SOME NOVEL APPROACHES TO CHROMATOGRAPHIC AND ELECTROPHORETIC SEPARATIONS IN BIOPHARMACEUTICAL ANALYSIS

By

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed Michaela Walshe ID No: 92700594

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Date 26/9/96

Dedicated to Liam

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ABBREVIATIONS

AUFS	Absorbance units full scale
BAM	Benzamidine
BSA	Bovine serum albumin
CD	Cyclodextrin
CZE	Capillary zone electrophoresis
DZM	Dual zone material
EDMA	Ethylene glycol dimethacrylate
EOF	Electric osmotic flow
HSA	Human serum albumin
IEC	Ion-exchange chromatography
ISRP	Internal surface reverse-phase
ITA	Itaconic acid
LSC	Liquid-solid chromatography
MAA	Methacrylic acid
MI	Molecular imprinting
MIP	Molecularly imprinted polymer
ODS	Octadecylsilane
7-OHC	7-hydroxycoumarin
PAH	Polyaromatic hydrocarbon
PS-DVB	Polystyrene-divinylbenzene
RP	Reversed-phase
RP-HPLC	Reversed-phase high performance liquid chromatography
SAX	Strong anion exchanger
SCX	Strong cation exchanger
SEC	Size exclusion chromatography
SHP	Shielded hydrophobic phase

SPE	Solid-phase extraction
TCA	Tri-cyclic antidepressant
TLC	Thin layer chromatography
PAM	Pentamidine

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ABSTRACT

Recent developments in the field of stationary phases for liquid chromatography and their use as solid-phase extraction(SPE) materials are discussed in chapter 1. In chapter 2, the use of a mixed-mode stationary phase (C18/SCX) for HPLC and its retention properties was then examined by variation of a number of parameters, including pH, ionic strength and buffer cation. A comparison was also carried out using a conventional C18 column.

The use of this mixed-mode column was further investigated in chapter 3 for the simultaneous determination of propranolol and furosemide in human plasma. Development of the chromatography and the extraction procedures from first principles are also described. Two sample clean-up procedures were examined: liquid/liquid extraction and column switching. Each of these methods was validated and a comparison of the methods was carried out.

Chapter 4 then describes how "molecular imprinting" was used to create a chiral environment which would allow the separation of one enantiomer from the other, and which might find application in the area of solid-phase extraction and capillary zone electrophoresis. This work was based on the monomer most commonly used in molecular imprinting, i.e. methacrylic acid. It was then decided to modify this monomer and incorporate a chiral group, and use this monomer for polymerization against the target molecule propranolol. Polymers were also prepared using methacrylic acid to 7-hydroxy-coumarin (7-OHC) under a number of conditions.

In chapter 5, the polymer prepared to 7-OHC was packed into cartridges and applied to the determination of 7-OHC in urine by capillary zone electrophoresis (CZE). The polymer prepared to S-propranolol was also used as an additive in the buffer to enhance the CZE separation between the R- and S- enantiomers of the parent drug.

The thesis concludes with a critical appraisal of the work carried out in the thesis and suggestions for future research.

CHAPTER ONE

STATIONARY PHASES AND PSEUDO-STATIONARY PHASES

1.1 INTRODUCTION

Chromatography was first reported as early as 1903, when Tswett separated chlorophylls from plants employing a calcium carbonate bed with petroleum as an eluting system.[1] Thin-layer chromatography (TLC)[2] was then developed, and following this, Martin first described the method of liquid/liquid partition chromatography and laid the foundation for gas-liquid chromatography (GLC).[3] Liquid chromatography developed further during the sixties due to the onset of high performance (or pressure) liquid chromatography (HPLC).[4] During the late 60's and early 70's, with the advent of solid-phases onto which moieties could be chemically bonded, the dominant position of inorganic oxides as stationary phases declined considerably. HPLC technology then evolved around the production of support materials which offered improved rates of mass transfer by reducing the distance over which solutes have to diffuse. Impervious glass beads were coated with the stationary phase resulting in shorter diffusion paths, and the second method involved the production of particles which were totally porous, but of smaller diameter (about 40 μm). Porous particles have lead the way forward, and today, columns contain particles in the range 3-10 μm in diameter. Many studies on the technology of bonding to a variety of stationary phases occurred with Halasz producing the first bonded phase by reacting silica with alcohols[5] and amines.[6] The 1980's saw a refinement in bonding procedures, especially with regard to phase reproducibility. Today, a wide range of bonded supports can be tailored to suit a variety of analytical needs, and the use of specialized phases has become more prominent, in particular for the separation of chiral compounds (i.e. different enantiomers). Other specialized phases have been developed, such as shielded hydrophobic phases, internal surface reversed phases and molecularly imprinted polymers (MIP's).

1.2 MECHANISMS IN HPLC

HPLC is a natural progression from classical column liquid chromatography and can encompass almost all types of chromatography, i.e. adsorption, partition, ion-exchange and size exclusion. Chromatography is a physical method of separation in which the compounds to be separated are distributed between a mobile phase and a stationary phase. The chromatographic process occurs as a result of repeated sorption onto and desorption from the stationary phase by sample components as they traverse the column bed. Separation occurs between individual components due to the differences in their distribution coefficients between the two phases. Components migrate at different rates due to differences in polarity, solubility, ionic charge or size.

1.2.1 *Liquid-Solid Chromatography*

The principle of adsorption chromatography is the same as the basis of classical column and thin layer chromatography. Separation occurs by the reversible adsorptive interaction of the solute with a solid adsorbent. It is dependent on the relative polarities of the solute and adsorbent. The stationary phases for liquid-solid chromatography (LSC) most commonly used are silica, alumina glass beads and polymer beads. It's main uses are for components which are soluble in non-polar or moderately polar solvents (e.g. heptane, hexane and methylene chloride).

1.2.2 *Reversed-phase Chromatography*

In this technique a hydrophobic stationary phase is bonded onto a solid support to produce a non-polar surface and used in conjunction with a polar

hydro-organic mobile phase. The name "reversed-phase" refers to the fact that the relative polarity of the stationary and mobile phases are opposite to those used in LSC. This particular type of chromatography is the most common, and is widely applicable to the separation of solutes of different polarity, molecular weight and chemical functionality. Reversed-phase (RP) chromatography has found a number of uses in recent years, including the trace analysis of basic drugs in biological fluids. Another factor in the widespread use of RP-HPLC is the convenience, versatility and high efficiency of silica-based, microparticulate, bonded-phase chromatographic supports. However, the selectivity of reversed-phase systems is insufficient for a number of reasons; severe peak tailing associated with the separation of basic compounds which has been attributed to polar interactions of the amino-functionalities of the analyte to active sites, notably unreacted silanol groups on the modified silica surface, and poor column efficiencies are commonly encountered problems. This chromatographic behaviour means that basic compounds are retained on modified silica by means other than lipophilic retention mechanisms. It is possible to improve selectivity for polar and ionic molecules by manipulation of secondary chemical equilibria. These equilibria are affected by changes in the mobile phase composition, the use of buffers and the adjustment of pH (ion suppression), and the addition of ion-pair reagents (ion-pairing). Complexing agents and chiral reagents can also be added to the mobile phase to achieve selectivity between optically active isomers.

1.2.2.1 *Reversed-phase Ion Suppression*

For organic compounds which contain ionisable groups, the method of choice has traditionally been reversed-phase chromatography employing a buffered mobile phase to control the degree of ionization, and hence the retention of the compound on the hydrophobic stationary phase. For separation of weak

acids by reversed-phase techniques, it is necessary to lower the pH and thus suppress ionization of the acid and drive the equilibrium of the reaction below to the left.

$$RCOOH + H_2O \rightleftharpoons RCOO^- + H_3O^+$$

For basic compounds the equilibrium is driven to the right, as shown in the equation below.

$$RNH_3^+ + OH^- \rightleftharpoons RNH_2 + H_2O$$

This technique is suitable for the determination of weak acids and bases in the pH range 2 - 8. At pH values below 2, stripping of the bonded phase occurs while at high pH dissolution of the silica packing occurs.

1.2.2.2 *Ion-Pair Chromatography*

Strong acids and bases cannot be separated by ion suppression. However, by forming an ion-pair with a suitable counter-ion, ionic or ionizable compounds can be converted to electrically neutral and non-polar compounds, and therefore can partition into the respective non-polar phase. A large organic counter-ion is added to the mobile phase (at a low concentration of the order 0.005 M) to form a reversible ion-pair complex with the ionized sample. The ion-pair reagent is itself ionized, and one ion of the reagent is retained by the stationary phase then providing the otherwise neutral stationary phase with its charge. This charged stationary phase can retain and separate organic solutes ions of the opposite charge by the formation of a reversible ion-pair complex. The following equilibrium represents the coulombic association species formed between two ions of opposite electrical charge with the ionized sample:

$$RCOO^- + R_4N^+ \rightleftharpoons [R_4N^+, {}^-OOCR]^0 \text{ ion-pair complex}$$

Therefore, by using a suitable counterion, ionic (or ionizable) compounds can be converted to electrically neutral compounds that will partition between the mobile and non-polar stationary phases, while simultaneously allowing the non-ionized organic substances to interact with the stationary phase. Ion-pairing, despite its flexibility and popularity, has a number of disadvantages associated with it, including long equilibration times, sample contamination from foreign ions, the inability to apply solvent gradients, decreased column lifetimes, and sometimes poor reproducibility. The most important factor to consider is the pH of the mobile phase, as it is necessary that both the counterion and solute are ionised. It is also necessary that the counterion should be univalent, aprotic and soluble in the mobile phase. Charged surfactants at concentrations below the critical micelle concentration are widely used as mobile phase modifiers in ion pairing chromatography. Bidlingmeyer *et al.*[7] have used ion-pairing to separate a number of ionic compounds, including phenacetin, phenylpropanolamine and pyrilamine.

1.2.2.3 *Micellar Liquid Chromatography*

Separation of compounds depends on the ability to control the analyte-solvent interactions by changing the composition of the mobile phase. One method of modifying the mobile phase is by the addition of surfactants at low concentrations to enhance the separation of oppositely charged solutions. When surfactant solutions are present at high concentrations and micelles co-exist with monomers and dimers, this can offer a further degree of selectivity to separations because of the possibility of interaction between the analyte and the micelle. This is known as micellar liquid chromatography.

Surfactants exist in a number of different forms in which the hydrophobic end varies from 8-20 carbon atoms and the hydrophilic portion can be a partially dissociable carboxylate ion, a fully-ionised moiety (such as anionic sulphate or cationic trimethylammonium ions), plus counterion, or an uncharged species such as polyethylene glycol. They can also vary in their charge type (zwitterionic, positive, negative or neutral), but the nature of the hydrophobic portion can also vary (single, double or multiple bonds). At low concentrations in aqueous solution, surfactant molecules are dispersed as monomers, and to a lesser extent as dimers and trimers. Above a certain concentration the monomers aggregate into micelles which are roughly spherical in shape, and consist of approx. 60-100 monomers (the aggregation monomer). As the surfactant concentration is increased above the critical micelle concentration (CMC), more micellar assemblies are formed with the monomer concentration remaining approximately constant and equal to the CMC. The most commonly used in chromatography are sodium dodecyl sulphate and cetyltrimethylammonium bromide. For instance, Armstrong *et al.*[8] employed aqueous based mobile phases, containing 0.1 - 0.2 M sodium dodecyl sulphate (SDS) to separate a range of phenols and polynuclear aromatic hydrocarbons. They showed that the presence of micelles in the mobile phase provides a hydrophobic site for interaction with the solute, and that surfactant solutions overcome the need for traditional organic modifiers, e.g. acetonitrile or methanol. In micellar systems, additional interactions of an electrostatic, hydrophobic and steric nature can occur, and these additional interactions can add an added degree of selectivity to the separation of a drug or other analytes by HPLC.

It has been found that retention of analytes generally decreases with increases in surfactant concentration, but the rate of decrease is strongly analyte dependent and this frequently produces reversals in orders of retention. These inversions are the result of two competing equilibria, solute-stationary phase interactions and solute-micelle interaction. Micellar liquid chromatography has found particular application

for the direct analysis of drugs in biological fluids by HPLC. For instance, Cline-Love *et al.*[9] determined a number of drugs in urine by direct injection. The surfactant used for the determination of furosemide, hydrochlorothiazide and propranolol was polyoxyethylene 23 lauryl ether (Brij 35). It was found suitable for the simultaneous determination of both hydrophilic and hydrophobic substances.

1.2.3 *Ion Exchange Chromatography*

While reversed-phase separation of charged analytes may be accomplished by ion-pairing techniques, recently, separations of charged species have been carried out on highly efficient HPLC phases, specifically designed to act as ion exchangers. Cations are separated on phases modified with a fixed sulphonic acid group [$-C_6H_4SO_3^-H^+$] with cationic mobile counter ions, while anions are separated on phases modified with quaternary ammonium group [$-(CH_2)_nNR_3^+X^-$] with anionic mobile counter ions. The mechanism by which these systems work is based on displacement of the counter ions from the charged sites. The analyte binding may be modulated by the inclusion of competing cations in a buffered aqueous mobile phase.

1.2.4 *Size Exclusion Chromatography*

This form of chromatography is essentially a separation method, based on the relative molecular mass of different molecules. The stationary phase consists of a pore structure of controllable size. Small molecules can enter these pores and will be retained longer, while larger molecules which cannot enter the pores will elute more quickly. Molecules which are of intermediate size will be moderately retained. These phases are mainly used for the separation of compounds which have molecular masses above 2000 daltons e.g. polymers and large biomolecules.

1.3 COLUMN PACKING SUPPORTS

A number of inorganic supports exist; however, the use of silica gel-based packings still dominates. The major limitation of silica gel, however, is that it cannot be used in systems (of pH above 8.0). This has encouraged the development of alternative materials, including alumina, carbon and polymer-based packings.

1.3.1 *Alumina*

Alumina is normally basic in nature, but with suitable processing acidic and neutral types can also be prepared, the latter finding use in adsorption chromatography. Alumina has also found application as an ion-exchange material and due to its amphoteric nature; basic alumina has found use as a weak cation exchanger while the acidic form is used for anion exchange. This material finds application at extremes of pH where silica is unsuitable, in particular for the determination of strongly basic compounds at pH values as high as 12. Retention can be controlled by a number of parameters including ionic strength, nature of the counter-ion and pH. The selectivity of ion-exchange chromatography can be substantially improved by addition of organic solvents to the aqueous mobile phase. It is more prone to chemisorption problems than is silica, particularly when acidic components are involved and tailing may result. It has, however, fewer theoretical plates than comparable silica columns.

Laurent *et al.*[10] used aluminium oxide as a cation-exchange material, by the addition of competing ions to a mobile phase consisting of an aqueous buffer and an organic modifier (acetonitrile or methanol). Determination of protonated amines was achieved with this type of ion exchange system. Although the discriminating power of the aluminium oxide system is comparable with that of

silica gel-based systems, the retention behaviour of organic cations is more complex, and therefore not easily predictable. This can be explained by the substantial number of parameters that influence the retention and the pronounced amphoteric character of aluminium oxide. Aluminium oxide shows anion-exchange properties at pH values below its zero point of charge (ZPC), and cation-exchange behaviour above this pH value. This means that for the analysis of positively charged compounds, the pH of the mobile phase must be higher than the ZPC.

1.3.2 *Carbon Sorbents*

Carbon sorbents[11] have a number of advantages in comparison with silica gels, including their high chemical resistance to most mobile phases and their total inertness towards separated substances. Both glassy carbon and porous graphitic carbon have proved to be very strong adsorbents; however, this can be moderated by a number of methods, including use of a properly chosen mobile phase composition, a polymer coating, or by chemical modification of the surface. The rate of mass transfer within carbon-based sorbents is usually somewhat lower than obtained within silica gels. Other advantages of carbon is that it has a wide pH range, that it shows good retention for polynuclear aromatic hydrocarbons, it exhibits different selectivity from reversed-phase silica, and is relatively inert. It has a number of disadvantages, however, including its lack of rigidity, and its poor inefficiency for strongly retained components.

1.3.3 *Polymer-based sorbents*

The use of rigid macroporous copolymers for HPLC has its origins in the work carried out by Peterson.[12] They described how proteins could be adsorbed

by diethylaminoethyl (DEAE) derivatized cellulose under certain conditions and then subsequently eluted by increases in the ionic strength of the mobile phase. Moore[13] synthesized macroporous poly(styrene-divinylbenzene) materials with a high percentage of crosslinked, divinylbenzene. The polymerization was carried out in the presence of a porogen, a compound which is soluble in the monomers but not in the polymer. This produced rigid spherical particles containing large pores/voids. The polymers were prepared by mixing the monomer, the crosslinker and the initiator in the presence of a suitable porogen. The mixture is stirred rapidly with water to produce organic phase droplets equivalent to the particle size of the polymer bead required. The polymerization proceeds in the droplets of the organic phase, with the growing polymer chains precipitating in the droplet as they reach a critical size. The crosslinker functions to produce a three-dimensional structure. The porogen voids in the polymer network are macropores; therefore by suitable choice of the porogen the pore size, pore size distribution and pore geometry can be optimised for HPLC separations.

