	Mobile Phase	Analytical Column	Backward/ Forward Flush	L.O.D.	Purge Time	Detection Mode	Internal External Standard	Ref.
Corticoster-oids	Serum	200ul direct injection	-	porous styrene-divinyl benzene co-polymer 10-30mm x 0.5mm of PTFE	Dichlormeth-ane - methanol 97:3	Zorbax SIL or Jasco SS-05	-	20 ppb		U.V.at 240mm	Ext.	33
Sulmazole + its two metabolites	Plasma Urine Bile	Direct injection of 5-50ul	Automated 3 valves with alterna-ting pre columns	Corasil C_{18} (30-50um) 40x4.6mm	A: Water B: Phosphate Buffer Acetonitrile 2:1	Hypersil ODS (5um) 125x4.6mm	Backflush	8ng/ml	2-4min	Fluorescence detection Xex = 345nm Xem = 515nm	Ext.	27
Cephalospo-rin	Plasma Urine	Deproteinizat-ion + Dilution	Automated 2x3 port valves 1x6 port valve	(a)1cm x 4mm ID anion exchange (5um) (b)1cm x 4mm RP (5um)	A: 0.03M ammonium dihydrogen phosphate + phosphoric acid B: 0.5mNaCl + acetonitrile + sulphuric acid 15:85 : 0.2	Toya Soda ODS 150x4.6um (5um)	Forward	50ng/ml for 300ul injecti-on of serum 500ng/ml for 50ul injecti-on of urine	75 secs	U.V at 295nm	Ext.	29

TABLE 1 (Contd.)

Name of Substance	Biological Medium	Sample Pre-treatment	Valve System	Size & Type of Pre-Column	Mobile Phase	Analytical Column	Backward/ Forward Flush	L.O.D.	Purge Time	Detection Mode	Internal External Standard	Ref
Biogenic Amines dopamine, norepinephrine, epinephrine and serotonin related metabolites	Urine		Automated 4 valves and 4 columns (Kontron system)	Nucleosil C_{18} (10um)	KH_2PO_4 Na_2 EDTA, SDS + 12.5% methanol	Nuclosil C_{18} (10um) 4 columns	-	-	- -	Electrochemical + Fluorescence Xex = 282nm Xem = 314nm	Int.	52
Doxorubicin + three metabolites	Plasma	Direct injection after high speed centrifugation	1 valve	23 x 3.9mm u Bondapak phenyl (37-50um)	A: Water B: Water Acetonitrile glacial acetic acid 69:30:1	R.P.	Backflush	2.3ng on column	0.5ml of water is the purge volume	Electrochemical + Fluorescence Xex = 480nm Xem = 560nm	Int.	41
Antiepileptic drugs ethosuximide primidone, phenobarbital phenytoin carbamazepine	Serum 10-100ul	ppt with acetonitrile Direct injection after centrifugation at 12,000 rpm for 1 min.	Automated 2 valves (Roth et al)	2 alternating pre-columns (50 x 4.6mm) Lichrosorb RP - 18 (10um)	A: Water B: acetonitrile water (21:80) low-step gradient	Spherisorb 250x4.6mm ODS (5um)	Backflush	200ng/ ml in serum	5 min at 2ml/min	U.V at 200nm	Ext.	42

TABLE 1 (Contd.)

Name of Substance	Biological Medium	Sample Pre-treatment	Valve System	Size & Type of Pre-Column	Mobile Phase	Analytical Column	Backward/ Forward Flush	L.O.D.	Purge Time	Detection Mode	Internal External Standard	Ref
Cephalosporin Ro 14-1761	Plasma Milk	Dilution + Precipitation with acetonitrile	1 valve automated	4cm x 4mm RP - 18 Lichrosorb	Acetonitrile TOAB	Hypersil C_8 (5um) 125mm x 4mm	Backflush	0.1ug/ml	0.8min	U.V. at 274nm	Ext.	47
Tripelennamine	Plasma - Milk -	Direct injection Centrifugation + Direct injection	1 valve manual	1cm x 1.5mm Corasil C_{18} (37-50um)	A: Water B: Acetonitrile Acetate Buffer 70:30	Techsphere 3 CN (100 x 4.6mm)	Backflush	2ng/ml 5ng/ml	3 min	U.V. at 246nm	Int.	36
Amitriptyline + its Metabolites	Plasma	Direct injection	1 valve manual	1cm x 1.5mm Corasil C_{18} (37-50um)	A: Water B: Acetonitrile Acetate Buffer 60:40	Techsphere 3CN 100x4.6mm	Backflush	5ng/ul	1.5min	U.V at 215nm	Int.	37

TABLE 1 (Contd.)

Name of Substance	Biological Medium	Sample Pre-treatment	Valve System	Size & Type of Pre-Column	Mobile Phase	Analytical Column	Backward/ Forward Flush	L.O.D.	Purge Time	Detection Mode	Internal External Standard	Ref
Antiepileptics - Primidone, - Phenobarbital, - Carbamazepine	Serum	Defribrination of plasma	Automated 2x6 port valves	25 x 3.9 mm dry packed with 30um RP Lichrosorb Si 60 bonded with C_{18} in the lab	A: Phosphate buffer pH3.5 B: Phosphate buffer: methanol 55:45 + 65 ul/l triethyamine	Fast LC-8 150x4.6mm 5 um particles of porous silica bonded dimethyl octylchlorosilane	Backflush	10ug/ml	4 min	U.V.at 214 nm	Ext.	19
Methaqualone	Plasma	Direct injection 500 ul	2 valves	2cm x 2mm Amberlite XAD - 2 (37-44 um)	A: Ammonium Chloride + Ammonia pH 9.3 B: Citrate buffer acetonitrile 68:32	Lichrosorb 250x4.6mm (10um)	Backflush	1-2ng/ml	5 min	U.V. at 254 nm or 265 nm	Ext.	21
Clobazam + Desmethylclobazam	Plasma or Serum	Direct 100 ul	Automated 2 valves	Micro pre-column inserted within the axis of a 6-port valve 40 um C_8 4.5mm x 1mm	A: Water	Spherisorb 20cm x 1mm ODS - 2 (3 um)	Forward	2.5ng/ml	3000ul 900 sec	U.V at 254 nm	Ext.	11

TABLE 1 (Contd.)

Name of Substance	Biological Medium	Sample Pre-treatment	Valve System	Size & Type of Pre-Column	Mobile Phase	Analytical Column	Backward/ Forward Flush	LOD	Purge Time	Detection Mode	Internal External Standard	Ref
AR-L 115 (Y) Rapenton (X) Dipyridamole	Plasma Urine Saliva	10ul - 150ul direct injection	Automated 3 valves	2 alternating pre-columns 25mm x 4.6mm C_{18} corasil (37 - 50um)	A: Water or buffer B: Methanol tris buffer	Lichrosorb RP-18 (5um) (X) Hypersil ODS (5um) (Y)	Backflush	50ng/ml	5 min	Fluorometric		7
Anti-Convulsants	Serum	Direct injection	Kontron "TRACER" MCS 670 column switching unit with 5 valves	Brownlee RP18 (20um)	A: Water B: Water Methanol Acetonitrile	Spherisorb S5 C_8 125x4.6mm	Backflush			U.V.	Int.	35
Phenytoin	Plasma	Direct injection	1 valve pneumatic	50 x 4 mm packed with an immuno-sorbent	A: 0.1mol/l phosphate buffer B: phosphate buffer ethanol (60:40, v/v)	Lichrosorb RP-18 (7um) (250 x 3.9mm)	Backflush	20umol/l	4 min	U.V. at 254nm	Ext.	25
Diazepam + Metabolites	Plasma	Addition of Salicylic acid + direct injection	1 valve	1cm x 1.5mm Separalyte C_8 (40um)	A: Water B: Methanol Phosphate Buffer 50:50	Lichroso-rb RP_8	Backflush	25ng/ml	2 min	U.V.at 246 nm	Ext.	34

TABLE 1 (Contd.)

Name of Substance	Biological Medium	Sample Pre-treatment	Valve System	Size & Type of Pre-Column	Mobile Phase	Analytical Column	Backward/ Forward Flush	L.O.D.	Purge Time	Detection Mode	Internal External Standard	Ref
Antiepileptics e.g. phenobarbital, phenytoin, etosuximide carbamazepine + metabolites	Serum 300-400 injections column	Direct injection 50ul	Automated 3 valves	Cartridge 5mm x 4.6mm filled with Nucleosil 30 C18 (30um)	A: 0.01% vol % phosphoric acid B: buffer/ acetonitrile gradient	Shandon ODS Hypersil (5um) 250mm x 4.6mm	Forward	ug/ml region	300 sec	U.V. at 205nm	Ext.	20
Hydroxyphenytoin	Urine 400-500 injections	Acid hydrolysis of glucuronic acid conjugate Dilution Centrifugation	Automated 3 valves	Lichrosorb RP - 18 (10um) 40mm x 4.6 mm	A: Water B: Water/ acetonitrile gradient	Zorbax ODS (5um) 250mm x 4.6mm	Backflush	50ug/ml	200sec	U.V. at 205nm	Int.	50
Etoposide Teniposide	Serum Plasma Urine	Hydrolysis with Sulstilisin A 0.2 - 2 mls of sample	Automated 4 valves 1 low pressure 3 high pressure	2mm x 4.6mm or 10mm x 2mm with Nucleosil C18 or PRP (divinylbenzene styrene CD polymer)	A: Water B: Water Methanol	Lichrocart 10 um Lichrosorb C18 (125mm x 40mm)	Backflush	100ng/ml	120sec	Fluorescence Xex = 230nm Xem = 328nm	Ext.	48

TABLE 1 (Contd.)

Name of Substance	Biological Medium	Sample Pre-treatment	Valve System	Size & Type of Pre-Column	Mobile Phase	Analytical Column	Backward/ Forward Flush	L.O.D.	Purge Time	Detection Mode	Internal External Standard	Ref
Secoverine	Plasma 500 ul	Enzymatic hydrolysis with Subtilisin - A	Kontron system	2 types (a) 30 x 4.6mm Lichrosorb CN (10 um) prepacked (b) (1.5-4mm) x4.6mm ID	A: Water B: Dioxan: Buffer 15:85	Polygosil CN (10 um)	Backflush	20ng/ml	2 ml of water	U.V. at 274.5nm and Fluorescence after post column ion-pairing with DAS	-	49
Spiramycin 1	Plasma	1ml of plasma + 1.5ml of 4% acetonitrile ---------- 1 ml of mixture in injected	1 valve automated	50 x 7 mm Perisorb RP-18 (30-40um)	A: 4% acetonitrile B: 26% acetonitrile in 2% perchloric acid	Nucleosil C8 (5um) 150x4.6mm	Backflush	50ng/ml	400sec	U.V. at 230mm	Int.	26
Aminopyrine + its Metabolites	Serum	50 - 1000 ul	1 valve	50 x 4.6 mm Lichroprep RP - 8 (25-40 um)	A: Water B: Gradient Acetonitrile Phosphate buffer	Lichrosorb RP - 18 (5 um) (250x4mm ID)	Backflush	2.2ug/ml	1ml purge volume	U.V. at 257nm	Int	31

TABLE 1 (Contd.)

Name of Substance	Biological Medium	Sample Pre-treatment	Valve System	Size & Type of Pre-Column	Mobile Phase	Analytical Column	Backward/ Forward Flush	L.O.D.	Purge Time	Detection Mode	Internal External Standard	Ref
Catecholamines i.e. noradrenaline, adrenaline, dopamine	Plasma Urine	Direct injection (60ul)	Kontron Valve switching unit	1. 10x4.6mm phenylboronic acid gel for plasma or 20x3.8mm dihydroxyboryl silica for urine 2. 20x4.6mm 5um Supelcosil LC - 18	A: Phosphate buffer with 2mM decylsulphate + 0.3mM EDTA B: Phosphoric acid + 2mm decylsulphate C: Phosphate buffer-citrate buffer-Methanol (37.5 37.5 25) with decylsulphate	Supelcosil LC - 18 (8um) 75 x 4.6mm	Backflush	0.05 nmol/l	6 min	Electrochemical + Fluorescence after derivatization	Ext.	38
Chloramphenicol	Serum	Direct injection (25ul)	1 valve automated	20 x 2.1mm Hypersil ODS (10um)	A: Water B: 3ml/l Triethylamine + Acetonitrile gradient	Hypersil ODS 100x2.1mm (5um)	-	5ug/ml	2 min	U.V. at 277 nm	Ext.	39
Procainamide + N. Acetyl Procainamide	Serum	Direct injection	1 valve automated	20 x 2.1mm ODS - Hypersil	A: Water + 3ml/l Triethylamine B: Water + 0.01M K_2HPO_4	Hypersil ODS 100x2.1mm (5um)	-	4ug/ml	0.8min	U.V at 270 nm	Ext.	40

TABLE 1 (Contd.)

Name of Substance	Biological Medium	Sample Pre-treatment	Valve System	Size & Type of Pre-Column	Mobile Phase	Analytical Column	Backward/ Forward Flush	L.O.D.	Purge Time	Detection Mode	Internal External Standard	Ref
Fluorpro-qualzone	Animal feed (mixture of cereals, potatoes, Amino acids + vitamins)	20ul injection after extraction into methanol.	2 valves automated	50 x 4.6mm RP - 18 (10 um)	A: Water B: Acetonitrile Water 55:45	Lichrosorb RP - 8 (5um) 25cmx4.6mm	Backflush	50 ppm	2 min.	U.V. at 240mm	Ext.	51
Pimobendene + O-Desmethyl-ated metabolite + Polar Conjugate	Rat Bile	Direct injection - various amounts	2 valves fully automated	Variety of packings (30-50um)	A: Water with 1% ammonium acetate B: Methanol ammonium acetate (2g/l)	Hypersil ODS (5um) 125x4.6mm	Backflush	-	3 min.	Fluorescence detection Xex = 332nm Xem = 405nm	Ext.	12

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TRICYCLIC ANTIDEPRESSANTS

CHAPTER 4

4.1. INTRODUCTION TO TRICYCLIC ANTIDEPRESSANTS

Tricyclic Antidepressants (TCA´s) (Fig. 1) are widely used in the treatment of patients suffering from depression. The concentration of these drugs in plasma and their effect on depression symptoms is controversial [1] but it is very important that their therapeutic levels be monitored so that therapy can be made optimal. Several reports have shown that there are large individual variations in plasma concentrations among patients receiving the same dosage of these drugs [2-4].

Many methods have been described for the analysis of these drugs since 1960[5]. Earlier techniques had neither the sensitivity or selectivity demanded for the analyses. The problem of achieiving good selectivity is important since, because of their extensive metabolism[6], a large number of active metabolites of antidepressants are produced and in assessing relationships to clinical effects these must be quantified.

Methods employed for analysis include spectrofluorimetry[7] GC-FID[8-12], GC-ECD[3-17], GC-NPD[18-27], radioimmunoassay [28-31], GC-MS[32], isotope derivatization dilution analysis and thin layer chromatography[33-34].

Spectrophotometric and fluorimetric techniques lack adequate sensitivity and or selectivity for routine application. Isotope dilution assays are tedious but no more so than the extraction required for GC. Radioimmunoassays provide a

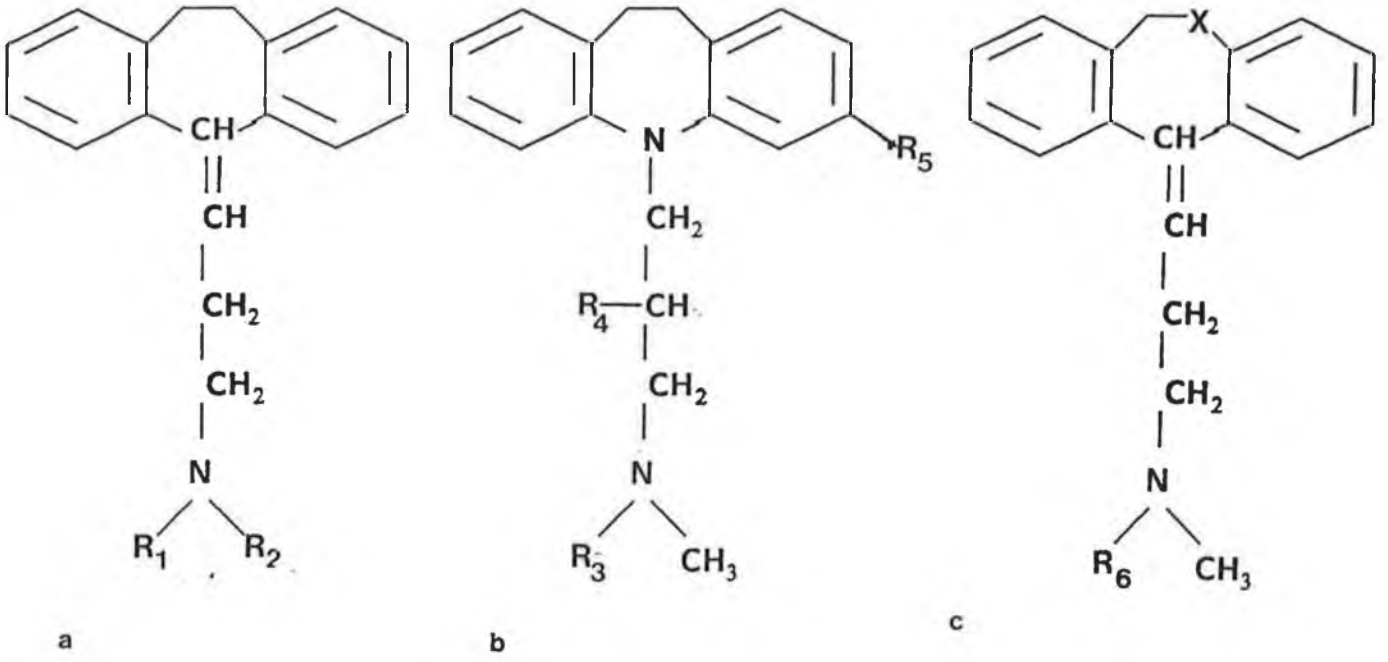

Fig 1

(a)	Amitriptyline	$R_1 = CH_3$, $R_2 = CH_3$
	Nortriptyline	$R_1 = CH_3$, $R_2 = H$
	Desmethylnortriptyline	$R_1 = H$, $R_2 = H$
(b)	Imipramine	$R_3 = CH_3$, $R_4 = H$, $R_5 = H$
	Desipramine	$R_3 = H$, $R_4 = H$, $R_5 = H$
	Clomipramine	$R_3 = CH_3$, $R_4 = H$, $R_5 = Cl$
	Desmethylclomipramine	$R_3 = H$, $R_4 = H$, $R_5 = Cl$
	Trimipramine	$R_3 = CH_3$, $R_4 = CH_3$, $R_5 = H$
(c)	Doxepin	$R_6 = CH_3$, $X = O$
	Desmethyldoxepin	$R_6 = H$, $X = O$
	Dothiepin	$R_6 = CH_3$, $X = S$
	Northiaden	$R_6 = H$, $X = S$

sensitive and rapid analysis from a small plasma volume. They are usually direct assays eliminating the need for plasma extraction but have the disadvantage that they are often not selective since the antisera raised against a secondary amine will cross react to varying degrees with tertiary amines[35].

Although all the above techniques can be applied to analyses in clinically relevant situations, by far the most commonly used methods for antidepressant analysis in plasma are those based on chromatographic methods i.e. GC and HPLC.

Gas chromatography is a highly selective technique, sensitivity depending on the type of detector used. However, an extensive clean up of plasma samples is required with a three step extraction procedure being commonly employed.

The flame ionization detector[8-12] is not highly sensitive hence large volumes of plasma must be extracted in order to give adequate sensitivity and detection limits. The limit of detection of most FID methods is around 10 - 20 ng/ml, so it is more suitable for routine monitoring and forensic applications than for pharmacokinetic studies.

Gas chromatography with electron capture detection[13-17] offers a lower limit of detection and increased sensitivity but a limitation of its use is the need to convert drugs into a species which rapidly captures electrons. While secondary amines can be easily converted into a

trifluroacetyl or heptafluorobutyryl derivative, this proves to be a more complex procedure for tertiary amines. These may either undergo quantitative oxidation to a species capable of electron capture or derivatization to form a halogenated compound, the nature of the product is not important once quantitative conversion occurs. ECD is well suited to all applications of antidepressant measurement even if it is somewhat difficult technically.

The nitrogen phosphorous detector is a modification of a conventional flame ionization detector with a high selectivity for organic compounds containing nitrogen or phosphorous and a much lower detection limit than FID[18-27]. A three step extraction procedure is usually required for plasma but smaller volumes i.e. 1ml can be used.

The majority of recently published methods for TCA's are based on NPD and this would appear to be the technique of choice for routine monitoring and pharmacokinetic studies.

The combination of the separative power of GC and the selectivity of mass fragmentography has produced one of the most powerful analytical techniques available but it is not suitable for routine use since highly sophisticated equipment is required.

HPLC now rivals GC-NPD as the method of choice for the analysis of the tricyclics. Separation of antidepressants by HPLC was first achieved in 1975[39-40] but is was not until 1976 that the practicality of measuring clinical

samples by this technique was demonstrated[41]. Most of the existing methods are similar, they require solvent extraction from basic plasma followed by ion-pair partition [42-46], adsorption[47-55], bonded or RP chromatography [56-75] with a number of different types of stationary and mobile phases in each mode.

Of these, RP appears to be the most popular. HPLC methods provide good sensitivity and the chromatographic run is usually 10-20 minutes.

Quantitation is usually done using UV absorption at 254nm, however an enhancement in sensitivity can be achieved by monitoring at 215nm. Fluorimetric detection has also been used[50, 59, 67] and generally lower detection limits can be achieved using this detection mode.

Tricyclic antidepressants undergo extensive metabolism with major metabolic pathways being demethylation and hydroxylation[76]. More recently quantitation of the hydroxylated metabolites has become important with the demonstration that they are pharmacologically active[77]. For amitriptyline the metabolic pathway is shown in fig. 2. All of its metabolites have shown pharmacological activity[51, 70].

In any of the HPLC methods described to date for the analysis of the tricyclics, direct injection of the plasma or serum sample has not been possible. Consequently the drugs must be extracted from the biological medium prior to their determination. The majority of antidepressants are

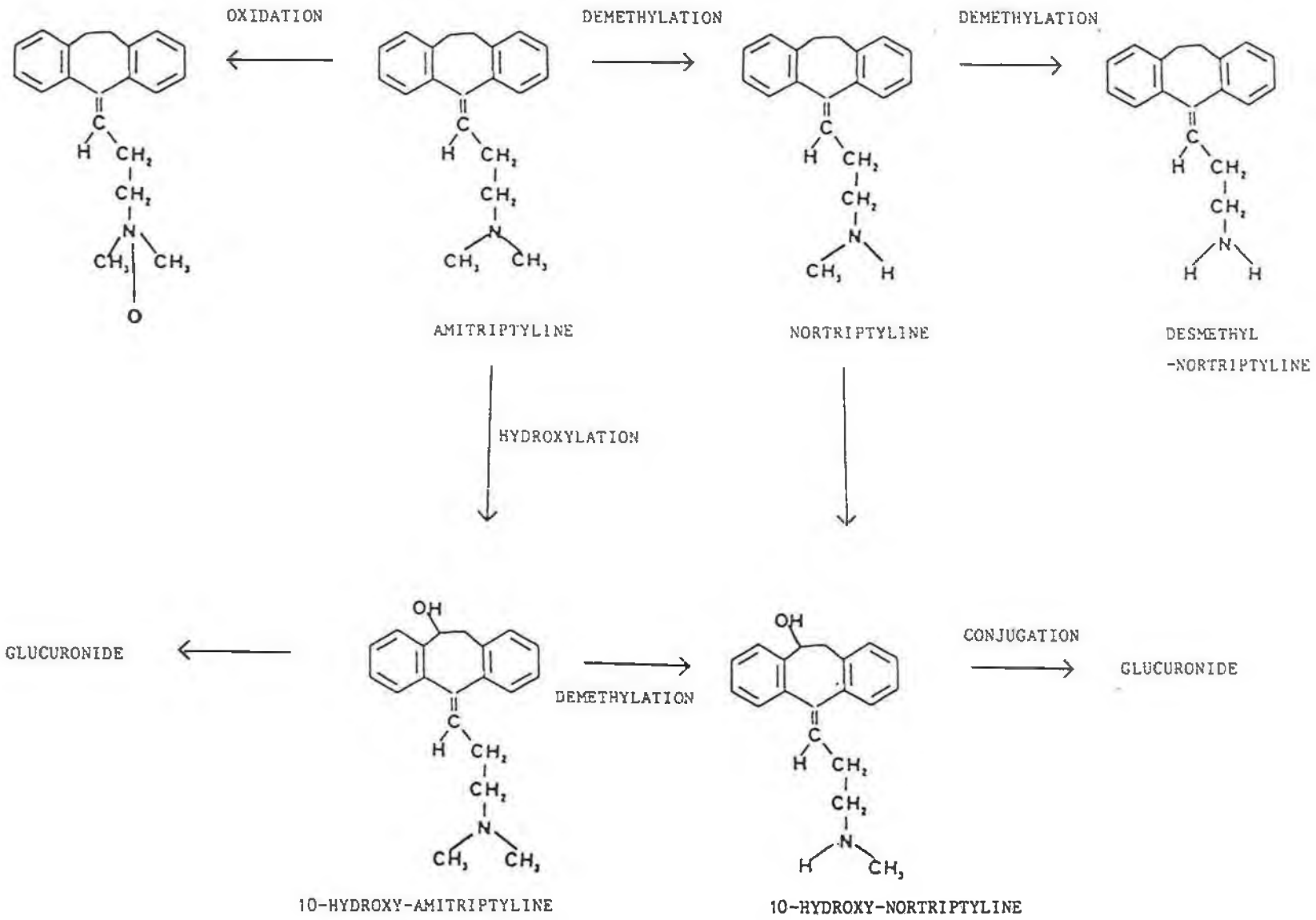

Fig. 2: Metabolic Pathway for Amitriptyline.

secondary or tertiary amines, lipophilic strong bases so that at high pH the free base can be extracted into an organic solvent from aqueous solution. This behaviour allows for the separation of antidepressants from plasma constituents and provides a relatively pure sample for chromatographic analysis.

It is worth noting here that for the collection of plasma samples containing the tricyclics, it is important that standardization of blood collection techniques occurs. It has been shown[79] that significant decreases in tricyclic analytical concentrations have been attributed to sample contact with the plasticizer tris (2 butoxy-ethyl) phosphate which may be present in the rubber stoppers of collection tubes, these losses are more significant at lower concentrations[79].

For extraction of the collected samples, a three step procedure is usually employed but as little as one step may be sufficient for some applications. Various solvents have been applied, n-hexane or n-heptane being the most common. Others include diethyl ehter, ethyl acetate or acetonitrile. A small amount (1 - 5%) of isoamyl alcohol is sometimes used in the extracting solvent to increase the polarity of the medium and to inhibit emulsion formation. Overall recovery for a three step extraction of the order 60 - 80%, while for the individual steps it is around 90%.

Solid - phase extraction procedures are becoming increasingly popular for sample clean-up prior to drug

analysis and these have advantages over the three step procedures in terms of analysis time.

The use of C_{18} bonded-phase columns for sample clean-up has been described[55, 71, 80, 81]. and equivalent results obtained using these and the three step method have been demonstrated[80, 25].

In the following chapter, two analytical methods for the determination of amitriptyline and its metabolites in plasma are described, validated and compared. The first method allows direct injection of plasma using a column switching valve. The method is based on the enrichment of the drugs on a reverse phase concentration column packed with Corasil RP C_{18}. The enriched drugs are then separated, using back flush mode on a bonded phase CN column using an isocratic acetonitrile - acetate buffer (60:40, v/v) mobile phase. In the second method, the drugs are extracted by conventional liquid-liquid extraction and separated using a silica column with methanolic mobile phase.

4.2. A COLUMN SWITCHING DIRECT/INJECTION METHOD

4.2.1. EXPERIMENTAL

4.2.1.1. Reagents

10-Hydroxynortriptyline, 10-hydroxyamitriptyline and desmethyl-doxepin were obtained as a gift from the laboratory of Clinical Pharmacology and Toxicology, Groot Ziekengasthuis, The Netherlands.

Amitriptyline, nortriptyline, doxepin and protriptyline were obtained from Jervis Street hospital, Dublin and the other members of the tricyclics were obtained as gifts from I.C.P. Dublin. Hexane, methanol, acetonitrile and isopropanol (all HPLC grade) were purchased from Fisons, England. Ammonia solution, sodium hydroxide, sodium acetate and zinc sulphate were obtained from BDH Chemicals Ltd., Poole, England. Trichloroacetic acid was purchased from May and Baker, Dagenham, England and perchloric acid from Riedel-de-Haen, Germany.

For the preparation of the plasma samples, dried human plasma (from the Blood Transfusion Service Board, Ireland) was dissolved in deionised water (obtained by the Mili-Q water purification system). The control plasma obtained was examined for the presence of endogenous components which might interfere with the tricyclic drugs in the assay system. The reconstituted plasma was stored frozen and used within two weeks of preparation.

4.2.1.2. Instrumentation

The HPLC system consisted of two Waters Assoc. P-45 liquid chromatograph solvent delivery systems equipped with a Waters U6K manual injector and fitted to a Shimadzu SPD-6A variable wavelength U.V. detector. The chromatograms were recorded on a Philips PM 8251 single-pen recorder. A Rheodyne 7000, 6-port switching valve was used.

4.2.1.3. Chromatography

The instrumental arrangement for the chromatography is shown in Fig. 3. The chromatographic conditions for the separation were as follows. Mobile phase pump A: water; concentration column (10x1.5mm) dry packed with Corasil RP C_{18} packing (37-50μm) in our laboratory; wash time, 1.5 min.; flow rate, 0.8 ml/min. Mobile phase PUMP B: acetonitrile - 0.05M acetate buffer (60 : 40, v/v) pH 7; stationary phase, Techsphere 3CN (10cm x 4mm); flow rate, 0.9ml/min; recorder chart speed, 0.5cm per min; detection wavelength, 215nm; injection volume 250μl of plasma. Under the described chromatographic conditions the mean retention times, (fig. 4) were as follows, 10-hydroxynortriptyline 6.0 mins; 10-hydroxyamitriptyline, 7.5 mins; nortriptyline, 10.0 mins and amitriptyline 12.5 mins.

OPERATION OF COLUMN SWITCHING VALVE FOR SAMPLE ENRICHMENT

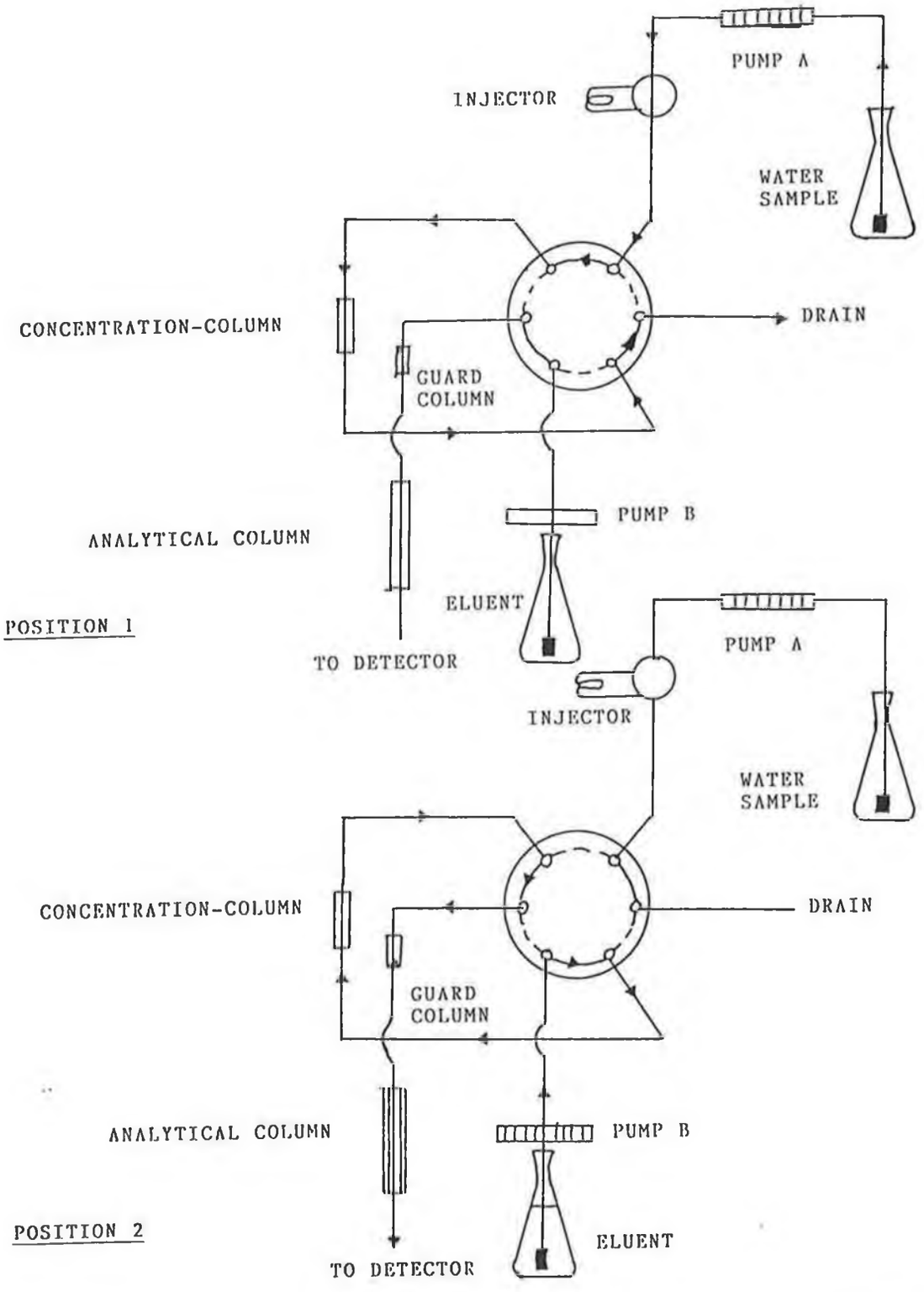

Fig. 3

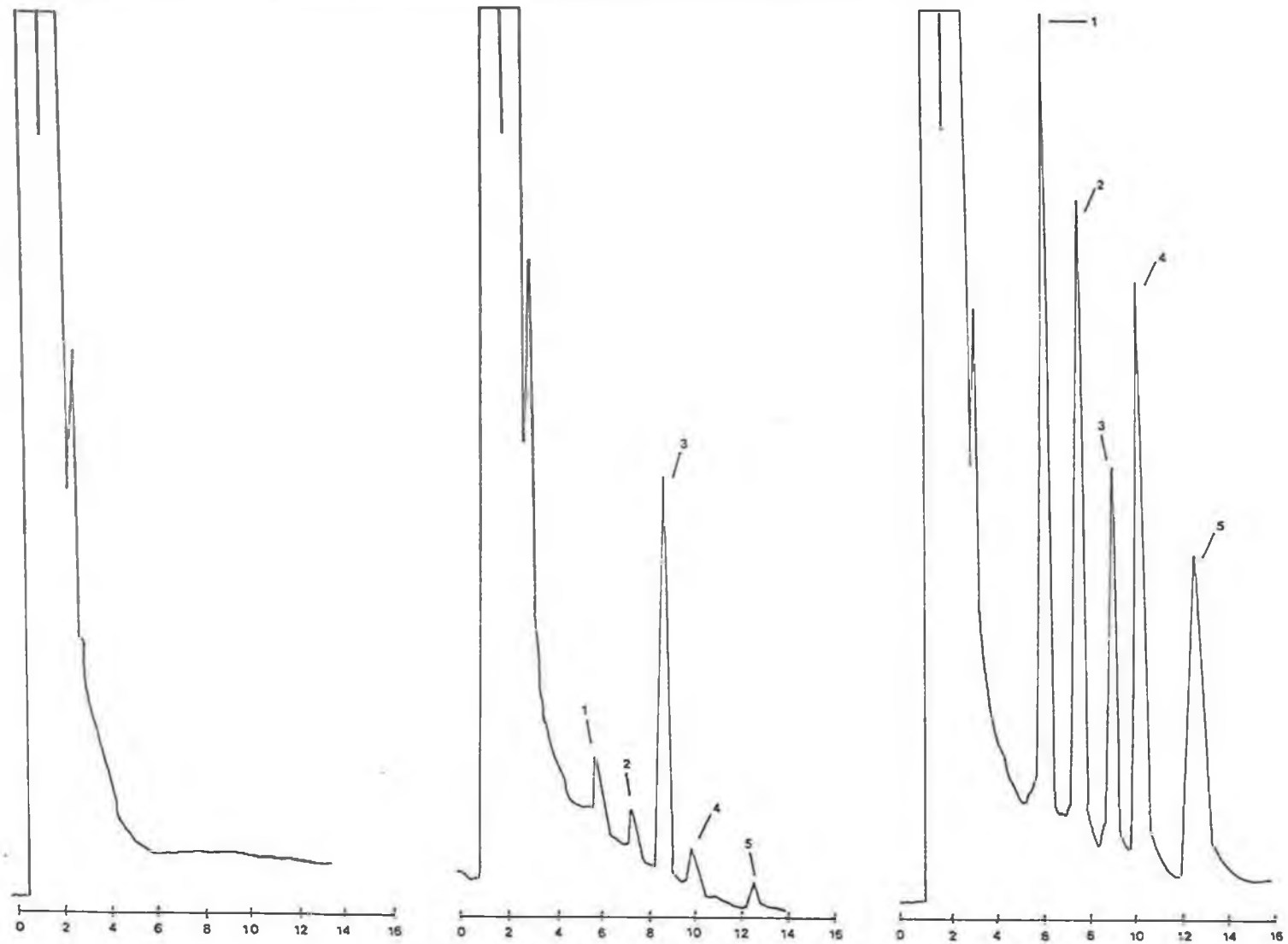

Fig.4 Chromatograms of (a) a small pooled human drug-free plasma, (b) plasma spiked with 10ng/ml of each of the drugs and (c) plasma spiked with 150 ng/ml of each of the drugs. Peaks: 1 = 10-hydroxynortriptyline; 2 = 10-hydroxyamitriptyline; 3 = desmethyldoxepin (internal standard); 4 = nortriptyline; 5 = amitriptyline.

4.2.1.4. Preparation of Standards

Amitriptyline HCl (11.32mg), Nortriptyline HCl (11.39mg), 10-hydroxyamitriptyline HCl (11.25mg) and 10-hydroxynortriptline HCl (11.31mg) were weighed and dissolved in 100ml of methanol to yield a stock solution of 100µg/ml of amitriptyline, nortriptyline, 10-hydroxyamitriptyline and 10-hydroxynortriptyline.

This stock solution was then diluted with water-methanol (1:1) to yield working standards ranging from 0.2 to 6µg/ml.

Desmethyl-doxepin (11.4mg) was weighed and dissolved in 100ml of methanol to yield a solution of 100µg/ml. This was then diluted with a methanol-water mixture to yield a solution of 10 µg/ml desmethyl-doxepin. Spiked plasma standards ranging for 10 to 300 ng/ml of the drugs in plasma were then prepared in each assay day by spiking 1ml of plasma with 50 µl of the working standards and 50 µl of the internal standard. Patient and blank plasma samples were spiked with 50 µl of a methanol-water mixture to compensate for any changes in the composition of plasma.

4.2.1.5. Column Switching Procedure

The spiked plasma sample is injected through the injector port and washed by the water in pump A onto the concentration column (position 1, Fig. 3). The drugs are held on the concentration column while the other components

in plasma are eluted to the drain. Meanwhile the eluent from pump B is passing through the analytical column and out to waste. On switching the valve (position 2, Fig. 3), the eluent from pump B elutes the drugs in back flush mode, which have been held on the concentration column, onto the analytical column where they are separated. In using the column switching valve the following variations in sample handling were studied:

Sample pretreatment
Concentration column packing
Wash time and flow rate
Analytical column
Buffer in the mobile phase

4.2.1.5.1. Sample Pretreatment

Protein precipitation prior to injection was investigated using:

(i) Acid precipitation with perchloric acid or trichloroacetic acid
and
(ii) base precipitation of proteins with NaOH and $ZnSO_4$.

The proteins were precipitated by addition of the reagent to plasma in tubes which were then centrifuged for 15 minutes and the supernatant injected for analysis according to the method described by Blanchard [81]. Direct injection with no sample pre-treatment was also tried. For all

concentrations, acid precipitation gave the lowest recovery due to ionization of the basic tricyclic drugs in the acid solution which were then eluted with water from pump A and not retained on the RP concentration column.

Base precipitation also gave poor recovery probably due to co-precipitation of the drugs with plasma. The strong acids and base also dissolved the packing in the concentration column. Over the range of packings tried therefore, best recovery, sensitivity and reproducibility were achieved using a 250 µl direct injection of plasma (Fig. 5a). However, as has been outlined in other publications direct injection is not without its problems.

These problems include (a) deterioration in the performance of the concentration column due to precipitation of plasma proteins when they come into contact with the buffers and organic solvents from pump B, i.e. the mobile phase and, (b) build up of back pressure due to accumulation of particulate matter from plasma, however this can be minimized by using the backflush mode.

4.2.1.5.2. Concentration Column Packings

The following packings were tried in the concentration column.

(a)	Lichrosorb RP C_{18}	10µm
(b)	Hypersil phenyl	10µm
(c)	Vydak cyano	30µm
(d)	Corasil RP C_{18}	37 - 50 µm

The main factor is the size of the packing particles.

Packings with particle size 10um do not allow direct injection since the small size particles act as an efficient filter and after 1-2 injections become clogged with particulate matter from plasma (Fig. 5b and 5c). Using 10µm packings, acid or base precipitation must be used and as outlined above these give poor recovery. A 30µm Vydak cyano packing allowed direct injection, however, the column capacity was only 150µl of plasma. No enhancement in sensitivity was achieved by using larger injection volumes (Fig. 5d).

Corasil R.P. packing, particle size 37 - 50µm proved to be the best among the packings tried in terms of recovery and cleanliness of chromatogram. Although greater injection volumes of plasma provided endhanced sensitivity, it limited the number of injections per concentration column. An injection volume of 250µl plasma allowed for at least fifty consecutive injections.

4.2.1.5.3. Wash Times

Wash times were varied from 1 minute up to 10 minutes, and flow rates from 0.5 to 1.5ml/min. Variation in these factors did not affect the chromatograms obtained to a large extent, but the sharpest peaks and cleanest chromatograms were obtained for a wash time of 90 seconds and a flow rate of 0.8ml/min. Shorter wash times result in very large plasma peaks while longer wash times give rise to band broadening.

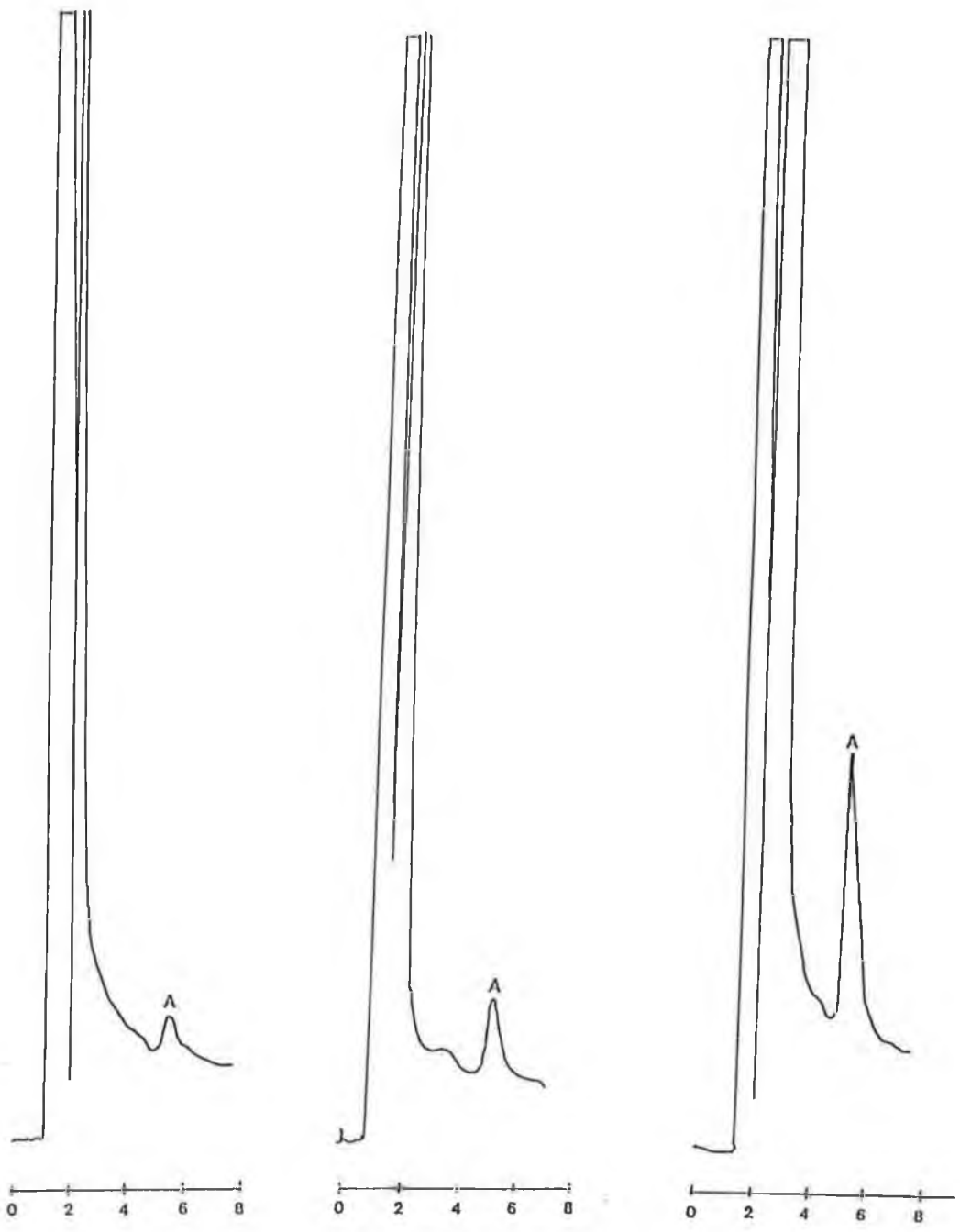

Fig. 5a Reverse phase concentration column:
Chromatograms obtained for 100ng/ml of amitriptyline in plasma using (a) acid precipitation (b) base precipitation and (c) direct injection of plasma.

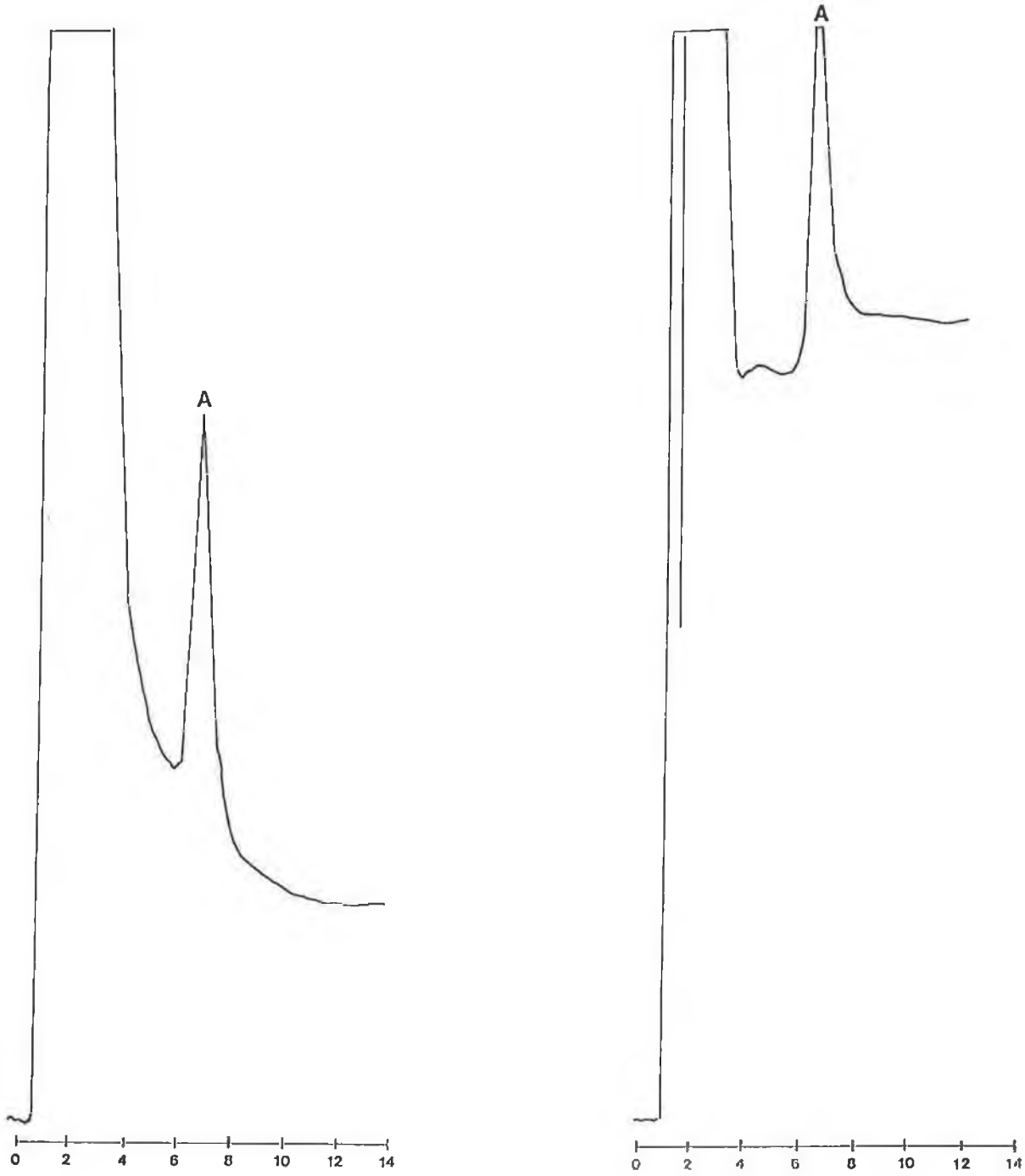

Fig. 5b Concentration column containing Lichrosorb Reverse Phase 10 µm packing -
Chromatograms showing a 250 µl Direct Injection of Plasma containing 100ng/ml Amitriptyline (A).

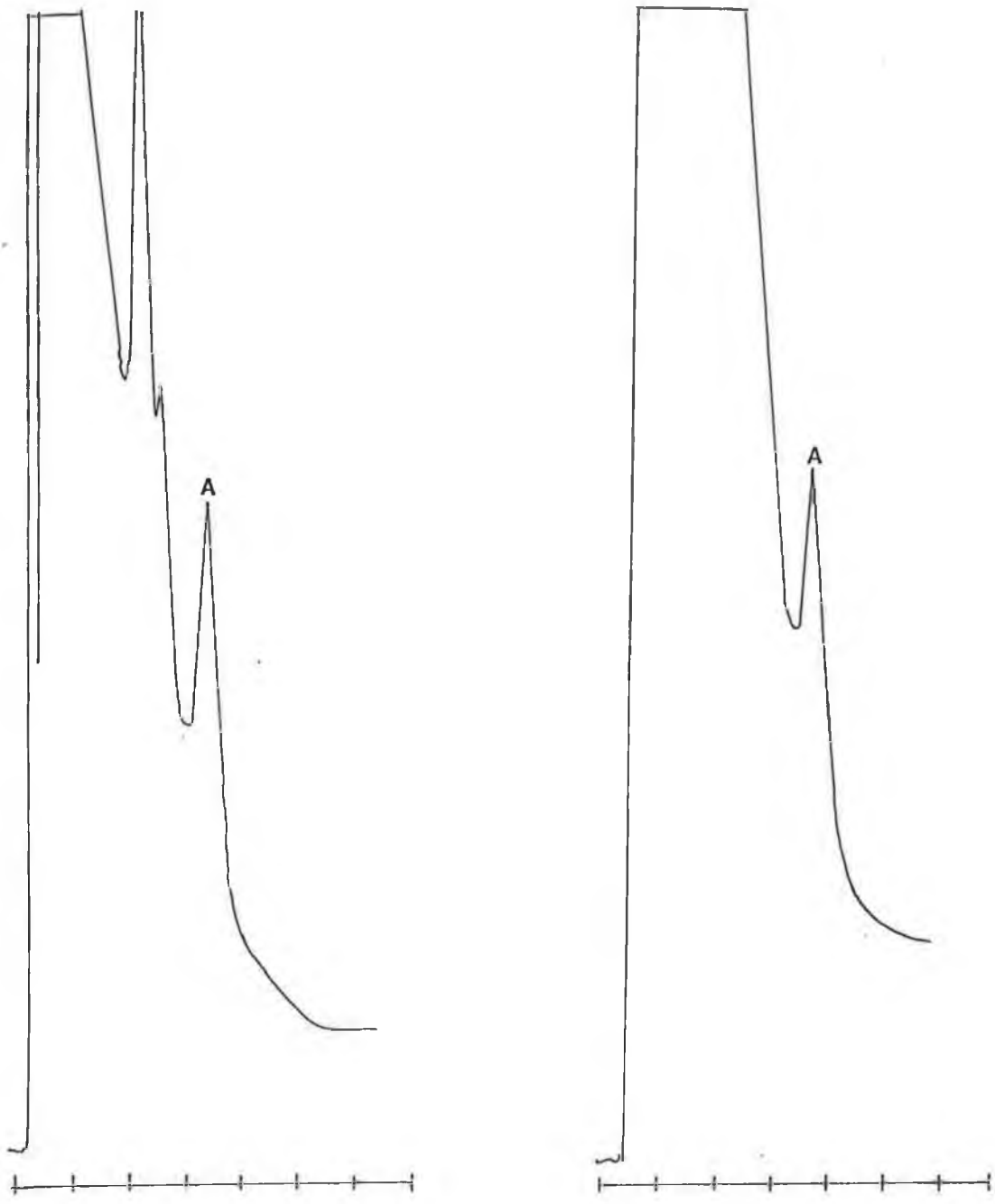

Fig. 5c Concentration column containing Hypersil Phenyl 10 µm packing - Chromatograms showing the
(A) The First and (B) The Third
250 µl direct injection of plasma containing 100ng/ml Amitriptyline (A).

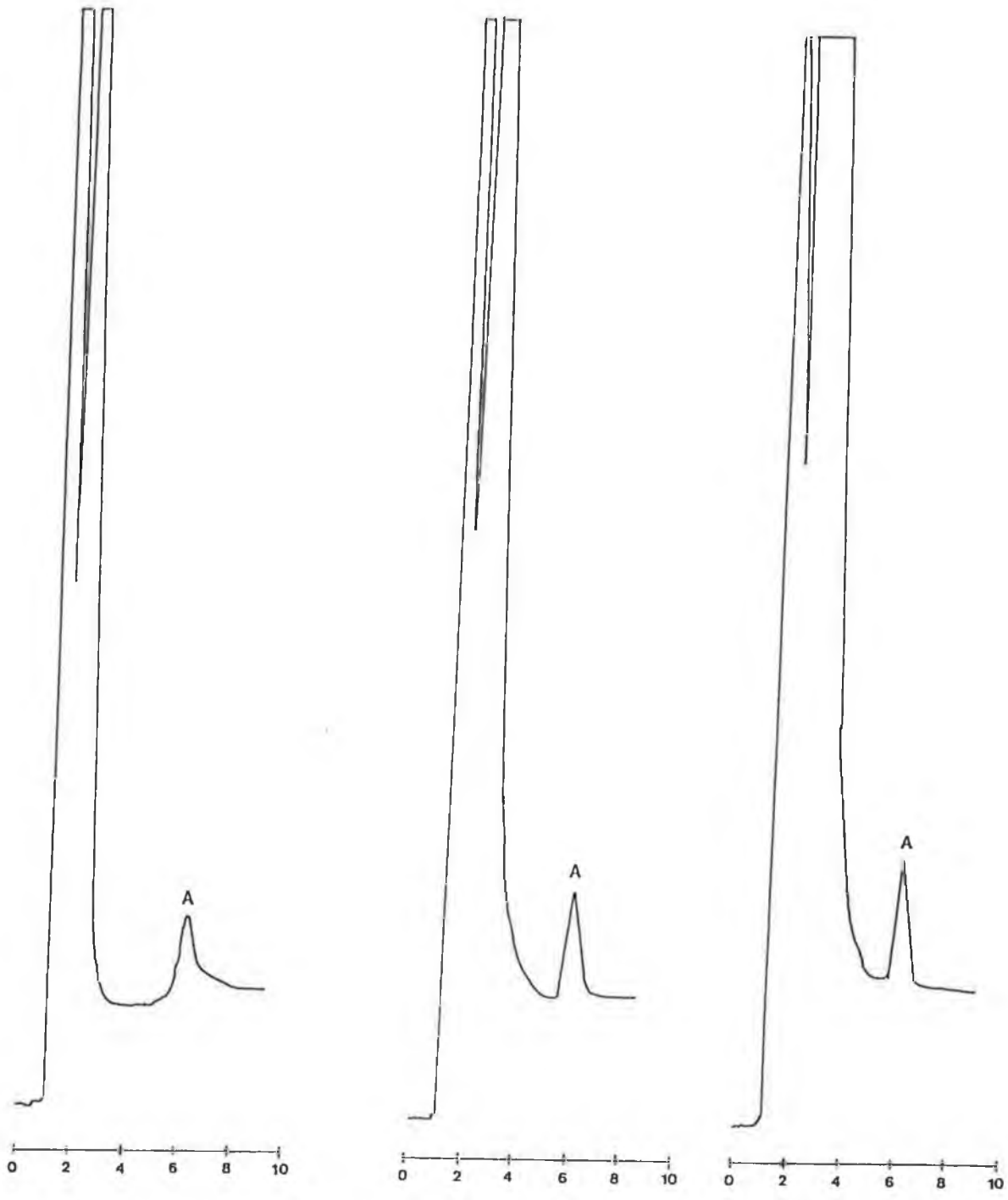

Fig. 5d Chromatograms showing

(A) 100 µl injection

(B) 200 µl injection

(C) A 400 µl injection of 100ng/ml Amitriptyline in plasma using a Cyano concentration column.

4.2.1.5.4. Other CN Columns

Separation of the drug and its metabolites may also be achieved using other CN columns.

A μBondapak CN column (300 x 3.9mm) was tried but it was necessary to vary both the composition of the mobile phase from 60:40 to 70:30 acetonitrile-acetate buffer and also to vary the molarity of the buffer from 0.05M to 0.03M in order to obtain separation.

4.2.1.5.5. Phosphate Buffer

Phosphate buffers were also used to buffer the mobile phase to pH7 but the chromatograms obtained were not as clean as when acetate buffers was used Fig. 5e.

4.2.1.6. Calibration and Calculation

Evaluation of the assay was carried out using four-point calibration standards in the concentration range 10-300ng/ml of the drugs in plasma. The slope and intercept of the calibration curves were obtained by linear regression of the peak height ratios of drug/internal standard versus the concentration of the drug (internal standard method).

The internal standard (IS) used was desmethyl-doxepin which had a suitable retention time (t_r = 8.5min). These calibration curves were then used to interpolate the concentration of drugs in patient plasma from the measured peak height ratios. The chromatograms obtained are shown in Fig. 4.

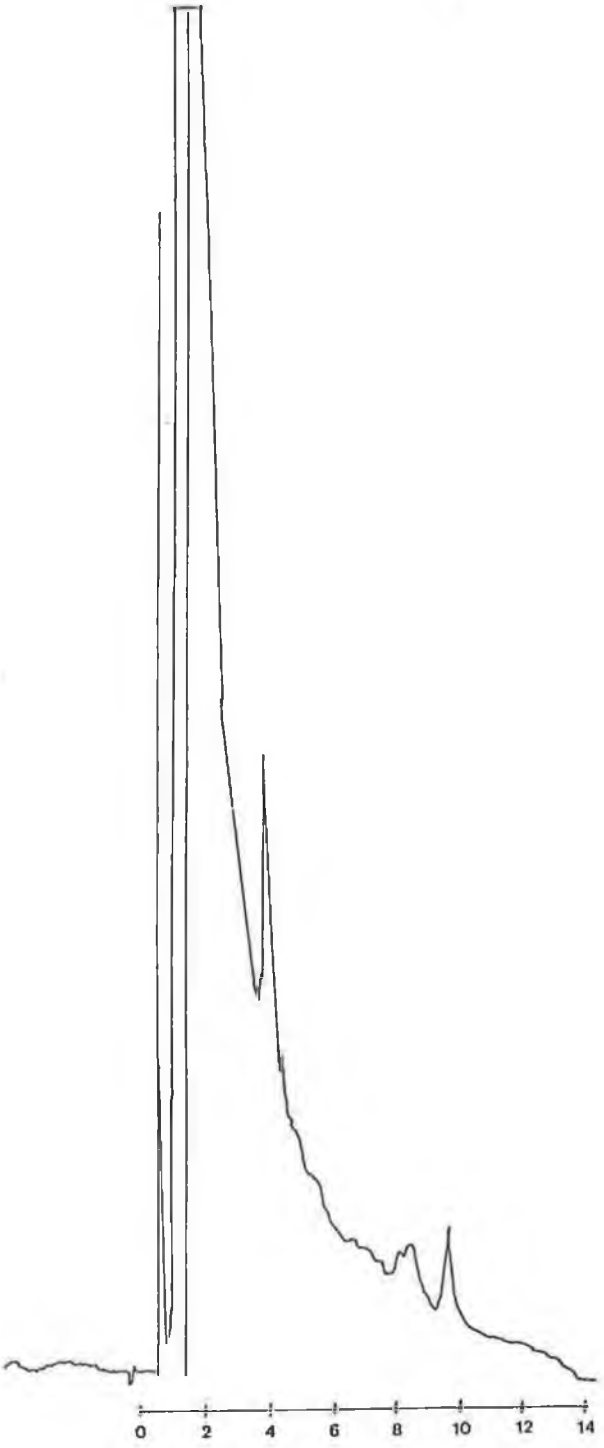

Fig. 5e Chromatogram of blank plasma obtained using a mobile phase containing phosphate buffer.

4.2.2. RESULTS AND DISCUSSION

4.2.2.1. Limit of Detection

Using a 250µl direct injection of plasma, and under the procedural conditions outlined, the limit of detection for each of the four drugs was between 5 - 10ng/ml. The variation in detection limit was due to day to day changes in operational conditions and detection system. The detection limit was taken as the amount of compound giving a signal to noise ratio greater than 3:1.

4.2.2.2. Precision

The data presented in tables Ia-d demonstrate the within-batch (intra-assay) and tables IIa-d between batch (inter-assay) variation. Intra-assay variability was determined at four concentrations in quadruplicate at levels of 10, 50, 150 and 300 ng/ml of each drug in plasma.

Inter-assay variability was determined singly and at the same four concentrations in four replicate runs.

The precision of the method (mean coefficient of variation) for the values of the recovered determinate standards when calculated as "unknowns" against the linear regression lines for intra-assay and inter-assay respectively were 3.5% and 4.2% for amitriptyline, 4.2% and 5.7% for nortriptyline, 3.7% and 8.0% for 10-hydroxyamitriptyline and 3.0% and 1.7% for 10-hydroxy-nortriptyline. Tables I + II, a, b, c, d and Table III.

Table I(a) Intra - Assay Amitriptyline

* AT = Amitriptyline
DD = Desmethyl doxepin

Concentration added (ng/ml)	1 Peak Height (cm)		Peak Rt	2 Peak Height (cm)		Peak Rt	3 Peak Height (cm)		Peak Rt	4 Peak Height (cm)		Peak Rt	MEAN RATIO
	AT *	DD *	AT/DD	AT *	DD	AT/DD	AT *	DD	AT/DD	AT *	DD	AT/DD	AT/DD
0	-	-		-	-		-	-		-	-		-
10	0.5	10.3	0.0485	0.5	9.8	0.0459	0.4	8.2	0.0488	0.4	8.3	0.0482	0.0479
50	2.5	9.8	0.2551	2.4	9.8	0.2449	2.0	9.5	0.2105	2.2	9.5	0.2316	0.2355
150	6.4	8.8	0.7273	6.2	8.5	0.7294	6.2	8.2	0.7561	5.9	8.0	0.7375	0.7376
300	6.3	4.1	1.5366	6.3	4.0	1.5750	5.5	3.7	1.4865	5.7	3.6	1.5833	1.5454

Slope = 5.18 e-03 I = -.0186

Concent. C.C. = 0.999

Concentration added (ng/ml)	Concentration found (ng/ml) 1	2	3	4	Mean Concentration found (ng/ml)	S.D.	% C.V.	% Difference
0	-	-	-	-	-	-	-	-
10	12.96	12.46	13.02	12.90	12.85	0.25	1.95	28.5
50	52.85	50.84	44.25	48.32	49.08	3.72	7.58	1.84
150	144.05	144.46	149.61	146.02	146.04	2.53	1.73	2.64
300	300.34	307.76	290.67	309.36	302.04	8.53	2.82	0.68

Mean % C.V. = 3.5%

Table I(b) Intra - Assay Nortriptyline

* NT = Nortriptyline
DD = Desmethyl doxepin

Concentration added (ng/ml)	1 Peak Height (cm)		Peak Rt	2 Peak Height (cm)		Peak Rt	3 Peak Height (cm)		Peak Rt	4 Peak Height (cm)		Peak Rt	MEAN RATIO
	NT *	DD *	NT/DD	NT *	DD	NT/DD	NT *	DD	NT/DD	NT *	DD	NT/DD	NT/DD
0	-	-		-	-		-	-		-	-		-
10	1.0	10.3	0.0971	0.8	9.8	0.0816	0.8	8.2	0.0976	0.8	8.3	0.0964	0.0932
50	4.5	9.8	0.4592	4.5	9.8	0.4592	3.9	9.5	0.4105	3.9	9.5	0.4105	0.4349
150	12.3	8.8	1.3977	12.3	8.5	1.4471	11.9	8.2	1.4512	11.2	8.0	1.4000	1.4240
300	12.2	4.1	2.9756	12.0	4.0	3.0000	10.7	3.7	2.8919	11.4	3.6	3.1667	3.0086

Slope = .0101 I = -.0491

C.C. = 0.999

Concentration added (ng/ml)	Concentration found (ng/ml) 1	2	3	4	Mean Concentration found (ng/ml)	S.D.	% C.V.	% Difference
0	-	-	-	-	-	-	-	-
10	14.46	12.93	14.51	14.39	14.07	0.76	5.40	40.70
50	50.27	50.27	45.45	45.45	47.87	2.78	5.81	4.26
150	143.08	147.96	148.37	143.31	145.67	2.88	1.98	2.89
300	299.11	301.53	290.84	318.01	302.38	11.39	3.77	0.79

Mean % C.V. = 4.2%

Table I(c) Intra - Assay OH - Amitriptyline

* OH AT = Hydroxy-amitriptyline
DD = Desmethyl doxepin

Concentration added (ng/ml)	1 Peak Height (cm)		Peak Rt	2 Peak Height (cm)		Peak Rt	3 Peak Height (cm)		Peak Rt	4 Peak Height (cm)		Peak Rt	MEAN RATIO
	OH-AMI *	DD *	OH-AMI / DD	OH-AMI *	DD	OH-AMI / DD	OH-AMI *	DD	OH-AMI / DD	OH-AMI *	DD	OH-AMI / DD	OH-AMI / DD
0	-	-		-	-		-	-		-	-		-
10	0.8	10.3	0.0777	0.8	9.8	0.0816	0.7	8.2	0.0854	0.7	8.3	0.0843	0.0823
50	4.6	9.8	0.4694	4.6	9.8	0.4694	3.9	9.5	0.4105	4.0	9.5	0.4211	0.4426
150	11.8	8.8	1.3409	11.9	8.5	1.4000	11.6	8.2	1.4146	11.5	8.0	1.4375	1.3983
300	12.1	4.1	2.9512	11.2	4.0	2.8000	10.5	3.7	2.8378	10.4	3.6	2.8889	2.8695

Slope = 9.635 e-03 I = -.0303

C.C. = .9999

Concentration added (ng/ml)	Concentration found (ng/ml) 1	2	3	4	Mean Concentration found (ng/ml)	S.D.	% C.V.	% Difference
0	-	-	-	-	-	-	-	-
10	11.21	11.61	12.01	11.89	11.69	0.36	3.08	16.90
50	51.86	51.86	45.75	46.85	49.08	3.24	6.60	1.84
150	142.31	148.44	149.96	152.34	148.27	4.28	2.89	1.15
300	309.44	293.75	297.67	302.98	300.96	6.80	2.26	0.32

Mean % C.V. = 3.7%

Table I(d) Intra - Assay OH - Nortriptyline

* OH-NOR = Hydroxy-nortriptyline
DD = Desmethyl-doxepin

Concentration added (ng/ml)	1 Peak Height (cm)		Peak Rt	2 Peak Height (cm)		Peak Rt	3 Peak Height (cm)		Peak Rt	4 Peak Height (cm)		Peak Rt	MEAN RATIO
	OH-NT*	DD*	OH-NOR / DD	OH-NOR*	DD	OH-NOR / DD	OH-NOR*	DD	OH-NOR / DD	OH-NOR*	DD	OH-NOR / DD	OH-NOR / DD
0	-	-	-										
10	0.8	10.3	0.0777	0.8	9.8	0.0816	0.6	8.2	0.0732	0.6	8.3	0.0723	0.0762
50	4.6	9.8	0.4694	4.9	9.8	0.5000	4.5	9.5	0.4737	4.6	9.5	0.4842	0.4818
150	14.4	8.8	1.6364	14.6	8.5	1.7176	14.1	8.2	1.7195	13.9	8.0	1.7375	1.7028
300	13.6	4.1	3.3171	13.3	4.0	3.3250	12.7	3.7	3.4324	12.9	3.6	3.5833	3.4145

Slope = 0.0116 I = -.0606

C.C. = 0.999

Concentration added (ng/ml)	Concentration found (ng/ml) 1	2	3	4	Mean Concentration found (ng/ml)	S.D.	% C.V.	% Difference
0	-	-	-	-	-	-	-	-
10	11.92	12.25	11.53	11.45	11.79	0.37	3.14	17.90
50	45.67	48.31	46.05	46.95	46.74	1.17	2.50	6.52
150	146.25	153.25	153.41	154.97	151.97	3.89	2.56	1.31
300	291.10	291.78	301.04	314.04	299.50	10.71	3.58	0.18

Mean % C.V. = 3.0%

Table II(a) Inter - Assay Amitriptyline

* AT = Amitriptyline
DD = Desmethyl doxepin

Concentration added (ng/ml)	DAY I Peak Height (cm) AT	DD	Peak Rt AT/DD	DAY II Peak Height (cm) AT	DD	Peak Rt AT/DD	DAY 3 Peak Height (cm) AT	DD	Peak Rt AT/DD	DAY 4 Peak Height (cm) AT	DD	Peak Rt AT/DD
0	-	-		-	-		-	-		-	-	
10	0.5	6.7	0.0746	0.6	9.6	0.0573	0.5	7.7	0.0649	0.5	6.2	0.0806
50	2.0	6.6	0.3030	2.5	9.5	0.2632	2.4	7.8	0.3077	1.8	5.4	0.3333
150	6.3	6.6	0.9545	7.9	9.7	0.8144	7.1	7.9	0.8987	5.6	5.4	1.0370
300	5.9	3.2	1.8438	7.9	4.8	1.6458	6.9	3.9	1.7692	12.6	6.0	2.1000

Concentration added (ng/ml)	Concentration found (ng/ml) DAY 1	DAY 2	DAY 3	DAY 4	Mean Concentration found (ng/ml)	Standard Deviation	Coefficient of Variation	% Difference
0	-	-	-	-				
10	10.27	11.38	9.06	12.12	10.70	1.34	12.52	7.00
50	47.49	48.87	50.43	48.24	48.76	1.25	2.56	2.48
150	153.66	149.20	151.10	148.84	150.70	2.21	1.47	0.47
300	298.58	300.54	299.41	300.80	299.83	1.03	0.34	0.06

Mean % C.V. = 4.2%

Table II(b) Inter - Assay Nortriptyline

Concentration added (ng/ml)	DAY I Peak Height (cm) NT	DAY I Peak Height (cm) DD	DAY I Peak Rt NT/DD	DAY II Peak Height (cm) NT	DAY II Peak Height (cm) DD	DAY II Peak Rt NT/DD	DAY 3 Peak Height (cm) NT	DAY 3 Peak Height (cm) DD	DAY 3 Peak Rt NT/DD	DAY 4 Peak Height (cm) NT	DAY 4 Peak Height (cm) DD	DAY 4 Peak Rt NT/DD
0	-	-		-	-		-	-		-	-	
10	0.8	6.7	0.1194	1.0	9.6	0.1042	0.7	7.7	0.0909	0.8	6.2	0.1290
50	3.7	6.6	0.5606	4.7	9.5	0.4947	4.0	7.8	0.5128	3.0	5.4	0.5556
150	11.2	6.6	1.6970	14.6	9.7	1.5052	12.5	7.9	1.5823	9.4	5.4	1.7407
300	10.2	3.2	3.1875	14.6	4.8	3.0417	12.3	3.9	3.1538	18.8	5.8	3.2414

Concentration added (ng/ml)	Concentration found (ng/ml) DAY 1	DAY 2	DAY 3	DAY 4	Mean Concentration found (ng/ml)	Standard Deviation	Coefficient of Variation	% Difference
0	-	-	-	-				
10	7.47	10.96	9.79	8.11	9.08	1.59	17.51	9.20
50	49.11	49.45	49.71	47.66	48.98	0.92	1.88	2.04
150	156.37	149.06	150.90	157.54	153.47	4.12	2.68	2.31
300	297.05	300.52	299.60	296.68	298.46	1.89	0.63	0.51

Mean % C.V. = 5.7%

Table II(c) Inter - Assay OH-Amitriptyline

Concentration added (ng/ml)	DAY I			DAY II			DAY 3			DAY 4		
	Peak OH-AMI	Height DD (cm)	Peak Rt OH-AMI / DD	Peak OH-AMI	Height DD (cm)	Peak Rt OH-AMI / DD	Peak OH-AMI	Height DD (cm)	Peak Rt OH-AMI / DD	Peak OH-AMI	Height DD (cm)	Peak Rt OH-AMI / DD
0	-	-		-	-		-	-		-	-	
10	0.8	6.7	0.1194	0.9	9.6	0.0938	0.8	7.7	0.1038	0.8	6.2	0.1290
50	3.3	6.6	0.5000	4.4	9.5	0.4632	3.9	7.8	0.5	3.0	5.4	0.5555
150	10.6	6.6	1.6061	13.7	9.7	1.4124	11.9	7.9	1.5063	9.3	5.4	1.722
300	9.4	3.2	2.9375	14.0	4.8	2.9166	11.8	3.9	3.0256	18.3	5.8	3.1555

Concentration added (ng/nl)	Concentration found (ng/nl) DAY 1	DAY 2	DAY 3	DAY 4	Mean Concentration found (ng/nl)	Standard Deviation	Coefficient of Variation	% Difference
0	-	-	-	-				
10	8.34	11.03	10.40	8.95	9.68	1.25	12.87	3.22
50	48.39	49.07	49.70	49.31	49.12	0.55	1.12	1.77
150	160.35	146.81	149.81	159.69	154.09	6.93	4.50	2.73
300	295.15	301.72	300.27	295.30	297.51	3.21	1.08	0.83

Mean % C.V. = 8.0%

Table II(d) Inter - Assay OH - Nortriptyline

Concentration added (ng/ml)	DAY I Peak OH-NOR (cm)	DAY I Height DD (cm)	DAY I Peak Rt OH-NOR / DD	DAY II Peak OH-NOR (cm)	DAY II Height DD (cm)	DAY II Peak Rt OH-NOR / DD	DAY 3 Peak OH-NOR (cm)	DAY 3 Height DD (cm)	DAY 3 Peak Rt OH-NOR / DD	DAY 4 Peak OH-NOR (cm)	DAY 4 Height DD (cm)	DAY 4 Peak Rt OH-NOR / DD
0		-									-	
10	1.0	6.7	0.1493	0.9	9.6	0.0938	0.8	7.7	0.1039	0.9	6.2	0.1452
50	4.2	6.6	0.6264	4.5	9.5	0.4737	4.0	7.8	0.5128	3.3	5.4	0.6111
150	13.2	6.6	2.0000	14.4	9.7	1.4845	12.8	7.9	1.6202	10.3	5.4	1.9074
300	12.9	3.2	4.0313	14.8	4.8	3.0833	13.0	3.9	3.3333	20.5	5.4	3.7963

Concentration added (ng/ml)	Concentration found (ng/ml) DAY 1	DAY 2	DAY 3	DAY 4	Mean Concentration found (ng/ml)	Standard Deviation	Coefficient of Variation	% Difference
0	-	-	-	-				
10	12.21	12.35	12.17	11.29	12.01	0.48	4.00	20.10
50	47.64	49.11	48.76	48.13	48.41	0.65	1.34	3.18
150	149.65	146.92	147.88	150.62	148.76	1.67	1.12	0.83
300	300.50	301.61	301.20	299.96	300.82	0.73	0.24	0.27

C.V. = 1.7%

TABLE III
PRECISION AND REPRODUCIBILITY

INTRA-ASSAY

Conc added (ng/ml)	Mean (n=4) concentration found ± S.D. (ng/ml)	% C.V.	Difference between added and found Concent. (ng/ml)
AMITRIPTYLINE			
10	12.85 ± 0.25	1.95	28.50
50	49.08 ± 3.72	7.58	1.84
150	146.04 ± 2.53	1.73	2.64
300	302.04 ± 8.53	2.82	0.86
	Mean % C.V. = 3.5%		
NORTRIPTYLINE			
10	14.07 ± 0.76	5.40	40.70
50	47.87 ± 2.78	5.81	4.26
150	145.67 ± 2.88	1.98	2.89
300	302.38 ± 11.39	3.77	0.79
	Mean % C.V. = 4.2%		
10-HYDROXY-AMITRIPTYLINE			
10	11.69 ± 0.36	3.08	16.90
50	49.08 ± 3.24	6.60	1.84
150	148.27 ± 4.28	2.89	1.15
300	300.96 ± 6.80	2.26	0.32
	Mean % C.V. = 3.7%		
10-HYDROXY-NORTRIPTYLINE			
10	11.79 ± 0.37	3.14	17.90
50	46.74 ± 1.17	2.50	6.52
150	151.97 ± 3.89	2.56	1.31
300	299.50 ± 10.71	3.58	0.18
	Mean % C.V. = 3.0%		

Linear regression line for intra-assay precision

(a) for AMITRIPTYLINE i Y = .0051 x -.0186 and correlation coefficient (r) = 0.999

(b) for NORTRIPTYLINE: Y = .0100x - .0491 and the correlation coefficient (r) = 0.999

(c) for 10-HYDROXY-AMITRIPTYLINE: Y = .0096X - .0303 and the correlation coefficient (r) = 0.999

(d) for 10-HYDROXY-NORTRIPTYLINE: Y = 0.0116 x - .0606 and the correlated coefficient (r) = 0.999

TABLE III Contd.

INTER-ASSAY

Conc added (ng/ml)	Mean (n=4) concentration found + S.D. (ng/ml)	C.V. %	Difference between added and found Concentration (%)
AMITRIPTYLINE			
10	10.70 ± 1.32	12.52	7.00
50	48.76 ± 1.25	2.56	2.48
150	150.70 ± 2.21	1.47	0.47
300	299.83 ± 1.03	0.34	0.06
		Mean % C.V. = 4.2%	
NORTRIPTYLINE			
10	9.08 ± 1.59	17.51	9.20
50	48.98 ± 0.92	1.88	2.04
150	153.47 ± 4.12	2.68	2.31
300	298.46 ± 1.89	0.63	0.51
		Mean % C.V. = 5.7%	
10-HYDROXYAMITRIPTYLINE			
10	9.29 ± 2.19	23.57	7.10
50	48.49 ± 1.49	3.07	3.02
150	153.88 ± 6.50	4.22	2.59
300	298.34 ± 2.95	0.99	0.55
		Mean % C.V. = 8.0%	
10-HYDROXYNORTRIPTYLINE			
10	12.01 ± 0.48	4.00	20.10
50	48.41 ± 0.65	1.34	3.18
150	148.76 ± 1.67	1.12	0.83
300	300.82 ± 0.73	0.24	0.27
		Mean % C.V. = 1.7%	

4.2.2.3. Linearity

Measures of linearity as defined by the correlation coefficient of the regression lines for all drugs and the difference between added and found concentrations are given in intra-assay precision Table I (a), (b), (c), (d) and Table III. In all cases the correlation coefficients were better than 0.999 and the intercepts did not differ greatly from the origin.

4.2.2.4. Recovery

The overall recovery was calculated in two different ways (Table IV)

First by comparing the peak heights of a series of spiked plasma samples after they had been taken through the entire procedure with a series of reference standards. Secondly, by comparing the slopes of the regression lines obtained from the two sets used in the first procedure. Using these methods in the concentration range 10-300 ng/ml, the mean overall recoveries were 90.7%, 92.6%, 83.9% and 90.5% for amitriptyline, nortriptyline, 10-hydroxynortriptyline and 10-hydroxyamitriptyline respectively.

4.2.2.5. Interference Study

Eight other members of the tricyclic drugs were tested for possible interferences with the measured substances. As can be seen from the chromatogram in Fig. 6, no other member of

TABLE IV

RECOVERY

METHOD 1 — AMITRIPTYLINE

Concentration (ng/ml)	Amitriptyline peak height (cm) (mean of duplicates) Set A Authentic Standards	Set B Recovered	Recovery (%)
Blank	N.D.*	N.D.	-
10	0.50	0.53	105.00
50	2.35	2.45	104.25
150	8.00	7.50	93.75
300	17.50	14.80	84.57

Mean overall % recovery $\pm$ S.D. = 96.89% $\pm$ 9.68

Method 2

Regression line for Set A : Y = .05835 x - .28183 , r = .9988
Regression line for Set B : Y = .04936 x + .02029 , r = .9999

$$\text{Overall recovery} = \frac{.04936}{.05835} \times 100 = 84.59\%$$

Mean recovery determined by both methods = 90.7%

METHOD 1 — NORTRIPTYLINE

Concentration (ng/ml)	Nortriptyline peak height (cm) (mean of duplicates) Set A Authentic Standards	Set B Recovered	Recovery (%)
Blank	N.D.*	N.D.	-
10	0.90	0.85	94.44
50	4.00	4.35	108.75
150	14.20	13.55	94.42
300	30.40	26.90	88.48

Mean overall % recovery $\pm$ S.D. = 96.77% $\pm$ 8.55

METHOD 2

Regression line for Set A : Y = .10162 - 0.06493, r = 0.999
Regression line for Set B : Y = .08992 - 0.04191, r = 0.9999

$$\text{Overall recovery} = \frac{.08992}{.10162} \times 100 = 88.49\%$$

Mean recovery determined by both methods = 92.6%

TABLE IV Contd.

METHOD 1 — 10-HYDROXY-AMITRIPTYLINE

Concentration (ng/ml)	10-hydroxy-amitriptyline peak height (cm) (mean of duplicates)		Recovery (%)
	Set A Authentic Standards	Set B Recovered	
Blank	N.D.*	N.D.	-
10	0.90	0.85	94.44
50	3.90	4.15	106.41
150	13.45	12.80	96.16
300	30.20	25.80	85.43

Mean overall % recovery $\pm$ S.D. = 95.36% $\pm$ 8.59

METHOD 2

Regression line for Set A: Y = .10050 x - .56109, r = .998
Regression line for Set B: Y = .08606 x - .05833, r = .9999

$$\text{Overall recovery} = \frac{.08606}{.10050} \times 100 = 85.63$$

Mean recovery determined by both methods = 90.5%

METHOD 1 — 10-HYDROXY-NORTRIPTYLINE

Concentration (ng/ml)	10-hydroxy-nortriptyline peak height (cm) (mean of duplicates)		Recovery (%)
	Set A Authentic Standards	Set B Recovered	
Blank	N.D.*	N.D.	-
10	1.00	0.85	85.00
50	4.55	4.25	93.40
150	15.40	13.60	88.31
300	34.50	27.80	80.58

Mean overall % recovery $\pm$ S.D. = 86.82% $\pm$ 5.41

METHOD 2

Regression line for Set A: Y = .11480 x - .6196, r = .9983
Regression line for Set B: Y = .09286 x - .1724, r = .99988

$$\text{Overall recovery} = \frac{.09286}{.11480} \times 100 = 80.88\%$$

Mean recovery determined by both methods = 83.8%

* N.D. = Not detectable, below limit of detection.

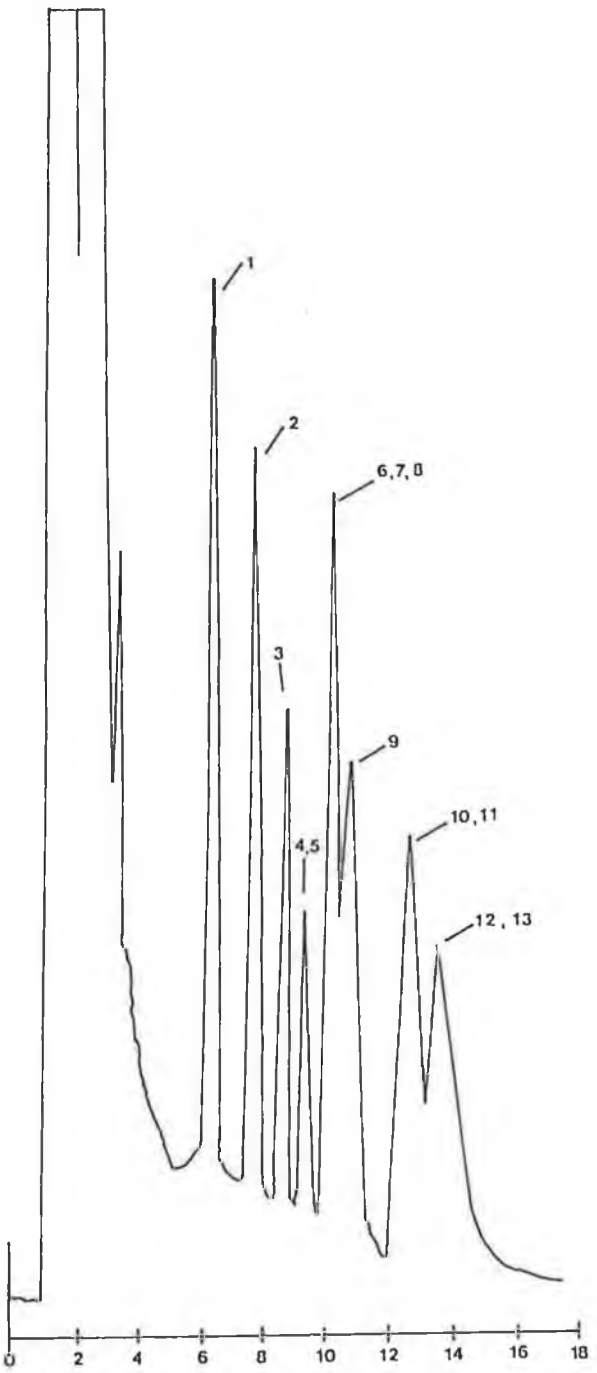

Fig. 6 Chromatogram of a small pooled human drug-free plasma spiked with tricyclic antidepressant drugs. Peak 1 = 10-hydroxy-nortriptyline; 2 = 10-hydroxyamitriptyline; 3= desmethyldoxepin; 4 and 5 = protriptyline and desipramine; 6,7 and 8 = trimipramine, cianopramine and nortriptyline; 9= doxepin; 10 and 11 = amitriptyline and imipramine; 12 and 13 = chlomiprimine and chlorapramine.

the tricyclics studied interfered with the determination of 10-hydroxyamitriptyline or 10-hydroxynortriptyline. Imipramine interfered with amitriptyline and likewise cianopramine and trimipramine interfered with nortriptyline, but it is unlikely that any of these combinations would be administered simultaneously.