The driving force behind the development of polymeric columns is the inherent pH instability of silica-based columns, especially above pH 7, which places severe limitations on their practical use. Rigid macroporous copolymers of styrene and divinylbenzene have been developed which have the high physical /mechanical stability necessary to operate under HPLC conditions of pressure and flow rate. These matrices can be produced in a range of porosities 10 to 400 nm each, with the controlled uniform pore geometry and pore size distribution essential for high efficiency separations. The unmodified form can be used for HPLC separations of small molecules or for the analysis of biological macromolecules by increasing the pore size. Polystyrene-divinylbenzene (PS-DVB) packings permit operation over a wide pH range (1 - 13), but give poorer efficiency an equivalent than silica-based packing.

Over the last decade a number of polymer stationary phases have been prepared, but these differ in the way they are prepared. In recent years, there has been much interest in molecularly imprinted polymers (MIP's). This concept was first developed by Wulff,[14] and involves polymerization of the monomer in the presence of the target molecule, and once the polymer is prepared, the target molecule is removed. The resulting polymer has a rigid structure with an affinity for the original print molecule. The MIP can then be used as a HPLC stationary phase, solid-phase extraction material or as a sensor. These polymers have found application for a large number of compounds including amino acids, amino acid derivatives and a number of drug components. Further discussion of this concept is given in Chapter 4.

1.4 SILICA

1.4.1 *Unmodified Silica*

The surface of silica is covered with a layer of silanol (Si-OH) groups and reactions at these sites are used to introduce organic groups onto the adsorbent surface. Numerous attempts have been made to improve the pH stability of silica-based columns, including the use of bulky silane reagents and the coating of polymers on the surface of the silica. Instead of trying to eliminate the surface contribution to retention on bonded columns, an unmodified silica column can also been employed in conjunction with a reversed-phase eluent. As first reported by Jane,[15] the amine-silanol interaction is exploited in this situation, and efficient separations were obtained using a buffered aqueous methanol-rich mobile phase at high pH in a chromatographic mode which has since been described as "pseudo reversed-phase" chromatography. This system was found suitable for the analysis of basic drugs of abuse, including opium and heroin, indicating the potential of

separating drugs containing amino functionalities. The retention mechanisms of such polar separations on unmodified silica with aqueous eluents have not been fully explained, and seem to be multi-functional in nature. It has, however, been suggested that ion-exchange interactions with silanol groups on the silica surface are the most prominent interaction.

The effects of the variation of pH, methanol concentration, ionic strength and nature of the ion in aqueous methanol mobile phases on the k′ (Section 2.2.5) values of various amino compounds, e.g.amprolium, amylocaine benzocaine, butacaine and cocaine, were studied using both silica and a silica surface modified by treatment with trichloro(octadecyl)silane (ODS-silica).[16] The retention mechanisms of the species are complex, and involve ion-exchange with the proton of a surface silanol site (particularly in the case of amprolium), ion-pair partition and salting-out effects. The addition of a salt to an aqueous methanol mobile phase employed in RP-HPLC has been shown to have a significant effect on the capacity ratios of amines. Both the nature and concentration of the added salt influence retention characteristics, and with careful control of pH, the separation of species can be optimized. The retention mechanism is complex but may involve both ion-pair partition and participation of unsilanized surface silanol groups.

Law *et al.* have also described a method using a silica column and a methanol-aqueous ammonium nitrate eluent for the determination of two classes of drugs. These drugs include the narcotic analgesics (including antagonists, metabolites and analogues) and drugs structurally and pharmacologically related to amphetamine.[17] Different k′ values were obtained depending on the brand of silica used, but the elution order remained the same.

Flanagan *et al.*[18] examined the addition of ionic modifiers at low concentrations to non-aqueous, primarily methanolic eluents for the determination of basic compounds on silica columns. Characteristics of this type of system

were that basic compounds were retained under conditions where they were appreciably ionized and increases in the ionic strength of the buffer produced decreases in retention. The pH was also found to influence the selectivity of the system and the retention volumes of individual analytes. Changing the organic solvent can give useful changes in the selectivity. These non-aqueous systems show high efficiency, stability and reproducibility and give long column life.

Flanagan *et al.*[19] examined further the factors influencing retention and peak shape while using a silica column in conjunction with a non-aqueous ionic eluent and found that retention occurred mainly by cation-exchange with surface silanols. At constant ionic strength, changes in eluent pH influence retention *via* ionization of surface silanols and protonation of basic analytes. Conversely, at constant pH, increases in the ionic strength result in decreases in the retention times, as would be expected for a cation exchanger. Further studies have shown that silica columns in combination with non-aqueous, primarily methanolic, eluents modified by ionic compounds which are highly dissociated in organic media provide a stable yet flexible system for the analysis of basic drugs.

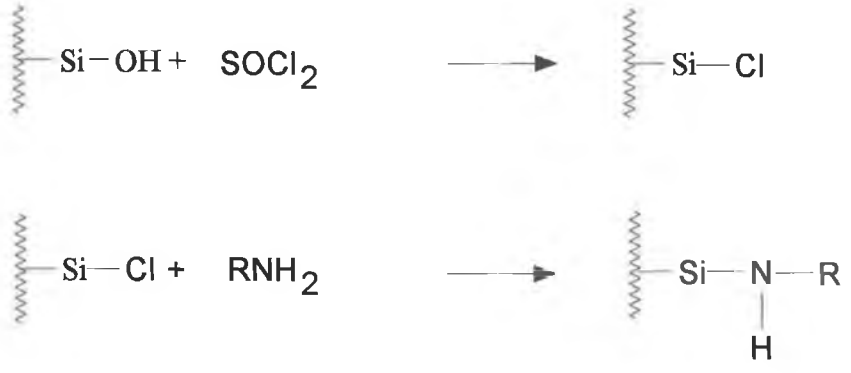
1.4.2 *Modified Silica*

A number of modified silicas exist and these have been prepared by chemical modification of the bare silica. These modifications can be accomplished by one of two methods: a) by a reaction known as "surface modification" between an organosilane and the silicate or by b) hydrolytic polycondensation of organosilanes. The former method is more often employed, as the latter method is often difficult to perform, is uncontrollable, and gives rise to undefined support materials. The method of surface modification can be further divided into three groups; modifications using (i) hydrophobic chains including octadecyl (C_{18}), C_8 and C_2 functionalities, (ii) polar groups, including

cyanopropyl, glycol and aminopropyl functionalities and (iii) ionic groups including amino, quaternary ammonium and sulphonic acid groups. These phases are prepared by a reaction of the organic groups with the silanol sites on the silica surface. They can be prepared by a number of methods including

(a) esterification of the silanol group with an alcohol ROH[5] where R may be an alkyl or any other functional group:

(b) reaction with thionyl chloride $SOCl_2$ which produces a chloride, which can then combine with an amine[6] to give an Si-N bond:



(c) reaction of the silanol groups with a mono- or dichlorosilane[20,21] to give an Si-O-Si-C bond. Octadecylsilane, in which R= $-(CH_2)_{17}CH_3$, is the most widely

used of these, and has been the preferred choice for reversed-phase chromatography:

The surface of the silica has about five -OH groups per nm^2 which corresponds to 8 - 9 $\mu mol/m^2$, and it is impossible to react all of them completely, even while using compounds of relatively small size, e.g. trimethylsilane. Colin *et al.*[22] found that the maximum concentrations of trimethylsilyl, octadecylsilyl and triphenylmethylsilyl are found to be about 4.5, 3.5 and 1.5 $\mu mol/m^2$ respectively, indicating that at best about half the available -OH groups can be reacted. As a number of these sites remain unreacted and accessible to analyte molecules, interaction with these residual groups can lead to poor chromatographic results; which is an ongoing problem with modified silica.

Triethylamine (TEA) has been used by Roos *et al.*[23] as a competing base for retention control and peak shape improvement in the reversed-phase HPLC analysis of a number of components. The alkylamine moiety acts primarily by nitrogen bonding to non-derivatised silanol sites, thereby reducing adsorption and ion-exchange effects. The addition of an alkylamine compound to a mobile phase can cause improvement in peak shape with little loss of retention. In addition to their ability to reduce peak tailing, alkylamines have useful selectivity-enhancing effects. Normalization of the retention behaviour of a solute can be achieved by incorporating TEA[24] in the eluent to serve as a competing base for masking

accessible surface silanol groups and to provide heterogeneity on the RP bonded surface. Short chain tertiary amine modifiers such as TEA are highly effective in reducing or eliminating silanophilic interactions.

RP-HPLC on chemically bonded phases has become the most popular technique in HPLC finding use in pharmaceutical and biological chemistry. However, it is unfortunate that standardization of RP-HPLC materials is not possible when developed on column packing materials from different manufacturers, even though they are in theory identical chemically bonded phases (e.g. octadecylsilyl). As different k′ values were obtained depending on the brand of silica used, a technique was developed which involved '*in-situ*' modification of the silica.[25] Bare silica was obtained from different manufacturers and the silica surfaces were dynamically modified with long-chain quaternary ammonium ions generating the reversed-phase system. High reproducibility of selectivity could be obtained with these types of phases with good peak symmetries been obtained for amine compounds chromatographed on these systems. The amount of CTMA (cetyltrimethylammonium) adsorbed on the silica surface increases with increasing alkyl chain length of the alkyl-trimethylammonium ion. As the amount of quaternary ammonium ions in the eluent is increased, a plateau is ultimately attained. The point at which this occurs coincides with the critical micellar concentration (CMC) of the ammonium ions in the eluent used and above this value the concentration in the eluent is constant.

When chromatographing basic analytes on chemically bonded phases, severe tailing problems are often seen in connection with poor reproducibility. These problems may also be solved by using dynamically modified silica, due to the deactivation of the active silanol groups. This technique wasfound to be superior to RP chromatography on chemically bonded phases with regard to reproducibility of selectivity. It should therefore be taken into consideration

when high reproducibility is needed, as such in the stability testing of drugs over long periods of time, and the international standardization of methods.

Hansen[26] examined a number of other parameters associated with this technique including varying the concentration or nature of the quaternary ammonium ion, the ionic strength or the pH of the buffer, or by changing the concentration or nature of the organic modifier. He found that retention and selectivity could be controlled by manipulation of these parameters. Other retention mechanisms such as ion exchange and ion-pair formation were found to be involved in the separation of analytes. Long-chain quaternary ammonium salts have been used in water rich eluents, with silica as the support, giving chromatographic separations similar to those obtained with chemically bonded reversed-phase materials.

Hansen *et al.* then examined a number of silica packings which were dynamically modified with cetyltrimethylammonium bromide[27]. Thirteen different silica packings exhibited the same selectivity towards a test mixture which included acids, bases and non-ionic compounds. As the selectivities obtained were the same regardless of origin of the column material, it was thus demonstrated that it is possible to standardize an HPLC system using dynamically modified silica.

Further work involved examining the influence of the nature of quaternary ammonium[28] compounds on retention in HPLC. Adsorption isotherms were determined on bare silica using four alkyltrimethylammonium bromides and two symmetrical tetra alkylammonium bromides, each containing 15 - 21 carbon atoms. It was found that only the long-chain quaternary ammonium ions are adsorbed on to the silica surface in appreciable amounts, and that the affinity for the silica increases with increasing number of carbons in the alkyl chain. Dynamic coating has the advantage over chemically bonded phases in that only

slight variations in selectivity are observed for different brands of column material.

A retention model based on the distribution of ion-pairs to the solid stationary phase was proposed by Crommen.[29] The ion-pair reversed-phase system was suitable for various kinds of drugs and other compounds of biological interest. Samples of widely different hydrophobic character could be chromatographed with suitable retention on the same silica columns by changing the composition of the aqueous mobile phase. It has been shown that the retention of ionic samples can be increased by the addition of hydrophobic counterions to the mobile phase. This "ion-pairing" effect was used to regulate the retention of the most hydrophilic analytes. The retention of hydrophobic samples is more conveniently regulated by the addition of a competing ion or uncharged compound to the mobile phase.

1.5 MODIFIED POLYMERS

There has been an increasing demand for packing materials more stable than C_{18} materials in RP-HPLC, and this has prompted the recent development of new classes of materials, in particular polymer-coated silica packings[30,31] and organic polymer-based[32] packing materials. These include polymers such as poly(styrene-divinylbenzene) (PS), esterified poly(vinyl alcohol) (PVA), poly (alkylmethacrylate) (PAM), alkylated poly(acrylamide) and poly(hydroxyalkyl acrylate or methacrylate) (PHA). These have all been substituted for silica-based C_{18} materials under appropriate conditions. In the same way as a silica matrix can be derivatised and/or coated to alter surface characteristics, so can a polymeric support. However, when a chemically stable polymer is coated it is possible to use functionalities which are themselves exceptionally stable. Thus a HPLC adsorbent is produced which is capable of with standing aggressive eluents and

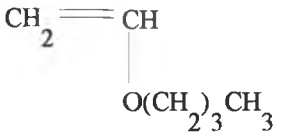
extremes of pH. Porous poly(styrene-divinylbenzene) packings (PS-DVB) have the advantage over silica-based materials of much greater chemical stability. The totally organic adsorbents are operable over a much wider pH range, typically 1-14. However, styrene-divinylbenzene copolymers do have the disadvantages of the appearance of micropores caused by the penetration of solutes into the polystyrene matrix. A new copolymer has been obtained by the copolymerisation of two cross-linking agents: a mixture of 1,4 and 1,5-di(methacryloyloxymethyl) naphthalenes (DMN) and divinylbenzene (DVB). This polymer has already found use as a stationary phase in gas chromatography and in solid-phase extraction (SPE).

The hydrophobic character of the poly(styrene-divinylbenzene) matrix is a serious limitation to its suitability for macromolecule separations other than in a reversed-phase mode. Even after derivatisation, the hydrophobic interactions between polymers and proteins can be sufficiently strong that the protein may be denatured either when adsorbed onto the surface of the stationary phase or when subsequently eluted. Therefore it is essential that the hydrophobic character of the poly(styrene-divinylbenzene) matrix is masked for the chromatographic separation of biological macromolecules in all retention modes except reversed-phase separations.

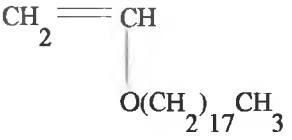
Tanaka *et al.*[33] examined the performance and retention characteristics for a variety of hydrocarbons in aqueous-organic mobile phases using a number of polymer-based packing materials with alkyl backbones, including poly(styrene-divinylbenzene), poly(alkyl methacrylate) and esterified poly(vinyl alcohol) by reversed-phase liquid chromatography. Materials with alkyl backbones showed performance comparable with C_{18} materials under optimized conditions. All of the polymer based packing materials showed preferential retention of aromatic analytes, in particular those with a rigid planar structure rather than those with flexible bulky groups. The molecular shape, rigidity and aromatic character of

the analytes as well as the organic solvents were found to effect the performance of the column.

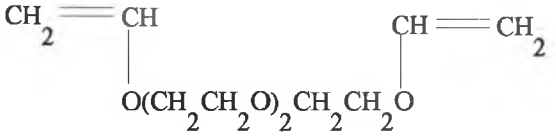
A spherical porous polymer for RP-HPLC was prepared from vinyl ether (Figure 1.1) derivatives by Hirayama *et al.*[34] by suspension copolymerization of alkylvinyl ether with triethyleneglycol divinyl ether. By changing the monomer ratio, the hydrophobicity of the packings could be easily adjusted. The packings showed the usual reversed-phase HPLC properties, but did not show abnormal retention, tailing and broadening of peaks, or irreversible adsorption of ionic and aromatic substances. This was due to the lack of ionic or aromatic groups. In addition, the packings were found to be stable in alkaline solutions because of the relative alkali stability of C-O-C as compared with CO-O and Si-O-C bonds in conventional packings.



n - Butylvinyl ether



n -Octadecylvinyl ether



Triethyleneglycol divinyl ether

Figure 1.1: ***Vinyl ethers used in polymerization reactions.***

Poly(vinylpyrrolidone) (PVP) has been immobilized on both small and large-pore silicas by Köhler by a number of methods including thermal treatment, or peroxide-initiated polymerization, or by γ-radiation.[35] These phases were

found to be hydrolytically stable compared to phases prepared by the chemical reaction of silica with a pyrrolidone ethyl dimethylchlorosilane silanization reagent. Columns of these particular PVP-silica packings have found application in several modes including: a) under normal-phase conditions as a polar bonded stationary phase, b) under RP conditions, for the separation of organic proton-donor and hydrogen-bonding compounds, c) for the aqueous size exclusion chromatography of proteins, and d) with salt gradients for the hydrophobic interaction chromatography of proteins. The use of a double-layer polymer has also been examined.

A thin layer of the organic polyamine was adsorbed onto the surface of the silica through ion-pair formation between the silanols and amine groups, and subsequent cross-linking has been used to produce silica-based anion-exchangers for protein chromatography.[36] The PL-SAX strong anion exchange material has been produced by coating PLRP-S poly(styrene-divinylbenzene) matrices, which have been shown to have the required pore geometry for high efficiency separations of biological macromolecules, with polyethyleneimine. This coating is then cross-linked in position to give chemical stability and quaternized to provide the strong anion-exchange functionality. The chemical stability of the matrix coupled with the quaternary amine functionality which is ionized over a broad pH range, enables anion-exchange separations to be performed over a wider pH range than previously achieved using weak ion-exchangers based on a silica matrix. With this polymer system it is possible to strip denatured proteins from the column or suppress the ionization of the quaternary amine group by flushing the column with strong alkali (sodium hydroxide) or acid (hydrochloric acid). As the coating is not only adsorbed onto the surface of the matrix but covalently cross-linked in position, organic modifiers can also be used in the eluent or for column clean-up. A strong cation-exchange material, PL-SCX, has been produced with sulphonic acid functionalities and a masked hydrophobic surface.