Tranylcypromine which was given in combination with amitriptyline to some patients did not interfere and eluted with plasma peaks.

4.2.2.6. Plasma Levels

The described method has been successfully applied to the measurement of amitriptyline, nortriptyline, 10-hydroxyamitriptyline and 10-hydroxynortriptyline in patients with neurotic and endogenous depression receiving oral doses of placebo, 50, 75, 100 or 150 mg of amitriptyline alone or in combination with tranylcypromine, (table V). The exact amount of dosage could not be revealed to us at this stage and therefore, results in table V are only indicative of the capability of the described method for the analysis of patient samples receiving the above doses of amitriptyline. Chromatograms from patients are shown in Fig. 7.

4.2.2.7. Discussion

As mentioned previously, HPLC procedures for the determination of tricyclic antidepressants generally involve extraction of the drugs from plasma or serum,

TABLE V

TIME*	PATIENT 1 CONCENTRATION (ng/ml)				PATIENT 2 CONCENTRATION (ng/ml)				PATIENT 3 CONCENTRATION (ng/ml)				PATIENT 4 CONCENTRATION (ng/ml)			
	AMI	NOR	OH-AMI	OH-NOR	AMI	NOR	OH-AMI	OH-NOR	AMI	NOR	OH-AMI	OH-NOR	AMI	NOR	OH-AMI	OH-NOR
DAY 1	31.9	22.9	N.D.	33.2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	24.6	7.2	N.D.	22.4
DAY 14	54.5	42.2	N.D.	60.1	36.7	55.7	10.6	84.5	N.D.	N.D.	N.D.	N.D.	34.2	30.4	N.D.	115.9
DAY 28	106.7	86.1	6.3	110.4	50.1	47.3	5.9	56.7	N.D.	N.D.	N.D.	N.D.	36.3	50.4	7.4	114.5
DAY 42	74.0	61.6	N.D.	82.6	47.2	56.7	9.6	64.3	N.D.	N.D.	N.D.	N.D.	50.5	49.2	6.9	138.7

AMI = AMITRIPTYLINE

NOR = NORTRIPTYLINE

OH-AMI = 10-HYDROXY-AMITRIPTYLINE

OH-NOR = 10-HYDROXY-NORTRIPTYLINE

* Blood samples were taken 10 hours after administration.

\+ N.D. = Not detectable, below limit of detection.

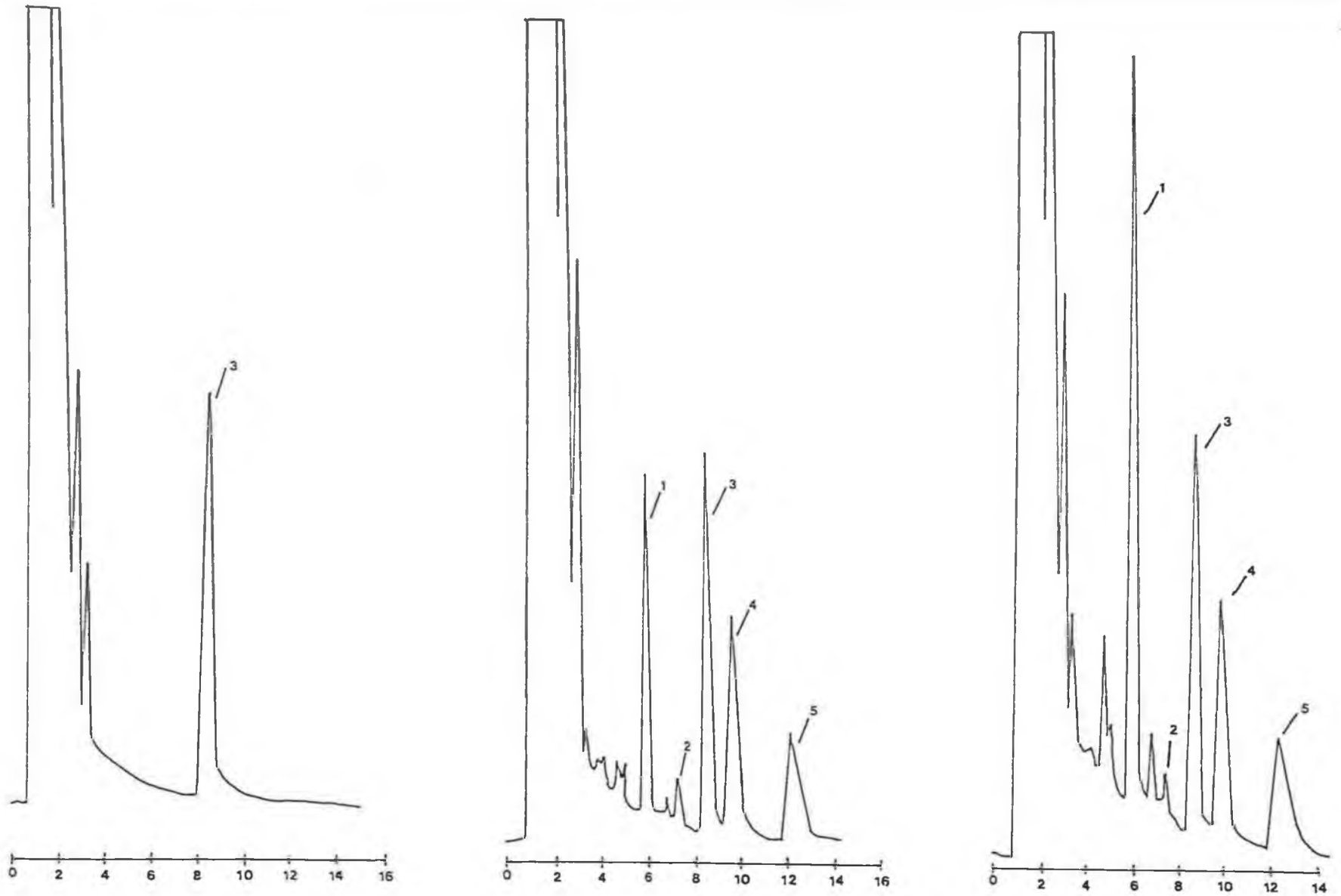

Fig. 7 Chromatograms of plasma from a patient receiving (a) a placebo and (b) a 50 mg oral dose of amitriptyline and (c) a 100 mg oral dose of amitriptyline.

followed by various modes of chromatography. Most recent publications, although different in many aspects, exhibit similar results in terms of validation and applications.

The use of on-line column switching with direct injection of plasma provides a new alternative for sample preparation of tricyclic drugs prior to analysis with HPLC.

Sample preparation procedures based on extraction are often tedious and time consuming, susceptable to errors and often can result in loss of sample because of incomplete extraction or formation of artifacts.

In chapter 2, the advantages of column switching as an on-line method for sample preparation are fully discussed. In setting up the system, a major consideration is the nature of packing material in the concentration column and the mobile phase passing through it during the sample enrichment process. They have to be selected in such a way that the drug is almost completely retained while the interfering substances in plasma are removed.

The packing eventually chosen was pellicular Corasil C_{18} (37-50μm) with water as the mobile phase for enrichment. This allowed at least fifty injections of 250μl of plasma. More injections resulted in the loss of resolution, band broadening and lesser recovery and hence lesser sensitivity. This may not allow automatic injection of sample batches exceeding fifty in number. Except for this limitation, direct injection/column switching proved to be a superior

technique as virtually no sample preparation is necessary and the results obtained showed excellent precision and reproducibility with high recovery and limits of detection of between 5-10 ng/ml of each drug in plasma.

Detection at 215nm also gave a four-fold enhancement in sensitivity as compared to 254nm.

The method allowed separation and quantitation of amitriptyline and its three major metabolites, nortriptyline, 10-hydroxynortriptyline and 10-hydroxyamitriptyline. The large differences in concentrations added and found at the low concentration of 10ng/ml can be accounted for by the fact that the method is based on peak height measurements. At these concentrations a large difference in peak height ratio of drug/internal standard, hence concentration of the drug, is obtained for a very small difference, e.g. 1mm, in peak height measurement.

4.2.3. CONCLUSION

For routine analysis of the tricyclic drugs in plasma, a new method of analysis based on a direct injection/column switching technique was developed. The advantages of this method over conventional extraction methods is that it was less time consuming, needed a smaller volume of plasma, and gave better recovery. It has a comparable precision and detection limit with conventional extraction methods. The disadvantages are that the concentration column had to

be changed after every fifty injections and it needed more elaborate instrumentation i.e. two pumps and a column switching valve.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF AMITRIPTYLINE AND ITS METABOLITE NORTRIPTYLINE IN HUMAN PLASMA USING RAPID LIQUID EXTRACTION FOR SAMPLE CLEAN-UP

4.3. INTRODUCTION TO THE EXTRACTION METHOD FOR TRICYCLIC DETERMINATION

A new, sensitive and simple HPLC method for the determination of plasma levels of amitriptyline (AT) and nortriptyline (NT) is described. Plasma spiked with the drugs and internal standards, imipramine (IP) and desmipramine (DP) for AT and NT respectively was mixed with 0.1M NaOH and the drugs were extracted into hexane-isopropanol (99:1). After evaporation of the extracting solvent, the residue was taken up in methanol and an aliquot was injected for chromatography. The separation of the drugs was carried out on an adsorption column 300 x 3.9mm commercially packed with μ Porasil, (10μm), using methanol containing 0.1% ammonia as the mobile phase at a flow rate of 1.5ml/min. The drugs were detected by absorption at 215nm. The limit of detection for AT and NT in plasma was 5 and 10ng/ml respectively. Mean percentage coefficients of variation for intra-assay and inter-assay precision were respectively 3.2% and 8.6% for AT and 3.0% and 5.6% for NT. Linearity as measured by the correlation coefficient for the intra-assay linear regression lines was 0.9999 for both drugs. The mean overall recoveries from plasma for AT and NT were respectively 80.8% and 71.0%. The assay described has been successfully applied to the determination of plasma levels of AT and NT in man after oral administration.

The advantages of this method over previously published methods are:

(a) a single extraction step with little adsorption onto the glass.

(b) use of two internal standards.

(c) detection at 215nm.

(d) simple mobile phase, and

(e) use of pre-column and guard column packed with Lichrosorb Si 100.

All these points have contributed to the sensitivity, reproducibility and reliability of the described method.

4.3.1. EXPERIMENTAL

4.3.1.1. Reagents

Amitriptyline, nortriptyline, imipramine, desipramine, all in the form of the HCL salt, and trimipramine as maleate were gifts from I.C.P. Dulbin. Hexane, methanol, acetonitrile and iso-propanol (all HPLC grade) were purchased from Fisons, England. Ammonia solution, sodium hydroxide, sodium acetate and zinc sulphate were obtained from B.D.H. Chemicals Ltd., Poole, England.

Trichlorocetic acid was purchased from May & Baker, Dagenham, England and perchloric acid from Riedel-de-Haen,

Germany.

For the preparation of the plasma samples dried human plasma (from the Blood Transfusion Service Board, Ireland) was dissolved in Millipore water (deionized water obtained by the MILLI-Q water purification system). The blank plasma obtained was examined for the presence of endogenous components which might interfere with the tricyclic drugs in the assay system. The reconstituted plasma was stored frozen and used within two weeks of preparation.

4.3.1.2. Instrumentation

The HPLC system consisted of Waters P-45 liquid chromatograph solvent delivery system equipped with a Waters U6K manual injector and fitted to a Shimadzu SPD-6A variable wavelength UV detector. The chromatograms were recorded on a Philips PM 8251 single pen recorder.

4.3.1.3. Chromatography

The chromatographic conditions for the separation were as follows, the stationary phase was an adsorption μ Porasil column (300 x 3.9mm) commercially packed with Porasil, (particle size 10μm). Mobile phase was methanol containing 0.1% ammonia, flow rate 1.5ml/min, recorder chart speed 0.5 cm/min., detection wavelength 215nm and an injection volume of 70μl.

Under the described chromatographic conditions the following retention times were obtained, amitriptyline 3.1 min, nortriptyline 5.8 min, imipramine 3.4 min, and desipramine 7.5 min. (fig. 8).

4.3.1.4. Preparation of Standards

Amitriptyline hydrochloride (11.23 mg) and nortriptyline hydrochloride (11.39 mg) were weighed and dissolved in 100ml of methanol to yield a stock solution of 100ug/ml amitriptyline and nortriptyline. This stock solution was then diluted to yield working standards ranging from 0.30 to 6.0 µg/ml. Imipramine hydrochloride (5.65mg) was weighed and dissolved in 50ml of methanol to yield a stock solution of 100 µg/ml of imipramine. Desipramine hydrochloride (11.37 mg) was weighed and dissolved in 100 ml of methanol to yield a stock solution of 100 µg/ml. These were then diluted with a methanol-water (1:1) mixture to yield a solution of 4 µg/ml imipramine and 20 µg/ml desipramine. Spiked plasma standards were then prepared each day by addition of 50 µl of the working standards and 50 µl of the internal standards to 1.5ml of plasma to provide standards ranging from 10 to 200 ng/ml of the drugs in plasma.

4.3.1.5. Extraction Procedure

For several of the tricyclic drugs, irreversible adsorption onto glass is a problem.

Methods to prevent adsorption [42, 82-85], such as silanization and siliconizing of glassware, replacement by plastic,

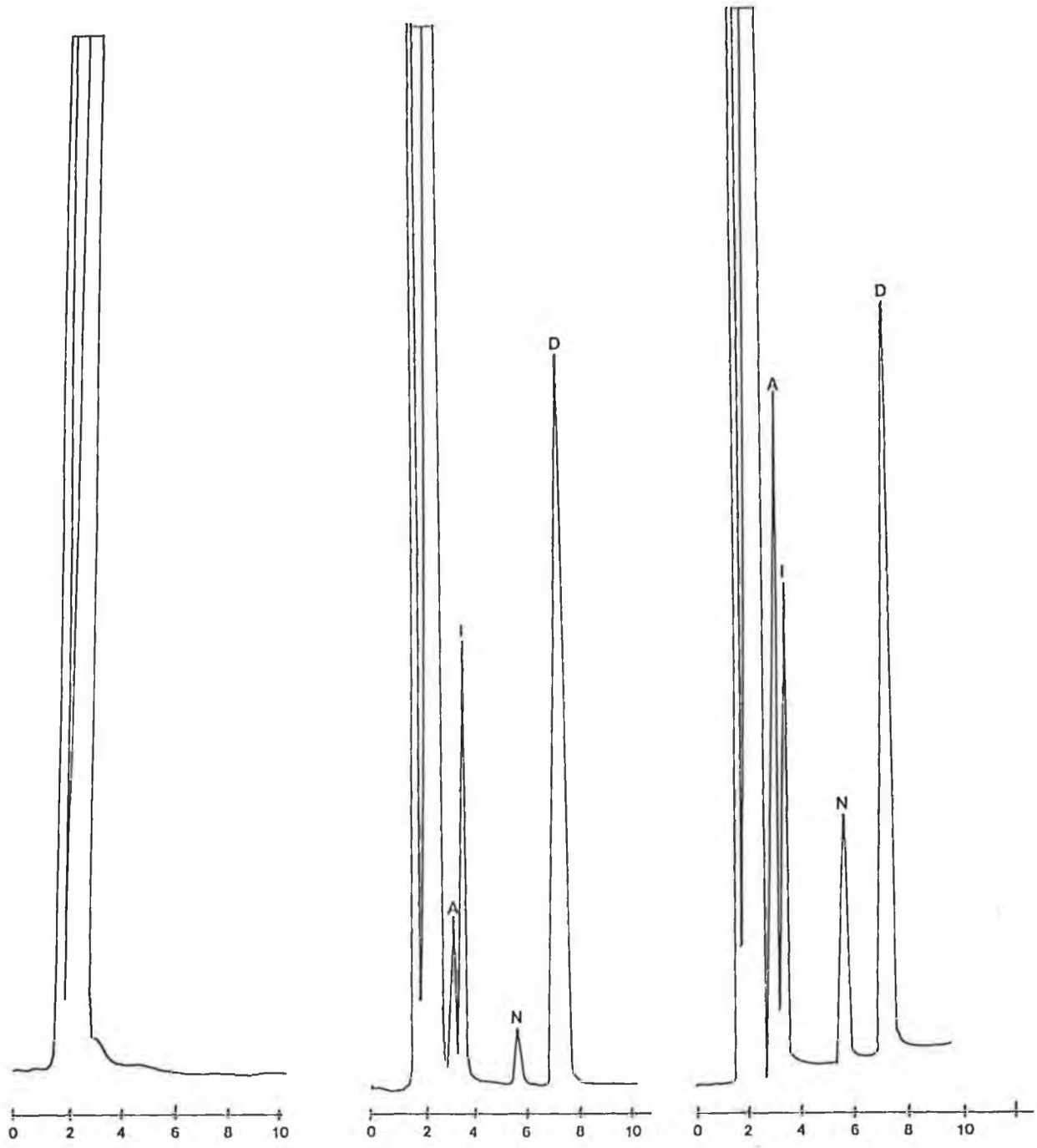

Fig.8 Chromatograms of (a) a small pooled human drug free plasma (b) the same plasma spiked with 10 µg/ml of each of the drugs and (c) spiked with 50 ng/ml of each of the drugs.

A = Amitriptyline, I = Imipramine, N = Nortriptyline and D = Desipramine.

pretreatment of glassware with alkylamines or alkyl alcohols, or addition of these reagents to the solution for evaporation all gave variable results. In the present method plasma (1.5 ml) spiked with 50 µl of the working standards and 50 µl of the internal standard was mixed with 0.4 ml of 0.1M NaOH and vortex mixed for 20 seconds in polypropylene tubes. After the addition of 10ml of hexane: isopropanol (99:1), the drugs were extracted by vortexing the tubes for 1 min, followed by centrifugation for 15 mins at 500 g. The supernatant was then transferred into clean polypropylene tubes and placed in a water bath at 40°C and the solvent was evaportated under a gentle stream of oxygen free nitrogen. The residue was reconstituted, just before injection, in 100 µl of methanol and an aliquot of 70 µl was injected for HPLC analysis. The major problem of adsorption onto glass was minimized by (a) the use of polypropylene tubes and (b) the presence of 1% isopropanol in the extraction mixture. The patient samples were treated exactly as described for standards except that in place of authentic standards, 50 µl of methanol : water (1:1) and 50 µl of internal standard was added prior to extraction.

4.3.1.6. CALIBRATION AND CALCULATION

The slope and intercept of the calibration curves were obtained by linear regression of the peak height ratios of drug/internal standard versus the concentration of the drug (internal standard method). For this method, based on extraction, imipramine and desipramine were used as internal

standards for amitriptyline and nortriptyline respectively. The concentration of amitriptyline and nortriptyline in plasma is then calculated from the measured peak height ratios - (table VIa and VIb and table VIIa and VIIb).

4.3.2. RESULTS AND DISCUSSION

4.3.2.1. Limit of Detection

Under the procedural conditions, the limit of detection using a 1.5ml plasma sample and 70µl injection from a reconstituted volume of 100µl was between 1-5ng/ml for amitriptyline in plasma. The variation in detection limit was due to day-to-day changes in operational conditions and detection system. The detection limit was taken as the amount of compound giving a signal to noise ratio greater than 3:1.

4.3.2.2. Precision

The data presented in table VIa, VIb, VIIa, VIIb and VIII demonstrate the within-batch (intra-assay) and between batch (inter-assay) variation of this method. Using this extraction procedure, variability was determined at five concentrations and in quadruplicate at levels of 10, 25, 50, 100 and 200 ng/ml of each drug in plasma. Inter-assay variability was determined singly at the same five concentrations in four replicate runs. The precision of the method (mean coefficient of variation +\- standard deviation) for the values of the recovered determinate standards when calculated as unknown against the linear

TABLE VI (a)

INTRA-ASSAY

AMITRIPTYLINE

Peak Ratios

Concentration added (ng/ml)	Peak Ht (cm) AT	Peak Ht (cm) IP	Peak RT AT/IP	Peak Ht (cm) AT	Peak Ht (cm) IP	Peak RT AT/IP	Peak Ht (cm) AT	Peak Ht (cm) IP	Peak RT AT/IP	Peak HT (cm) AT	Peak HT (cm) IP	Peak Rt AT/IP	Mean Ratio AT/IP
0	ND	ND		ND	ND		ND	ND		ND	ND		
10	0.4	3.1	0.1290	0.5	3.2	0.1563	0.4	3.2	0.1250	0.3	2.2	0.1364	0.1367
25	0.8	2.5	0.3200	0.8	2.3	0.3478	0.6	1.8	0.3333	0.7	2.3	0.3261	0.3318
50	2.3	3.6	0.6389	1.6	2.5	0.6400	1.6	2.5	0.6400	1.1	1.7	0.6471	0.6415
100	2.9	2.2	1.3182	6.2	4.7	1.3191	2.9	2.2	1.3182	3.3	2.5	1.3200	1.3189
200	9.6	3.7	2.5946	7.0	2.7	2.5926	11.0	4.3	2.5581	6.0	2.3	2.6086	2.5885

$Y = .0130X + .0076$, $(r) = .9999$

Ht = Height
Rt = Ratio
N.D. = Not Detectable

Concentration added (ng/ml)	Concentration found (ng/ml) 1	2	3	4	Mean concentration found (ng/ml)	Standard Deviation	Coefficient of variation (%)
0	-	-	-	-			
10	9.39	11.50	9.08	9.96	9.99	1.08	10.81
25	24.16	26.31	25.19	24.63	25.07	0.93	3.71
50	48.81	48.90	48.90	49.45	49.01	0.29	0.59
100	101.33	101.40	101.33	101.47	101.39	0.07	0.07
200	200.02	199.86	197.20	201.10	199.55	1.66	0.83

Mean % C.V. = 3.2%

TABLE VI (b)

INTRA-ASSAY

NORTRIPTYLINE

Concentration added (ng/ml)	1 Peak Ht NT (cm)	1 Peak Ht DP (cm)	1 Peak RT NT/DP	2 Peak Ht NT (cm)	2 Peak Ht DP (cm)	2 Peak RT NT/DP	3 Peak Ht NT (cm)	3 Peak Ht DP (cm)	3 Peak RT NT/DP	4 Peak HT NT (cm)	4 Peak HT DP (cm)	4 Peak Rt NT/DP	Mean Ratio NT/DP
0	ND	ND		ND	ND		ND	ND		ND	ND		
10	0.2	7.5	0.0267	0.1	3.7	0.0270	0.1	4.4	0.0227	0.1	3.9	0.0256	0.0255
25	SL	SL	SL	0.3	4.5	0.0667	0.3	4.5	0.0667	0.3	4.7	0.0638	0.0657
50	0.6	4.6	0.1304	0.4	3.0	0.1333	0.4	3.0	0.1333	0.3	2.3	0.1304	0.1319
100	0.7	2.6	0.2692	0.9	3.2	0.2813	0.9	3.2	0.2813	1.2	4.5	0.2667	0.2746
200	2.3	4.1	0.5610	2.3	4.1	0.5610	3.0	5.3	0.5660	2.0	3.7	0.5405	0.5571

Y = .0028X -.0051, (r) = .9999

* SL = Sample Lost
ND = Not Detectable
Ht = Height
Rt = Ratio

Concentration added (ng/ml)	Concentration found (ng/ml) 1	2	3	4	Mean concentration found (ng/ml)	Standard Deviation	Coefficient of variation (%)
0	-						
10	11.32	11.43	9.90	10.93	10.89	0.70	6.43
25	SL	25.69	25.69	24.55	25.31	0.66	2.61
50	48.29	49.32	49.32	48.29	48.82	0.60	1.23
100	97.76	102.07	102.07	96.86	99.68	2.77	2.78
200	201.77	201.77	203.55	194.46	200.38	4.04	2.02

Mean % C.V. = 3.0 %

TABLE VII (a)

INTER-ASSAY

AMITRIPTYLINE	Day 1			Day 2			Day 3			Day 4		
Concentration added (ng/ml)	Peak Ht AT (cm)	IP (cm)	Peak RT AT/IP	Peak Ht AT (cm)	IP (cm)	Peak RT AT/IP	Peak Ht AT (cm)	IP (cm)	Peak RT AT/IP	Peak HT AT (cm)	IP (cm)	Peak Rt AT/IP
0												
10	0.3	2.4	0.1250	0.5	3.9	0.1282	0.3	2.2	0.1364	0.4	3.9	0.1026
25	0.8	2.6	0.3077	1.2	3.6	0.3333	0.7	2.0	0.3500	1.2	4.7	0.2553
50	1.2	2.3	0.5217	1.8	3.0	0.6000	1.5	2.0	0.7500	1.4	2.7	0.5185
100	5.7	4.5	1.2667	4.4	3.6	1.2222	3.0	2.0	1.5000	7.0	6.4	1.0938
200	11.8	4.6	2.5652	7.5	3.0	2.5000	3.1	1.1	2.8182	11.9	5.4	2.2037

Concentration added (ng/ml)	Concentration found (ng/ml) Day 1	Day 2	Day 3	Day 4	Mean concentration found (ng/ml)	Standard Deviation	Coefficient of variation (%)
0	0	0	0	0	-		
10	12.90	10.43	8.11	8.78	10.06	2.13	21.17
25	26.98	26.91	23.20	23.69	25.20	2.03	8.06
50	43.46	48.34	51.49	49.38	48.17	3.40	7.06
100	100.83	98.33	104.51	105.54	102.30	3.33	3.26
200	200.83	200.99	197.69	213.88	203.35	7.18	3.53

Mean % C.V. = 8.6 %

TABLE V11(b)

INTER-ASSAY

NORTRIPTYLINE	Day 1			Day 2			Day 3			Day 4		
Concentration added (ng/ml)	Peak Ht NT (cm)	Peak Ht DP (cm)	Peak RT NT/DP	Peak Ht NT (cm)	Peak Ht DP (cm)	Peak RT NT/DP	Peak Ht NT (cm)	Peak Ht DP (cm)	Peak RT NT/DP	Peak HT NT (cm)	Peak HT DP (cm)	Peak Rt NT/DP
0	N.D.	N.D.		N.D.	N.D.		N.D.	N.D.		N.D.	N.D.	
10	0.2	6.1	0.0328	0.15	5.1	0.0294	0.1	2.8	0.0357	0.2	6.25	0.0320
25	0.4	4.9	0.0816	0.35	4.7	0.0745	0.3	3.3	0.0909	0.7	8.9	0.0787
50	0.9	5.4	0.1667	0.7	4.4	0.1591	0.5	2.9	0.1724	0.9	5.8	0.1552
100	2.2	7.3	0.3014	1.4	4.9	0.2857	1.3	4.0	0.3250	3.0	9.2	0.3261
200	4.9	8.0	0.6125	2.2	3.7	0.5946	1.8	2.5	0.7200	6.0	9.6	0.6250

Concentration added (ng/ml)	Concentration found (ng/ml) Day 1	Day 2	Day 3	Day 4	Mean concentration found (ng/ml)	Standard Deviation	Coefficient of variation (%)
0	-	-	-	-			
10	8.83	9.46	11.77	9.59	9.91	1.28	12.91
25	24.97	24.75	27.22	24.48	25.36	1.26	4.97
50	53.10	53.42	50.02	48.88	51.36	2.25	4.38
100	97.63	96.33	92.73	103.37	97.52	4.42	4.53
200	200.47	201.04	203.27	198.68	200.87	1.89	0.94

Mean % C.V. = 5.6%

TABLE VIII

PRECISION AND REPRODUCIBILITY

INTRA-ASSAY

AMITRIPTYLINE

Conc added (ng/ml)	Mean (n=5) concentration found ± S.D. (ng/ml)	C.V. %	Difference between added and found Concentration %
10	9.99 ± 1.08	10.81	0.10
25	25.07 ± 0.93	3.71	0.28
50	49.01 ± 0.29	0.59	1.98
100	101.39 ± 0.07	0.07	1.39
200	199.55 ± 1.66	0.83	0.23
		Mean % C.V. = 3.2 %	
NORTRIPTYLINE			
10	10.89 ± 0.70	6.43	8.90
25	25.31 ± 0.66	2.61	1.24
50	48.82 ± 0.60	1.23	2.36
100	99.68 ± 2.77	2.78	0.32
200	200.38 ± 4.04	2.02	0.19
		Mean % C.V. = 3.0 %	
INTERASSAY			
AMITRIPTYLINE			
10	10.06 ± 2.13	21.17	0.60
25	25.20 ± 2.03	8.06	0.86
50	48.17 ± 3.40	7.06	3.66
100	102.30 ± 3.33	3.26	2.30
200	203.35 ± 7.18	3.53	1.68
		Mean % C.V. = 8.6 %	
NORTRIPTYLINE			
10	9.91 ± 1.28	12.91	0.90
25	25.36 ± 1.26	4.97	1.44
50	51.36 ± 2.25	4.38	2.72
100	97.52 ± 4.42	4.53	2.48
200	200.87 ± 1.89	0.94	0.44
		Mean % C.V. = 5.6 %	

regression line were 3.2% and 8.6% for amitriptyline for intra- and inter-assay variation respectively. The values were 3.0% and 5.6% for nortriptyline for intra-assay and inter-assay respectively.

4.3.2.3. Linearity

Measures of linearity as defined by the correlation coefficient of the regression lines for both drugs and the difference between added and found concentrations are given in intra-assay precision (Table VIa and VIb).

4.3.2.4. Recovery

The overall recovery was calculated in two different ways (Table VIII). First by comparing the peak heights of a series of spiked plasma standards after extraction with the peak heights of a series of unextracted authentic standards. Secondly, by comparing the slopes of the regression lines obtained from the two sets used in the first procedure. Using these methods in the concentration range 10-200 ng/ml, the mean overall recoveries were 80.8% and 71.0% for amitriptyline and nortriptyline respectively.

4.3.2.5. Plasma levels of Amitriptyline and Nortriptyline

The described method was successfully applied to the measurement of anitriptyline and nortriptyline in patients with neurotic and endogenous depression receiving oral doses of placebo, 50, 75, 100 or 150mg of anitriptyline. For

TABLE V

RECOVERY

Concentration (ng/ml)	Amitriptyline peak height (cm) (mean of duplicates)		Recovery (%)
	Set A Authentic Standards	Set B Extracts	
Blank	N.D.*	N.D.	-
10	2.1	1.6	76.19
25	5.2	3.7	71.15
50	10.5	8.5	80.95
100	22.4	17.9	79.91
200	45.7	38.0	83.15

Mean overall recovery $\pm$ S.D. = 78.27 $\pm$ 4.21

METHOD 2

Regression line for set A: Y = .2294 x -.4063, r = 0.999
Regression line for set B: Y = .1911 x -.6473, r = 0.999

Overall recovery = $\frac{.1911}{.2294}$ x 100 = 83.30%

Mean recovery determined by methods 1+2 = 80.79%

NORTRIPTYLINE

METHOD 1

Concentration (ng/ml)	Nortriptyline peak height (cm) (mean of duplicates)		Recovery (%)
	Set A Authentic Standards	Set B Extracts	
Blank	N.D.	N.D.	
10	0.80	0.60	75
25	2.05	0.40	68.28
50	4.05	3.05	75.30
100	8.55	5.25	61.40
200	18.00	13.10	72.77

Mean overall recovery $\pm$ S.D. = 70.55 $\pm$ 5.22

METHOD 2

Regression line for set A: Y = .0901 x -.2079, r = 0.999
Regression line for set B: Y = .0644 x -.2329, r = 0.994

Overall recovery = $\frac{.0644}{.0901}$ x 100 = 71.48%

Mean recovery determined by methods 1 + 2 = 71.02%

* N.D. Not detectable.

quality control, each batch of samples received from the hospital contained a couple of split samples whose indentities were not revealed to us. The results of the split samples from patients are shown in Table X. These results indicated excellent precision of the described method.

4.3.2. CONCLUSION

The tricyclic drugs are lipophilic strong bases, thus at high pH´s the free base can be extracted from an aqueous phase into an organic solvent such as hexane. This method which is based on a single extraction step is comparable or better in most of its features to other published methods. The features of the method are (a) a single extraction step with little adsorption onto the glass, achieved by using inexpensive polypropylene tubes wherever possible and the use of 1% iso-propanol in the extraction solvent which enhances recovery and reproducibility. (b) the use of the double internal standard for improving precision. (c) detection at 215 nm which improves sensitivity and was possible to use because of the simple mobile phase and the cleanliness of the extracts. (d) use of normal phase chromatography - the tricyclic antidepressants become ionized to some extent when they are in aqueous solution, that is they act as weak bases. The basic properties of these secondary and tertiary amines indicate that maximum separation potential would be attained with a mobile phase of pH9, since at higher pH´s the ionization of these weak bases is suppressed.

TABLE X

Plasma concentrations of At and NT in patients with neurotic and endogenous depression receiving oral doses of amitriptyline.
Samples were split into two parts and their code revealed after analysis.

	Concentration found (ng/ml)			
DOSAGE	Part 1		Part 2	
mg	AT	NT	AT	NT
100	58.2	107.4	66.0	102.9
100	76.4	62.4	85.5	66.9
100	12.8	17.5	12.8	17.5
75	169.9	44.4	150.1	44.4
75	21.9	31.0	21.9	31.0
PLACEBO	N.D.	N.D.	N.D.	N.D.
150	248.8	187.8	226.6	211.4
PLACEBO	N.D.	N.D.	N.D.	N.D.

N.D. = NOT DETECTABLE (Below limit of detection).

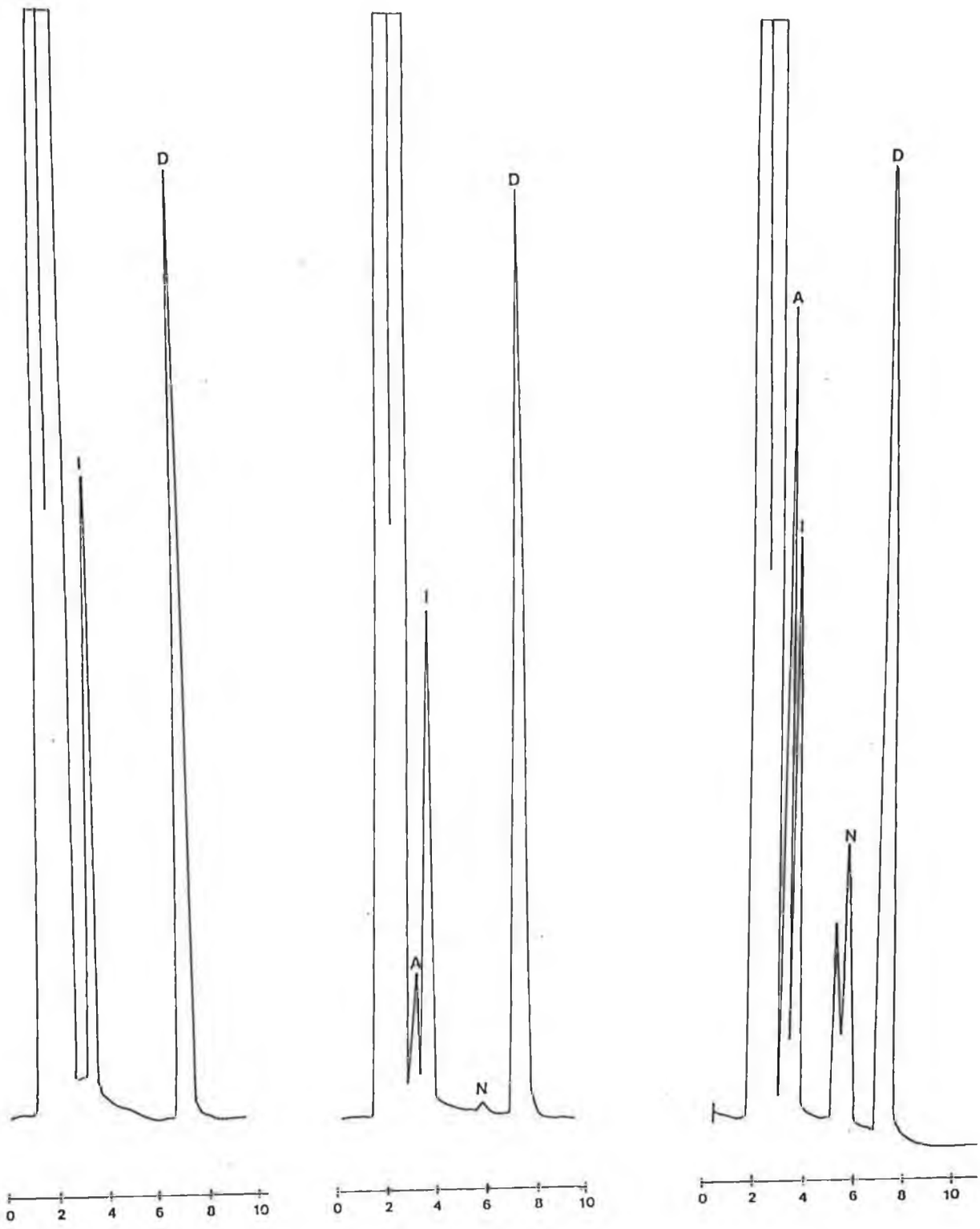

Fig.9

Chromatograms of human plasma samples 10 hours after receiving a single oral dose of (1) Placebo and (2) tablets and containing 50 mg of Amitriptyline and (3) 150 mg of amitriptyline.

Use of a Lichrosorb Si 100 column means that a high pH mobile phase can be used but the column must be kept free of water. Peak tailing was minimized by the addition of 0.1% ammonia solution to the mobile phase. (e) use of A pre-column and guard column packed with silica in order to avoid formation of a void in the analytical column due to dissolution of silica in the mobile phase which could occur due to the high pH of this mobile phase. More recent publications [53, 73, 75] report recoveries of 90 - 100% for the extraction of amitriptyline and nortriptyline which involves time consuming extraction/back extraction and the use of higher volumes of plasma. Although the recovery of our extraction scheme is lower, the detection limits are similar (1-5 ng/ml) and the baseline is more stable in terms of noise and drift at the most sensitive detector setting. This can be attributed to the use of the lower detection wavelength (215 nm) for the measurement of the drugs. The use of the double internal standards imipramine and desipramine, not only improve precision but also offers the possibilities of determining other important tricyclic drugs using the described method. Some typical chromatograms are shown in fig. 8 + 9 and it can be seen that chromatograms of control plasma and patients plasma are quite similar with no interfering peaks in the region where the drugs and internal standards elute.

4.4. COMPARISON

For the HPLC determination of amitriptyline and nortriptyline in human plasma, the two procedures for sample preparation are compared.

The first method used on-line, direct injection of plasma and a column switching technique followed by reverse bonded phase chromatography whereas the second is based on conventional liquid - liquid extraction and adsorption chromatography. Validation of both techniques showed excellent sensitivity, precision and reproducibility. For the method based on extraction the limit of detection was between 1-5 ng/ml of the drugs in plasma and the mean % coefficient of variation for intra and inter - assays was better than 8% for both drugs. The column switching/direct injection method showed a limit of detection of 5 ng/ml and the mean % coefficient of variation for intra and inter -assay better than 5%. This method shows obvious advantages in terms of speed and ease of sample handling. Both methods have been sucessfully applied to samples from patients receiving oral doses of amitriptyline and results were in good agreement.

As mentioned previously, HPLC procedures in the determination of tricyclic antidepressants generally involve extraction of drugs from plasma or serum, followed by various modes of chromatography. These extraction schemes are often tedious and time consuming, susceptible to errors and often can result in loss of sample because of incomplete extraction or formation of artifacts.

On the other hand, the use of on-line column switching with direct injection of plasma provides a new rapid alternative for sample preparation of tricyclic drugs prior to analysis with HPLC. The recovery of amitriptyline and nortriptyline was 90.7% and 92.6% respectively whereas using the extraction scheme recoveries were only 80.8% and 71.0%

Erni et al. [86] fully discuss the advantages of column switching as an on-line method for sample preparation and points out its many possibilities. In setting up the system, a major consideration is the nature of packing materials in the concentration column and the mobile phase passing through it during sample enrichment process. These have to be selected in such a way that the drug is almost completely retained while the interfering substances in plasma are removed.

The packing eventually chosen was pellicular Corasil C_{18} (37-50 μm). Water was chosen as the mobile phase for enrichment since it gave good recovery and eliminated the need for buffer salts which can cause precipitation of plasma proteins. This allowed at least 30 or 50 injections of 250 μl plasma. More injections resulted in the loss of resolution, band broadening and lesser recovery hence lesser sensitivity. This may not allow automatic injection if total number of samples exceed thirty or fifty. Except for this limitation, direct injection/column switching proved to be a superior technique as virtually no sample preparation was necessary.

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HPLC DETERMINATION OF ANTIHISTAMINES IN BIOLOGICAL FLUIDS

CHAPTER 5

5.1. INTRODUCTION TO ANTIHISTAMINES

Drugs that are customarily classified as antihistamines have in common the property of antagonising the actions of histamine that are now recognised as involving H_1 - receptors. They could therefore be termed 'H_1 - receptor antagonists'. Their development began in 1937 when Bovet discovered that an adrenaline antagonist - thymoxyethyldiethylamine also antagonised the actions of histamine. A large series of related compounds were then prepared to obtain increased potency and specificity and lower toxicity. In 1942, antergan was prepared and this became the first antihistamine to be used in medicine and found to be of value. This was followed by mepyramine in 1944 which even today has retained its position among the drugs used in the treatment of allergies. At about the same time in the U.S.A. another parent compound was developed i.e. tripelennamine which has also remained in use today. From this start many other type of antihistamines have been developed and it is worth noting that two other important drug groups i.e. neuroleptic phenothiazines and tricyclic antidepressants had their origins in research into antihistamines.

Chemically, antihistamines are a diverse group of compounds. The only common feature is that they all possess at least one tertiary nitrogen atom and have an aromatic portion consisting of two phenyl rings. They are all weak bases,

consequently they are well absorbed, widely distributed in tissues and cross the blood, brain and placental barriers readily. They are rapidly eliminated in the urine partly unchanged and partly after metabolism in the liver. Antihistamines can be divided into groups on the basis of their chemical structures as follows, ethylenediamine, imidazoline and piperidine derivatives, oxyethylamines arylalkylamines and phenindine derivatives.