Other coating experiments involved preparing a polymer with a double layer. These were carried out using PVP-silica covered with poly (methyl octadecylsiloxane) and have been used to study diffusion and shielding effects of different polymer layers in the stationary phase. It appears that by coating the silica with various polymers of different polarities and properties, the retention and selectivity properties could be adjusted. Depending on separation conditions, either polymer may govern the retention process; however, the homogenity of the different films are difficult to control.

Figge *et al.*[37] coated silicas with a number of polymers of different polarities. They were synthesised by equilibration of different mixtures of methylhydropolysiloxanes with octamethyltetrasiloxane, with subsequent hydrosilylation of l-alkenes of different chain length. The amount of the coating polymer to be deposited on the surface can be varied. This results in the preparation of stationary phases of different phase ratios. The efficiency and sample capacity of the column obtained with these phases were comparable with efficiency and sample capacity obtained with silanized silica.

1.5.1 *XAD Resins*

Amberlite XAD-2 resin is a macroporous styrene-divinyl benzene copolymer which has found application as a versatile liquid chromatographic packing. XAD-2 is nonionic but is capable of adsorbing both neutral and ionic species.[38] Columns of this material have been prepared with 20,000 plates per metre (15 cm length); however, it is necessary to use slower flow rates. It has found application in the analysis of preservatives[39] and active drugs in pharmaceutical syrups[40]. It has also been used in the separation of a variety of organic acids[41], bases, nitro- and chloro-phenols and other aromatic compounds, peptides, amino acids, nucleosides and nucleotides. It is suitable for analysis over

a wide pH range (0 - 14), is compatible with virtually all solvents and has relatively strong adsorbent properties. Its hydrophobic surface which finds use in a number of separations that can be normally carried out on reversed-phase materials. The XAD resin has also been used for ion-pair chromatography, in particular for the determination of anionic samples while using a large quaternary ammonium ion. However, there a number of disadvantages associated with this material; in particular it has a relatively low permeability which translates into either longer analysis time or higher operating pressure. XAD-2 has been used by Hux *et al.*[42] for the determination of methaqualone from blood plasma. The pre-column allowed the direct injection of a relatively large volume (0 - 4 ml) of undiluted plasma. A high pH buffer was used to wash out plasma components and detection limits in the region 1 - 2 ng/ml were obtained.

1.6 MIXED MODE CHROMATOGRAPHY

Stationary phases in HPLC are usually accepted as functioning by a single mode, i.e. that chromatographic separations on these phases are achieved through one simple retention mechanism. For reversed-phase materials, this mechanism is normally based on hydrophobic interactions, whereas retention in normal-phase materials is *via* hydrophilic, such as dipole-dipole and adsorption interactions, with the polar stationary phase. Ion-exchange phases separate ionized solutes on the basis of their interactions with the charged sites on the stationary phase.

The preparation of these phases, however, can lead to differing functionalities on the chromatographic matrix compared to the support. Free silanol groups on the silica gel-based matrices are known to affect resolution. In the case of the ion-exchange materials, where the charged site is linked to the support by an alkyl chain, hydrophobic interactions can alter retention. The premise is that better chromatographic separations are achieved on unimode

phases. Steps are often taken therefore to minimize the effect of residual silanols by endcapping reversed-phase materials and to maximize the exchange capacity on ion-exchange phases. These steps aim to remove the so called deleterious presence of secondary retention mechanisms. In some situations, however, a separation may actually be enhanced by use of a more heterogeneous support. Several workers have examined this enhanced separation by supports which show retention mechanisms other than that expected for their monofunctional phase.

This work led to the preparation of multifunctional phases to achieve a better degree of separation. Hydrophobic and ionic interactions were the most commonly investigated. The direct approach to the preparation of a chromatographic material that contains sites for both these interactions involves modifying a matrix containing one class of residues with a second functionality in order that it takes on the necessary multifunctional character. However, this can cause problems with batch-to-batch reproducibility. Mixed-mode columns may also be prepared by mixing together individual phases which exhibit little batch-to-batch variation and also contain the appropriate functionalities to obtain a mixed-bed with the required properties.

The combination of separation modes would hopefully both increase the number of parameters that could be varied to optimize retention and resolution, and in addition enable the separation of non-ionic and ionic compounds simultaneously. Mixed-mode chromatoraphy will be discussed in greater detail in Chapter 2.

1.7 DUAL ZONE CHROMATOGRAPHIC MATERIALS

Most types of columns and adsorbents, regardless of the separation mode or of the pore diameter, must be periodically renewed after exposure to large concentrations of protein. Injection of clarified plasma or serum onto RP-HPLC

columns for drug analysis is especially problematic. Rapid efficiency loss occurs because the high organic content of the mobile phase usually required during RP-HPLC chromatography also causes the highly concentrated proteins to coagulate and block the column. Furthermore, even when such coagulation can be avoided by lowering the organic content of the mobile phase, the serum protein rapidly builds up as an adsorbed layer on the reversed-phase adsorbent. This problem has been overcome by the use of dual zone materials.

Dual zone materials are those in which one type of organosilane group is attached to the external zone and another to the internal zone. Attachment of a first residue occurs primarily in the external zone. This is followed by a second reaction with another silylating agent which finds few unreacted exterior silanols, and hence reacts preferentially with the internal silanols. Candidates for the weakly adsorptive external group include the non-ionic, hydrophilic groups used for gel filtration. In the first instance, a packing is chosen with small mesopores so that large solutes are excluded by a steric exclusion mechanism. At the same time, the outer surface bears hydrophilic ligands which interact weakly with the proteinaceous constituents. The stationary phase on the inside of the particles has a different surface chemistry bearing ligands which interact selectively with the analytes. In the second case, the surface barrier is located at the interface between the stagnant mobile phase and the stationary phase, and functions as a semi-permeable surface or interface. The packings allow access of the analytes to the stationary phase and exclude the high molecular weight proteins. The internal surface exerts a dual chemical functionality with hydrophilic ligands externally and hydrophobic or ionic ligands internally, embedded in a polymer network. These phases are suitable for analysis of low-molecular weight, biologically active analytes which need to be separated in complex mixtures.

To overcome the problems of these techniques, new packing materials were designed which retained the analytes of interest while eluting the water

soluble proteins in or near the void volume. Exclusion of the larger proteins from the pores of the packing was the first approach tried. If the retentive interaction could be made to occur only inside the pores, then the excluded proteins would elute unretained, while the smaller analytes would be retained and eluted later. The principle behind the internal surface reverse-phase (ISRP) is to confine the hydrophobic partitioning phase exclusively to the internal particulate region of the porous silica, with the external surface being maintained hydrophilic and non-adsorptive to proteins. As the pores have small diameters, the analytes are capable of penetrating the particulates and interacting with the internal hydrophobic partitioning phase while all the proteinaceous substances are eluted in the void volume. These supports have found use as extraction pre-columns or HPLC columns. They have a number of advantages over alkyl bonded pre-column methods, notably in allowing for the complete passage of proteins. Additionally, the use of small particulates (5 μm) allows the direct injection of serum or plasma samples onto HPLC ISRP separation columns. A further advantage of these supports is the use of polypeptide partitioning phase which is relatively weak in comparison. This allows the use of a lower proportion of organic modifier in the mobile phase used to elute drugs, thus ensuring against protein precipitation. Mobile phases with 20% or less have been used.

The first support was developed by Hagestam and Pinkerton,[43] who developed the internal surface reversed-phase (ISRP) silica support. This suppport consisted of a hydrophobic oligopeptide phase and a hydrophilic diol phase bound to silica with a pore size of less than 80 Å as internal and external surfaces respectively. This was prepared by derivatization of the silica with glycerylpropyl silane followed by the attachment of glycine-L-phenylalanine or glycine-L-phenylalanine-L-phenylalanine to a given fraction of the glycerylpropyl groups, using carbonydiimidazole as a coupling reagent. The phenylalanine moieties were then removed from the external surface of the silica particulates by

treatment with carboxypeptidase. The pore diameters are kept small to exclude proteins from the internal surface region. This support was found suitable in the analysis of hydrophobic drugs in serum or plasma by direct injection. An ISRP analytical column has also been found suitable for the determination of an anticonvulsant drug mixture in serum by direct injection.

In another method for the preparation of these supports, the hydrophobic partitioning phase (butoxy-L-phenylalanine, Boc-L-Phe) was bound to the silica via an amine spacer.[44] This hydrophobic partitioning moiety was then cleaved from the external surface using α-chymotrypsin, thereby leaving the amine spacer. By attaching glycol groups to the freed amino spacers, this leaves the external surface non-adsorbtive to proteins. With this approach greater partitioning phase capacity can be achieved due to the high yield of silanization reactions. This stationary phase has found use in the determination of phenytoin, carbamazepine and phenobarbital; however, it has the disadvantage that it can retain almost no hydrophilic drugs such as amphoteric drugs, cephalosporins and penicillins within the recommended eluent pH range (6.0 - 7.5).

To overcome this problem a new support has been developed. The external surface has a *N*-(2,3-di-hydroxypropyl)aminopropyl phase (which is a nonadsorptive surface for proteins) and the internal surface has a *N*-octanoylaminopropyl phase (Figure 1.2). The average pore diameter of the ISRP silica is 50 Å, particles of this size being small enough to exclude macromolecules such as serum proteins, while still allowing access for hydrophilic and hydrophobic drugs from plasma without the destructive accumulation of the proteins in the pH range 3 - 7. Cleavage of the *N*-octanoylaminopropyl group from the external surface was achieved using a novel enzyme, polymyxin acylase. Results obtained demonstrated that it is suitable for direct injection analysis of hydrophilic and hydrophobic drugs in serum or plasma without destructive accumulation of proteins over the eluent pH range 3 - 7.

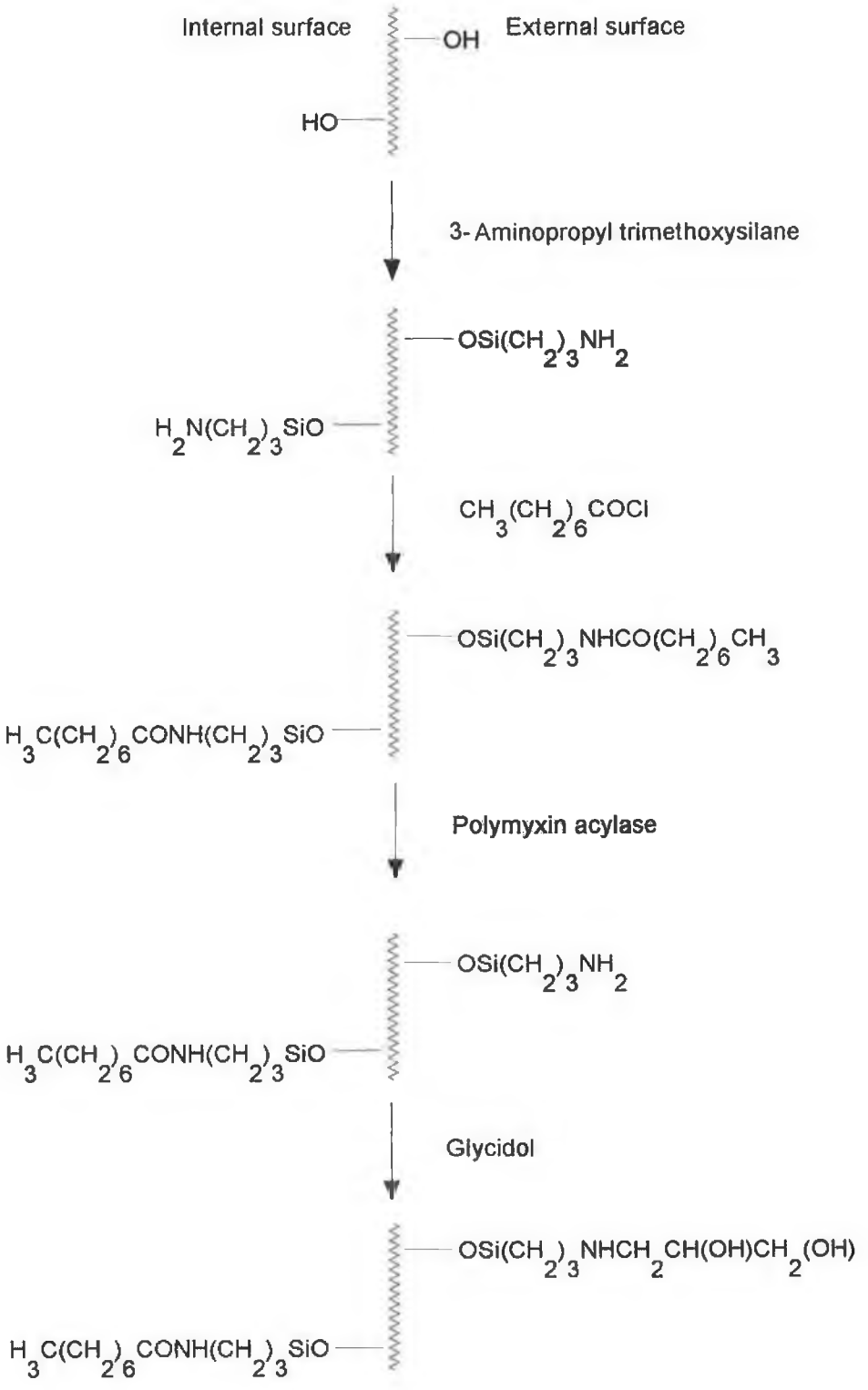


Figure 1.2: ***Synthetic scheme for preparation of the ISRP silica support.***

The recovery of drugs from serum was almost 100 %, regardless of the difference in their protein binding. A method for the analysis of barbital, sulfamethoxazole and lidocaine has also been described by Haginka *et al.*[45] These columns were found to be stable even after continuous use at low pH values.

1.7.1 *Shielded Hydrophobic Phase Columns*

Shielded hydrophobic phases (SHP's) are similar to ISRPs, but consist of a different chemistry, in that the same phase covers all the support, while the restricted-access phases have a dual phase. This is a relatively new concept for direct analysis of biological fluids using HPLC developed by Gisch *et al.*[46] which excludes proteins while interacting with small molecules. The support material consists of a polymeric bonded phase containing a hydrophobic region (a phenyl group) enclaved by a hydrophilic poly(oxyethylene)network (Figure 1.3). The hydrophilic network forms a water-solvated interface which operates as a shielding barrier for the lipophilic groups through which small analytes, such as drugs, penetrate and interact with the hydrophobic groups. At the same time, larger water-solvated molecules, such as proteins, are prevented from such interactions by hydrophilic shielding. This results in the bulk of the protein matrix being eluted virtually unretained in the void volume without affecting the retention of the smaller analytes.

The technique has been used in the analysis of carbamazepine and phenobarbital in human plasma. The bulk of the proteins were eluted unretained from the serum and plasma matrices. Retention of the low molecular mass components of such matrices was adjusted by modifying mobile phase conditions. A number of mobile phases have been used, with the portion of the organic component being rarely greater than 15 - 20%. As the shielded hydrophobic phases are based on silica, the pH range is limited to 2.5 - 7.0.

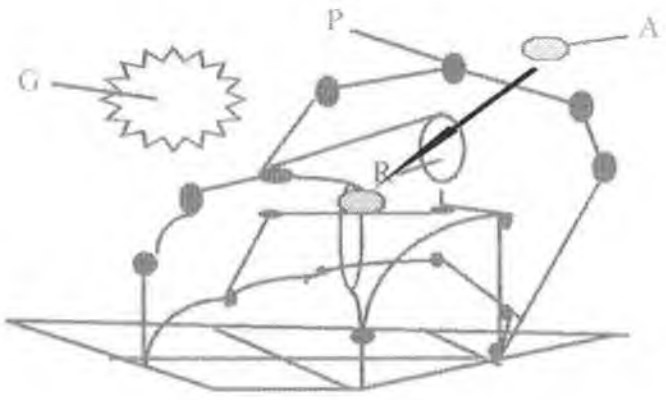


Figure 1.3: ***A typical shielded hydrophobic phase. R = hydrophobic pocket, P = hydrophilic network, G = large unretained protein and A = small retained analyte.***

Gisch *et al.*[47] developed SHPs and by two separate methods creating both bonded micellar and embedded polymeric phases. In the first method the bonded hydrophobic alkyl ligands are substituted at the ω position with a polar hydrophilic group. This phase resembles a low-density micellar phase bonded to the support through the hydrophobic tails. By the latter method a polymeric hydrophilic network embeds hydrophobic moieties. A number of other SHP's were prepared by varying the shielding group R, which could be basic or acidic in nature, or bind any other group giving rise to additional selectivity. Further selectivity and retention could be achieved by the use of ion-pair groups, in particular for analyses where hydrophobic retention alone is insufficient. In an attempt to solve the problems inherent in the use of surfactant-coated n-alkyl silica, micellar bonded phases were prepared by Gisch *et al.*[41] A 10 nm pore size silica with N,N-bis-(2'-methoxyethyl)-11-silylundecamide [$=Si(CH_2)_{10}CON(CH_2CH_2OCH_3)_2$] groups was prepared. The bonded micellar layer was

composed of -CON($CH_2CH_2OCH_3$)$_2$ groups with hydrophilic properties and decamethylene groups with hydrophobic properties. Wang *et al.*[48] used a SHP to determine catecholamines in urine by direct injection. The HISEP™ SHP, allowed the retention of the drug components while eluting the proteins to waste. Uno *et al.*[49] also used a HISEP column to analysis sulphamonomethoxine (SMM) and its metabolite in blood serum by direct injection. This HPLC method allowed rapid analysis without prior sample pretreatment.