The main use of antihistamines that is directly related to their antagonistic activity at H_1 - receptors is in the relief of histamine - mediated effects in allergic and anaphylactic reactions. Thus they are useful for the symptomatic relief of urticarial lesions, angioneurotic oedema, hay fever and serum sickness. Antihistamines are also useful in treating reactions to venoms which contain histamine and substances causing histamine release in tissues (e.g. certain insect stings).

In the treatment of allergic skin rashes antihistamines may be applied topically in ointments or creams. Those with local anaesthetic activity are particularly effective in allying itching. Other actions include antinauseant and antiemetic activity, relief of vertigo and nausea in Menieres Syndrome and other labyrinthine disorders, the treatment of Parkinson´s disease and sedative activity.

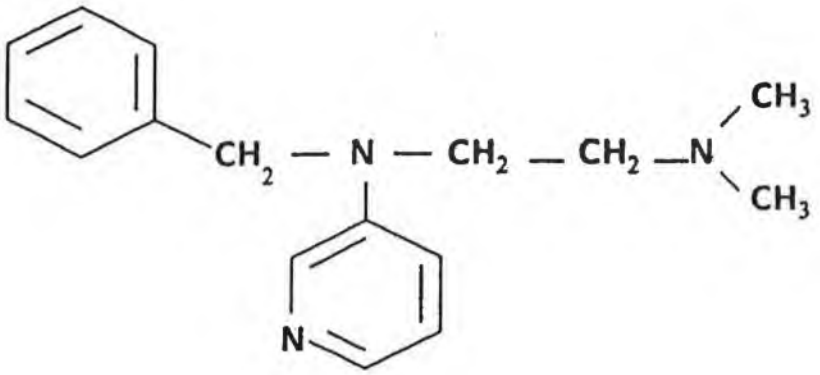
Side effects may be drowsiness, atrophine like effects e.g. dryness of the mouth and the mucosa of the upper respiratory

tract. A variety of other effects have been reported including anorexia, nausea, vomiting, diarrhoea, dizziness, tinnitus and disorders of perceptors.

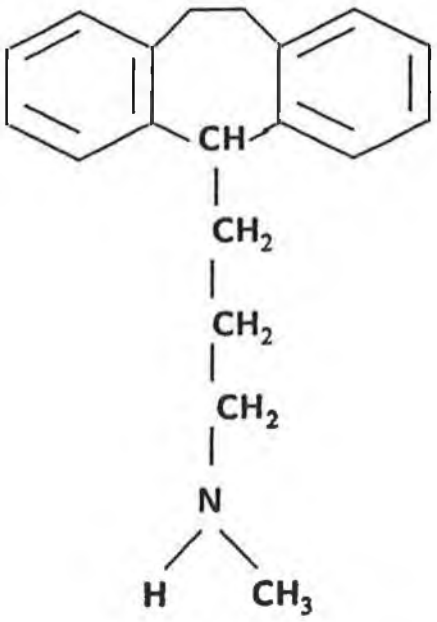
High Performance Liquid Chromatographic Determination of Tripelennamine in bovine plasma and milk using a Column Switching Technique.

5.2.1. INTRODUCTION

A new, sensitive and selective HPLC method for the determination of tripelennamine, an antihistamine, in bovine plasma and milk is described which uses direct injection and a column switching valve. The method is based on the enrichment of the drug on a small reverse phase concentration column, packed with Corasil RP, 37-50 μm. The enriched drug is then eluted from the concentration column, in back flush mode, and separated on a bonded phase Techsphere CN column, 3 μm, using an isocratic acetonitrile - 0.05 M acetate buffer (70:30 v/v) mobile phase. Tripelennamine was detected by absorption at 246 nm. The validation of the method showed excellent sensitivity, precision and reproducibility. The limit of detection, using a 250 μl direct injection of biological sample was 2 ng/ml. The mean percentage coefficient of variation for intra-and inter-assay were 5.6% and 7.4% respectively. The linearity, as measured by the correlation coefficient of the intra-assay linear regression curve and calibration curves throughout the analyses was consistently better than 0.999. The mean overall recovery from plasma was 78% and from milk was 73.5%. The method showed obvious advantages over existing methods in terms of speed and convenience of sample handling. The method has been successfully applied to the samples from cattle receiving an intravenous injection of a formula containing tripelennamine.



TRIPELENNAMINE

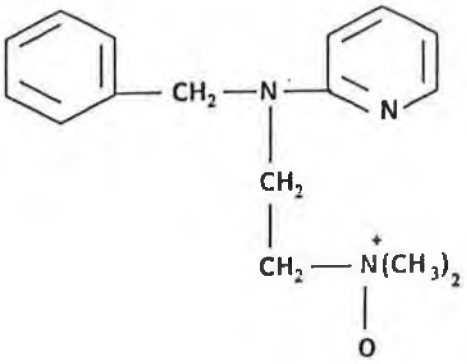


PROTRIPTYLINE

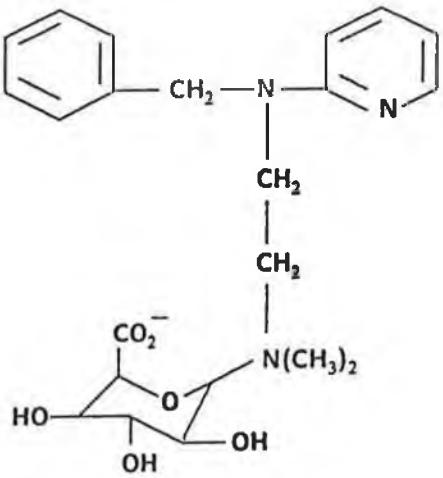
Fig. 1 Chemical structure for Tripelennamine and the internal standard Protriptyline.

Tripelennamine 2 - [benzyl (2 - dimethylaminoethyl) amine] pyridine, (fig 1) is an antihistamine which acts as a competitive antagonist of histamine at H_1 receptors [1]. Tripelennamine is used to control allergic manifestations in humans and animals and it has also been used as a stimulant in cows who refuse to stand because of various disorders [2]. The recommended dosage of tripelennamine for an adult is 50 to 100 mg once or twice daily, it is administered to other animals e.g. cattle in certain pharmaceutical preparations and in animal feed. In humans, pharmacological effects due to the drug are evident within 15 to 30 minutes and maximal effects are reported 1 to 2 hours after dosage. The duration of action is 4 to 6 hours. The drug is rapidly absorbed from the gastrointestinal tract and the tissues are normally free of it in about 6 hours. The majority of tripelennamine is biotransformed primarily in the liver, although some metabolic conversion has been noted in the lung and kidney [3]. Tripelennamine is then excreted in the urine and in bile. Chadhuri et al, [4] isolated four metabolites from human urine (fig 2), the drug is metabolised by N-demethylation and aromatic hydroxylation followed by conjugation with glucuronic acid.

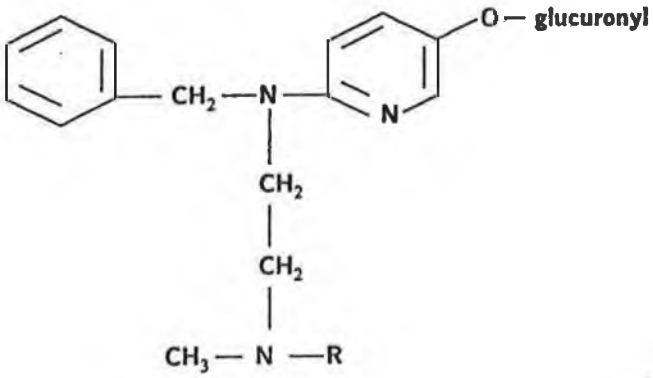
Methods employed for the determination/detection of tripelennamine include, UV spectrophotometry [5,6] fluorimetry [5], and chromatographic methods. These chromatographic methods include thin layer chromatography [6-8] to detect tripelennamine in urine when screening for drug abuse. Gas liquid chromatography (GLC) with flame



N-oxide of tripelennamine.



Quaternary ammonium N-glucuronide of tripelennamine.



R=CH_3; O-glucuronide of hydroxytripelennamine.
R=H; O-glucuronide of hydroxydesmethyltripelennamine.

Fig. 2: Metabolites of tripelennamine.

ionization detection for the identification of the drug in urine [6,7] and to determine tripelennamine residues in bovine milk [2] and in pharmaceutical formulations [9]. GLC has also been used with nitrogen specific detection [10,11] to determine tripelennamine in the blood of addicts taking a combination of pentazocine and tripelennamine known as "T´s and Blues".

Spectrophotometric methods and all but one of the GC methods mentioned above while giving adequate sensitivity for measurement of tissue levels in cases of massive overdose [5,13] or among "T´s and Blues" addicts [6,12] do not allow quantitation in the low nanogram region which is required for bioavailibility studies. Only the method described by Luders et al. [2] achieves this. GC with flame ionization detection is used giving a limit of detection of 10 ppb for a 2µl injection, however a major drawback of this method is the very lengthy and elaborate extraction procedure used requiring specially made tubes. This would render it impracticable for routine monitoring in the laboratory.

Although GC methods have been the most use for quantitative determination of tripelennamine, high performance liquid chromatographic methods have been reported for the determination of basic antihistamines including tripelennamine [14] in animal feed, human urine and waste water [15]. In one of these methods [15], after liquid - liquid partitioning of the drug and silica gel column clean-up, levels of 500ng/ml of the drug could be determined.

However, this method suffers from the same drawback as the GC method [2], i.e. lengthy sample preparation technique.

The objective of the present study was to develop a HPLC method for the determination of tripelennamine in bovine plasma and milk that was sensitive, selective and reproducible and yet convenient in terms of sample handling and speed of analysis. In the following section a technique will be described which fully meets the objectives set out above. Each sample is processed and chromatographed inside 15 minutes, giving a limit of detection of 2ng/ml.

5.2.2. EXPERIMENTAL

5.2.2.1. Reagents and Solvents

Tripelennamine hydrochloride was obtained from Sigma Chemical Company, Dorset, England. Protriptyline hydrochloride (internal standard) (IS), was obtained as a gift from Jervis Street Hospital, Dublin. Methanol and acetonitrile (HPLC grade) were purchased from Fisons, Loughborough, England and sodium acetate (AnalaR grade) was purchased from BDH Chemicals Ltd., Poole, England. Pure water was obtained by the Milli-Q water purification system. Drug free bovine plasma and milk were provided by Bimeda Chemicals Ltd., Dublin, stored frozen at -4^{o}C and thawed at room temperature prior to use. The control plasma and milk showed no interference from endogenous components when examined for possible interferences with tripelennamine in the assay system.

5.2.2.2. Instrumentation

The HPLC system consisted of two Waters Assoc. (Milford, MA, USA) P-45 liquid chromatograph solvent devilvery systems equipped with a Waters U6K (Milford, MA, USA) manual injector and a Rheodyne (California, USA) 7000, 6 port switching valve. A Shimadzu (Tokyo, Japan) SPD -6A variable wavelength UV detector was used and the chromatograms were recorded on a Philips (Endhoven, Holland) PM 8251 single pen recorder.

5.2.2.3. Preparation of Standards

Tripelennamine HCl (11.43mg) was weighed and dissolved in 100ml of methanol to yield a stock solution of 100µg/ml. This stock solution was then diluted with methanol-water (1:1) to yield working standards of the following concentrations 0.04, 0.1, 0.2, 0.5, 1.0, 1.5, 3.0 and 6.0µg/ml. Protriptyline HCI (11.39mg) was weighed and dissolved in 100ml of methanol to yield a stock solution of 100µg/ml. This was then diluted with a methanol-water (1:1) mixture to obtain a working internal standard solution of 4µg/ml. Spiked plasma standards ranging in concentration from 2 to 50ng/ml of the drug were prepared in each assay day by spiking 1ml of plasma with 50µl of working standards (0.04 - 6.0µg/ml) and 50ul of IS (4µg/ml) solution.

Spiked milk samples ranging from 5-300ng/ml of the drug in milk were prepared by spiking 1ml of milk with 50µl of the working standards (0.1 - 6.0 µg/ml) and 50ul of IS (4 µg/ml) solution. It should be noted that tripelennamine, although stable for at least 5 years at room temperature should be protected from light as on exposure it slowly darkens [1] and appears to undergo light catalyzed oxidation to yield benzaldehyde and 2-[2- (dimethylamino) ethylamino] pyridine.

Samples from cattle undergoing treatment were spiked with 50µl of methanol-water (1:1) mixture to compensate for any changes in the composition of the sample, plus 50ul of IS (4µg/ml).

5.2.2.4. Chromatography

The instrument arrangement for the chromatography was exactly as described in section 4.2.1.5. The chromatographic conditions for the separation were as follows: mobile phase pump A: water; concentration column: (10 x 1.5mm) dry packed in-house with Corasil (Waters Assoc. USA) RP 18, 37-50 µm packing; wash time: 3.0 min.; flow rate: 0.8 ml/min. Mobile phase pump B: acetonitrile - 0.05 M acetate buffer (70:30 v/v) pH 7.2; analytical column: Techsphere (HPLC Technology, Cheshire, UK) 3CN (10 cm x 4.6 mm); flow rate: 0.8 ml/min, detection wavelength: 246 nm; recorder chart speed: 0.5 cm/min and an injection volume of 250 µl of plasma or milk sample.

Under the described chromatographic conditions the mean retention times were 5.2 min and 6.8 min for tripelennamine and protriptyline respectively (fig. 3).

5.2.2.5. Column Switching Procedure

5.2.2.5.1. (a) Plasma

The column switching procedure described in section 4.2.1.5. was adopted for use in the present study. The bovine plasma was more viscous than human plasma and the wash time was therefore extended to 3.0 minutes per sample to avoid cloggin gof the pre-column and the resulting high back pressure. It was possible to inject up to thirty samples per concentration column. Chromatography deteriorated where sample injections exceeded thirty.

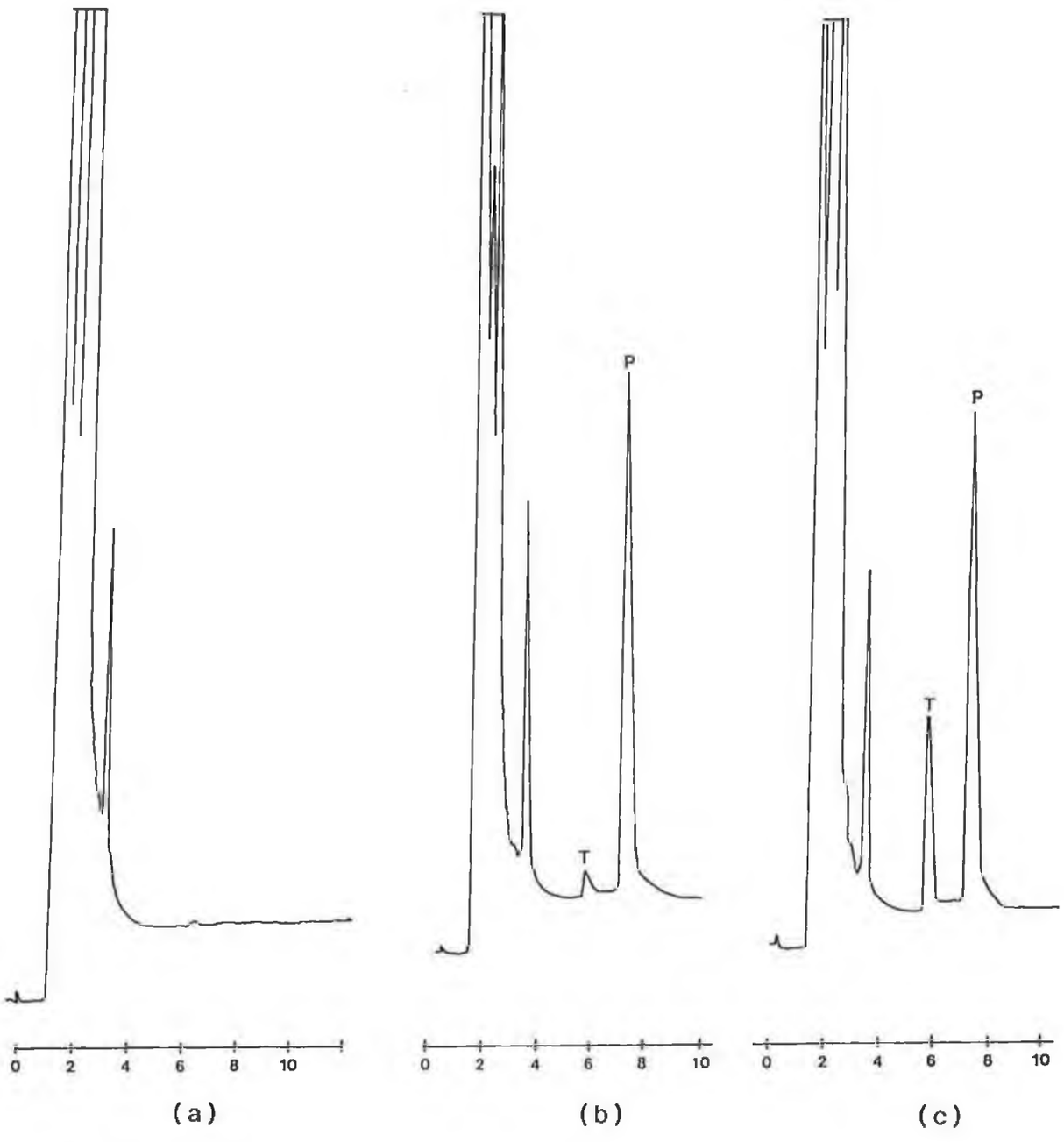


Fig. 3: Chromatograms of (a) drug free bovine plasma, (b) drug free plasma spiked with 2ng/ml of tripelennamine and the internal standard and (c) drug free plasma spiked with 25ng/ml of tripelennamine and the internal standard.

5.2.2.5.2. (b) Milk

Because of the relatively high amount of fats in milk it was not possible to directly inject the milk samples onto the concentration column. Their subsequent elution from this column onto the analytical column gives rise to the presence of interfering peaks on the chromatogram. Removal of the fats from the milk was attempted using three different procedures:

(a) Extraction of the fats into hexane (6ml), followed by centrifugation.

(b) Addition of 50 µl of a 0.1 M HCl solution followed by extraction of the fats into hexane followed by centrifugation. After removal of the hexane, 50 µl of 0.1 M NaOH is added to the lower milk layer.

(c) Centrifugation of the milk sample so that the fats rise to the top.

Spinning of the milk sample in a centrifuge at 1200 g to separate fats from the sample proved to be as effective as extraction of the fats into hexane. Upon centrifugation of milk samples three distinct layers were formed. The top layer was the fat and the bottom layer was precipitated protein. Hence care was taken to inject the middle layer for chromatrography. The analysis of separated fat and protein showed no detectable amount of tripelennamine (at

a spiked level of 300 ng/ml tripelennamine in milk) in either fat or protein.

5.2.2.6. Calibration and Calculation

Evaluation of the assay was carried out using five point calibration standards in the concentration range 2-50 ng/ml of tripelennamine in plasma and six point calibration standards in the concentration range 5-300 ng/ml of the drug in milk. The slope and intercept of the calibration curves were obtained by linear regression of the peak height ratios of drug / IS versus the concentration of the drug (internal standard method). These calibration curves were then used to interpolate the concentration of tripelennamine in bovine plasma and milk from the measured peak height ratios.

5.2.3. RESULTS AND DISCUSSION

In this study, the extension of the use of column switching/direct injection was investigated for the clean-up of milk samples. With some modification i.e. centrifugation of the samples for removal of fats prior to injection, this proved successful. On line sample clean-up followed by chromatographic analysis proved to be a rapid, sensitive and selective method for the determination of tripelennamine in the low nanogram range in milk, giving a comparable if not better limit of detection than more elaborate clean-up procedures, [2]. The use of column switching as a method for analysis of organopesticides in milk has been

reported previously using a silica pre-column for sample clean-up [16]. Fats from the milk are adsorbed onto the pre-column while the organopesticides are eluted onto the analytical column. In a regeneration phase the fats are then backflushed off the pre-column using 1% isopropanol in hexane, followed by hexane leaving it ready for the next injection.

For the determination of tripelennamine in plasma on line solid phase extraction gave good sample clean-up coupled with a low limit of detection. In all the previously described methods liquid - liquid extraction prior to analysis was used with some of the procedures being somewhat lengthy. Monteford et al, [5] evaluated the extraction efficiencies of various solvents for extraction of tripelennamine from plasma and these are shown in Table 1.

TABLE 1

SOLVENT	% RECOVERY OF TRIPELENNAMINE
Ethyl Acetate	47
Diethyl Ether	75
Toluene	68
n-butyl chloride	40
Chloroform	62
Methylene Chloride	62
Ethylene Chloride	60

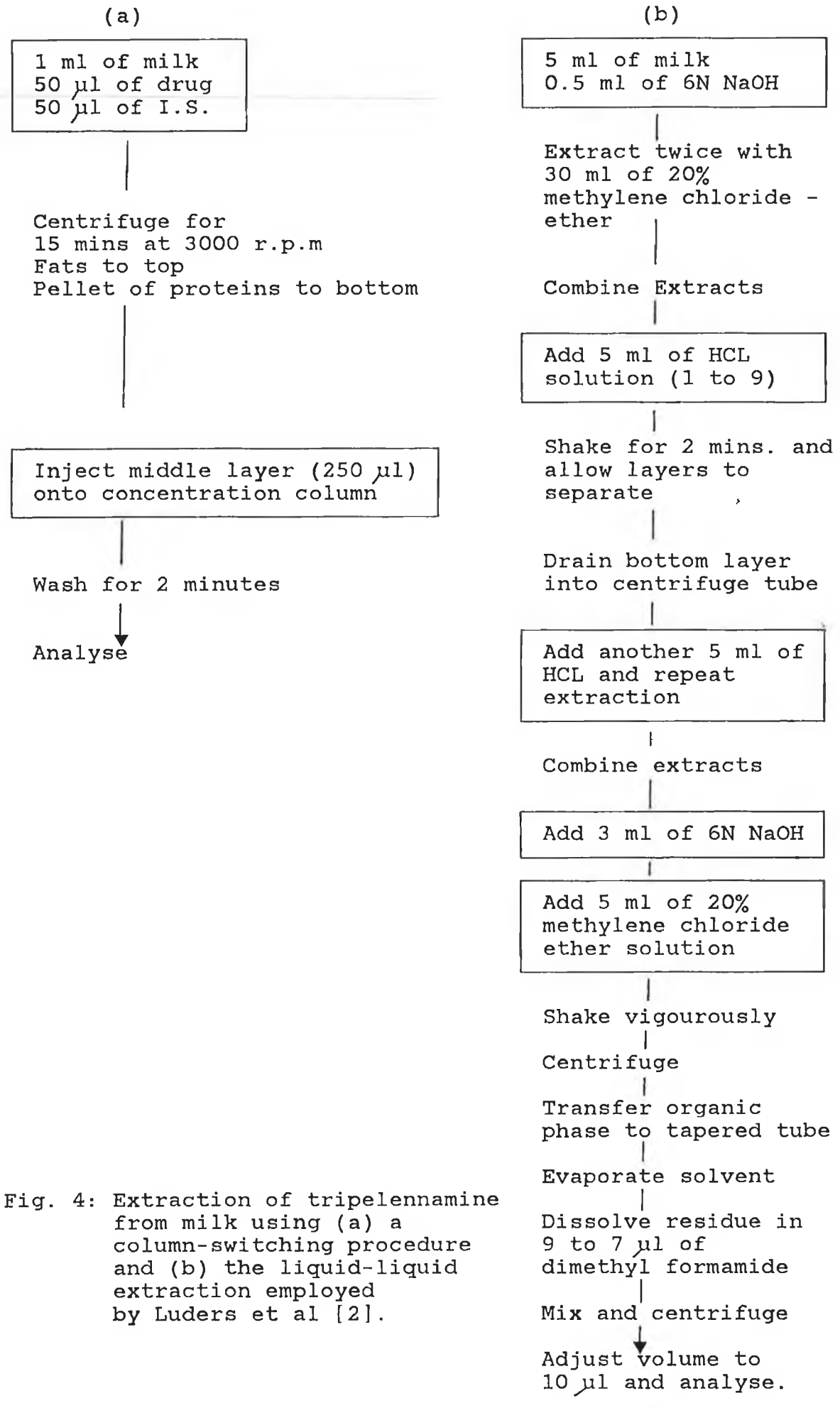


Fig. 4: Extraction of tripelennamine from milk using (a) a column-switching procedure and (b) the liquid-liquid extraction employed by Luders et al [2].

5.2.3.1. Limit of Detection

Under the described procedural conditions, the limit of detection using a 250 µl direct injection of plasma or milk was 2 ng/ml of tripelennamine in the biological fluid. The detection limit was taken as the amount of compound giving a signal-to-noise ratio greater than 3:1.

5.2.3.2. Precision

The data presented in table II, III and IV demonstrates the within batch (intra-assay) and between batch (inter-assay) variation of the method. Intra-assay variability was determined at five concentrations in quadruplicate at levels of 2,5,10,25 and 50 ng/ml of the drug in plasma. Inter-assay variability was determined singly at the same five concentrations in four replicate runs. The precision of the method (mean coefficient of variation) for the values of the recovered determinate standards when calculated as unknowns against the generated linear regression line were 5.6% and 7.4% for intra- and inter-assay respectively.

A full validation was not carried out for milk samples as the precision of the assay for milk was found to be very similar to that of plasma.

TABLE II

INTRA-ASSAY - PLASMA

TRIPELENNAMINE

Concentration added (ng/ml)	Peak HT (cm)		Peak RT T/P	Peak HT (cm)		Peak RT T/P	Peak HT (cm)		Peak RT T/P	Peak HT (cm)		Peak RT T/P	Mean Ratio T/P
	T	P		T	P		T	P		T	P		
0	N.D.		N.D.	N.D.		N.D.							
2	0.2	5.1	0.0392	0.2	4.9	0.0408	0.15	4.5	0.0333	0.15	3.9	0.0385	0.0380
5	0.4	4.7	0.0851	0.4	4.7	0.0851	0.4	4.7	0.0851	0.5	5.2	0.0962	0.0879
10	1.1	6.1	0.1803	0.8	4.6	0.1739	0.8	4.5	0.1778	0.7	4.1	0.1707	0.1757
25	2.4	5.4	0.4444	2.0	4.7	0.4255	2.1	4.8	0.4375	1.9	4.4	0.4318	0.4348
50	3.2	4.0	0.8000	3.2	4.0	0.8000	2.6	3.2	0.8125	4.4	5.0	0.8800	0.8231

$Y = 0.0164\,x + 0.0102$ $\qquad r = .999$

Concentration added (ng/ml)	Concentration found (ng/ml)				Mean Concentration found (ng/ml)	Standard Deviation	Coefficient of Variation %
	1	2	3	4			
0							
2	1.77	1.87	1.41	1.73	1.70	0.199	11.71
5	4.57	4.57	4.57	5.24	4.74	0.335	7.07
10	10.38	9.99	10.22	9.79	10.09	0.259	2.57
25	26.48	25.33	26.06	25.71	25.89	0.491	1.90
50	48.16	48.16	48.92	53.04	49.57	2.340	4.72

Mean % C.V. = 5.6%

N.D. = not detectable
HT = height
RT = ratio
T = Tripelennamine

TABLE III

INTER-ASSAY - PLASMA

TRIPELENNAMINE

Concentration added (ng/ml)	DAY 1			DAY 2			DAY 3			DAY 4		
	Peak HT (cm)		Peak RT $\frac{T}{P}$	Peak HT (cm)		Peak RT $\frac{T}{P}$	Peak HT (cm)		Peak RT $\frac{T}{P}$	Peak HT (cm)		Peak RT $\frac{T}{P}$
	T	P		T	P		T	P		T	P	
0	N.D.		N.D.	N.D.		N.D.						
2	0.3	9.3	0.0323	0.3	8.7	0.0345	0.4	11.2	0.0357	0.2	6.6	0.0303
5	0.7	9.5	0.0737	0.7	8.5	0.0824	1.0	10.3	0.0971	0.3	4.1	0.0732
10	1.4	9.1	0.1538	1.5	8.4	0.1786	2.1	12.9	0.1628	0.7	5.3	0.1321
25	3.3	8.8	0.3750	3.1	8.1	0.3827	3.9	10.2	0.3824	1.4	4.5	0.3111
50	7.1	9.4	0.7553	6.1	8.1	0.7531	8.4	10.1	0.8317	2.8	4.4	0.6364

Concentration added (ng/ml)	Concentration found (ng/ml)				Mean Concentration found (ng/ml)	Standard Deviation	Coefficient of Variation %
	1	2	3	4			
0	N.D.						
2	2.10	1.42	2.13	1.95	1.9	0.330	17.37
5	4.85	4.65	5.88	5.37	5.2	0.552	10.62
10	10.16	11.14	9.90	10.06	10.3	0.561	5.45
25	24.83	24.90	23.32	24.34	24.4	0.729	2.99
50	50.06	49.88	50.77	50.28	50.2	0.385	0.77

Mean % C.V. = 7.4%

N.D. = not detectable
HT = height
RT = ratio
T = Tripelennamine

TABLE IV

PRECISION (REPEATABILITY AND REPRODUCIBILITY)

Concentration added (ng/ml)	Mean (n=4) concentration found ± S.D. (ng/ml)	C.V. %	Difference between added and found concentration %
INTRA-ASSAY			
2	1.7 ± 0.20	11.7	15.0
5	4.7 ± 0.34	7.1	5.2
10	10.1 ± 0.26	2.6	0.9
25	25.9 ± 0.49	1.9	3.2
50	49.6 ± 2.34	4.7	0.9
	Mean % C.V. = 5.6%		
INTER-ASSAY			
2	1.9 ± 0.33	17.4	5.0
5	5.2 ± 0.55	10.6	4.0
10	10.3 ± 0.56	5.5	3.0
25	24.4 ± 0.73	2.0	2.4
50	50.2 ± 0.39	0.8	0.4
	Mean % C.V. = 7.4%		

MILK SAMPLES - TRIPELENNAMINE

Duplicate Standards

Standard Curve

Concentration ng/ml	Peak Ratios	Mean Ratio	Concentrations found ng/ml
0			
5	$\frac{0.5}{5.7} = .0877$	0.0890	6.32
	$\frac{0.55}{6.1} = .0902$		
10	$\frac{0.6}{3.4} = 0.1765$	0.1820	11.34
	$\frac{0.6}{3.2} = 0.1875$		
25	$\frac{1.5}{3.4} = 0.4412$	0.4394	25.24
	$\frac{1.4}{3.2} = 0.4375$		
75	$\frac{4.4}{3.4} = 1.2941$	1.3190	72.76
	$\frac{4.3}{3.2} = 1.3438$		
150	$\frac{9.7}{3.5} = 2.7714$	2.7071	147.74
	$\frac{11.1}{4.2} = 2.6428$		
300	$\frac{15.0}{2.7} = 5.5555$	5.5556	301.60
	$\frac{20.0}{3.6} = 5.5555$		

Slope = .0185 I = -.0279, r = .99988

5.2.3.3. Linearity

Measures of linearity as defined by the correlation coefficients (r) of the regression lines generated based on duplicate calibration curves obtained for plasma and milk were consistantly better than 0.999.

5.2.3.4. Recovery

In order to establish the recovery, two calibration curves based on the external standard method, were set up. One calibration curve was based on spiked plasma samples in the concentration range 2-50 ng/ml and the other was based on working reference standards in the same concentration range. The overall recovery was calculated by comparing the slopes of the regression lines generated for the two sets. The same procedure was applied to the milk samples in the concentration range 5-300 ng/ml tripelennamine. The overall recovery was also calculated by direct comparison of the peak heights. The results obtained are shown in Table V.

5.2.4. APPLICATIONS OF THE METHOD

The described method has been successfully applied to the samples from cattle receiving an intravenous injection of a formula containing tripelennamine.

TABLE V

RECOVERY FROM PLASMA

CONCENTRATION (ng/ml)	TRIPELENNAMINE PEAK HEIGHTS (cm) (mean of duplicates)		RECOVERY %
	Set A: Working standards	Set B: Standards extracted from plasma/milk	
2*	0.9	0.7	78
5	0.9	0.7	78
10	1.9	1.5	79
25	4.5	3.5	79
50	9.1	7.1	77

Mean overall recovery = 78%

Regression line for Set A : $Y = 0.1805X + 0.0480$, $r = 0.9999$

Regression line for Set B : $Y = 0.1407X + 0.0402$, $r = 0.9999$

Overall recovery = $\frac{0.1407}{0.1805} \times 100 = 78\%$

Mean recovery determined by the two methods: 78%

RECOVERY FROM MILK

5	0.50	0.35	70
10	0.85	0.65	77
25	2.30	1.75	76
75	7.90	5.70	72
150	15.75	11.90	76
300	31.00	22.60	73

Mean overall recovery = 74%

Regression line for Set A: $Y = 0.1040 \times -0.0821$, $r = 0.999$

Regression line for Set B: $Y = 0.0761 \times -0.0113$, $r = 0.999$

Overall recovery = $\frac{0.0761}{0.1040} \times 100 = 73\%$

Mean recovery determined by the two methods: 73.5%

* More sensitive detector setting.

Results of the analysis of plasma and milk samples are shown in Table VI and VII. Chromatograms from the actual plasma and milk samples are included in figures 5 and 6. As can be seen, these chromatograms resemble the chromatograms obtained from spiked samples and no interferences were observed from endogenous components of plasma or milk.

5.2.5. CONCLUSION

For routine anlaysis of tripelennamine in bovine plasma and milk, a new method of analysis based on direct injection/column-switching technique was developed. Plasma samples could be injected directly while milk samples had to be spun first, to separate fat, prior to injection. The method is rapid and eliminates the need for lengthy and tedious liquid-liquid extraction procedures with good recovery and low running cost. The method achieves a low limit of detection which compares with some reported GC methods which employ long and elaborate extraction procedures. The chromatrography was selective, i.e. there were no interferences from endogenous plasma components, and sharp peaks were obtained for the drug and the internal standard. This procedure illustrates the extension of the use of the column switching technique for clean up of biological fluids other than plasma.

TABLE VI - Results of plasma samples from cattle undergoing treatment with a complex, containing tripelennamine.

	Concentrations of Tripelennamine in Plasma (ng/ml)				
	Cow 1	Cow 2	Cow 3	Cow 4	Cow 5
Time after dosage					
0 min (pre-treatment)	N.D.	N.D.	N.D.	N.D	N.D.
15 mins	18.3	21.6	12.6	14.3	15.6
30 mins	27.2	27.5	18.2	15.5	23.6
45 mins	25.6	30.6	17.9	16.5	32.8
1 hr	14.8	24.7	15.9	22.8	23.9
2 hr	9.7	15.7	12.1	18.1.	17.2
4 hr	3.0	S.L.			
6 hr	N.D.	3.9		*	
12 hr	N.D.	N.D.			
24 hr	N.D.	N.D.			

* Samples not given

N.D. = not detectable

S.L. = sample lost

TABLE VII - Results of milk samples from cattle undergoing treatment with a complex, containing tripelennamine.

	Concentration found of tripelennamine (ng/ml)					
Cow No.	1	2	10	22	536	545
Sample Number						
0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
1	75.83	71.26	83.41	156.57	90.58	46.06
2	68.15	21.45	48.90	47.89	28.22	18.78
3	17.08	7.17	8.05	9.58	6.80	10.18
4	6.08	4.17	5.67	N.D.	N.D.	N.D.
5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

N.D. = not detectable.

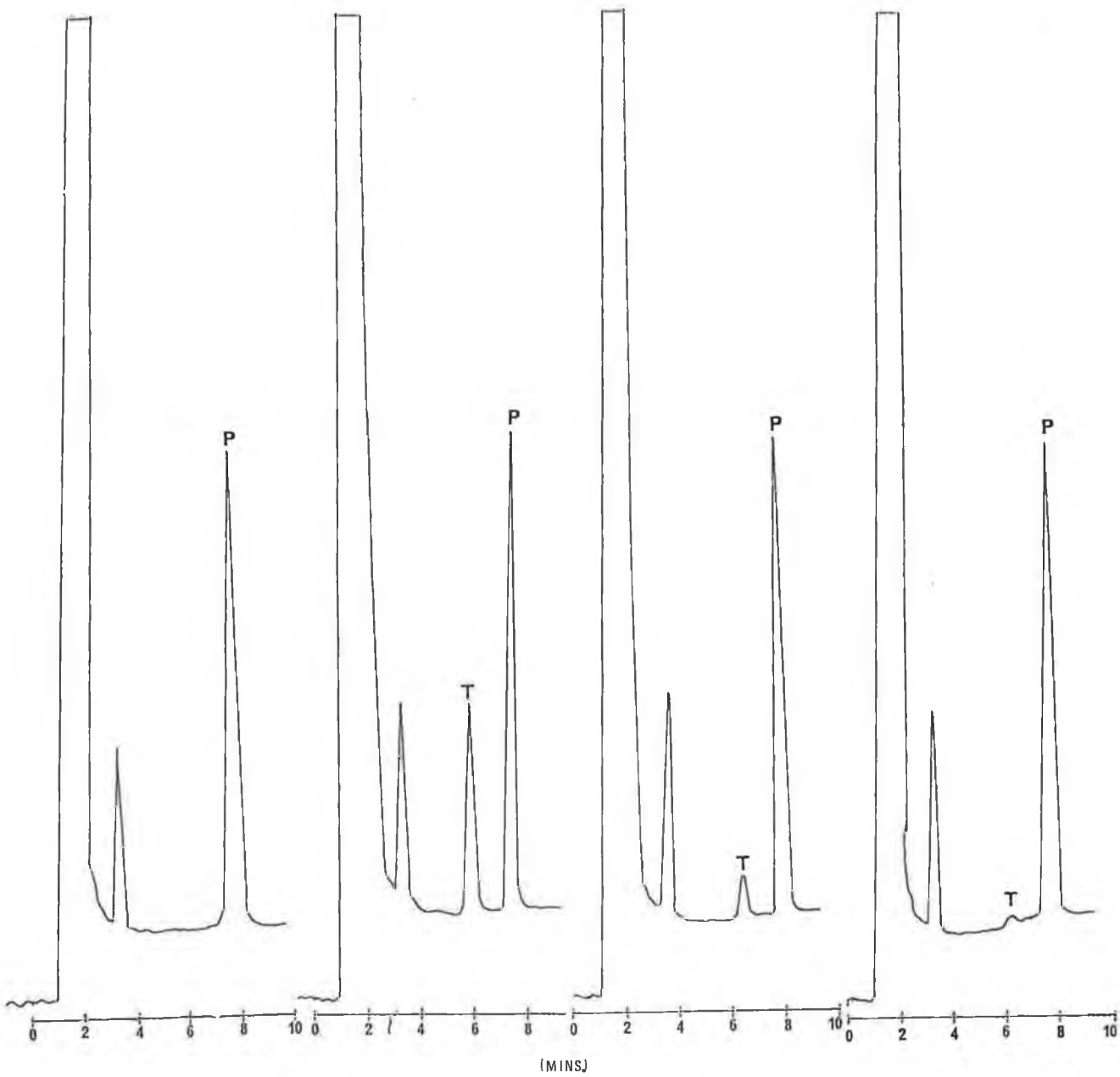


Fig. 5: Chromatograms from cow 1 showing levels of tripelennamine in plasma (a) pre-dose, (b) 30 mins. after dosage, (c) 4 hours after dosage and (d) 24 hours after dosage. Each cow received a 15mg/kilo intravenous dose of a complex containing tripelennamine.

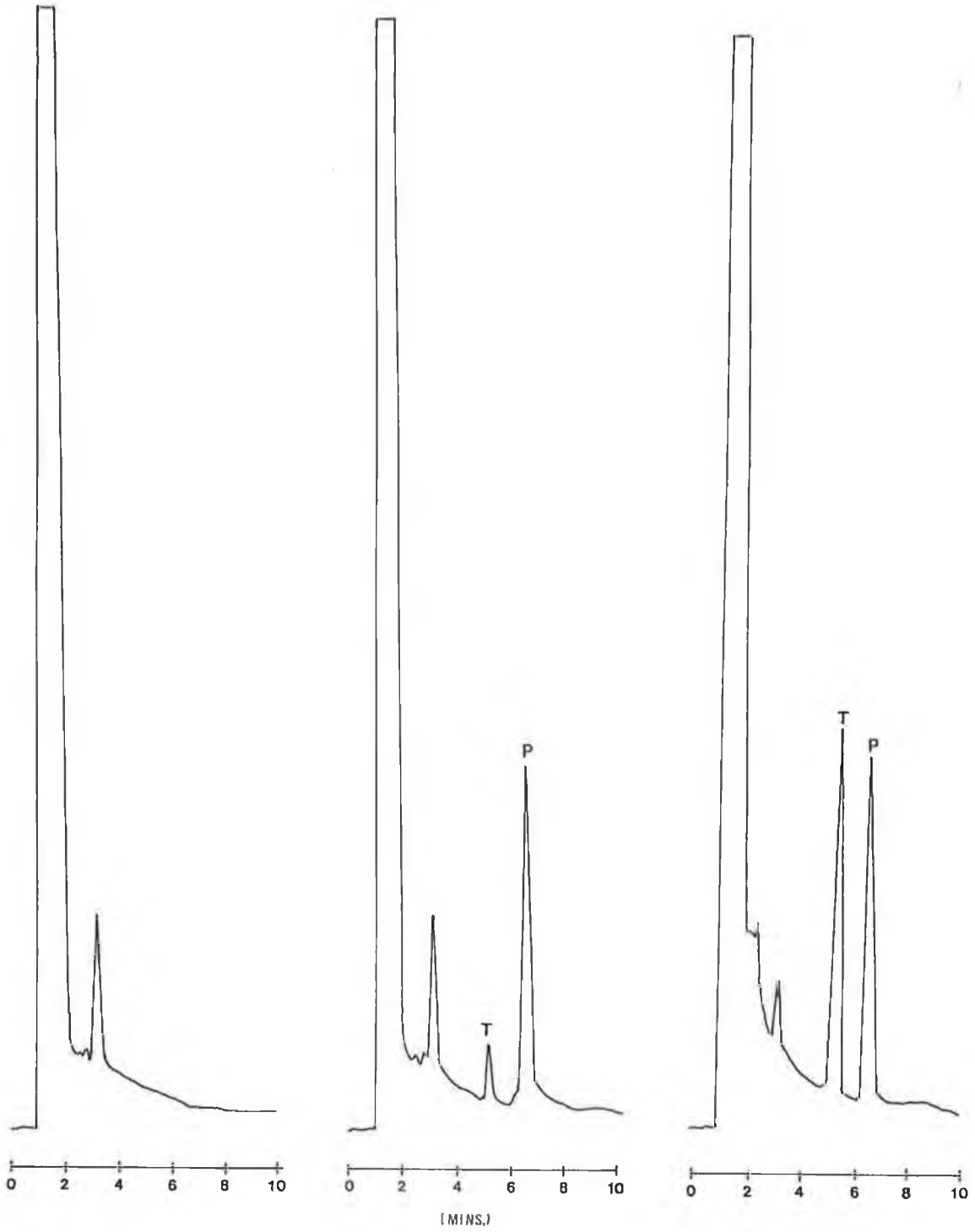
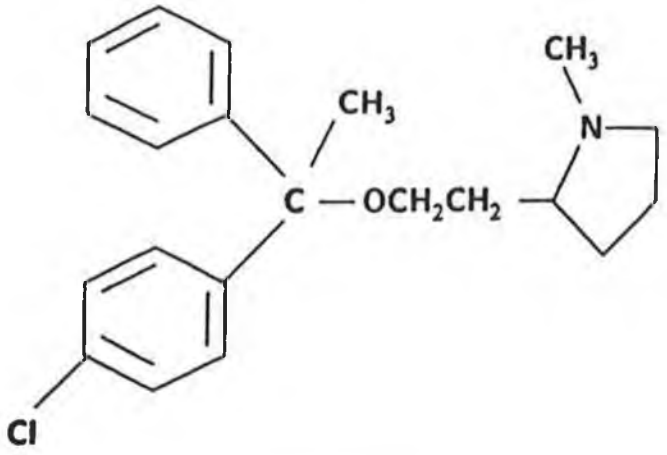


Fig. 6: Chromatograms of (a) Bovine drug free milk, (b) drug free milk spiked with the internal standard and 5ng/ml tripelennamine and (c) milk of a cow 30 mins. after receiving an intravenous injection of a formula containing tripelennamine.