Williams and Kabra[50] proposed a further synthetic route for the synthesis of a dual zone material (DZM) with a hydrophilic external and a lipophilic internal surface. In the first step, perflurobutylethylenedimethylsilyl (PFB) groups were bound exclusively at the external surface of a 6 nm pore size silica. Perflurobutylethylenedimethylsilyl-*N*-methylacetamide groups were employed as the silylating reagent, which was found to react rapidly with silica leaving *N*-methylacetamidyl groups. In the second step the remaining unreacted internal surface was covered with n-octadecyl groups using n-octadecyldimethyl chlorosilane as a reagent. As with the previously described packings, proteins are prevented from reaching the bonded phase by a size exclusion mechanism.

Kimata *et al.*[51] employed an approach in which a reversed-phase silica was subjected to a controlled acid treatment, whereby the bonded n-alkyl groups were hydrolytically cleaved exclusively at the external surface. After washing, the rejuvenated external surface was treated with 3-glycidoxypropylsilane to introduce a diol functionality. These phases posess the properties of ISRP packing materials.

Boos *et al.*[52] prepared tailor-made bonded packings based on polyvinyl-copolymers and glycerol-coated porous glasses as pre-column materials. Due to the small pore size the packings showed steric exclusion properties and excluded the proteinaceous constituents. Depending on the desired selectivity, a family of

packings were synthesized with distinct internal stationary phases such as n-octadecyl, phenyl and boronic acid.

1.8 CHIRAL STATIONARY PHASES

The determination of enantiomers is of great importance for the pharmaceutical industry, as it is necessary to determine the purity of both enantiomers. For the synthesis of new drug components it is essential for them to be separated in order to subject them to individual pharmaceutical testing.[53] Therefore it is essential to have reliable methods for the determination of the optical purity of both the enantiomers. It is possible to have different intensities of activity and in some cases opposite activities; a well known example being thalidomide where one isomer is dangerous (mutagenic) while the other shows no adverse effects. A number of drugs are marketed as racemates, although only one enantiomer may contribute to virtually all of the therapeutic activity. In a few instances, the less active isomer happens to be the toxic one.

The enantiomers can be separated by a number of methods, including (1) reacting the enantiomers with a suitable derivatizing agent, (2) a chiral stationary phase and (3) the use of a chiral additive in the mobile phase. The first method involves derivatization with a suitable reagent forming a pair of diastereomers, which may be then separated on a non-chiral phase.[54] There are a number of problems associated with this method, including a requirement for the optical purity of the reagent to be known (it must be 100 % pure). In addition, the reaction between the enantiomer and the reagent must go to completion (or at least 99.99 %), otherwise differences in product yields may result in large errors. The last factor to take into condsideration is the distance between the two chiral centres in the derivatives; they should be as close as possible to each other to maximise the difference in chromatographic properties. A number of derivatizing

agents have been used, which include N-carboxy anhydrides for determination of amino acids, isocyanates for the determination of amines and amide and ester derivatives for determination of carboxylic acids.

It is also possible to determine these enantiomers without prior derivatisation; a number of stationary phases exist today which allow the direct determination of a number of compounds as their enantiomers (one of the enantiomers been selectively retained by the chiral stationary phase over the other). These phases have been prepared based on the theory of the "three point interaction" introduced by Dalgleish[55] in 1952. According to this theory, chiral discrimination is obtained when three simultaneous points of interaction occur between the enantiomer and the chiral stationary phase. The types of interaction that can occur include hydrogen bonding, as well as ionic or dipole attraction; these effects been enhanced by non-polar solvents, whereas hydrophobic interactions may be important in aqueous media.

There are a number of different types of stationary phases including those based on ligand exchange process, protein phases, polymers, chiral cavity phases and brush-type phases. The first type consists of an immobilized chiral ligand forming a co-ordination complex with a transition metal ion. Resolution of enantiomers by ligand-exchange chromatography occurs through the formation of a diastereomeric mixed-ligand complexes using an optically active ligand. This is bonded to the support and is co-ordinated to a transition metal ion, resulting in resolution of the enantiomers. The stability difference between the mixed-ligand complexes affects the chiral discrimination of the two enantiomeric molecules. Only components which have two polar functional groups with the correct distance between them are capable of interacting with the metal. Carunchio *et al.*[56] used this method to prepare three stationary phases by grafting silica gel with (-)-*trans*-1,2-cyclohexanediamine. These were used for the resolution of amino

acids, and separation was due to the addition of an eluent containing a constant concentration of copper (II) acetate.

It is possible to bind proteins to silica and obtain a valuable class of CSPs, that are mainly suitable for the separation of chiral drugs. There are a number of protein phases available and these have numerous bonding sites for smaller molecules and different degrees of bonding affinity with many enantiomers. The more commonly used proteins are bovine serum albumin (BSA), human serum albumin (HSA),[57] ovomucoid, cellulase and α-acid-glycoprotein. These phases differ in their chromatographic and enantioselective properties, due to the differences in their biological functions and size, shape and isoelectric point. These phases are expensive and delicate in handling, with low performance and loadability; however, they can offer excellent enantioselectivity.

α1-Acid glycoprotein columns[58,59] have found use in a number of applications including the determination of β-blockers.[60] These phases were capable of tolerating organic solvents as well as high temperatures,[61] and were suitable over a wide pH range without been denatured.

Mano *et al.*[62] described a phase which was prepared from conalbumin-conjugated silica gel (from chicken egg white) and used for the determination of racemic azelastine, an antiallergic drug. The column was found to be stable and capable of separating the enantiomers using an aqueous mobile phase. The disadvantage of this phase, however, is its lability to heat and acid. Miwa *et al.*[63] prepared a phase based on ovomucoid-acid glycoprotein (obtained from chicken egg white) which has been used in the resolution of both acidic and basic compounds. It was found to be resistant to variation in pH and organic solvents and demonstrated some degree of heat resistance.

The synthesis of a chiral stationary phase based on human serum albumin (HSA) was described by Domenici *et al.*[64] The phase was synthesized "*in situ*" by covalent immobilization of HSA. The protein was immobilized on a

commercially available diol column which had first been activated with 1,1-carbonyldiimidazole (Figure 1.4). The phase was found to be suitable for chiral separation of enantiomeric analytes and the effects of mobile phase composition and temperature on the stereochemical resolutions was examined. Drugs like benzodiazepines, warfarin and leucovorin can be resolved using this method.

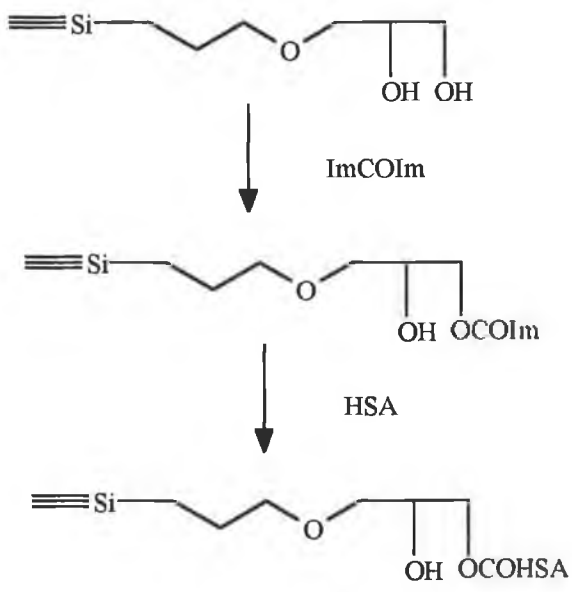


Figure 1. 4: ***The synthesis of the HSA-CSP using 1,1-carbonyldiimidazole (ImCOIm) activation of a commercially available diol HPLC column.***

Thelohan *et al.* have described the immobilization of enzymes onto supports and used them as chromatographic phases for HPLC. A new stationary phase was synthesized by first covalently bonding a hydrophilic polymer to silica particle.[65] Glutaraldehyde was then covalently attached to the polymer. This resulted in a surface of negligble hydrophobic character. Trypsin (TRYP) was then immobilised on this support through covalent binding to the glutaraldehyde moieties. The hydrolytic activity of the immobilized enzyme was 72% of the activity of an equivalent molar amount of free TRYP. The initial

chromatographic studies indicated that this phase could be used for chiral separations of enantiomeric O- and N, O-derivatised racemic amino acids which are natural substrates on TRYP and that the stereochemical resolutions are a result of the activity of the enzyme. Separations were due to the enzymatic activity of the support. Although selectivity is high for these protein phases, normally the efficiency of these is quite low resulting in broad peaks.

A number of helical polymers have been used including cellulose[66] and derivatives of cellulose[67,68] and these were found to retain enantiomers to different degrees between the layers of the helix. The underivatised form was used by Gübitz *et al.*[69] to separate *D,L*-tryptophan, *D,L*-5-hydroxytryptophan and *D,L*-dopa; however, it was necessary to use extremely low flow rates (0.1 ml/min). Derivatization of the -OH groups of cellulose does not destroy the helical structure and forms a tertiary structure, and these chiral cavities are able to include molecules stereoselectively. Helical polymers are suited for the resolution of enantiomers of twisted molecules; however, a number of flat molecules may also be resolved.

The phases described as cavity phases can be divided into two classes, namely cyclodextrins and crown ethers. They can form host-guest complexes with small molecules if they are capable of fitting into the ring structure; this needs too be stereochemically controlled for enantioselectivity. Cyclodextrins are oligoglucose derivatives with six, seven or eight units, linked through a 1,4-glycosidic linkage. Glucose itself is chiral, and the cyclodextrins form a hollow truncated cone with a cavity diameter determined by the number of glucose units. At one opening, the secondary hydroxy groups of the glucose are found. The interior of the cavity contains no hydroxy groups and is rather hydrophobic, while the external surface is hydrophilic. Chiral recognition based on the inclusion of an aromatic into the hydrophobic cavity. The chiral centre must be close to the cavity entrance and it is necessary to have interaction between the analyte and the

mouth of the cyclodextrin, i.e. that is from the hydrogen bonding to the chiral hydroxyl moieties. Cyclodextrins [70,71] have been bound to silica, and found use for the separation of a range of chiral compounds, but it is difficult to predict their suitability for a given compound. There are a number cyclodextrin derivatives (Figure 1.5) available and a number of these have found use as stationary phases.

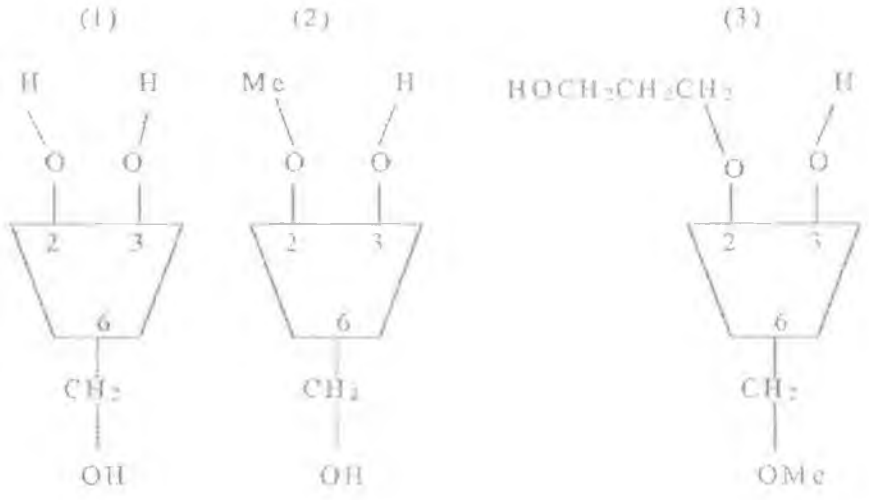


Figure 1.5: ***Structures of (1) β-cyclodextrin, (2) 2,6-dimethyl-β-cyclodextrin and (3) 2-hydroxypropyl-β-cyclodextrin.***

Chiral crown ethers of the 18-crown-6 type have found use for the resolution of amino acids. The interaction occurs between the amino protons and the crown ether oxygens (Figure 1.6). The R and R′ groups of the crown ether[72] need to be large and rigid in order to force the small guest molecules into a well defined interaction with the host.

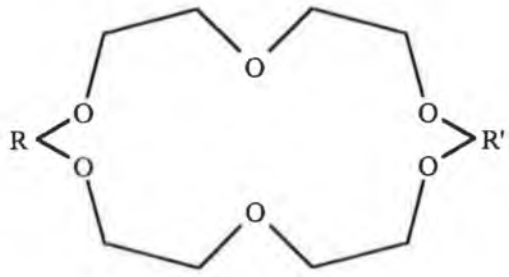


Figure 1.6 : ***Structure of a typical crown ether.***

The final type of chiral stationary phase is the brush-type, and these phases are based on silica gel which may be derivatized with almost any functional group and the resulting monomer structures being known as "brushes". The most widely characterised chiral stationary phases are those developed by Pirkle *et al.*[73] These phases are derived from *N*-(3,5-dinitrobenzoylphenylglycine (DNBPG))[74] (Figure 1.7). This molecule has two amide groups which are rigid (planar), which allows the chiral moiety a limited number of conformations, which is important for chiral recognition. The amide groups can undergo dipole-dipole interaction as well as hydrogen bonding. The dinitrobenzoyl group acts as a π-acceptor and will interact with π donors, e.g. anilines and napthalenes. This interaction is assumed to be the most important for the separation of enantiomers, which will not occur unless they have this type of group. In most cases, however, it is necessary to introduce these groups by derivatisation steps. Separations carried out using these phases are normally in the normal phase mode.[75]

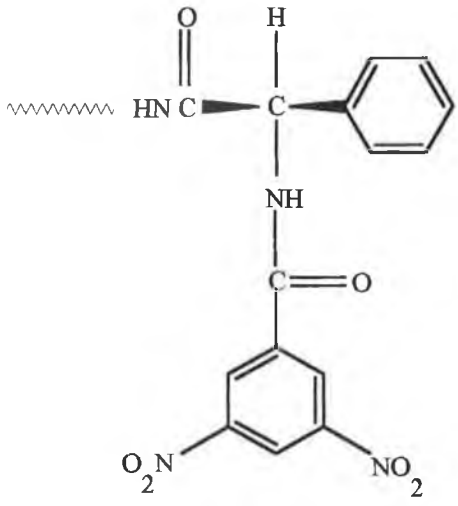


Figure 1.7: ***Structure of 3,5-dinitrobenzoylphenylglycine used in the preparation of Pirkle phases.***

If a chiral additive is capable of forming a complex, ion-pair, or any other adduct with the enantiomers in the sample, and is added to the mobile phase, there is a chance that the distribution coefficients of the diastereomers formed between the mobile and stationary phases will be different and, therefore, these can be separated using a non-chiral phase.[76,77,78] The chiral additive does not need to be optically pure and there is no restrictions on the stationary phase used. One disadvantage is that the enantiomers exists as diastereomeric associates after separation, whose dissociation may be impossible.

1.9 SAMPLE PREPARATION

The analysis of drugs in biological media has developed over the last 30 years, as the number of drug compounds introduced yearly onto the market increases. It is also necessary to determine these compounds at increasingly lower levels, hence, the methods for their determination are stretched to their

fullest. The number of matrices which can be used as a source of these compounds is expanding, and these include serum, plasma, urine, tissue (kidney, liver) cerospinal fluid.

HPLC analysis is often encounters problems by interferences from endogenous compounds and a number of different approaches can be applied to overcome these problems. These include changes in the eluent, or the column packing materialor even chemical derivatization to enhance the relative detector response of the drug. An alternative and more commonly used approach involves a sample preparation, removing the interferents prior to the analysis. It is therefore neccessary to carry out some clean-up/pre-concentration step(s), and there are a number of these available which include liquid/liquid extraction and solid-phase extraction.

Liquid/liquid (partitioning) extraction was for a long time the standard method for isolation of drug components from biological matrices.[79] This technique is based on the extraction of an analyte depending on its partition between an aqueous and an immiscible organic phase. The components are extracted according to their acidity/alkalinity and their pK_a values; basic compounds are extracted by increasing the pH of the solution to a value 1 - 2 pH units above their pK_a, whereas for acidic compounds the pH is adjusted to a value which is 1 - 2 pH units below the pK_a value of the component in question. This method permits concentration of the analyte, however, it is well known that repeated extraction (2 or more times) with small portions of solvent can recover much more analyte than a single batch extraction. For practical purposes, however, an extraction procedure would involve no more than 2 extractions. There are disadvantage is relatively time-consuming and is unsuitable for components with various chemical properties or of polar origin. A number of salts have also been used to increase the selectivity of the extraction by increasing the transfer of a drug from the aqueous to the organic phase.

In order to achieve higher efficiency new techniques were developed, e.g. solid-phase extraction. The extraction is carried out in small columns or cartridges packed with a suitable sorbent e.g. silica gel, bonded silica, ion exchange materials, polymers and mixed functional phases (MFP). Columns containing restricted access packings are also available, these materials being particularly suitable for the determination of plasma samples due to the surface barrier which allows proteins to be eluted in the void volume. These phases (previously mentioned) include ISRP, SHP and DZP. The sample is applied to the column and any interferents present are removed by a washing procedure. This is then followed by an elution step which removes the component(s) of interest. They have the advantage that they can be easily automated, have low solvent usage and less time consuming.

SPE can be further divided into two areas of off-line and on-line extraction. Off-line SPE deals with cartridges packed with a suitable material for the component(s) of interest. These columns have a high sample loading capacity, and single use analysis as components may be retained on the column from a previous sample. The most commonly used phases are the reversed-phase materials, C_{18} and C_8. Whereas on-line involves the use of a pre-concentration column positioned between the injection valve and the analytical column. The column is usually prepared with the same material as the analytical column, but of larger particle size (usually in the range 30 to 50 μm). A dual pump system allows the components to be absorbed onto the pre-column, while interfering compounds are eluted to waste. On switching the injection valve, the drug can be transferred from the precolumn to the analytical column by means of the mobile phase. The advantages of this method are the high speed of analysis, simplicity and accuracy. Efficiency and reproducibility are as good, or better than those of liquid/liquid extraction. SPE also may be preformed on compounds which are difficult to extract at any pH e.g. antibiotics.

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CHAPTER TWO

RETENTION STUDIES ON A MIXED-MODE COLUMN IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

2.1 INTRODUCTION

The basic principle behind mixed-mode chromatography has been described briefly in chapter 1, while this chapter will detail mostly with the applications of such phases.

Prior to the use of mixed-mode phases, Halfpenny *et al.*[1] utilized column switching to combine two modes (an ion-exchange and a reversed-phase). However, this system involved the use of two analytical columns coupled by a number of switching valves thus allowing the isolation of either column during the analysis. In this manner the simultaneous determination of purine and pyrimidine nucleotides, nucleosides and bases was achieved in one analysis. The nucleotides were separated on the anion-exchange column and the nucleosides and bases on the reversed-phase C_{18} column. As only one analysis was required instead of two, smaller amounts of sample were required. The analysis times using this system were in the region of one hour.

Difeo *et al.*[2] also used a tandem system of a LCSCX and a Novapak for the determination of RG-14620 and its potential impurities/ degradation products in a topical formulation. The analytes were eluted using a gradient firstly from a Supelcosil LC-SCX cation exchanger and were then further purified on a Nova-Pak dimethyloctadecylsilyl analytical column. This system was found useful when the mixture under analysis contained a large number of components of varying polarity.

Johnson *et al.*[3] used coupled column chromatography (CCC), with exclusion chromatography as the preliminary step and reversed-phase chromatography as the secondary step. This system was found suitable for the determination of (a) additives in compounded rubber, (b) a pesticide (malathion) in vegetable matter and (c) limonin in grapefruit peel. This system could be used by either of two methods; on-line or off-line. The off-line is carried out by

collection of the analytes as they exit from the detector and these are then injected onto the second column, however, this is complicated and tedious. On-line is achieved by coupling the second column *via* a switching valve. The valve can serve to trap a defined volume of collected sample, usually in a loop, and direct it onto the second column. It can also divert the mobile phase containing the analyte of interest from the first column to the second column for a defined period of time. Although on-line would be the preferred method, it is not always suitable for the application at hand. A number of complex plant extracts[4] were separated by combining GPC and reversed-phase chromatography.

Solid-phase extraction may also be carried out in tandem; Mills *et al.*[5] have used two resins in tandem to determine triazine metabolites from soil and aquifer sediments. The samples are initially applied to a C_{18} phase, then after elution of the components of interest they are applied to an anion-exchanger to remove any further interfering components. It was possible to automate this method and hence make it a viable method for studying triazine metabolites in the environment.

In recent years a number of new types of selective bonded phases with mixed functional groups have been introduced. Polar and non-polar groups can be bonded onto the same sorbent, introducing separation of sample components depending on both adsorption and partition chromatography. The preparation of these phases can be achieved by various modification methods, such as (a) the bonding of a multifunctional reagent, (b) by multistep modification of the silica gel; different functional groups being bonded in each step and finally (c) by modification using mixtures of reagents.

Crowther *et al.*[6] has prepared phases which consist of chemically dissimilar ligands bonded to silica supports. The aim was to balance the ionic and hydrophobic properties of the stationary phase in a reproducible manner, producing stationary phases which are similar to ion-pairing in selectivity, but

more like reversed-phase packings in stability, reproducibility and efficiency. These phases are mainly reversed-phase in nature, but also contain significant ion-exchange properties. Mixed retention mechanisms were created by bonding ionic and hydrophobic groups in the correct proportions resulting in a unique selectivity; Figure 2.1 shows a schematic of the synthesis of this mixed-mode phase. The ratio of ionic to hydrophobic content could be varied during the synthesis step and hence allowed changes in the separation other those obtained by varying the mobile phase only. The selectivity could be enhanced without the use of strong buffer salts or contamination due to ion-pair reagents. The preparation of both anionic and cationic/ reversed materials has been described. They have been used in the simultaneous separation of nucleosides and nucleotides, and also for the separation of the catecholamines. A number of phases were also prepared which found use in the determination of oligonucleotides.[7]

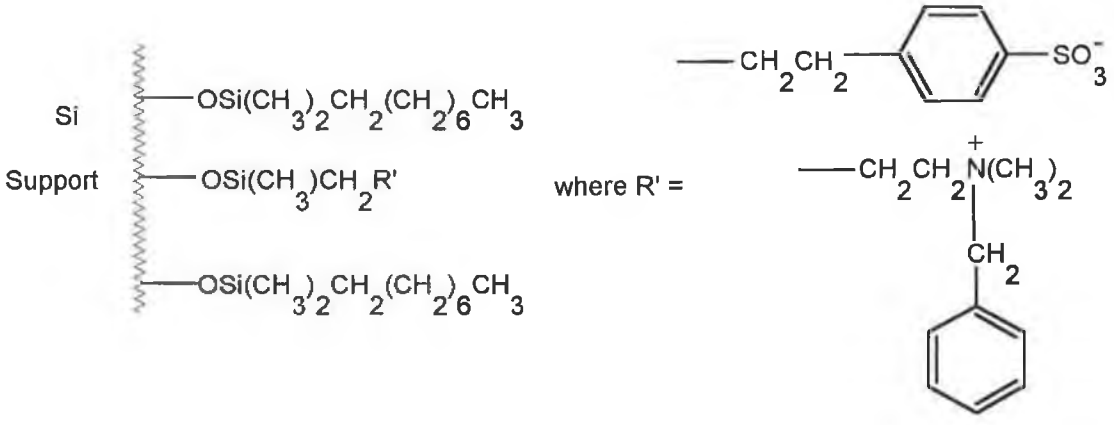


Figure 2.1: ***Schematic of a "mixed-mode" stationary phase synthesised by bonding both functionalised ionic (R') and reversed-phase groups onto the silica support. The result is a stationary phase with well-defined hydrophobic and ionic characteristics.***

Colmsjö *et al.*[8] examined a number of different cyano/ODS polymeric modified silica gel phases. These were prepared by the simultaneous bonding of octadecylsilane and cyanoalkylsilanes. This modified silica was suitable for separation in both normal and reversed-phase modes and found use for the determination of PAH's with MW's over 300. A significant decrease in retention time was observed for these compounds compared to the values obtained on a single phase polymeric ODS phase.

A number of mixed-mode sorbents have found use as solid-phase extraction materials since the multiple interactions have found use in the isolation and purification of analytes. Patel *et al.*[9] described a mixed-mode poly(styrene-divinylbenzene) sorbent containing both C_{18} (reversed-phase) and sulfonic acid sites, and this was compared with a silica-based sorbent that also showed mixed-mode behaviour. Two different test compounds were used to assess each of the functionalities; valerophenone was used to test the reversed-phase interaction while phenylpropanolamine was used to measure the cation-exchange interactions. This phase was found to be suitable for the determination of barbituates from urine by reversed-phase chromatography, while the cation-exchange interaction was used for the determination of antihistamines in cough syrups and also for preconcentrating triazine herbicides from water. Pheniramine was used to test reusability of the polymeric sorbent, and it was found that the polymeric mixed-mode sorbents have a much higher reversed-phase and cation-exchange capacity than silica-based mixed-mode sorbents. The polymer-based sorbents are also stable from pH 0 - 14 and are suitable for isolating organic amines with high pK_a values by cation exchange chromatography.

Chen *et al.*[10] used a mixed-mode bonded-phase silica column at pH 6 for the isolation of acidic, neutral and basic drugs from whole calf blood. The cartridge used was a Bond Elut Certify™ material which contained both hydrophobic and cation-exchange functional groups. By adjustment of the

extraction system pH, two different eluate fractions could be collected; the first being acidic, neutral and weakly basic drugs with lower pK_a values, the second been the other basic drugs, with recoveries of > 81 % being obtained.

Mills *et al.*[11] compared silica- and styrene-divinylbenzene-based sorbents which contained a mixture of C_8, C_{18} and sulphonated cation-exchange groups for their efficiency in the isolation of neutral triazine compounds from water samples. Their efficiencies for the isolation of benzoylecgonine from urine was also examined. Larger recoveries (95 %) were obtained on copolymerized mixed-mode resins in which C_{18} and sulfonic acid are in closer proximity than on blended mixed-mode resins, where recoveries of 60 - 70 % were obtained.

Collins *et al.*[12] described a method using a mixed-phase (XtrackT) for the determination of clenbuterol, malbuterol and terbutaline from bovine urine and liver samples. The columns exhibit two retention mechanisms based on octyl and benzosulfonic acid groups bound to silica particles, and recoveries obtained were >75 % and 85 %, for urine and liver, respectively.

Dixit *et al.*[13] described the extraction of a number of catecholamines and their metabolites from urine using a bonded silica gel solid-phase extraction column. The AccuCAT material is a chemically modified silica gel which exhibits multiple interactions including polar, nonpolar, anion-exchange and cation-exchange properties. These properties enabled the extraction of free catecholamines and their acidic and basic metabolites on a single column, by following a specific procedure for each class yielding clean extracts with high recoveries. A number of acidic, neutral and basic compounds were isolated using a Bond Elut Certify™ mixed-mode extraction column.[14] Elution of the acidic and neutral components was achieved using methylene chloride and the basic components were eluted using a 2 % ammonium hydroxide solution in ethyl acetate. Recoveries obtained by this method varied in the range 60 - 100 %, the lower values being obtained for the recovery of the acidic/neutral fraction. A

number of other drug components were determined by mixed-mode solid-phase extraction and these include iloperidone[15] and codeine.[16]

Brown *et al.*[17] described a mixed-bed ion-exchange (MBIE) column containing alumina and silica. The MBIE column was evaluated for the simultaneous separation of anionic and cationic analytes. Adjustment of the mobile phase pH allowed the alumina to provide anion-exchange sites while simultaneously providing cation-exchange sites on the silica. The elution order was effected by pH and type and concentration of counter-anions and counter-cations. The weight ratio of the two sorbents could be altered to alter resolution and elution order. The column was found suitable for the simultaneous determination of inorganic mono- and divalent anions and cations.

A method was described by Saari-Nordhaus *et al.*[18] whereby inorganic anions and carboxylic acids were separated using a mixed-mode stationary phase. This phase contained reversed-phase (C_{18}) and ion-exchange (anion) ligands mixed in a 1:1 ratio and attached to a silica base. The compounds analysed on this phase included bromide, nitrate, phosphate and carboxylic acids such as acetic, lactic, formic and propionic acids. The separation of these analytes was effected by several factors such as type of the alkyl group in the support, ionic strength, eluent pH and percent organic modifier in the eluent. By careful manipulation of these parameters, the simultaneous determination of inorganic anions and carboxylic acids could be achieved.

A polymeric fluorocarbon-diamine silica (weak anion-exchanger/reversed-phase) column packing was prepared by Danielson *et al.*[19] and the separation of a number of aromatic compounds were examined on this column and on a hydrocarbon (C_8) column. The fluorocarbon column was generally less retentive than the hydrocarbon one, with good resolution and fast analysis of the sample mixtures. It also had the advantage of being stable under alkaline conditions.

Weatherall[20] also used a mixed-mode column (anion-exchange/ reversed-phase), for the determination of sulphonated azo dyes. Previous reverse-phase methods required the use of aggressive acidic solvents or the use of ion-pair reagents. Separation of the structural isomers was obtained using isocratic elution with acetonitrile and near neutral phosphate buffers.

A mixed-mode column has been used by Mc Laughlin[21] to analyze nucleic acids. He found that the mixed-mode materials relied upon both the hydrophobic interaction with the nucleobases and ionic interactions with the phosphodiesters for determination of these compounds. The resolution in some cases surpassed that observed with either ion-exchange or reversed-phase chromatography. Other work includes the determination of oligonucleotides[22,23] and tRNAs.[24]

Haginaka *et al.*[25] synthesized a mixed-functional silica support which was found suitable for the direct injection of drugs in serum. The phase is prepared from porous silica; 3-glycidoxypropyl groups are introduced, then phenyl groups are introduced and finally the oxirane is hydrolysed to diol groups. This phase was found suitable for the direct injection analysis of hydrophobic and hydrophilic drugs in serum, with recoveries in the region of 100 % being obtained.

Mixed-bed HPLC columns packed with equal proportions (w/w) of C_{18}- and SAX- bonded silica have been used for the chromatography of a range of substances, including phenols, substituted aromatic acids and sulphates, glycine and glucuronide conjugates.[26] It was found that columns prepared in this way showed chromatographic properties which were intermediate between those of the individual phases. The dual nature of the retention mechanism allowed the retention of ionizable molecules to be adjusted by varying the pH. This did not affect the retention of the uncharged compounds. The simultaneous chromatography of model compounds and their glucuronide, sulphate and glycine conjugates was demonstrated under a variety of conditions.

Other mixed-phases included C4 alkyl/phenyl sulfonate[27] bound to a silica support, a Poly-RP[28] and its cyano and diol derivatives which could be used with polar and non-polar solvent systems.

The work described in this chapter relates to the retention properties of a column containing equal proportions (w/w) of ion-exchange and reversed-phase materials, since the combined separation modes would increase the number of parameters that could be varied to optimize retention and resolution and permit the separation of both non-ionic and ionic compounds simultaneously. It describes experiments with a mixed-mode column containing both reversed-phase (C_{18}) and a strong cation exchange material (SCX). The chromatographic characteristics of a range of drug compounds, including benzodiazepines, tricylic antidepressants and barbiturates was investigated utilizing typical "reversed-phase" mobile phases at various pH's, with different buffer ions, buffer ion concentrations and various percentages of organic component. The chromatographic characteristics of these compounds were also determined on a C_{18} column for a comparison study.

2.2 EXPERIMENTAL

2.2.1 *Reagents and solvents*

HPLC grade methanol and acetonitrile were obtained from Labscan Analytical Sciences (Dublin, Ireland). Analar grade disodium hydrogen phosphate, sodium acetate, ammonium acetate, and dipotassium hydrogen phosphate, which were used as buffer salts, were obtained from Merck (Darmstadt, Germany). Deionized water was obtained from an Elgastat spectrum water purification unit.

2.2.2 *Test solutions*

Phenolphthalein and phenol were obtained from Sigma Chemical Co. (Poole, UK). The remaining compounds were obtained from commercial suppliers and were of pharmaceutical quality. Stock solutions were prepared by dissolving the appropriate amount of drug in methanol (100 %) to give a concentration equivalent to 1 mg/ml. The solutions were diluted to 100 μg/ml with deionized water and were stored at 4 °C. These stock solutions were freshly prepared on a weekly basis.

2.2.3 *HPLC Eluents*

A number of eluents were required for evaluation of this column; the organic component included acetonitrile or methanol. The buffer salts included sodium acetate, ammonium acetate, disodium hydrogen phosphate, dipotassium hydrogen phosphate. The pH was adjusted with 10 % phosphoric acid, 10 % acetic acid or 0.1 M sodium hydroxide.

2.2.4 *HPLC separations*

The HPLC system consisted of a Waters Associates (Milford, MA, USA) dual piston chromatography pump (Model 510) fitted with a Rheodyne (Cotati, CA, USA) injection port with a 20 μl injection loop. Detection was achieved with a Waters Model 486 spectrophotometric detector set at 254 nm. The sensitivity was 0.5 AUFS and retention times were determined using an integrator (Waters 746 Data Module). The column under evaluation was a Hypersil (250 mm x 4.6 mm I.D.) SCX/C_{18} column (Shandon Scientific Ltd., Cheshire, UK).

The column contained equal quantities of 5 μm C_{18} and 5 μm sulphonate modified silica. The column used for the comparison study was a Hypersil (250 x 4.6 mm i.d., 5 μm) single-mode column containing the same batch of C_{18} silica (Shandon Scientific Ltd., Cheshire, UK). All separations were carried out at ambient temperature.

2.2.5 *Calculations*

The capacity factors (k') of components were calculated from the equation:

$$k' = (t_r - t_o)/t_o$$

where t_r is the retention time of the analyte and t_o is the retention time of the first deviation of the baseline following injection of the organic component of the mobile phase.