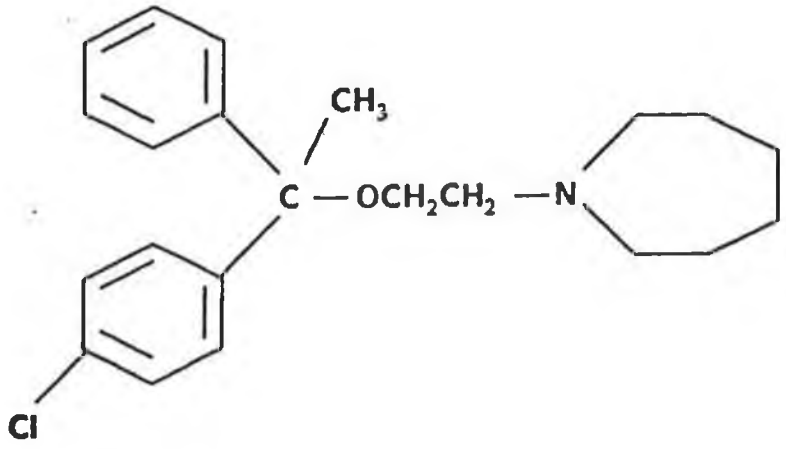
High Performance Liquid Chromatographic Determination of Setistine in plasma using a Column Switching Technique.

5.3.1. INTRODUCTION TO SETISTINE

Setistine (fig 7) is an antihistamine, developed recently in Eastern Europe. No literature is available at present. It bears structural similarity to clemestine (fig 7) which is used as the internal standard.



CLEMASTINE



SETISTINE

Fig. 7: Chemical Structures of Setistine and the Internal Standard Clemastine.

5.3.2. EXPERIMENTAL

5.3.2.1. Reagents and Solvents

Setistine and clemastine were obtained from The Institute of Clinical Pharmacology, Dublin. Methanol and acetonitrile (HPLC grade) were purchased from Fisons, Loughborough, England and sodium acetate (AnalaR grade) was purchased from BDH Chemicals Ltd., Poole, England. Pure water was obtained by the Milli Q water purification system. Drug free human plasma was obtained from the Blood Transfusion Board, Dublin and stored at -4^{0}C.

5.3.2.2. Preparation of Standards

Setistine (10mg) was weighed and dissolved in methanol to yield a stock solution of 100 ug/ml. This stock solution was then diluted with methanol to yield working standards in the range 0.1 to 1 ug/ml. Clemastine (10mg) was weighed and dissolved in 100 ml of methanol to yield a stock solution of 100 ug/ml. This was then diluted with methanol water (1:1) to obtain a working internal standard solution of 2 ug/ml. Spiked plasma samples ranging in concentration from 5 to 50 ng/ml of the drug in plasma were prepared in each assay day by spiking 1 ml of plasma with 50 ul of the working standards (0.1 to 1ug/ml) and 50 ul of internal standard (2ug/ml) solution. Samples from patients undergoing treatment were spiked with 50 ul of a methanol - water solution (1:1) to compensate for any change in the samples plus 50 ul of internal standard (2ug/ml).

5.3.2.3. Instrumentation and Chromatography

The HPLC system consisted of two Waters Assoc. P-75 liquid chromatograph solvent delivery systems equipped with a Waters U6K manual injector and a Rheodyne 7000 six port switching valve. A Shimadzu SPD-6A variable - wavelength UV detector was used and the chromatograms were recorded on a Philips PM 8251 single pen recorder. A Perkin-Elmer fluorescence spectrometer with flow cell was used. Under these chromatrogaphic conditions the mean retention times were 5.9 min and 7.0 min for setistine and clemastine respectively. The instrumental arrangement is as described in section 4.2.1.5. The addition the fluorescence detector in line with the UV detector was used.

The chromatographic conditions for the detection were as follows: mobile phase pump A, water with 1% acetonitrile, concentration column (10 x 1.5 mm) dry packed in house with Corasil RP 18, (37-50 μm) packing. The mobile phase from pump A was passed through the concentration column for 90 seconds (wash time) at a flow rate of 1 ml/min. Mobile phase pump B, acetonitrile -0.05M acetate buffer adjusted to PH 6.5 with acetic acid (60:40 v/v). Analytical column: Techsphere 3CN (10cm x 4.6mm) flow rate: 0.8 ml/min; detection wavelength: 225nm; injection volume: 300 ul of plasma. Under the described chromatographic conditions the mean retention times were 5.9 min and 7.0 min for setistine and clemastine respectively (fig 8).

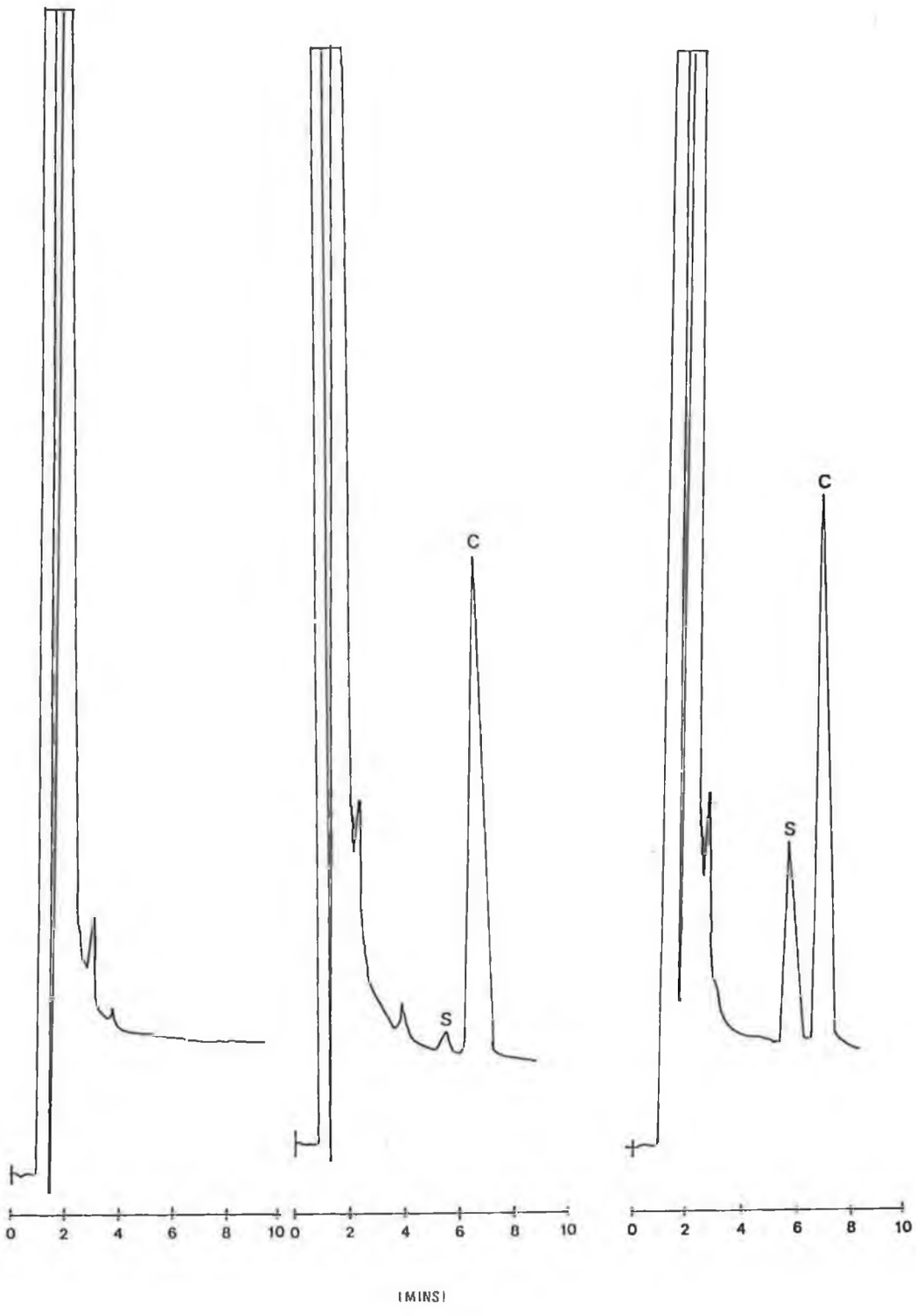


Fig. 8: Chromatograms showing (a) drug free human plasma (b) drug free plasma spiked with 5ng/ml of setistine and the internal standard and (c) drug free plasma spiked with 25ng/ml of setistine and the internal standard.

5.3.2.4. Column Switching Procedure

The column switching procedure is described in section 4.2.1.5. The injection volumn was increased to 300 µl in an attempt to lower the limit of detection.

5.3.2.5. Off-line Solid-Phase Extraction using Sep-pak

A Sep-pak cartridge was placed above each tube. Each cartridge was conditioned using 2 volumes of methanol followed by 2 volumes of deionized water. 1 ml of each plasma standard was then added to the cartridge and eluted using different solvents as outlines in Table VIII. Each was eluted with 3 x 2 ml portions of the relevant solvent and the extracts then combined and evaporated to dryness. The residue was then reconstututed in the mobile phase prior to injection. The recoveries are shown in table VIII. Other cartridges were also investigated but C_{18} gave the best recovery among the packings tried.

If any residual silanol groups were present on the packing these could irreversibly bind the drug. To prevent this happening (a) diethylamine and (b) octylamine were added to the 1st portion of methanol but this did not improve recovery. Recovery may be improved by the addition of 0.1% acetic acid, but this results in dirty chromatograms.

TABLE VIII

Results for recovery using a C_{18} cartridge.

ELUENT	% RECOVERY
Methanol	46.0
Acetonitrile	42.0
Methanol/Chloroform	22.9
Methanol/Water	6.4
Diethylamine in Methanol	40.3
Octylamine in Methanol	36.3
Addition of Acetic Acid to Methanol	75.4

5.3.2.6. Calibration and Calculation

Evaluation of the assay using the column-switching procedure was carried out using four-point calibration standards in the concentration range 5 to 50 ng/ml of the drug in plasma. The slope and the intercept of the calibration curves were obtained by linear regression of the peak height ratios of drug/internal standard versus the concentration of the drug (internal standard method).

The internal standard (I.S.) used was clemastine a drug which bears structural resemblance to setistine and which has a suitable retention time (t_r = 7.0 min). These calibration curves were then used to interpolate the concentrations of drug in patient plasma from the measured peak - height ratios.

5.3.3. DISCUSSION

5.3.3.1. Off-line Solid Phase Extraction

The Sep-pak column extraction procedure has several steps that can cause variability. Firstly, it is essential that the columns be activated thoroughly with 100% methanol, a two-column volume wash will achieve this. The columns then need to have all traces of methanol removed by using another 2 column volume wash of distilled, deionized water. The plasma standard is then loaded onto the column and proteins removed by washing the column with water. A wash with methanol-water (50:50) is then included. Finally using 0.1% acetic acid methanolic solution the drugs are then eluted from the column in 3 x 2 ml portions.

The rigid silica substrate enables the user to apply either positive or negative pressure to draw the sample rapidly through the cartridge without damaging the material.

5.3.3.1.1. Protein Binding Study

The peak heights obtained for a series of authentic standards were compared with the peak heights obtained for a series of plasma standards in the range of 5 - 50 ng/ml. Similar recoveries for both indicated that the loss in recovery of the drug from the Sep-pak is not due to protein binding.

5.3.3.1.2. Cartridge Selection

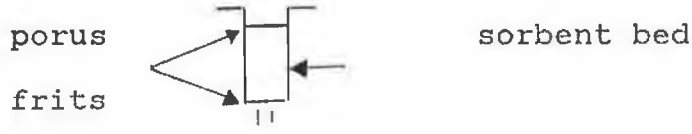
Sep-pak cartridges containing C_8 and CN packing were also investigated with a new to improving recovery but C_{18} proved to be the best among the packings tried. The use of Bond-Elut cartridges did not improve recovery.

5.3.3.1.3. Detector Settings

Using this off-line solid phase extraction procedure the lowest detector setting which could be achieved was .02 AUFS because of the co-extraction of interferents from the cartridge. This proved to be a severe limitation in the case of the present study since a limit of quantitation of 5 ng/ml was desired and this requires a setting of .005 AUFS.

The amount of interfering peaks in the chromatogram may be due to the dimension of the sep-pak cartridge since we also recorded similar types of chromatograms with larger size concentration columns used in column switching procedures in our laboratory. Smaller size commercial cartridges are now available. A sample chromatogram is shown in figure 9.

Typical Non-polar Extraction Procedure



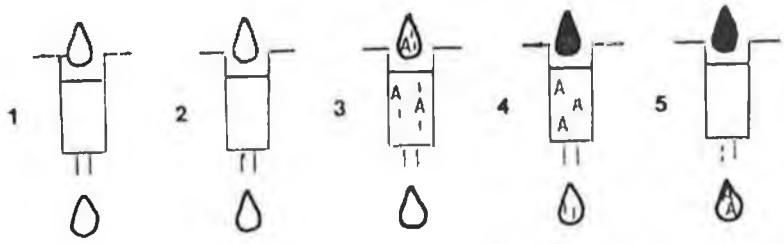


Fig. 10.

1. Activation of Sorbent
2. Removal of activation solvent
3. Application of sample
4. Removal of interferences (I)
5. Elution of the concentrated, purified drug (D)

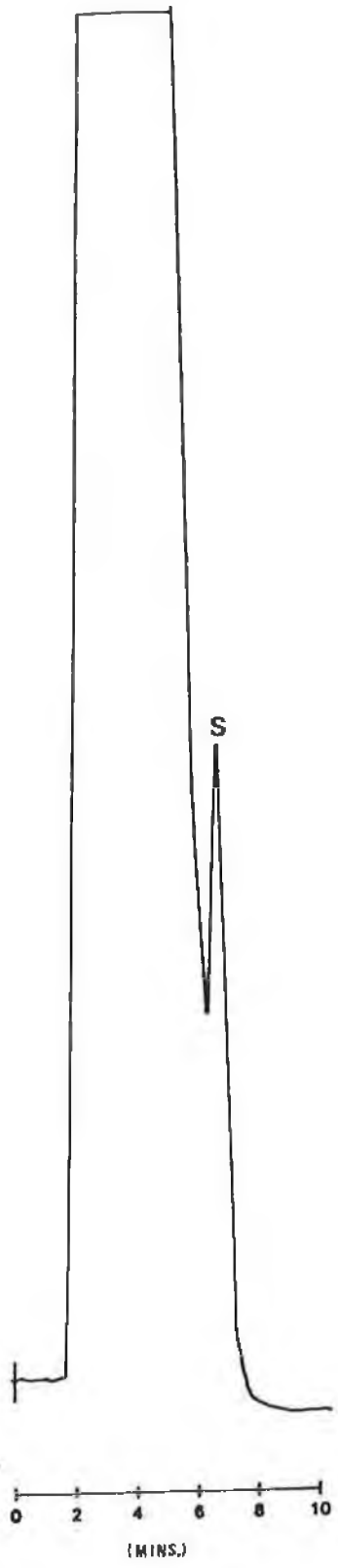


Fig. 9: Chromatogram obtained for a drug free human plasma spiked with 100ng/ml of setistine using a Bond-elut cartridge for sample clean up.

5.3.3.2. Column switching

5.3.3.2.1. Concentration column packings

The following packings were investigated in the concentration column, Vydak cyano (30 μm), Corasil RP C_{18} (37-50 μm) and Separalyte C_8 (40 μm). Smaller size packings were not investigated. With regard to recovery of setistine and cleanliness of chromatograms C_{18} proved the best among the packings tried. An injection volumn of 300 μl of plasma was used and this allowed thirty consecutive injections before changing the concentration column.

5.3.3.2.3. Wash time and Flow-rates

The sharpest peaks and cleanest chromatograms were obtained for a wash period of 90 seconds at 1 ml/min.

5.3.3.2.3. Limit of Detection

Using a 300 μl direct injection of plasma and under the procedural conditions outlined, the limit of detection for setistine in plasma was 5 ng/ml. Using the off-line extraction procedures and for a 75 μl injection from a reconstituted volume of 100 μl, the limit of detection was 500 ng/ml because of the limitation in detector setting outlined above.

The assay was then validated for the column switching procedure since this proved to be superior in terms of selectivity, sensitivity, precision and reproducibility.

5.3.3.2.4. Precision

Intra-assay variability was determined at four concentrations and in quadruplicate at levels of 5, 10, 25 and 50 ng/ml of the drug in plasma. Inter-assay variability was determined singly and at the same four concentrations infour replicate runs. The precision of the method (mean coefficient of variation) for the values of the recovered determinate standards when calculated as unknowns against the linear regression lines for intra-assay and inter-assay were 2.4% and 1.2% respectively (tables IX, X and XI).

5.3.3.2.5. Linearity

A measure of linearity as defined by the correlation coefficient of the regression line for the intra-assay was better than 0.999 and the intercept did not differ greatly from the origin.

5.3.3.2.6. Recovery

The overall recovery was calculated in two different ways. First by comparing the peak heights of a series of spiked plasma samples after they had been taken through the entire procedure with a series of reference standards. Secondly, by comparing the slopes of the regression lines obtained from the two sets used in the first procedure. Using these methods in the concentration range 5-50 ng/ml, the mean overall recovery was 86.5% (Table XII).

TABLE IX

INTRA-ASSAY - PLASMA

Concentration added (ng/ml)	Peak HT (cm)		Peak RT S/C	Peak HT (cm)		Peak RT S/C	Peak HT (cm)		Peak RT S/C	Peak HT (cm)		Peak RT S/C	Mean Ratio S/C
	S	C		S	C		S	C		S	C		
0	-	-		-	-		-	-		-	-		
5	0.4	8.5	0.0471	0.4	8.6	0.0465	0.4	8.8	0.0455	0.3	6.6	0.0455	0.0462
10	0.7	8.2	0.0854	0.7	8.2	0.0854	0.8	8.8	0.0909	0.6	7.1	0.0845	0.0866
25	2.3	9.7	0.2371	1.9	8.4	0.2262	2.0	8.9	0.2247	1.8	7.7	0.2338	0.2305
50	3.2	7.1	0.4507	3.1	6.9	0.4493	3.4	7.5	0.4533	3.3	7.6	0.4342	0.4469

Y = 0.00895 + .00096 r = .9997

Concentration added (ng/ml)	Concentration found (ng/ml)				Mean Concentration found (ng/ml)	Standard Deviation	Coefficient Variation %
	1	2	3	4			
0	-	-	-	-			
5	5.15	5.08	4.97	4.97	5.05	0.09	1.78
10	9.42	9.42	10.03	9.32	9.55	0.32	3.35
25	26.36	25.14	24.97	25.99	25.62	0.67	2.62
50	50.20	50.04	50.49	48.36	49.77	0.96	1.93

Mean % C.V. = 2.4%

TABLE x

INTER-ASSAY - PLASMA

SETISTINE

Concentration added (ng/ml)	DAY 1			DAY 2			DAY 3			DAY 4		
	Peak HT (cm)		Peak RT $\frac{S}{C}$	Peak HT (cm)		Peak RT $\frac{S}{C}$	Peak HT (cm)		Peak RT $\frac{S}{C}$	Peak HT (cm)		Peak RT $\frac{S}{C}$
	S	C		S	C		S	C		S	C	
0	-	-		-	-		-	-		-	-	
5	0.4	9.5	0.0421	0.6	12.8	0.0469	0.3	8.2	0.0366	0.3	7.5	0.0400
10	0.7	8.8	0.0795	1.0	11.8	0.0847	0.6	7.7	0.0779	0.7	8.3	0.0843
25	1.6	8.0	0.2000	2.5	12.5	0.2000	1.6	8.5	0.1882	1.6	7.5	0.2133
50	3.7	9.7	0.3814	4.7	12.2	0.3852	2.7	7.2	0.3750	3.4	8.0	0.4250

Concentration added (ng/ml)	Concentration found (ng/ml)				Mean Concentration found (ng/ml)	Standard Deviation	Coefficient Variation %
	1	2	3	4			
0	-	-	-	-		-	
5	4.82	4.90	4.75	4.87	4.84	0.07	1.45
10	9.76	9.94	10.27	10.05	10.01	0.21	2.10
25	25.70	25.27	25.01	25.15	25.28	0.30	1.19
50	49.71	49.88	49.96	49.92	49.86	0.11	0.22

Mean % C.V. = 1.2%

TABLE XI

PRECISION (REPEATABILITY AND REPRODUCIBILITY)

Concentration added (ng/ml)	Mean (n=4) concentration found ± S.D. (ng/ml)	C.V. %	Difference between added and found concentration %
INTRA-ASSAY (REPEATABILITY)			
0			
5	5.05 ± 0.09	1.8	1.0
10	9.55 ± 0.33	3.4	4.5
25	25.62 ± 0.67	2.6	2.5
50	49.77 ± 0.96	1.9	0.5
	Mean % C.V. = 2.4%		
INTER-ASSAY (REPRODUCIBILITY			
0			
5	4.84 ± 0.07	1.5	3.2
10	10.01 ± 0.21	2.1	0.1
25	25.28 ± 0.30	1.2	1.12
50	49.86 ± 0.11	0.2	0.28
	Mean % C.V. = 1.2%		

TABLE XII

RECOVERY FROM PLASMA

METHOD I

Concentration (ng/ml)	Peak Heights (cm) (mean of duplicates)		Recovery (%)
	Set A Working Standards	Set B Standards Extracted from plasma	
0	-	-	
5	0.4	0.38	93.75
10	0.75	0.70	93.33
25	1.8	2.00	90.00
50	3.9	3.25	83.33

Mean overall recovery = 90.10%

METHOD II

Regression line for set A : $y = .0779X - 0.0393$

Regression line for set B : $y = .0645X + 0.1291$

Overall recovery = $\% = \frac{0.0645}{0.0779} \times 100 = 82.80\%$

Mean recovery determined by two methods = 86.5%.

TABLE XIII
RESULTS OF SETISTINE ANALYSIS

Comparison of on-line and off-line solid phase extraction

	ON-LINE	OFF-LINE BOND - ELUT
LIMIT OF DETECTION ng/ml	5	500
INTRA-ASSAY PRECISION MEAN % C.V.	2.5	NOT VALIDATED
INTER-ASSAY PRECISION MEAN % C.V.	2.2	
% DIFFERENCE BETWEEN ADDED & FOUND (intra-assay)	0.12 - 4.5	
% DIFFERENCE BETWEEN ADDED & FOUND (inter-assay)	0.1 - 1.9	
RECOVERY	87.7%	46.0%

5.3.3. CONCLUSION

When the described method was applied to plasma from patients receiving an 8 mg oral dose of the drug. However, it was found that the highest level in plasma was 5 ng/ml after 1 hour, the other amounts were not quantifiable so in this regard the method as it stands at present is not adequate for bioavailability studies of setistine. A more sensitive dectection system is required.

Fluorescence detection was investigated but did not lower the detection limit. The limit of detection using the fluorescence detector was 20 ng/ml.

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HPLC DETERMINATION OF DIAZEPAM AND ITS METABOLITES IN PLASMA.

A COMPARISON OF DIRECT/INJECTION COLUMN SWITCHING WITH CONVENTIONAL LIQUID-LIQUID EXTRACTION

CHAPTER 6.

6.1. INTRODUCTION TO BENZODIAZEPINES

Benzodiazepine derivatives are widely used as tranquillisers, hypnotics, anticonvulsants, muscle relaxants and in the treatment of acute alcohol withdrawal [1]. Used singly they appear to be relatively safe but care must be exercised when they are taken in conjunction with other drugs [2]. They are clinically effective at low doses ranging from 1 to 25mg and this results in blood concentrations in the range 10 - 500 ng/ml. Metabolism of the drugs is extensive and many of the metabolites are pharmacologically active [3-6]. Thus it is essential that any assay method be both sensitive and specific and capable of monitoring the parent drug and the metabolites. Methods for the analysis of these drugs have been extensively reviewed [6-10].

The 1-4 benzodiazepines can be structurally generalised by the formula shown in fig. 1 where substitution with different groups leads to different benzodiazepines. All the members of importance contain a 5 phenyl ring, a halogen or nitro group at position 7 and in some cases an additional halogen in the R_4 position. These compounds with a substituent at position 7 on ring A are highly pharmacologically active. Substitution with electron withdrawing groups increases this activity but substitution with electron donating groups reduces it. Substitution with a methyl group at position 1 (in ring B) generally increases the activity while side chains or bulky groups at the same position have the opposite effect. Any substitution in the para position in ring C causes a complete disappearence of biological activity [11].

The structure of chlordiazepoxide differs from other benzodiazepines in that it is an N - oxide with a methyl amino group in position 2 and no alkyl group at position 1 (Fig. 2).

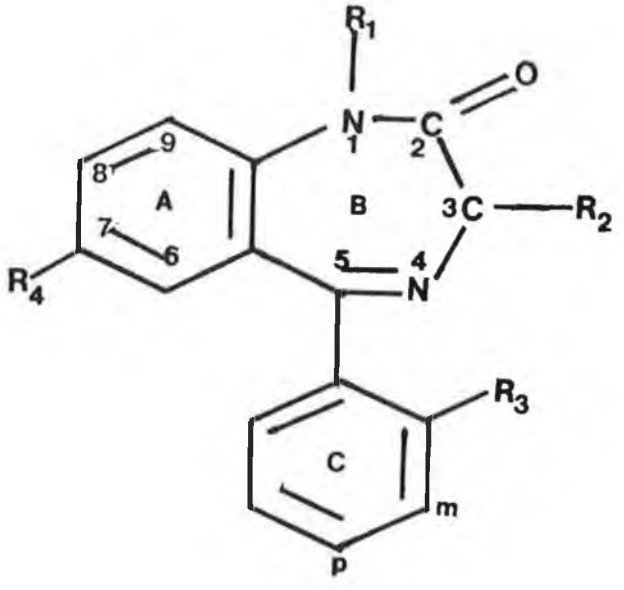


Fig 1

Benzodiazepine Structure

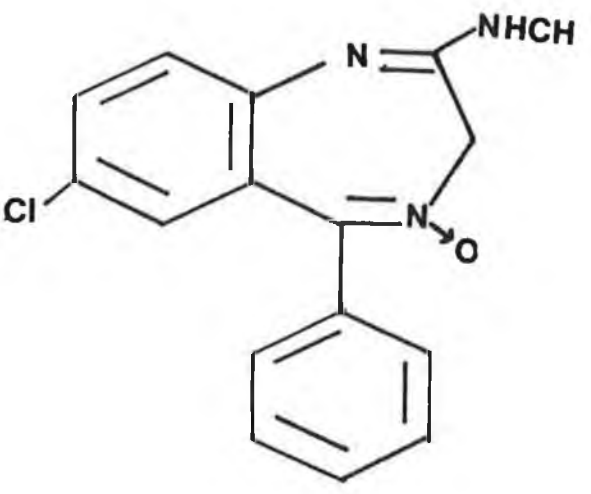


Fig 2

Chlordiazepoxide Structure

Benzodiazepine	R_1	R_2	R_3	R_4
Diazepam	CH_3	H	H	Cl
Desmethyl diazepam	H	H	H	Cl
Temazepam	CH_3	OH	H	Cl
Oxazepam	H	OH	H	Cl
Nitrazepam	H	H	H	NO_2
Flurazepam	$-(CH_2)-NEt_2$	H	F	Cl
Lorazepam	H	OH	Cl	Cl

Benzodiazepines are basic drugs and in the free form are lipid soluble and water insoluble, the salts of the drugs, however, are water soluble.

Because the benzodiazepines are such a widely prescribed group of drugs many years of work and research has gone into the development of sensitive and specific assays for their determination. Results of benzodiazepine assays can be used to evaluate and care for a toxic patient (clinical toxicology), to check on a patients drug compliance with the prescribed regimen (compliance) and to adjust the drug dosage or schedule to achieve maximum therapeutic benefit (pharmacokinetics). For clinical toxicological testing, a rapid method that measures total active drug is very useful. The precision of the method is less important in this case and a between assay CV of 10 to 15% can be tolerated. For compliance testing, measurement of a single representative compound, either parent drug or metabolite suffices. However the precision required for perodic monitoring of a patient for compliance in somewhat greater than for clinical toxicological studies. A between-day assay C.V. of 5 to 10% would be acceptable for this purpose [12].

Pharmacokinetic studies are the most demanding in terms of specificity and precision. Because each bioactive metabolite may have a different distribution and excretion pattern and biological half-life, each one should be measured individually. Furthermore the monitoring of changes resulting from dosage adjustments requires a between assay CV of 5% although a CV as large as 10% can often be tolerated. [12]

Many different analytical methods have been reported for the determination of benzodiazepines in pharmaceutical preparations, serum and other body fluids. These include spectrophotometry [13-15], colorimetry [16], TLC [17-19], radioimmunoassay [20,21], with polarography [22-27], GLC [6, 11, 28-46] and HPLC [47-61] being the most widely applied. Spectrophotometry is not specific and only gives information about the overall concentration of a drug and its metabolites, and radioimmunoassay is limited not only by the low linear range available but also by the cross reaction of diazepam with antibodies specific for desmethyl diazepam and by the time (24 hrs) needed to complete the assay [20].

Polarography requires large amounts of serum and lacks the selectivity required for metabolic studies. The polarographic reduction waves of the 1-4 benzodiazepines, corresponding to the C=N group have been used mostly for trace analysis[62]. Metabolism of the benzodiazepines leads to structural changes distinct from this group and hardly affects its electron density. Therefore electroanalytical methods based on these reduction waves possess little selectivity [63]. Nitrazepam, clonazepam and flunitrazepam structures do contain a readily reducible NO_2 group which can be modified to electroinactive - NH_2 and -N-C-CH_3 groups on metabolism. Some improvement in selectivity can be obtained by introducing a solvent extraction step prior to differential pulse polarographic

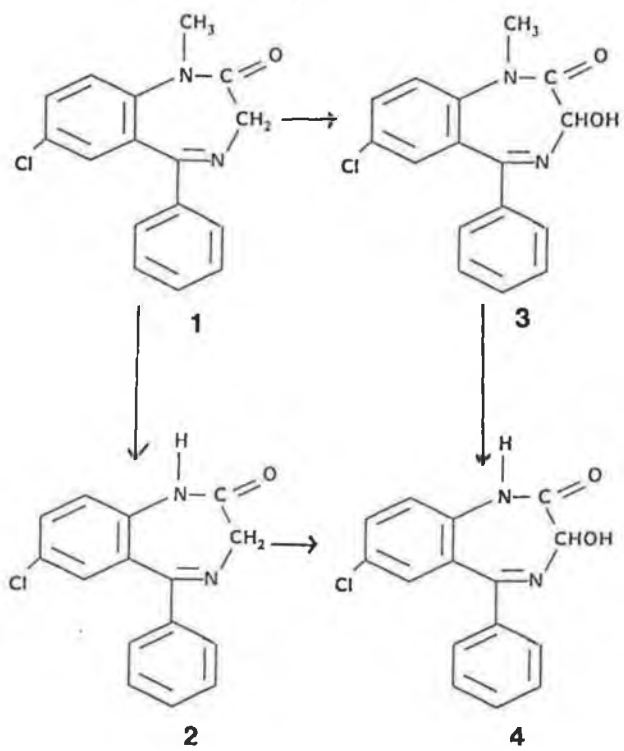
analysis. A recent publication [64] describes solvent extraction for flurazepam and its metabolites but the method was found to be somewhat time-consuming.

On-line electrochemical detection of 1,4 benzodiazepines has been investigated in both oxidative [69] and reductive modes, the latter being used in conjunction with reversed phase HPLC for the separation and determination of nanogram amounts of nitrazepam, diazepam and chlordiazepoxide in mixtures [66]. Such on-line methods, when linked with HPLC, result in optimization of the selectivity and time of analysis of the overall analytical method. Additional selectivity over U.V. detection using electrochemical detection in either mode can be obtained by control of the detector potential.

A very widely applied technique for benzodiazepine analysis is gas - chromatography. Many of the earlier GC methods depended on the acid hydrolysis of the benzodiazepines to their corresponding benzophenones [7] followed by gas-liquid or thin layer chromatography. Unfortunately hydrolysis of several of the benzodiazepines results in the formation of the same benzophenone [6,67] so that the individual parent drugs cannot be identified. More lately the technique most extensively used has been GLC with electron captive detection. De Silva et al. [11] have described a comprehensive extraction scheme using GLC - ECD for the determination of the compounds in blood. However the procedure has major disadvantages such as lengthy purification steps and the necessity for relatively large sample volumes (up to 2ml). Rutherford [40] has described a

rapid micro method using GLC - ECD for the determination of diazepam and its metabolites in plasma. The method involves a direct solvent extraction followed by GLC. A similar procedure is also reported [68] and extended to include the

Fig. 3 Metabolic Pathway for Diazepam



1 Diazepam (7 - chloro - 1,3 dihydro - 1 - methyl - 5 phenyl - 2H - 1,4 benzodiazepine - 2 - one)

2 Desmethyl diazepam (7 - chloro - 1,3 dihydro - 5 - phenyl - 2H - 1,4 - benzodiazepine - 2 - one)

3 Temazepam (7 - chloro - 1,3 dihydro - 3 hydroxy - 1 methyl - 5 phenyl 2H - 1, 4 - benzodiazepine - 2 - one)

4 Oxazepam (7 - chloro - 1,3 dihydro - 3 hydroxy - 5 phenyl - 2H, - 1,4 benzodiazepine - 2 - one)

quantitative determination of flurazepam and its major metabolite N-desalkylflurazepam.

With regard to the analysis of diazepam and its metabolites (Fig 3) earlier methods pointed to GC - ECD as the method of choice [29, 30, 31, 39, 40] for its determination and that of its main metabolites, oxazepam, temazepam and desmethyldiazepam. Although GC - ECD is inherently more sensitive than HPLC analysis with U.V. detection for the analysis of diazepam and desmethyldiazepam per se, the analysis of temazepam and oxazepam requires silylation to the trimethylsilyl ether derivative not only for enhanced sensitivity and chromatographic behaviour but also to ensure thermal stability, especially of oxazepam. The procedural details required make the simultaneous analysis of all four metabolites by EC - GLC [28] a difficult process.

Due to convenience and ease of operation HPLC has now become the method of choice for the analysis of the benzodiazepines. Use of electrochemical detection or U.V. absorbance in the 230 - 260 nm range gives sensitivity in the nanogram range.

Most of the current HPLC methods for benzodiazepines use reverse phase columns with U.V. detection and mobile phases consisting of organic solvents mixed with water or weakly acidic buffers. [9]. Using reverse phase chromatography drugs of varying polarity can be separated and the columns require little maintenance. The most popular column packing material for benzodiazepine analysis is chemically

bonded silica containing octadecyl (C_{18}), or octyl (C_8)groups, although hexyl (C_6) and cyano - bonded R.P. columns as well as ordinary silica have also been used. Radial compression columns have been used for the determination of diazepam and its metabolites [69], these columns afford short analysis time and can be operated at lower pressures. The most common solvent system used as mobile phase is a mixture of acetonitrile or methanol and an aqueous acetate or phosphate buffer or water [69]. Combinations of two organic solvents and an aqueous buffer have also been employed to achieve optimum conditions for the separation of closely related solutes such as diazepam [53, 69, 70], chlordiazepoxide [70, 71] and their metabolites.

Before analysis, however, the benzodiazepines must be extracted from the biological medium in which they occur. They are usually extracted as neutral molecules with a range of organic solvents under weakly alkaline conditions [7]. Some workers have found it unnecessary to alkalize samples since the pK values for the benzodiazepines are considerably below the physiological pH. A wide range of solvents have been used, some workers choose diethyl ether, ethyl acetate and chloroform in that order [7] while others prefer a relatively non-polar solvent such as methylene chloride. One advantage of the low boiling point solvents is that they can be readily evaporated for recovery of the drugs. A single extraction step is sufficient for all benzodiazepines except oxazepam and lorazepam. Owing to their lower lipid solubility they need a double extraction. [72]

Solid phase extraction techniques using disposible C_{18} bonded phase columns (Bond - Elut) have been applied to the isolation of the benzodiazepines from biological fluids, specifically to clonazepam [59], temazepam [60] and in particular to the determination of diazepam and its metabolites [56]. These columns selectively retain benzodiazepines and their metabolites from biofluids at a pH of 9.0. The compounds are then eluted with methanol, making sample preparation more convenient than the traditional solvent extraction procedure. These methods give good sensitivity, selectivity and recoveries, allow a smaller volume of plasma to be used but are expensive.

More recently Koenigbauer et al. have developed a method for trace analysis of diazepam using on-line preconcentration [61]. However, the method requires ultracentrifugation of plasma prior to injection and does not simultaneously assay all three metabolites.

6.2 Introduction to a column switching/direct injection method for determination of diazepam and its metabolites

The following work describes an on-line solid phase extraction scheme for the determination of diazepam and its metabolites. It uses a switching valve to allow direct injection of plasma onto a concentration column where the drugs are enriched and held. They are then eluted in backflush mode onto the analytical column for separation and quantitation. This method is compared to a conventional extraction procedure in terms of speed of analysis, precision, accuracy, sensitivity and specificity.

6.2.1. EXPERIMENTAL

6.2.1.1. Reagents

Diazepam, temazepam, oxazepam, desmethyl-diazepam, nitrazepam and salicylic acid were obtained as a gift from the Institute of Clinical Pharmacology, Dublin. Methanol, acetonitrile, diethyl ether (all HPLC grade) were obtained from Lab-Scan Ireland Ltd. Chloroform and sodium hydrogen phosphate were obtained from Riedel-de -Haen (Hanover, F.R.G.). Sodium carbonate, sodium bicarbonate and sodium hydroxide were obtained from May and Baker (Dagenham U.K.) Ammonia solution and phosphoric acid were purchased from BDH, Poole U.K.

For the preparation of the plasma samples, dried human plasma (from the Blood Transfusion Service Board, Dublin , Ireland) was dissolved in deionised water (obtained by the Milli-Q-water purification system). The control plasma obtained was examined for the presence of endogenous components which might interfere with the benzodiazepines in the assay system. The reconstituted plasma was stored frozen and used within two weeks of preparation.

6.2.1.2. Intrumentation

The HPLC system consisted of two Waters Assoc. (Milford, MA. USA.) P-45 liquid chromatographic systems equipped with a Rheodyne 7125 injector fitted to a Shimadzu (Tokyo, Japan) SPD-6A variable wavelength U.V. detector. The chromatograms

were recorded on a Philips (Eindhoven, The Netherlands) PM 8251 single pen recorder. A Rheodyne (CA ; USA) 7000 six - port switching valve was used.

6.2.1.3. Chromatography

The instrumental arrangement for the chromatography is described in section 4.2.1.5. The chromatographic conditions for the separation were as follows.
Mobile phase pump A: Water; concentration column (10 x 1.5mm) dry packed with Separalyte (Analytichem International) RP C_8 packing (40µm) in our laboratory; wash time, 2.0 min; flow rate: 1ml/min. Mobile phase pump B: methanol - 2mM NaH_2PO_4 buffer (50:50, V/V) adjusted to pH3 with phosphoric acid; stationary phase: Lichrosorb 10 RP8, 5um (HPLC Technology, Macclesfield, U.K.) (25cm x 4.6mm), flow rate: 1.6ml/min, recorder chart speed: 120mm/hr, detection wavelength: 246nm, injection volume: 50µl or 250µl of plasma. Under the described chromatographic conditions the mean retention times were as follows, oxazepam 10.5 min, temazepam 12.5 min, desmethyl - diazepam 15.5 min and diazepam 20.5 min (fig 4).

Preparation of Standards:

Oxazepam (10 mg), temazepam (10 mg), desmethyl diazepam (10 mg) and diazepam (10 mg) were weighed and dissolved in 100 ml of methanol to yield a stock solution of 100 µg/ml of each drug. This stock solution was then diluted with

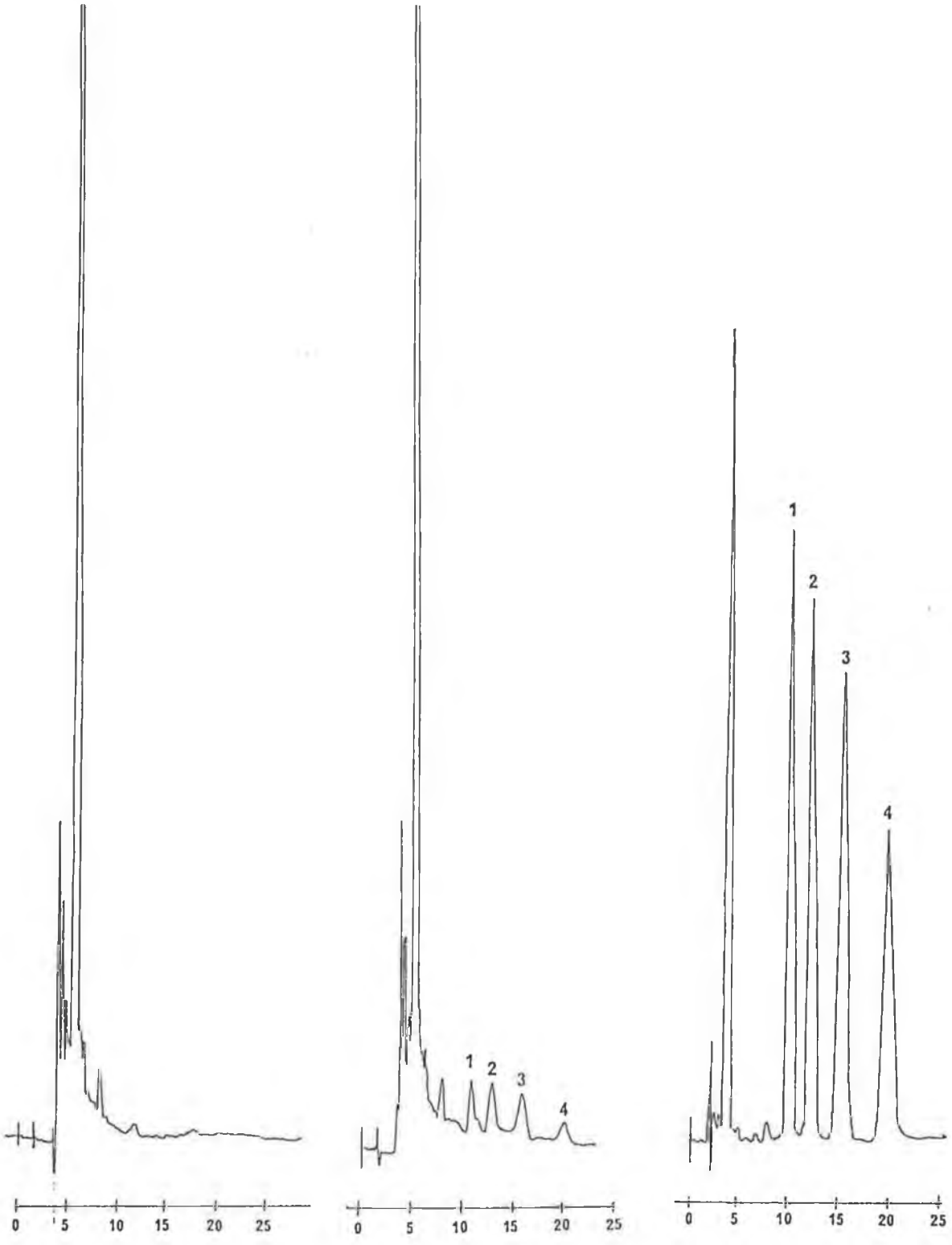


Fig. 4: Chromatograms obtained for (a) human drug free plasma, (b) the same plasma spiked with 25ng/ml of each drug (250µl injection, 0.02 AUFS) and (c) spiked with 250ng/ml of each drug (50µl injection 0.02 AUFS)

1 = oxazepam, 2 = temazepam, 3 = desmethyl-diazepam, 4 = diazepam.

water - methanol (1:1) to yield working standards ranging from 0.5 to 50 µg/ml. Spiked plasma standards ranging from 25 to 2500 ng/ml of the drugs were then prepared in each assay day by spiking 1 ml of plasma with 50 µl of the working standards.

6.2.1.5. Column Switching Procedure

Prior to injection 50 µl of a 4mg/ml solution of salicylic acid is added to the spiked plasma samples (this frees the bound drugs from plasma), and the sample is centrifuged for 5 minutes. It is then injected through the injector port and washed by water from pump A onto the concentration column. The drugs are held on the concentration column while the other components in plasma are eluted to the drain. Meanwhile the eluent from pump B is passing through the analytical column and on to waste. On switching the valve the eluent from pump B elutes the drugs - which have been enriched on the concentration column - in backflush mode onto the analytical column where they are separated.

With regard to optimising the analytical procedure in terms of precision, accuracy, reproducability, recovery, sensitivity and selectivity, variations in the following experimental parameters were investigated.

Concentration Column packing - (i) recovery
- (ii) protein binding study.

(a) Concentration Column packing - (i) recovery
- (ii) protein binding study.

(b) Sample pre-treatment prior to injection

(c) Concentration column size

(d) Sample injection volume

(e) Wash time and flow rate

(f) Analytical column

Concentration Column Packing

The concentration column packing should be chosen in such a way that the drugs are completely retained while interfering substances from plasma are removed. The following packings were tested in the concentration column, CN and DIOL from a Sep-pak (50-100μm), Lichroprep CN (40μm), u Bondapak C_{18} (40μm) and Separalyte C_8 (40μm). Smaller size packings were not investigated as from a previous study it had been found that they did not allow direct injection of plasma [73]. The recovery of the four drugs i.e. diazepam and its three metabolites from each of the packings was calculated by comparing the peak heights of a standard injected directly onto the analytical column with the peak heights obtained for the recovered authentic standards using the valve (Table 1).

The only packing which gave good recovery of the most polar metabolite i.e. oxazepam was the C_8 packing. This was then chosen for the assay. Other packings gave better results in terms of cleanliness of chromatograms for blank plasma but C_8 gave adequate sample clean up.

TABLE 1

% Recovery of the Authentic Standards from the Concentration Column

	Peak Heights (cm)			
	C_{18}	C_8	CN	Diol
Oxazepam	$\frac{6.2}{11.1}$ = 55.8%	$\frac{11.0}{11.1}$ = 99%	$\frac{0.6}{11.1}$ = 5.4%	$\frac{0.6}{11.1}$ = 5.4%
Temazepam	$\frac{7.4}{8.1}$ = 91.3%	$\frac{8.0}{8.1}$ = 98.7%	$\frac{0.2}{8.1}$ = 2.5%	$\frac{0.3}{8.1}$ = 3.7%
Desmethyl Diazepam	$\frac{12.7}{13.2}$ = 96.2%	$\frac{14.0}{13.2}$ = 106%	$\frac{1.6}{13.2}$ = 7.5%	$\frac{1.4}{13.2}$ = 10.6%
Diazepam	$\frac{9.3}{9.5}$ = 97.9%	$\frac{10.4}{9.5}$ = 109%	$\frac{1.6}{9.5}$ = 16.8%	$\frac{1.0}{9.5}$ = 10.5%

Calculation: $\frac{\text{Peak Height of Recovered Authentic Standard}}{\text{Peak Height of Authentic standard}} \times 100 = \%\ \text{Recovery}$

6.2.1.5.2. Protein Binding Study

Having chosen the concentration column packing, ideally the drug should have a greater affinity for this packing than for the proteins of plasma, otherwise recovery of the drug from plasma will be very poor. In the present study when the peak heights obtained for the recovered authentic standards were compared with the peak heights for the recovered plasma standards from the concentration column it was found that the recovery of diazepam from plasma was only 35% (table II). This is an expected result since it is well known that the benzodiazepines and in particular diazepam are extensively protein bound. Other workers have reported the use of proteolytic enzymes i.e. subtilisin carbergensis [47] or subtilisin A[74] to overcome this problem in tissue and plasma, however, this renders the procedure somewhat elaborate. We searched for a cheaper and more convenient solution to the problem of protein binding.

It was found that the addition of 50µl of a 4mg/ml solution of salicylic acid which is very strongly protein bound frees the diazepam and improves recovery of the drug to 100% but does not interfere with the assay as it is washed off the concentration column bound to plasma. Use of a lower concentration of salicylic acid does not give full recovery or reproducible results (table III).

Table II

Protein binding studies using C_8 packing in the concentrated column.

	Peak Heights (cm)			
Drug	No valve authentic standard	Valve + authentic standard	Valve + plasma standard	Overall % recovery
Oxazepam	6.5	5.9	5.3	81.5
Temazepam	4.5	4.1	3.3	73.3
Desmethyl Diazepam	7.6	6.9	6.4	84.2
Diazepam	5.5	4.8	1.9	34.5

Table III

Use of Salicylic acid to improve recovery of the drugs from plasma.

	Peak Heights in cm			
	I	II	III	IV
Authentic	13.7	9.6	9.7	6.5
No Salicylic acid	10.1	6.6	7.4	2.9
2.0 mg/ml Salicylic acid	10.1	6.6	8.5	4.1
4.0 mg/ml Salicylic acid	11.2	7.6	8.7	4.8
6.0 mg/ml Salicylic acid	11.4	7.6	8.7	4.8

I = Oxazepam
II = Temazepam
III = Desmethyl-Diazepam
IV = Diazepam

6.2.1.5.3. Sample Pretreatment Prior to Analysis

One of the major drawbacks in using a direct injection of plasma is the build up of backpressure due to accumulation of particular matter from plasma and the resultant clogging of frits. Also proteins from plasma may precipitate on the concentration column when they come into contact with the buffer salts and organic solvents from the mobile phase. With a view to overcoming these problems protein precipitation prior to injection using a range of precipitating agents was examined, as outlined by Blanchard et al [75] (Table IV).

Table IV

Table of Precipitating Agents

Precipitant	Volume added per ml of plasma
10% Trichloroacetic acid	0.2 ml
Methanol	1.0 ml
Ethanol	1.0 ml
1:1 10% $ZnSO_4$:0.1M NaOH	0.4 ml
1:1 6% Na_2WO_4:0.7N H_2SO_4	0.6 ml

For each precipitant, the required volume was added to 1ml of plasma and the resulting mixture vortexed for twenty seconds and then allowed to settle for fifteen minutes. This mixture was then centrifuged at 3,500 rpm for fifteen minutes and 250 μl of the supernatant injected onto the concentration column. However protein precipitation did not give good results. Precipitation with acid leads to ionization of the basic benzodiazepines and results in their not being held on the C_8 packing, also addition of the acid gives dirty chromatograms.

The organic solvents proved to be very efficient at deproteinizing the plasma, giving clean chromatogams for blank plasma. However, the organic solvent of the supernatant is a strong eluent i.e. MeOH or EtOH and result in almost complete elution of the drug from the concentration column immediately after injection. Also the large volume of the organic solvent required for efficient deproteinization leads to dilution of the drugs.

Precipitation with base and heavy metal i.e. $ZnSO_4$ and NaOH leads to co-precipitation of the drugs with the plasma proteins. Best recovery, sensitivity and reproducibility were achieved using direct injection of plasma. The problems associated with direct injection i.e. build up of back-pressure etc. can be minimized by using backflush mode.

6.2.1.5.4. Concentration Column Size

The size of the concentration column is also an important experimental consideration in the chromatographic system.

Two different sizes were investigated

(i) 2.5 x 0.4 cm (large)

(ii) 1.1 x 0.15 cm (small)

It was found that a large concentration column allows a larger injection volume without a decrease in the lifetime of the column but a longer wash time is necessary. This is because the plasma has further to travel on the larger column. This leads to band broadening and the greater volume of packing in the larger column retains more interferents from plasma giving dirty chromatograms. The "small" concentration column was chosen since, while giving good recovery of the drugs, less wash time is required for plasma clean-up and it is more economical as less packing is used.

6.2.1.5.5. Sample Injection Volume

For the higher concentration of diazepam and its metabolites in plasma a 50 µl injection of plasma gives an adequate limit of detection. For lower concentrations a larger injection volume is required. A direct injection of 250 µl allows quantitation at the 25 ng/ml level. Larger volume injections are possible but these will significantly lower the lifetime of the concentration column.

6.2.1.5.6. Wash Time and Flow Rate

One of the applications of column switching is sample

clean-up, however, for an efficient sample clean-up the wash phase must be optimized. A good washing solvent should eliminate interferences and larger molecules present in the sample and prevent contamination of the analytical column. Water was found to best meet these requirements. Variations in wash time and flow rate do not affect the chromatography to a large extent. Best results in terms of cleanliness of chromatogram and peak shape were obtained for a 2.0 minute wash time at 1ml/min. These conditions allow adequate sample clean-up with little or no band broadening. Longer wash times do not cause breakthrough of the drugs. Breakthrough volume was estimated to be 20 mls.

6.2.1.5.7. Analytical Columns

Using a range of mobile phases at various pH's, separation of diazepam and its metabolites was attempted using a Nucleosil C_{18} (HPLC Technology), a Lichrosorb C_{18} (HPLC Technology), a Resolve C_{18} (Waters) a Techsphere Phenyl (HPLC Technology), a Pierce C_8 and a Lichrosorb C_8 (HPLC Technology) column. The C_{18} columns did not provide the selectivity required to separate diazepam and its metabolites in a reasonable analysis time. The phenyl column retained the drugs for too short a time resulting in their co-elution with endogenous compounds from plasma (Fig 5). Best results were achieved using a Lichrosorb C_8 (10 μm).

6.2.1.6. Calibration and Calculation

Evaluation of the assay was carried out using 7 - point

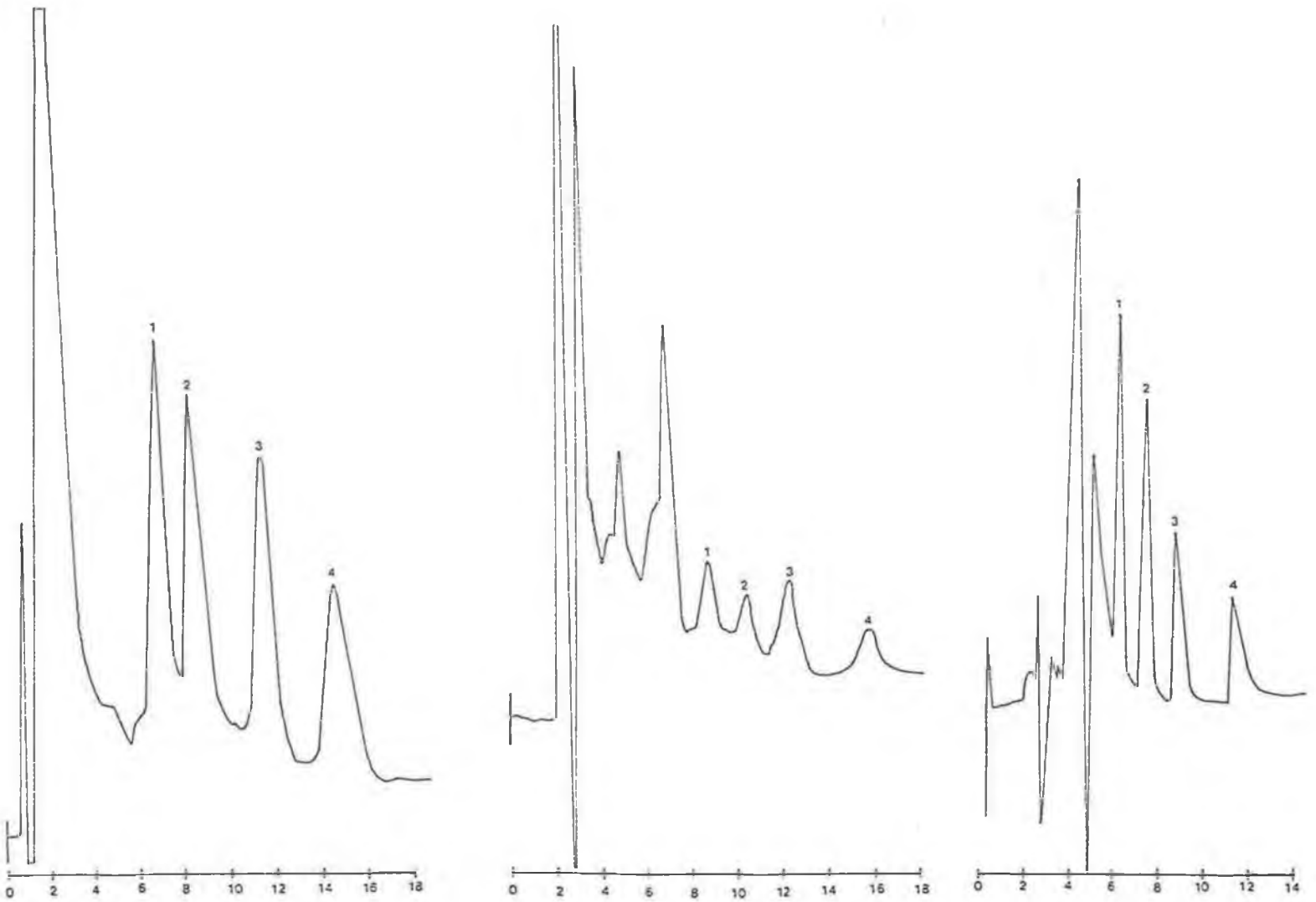


Fig. 5: Chromatograms showing separation of oxazepam (1), temazepam (2), desmethyl-diazepam (3) and diazepam (4) on (a) A Nova-pak C_{18} column (b) A nucleosil C_{18}

calibration standards in the range 25 - 2500 ng/ml of the drugs in plasma. The slope and intecept of the calibration curves were obtained by linear regression of the peak heights versus the concentration of the drug. These calibration curves were then used to interpolate the concentration of the drug in plasma from the measured peak heights (External standard method).

No internal standard was used, nitrazepam has a suitable retention time but interferents eluting with similar retention times were found in some plasma samples.

6.2.2. RESULTS AND DISCUSSION

6.2.2.1. Limit of Detection

For the higher concentrations (250 - 2500 ng/ml) a 50 µl direct injection of plasma was used. For the lower concentrations (25 - 125ng/ml) a 250 µl direct injection was used. Using a 250 µl direct injection of plasma and under the procedural conditions outlined, the limit of detection for each of the four drugs was 25 ng/ml. The detection limit was taken as the amount of compound giving a signal to noise ratio greater than 3:1.

6.2.2.2. Precision

Intra-assay variability was determined at seven concentrations and in quadruplicate at levels of 25, 50,

125, 250, 750, 1500, 2500 ng/ml of each drug in plasma. Inter-assay variability was determined singly and at the same seven concentrations in four replicate runs. The precision of the "column-switching method" (mean coefficient of variation) for the recovered determinate standards when calculated as "unknowns" against the linear regression lines for intra-assay and inter-assay were, respectively, 0.6 and 2.8% for diazepam, 0.5% and 2.8% for desmethyl diazepam, 1.4% and 3.2% for temazepam and 2.0% and 1.9% for oxazepam. (Tables V and VI (a), (b), (c) and (d) and Table VII).

6.2.2.3. Linearity

Measures of linearity as defined by the correlation coefficient of the regression lines of intra-assay for all drugs were better than 0.999 and the intercepts did not differ greatly from the origin.

6.2.2.4. Recovery

The overall recovery was calculated in two different ways. First by comparing the peak heights of a series of spiked plasma samples after they had been through the entire procedure (either column switching or extraction) with a series of reference standards. Secondly by comparing the slopes of the regression lines obtained from the two sets i.e. the reference and the processed samples. Using these methods in the concentration range 25-2500 ng/ml the mean overall recoveries were 100.8%, 98.5%, 101.5%, 104.1% for diazepam, desmethyl diazepam, temazepam and oxazepam respectively using the column switching procedure (Table VIII)

Table V(a)

Intra - Assay

Diazepam

Slope = .0122
I = - .0153
r = .9999

Concentration ng/ml added	1		2		3		4		Mean		S.D.	% CV	% Differ. between added and found
	Peak H	Conc. found	Peak H	Conc. found	Peak H	Conc. found	Peak H	Conc. found	Peak H	Conc. found			
25	0.30	25.77	0.30	25.77	0.30	25.77	0.30	25.77	0.30	25.77	0.0	0.0	3.08
50	0.60	50.29	0.60	50.29	0.60	50.29	0.60	50.29	0.60	50.29	0.0	0.0	0.58
125	1.50	123.84	1.50	123.84	1.50	123.84	1.50	123.84	1.50	123.84	0.0	0.0	0.93
250	3.00	246.42	3.00	246.42	3.00	246.42	3.00	246.42	3.00	246.42	0.0	0.0	1.43
750	9.00	736.76	9.00	736.76	9.00	736.76	9.00	736.76	9.00	736.76	0.0	0.0	1.77
1500	18.00	1472.26	18.00	1472.26	18.50	1513.12	18.50	1513.12	18.25	1492.69	23.59	1.58	0.49
2500	32.00	2616.38	30.50	2493.80	31.00	2534.66	30.00	2452.93	30.88	2524.85	69.79	2.76	1.00

Peak H = Peak Height (cm)
S.D. = Standard Deviation
C.V. = Coefficient of Variation

Mean % C.V. = 0.6%

Table V(b)

Intra - Assay

Desmethyl Diazepam

Slope = 0.0221
I = 0.0003
r = 0.9999

Concentration added ng/ml	1		2		3		4		Mean		S.D.	% CV	% Differ. between added and found
	Peak H	Conc. found	Peak H	Conc. found	Peak H	Conc. found	Peak H	Conc. found	Peak H	Conc. found			
25	0.55	24.91	0.55	24.91	0.55	24.91	0.55	24.91	0.55	24.91	0	0	0.36
50	1.10	49.83	1.10	49.83	1.10	49.83	1.10	49.83	1.10	49.83	0	0	0.34
125	2.75	124.59	2.75	124.59	2.75	124.19	2.75	124.59	2.75	124.59	0	0	0.33
250	5.50	249.19	5.50	249.19	5.50	249.19	5.50	249.19	5.50	249.19	0	0	0.32
750	17.00	770.25	17.00	770.25	16.50	747.60	16.50	747.60	16.75	758.93	13.08	1.72	1.19
1500	33.00	1495.21	33.50	1517.86	34.00	1540.52	33.50	1517.86	33.50	1517.86	18.50	1.22	1.19
2500	55.00	2492.02	54.50	2469.36	54.50	2469.36	54.50	2469.36	54.63	2475.26	11.33	0.46	0.99

Peak H = Peak Height (cm)
S.D. = Standard Deviation
C.V. = Coefficient of Variation

Mean % C.V. = 0.5%

Table V(c)

Intra - Assay

Temazepam

Slope = .0189
I = - .0840
r = .9999

Concentration added ng/ml	1		2		3		4		Mean		S.D.	% CV	% Differ. between added and found
	Peak H	Conc. found	Peak H	Conc. found	Peak H	Conc. found	Peak H	Conc. found	Peak H	Conc. found			
25	0.40	25.56	0.40	25.56	0.40	25.56	0.40	25.56	0.40	25.56	0.0	0.0	2.24
50	0.90	51.95	0.90	51.95	0.80	46.67	0.90	51.95	0.88	50.90	2.64	5.19	1.80
125	2.25	123.23	2.25	123.23	2.25	123.23	2.25	123.23	2.25	123.23	0.0	0.0	1.42
250	4.50	240.02	4.50	240.02	4.50	240.02	4.50	240.02	4.50	242.02	0.0	0.0	3.19
750	14.50	769.99	14.00	743.59	14.00	743.59	14.00	743.59	14.13	750.46	13.20	1.76	0.06
1500	28.50	1509.15	28.00	1482.75	28.50	1482.75	28.00	1509.15	28.25	1495.95	15.24	1.02	0.27
2500	48.50	2565.08	47.50	2512.29	47.50	2459.49	46.50	2512.29	47.50	2512.29	43.11	1.72	0.49

Peak H = Peak Height (cm)
S.D. = Standard Deviation
C.V. = Coefficient of Variation

Mean % C.V. = 1.4%

Table V(d)

Intra - Assay

Oxazepam

Slope = .0283
I = - .0317
r = .99999

Concentration added ng/ml	1		2		3		4		Mean		S.D.	% CV	% Diff between added and found
	Peak H	Conc. found	Peak H	Conc. found	Peak H	Conc. found	Peak H	Conc. found	Peak H	Conc. found			
25	0.70	25.90	0.60	22.36	0.70	25.90	0.70	25.90	0.68	25.19	1.77	7.03	0.76
50	1.40	50.67	1.30	47.13	1.40	50.67	1.40	50.67	1.38	49.79	1.77	3.55	0.06
125	3.50	125.00	3.50	125.00	3.50	125.00	3.50	125.00	3.50	125.00	0.0	0.0	0.00
250	7.00	248.88	7.00	248.88	7.00	248.88	7.00	248.88	7.00	248.88	0.0	0.0	0.45
750	21.50	762.08	21.00	744.39	21.00	744.39	21.00	744.39	21.13	748.99	8.85	1.18	0.13
1500	42.00	1487.65	42.50	1505.35	42.50	1505.35	41.50	1469.95	42.13	1492.25	16.95	1.14	0.52
2500	72.00	2549.46	70.50	2496.37	71.00	2514.06	70.00	2478.67	70.88	2509.64	30.22	1.20	0.35

Peak H = Peak Height (cm)
S.D. = Standard Deviation
C.V. = Coefficient of Variation

Mean % C.V. = 2.0%

Table VI(a)

Inter - assay - Diazepam

Concentration added ng/ml	Day 1		Day 2		Day 3		Day 4		Mean Concentration found	S.D.	%C.V.	% Difference between added + found
	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found				
25	0.30	24.81	0.25	25.71	0.30	27.26	0.20	25.39	25.79	1.05	4.07	3.16
50	0.60	50.25	0.50	50.20	0.55	49.93	0.50	53.02	50.85	1.45	2.85	1.70
125	1.50	126.56	1.25	123.67	1.25	113.42	1.25	122.10	121.44	5.66	4.66	2.85
250	3.00	253.75	2.50	246.13	2.75	249.47	2.50	237.22	246.64	7.01	2.84	1.34
750	8.50	720.09	7.50	735.95	8.50	770.10	8.00	743.79	742.48	20.88	2.81	1.00
1500	18.00	1525.60	15.50	1519.66	16.50	1496.59	16.00	1480.61	1505.62	20.84	1.38	0.37
2500	29.50	2500.69	25.50	2499.30	27.50	2494.29	27.50	2539.80	2508.52	21.03	0.84	0.34

Mean % C.V. = 2.8%

P.H. = Peak Height (cm)
S.D. = Standard Deviation
C.V. = Coefficient of Variation

Table VI(b)

Inter - assay Desmethyl - diazepam

Concentration added ng/ml	Day 1		Day 2		Day 3		Day 4		Mean Concentration found	S.D.	%C.V.	% Difference between added + found
	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found				
25	0.60	26.16	0.55	24.88	0.60	25.43	0.60	23.32	24.95	1.21	4.15	0.20
50	1.10	48.08	1.10	50.00	1.10	48.73	1.30	52.06	49.72	1.75	3.52	0.56
125	3.00	131.37	2.75	125.35	2.75	125.64	3.00	121.87	126.06	3.93	3.12	0.85
250	5.50	241.00	5.50	250.94	5.50	253.82	6.50	265.59	252.84	10.12	4.00	1.14
750	16.50	723.18	16.50	753.33	16.00	743.24	18.50	758.34	744.52	15.55	2.09	0.73
1500	35.00	1534.17	32.50	1484.07	32.00	1489.01	36.00	1476.95	1496.05	25.89	1.73	0.26
2500	57.00	2498.60	55.00	2511.67	54.00	2514.44	61.00	2503.52	2507.06	7.30	0.29	0.28

% C.V. = 2.8%

P.H. = Peak Height (cm)
S.D. = Standard Deviation
C.V. = Coefficient of Variation

Table VI(c)

Inter - assay Temazepam

Concentration added ng/ml	Day 1		Day 2		Day 3		Day 4		Mean Concentration found	S.D.	%C.V.	% Difference between added + found
	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found				
25	0.50	26.88	0.40	26.84	0.50	24.18	0.50	25.26	25.79	1.31	5.08	3.16
50	0.90	48.64	0.80	50.21	0.90	47.69	1.00	48.96	48.88	1.04	2.13	2.24
125	2.25	122.08	2.00	120.32	2.25	127.04	2.50	120.06	122.38	3.24	2.65	2.10
250	4.50	244.50	4.00	237.16	4.50	259.28	5.50	262.25	250.80	11.96	4.77	0.32
750	14.00	761.34	12.50	733.74	13.50	788.24	16.00	759.93	760.81	22.26	2.93	1.44
1500	27.00	1468.61	27.00	1580.85	25.00	1461.13	31.50	1494.59	1501.30	54.94	3.66	0.09
2500	46.50	2529.50	42.00	2457.17	42.50	2492.66	52.50	2489.94	2492.07	29.60	1.19	0.32

Mean % C.V. = 3.2%

P.H. = Peak Height (cm)
S.D. = Standard Deviation
C.V. = Coefficient of Variation

Table VI(d)

Inter - assay C.S. Oxazepam

Concentration added ng/ml	Day 1		Day 2		Day 3		Day 4		Mean Concentration found	S.D.	%C.V.	% Difference between added + found
	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found				
25	0.70	24.49	0.70	25.66	0.80	25.52	0.70	24.16	24.96	0.75	3.01	0.16
50	1.40	49.78	1.40	50.11	1.40	47.50	1.50	49.59	49.12	1.13	2.30	1.76
125	3.50	125.65	3.50	123.49	3.50	124.45	4.00	129.05	125.66	2.43	1.93	0.53
250	7.00	252.10	7.00	245.77	7.00	252.69	8.00	256.19	251.69	4.34	1.72	0.68
750	21.00	757.91	21.50	752.40	21.50	783.96	23.50	748.85	760.78	15.90	2.09	1.44
1500	42.00	1516.61	42.50	1486.12	40.50	1480.12	46.50	1479.90	1490.69	17.52	1.18	0.62
2500	68.50	2474.02	72.00	2516.82	68.00	2487.71	79.00	2512.90	2497.90	20.46	0.82	0.09

Mean % C.V. = 1.9%

P.H. = Peak Height (cm)
S.D. = Standard Deviation
C.V. = Coefficient of Variation

Table VII

Diazepam — Precision and Reproducibility

Concentration added (ng/ml)	Mean (n=4) Concentration found ± S.D. (ng/ml)	C.V. %	Difference between added + found
INTRA-ASSAY	PRECISION (REPEATABILITY)		
25	25.77 ± 0.0	0.0	3.08
50	50.29 ± 0.0	0.0	0.58
125	123.84 ± 0.0	0.0	0.93
250	246.42 ± 0.0	0.0	1.43
750	736.76 ± 0.0	0.0	1.77
1500	1492.69 ± 23.59	1.58	0.49
2500	2524.85 ± 69.79	2.76	1.00
	Mean % C.V. = 0.6%		
INTER-ASSAY	REPRODUCIBILITY		
25	25.79 ± 1.05	4.07	3.16
50	50.85 ± 1.45	2.85	1.70
125	121.44 ± 5.66	4.66	2.85
250	246.64 ± 7.01	2.84	1.34
750	742.48 ± 20.88	2.81	1.00
1500	1505.62 ± 20.84	1.38	0.37
2500	2508.52 ± 21.03	0.84	0.34
	Mean % C.V. = 2.8%		

Table VII contd

Desmethyl diazepam

Concentration added (ng/ml)	Mean (n=4) Concentration found ± S.D. (ng/ml)	C.V. %	Difference between added + found
INTRA-ASSAY	PRECISION (REPEATABILITY)		
25	24.91 ± 0.0	0.0	0.36
50	49.83 ± 0.0	0.0	0.34
125	124.59 ± 0.0	0.0	0.33
250	249.19 ± 0.0	0.0	0.32
750	758.93 ± 13.08	1.72	1.19
1500	1517.86 ± 18.50	1.22	1.19
2500	2475.26 ± 11.33	0.46	0.99
	Mean % C.V. = 0.5%		
INTER-ASSAY	REPRODUCIBILITY		
25	24.95 ± 1.21	4.85	0.20
50	49.72 ± 1.75	3.52	0.56
125	126.06 ± 3.93	3.12	0.85
250	252.84 ± 10.12	4.00	1.14
750	744.52 ± 15.55	2.09	0.73
1500	1496.05 ± 25.89	1.73	0.26
2500	2507.06 ± 7.30	0.29	0.28
	Mean % C.V. = 2.8%		

Table VII contd

Temazepam — Precision and Reproducibility

Concentration added (ng/ml)	Mean (n=4) Concentration found ± S.D. ng/ml	C.V. %	Difference between added + found Concentration (%)
INTRA-ASSAY	PRECISION (REPEATABILITY)		
25	25.56 ± 0.00	0.00	2.24
50	50.90 ± 2.64	5.19	1.80
125	123.23 ± 0.00	0.00	1.42
250	242.02 ± 0.00	0.00	3.19
750	750.46 ± 13.20	1.76	0.06
1500	1495.95 ± 15.24	1.02	0.27
2500	2512.29 ± 43.11	1.72	0.49
	Mean % C.V. = 1.4%		
INTER-ASSAY	REPRODUCIBILITY		
25	25.79 ± 1.31	5.08	3.16
50	48.88 ± 1.04	2.13	2.24
125	122.38 ± 3.24	2.65	2.10
250	250.80 ± 11.96	4.77	0.32
750	760.81 ± 22.26	2.93	1.44
1500	1501.30 ± 54.94	3.66	0.09
2500	2492.07 ± 29.60	1.19	0.32
	Mean % C.V. = 3.2%		

Table VII contd

Oxazepam — Precision and Reproducibility

Concentration added (ng/ml)	Mean (n=4) Concentration found ± S.D. (ng/ml)	C.V. %	Difference between added + found (%) Concentration
INTRA-ASSAY	PRECISION (REPEATABILITY)		
25	25.19 ± 1.77	7.03	0.76
50	49.79 ± 1.77	3.55	0.06
125	125.00 ± 0.00	0.00	0.00
250	248.88 ± 0.00	0.00	0.45
750	748.99 ± 8.85	1.18	0.13
1500	1492.25 ± 16.95	1.14	0.52
2500	2509.64 ± 30.22	1.20	0.35
	Mean % C.V. = 2.0%		
INTER-ASSAY	REPRODUCIBILITY		
25	24.96 ± 0.75	3.01	0.16
50	49.12 ± 1.13	2.30	1.76
125	125.66 ± 2.43	1.93	0.53
250	251.69 ± 4.34	1.72	0.68
750	760.78 ± 15.90	2.09	1.44
1500	1490.69 ± 17.52	1.18	0.62
2500	2497.86 ± 20.46	0.82	0.09
	Mean % C.V. = 1.9%		

Table VIII

Recoveries - Diazepam

Method 1

Concentration ng/ml	PEAK HEIGHTS						% Recovery
	Authentic Standards			Recovered Standards			
	1	2	Mean	1	2	Mean	
25	0.3	0.3	0.3	0.3	0.3	0.3	100 %
50	0.6	0.6	0.6	0.6	0.6	0.6	100 %
125	1.5	1.5	1.5	1.5	1.5	1.5	100 %
250	3.0	3.0	3.0	3.0	3.0	3.0	100 %
750	9.0	9.5	9.25	9.0	9.0	9.0	94.7%
1500	19.0	19.0	19.0	18.0	18.0	18.0	94.7%
2500	30.0	30.0	30.0	32.0	30.5	31.25	104.2%

Mean Percentage Recovery $\pm$ SD = 99.1%

Method 2

Regression line for set A: $y = 0.0121 X + 0.0576$

Regression line for set B: $y = 0.0124 X - 0.1304$

$$\% \text{ Recovery} = \frac{0.0124}{0.0121} \times 100 = 102.48\%$$

Mean recovery by both methods = 100.79%

Table VIII contd

Recovery - Desmethyl - diazepam

Method 1

Concentration ng/ml	PEAK HEIGHTS						% Recovery
	Authentic Standards			Recovered Standards			
	1	2	Mean	1	2	Mean	
25	0.5	0.5	0.5	0.45	0.45	0.45	90
50	0.9	0.9	0.9	0.9	0.9	0.9	100
125	2.3	2.2	2.25	2.25	2.25	2.25	100
250	5.0	5.0	5.0	4.3	4.7	4.5	90
750	14.4	14.1	14.25	14.0	14.5	14.25	100
1500	29.1	28.4	28.75	27.9	28.6	28.25	98.3
2500	44	44	44	44	44	44	100

Mean Percentage Recovery = 96.9%

Method 2

Regression line for set A: $y = 0.0179 X + 0.3926$

Regression line for set B: $y = 0.0179 X + 0.2562$

$$\text{Recovery} = \frac{0.0179}{0.0179} \times 100 = 100\%$$

Mean recovery by both methods = 98.5%

Table VIII contd

Recoveries Temazepam

Method 1

Concentration ng/ml	PEAK HEIGHTS						% Recovery
	Authentic Standards			Recovered Standards			
	1	2	Mean	1	2	Mean	
25	0.5	0.5	0.5	0.4	0.5	0.45	90
50	0.9	0.9	0.9	0.9	0.9	0.9	100
125	2.5	2.0	2.25	2.25	2.25	2.25	100
250	5.0	5.0	5.0	4.5	4.5	4.5	90
750	14.0	14.5	14.25	14.5	14.0	14.25	100
1500	28.5	29	28.75	28.5	28.0	28.25	98.3%
2500	44.0	44.0	44.0	46.5	47.5	47	106.8 %

Mean Percentage Recovery = 97.9%

Method 2

Regression line for set A: $y = 0.0179 X + .3926$

Regression line for set B: $y = 0.0188 X - .0568$

$$\% \text{ Recovery} = \frac{0.0188}{0.0179} \times 100 = 105.3\%$$

Mean recovery by both methods = 101.47%

Table VIII contd

Recoveries Oxazepam

Method 1

Concentration ng/ml	PEAK HEIGHTS						% Recovery
	Authentic Standards			Recovered Standards			
	1	2	Mean	1	2	Mean	
25	0.6	0.7	0.65	0.7	0.6	0.65	100
50	1.3	1.4	1.35	1.4	1.3	1.35	100
125	3.5	3.5	3.5	3.5	3.5	3.5	100
250	7.0	7.5	7.25	7.0	7.0	7.0	96.6
750	21.5	20.5	21.0	21.5	21	21.25	101.2
1500	42.5	41.5	42	42	42.5	42.25	100.6
2500	65	65	65	70	71	70.5	108.5

Mean Percentage Recovery = 100.99%

Method 2

Regression line for set A: $y = 0.0263 X + 0.5504$

Regression line for set B: $y = 0.0282 X - 0.0318$

$$\% \text{ Recovery} = \frac{0.0282}{0.0263} \times 100 = 107.22\%$$

Mean % recovery by both methods = 104.11%

6.2.2.5. Interference Study

Seven other members of the benzodiazepines were tested for possible interference with the measured substances. As can be seen from the chromatogram in Fig 6 no other member of the benzodiazepines tested interfered with the determination of temazepam and desmethyl-diazepam. Lorazepam interfered with oxazepam and midozalam interfered with diazepam. Flurazepam and medazepam did not elute under these chromatographic conditions. Members of the tricyclics were also tested for possible interference but did not elute at similiar retention times to any of the drugs under study.

6.2.3. CONCLUSION

For routine monitoring of diazepam and its metabolites the on-line solid phase extraction technique described achieves a high recovery rate (>98%) for all four drugs. The method circumvents the need for lengthy liquid-liquid extraction and overcomes the problem of protein binding which often lowers the recovery for these compounds due to their being extensively protein bound. The addition of salicylic acid to preferentially bind the plasma proteins releases the benzodiazepines from the concentration column for subsequent analysis on the analytical column. This is a procedure which may be applied to other protein bound drugs and is a simple alternative to their release by hydrolysis using enzymes.

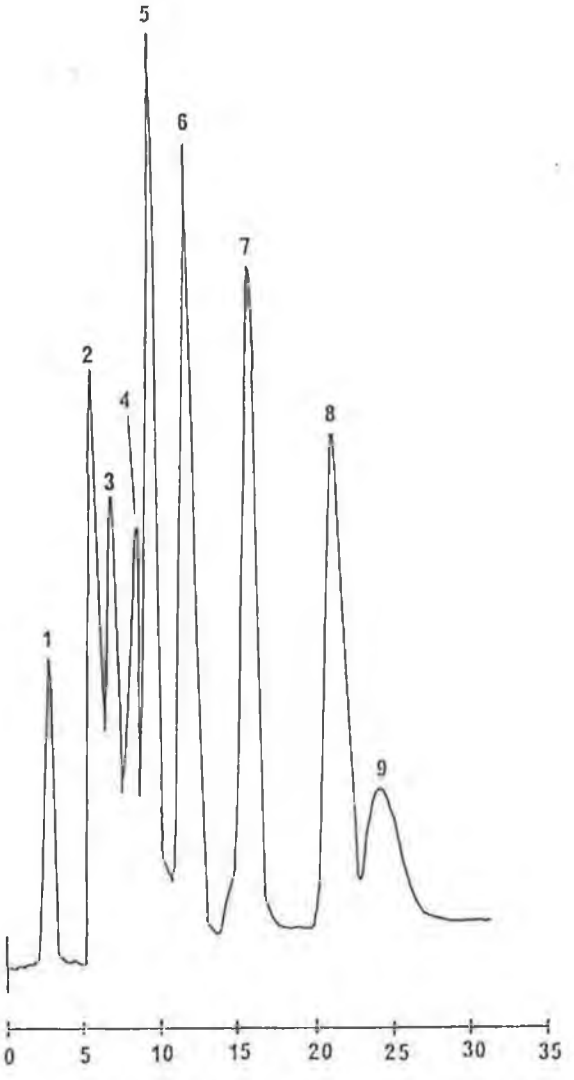


Fig. 6: Chromatogram obtained for members of the benzodiazepines showing interference with diazepam and its metabolites. 1 = 7-aminonitrazepam, 2 = clonazepam, 3 = nitrazepam, 4 = lorazepam, 5 = oxazepam, 6 = temazepam, 7 = desmethyl-diazepam, 8 = diazepam, 9 = midozalem.

The precision and accuracy of the method are excellent as denoted by the coefficients of variation and the percentage differences between added and found amounts of the drugs indicated above. The limit of detection may be improved by the use of a larger injection volume but this will shorten the lifetime of the concentration column considerably.

HPLC Determination of Diazepam and its metabolites in Plasma using an Extraction Technique.

6.3. INTRODUCTION TO THE EXTRACTION TECHNIQUE

A sensitive high-performance liquid chromatographic (HPLC) method for the determination of plasma levels of diazepam and its metabolites in human plasma is described. The drugs were extracted from plasma into diethyl ether. After evaporation of the solvent, the residue is reconstituted in the mobile phase and quantified by HPLC on a reverse phase Lichrosorb C_8 column using methanol - phosphate buffer (50:50) as the mobile phase with U.V. detection at 246 nm. The limit of quantitation was 25ng/ml in plasma, using a 1ml plasma sample and a 25 µl injection from a reconstituted volume of 100 µl. The mean overall recoveries of the drugs from plasma were 67.7%, 77.8%, 78.7% and 75.3% for diazepam, desmethyl diazepam, temazepam and oxazepam respectively. Mean percentage coefficients of variation for intra-assay and inter-assay precision respectively were 5.2% and 1.9% for diazepam, 4.1% and 2.8% for desmethyl diazepam, 5.1% and 3.2% for temazepam and 3.6% and 2.1% for oxazepam.

6.3.1. EXPERIMENTAL

Reagents, Instrumentation, Chromatography and Preparation of standards were as described in section 6.2.1.

6.3.1.1. Extraction Procedure

Plasma (1ml) spiked with 50 µl of each drug was mixed with 100 mg of a 3 : 1 dry mixture of $NaHCO_3$ and Na_2CO_3 by vortexing for 20 sec. After the addition of diethyl ether (7ml), the drugs were extracted by vortex mixing for 60 seconds followed by centrifugation for 10 minutes at 700g. The organic phase was then transferred into a clean poly-propylene tube which is placed in a water bath at 40^0 and the solvent evaporated under a gentle stream of oxygen free nitrogen. The residue was reconstituted in 100 µl of the mobile phase directly prior to injection and 25 µl injected for analysis onto the analytical column. The chromatograms are shown in fig 7.

6.3.1.2. Calibration and Calculation

Evaluation of the assay was carried out using seven-point calibration standards in the concentration range 25 - 2500 ng/ml of the drugs in plasma. No internal standard was used.