2.3 RESULTS AND DISCUSSION

Table 2.1 lists the compounds used in the study, with their pK_a values to aid the rationalization of their retention properties, while Figure 2.2 shows some typical structures of the different classes of compounds studied.

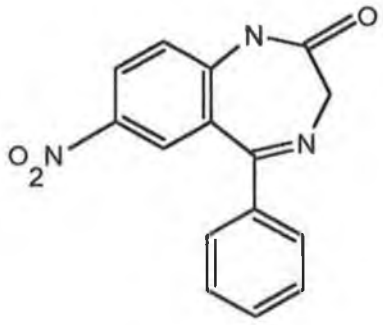
The compounds investigated were mostly of pharmaceutical or medical interest, and included strongly basic compounds such as tricyclic antidepressants (TCAs) and β-blockers, weakly basic compounds such as the benzodiazepines and compounds that are essentially acidic in character, such as the barbiturates.

TABLE 2.1

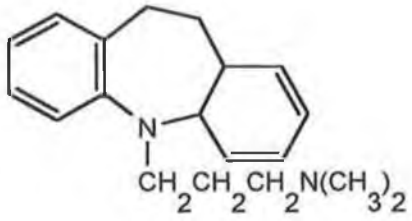
Compounds used in the study and their pK_a values

Compound	pK_a
Nitrazepam	3.2/10.8
Clonazepam	1.5/10.5
Diazepam	3.3
Flurazepam	1.9/8.2
Clomipramine	ND
Desmethyl-clomipramine	ND
Amitriptyline	9.4
Desipramine	10.2
Imipramine	9.5
Pindolol	8.8
Propranolol	9.5
Xylazine	ND
Terbutaline	8.7/10.0/11.0
Norephedrine	ND
N-Methyl-ephedrine	9.6
Barbitone	8.0
Phenobarbitone	7.3/11.8
Quinalbarbitone	7.9
Butobarbitone	8.0
Benzoic acid	4.2
Salicylic acid	3.0
Resorcinol	9.5/10.1
Furosemide	3.9
Phenolphthalein	9.7
Phenol	10.0

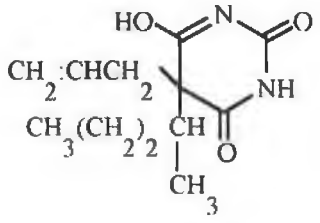
ND = not determined



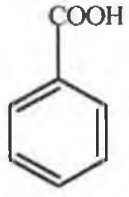
Nitrazepam



Imipramine



Quinalbarbitone



Benzoic acid

Figure 2.2: ***Structures of some compounds used in study: nitrazepam (weakly basic), imipramine (strongly basic), quinalbarbitone (weakly acidic) and benzoic acid (strongly acidic).***

2.3.1 *Chromatographic Elution Parameters*

2.3.1.1 *Effect of Eluent pH*

The aqueous component of the mobile phase used in this part of the study consisted of a 0.025 M sodium phosphate buffer adjusted to pH 3.0, 4.0, 5.0, 6.0 or 7.0 and mixed in a 50:50 (v/v) ratio with acetonitrile. The filtered degassed eluents were allowed to equilibrate on the column overnight to ensure that the desired on-column pH conditions were obtained.

Variation of the eluent pH alters the degree of ionization of ionizable compounds, resulting in different proportions of neutral and ionized forms. As only the unionized form of the compound will partition into the hydrophobic portion of the stationary phase, and the ionized form of the bases will interact with the sulphonate and the residual silanol groups, it is then to be expected that small changes in the pH of the eluent can elicit major changes in retention.

The benzodiazepines (nitrazepam, clonazepam, diazepam, flurazepam) are all weakly basic with pK_a values of less than 3.5. Hence, the retention times of these compounds are determined principally by their interaction with the C_{18} material over most of the pH range investigated. The data in Table 2.2 confirms that small increases in retention factors of these compounds occurred at lower pH values (3 - 4), while their retention indices were hardly affected by pH variation within the more alkaline eluents.

Phenobarbitone, barbitone, quinalbarbitone, butobarbitone, phenol and phenolphthalein are all weakly acidic with pK_a values varying between 7.2 and 11.8. These compounds only interact with the hydrophobic component in the column, and then only when unionized. Since they are largely unionized over the pH range investigated, it was found, as was expected, that their retention times

would remain unchanged over the pH range examined. Little variation in the retention times was indeed observed, as exhibited by the data shown in Table 2.2.

The tricylic antidepressants (clomipramine, desmethyl-clomipramine, amitriptyline, imipramine and desipramine) are all basic drugs with pK_a values greater than 8 and are thus mainly ionized over the pH range examined. The retention of these compounds decreased between pH 3 and pH 5 and then rose again sharply between pH 6 and pH 7 (Figure 2.3). In reversed-phase chromatography (RPC), the retention of basic compounds is strongly influenced by secondary equilibria between the oppositely charged amines and residual silanol groups on the stationary phase. These secondary equilibria will also contribute to the observed retention patterns on the mixed-mode phase, though the significance of these equilibra cannot be evaluated in terms of the primary ion exchange interactions with the SCX component in the column. It is possible that the decrease in retention observed between pH 3 and pH 5 is due to the decreasing percent ionization as the pH approaches the pK_a of the drugs.

However, as even the most weakly basic amine is still 99 % ionized at pH 7.0, the contribution from this source is probably not significant. It is more likely that since the fixed charge moiety is ionized over the entire pH range, the slight decrease in retention results from the increased molar concentration of competing cation (sodium) with increasing pH. Between pH 6 and pH 7, ionization of the silanol moieties becomes significant and the secondary silanophilic interaction could account for the dramatic increase in retention observed at this point.

Acidic compounds (benzoic acid, salicylic acid, resorcinol and furosemide) were found to be largely unaffected by eluent pH. In fact, they all had very low capacity factors at all pH's, which may be explained in terms of a low affinity for the hydrophobic component of the more acidic compounds (even when unionized) at low pH or, at high pH, in terms of electrostatic repulsion between the analytes and the similarly charged sulphonate sites.

TABLE 2.2

EFFECT OF ELUENT pH ON THE CAPACITY FACTORS

Eluent:-Acetonitrile :Disodium hydrogen phosphate 0.025 M (50:50)

Compound	Capacity Factor				
	pH				
	3	4	5	6	7
Nitrazepam	1.28	1.06	0.94	1.05	1.11
Clonazepam	1.35	1.13	1.03	1.24	1.20
Diazepam	2.32	2.19	1.98	2.32	2.37
Flurazepam	4.59	4.15	4.23	4.66	4.83
Clomipramine	3.99	3.75	3.59	4.61	8.59
Desmethyl-clomipramine	3.45	3.26	3.02	3.77	4.69
Amitriptyline	3.79	3.61	2.83	4.46	7.47
Desipramine	3.28	2.95	3.03	3.59	4.34
Imipramine	3.37	3.36	3.21	4.31	6.31
Pindolol	2.35	2.18	2.13	2.74	2.88
Propranolol	2.81	2.56	2.54	3.14	3.40
Xylazine	4.53	4.25	4.17	5.56	4.67
Terbutaline	2.72	2.47	2.35	2.92	2.89
Norephedrine	3.39	3.01	3.50	3.62	3.65
N-Methyl ephedrine	4.11	3.93	4.33	5.21	5.86
Barbitone	0.44	0.48	0.39	0.41	0.46
Phenobarbitone	0.77	0.73	0.67	0.96	0.79
Quinalbarbitone	1.17	1.08	0.90	1.07	1.20
Butobarbitone	0.85	0.80	0.72	0.82	0.78
Phenolphthalein	0.96	0.96	0.87	1.08	1.03
Phenol	0.79	0.83	0.78	0.85	0.86
Benzoic acid	0.69	0.62	0.45	0.52	0.37
Salicylic acid	0.50	0.36	0.39	0.61	0.36
Resorcinol	0.38	0.45	0.48	0.59	0.47
Furosemide	0.42	0.52	0.34	0.49	0.52

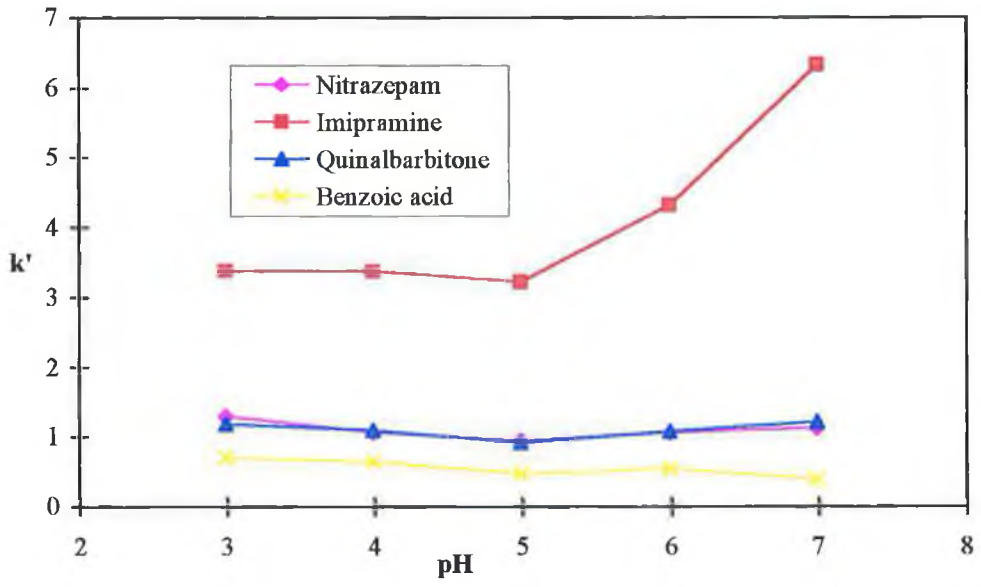


Figure 2.3: ***Effect of pH on the k′ values of nitrazepam(◆), imipramine (■), quinalbarbitone (▲) and benzoic acid (X) on a Hypersil C_{18}/SCX column. Mobile phase: acetonitrile-disodium hydrogen phosphate 25 mM (50:50 v/v). Flow rate 1.0 ml/min.***

The remaining basic compounds (pindolol, propranolol, terbutaline, xylazine and the catecholamines) were affected by pH in a manner similar to the TCA's: high retention values at low pH which decreased until pH 5, after which retention increased again.

The weakly basic and acidic analytes all have very low k′ values over the pH range, suggesting either that there is insufficient C_{18} material present to give them reasonable capacity factors, or that these compounds, even when unionized, have low affinities for the very hydrophobic C_{18} material.

2.3.1.2 *Effect of Ionic Strength*

The mobile phase used for this part of the study consisted of either 0.01, 0.025 or 0.05 M disodium hydrogen phosphate (pH 5.0) mixed in equal proportions with acetonitrile. The filtered degassed mobile phase was allowed to equilibrate on-column for at least 2 h.

It was expected that ionic strength would exert a considerable influence on the retention times of the cationic analytes since there are both primary (sulphonate) and secondary (silanophilic) cationic exchange mechanisms occurring. As indicated by the data in Table 2.3, the relative dominance of these two mechanisms was determined by the pH of the eluent. Either way, the concentration of competing cation in the mobile phase will influence retention in a characteristic ion exchange manner: as the concentration of competing ions in the mobile phase is increased, the number of ions with which the solute must compete for sites on the stationary phase is increased, resulting in lower retention times. Furthermore, compounds with different pK_a values (showing different degrees of ionization) are susceptible to different extents. The TCA's, catecholamines and a number of other basic compounds showed similar results: the retention values decreased as the ionic strength increased (Figures 2.4.a. and 2.4.b.)

The weakly basic benzodiazepines, unionized over the pH range examined, were largely unaffected by changes in the ionic strength (Figures 2.5.a. and 2.5.b.). There was a small decrease in retention with increasing ionic strength observed for the acidic compounds, though the origin of this observation has not been identified. Figure 2.6 indicates the effect the ionic strength has on the capacity factors of the components examined.

TABLE 2.3

EFFECT OF IONIC STRENGTH ON THE CAPACITY FACTORS

Eluent:-Acetonitrile:Disodium hydrogen phosphate pH 5.0 (50:50)

Compound	**Capacity Factor**		
	Relative Ionic Strength		
	0.01 M	**0.025 M**	**0.05 M**
Nitrazepam	0.99	0.94	0.91
Clonazepam	1.18	1.03	0.99
Diazepam	2.51	1.98	1.88
Flurazepam	>20	4.23	2.64
Clomipramine	8.02	3.59	2.26
Desmethyl-clomipramine	7.11	3.02	1.93
Amitriptyline	8.25	2.83	2.28
Desipramine	6.89	3.03	1.80
Imipramine	7.86	3.21	2.15
Pindolol	5.58	2.13	1.42
Propranolol	6.35	2.54	1.59
Xylazine	>20	4.17	2.76
Terbutaline	6.08	2.35	1.44
Norephedrine	8.25	3.50	1.86
N-Methyl ephedrine	>20	4.33	2.44
Phenobarbitone	0.78	0.67	0.62
Barbitone	0.61	0.39	0.35
Quinalbarbitone	1.30	0.90	0.84
Butobarbitone	0.92	0.72	0.62
Phenolphthalein	1.02	0.87	0.85
Phenol	0.91	0.78	0.74
Benzoic Acid	0.89	0.45	0.39
Salicylic Acid	0.55	0.32	0.39
Resorcinol	0.55	0.48	0.39
Furosemide	0.94	0.34	0.34

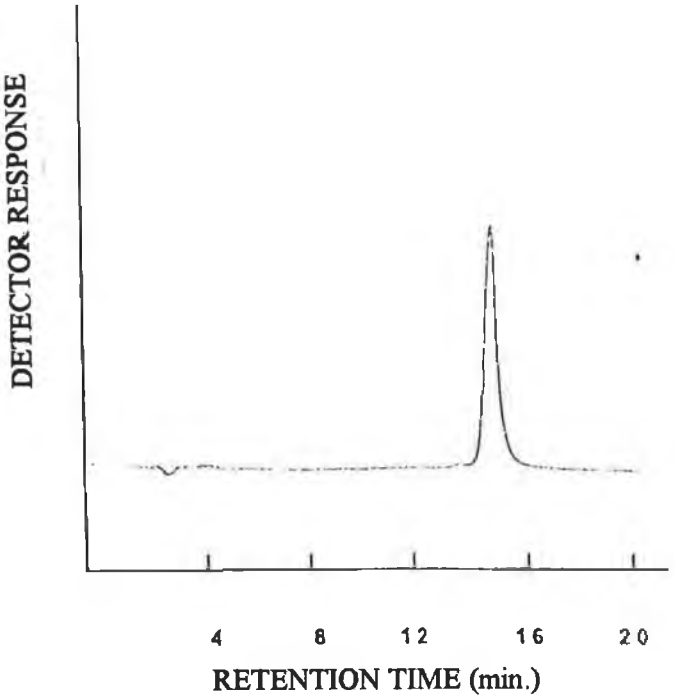


Figure 2.4.a: *Effect of eluent ionic strength on the capacity factor of the strongly basic compound clomipramine on a Hypersil C_{18}/SCXcolumn. Mobile phase: acetonitrile-disodium hydrogen phosphate 10 mM pH 5. (50:50 v/v). Flow rate 1.0 ml/min.*

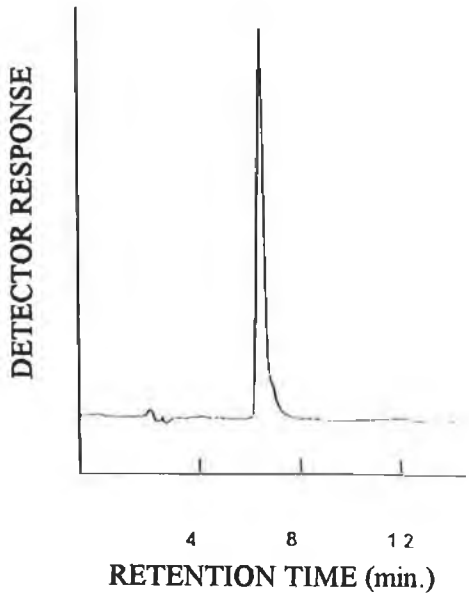


Figure 2.4.b: *Effect of eluent ionic strength on the capacity factor of the strongly basic compound clomipramine on a Hypersil C_{18}/SCX column. Mobile phase: acetonitrile-disodium hydrogen phosphate 50 mM pH 5. (50:50 v/v). Flow rate 1.0 ml/min.*

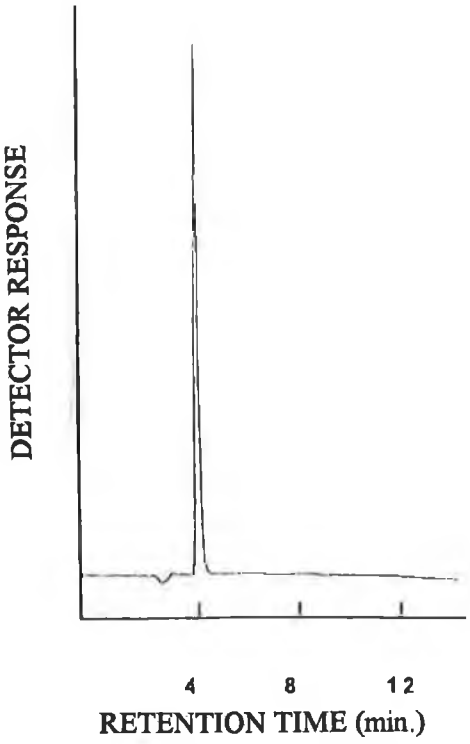


Figure 2.5.a: *Effect of eluent ionic strength on the capacity factor of the weakly basic compound clonazepam on a Hypersil C_{18}/SCX column. Mobile phase: acetonitrile-disodium hydrogen phosphate 10 mM pH 5. (50:50 v/v). Flow rate 1.0 ml/min.*

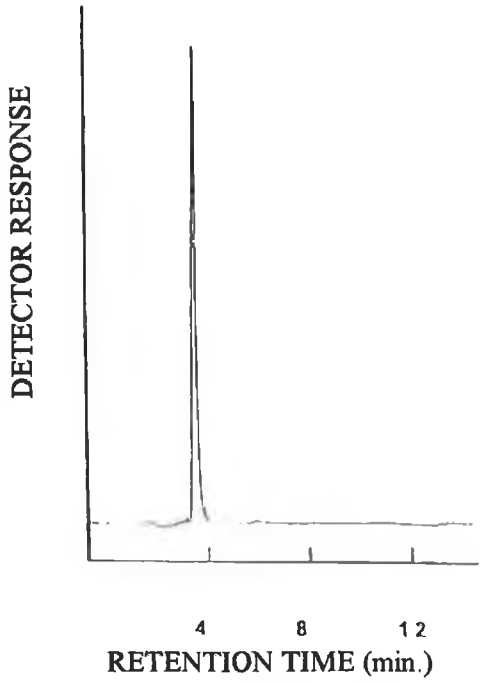


Figure 2.5.b: *Effect of eluent ionic strength on the capacity factor of the weakly basic compound clonazepam on a Hypersil C_{18}/SCX column. Mobile phase: acetonitrile-disodium hydrogen phosphate 50 mM pH 5. (50:50 v/v). Flow rate 1.0 ml/min.*

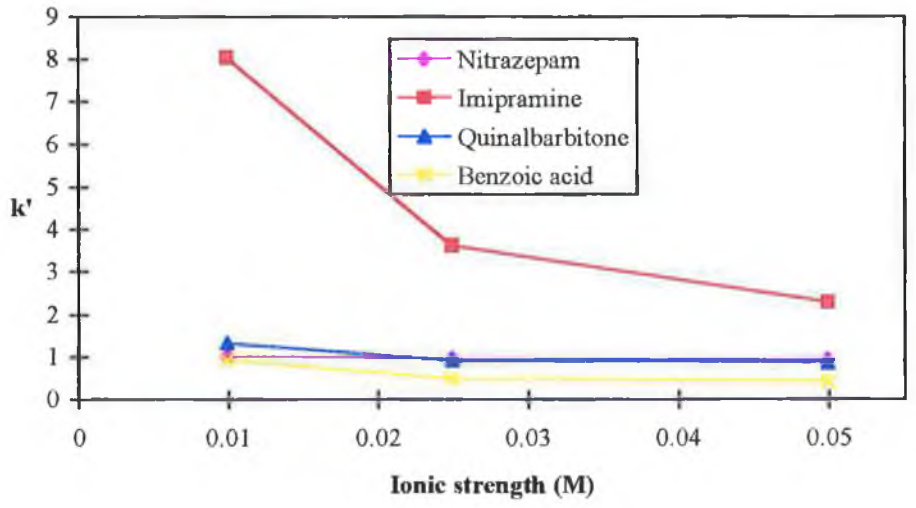


Figure 2.6: ***Effect of ionic strength on the k′ values of nitrazepam(◆), imipramine (■), quinalbarbitone (▲) and benzoic acid (X) on a Hypersil C_{18}/SCX column. Mobile phase: acetonitrile-disodium hydrogen phosphate 25 mM (50:50 v/v). Flow rate 1.0 ml/min.***

2.3.1.3 *Effect of Buffer Cation*

To examine the effect of buffer cation, the mobile phase consisted of either sodium, potassium phosphate or sodium or ammonium acetate. Each of these was prepared at two concentrations; 0.025 M and 0.05 M (pH 5.0) and mixed in equal proportions with acetonitrile. Changing the cation in the mobile phase will change retention values of ionized compounds. Increasing the ionic strength of the buffer cation results in a decreased retention of the compounds which are ionized at pH 5, regardless of the buffer cation. Varying retention values were obtained depending on the affinity of the cations for the fixed anionic sites (Figure 2.7). As expected, doubling the ionic strength caused the retention times

of the strongly basic TCA's and catecholamines to decrease dramatically (Table 2.4). The order of competing ion ability to displace the analytes from the charged sites was found to be:- $Na^+ << NH_4^+ << K^+$.

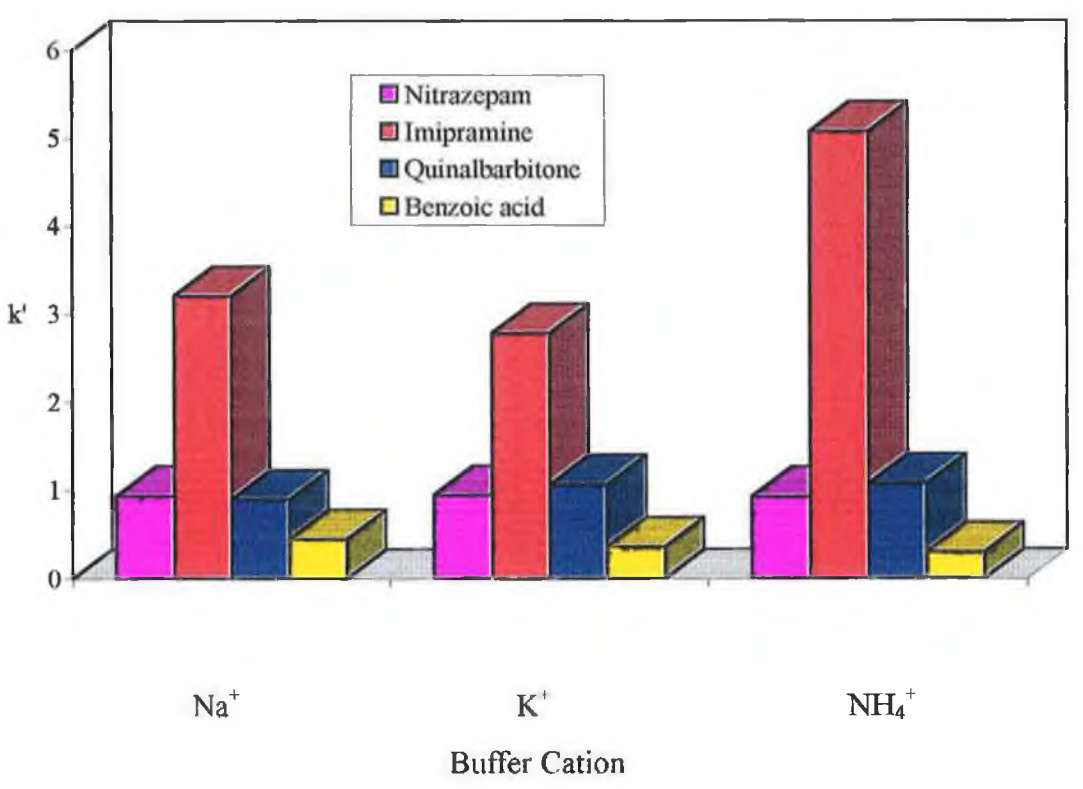


Figure 2.7: ***Effect of buffer cation on the k′ values of nitrazepam, imipramine, quinalbarbitone and benzoic acid on a Hypersil C_{18}/SCXcolumn. Mobile phase: acetonitrile-buffer 25 mM (50:50 v/v). Flow rate 1.0 ml/min.***

TABLE 2.4

EFFECT OF CATION ON THE CAPACITY FACTORS

Eluent:- Acetonitrile : Buffer pH 5.0 (50:50)

Compound	Capacity Factor/ Ionic Strength							
	Na^+		K^+		Na^+		NH_4^+	
	0.025	0.05	0.025	0.05	0.025	0.05	0.025	0.05
Nitrazepam	0.94	0.91	0.95	0.94	1.16	0.91	0.93	0.95
Clonazepam	1.03	0.99	1.03	1.00	1.24	0.99	1.01	0.97
Diazepam	1.98	1.88	2.00	1.94	2.34	1.96	2.00	1.87
Flurazepam	4.23	2.64	3.32	2.16	6.70	3.97	6.09	4.26
Clomipramine	3.59	2.26	3.17	2.20	5.84	3.74	5.22	3.81
Desmethyl-clomipramine	3.02	1.93	2.76	1.95	4.62	3.17	4.48	3.31
Amitriptyline	2.83	2.28	2.99	2.01	5.68	3.55	5.09	3.52
Desipramine	3.03	1.80	2.49	1.66	4.62	2.85	4.25	2.85
Imipramine	3.21	2.15	2.79	1.89	5.14	3.33	5.08	3.35
Pindolol	2.13	1.42	1.89	1.25	3.56	2.07	3.35	2.36
Propranolol	2.54	1.59	2.17	1.40	4.07	2.44	3.85	2.56
Xylazine	4.17	2.76	3.50	2.14	7.22	4.23	6.57	4.43
Terbutaline	2.35	1.44	2.01	1.31	3.69	2.20	3.54	2.42
Norephedrine	3.50	1.86	2.53	1.57	5.01	2.97	4.69	3.09
N-Methylephedrine	4.33	2.44	3.15	1.95	5.41	3.90	6.03	4.00
Phenobarbitone	0.67	0.62	0.65	0.64	0.80	0.61	0.62	0.63
Barbitone	0.39	0.35	0.43	0.37	0.56	0.42	0.46	0.40
Quinalbarbitone	0.90	0.84	1.07	0.98	1.28	1.04	1.08	1.12
Butobarbitone	0.72	0.62	0.72	0.65	0.88	0.69	0.72	0.77
Phenolphthalein	0.87	0.85	0.87	0.83	1.05	0.82	0.84	0.83
Phenol	0.78	0.74	0.75	0.74	0.92	0.72	0.72	0.78
Benzoic acid	0.45	0.39	0.36	0.33	0.43	0.33	0.30	0.30
Salicylic acid	0.39	0.39	0.29	0.27	0.41	0.33	0.29	0.33
Resorcinol	0.48	0.39	0.40	0.33	0.54	0.40	0.44	0.40
Furosemide	0.34	0.34	0.31	0.24	0.42	0.32	0.31	0.26

2.3.1.4 *Effect of Organic Component and Percent Organic Component*

The effect of both type and percentage of organic modifier was then investigated. Methanol and acetonitrile were compared by mixing either solvent with an equal volume of 0.025 M phosphate buffer, pH 5.0. The effect of changing the percentage organic component was investigated by adding acetonitrile to 0.025 M phosphate buffer, pH 5, in proportions ranging from 20 % to 80 % acetonitrile. Retention times were, on average, much shorter using acetonitrile as opposed to methanol in the mobile phase (Table 2.5). Acetonitrile has virtually no effect on selectivity, probably indicating that it does not modify the type, but rather the intensity of interaction between solutes and stationary phase (it competes with the solutes for occupation of the C_{18} groups). As acetonitrile changes only k' values, a decrease in acetonitrile concentration does not produce improved resolution of any two closely eluting solutes, but results in a longer analysis time. High retention values were observed with low proportions of acetonitrile, and these values decreased as the amount of acetonitrile present increased. For some compounds, retention values increased again between 60 % and 70 % acetonitrile (Table 2.6). This is in agreement with other workers who attributed the U-shape of the curve to hydrophobic interactions at low solvent concentrations and changes in the counter-ion solvation at high solvent concentrations.[29]

TABLE 2.5

EFFECT OF ORGANIC MODIFIER ON THE CAPACITY FACTORS

Eluent: Organic Modifier: Disodium hydrogen phosphate pH 5 (50:50)

Compound	**Capacity Factor**	
	Organic Component	
	Acetonitrile	**Methanol**
Nitrazepam	0.94	3.41
Clonazepam	1.03	3.45
Diazepam	1.98	>20
Flurazepam	4.23	>20
Clomipramine	3.59	>20
Desmethyl-clomipramine	3.02	>20
Amitriptyline	2.83	>20
Desipramine	3.03	>20
Imipramine	3.21	>20
Pindolol	2.13	4.62
Propranolol	2.54	>20
Xylazine	4.17	8.76
Terbutaline	2.35	3.01
Norephedrine	3.50	4.13
N-Methylephedrine	4.33	7.68
Phenobarbitone	0.67	1.39
Barbitone	0.39	0.85
Quinalbarbitone	0.90	4.89
Butobarbitone	0.72	2.11
Phenolphthalein	0.87	5.34
Phenol	0.78	1.17
Benzoic Acid	0.45	0.54
Salicylic Acid	0.39	0.67
Resorcinol	0.48	0.61
Furosemide	0.34	0.70

TABLE 2.6

EFFECT OF THE PERCENT ACETONITRILE ON THE CAPACITY FACTORS

Eluent:- Acetonitrile: Disodium hydrogen phosphate 0.025 M pH 5.0

Compound	**Capacity Factor**				
	% Organic Component				
	20	35	50	65	80
Nitrazepam	3.52	3.88	0.94	1.13	0.14
Clonazepam	4.07	4.46	1.03	1.24	0.23
Diazepam	>20	10.46	1.98	1.13	0.36
Flurazepam	>20	>20	4.23	2.67	1.48
Clomipramine	>20	>20	3.59	2.02	0.90
Desmethyl-clomipramine	>20	>20	2.83	1.14	0.83
Amitriptyline	>20	>20	3.62	1.74	0.94
Desipramine	>20	11.13	3.03	1.74	0.89
Imipramine	>20	>20	3.21	1.99	0.94
Pindolol	>20	4.76	2.13	1.80	1.34
Propranolol	>20	7.33	2.54	2.99	0.95
Xylazine	>20	>20	4.17	2.69	1.54
Terbutaline	3.81	3.68	2.35	2.36	2.27
Norephedrine	5.52	4.93	3.50	3.00	2.90
N-Methyl ephedrine	>20	3.68	4.33	3.47	2.55
Barbitone	1.76	0.94	0.39	0.57	0.18
Phenobarbitone	3.28	1.85	0.67	0.45	0.20
Quinalbarbitone	5.44	4.21	0.90	0.73	0.15
Butobarbitone	2.70	2.16	0.72	0.54	0.12
Benzoic acid	0.76	0.76	0.45	0.37	0.11
Salicylic acid	0.70	0.68	0.39	0.43	0.10
Resorcinol	1.18	1.08	0.48	0.52	0.20
Furosemide	1.39	0.93	0.34	0.43	0.05
Phenolphthalein	>20	4.05	0.87	0.60	0.28
Phenol	3.60	1.88	0.78	0.56	0.16

2.4 COMPARISON OF C_{18} WITH C_{18}/SCX

2.4.1 *Chromatographic Elution Parameters*

2.4.1.1 Effect of Eluent pH

The same experiments were carried out on a C_{18} column prepared from the same batch of silica base material. The column dimensions and particle size were the same as those previously described for the C_{18}/SCX column.

The benzodiazepines studied were found to interact strongly with the hydrophobic stationary phase above their pK_a values (unionized state), while below these values very little interaction occurred with the stationary phase. The data in Table 2.7 demonstrates that a large increase in the capacity factors occurs between pH 3 and pH 4, with further minor increases occurring between pH 4 and pH 7. The change in capacity factors between pH 3 and pH 4 on the C_{18} material is much larger than the change observed on the C_{18}/SCX column for this pH range, hence indicating that the primary mode of interaction of the benzodiazepines is with the hydrophobic stationary phase (Figures 2.8.a. and 2.8.b.). The capacity factors of the barbitones and the weak acids show little variation over the entire pH range, with only a slight increase on moving from pH 3 to pH 7 (Figure 2.9). The trend observed here is similar to that obtained on the C_{18}/SCX column.

The remaining acidic compounds (furosemide, benzoic acid) with low pK_a values show a decrease in capacity factors from pH 3 - 5 and then an increase from pH 5 - 7. As their pK_a values are low, these drugs are largely unionized at the lower pH values, and hence interact with the hydrophobic stationary phase. The trend observed here is similar to that obtained for these compounds on the C_{18}/SCX column.