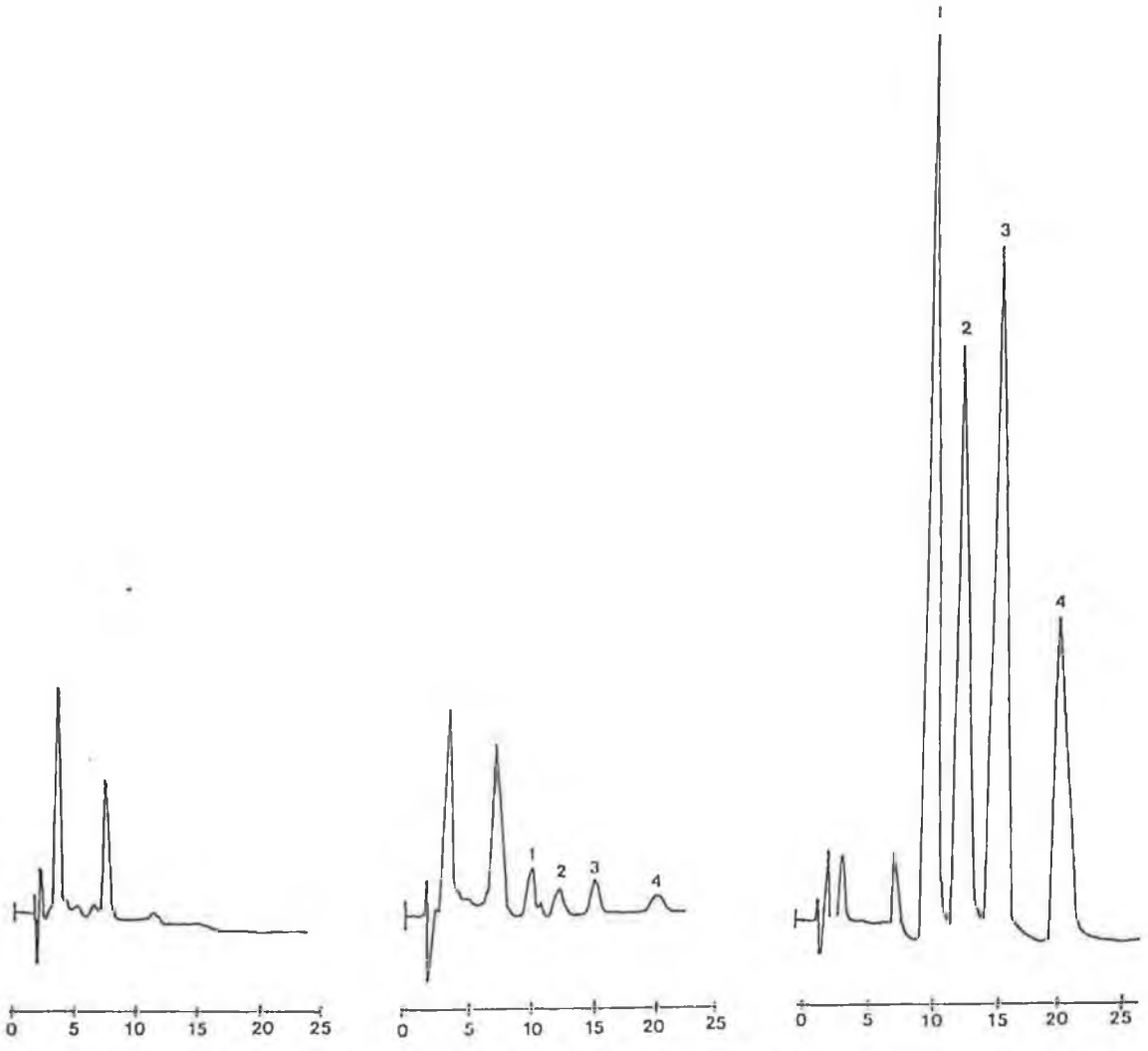


Fig. 7: Chromatograms obtained for (a) human drug free plasma, (b) the same plasma spiked with 25ng/ml of each drug and (c) spiked with 250ng/ml of each drug. The injection volume is 25ul from a reconstituted volume of 100ul.

6.3.1.3. Extraction Solvent

The majority of methods used for extracting benzodiazepines from biological fluids have been based on the partitioning of the drugs in favour of an organic solvent from a (usually alkaline) buffered aqueous solution. Organic solvents employed for this purpose have included n-hexane [76], toluene [77,78], chloroform [79,80], octanol [81], diethyl ether [82,83,84], n-butyl chloride [85,86] or n-butylacetate [87,40]. Mixed solvents have also been used for this purpose. In an article by Clifford and Smyth [7] over half of the extraction methods reviewed had been made using diethyl ether. Specifically for diazepam and its metabolites diethyl ether has been the solvent of choice with one publication reporting the use of a benzene-methylene chloride mixture which gave poor recovery.

Among the solvents tested in our own laboratory were ethyl-acetate, methylene chloride - toluene mixture (1:9), diethyl ether and chloroform. Ethyl acetate gave reasonable recovery but the chromatograms were not clean, also some of the samples emulsified upon vortexing. Interfering peaks eluting with similar retention times to temazepam and desmethyl diazepam were recorded from extracts using methylene chloride - toluene. Use of chloroform causes solid material to separate out resulting in low recovery of the drugs.

Diethyl ether was chosen as the extracting solvent as it is easily evaporated. Its limited polarity reduces the amount of endogenous material it extracts from biological fluids and being less dense than water, it is removed from centrifuged mixtures with less contamination from the aqueous layer than occurs with a denser liquid.

6.3.1.4. Choice of Buffer for extraction

Having chosen diethyl ether as the extracting solvent various buffers were investigated. In a review of extraction techniques by Stevens [58], he points to ether extraction of aqueous ammonical solutions as giving the cleanest extracts. The extraction procedure used by him is shown in Fig 8.

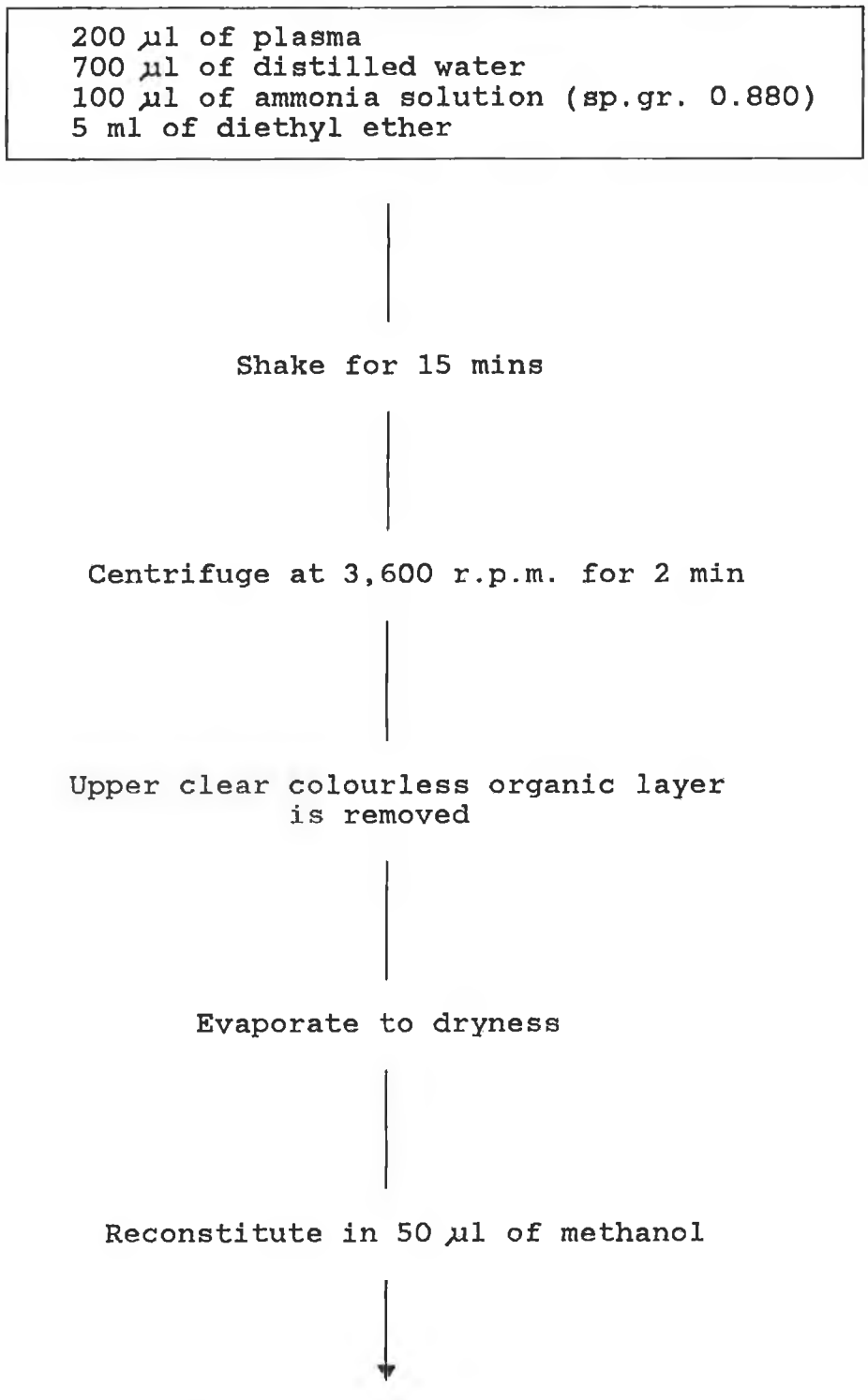


Fig 8: Extraction procedure for the benzodiazepines used by Stevens [58].

A necessary pre-requisite for successful extraction is the maintenance of the blood proteins in a water-soluble form, any protein precipitation tends to adsorb drug molecules and reduce recoveries [88]. The relatively large ammonical constituent/blood ratios employed ensured that all mixtures remain clear and free of solid matter during extraction. However, when we repeated this procedure in our laboratory we could not obtain clear chromatograms under the chromotographic conditions described in the assay.

Previously other workers in our laboratory had found LiCl to be useful for the clean up of plasma samples during extraction. However, in the present study we found a mixture of Na_2CO_3 and $NaHCO_3$ to be the most effective for sample clean up and good recovery of all four drugs.

6.3.1.5. Single vs Double Extraction

In order to improve the recovery of the drug and its metabolites a double extraction using diethyl ether may be carried out. This was found to improve recovery by up to 15%, however, it lengthens the procedure somewhat. A single step procedure was employed in this study.

6.3.2. RESULTS AND DISCUSSION

6.3.2.1. Limit of Detection

Under the procedural conditions the limit of detection, using a 25 ul injection from a reconstituted volume of 100 µl, was 25 ng/ml for each of the four drugs in plasma.

6.3.2.2. Precision and Accuracy

Intra-assay variability was determined at seven concentrations in quadruplicate at levels of 25, 50, 125, 250, 750, 1500 and 2500 ng/ml of each drug in plasma. Inter-assay variability was determined singly and at the same seven concentrations in four replicate runs. The precision of the method (mean coefficient of variation) for the values of the recovered determinate standards when calculated as "unknown" against the linear regression lines for intra-assay and inter-assay were respectively 5.2% and 1.9% for diazepam, 4.1% and 2.8% for desmethyl diazepam, 5.1% and 3.2% for temazepam, 3.6% and 2.1% for oxazepam. (Tables IX (a), (b), (c) and (d), X (a), (b), (c) and (d) and Table XI).

6.3.2.3. Linearity

The correlation coefficients under intra-assay precision were better than 0.999 and the intercepts did not differ significantly from zero.

6.3.2.4. Recovery

The recoveries of the four drugs was calculated as described in the column switching method. Using this method in the concentration range 25 - 2500 ng/ml, the mean overall recoveries were 67.7%, 77.8%, 78.7% and 75.3% for diazepam, desmethyl diazepam, temazepam and oxazepam respectively. (Table XII).

Table IX(a)

Intra-assay Extraction Diazepam

Slope = .0197
Intercept = - .1814

Concentration added ng/ml	1		2		3		4		Mean		S.D.	% CV	% Diff
	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found			
25	0.30	24.46	0.30	24.46	0.30	24.46	0.30	24.46	0.30	24.46	0	0	2.16
50	0.80	49.86	0.80	49.86	0.80	49.86	0.80	49.86	0.80	49.86	0	0	0.28
125	2.40	131.15	2.50	136.23	2.60	141.31	2.40	131.15	2.48	135.21	4.86	3.59	8.17
250	4.40	232.76	4.80	253.09	4.80	253.09	5.20	273.41	4.80	253.09	16.60	6.56	1.24
750	14.80	761.15	15.60	801.80	12.00	618.89	12.00	618.89	13.60	700.18	95.32	13.61	6.64
1500	27.20	1391.15	27.20	1391.15	31.60	1614.70	31.60	1614.70	29.40	1502.92	129.07	8.59	0.20
2500	48.00	2447.92	52.00	2651.15	51.20	2610.51	48.00	2447.92	49.80	2539.38	106.90	4.21	1.58

r = .999 Mean % C.V. = 5.2%

P.H. = Peak Height (cm)
S.D. = Standard Deviation
C.V. = Coefficient of Variation

Table IX(b)

Intra-assay Extraction Desmethyl Diazepam

Slope = .0297
Intercept = .0593

Concentration added ng/ml	1		2		3		4		Mean		S.D.	% CV	% Diff
	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found			
25	0.80	24.96	0.80	24.96	0.80	24.96	0.80	24.96	0.80	24.96	0.0	0.00	0.17
50	1.50	48.55	1.50	48.55	1.50	48.55	1.50	48.55	1.50	48.55	0.0	0.00	2.90
125	3.70	122.68	3.90	129.42	3.70	122.68	3.70	122.68	3.75	124.36	3.37	2.71	0.50
250	7.20	240.62	7.20	240.62	8.00	267.58	8.40	281.05	7.70	257.47	20.22	7.85	3.00
750	23.60	793.25	25.20	847.16	20.00	671.94	21.20	712.37	22.50	756.18	78.88	10.43	0.82
1500	47.20	1588.49	45.20	1521.10	43.60	1467.18	43.60	1467.18	44.90	1510.99	57.58	3.81	0.73
2500	71.20	2397.21	76.00	2558.96	76.00	2558.96	71.20	2397.21	73.60	2478.08	93.39	3.77	0.88

r = .9999 % C.V. = 4.1%

P.H. = Peak Height (cm)
S.D. = Standard Deviation
C.V. = Coefficient of Variation

Table IX(c)

Intra-assay Extraction Temazepam

Slope = 0.0306
Intercept = - .0138

Concentration added ng/ml	1		2		3		4		Mean		S.D.	% CV	% Diff
	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found			
25	0.80	25.31	0.80	25.31	0.80	25.31	0.80	25.31	0.80	25.31	0.00	0.00	1.22
50	1.60	50.18	1.60	50.18	1.60	50.18	1.60	50.18	1.60	50.18	0.00	0.00	0.36
125	4.00	124.80	4.20	131.02	4.00	124.80	3.80	118.58	4.00	124.80	5.08	4.07	0.16
250	7.20	224.30	8.00	249.17	8.00	249.17	8.60	267.83	7.95	247.62	17.86	7.21	0.95
750	25.20	783.96	27.60	858.58	20.80	647.15	22.00	684.46	23.90	743.54	96.00	12.91	0.86
1500	44.40	1380.93	48.80	1517.74	49.20	1530.17	50.80	1579.92	48.30	1502.19	85.19	5.67	0.15
2500	76.80	2388.32	84.00	2612.19	84.80	2637.06	76.80	2388.32	80.60	2506.47	136.81	5.46	0.26

R = .99999 % C.V. = 5.1%

P.H. = Peak Height (cm)
S.D. = Standard Deviation
C.V. = Coefficient of Variation

Table IX(d)

Intra-assay Extraction Oxazepam

Slope = .0355
Intercept = .1513

Concentration added ng/ml	1		2		3		4		Mean		S.D.	% CV	% Diff
	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found			
25	1.00	23.94	1.00	23.94	1.00	23.94	1.00	23.94	1.00	23.94	0	0	4.24
50	2.00	52.15	2.00	52.15	2.00	52.15	2.00	52.15	2.00	52.15	0	0	4.30
125	4.10	111.39	4.50	122.67	4.50	122.67	4.60	125.49	4.43	120.70	6.25	5.18	3.44
250	8.40	232.69	9.20	255.25	9.60	266.54	9.60	266.54	9.20	255.25	15.96	6.25	2.10
750	28.00	785.59	28.00	785.59	26.00	729.17	27.60	774.30	27.40	768.66	26.86	3.49	2.49
1500	54.40	1530.31	56.00	1575.44	50.80	1428.75	50.80	1428.75	53.00	1490.81	73.99	4.96	0.61
2500	92.80	2613.54	81.60	2297.60	88.80	2500.70	90.40	2545.84	88.40	2489.42	136.03	5.46	0.42

r = .9999 % C.V. = 3.6%

P.H. = Peak Height (cm)
S.D. = Standard Deviation
C.V. = Coefficient of Variation

Table X (a)

Inter-assay Extraction Diazepam

Concentration added ng/ml	1		2		3		4		Mean	S.D.	%C.V.	% Diff
	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found	Conc. found			
25	0.30	24.77	0.40	25.57	0.60	24.82	0.50	25.87	25.26	0.55	2.18	1.04
50	0.80	51.63	0.80	50.44	1.10	49.41	1.00	51.67	50.79	1.08	2.13	1.58
125	2.20	126.84	2.00	125.03	2.70	128.10	2.50	129.09	127.27	1.75	1.38	1.82
250	4.40	245.03	4.00	249.36	5.20	251.06	4.40	227.15	243.15	10.96	4.51	2.74
750	13.60	739.27	11.60	721.79	15.20	742.90	14.40	743.25	732.30	10.55	1.44	2.36
1500	27.20	1469.89	24.00	1492.59	30.40	1490.49	29.20	1507.08	1490.01	15.31	1.03	0.67
2500	47.20	2544.33	40.80	2536.92	51.20	2513.50	48.80	2518.64	2528.35	14.65	0.58	1.13

C.V. = 1.9%

P.H. = Peak Height (cm)
S.D. = Standard Deviation
C.V. = Coefficient of Variation

Table X(b)

Inter-assay Extraction Desmethyl - diazepam

Concentration added ng/ml	1		2		3		4		Mean	S.D.	%C.V.	% Diff
	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found	Conc. found			
25	0.70	24.74	0.70	24.93	0.80	24.42	0.90	24.02	24.53	0.40	1.63	1.88
50	1.40	50.65	1.30	49.19	1.40	45.64	1.80	53.84	49.83	3.40	6.82	0.34
125	3.40	124.69	3.20	126.01	4.10	141.10	3.90	123.42	128.81	8.27	6.42	3.05
250	6.80	250.56	6.40	255.40	7.20	250.71	7.60	246.02	250.67	3.83	1.53	0.27
750	20.40	754.02	18.40	740.58	21.20	745.72	22.40	736.42	744.19	7.58	1.02	0.77
1500	40.00	1479.61	37.20	1500.71	42.80	1509.44	46.40	1531.67	1505.36	21.55	1.43	0.36
2500	68.00	2516.16	62.00	2503.43	70.40	2485.32	75.20	2485.96	2497.72	14.89	0.60	0.09

Mean % C.V. = 2.8%

P.H. = Peak Height (cm)
S.D. = Standard Deviation
C.V. = Coefficient of Variation

Table X(c)

Inter-assay Extraction Temazepam

Concentration added ng/ml	1		2		3		4		Mean	S.D.	%C.V.	% Diff between added and found
	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found	Conc. found			
25	0.70	25.08	0.70	25.04	1.00	23.12	0.90	25.55	24.70	1.08	4.37	1.20
50	1.40	50.91	1.40	50.95	1.70	47.12	1.80	52.56	50.39	2.31	4.58	0.78
125	3.40	124.71	3.40	124.97	4.50	143.12	4.20	124.59	129.35	9.18	7.10	3.48
250	6.80	250.17	6.80	250.80	7.60	249.43	8.00	238.64	247.26	5.77	2.33	1.10
750	20.00	737.23	19.60	724.51	22.00	743.21	24.00	718.83	730.95	11.23	1.54	2.54
1500	40.00	1475.21	40.80	1509.09	44.00	1497.58	51.20	1535.15	1504.26	24.94	1.66	0.28
2500	68.80	2537.91	68.00	2515.72	73.20	2498.85	83.60	2507.53	2515.00	16.75	0.67	0.60

Mean C.V. = 3.2%

P.H. = Peak Height (cm)
S.D. = Standard Deviation
C.V. = Cofficient of Variation

Table X(d)

Inter-assay Extraction Oxazepam

Concentration added ng/ml	1		2		3		4		Mean	S.D.	%C.V.	% Diff
	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found	P.H.	Conc. found	Conc. found			
25	0.80	24.82	0.70	24.39	0.90	23.88	0.90	24.92	24.50	0.47	1.92	2.00
50	1.60	51.17	1.50	50.67	1.70	47.59	1.90	50.87	50.08	1.67	3.33	0.16
125	3.80	123.63	3.80	126.22	4.80	139.45	4.80	126.13	128.86	7.16	5.56	3.09
250	7.60	248.80	7.60	251.05	8.40	246.13	9.60	250.70	249.17	2.25	0.90	0.33
750	22.80	749.45	22.80	750.37	25.60	755.82	28.00	728.21	745.96	12.16	1.63	0.54
1500	45.60	1500.43	45.60	1499.35	50.80	1502.57	57.20	1486.00	1497.09	7.51	0.50	0.19
2500	76.00	2501.74	76.00	2498.00	84.00	2486.39	97.60	2534.46	2505.15	20.61	0.82	0.21

Mean % C.V. = 2.1%

P.H. = Peak Height (cm)
S.D. = Standard Deviation
C.V. = Coefficient of Variation

Table XI

Extraction

Diazepam

Concentration added (ng/ml)	Mean (n=4) Concentration found ± S.D. (ng/ml)	C.V. %	Difference between added + found
INTRA-ASSAY	PRECISION (REPEATABILITY)		
25	24.46 ± 0.00	0.00	2.16
50	49.86 ± 0.00	0.00	0.28
125	135.21 ± 4.86	3.59	8.17
250	253.09 ± 16.60	6.56	1.24
750	700.18 ± 95.32	13.61	6.64
1500	1502.92 ± 129.07	8.59	0.20
2500	2539.38 ± 106.90	4.21	1.58
	Mean % C.V. = 5.2%		
INTER-ASSAY	REPRODUCIBILITY		
25	25.26 ± 0.55	2.18	1.04
50	50.79 ± 1.08	2.13	1.58
125	127.27 ± 1.75	1.38	1.82
250	243.15 ± 10.96	4.51	2.74
750	732.30 ± 10.55	1.44	2.36
1500	1490.01 ± 15.31	1.03	0.67
2500	2528.35 ± 14.65	0.58	1.13
	Mean % C.V. = 1.9%		

Table XI contd

Extraction

Temazepam

Concentration added (ng/ml)	Mean (n=4) Concentration found ± S.D. (ng/ml)	C.V. %	Difference between added + found
INTRA-ASSAY	PRECISION (REPEATABILITY)		
25	25.31 ± 0.00	0.0	1.22
50	50.18 ± 0.00	0.0	0.36
125	124.80 ± 5.08	4.07	0.16
250	247.62 ± 17.86	7.21	0.95
750	743.54 ± 96.00	12.91	0.86
1500	1502.19 ± 85.19	5.67	0.15
2500	2506.47 ± 136.81	5.46	0.26
	Mean % C.V. = 5.1%		
INTER-ASSAY	Reproducibility		
25	24.70 ± 1.08	4.37	1.20
50	50.39 ± 2.31	4.58	0.78
125	129.35 ± 9.18	7.10	3.48
250	247.26 ± 5.77	2.33	1.10
750	730.95 ± 11.23	1.54	2.54
1500	1504.26 ± 24.94	1.66	0.28
2500	2515.00 ± 16.75	0.67	0.60
	Mean % C.V. = 3.2%		

Table XI contd

Extraction

Desmethyl Diazepam

Concentration added (ng/ml)	Mean (n=4) Concentration found ± S.D. (ng/ml)	C.V. %	Difference between added + found
INTRA-ASSAY	PRECISION (REPEATABILITY)		
25	24.96 ± 0.00	0.00	0.17
50	48.55 ± 0.00	0.00	2.90
125	124.36 ± 3.37	2.71	0.50
250	257.47 ± 20.22	7.85	3.00
750	756.18 ± 78.88	10.43	0.82
1500	1510.99 ± 57.58	3.81	0.73
2500	2478.08 ± 93.39	3.77	0.88
	Mean % C.V. = 4.1%		
INTER-ASSAY	REPRODUCIBILITY		
25	24.53 ± 0.40	1.63	1.88
50	49.83 ± 3.40	6.82	0.34
125	128.81 ± 8.27	6.42	3.05
250	250.67 ± 3.83	1.53	0.27
750	744.19 ± 7.58	1.02	0.77
1500	1505.36 ± 21.55	1.43	0.36
2500	2497.72 ± 14.89	0.60	0.09
	Mean % C.V. = 2.8%		

Table XI

Extraction

Oxazepam

Concentration added (ng/ml)	Mean (n=4) Concentration found ± S.D. (ng/ml)	C.V. %	Difference between added + found
INTRA-ASSAY	PRECISION (REPEATABILITY)		
25	23.94 ± 0.00	0.00	4.24
50	52.15 ± 0.00	0.00	4.30
125	120.70 ± 6.25	5.18	3.44
250	255.25 ± 15.96	6.25	2.10
750	768.66 ± 26.86	3.49	2.49
1500	1490.81 ± 73.99	4.96	0.61
2500	2489.42 ± 136.03	5.46	0.42
	Mean % C.V. = 3.6%		
INTER-ASSAY	REPRODUCIBILITY		
25	24.50 ± 0.47	1.92	2.00
50	50.08 ± 1.67	3.33	0.16
125	128.86 ± 7.16	5.56	3.09
250	249.17 ± 2.25	0.90	0.33
750	745.96 ± 12.16	1.63	0.54
1500	1497.09 ± 7.51	0.50	0.19
2500	2505.15 ± 0.82	0.82	0.21
	Mean % C.V. = 2.1%		

Table XII

Extraction Recoveries

Method 1

DIAZEPAM

Concentration Added ng/ml	Peak Heights (cm)						% Recovery
	Authentic standards			Recovered standards			
	(1)	(2)	Mean	(1)	(2)	Mean	
25	0.6	0.6	0.6	0.4	0.4	0.4	66.7
50	1.3	1.3	1.3	0.9	0.7	0.8	61.6
125	3.2	3.4	3.3	2.2	1.8	2.0	60.6
250	6.8	6.0	6.4	4.4	3.6	4.0	62.5
750	18.0	18.4	18.2	13.2	11.2	12.2	67.0
1500	38.4	39.2	38.8	27.2	23.6	25.4	65.5
2500	61.6	61.6	61.6	47.2	40.8	44.0	71.4

Mean % Recovery = 65.0%

Method 2

Regression line for set A: $0.0249 X + 0.1131$

Regression line for set B: $0.0175 X - 0.2892$

$$\% \text{ Recovery} = \frac{0.0175}{0.0249} \times 100 = 70.35\%$$

Mean recovery by both methods = 67.7%

Table XII contd

Extraction Recoveries

Method 1

DESMETHYL DIAZEPAM

Concentration Added ng/ml	Peak Heights (cm)						% Recovery
	Authentic standards			Recovered standards			
	(1)	(2)	Mean	(1)	(2)	Mean	
25	0.9	0.9	0.9	0.7	0.7	0.7	77.77
50	1.7	1.8	1.75	1.4	1.3	1.35	77.14
125	4.5	4.5	4.5	3.4	3.2	3.3	73.33
250	9.2	9.6	9.4	6.8	6.4	6.6	70.21
750	24.4	24.4	24.4	20.4	18.4	19.4	79.51
1500	50.0	50.8	50.4	40.0	37.2	38.6	76.58
2500	81.6	81.6	81.6	68.0	62.0	65.0	79.65

Mean % Recovery = 76.31%

Method 2

Regression line for set A: $0.0327 X + 0.3603$

Regression line for set B: $0.0259 X + .0226$

$$\% \text{ Recovery} = \frac{0.0259}{0.0327} \times 100 = 79.24\%$$

Mean recovery by both methods = 77.8%

Table XII contd

Extraction Recoveries

Method 1

TEMAZEPAM

Concentration Added ng/ml	Peak Heights (cm)						% Recovery
	Authentic standards			Recovered standards			
	(1)	(2)	Mean	(1)	(2)	Mean	
25	0.9	0.9	0.9	0.7	0.7	0.7	77.8
50	1.8	1.8	1.8	1.4	1.4	1.4	77.8
125	4.4	4.8	4.6	3.4	3.4	3.4	73.9
250	9.2	9.2	9.2	6.8	6.8	6.8	73.9
750	25.2	25.2	25.2	20.0	19.6	19.8	78.6
1500	52.0	50.8	51.4	40.0	40.8	40.4	78.6
2500	85.2	85.2	85.2	68.8	68.0	68.4	80.3

Mean % Recovery = 77.25%

Method 2

Regression line for set A: $0.0340 X + 0.1734$

Regression line for set B: $0.0272 x - 0.1017$

$$\% \text{ Recovery} = \frac{0.0272}{0.0340} \times 100 = 80.10\%$$

Mean recovery by both methods = 78.7%

Table XII

Extraction Recoveries

Method 1

OXAZEPAM

Concentration Added ng/ml	Peak Heights (cm)						% Recovery
	Authentic standards			Recovered standards			
	(1)	(2)	Mean	(1)	(2)	Mean	
25	0.9	1.0	0.95	0.7	0.7	0.7	73.68
50	2.0	2.0	2.0	1.6	1.5	1.55	77.50
125	5.3	5.4	5.35	3.8	3.8	3.8	71.03
250	10.8	10.8	10.8	7.6	7.6	7.6	70.37
750	29.2	29.2	29.2	22.8	22.8	22.8	78.08
1500	60.0	61.2	60.6	45.6	45.6	45.6	73.55
2500	100.0	99.2	99.6	76.0	76.0	76.0	76.30

Mean % Recovery = 74.4%

Method 2

Regression line for set A: $0.0399 X + 0.1507$

Regression line for set B: $0.0304 X - 0.0059$

$$\% \text{ Recovery} = \frac{0.0304}{0.0399} \times 100 = 76.27\%$$

Mean recovery by both methods = 75.3%

6.4. COMPARISON

The aim of any sample preparation technique is the isolation of the drug from the biological medium in which it occurs in a way that is selective and provides the drug in solution in a quantity appropriate for detection. In this study we compared an on-line solid phase extraction procedure (method A) with a liquid-liquid extraction procedure (method B).

Both methods provide a comparable limit of detection i.e 25ng/ml, but for method A a sample volume of only 250 µl is required, for method B, 1 ml of plasma is used. This is an important factor in pharmacokinetic studies of diazepam and its metabolites in small animals.

The reproducibility of the assays using the two methods as denoted by the % C.V. under intra-assay precision show that method A is superior in terms of precision. It is a simple one step procedure so that losses due to such factors as incomplete extraction and losses during evaporation are eliminated rendering it more precise. The reproducibility of the extraction procedure could be improved by the use of an internal standard but this highlights yet another advantage of the column switching procedure i.e the elimination of the search for a suitable internal standard, giving adequate reproducibility using external standardisation.

Recovery is around 100% using this on-line solid phase extraction procedure. It is much less using liquid - liquid extraction e.g. 67.7% for diazepam, 77.8% for desmethyl diazepam, 78.7% for temazepam and 75.3% for oxazepam.

Column - switching procedures may take longer to develop than extraction procedures but are much less time consuming to operate. The fewer steps and methods used in any given protocol, the simpler, more convenient, cost effective and less time-consuming it is. Simple protocols lead to increased accuracy, selectivity, reproducibility and safety.

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\PPLICATIONS OF COLUMN-SWITCHING TECHNIQUE IN 3IOPHARMACEUTICAL ANALYSIS

. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC)ETERMINATION OF AMITRIPTYLINE AND ITS METABOLITES IN :HUMAN PLASMA

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SUMMARY

A new high-performance liquid chromatographic method for the determination of amitriptyline and its metabolites, nortriptyline, 10-hydroxynortriptyline and 10-hydroxyamitriptyline, in plasma is described which uses direct injection and a column-switching valve. The method is based on the enrichment of drugs on a reversed-phase concentration column, packed with Corasil RP. The enriched drugs were then separated, using back-flush mode on a bonded-phase CN column using an isocratic acetonitrile–acetate buffer (60:40, v/v) mobile phase. The validation of the method showed excellent sensitivity, precision and reproducibility. The limit of detection, using a 250-μl direct injection of plasma, was between 5 and 10 ng/ml for each of the four drugs. The mean coefficient of variation for intra- and inter-assay was better than 5%. The method showed obvious advantages over conventional extraction procedures in terms of speed and ease of sample handling. The method has been successfully applied to the samples from patients receiving oral doses of amitriptyline.

INTRODUCTION

Tricyclic antidepressants, TCAs (Fig. 1), are widely used in the treatment of patients who are suffering from depression. The concentration of these drugs in plasma and the effect on depression symptoms is controversial [1] but it is very important that their therapeutic levels be monitored so that therapy can be made optimal. Several reports have shown that there are large individual variations in plasma concentrations among patients receiving the same dosage of these drugs [2–4].

Most existing methods for the determination of TCAs have been reviewed [5,6]. Gas chromatography (GC) using a variety of detection systems has been applied.

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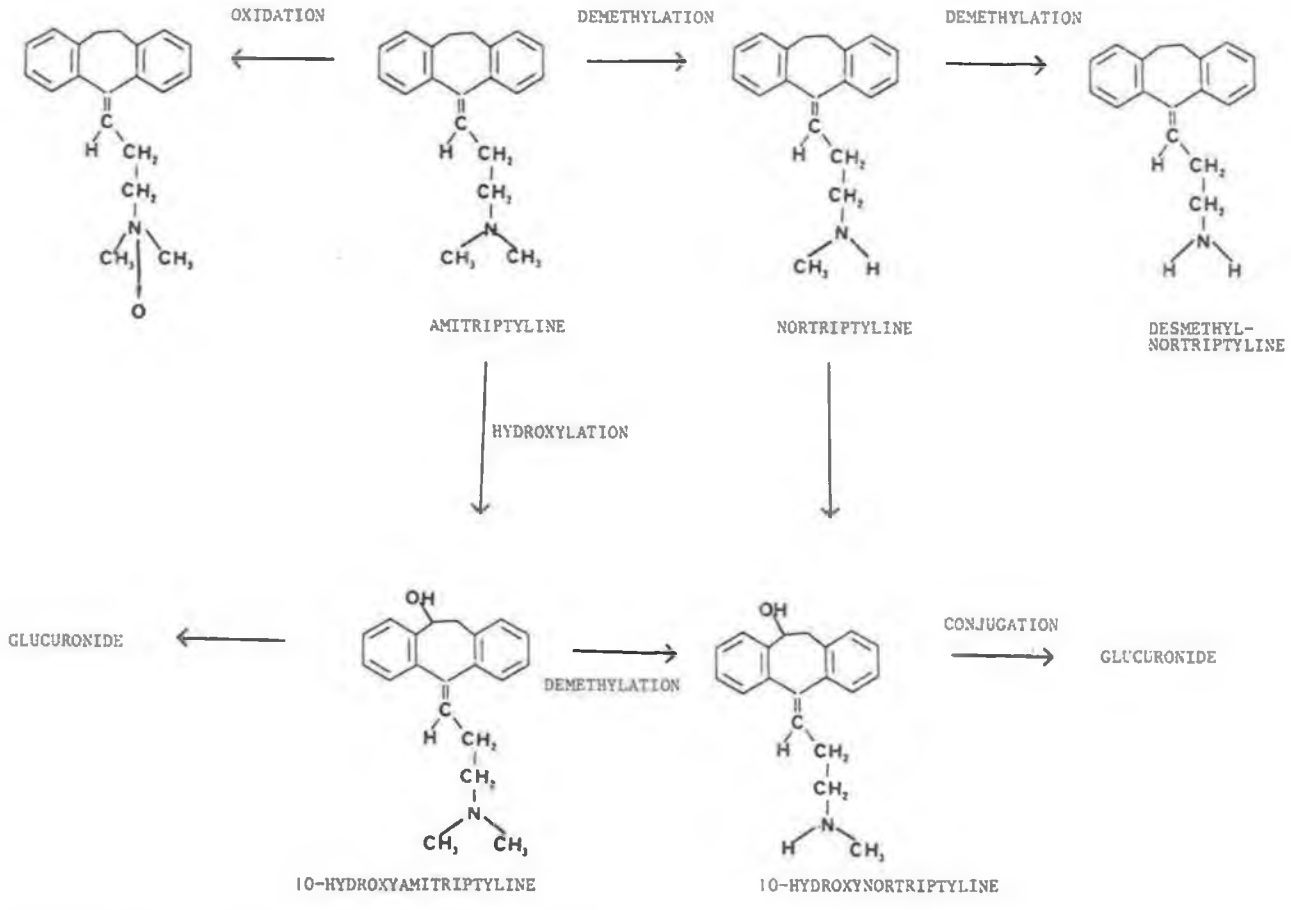

Fig. 1. Chemical structure of amitriptyline and its metabolic pathway.

hese include flame-ionization detection (FID) [7–10], election-capture detecon (ECD) [10–13], nitrogen-phosphorus detection (NPD) [14–16] and mass agmentography [17]. Since 1975 there has been extensive development of high-erformance liquid chromatography (HPLC) as a method of determination for lasma levels of tricyclics and currently rivals GC–NPD as the method of choice 18–26].

Common to all these methods is the need for the extraction of the drugs, which re lipophilic strong bases, from the biological medium at high pH. Although ngle-step extraction may be sufficient for some applications, usually a three-:ep extraction procedure is used with overall recovery of ca. 60–80% while the ecovery for each individual step is around 90%.

Recently, sample clean-up using solid-phase extraction techniques has become opular. The approach, which overcomes the need for lengthy extraction techiques has been successfully applied to the TCAs [20,21,27,28]. Measurement of nese drugs in plasma based on solid-phase extraction on disposable C_{18} bonded-hase columns yielded results in agreement with those obtained using a three-tep extraction procedure [27,28].

This application involves the use of a switching valve which allows on-line ample loading and isolation of the analytes on a "concentration column", fol-owed by rapid elution and direct analysis on the analytical column. The use of olumn-switching techniques has become a major area of interest and its appliation to drug analysis in biological samples has been the subject of a number of ecent publications [29–34].

EXPERIMENTAL

Reagents

10-Hydroxynortriptyline, 10-hydroxyamitriptyline and desmethyldoxepin were btained as a gift from the Laboratory of Clinical Pharmacology and Toxicology, Groot Ziekengasthuis, 's-Hertogenbosch, The Netherlands. Doxepin and protriptyline were obtained from Jervis Street Hospital (Dublin, Ireland) and the other nembers of the tricyclics were obtained as gifts from I.C.P. Dublin. Hexane, nethanol, acetonitrile and isopropanol (all HPLC grade) were purchased from Fisons (Loughborough, U.K.). Ammonia solution, sodium hydroxide, sodium cetate and zinc sulphate were obtained from BDH (Poole, U.K.). Trichloroicetic acid was purchased from May and Baker (Dagenham, U.K.) and perchoric acid from Riedel-de-Haen (Hannover, F.R.G.).

For the preparation of the plasma samples, dried human plasma (from the Blood Transfusion Service Board, Dublin, Ireland) was dissolved in deionised vater (obtained by the Milli-Q water purification system). The control plasma btained was examined for the presence of endogenous components which might nterfere with the tricyclic drugs in the assay system. The reconstituted plasma vas stored frozen and used within two weeks of preparation.

Instrumentation

The HPLC system consisted of two Waters Assoc. (Milford, MA, U.S.A.) P-45 liquid chromatograph solvent delivery systems equipped with a Waters U6K

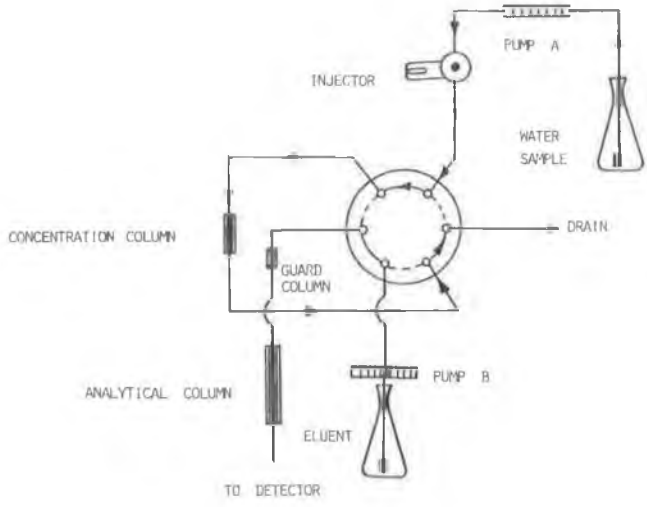

Fig. 2. Operation of column-switching valve for sample enrichment.

manual injector and fitted to a Shimadzu (Tokyo, Japan) SPD-6A variable-wavelength UV detector. The chromatograms were recorded on a Philips (Eindhoven, The Netherlands) PM 8251 single-pen recorder. A Rheodyne (CA, U.S.A.) 7000 six-port switching valve was used.

Chromatography

The instrument arrangement for the chromatography is shown in Fig. 2. The chromatographic conditions for the separation were as follows. Mobile phase pump A: water; concentration column (10×1.5 mm) dry-packed with Corasil (Waters Assoc.) RP C_{18} packing (37–50 μm) in our laboratory; wash time, 1.5 min; flow-rate, 0.8 ml/min. Mobile phase pump B: acetonitrile – 0.05 *M* acetate buffer (60:40, v/v) pH 7; stationary phase, Techsphere (HPLC Technology, Macclesfield, U.K.) 3CN (10 cm×4 mm); flow-rate, 0.9 ml/min; recorder chart-speed, 0.5 cm/min; detection wavelength, 215 nm; injection volume, 250 μl of plasma. Under the described chromatographic conditions the mean retention times (Fig. 3) were as follows: 10-hydroxynortriptyline, 6.0 min; 10-hydroxyamitriptyline, 7.5 min; nortriptyline, 10.0 min; amitriptyline, 12.5 min.

Preparation of standards

Amitriptyline hydrochloride (11.32 mg), nortriptyline hydrochloride (11.39 mg), 10-hydroxyamitriptyline hydrochloride (11.25 mg) and 10-hydroxynortriptyline hydrochloride (11.31 mg) were weighed and dissolved in 100 ml of methanol to yield a stock solution of 100 μg/ml amitriptyline, nortriptyline, 10-hydroxyamitriptyline and 10-hydroxynortriptyline. This stock solution was then diluted with water–methanol (1:1) to yield working standards ranging from 0.2 to 6 μg/ml.

Desmethyldoxepin (11.4 mg) was weighed and dissolved in 100 ml of methanol to yield a solution of 100 μg/ml. This was then diluted with a methanol–water

ixture to yield a solution of 10 μg/ml desmethyldoxepin. Spiked plasma standards ranging from 10 to 300 ng/ml of the drugs in plasma were then prepared in ach assay day by spiking 1 ml of plasma with 50 μl of the working standards and) μl of the internal standards. Patient and blank plasma samples were spiked ith 50 μl of methanol–water mixture to compensate for any changes in the composition of plasma.

olumn-switching procedure

Fig. 2 shows a simple six-port switching valve. The spiked plasma sample is ıjected through the injector port and washed by the water from pump A onto the ɔncentration column. The drugs are held on the concentration column while the ther components in plasma are eluted to the drain. Meanwhile the eluent from ump B is passing through the analytical column and out to waste. On switching ıe valve, the eluent from pump B elutes the drugs, which have been held on the ɔncentration column, in back-flush mode onto the analytical column where they re separated.

ESULTS AND DISCUSSION

In using the column-switching valve the following variations in sample handling were studied: sample pretreatment; concentration column packing; wash ime; flow rates; and analytical column.

ample pretreatment

Protein precipitation prior to injection was tried using (i) acid precipitation sing perchloric acid or trichloroacetic acid and (ii) base precipitation of proeins with sodium hydroxide and zinc sulphate. The proteins were precipitated y addition of the reagent to plasma in tubes which were centrifuged for 15 min; he supernatant was injected for analysis. Direct injection with no sample prereatment was also tried. For all concentrations, acid precipitation gave the lowst recovery, probably due to ionization of the basic tricyclic drugs in the acid olution which were then eluted with the water from pump A and not retained on he concentration column. Base precipitation also gave poor recovery probably lue to co-precipitation of the drugs with plasma. The strong acids and base also lissolved the packing in the concentration column. Over the range of packings ried therefore, best recovery, sensitivity and reproducibility was achieved using ı 250-μl direct injection of plasma. However, as it has been outlined in other ɔublications [29], direct injection is not without its problems. These problems nclude (i) deterioration in the performance of the concentration column due to ɔrecipitation of plasma proteins when they come into contact with the solvents rom pump B, i.e. the mobile phase, and (ii) build up of back-pressure due to ıccumulation of particulate matter in plasma which can be minimized by using he backflush mode.

Concentration column packings

The following packings were tried in the concentration column: LiChrosorb RP C_{18}, 10 μm; Hypersil phenyl, 10 μm; Vydak cyano, 30 μm; Corasil RP C_{18}, 37-

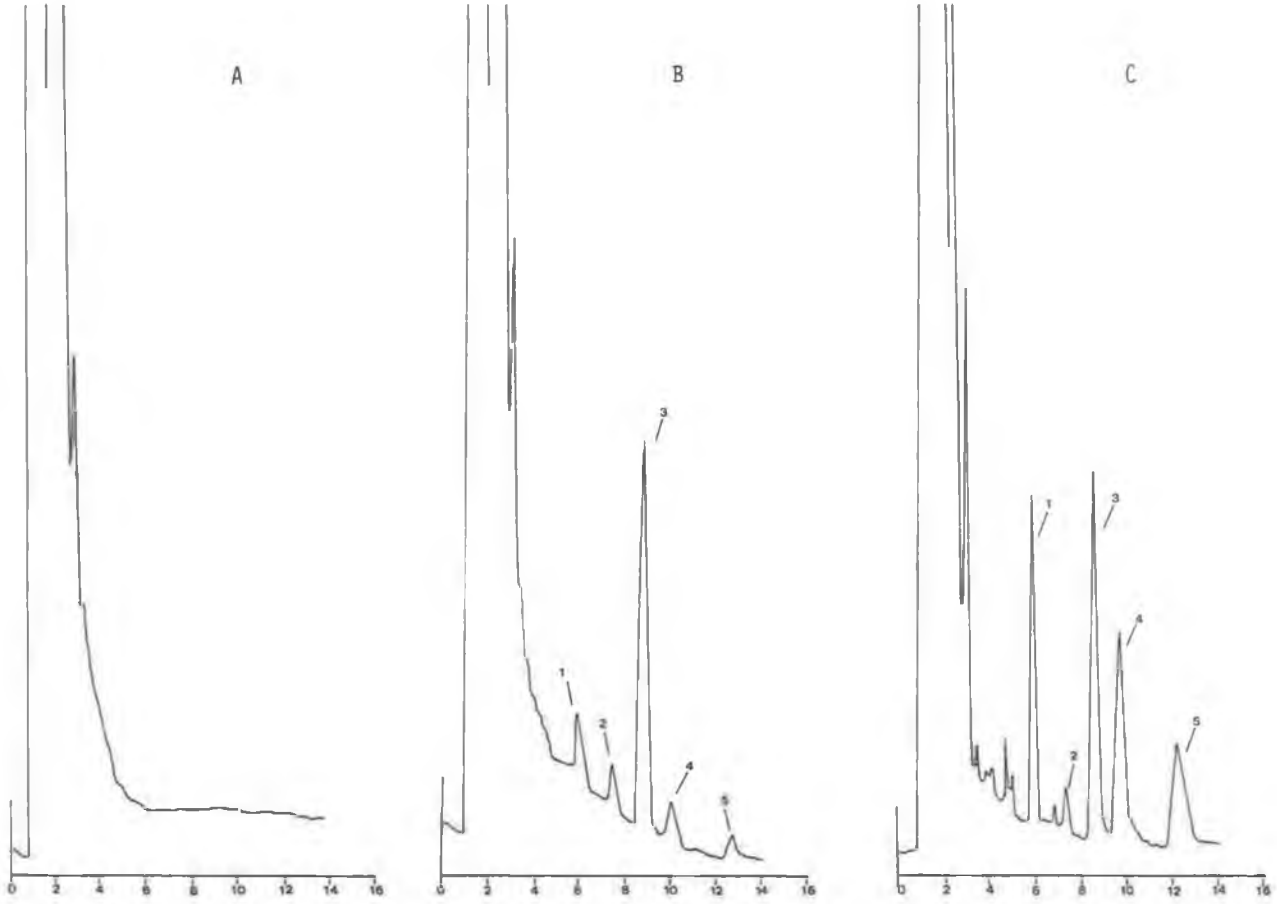

Fig. 3. Chromatograms of (A) a small pooled human drug-free plasma, (B) the same plasma spiked with 10 ng/ml of each of the drugs and (C) plasma of a patient receiving a 50-mg oral dose of amitriptyline. Peaks: 1 = 10-hydroxynortriptyline; 2 = 10-hydroxyamitriptyline; 3 = desmethyldoxepin (internal standard); 4 = nortriptyline; 5 = amitriptyline.

0 μm. The main factor is the size of the packing particles. Packings with particle ize 10 μm do not allow direct injection since the small-size particles act as an fficient filter and after one to two injections become clogged with particulate natter from plasma.

Using 10-μm packings, acid or base precipitation must be used and, as outlined bove, these gave poor recovery. A 30-μm Vydak cyano packing allowed direct njection; however, the column capacity was only 150 μl of plasma. No enhance- nent in sensitivity was achieved by using larger injection volumes.

Corasil RP packing, 37–50 μm particle size, proved to be the best among the ackings tried. Although injection volumes of greater than 250 μl of plasma pro- ided enhanced sensitivity, it limited the number of injections per concentration olumn. An injection volume of 250 μl plasma allowed for at least fifty consecutive njections.

Vash times and flow-rates

Wash times were varied from 1 to 10 min and flow-rates from 0.5 to 1.5 ml/min. 'ariation in these factors did not affect the chromatograms obtained to a large xtent, but the sharpest peaks and cleanest chromatograms were obtained with wash time of 90 s and a flow-rate of 0.8 ml/min. Shorter wash times result in ery large plasma peaks and longer wash times give rise to band broadening.

)ther CN columns

Separation of the drug and its metabolites may also be achieved using other 'N columns. A μBondapak CN column (300×3.9 mm) was tried but it was nec- ssary to vary both the composition of the mobile phase from 60:40 to 70:30 ace- onitrile–acetate buffer and also to vary the molarity of the buffer from 0.05 to .03 *M* in order to obtain separation. However, the peaks obtained were some- vhat broadened and not as well resolved.

Calibration and calculation

Evaluation of the assay was carried out using four-point calibration standards n the concentration range 10–300 ng/ml of drugs in plasma. The slope and inter- ept of the calibration curves were obtained by linear regression of the peak- eight ratios of drug/internal standard versus the concentration of the drug internal standard method).

The internal standard (I.S.) used was desmethyldoxepin which had a suitable etention time (t_R=8.5 min). These calibration curves were then used to inter- olate the concentrations of drugs in patient plasma from the measured peak- eight ratios.

Limit of detection

Using a 250-μl direct injection of plasma, and under the procedural conditions utlined, the limit of detection for each of the four drugs was between 5 and 10 g/ml in plasma. The variation in detection limit was due to day-to-day changes n operational conditions and detection system. The detection limit was taken as he amount of compound giving a signal-to-noise ratio greater than 3:1.

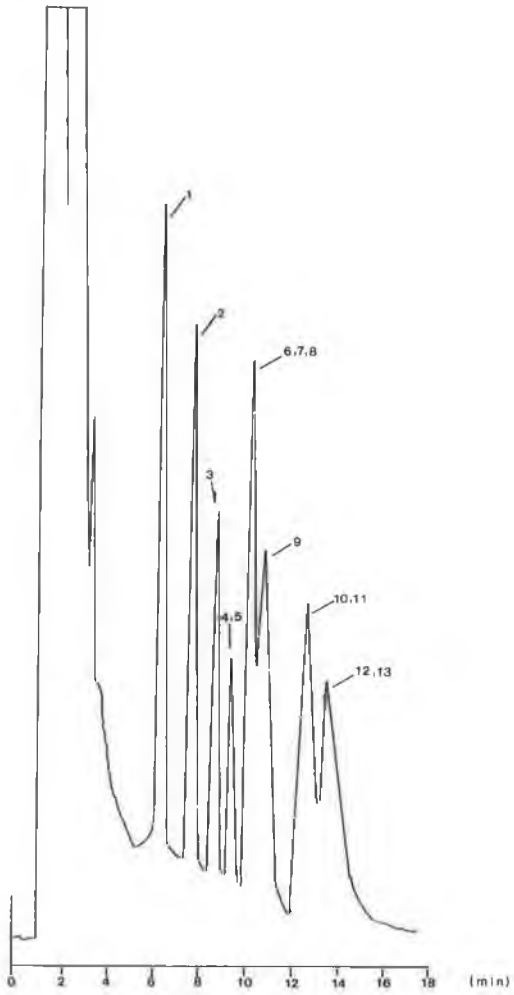

Fig. 4. Chromatogram of a small pooled human drug-free plasma spiked with tricyclic antidepressant drugs. Peaks: 1 = 10-hydroxynortriptyline; 2 = 10-hydroxyamitriptyline; 3 = desmethyldoxepin; 4 and 5 = protriptyline and desipramine; 6, 7 and 8 = trimipramine, cianoprimine and nortriptyline; 9 = doxepin; 10 and 11 = amitriptyline and imipramine; 12 and 13 = chlomiprimine and chloripramine.

Precision

Intra-assay variability was determined at four concentrations in quadruplicate at levels of 10, 50, 150 and 300 ng/ml of each drug in plasma. Inter-assay variability was determined singly and at the same four concentrations in four replicate runs. The precision of the method (mean coefficient of variation) for the values of the recovered determinate standards when calculated as "unknowns" against the linear regression lines for intra-assay and inter-assay were, respectively, 3.3 and 3.8% for amitriptyline, 4.5 and 3.3% for nortriptyline, 3.8 and 4.9% for 10-hydroxyamitriptyline and 3.1 and 5.0% for 10-hydroxynortriptyline.

ABLE I

LASMA CONCENTRATIONS OF AMITRIPTYLINE AND ITS METABOLITES IN HUMAN AMPLES

e text for dosage. Blood samples were taken 10 h after administration. N.D. = not detectable (below nit of detection).

ıtient	Compound	Concentration (ng/ml)			
		Day 1	Day 14	Day 28	Day 42
	Amitriptyline	31.9	54.5	106.7	74.0
	Nortriptyline	22.9	42.2	86.1	61.6
	10-Hydroxyamitriptyline	N.D.	N.D.	6.3	N.D.
	10-Hydroxynortriptyline	33.2	60.1	110.4	82.6
	Amitriptyline	N.D.	36.7	50.1	47.2
	Nortriptyline	N.D.	55.7	47.3	56.7
	10-Hydroxyamitriptyline	N.D.	10.6	5.9	9.6
	10-Hydroxynortriptyline	N.D.	84.5	56.7	64.3
	Amitriptyline	N.D.	N.D.	N.D.	N.D.
	Nortriptyline	N.D.	N.D.	N.D.	N.D.
	10-Hydroxyamitriptyline	N.D.	N.D.	N.D.	N.D.
	10-Hydroxynortriptyline	N.D.	N.D.	N.D.	N.D.
	Amitriptyline	24.6	34.2	36.3	50.5
	Nortriptyline	7.2	30.4	50.4	49.2
	10-Hydroxyamitriptyline	N.D.	N.D.	7.4	6.9
	10-Hydroxynortriptyline	22.4	115.9	114.5	138.7

inearity

Measures of linearity as defined by the correlation coefficient of the regression nes of intra-assays for all drugs were better than 0.999 and the intercepts did ot differ greatly from the origin.

ecovery

The overall recovery was calculated in two different ways. First by comparing ıe peak heights of a series of spiked plasma samples after they had been taken ırough the entire procedure with a series of reference standards. Secondly, by ɔmparing the slopes of the regression lines obtained from the two sets used in ıe first procedure. Using these methods in the concentration range 10–300 ng/ml, ıe mean overall recoveries were 90.74, 92.63, 83.85 and 90.50% for amitriptyline, ortriptyline, 10-hydroxynortriptyline and 10-hydroxyamitriptyline, respectively.

ıterference study

Eight other members of the tricyclic drugs were tested for possible interferıces with the measured substances. As can be seen from the chromatograms in ig. 4, no other member of the tricyclics studied interfered with the determination f 10-hydroxyamitriptyline or 10-hydroxynortriptyline. Imipramine interfered

with amitriptyline and likewise cianopramine and trimipramine interfered with nortriptyline, but it is unlikely that any of these combinations would be administered simultaneously. Tranylcypromine which was given in combination with amitriptyline to some patients did not interfere and eluted with the plasma peak.

Plasma levels

The described method has been successfully applied to the measurement of amitriptyline, nortriptyline, 10-hydroxyamitriptyline and 10-hydroxynortriptyline in patients with neurotic and endogenous depression receiving oral doses of placebo, 50, 75, 100 or 150 mg of amitriptyline alone or in combination with tranylcypromine (Table I). The exact amount of dosage could not be revealed to us at this stage and, therefore, results in Table I are only indicative of the capability of the described method for the analysis of patient samples receiving the above doses of amitriptyline (Fig. 3C).

CONCLUSION

For routine analysis of the tricyclic drugs in plasma, a new method of analysis based on direct-injection column-switching technique was developed. The advantages of this method over conventional extraction methods is that it was less time consuming, needed a smaller volume of plasma and gave better recovery. It has a comparable precision limit with conventional extraction methods. As compared to off-line solid-phase extraction [20,21] methods, it is less time-consuming and more selective, i.e. no unidentified peaks were observed with less running cost. The disadvantages are that the concentration column had to be changed after every fifty injections and it needed more elaborate instrumentation, i.e. two pumps and a column-switching valve.

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Note

Applications of column-switching techniques in biopharmaceutical analysis

II. High-performance liquid chromatographic determination of tripelennamine in bovine plasma and milk

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Tripelennamine, 2-[benzyl(2-dimethylaminoethyl)amino]pyridine (Fig. 1), is an antihistamine which acts as a competitive antagonist of histamine at H_1 receptors [1]. Tripelennamine is used to control allergic manifestations in humans and animals and it has also been used as a stimulant in cows who refuse to stand because of various disorders [2].

The chromatographic methods employed for the determination/detection of tripelennamine include thin-layer chromatography [3–5] to detect tripelennamine in urine when screening for drug abuse and gas chromatography (GC) with flame ionisation detection for the identification of the drug in urine [3,5], to determine tripelennamine residues in bovine milk [2] and in pharmaceutical formulations [6]. GC has also been used with nitrogen-specific detection [7,8] to determine tripelennamine in the blood of addicts taking a combination of pentazocine and tripelennamine known as T's and Blues. High-performance liquid chromatographic (HPLC) methods have been reported for the determination of basic antihistamines including tripelennamine [9] and in animal feed, human urine and waste water [10].

All the above methods either lack sufficient sensitivity or a lengthy and tedious extraction step is required prior to chromatography. The objective of the present study was to develop an HPLC method for the determination of tripelennamine in bovine plasma and milk that is sensitive, selective and reproducible, and yet convenient in terms of sample handling and speed of analysis. In the following sections we describe a technique which fully meets the objectives set out above.

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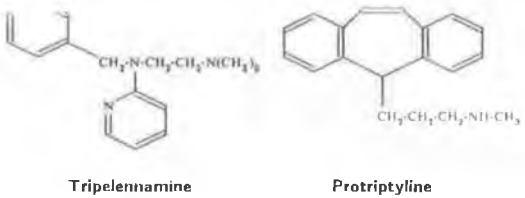

Tripelennamine Protriptyline

Fig. 1. Chemical structures of tripelennamine and protriptyline.

EXPERIMENTAL

Reagents and solvents

Tripelennamine hydrochloride was obtained from Sigma (Poole, U.K.). Protriptyline hydrochloride, internal standard, was obtained as a gift from Jervis Street Hospital (Dublin, Ireland). Methanol and acetonitrile (HPLC grade) were purchased from Fisons (Loughborough, U.K.) and sodium acetate (Analar grade) was purchased from BDH (Poole, U.K.). Pure water was obtained by the Milli-Q water purification system. Drug-free bovine plasma and milk were provided by the Bimeda Chemicals (Dublin, Ireland), stored frozen at $-4°C$ and thawed at room temperature prior to use. The control plasma and milk showed no interference for endogenous components when examined for possible interferences with the tripelennamine assay system.

Preparation of standards

Tripelennamine hydrochloride (11.43 mg) was weighed and dissolved in 100 ml of methanol to yield a stock solution of 100 μg/ml. This stock solution was then diluted with methanol–water (1:1) to yield working standards in the concentration range 0.04–6 μg/ml. Protriptyline hydrochloride (11.39 mg) was weighed and dissolved in 100 ml of methanol to yield a stock solution of 100 μg/ml. This was then diluted with methanol–water (1:1) to obtain a working internal standard solution of 4 μg/ml. Spiked plasma samples ranging in concentration from 2 to 50 ng/ml of the drug in plasma were prepared in each assay day by spiking 1 ml of plasma with 50 μl of working standards (0.04–1 μg/ml) and 50 μl of internal standard (4 μg/ml) solution. Spiked milk samples ranging from 5 to 300 ng/ml of the drug in milk were prepared by spiking 1 ml of milk with 50 μl of working standards (0.1–6 μg/ml) and 50 μl internal standard (4 μg/ml) solution.

Samples from cattle undergoing treatment were spiked with 50 μl of methanol–water (1:1) to compensate for any changes in the composition of the sample plus 50 μl of internal standard (4 μg/ml).

Instrumentation and chromatography

The HPLC system consisted of two Waters Assoc. (Milford, MA, U.S.A.) P-45 liquid chromatograph solvent delivery systems equipped with a Waters U6K manual injector and a Rheodyne (Cotati, CA, U.S.A.) 7000, six-port switching valve. A Shimadzu (Tokyo, Japan) SPD-6A variable-wavelength UV detector

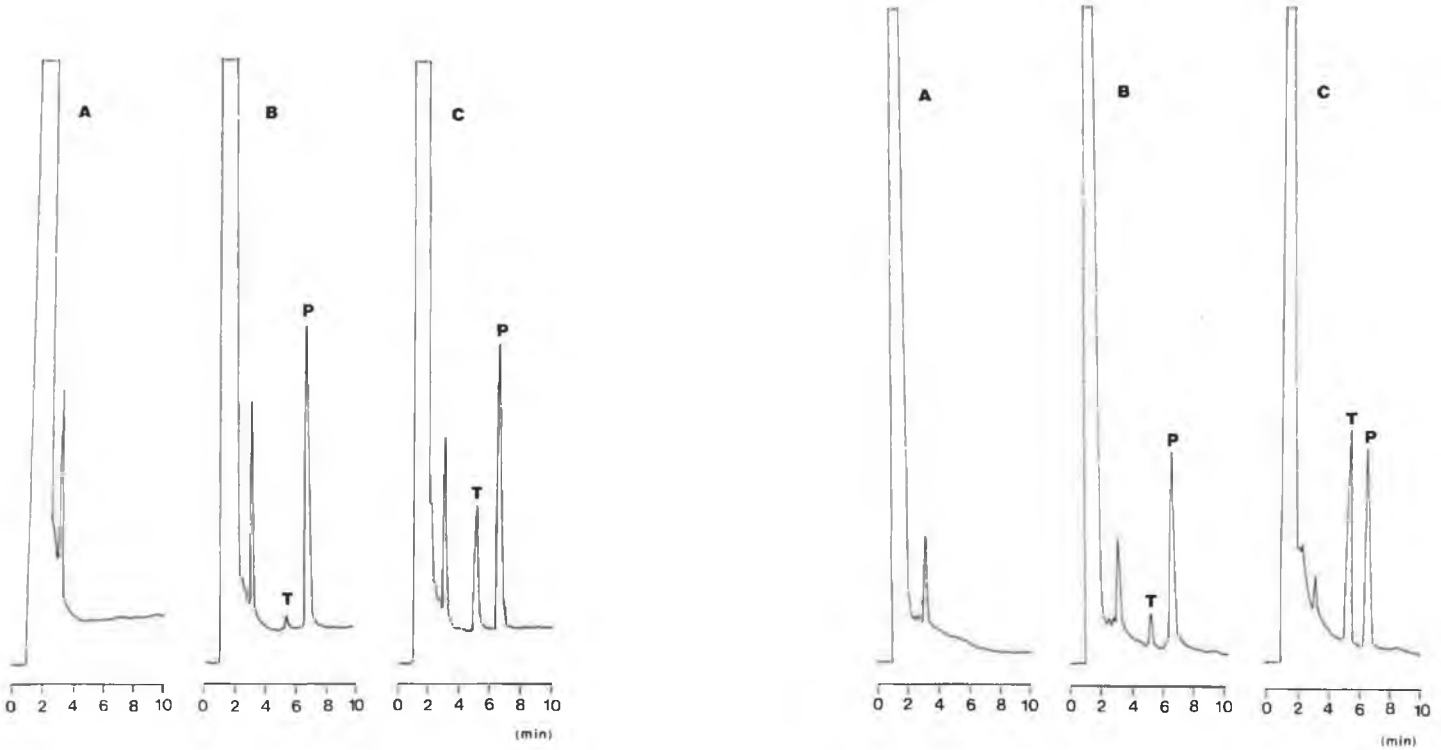

Fig. 2. (Left) Chromatograms of (A) a small pooled bovine drug-free plasma, (B) drug-free plasma spiked with internal standard and 2 ng/ml tripelennamine and (C) plasma of a cattle 30 min after receiving an intravenous injection of a formula containing tripelennamine. (Right) Chromatograms of (A) a pooled bovine drug-free milk, (B) drug-free milk spiked with internal standard and 5 ng/ml tripelennamine and (C) milk of a cattle 30 min after receiving an intravenous injection of a formula containing tripelennamine. Peaks: T = tripelennamine; P = protriptyline.

was used and the chromatograms were recorded on a Philips (Eindhoven, The Netherlands) PM8251 single-pen recorder.

The instrument arrangement for the chromatography was exactly as described in our previous publication [11]. The chromatographic conditions for the separation were as follows: mobile phase pump A, water; concentration column (10×1.5 mm), dry packed in house with Corasil (Waters Assoc.) RP 18, 37–50 μm packing. The mobile phase from pump A was passed through the concentration column for 3 min (wash time) at a flow-rate of 0.8 ml/min. Mobile phase pump B, acetonitrile–0.05 *M* acetate buffer adjusted to pH 7.2 with acetic acid (70:30, v/v); analytical column, Techsphere (HPLC Technology, Macclesfield, U.K.) 3CN (10 cm×4.6 mm); flow-rate, 0.9 ml/min; detection wavelength, 246 nm; injection volume, 250 μl of plasma or milk sample.

Under the described chromatographic conditions the mean retention times were 5.2 and 6.8 min for tripelennamine and protriptyline, respectively (Fig. 2).

Column-switching procedure

The column-switching procedure described by Dadgar and Power [11] was adopted for use in the present study. The bovine plasma was more viscous than human plasma and the wash time was therefore extended to 3.0 min per sample to avoid clogging of the pre-column and the resulting high back-pressure. It was possible to inject up to thirty samples per concentration column. Chromatography deteriorated where sample injections exceeded thirty. Because of high concentration of fat in milk, it was not possible to directly inject the milk samples. Spinning the milk samples in a centrifuge at ca. 1200 *g* to separate fat from the sample proved to be as effective as extraction of fat in hexane. Upon centrifugation of milk samples, three distinct layers were formed. The top layer was the fat and the bottom layer was the precipitated protein. Hence, care should be exercised to inject the middle layer for chromatography. The analysis of separated fat and protein showed no detectable amount of tripelennamine (at a spiked level of 300 ng/ml tripelennamine in milk) in either fat or protein.

Calibration and calculations

Evaluation of the assay was carried out using five-point calibration standards in the concentration range 2–50 ng/ml tripelennamine in plasma and six-point calibration standards in the concentration range 5–300 ng/ml of the drug in milk. The slope and intercept of the calibration curves were obtained by linear regression of the peak-height ratios of drug/internal standard versus the concentration of the drug (internal standard method). These calibration curves were then used to interpolate the concentration of tripelennamine in bovine plasma and milk from the measured peak-height ratios.

RESULTS AND DISCUSSION

Limit of detection

Under the described procedural conditions, the limit of detection using a 250-μl direct injection of plasma or milk was 2 ng/ml tripelennamine in the biological

TABLE I

PRECISION (REPEATABILITY AND REPRODUCIBILITY) ($n=4$)

Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)	Difference between added and found concentration (%)
Intra-assay (repeatability)			
2	1.90 ± 0.19	9.7	5.0
5	4.90 ± 0.33	6.8	2.2
10	10.30 ± 0.25	2.5	3.0
25	25.90 ± 0.49	1.9	3.6
50	49.50 ± 2.33	4.7	1.0
Mean		5.12	
Inter-assay (reproducibility)			
2	2.00 ± 0.18	9.2	0
5	5.30 ± 0.49	9.3	6.0
10	10.40 ± 0.59	5.7	4.0
25	24.30 ± 0.69	2.8	2.8
50	50.20 ± 0.37	0.7	0.4
Mean		5.5	

fluid. The detection limit was taken as the amount of compound giving a signal-to-noise ratio greater than 3:1.

Precision

The data presented in Table I demonstrate the within-batch (intra-assay) and between-batch (inter-assay) variation of the method. Intra-assay variability was determined at five concentrations in quadruplicate at levels of 2–50 ng/ml of the drug in plasma. Inter-assay variability was determined singly at the same five concentrations in four replicate runs. The precision of the method (mean coefficient of variation, C.V.) for the values of the recovered determinate standards when calculated as unknown against the generated linear regression line were 5.1 and 5.5% for intra- and inter-assay, respectively.

The precision study was not carried out for milk samples as the precision of the assay for milk was found to be very similar to that of plasma.

Linearity

Measures of linearity as defined by the correlation coefficients (r) of the regression lines generated based on duplicate calibration curves obtained for plasma and milk were consistently better than 0.999.

Recovery

In order to establish the recovery, two calibration curves based on the external standard method were set up. One calibration curve was based on spiked plasma samples in the concentration range 2–50 ng/ml and the other was based on working reference standards in the same concentration range. The overall recovery was calculated by comparing the slopes of the regression lines generated for the

TABLE II

RECOVERY FROM PLASMA AND FROM MILK

Concentration (ng/ml)	Tripelennamine peak heights (mean of duplicates) (cm)		Recovery (%)
	Set A: working standards	Set B: standards extracted from plasma/milk	
Plasma			
2*	0.9	0.7	78
5	0.9	0.7	78
10	1.9	1.5	79
25	4.5	3.5	79
50	9.1	7.1	77

Mean overall recovery = 78%

Regression line for set A: $y = 0.18133x + 0.0046$, $r = 0.9999$

Regression line for set B: $y = 0.14036x + 0.02612$, $r = 0.9999$

Overall recovery $= \frac{0.14036}{0.18133} \times 100 = 77\%$

Mean recovery determined by the two methods: 77.5%

Milk			
5	0.50	0.35	70
10	0.85	0.65	77
25	2.30	1.75	76
75	7.90	5.70	72
150	15.75	11.90	76
300	31.00	22.60	73

Mean overall recovery = 74%

Regression line for set A: $y = 0.10395x - 0.06310$, $r = 0.999$

Regression line for set B: $y = 0.07612x - 0.00869$, $r = 0.999$

Overall recovery $= \frac{0.07612}{0.10395} \times 100 = 73\%$

Mean recovery determined by the two methods: 73.5%

*More sensitive detector setting.

two sets. The same precedure was applied to the milk samples in the concentration range 5–300 ng/ml tripelennamine. The overall recovery was also calculated by direct comparison of the peak heights. The results obtained are shown in Table II.

Applications of the method

The described method has been successfully applied to the samples from cattle receiving an intravenous injection of a formula containing tripelennamine. For reasons of confidentiality, we were not given permission to reveal the results of the analysis of plasma and milk samples. However, chromatograms from the actual plasma and milk samples are included in Fig. 2. As can be seen, these chromatograms resemble the chromatograms obtained from spiked samples and no interferences were observed from endogenous components of plasma or milk.

CONCLUSION

For routine analysis of tripelennamine in bovine plasma and milk, a new method of analysis based on direct injection/column-switching technique was developed. Plasma samples could be injected directly while milk samples had to be centrifuged first, to separate fat, prior to injection. The method is rapid and eliminates the need for lengthy and tedious liquid–liquid extraction procedures with good recovery and low running cost. The method achieves a low limit of detection comparable with some reported GC methods which require long and elaborate extraction procedures. The chromatography was selective, i.e. there were no interferences from endogenous plasma components, and sharp peaks were obtained for the drug and the internal standard.

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Table 2. Calibration data for ATP - sulphurylase and pyrophosphate

ATP - sulphurylase			Pyrophosphate		
Activity/ U l^{-1}	Output/ V	RSD, %	Concentration/ mol l^{-1}	Output/ V	RSD, %
0	0.85	0	0	0.40	0
0.1	0.90	0	1×10^{-7}	0.60	0
1.0	1.88	3.2	1×10^{-6}	1.00	0
10.0	2.98	1.5	1×10^{-5}	1.73	5.0
20.0	3.56	3.4	1×10^{-4}	1.76	5.0
30.0	3.96	2.7	1×10^{-3}	1.10	0
40.0	4.30	2.6			
50.0	4.35	2.3			

luciferin (30 µl) were simultaneously injected into separate carrier streams, each pumped at 0.75 ml min^{-1} using a peristaltic pump. The two zones were synchronously merged 12.5 cm downstream and passed through a 2.2 cm mixing coil before passing into a glass coil (60 × 2.5 mm i.d.) containing firefly luciferase (0.1 g of beads). The detector was as described previously.[4] A similar manifold was used for the determination of PK. For the determination of PPi and PEP, ATP - sulphurylase and PK, respectively, were immobilised on Sepharose beads (0.1 g) and incorporated within the glass coil upstream of the firefly luciferase. All standards were injected in triplicate for each determination.

Results and Discussion

The reagent concentrations and flow-rates were optimised for each application to give maximum bioluminescent emission. The results obtained for aqueous PK standards (10–60 U l^{-1}) and PEP standards (1.0×10^{-5}–1.0×10^{-2} M) are given in Table 1 and the relative standard deviations (RSDs) for triplicate injections of each standard were in the range 0–5.1%. The results obtained for ATP - sulphurylase (0.1–50 U l^{-1}) and PPi (1.0×10^{-7}–1.0×10^{-3} M) are similarly presented in Table 2, with the RSDs ranging from 0 to 5.0%. Other important general characteristics of this technique include a wide dynamic range and a high sample throughput (>120 h^{-1}).

The results presented for PK and ATP - sulphurylase suggest that this is a useful technique for monitoring enzyme activity levels in serum for diagnostic purposes, the rapid response time (<10 s) and high sensitivity being of particular importance.

The results presented for PEP and PPi demonstrate the versatility and efficiency of the manifold design, whereby two enzymes are immobilised in series within the reaction coil. The range of applications could be extended further by using multi-step reaction sequences involving several enzymes, in which the enzymes could be immobilised in series or co-immobilised on a single support.

Conclusions

The flow injection manifold described above is suitable for the indirect determination of a wide range of enzymes and substrates that can be linked with the firefly luciferase - luciferin - ATP reaction.

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HPLC Determination of Tricyclic Antidepressants in Human Plasma

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Since 1975 there has been extensive development of HPLC as a method for the determination of plasma levels of the tricyclic drugs.[1–9] Most of the existing methods are similar. They require solvent extraction from basic plasma, followed by ion-pair partition, adsorption, bonded phase or reversed phase liquid chromatography with a number of different types of stationary and mobile phases in each mode. In this paper we describe an improved HPLC method based on the extraction of the drugs amitriptyline (A) and nortriptyline (N) from plasma (method 1), which is comparable with or better in most of its features than existing methods. Also, a new HPLC method based on a direct injection - column switching technique (method 2) will be described and the two methods will be compared.

Experimental

Method 1

Plasma (1.5 ml), spiked with the drugs and internal standards imipramine (I) and desipramine (D) for A and N, respectively, was mixed with 0.1 M sodium hydroxide solution (0.4 ml) and the drugs were extracted into hexane - isoamyl alcohol (99 + 1). After centrifugation, the supernatant was transferred into clean polypropylene tubes and the solvent evaporated under a stream of nitrogen. The residue was next reconstituted in 100 µl of methanol and an aliquot of 70 µl volume was injected for HPLC analysis. The separation of drugs was carried out on an adsorption column, 300 × 3.9 mm, commercially packed with µPorasil 10 µm, using methanol containing 0.1% ammonia as the mobile phase at a flow-rate of 1.5 ml min^{-1}. The drugs were detected by ultraviolet absorption at 215 nm. The limit of detection for A and N in plasma was between 1 and 5 ng ml^{-1}, where the signal to noise ratio was greater than 3:1. Mean percentage coefficients of variation for intra-assay and inter-assay precision were, respectively, 1.9 and 5.5% for A and 2.1 and 3.8% for N. Linearity as measured by the correlation coefficient of the intra-assay linear regression line was 0.999 for both drugs. The mean over-all recoveries from plasma for A and N were, respectively, 80.8 and 71.0%. All validations were carried out in the concentration range 10–200 ng ml^{-1} of drugs in plasma. Fig. 1 shows chromatograms of a control and a spiked plasma sample.

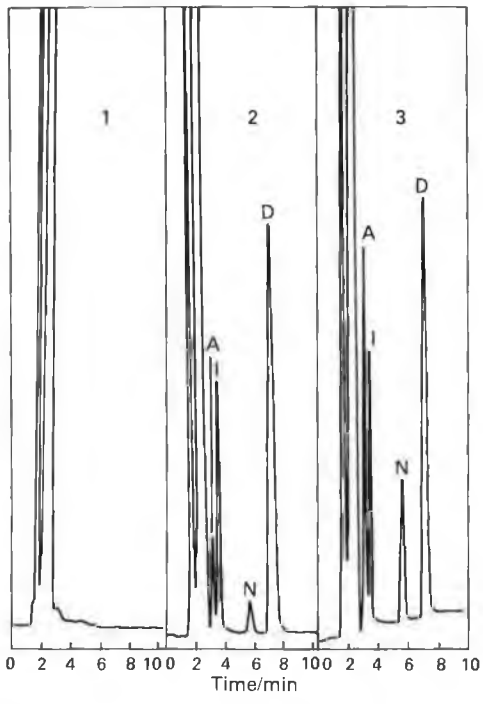

Fig. 1. Chromatograms of a human drug free plasma (1), and a human drug free plasma spiked with the drugs (25 ng ml^{-1}, 2, and 100 ng ml^{-1}, 3) and internal standards

Method 2

Plasma (250 µl) spiked with the drugs and the internal standard, trimipramine (T), was vortex mixed and directly injected through the injector port and washed by the water in pump 1 on to the concentration column (10 × 1.5 mm), packed with Corasil RP, 37–50 µm. On switching a simple 6-port switching valve, the eluent from pump 2 eluted the drug from the concentration column in back flush mode on to the analytical column, where they were separated. The mobile phase in pump 2 was acetonitrile - 0.03 M acetate buffer (70 + 30) and the analytical column was Waters µBondapak CN (300 × 3.9 mm).

The flow-rate from pump 2 was 2 ml min^{-1} and drugs were detected at 215 nm. The limits of detection for both drugs were between 8 and 12 ng ml^{-1}. The mean percentage coefficients of variation for intra-assay and inter-assay precision were, respectively, 3.4 and 4.5% for A and 4.1 and 4.4% for N. Linearity, as measured by the correlation coefficient of the intra-assay linear regression line, was 0.999 for both drugs. The mean over-all recoveries from plasma for A and N were, respectively, 85.4 and 89.0%. All validations were carried out in the concentration range 15–300 ng ml^{-1} of drugs in plasma. Fig. 2 shows chromatograms of a control and a spiked plasma sample.

Results

The method described based on extraction has been applied to the determination of plasma levels of A and N in man after oral administration of A. For quality control, each batch of samples received from the hospital contained a couple of split samples which were unknown to us. Some representative results of these split samples from the patients are given in Table 1.

Method 2 has also been applied to patient's plasma. Two extra peaks were observed, one as a shoulder after the internal standard peak and the other before the amitriptyline peak. These extra peaks were not present in any placebo or pre-dose samples and therefore could not constitute endogenous components of plasma. The natures of these interfering peaks are being studied at present as we have just received other metabolites of amitriptyline. The results of plasma samples based on method 2 were generally lower than those obtained by method 1.

Conclusions

For routine analysis of tricyclic drugs in plasma, two methods were developed. The advantages of direct injection were that it was less time consuming, needed a smaller volume of plasma and had better isolation power than extraction prior to chromatography with a comparable precision and detection limit. The disadvantages were that the concentration column had to be changed after every 30 injections and it needed an extra pump and a switching valve. Work is still being carried out to extend the capabilities of the direct injection - column switching technique for the analysis of other tricyclic drugs and their metabolites.

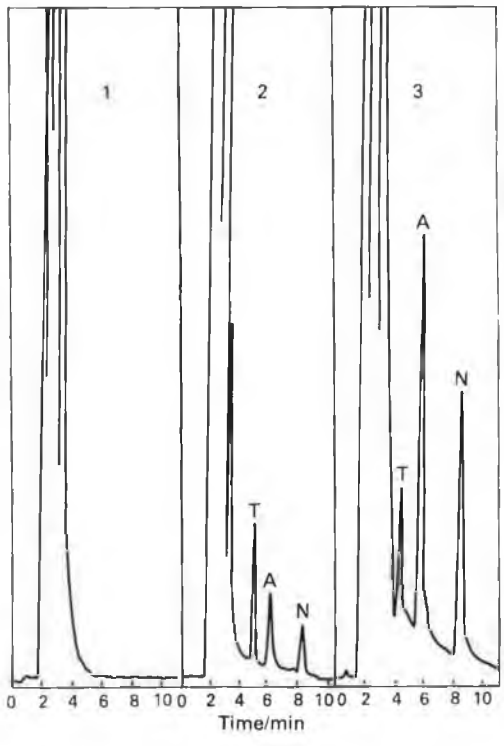

Fig. 2. Chromatograms of a human drug free plasma (1), and a human drug free plasma spiked with the drugs (37 ng ml^{-1}, 2, and 300 ng ml^{-1}, 3) and the internal standard

Table 1. Plasma concentration of A and N in patients with neurotic and endogenous depression receiving oral doses of amitriptyline. Samples had been split into two parts and their code was revealed after analysis

Dosage/ mg	Concentration found/ng ml^{-1}			
	Part 1		Part 2	
	A	N	A	N
100	58.2	107.4	66.0	102.9
100	76.4	62.4	85.5	66.9
100	12.8	17.5	12.8	17.5
75	169.9	44.4	150.1	44.4
75	21.9	31.0	21.9	31.0
Placebo	N.D.*	N.D.	N.D.	N.D.
150	248.8	187.8	226.6	211.4
Placebo	N.D.	N.D.	N.D.	N.D.

* N.D. = Not detectable (below limit of detection).

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Analytical Applications of Microemulsions

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Surfactant molecules, with their distinct hydrophilic and hydrophobic regions, have a tendency to collect at any interface where the hydrophobic groups (tail) can be partially or completely removed from contact with water and the hydrophilic groups (head) can remain wetted. Some of the possible structures that can result from oil - water - surfactant mixtures are shown in Fig. 1. The presence of such structures can modify chemical equilibria, reaction rates and reaction pathways[1,2] and their analytical applications extend to a variety of electrochemical, spectroscopic and chromatographic techniques.[3] The work reported here is concerned specifically with analytical applications of microemulsions prepared using the surfactant bis(2-ethylhexyl) sodium sulphosuccinate (Aerosol OT; AOT).

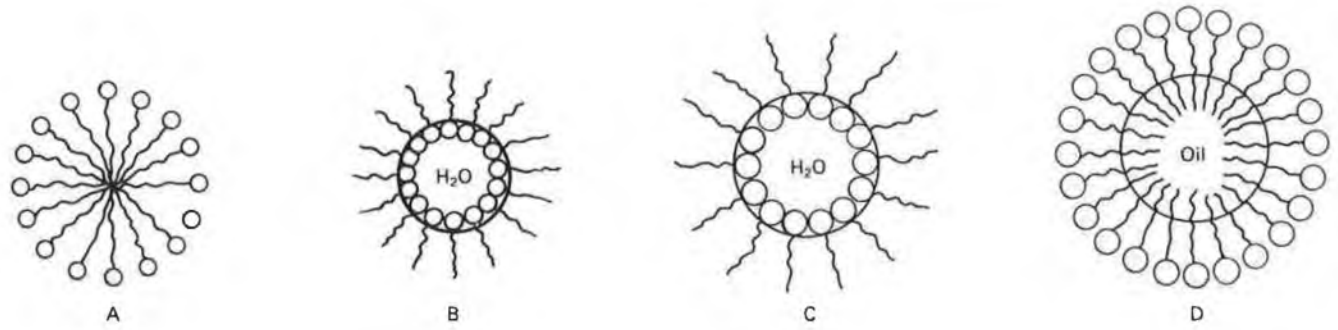

Fig. 1. Analytically useful organised surfactant structures. A, Aqueous micelle; B, reversed micelle; C, water in oil microemulsion; D, oil in water microemulsion

Microemulsions are thermodynamically stable, optically transparent, monodisperse droplets with droplet sizes in the range 5–100 nm.[4] These droplets can be formed by dispersing water in an apolar medium [water in oil (w/o) microemulsions] or by dispersing oil in an aqueous medium [oil in water (o/w) microemulsions] using a suitable surfactant or surfactant - co-surfactant mixture. The size of the water pool in w/o microemulsions can be altered by changing the water to surfactant molar ratio (R). This water pool has a low effective polarity[5] and a high microviscosity[6] relative to bulk water, which can lead to changes in equilibria, solubility, etc.

The work reported here demonstrates the analytical potential of microemulsions using a flow injection manifold with fluorimetric detection. In particular, the two systems studied demonstrate the possibility of reaction between water soluble reagents and oil solubie samples.

One is the reaction between *o*-phthalaldehyde - 2-mercaptoethanol (aqueous) and C_6–C_{10} primary amines (non-aqueous)[7] and the other is the enzymatic cleavage of 4-methyl umbelliferyl heptanoate (non-aqueous) by lipase (aqueous).[8]

Experimental

Reagents

OPA/2-ME reaction. Primary amine stock solutions (0.01 M) were prepared by dissolving hexylamine (133 µl), octylamine (166 µl) and decylamine (199 µl) in heptane (100 cm³) and

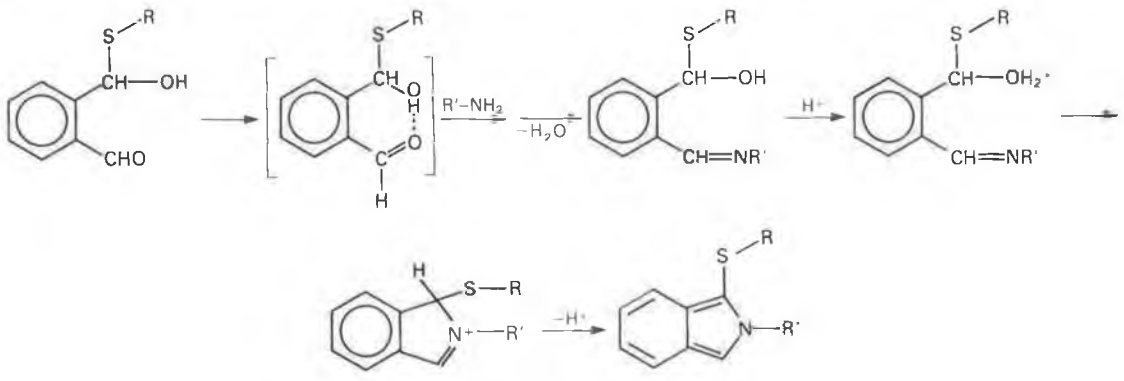

Fig. 2. Mechanism for the OPA/2-ME reaction with primary amines