TABLE 2.7

EFFECT OF ELUENT pH ON THE CAPACITY FACTORS

Eluent:-Acetonitrile :Disodium hydrogen phosphate 0.025 M (50:50)

Compound	**Capacity Factor**				
	pH				
	3	4	5	6	7
Nitrazepam	0.17	1.70	1.70	1.88	2.36
Clonazepam	0.67	1.85	1.86	2.07	2.54
Diazepam	0.52	3.81	3.80	4.26	5.07
Flurazepam	1.00	1.16	1.18	1.82	4.97
Clomipramine	2.71	2.91	3.08	3.96	8.87
Desmethyl-clomipramine	2.39	2.53	2.64	3.05	3.86
Amitriptyline	2.01	2.13	2.27	2.90	6.75
Desipramine	1.56	1.63	1.73	1.95	2.51
Imipramine	1.78	2.01	1.97	2.31	4.93
Pindolol	0.50	0.54	0.53	0.60	0.94
Propranolol	0.83	0.90	0.96	1.07	1.61
Xylazine	0.67	0.73	0.80	0.95	1.92
Terbutaline	0.46	0.51	0.56	0.70	0.71
Norephedrine	0.52	0.51	0.61	0.65	0.89
N-Methylephedrine	0.45	0.59	0.62	0.64	1.04
Barbitone	0.59	0.66	0.67	0.73	1.01
Phenobarbitone	0.94	1.08	1.08	1.14	1.44
Quinalbarbitone	1.67	1.99	2.00	2.13	2.71
Butobarbitone	1.17	1.26	1.26	1.15	1.75
Phenolphthalein	1.49	1.60	1.61	1.75	2.34
Phenol	1.59	1.76	1.68	1.72	1.87
Benzoic acid	0.83	0.48	0.40	0.48	0.79
Salicylic acid	0.83	0.55	0.43	0.51	0.87
Resorcinol	0.56	0.62	0.62	0.70	1.05
Furosemide	1.24	0.86	0.51	0.51	0.91

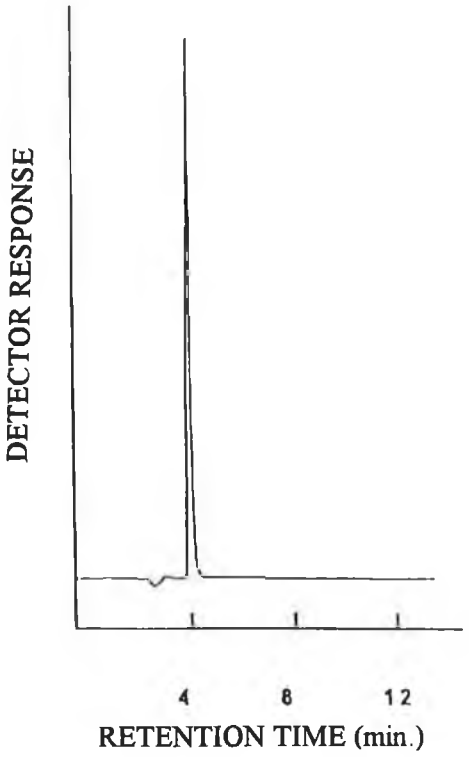


Figure 2.8.a: ***Influence of column packing material on the capacity factor of a weakly basic compound, clonazepam, on a Hypersil C_{18}/SCX column. Mobile phase: acetonitrile- disodium hydrogen phosphate 10 mM pH 5 (50:50 v/v). Flow rate 1.0 ml/min.***

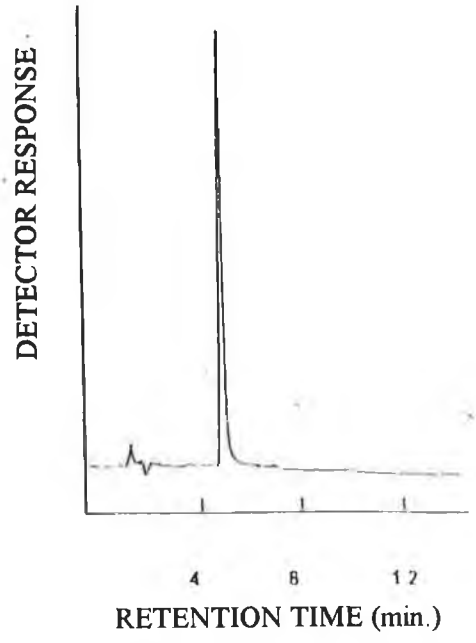


Figure 2.8.b: ***Influence of column packing material on the capacity factor of a weakly basic compound, clonazepam, on a Hypersil C_{18} column. Mobile phase: acetonitrile- disodium hydrogen phosphate 10 mM pH 5 (50:50 v/v). Flow rate 1.0 ml/min.***

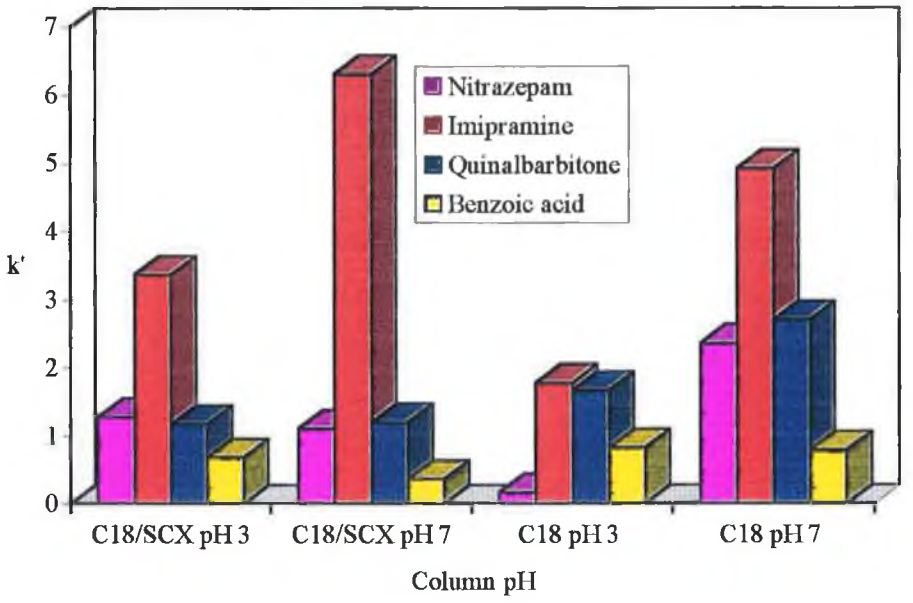


Figure 2.9: ***Comparsion between C_{18}/SCX and C_{18} columns at pH 3.0 and pH 7.0. Mobile phase 0.025 M disodium hydrogen phosphate (pH 3.0 or pH 7.0)-acetonitrile (50:50 v/v).***

The TCA's are ionized over this entire pH range and show increasing capacity values as pH increases. This is probably due to the secondary silanol interactions of the compounds with the stationary phase. The presence of dimethyl groups on the nitrogen (reduced steric hindrance around the nitrogen atom), and increased substitution on the nitrogen atom favour secondary interactions, the effects decreasing with decreasing pH.

The remaining basic compounds (pindolol, propranolol, terbutaline and the catecholamines) were affected in a manner similar to the TCA's (Figure 2.10). However the capacity factors obtained on this column compared to the C_{18}/SCX column are much lower, and hence this would indicate that the ion-exchange interaction of the analytes with the SCX material is an important factor in the retention of these compounds. Therefore the C_{18}/SCX would find particular

application in the simultaneous determination of these compounds (Figures 2.11.a and 2.11.b).

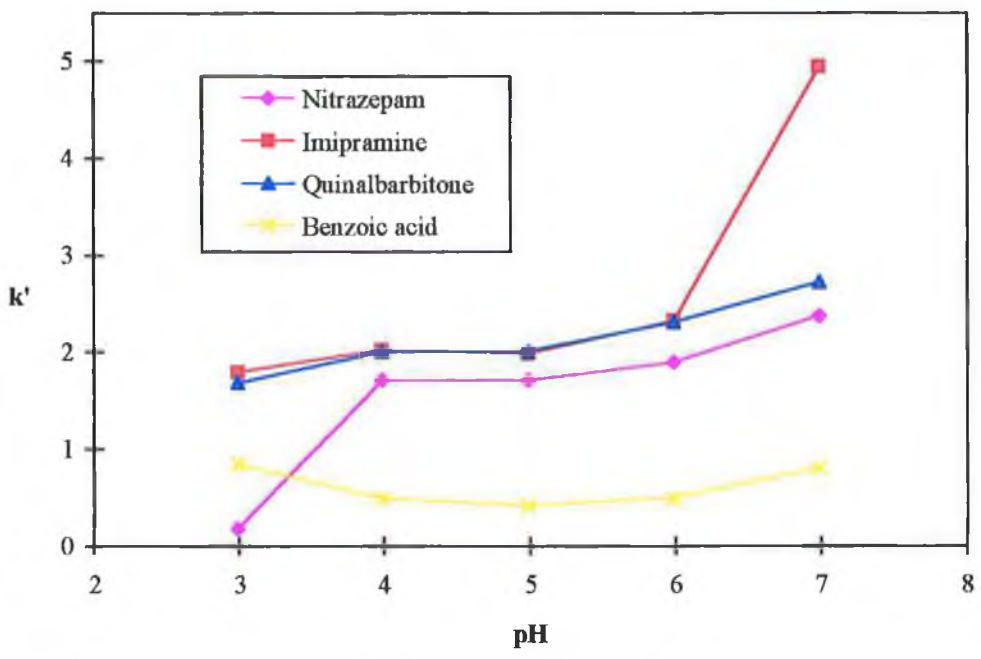


Figure 2.10: ***Effect of pH on the k' values of nitrazepam(◆), imipramine (■), quinalbarbitone (▲) and benzoic acid (✕) on a Hypersil C_{18} column. Mobile phase: acetonitrile-disodium hydrogen phosphate 25 mM (50:50 v/v). Flow rate 1.0 ml/min.***

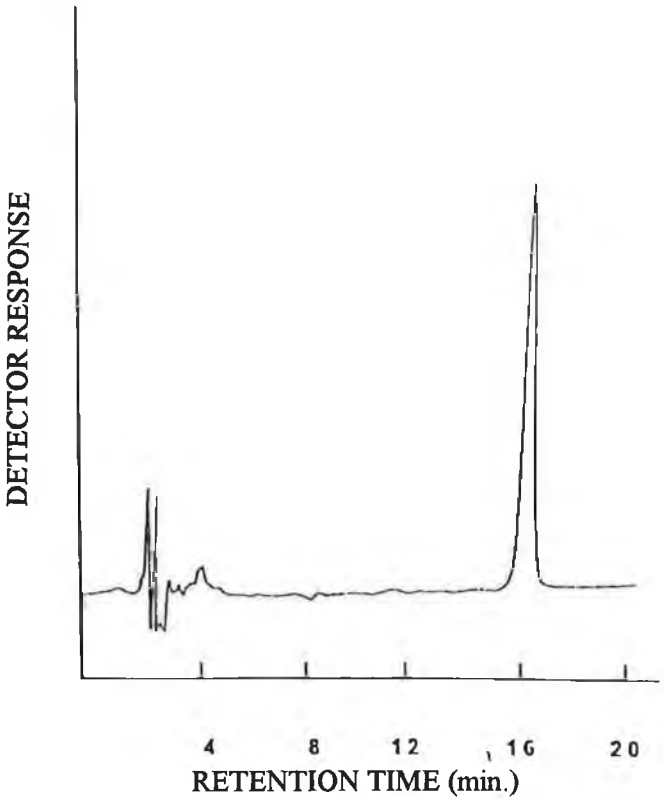


Figure 2.11.a: ***Influence of column packing material on the capacity factor of a weakly basic compound, clonazepam, on a Hypersil C_{18}/SCX column. Mobile phase: acetonitrile- disodium hydrogen phosphate 10 mM pH 5 (50:50 v/v). Flow rate 1.0 ml/min.***

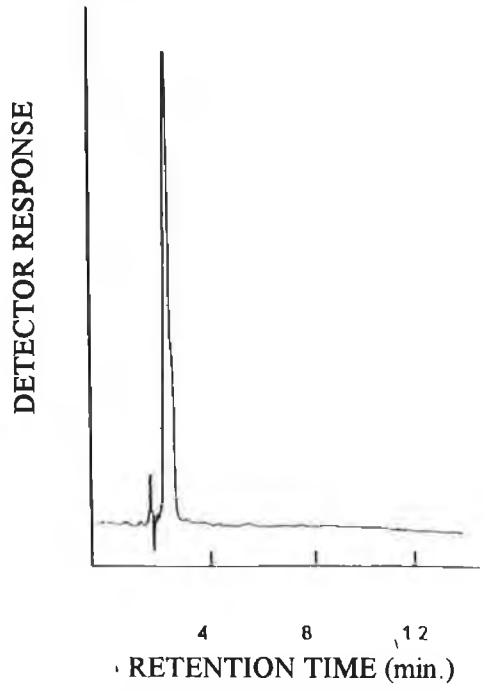


Figure 2.11.b: ***Influence of column packing material on the capacity factor of a strongly basic compound, norephedrine, on a Hypersil C_{18} column. Mobile phase: acetonitrile- disodium hydrogen phosphate 10 mM pH 5 (50:50 v/v). Flow rate 1.0 ml/min.***

2.4.1.2 *Effect of Ionic Strength*

It is expected that the ionic strength will affect the capacity factors of the cationic analytes due to the secondary (silanophilic) interactions. The largest variations in capacity factors with increasing ionic strength were observed for the TCA's (Table 2.8). The benzodiazepines were largely unaffected by changes in ionic strength and similar results were obtained on the C_{18}/SCX column. The catecholamines showed a reduction in capacity factors, although these changes were not as large as those observed for the TCA's or indeed the same compounds on the SCX/C_{18} column (Figure 2.12). The remaining compounds examined showed decreasing capacity factors with increasing ionic strength.

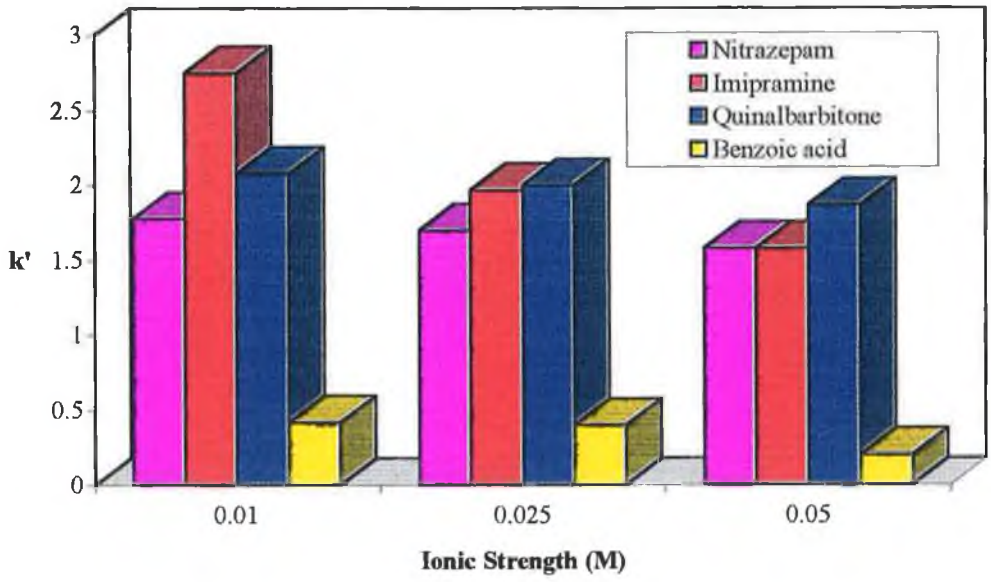


Figure 2.12: ***Effect of ionic strength on the k′ on nitrazepam, imipramine, quinalbarbitone and benzoic acid on a Hypersil C_{18} column. Mobile phase: acetonitrile-disodium hydrogenphosphate pH 5.0 (50:50 v/v). Flow rate 1.0 ml/min.***

TABLE 2.8

EFFECT OF IONIC STRENGTH ON THE CAPACITY FACTORS

Eluent:-Acetonitrile:Disodium hydrogen phosphate pH 5 (50:50)

Compound	**Capacity Factor**		
	Relative Ionic Strength		
	0.01M	**0.025M**	**0.05M**
Nitrazepam	1.78	1.70	1.58
Clonazepam	1.93	1.86	1.74
Diazepam	4.06	3.80	3.58
Flurazepam	1.60	1.18	0.93
Clomipramine	4.37	3.08	2.50
Desmethyl-clomipramine	3.85	2.64	2.11
Amitriptyline	3.15	2.27	1.83
Desipramine	2.46	1.73	1.37
Imipramine	2.75	1.97	1.58
Pindolol	0.68	0.53	0.42
Propranolol	1.34	0.96	0.75
Xylazine	1.02	0.80	0.60
Terbutaline	0.59	0.56	0.42
Norephedrine	0.62	0.61	0.41
N-Methyl ephedrine	0.62	0.62	0.45
Phenobarbitone	1.09	1.08	0.98
Barbitone	0.69	0.67	0.57
Quinalbarbitone	2.08	2.00	1.87
Butobarbitone	1.28	1.26	1.15
Benzoic Acid	0.42	0.40	0.20
Salicylic Acid	0.49	0.43	0.40
Resorcinol	0.64	0.62	0.54
Furosemide	0.63	0.51	0.44
Phenolphthalein	1.65	1.61	1.51
Phenol	1.78	1.68	1.62

TABLE 2.9

EFFECT OF THE PERCENT OF ACETONITRILE ON THE CAPACITY FACTORS Eluent:- Acetonitrile: Disodium hydrogen phosphate 0.025M pH 5

Compound	Capacity Factor % Organic Component				
	20	35	50	65	80
Nitrazepam	14.40	2.71	1.70	1.03	0.91
Clonazepam	11.20	2.11	1.86	0.52	0.51
Diazepam	>20	8.64	3.80	2.02	1.10
Flurazepam	>20	2.71	1.18	0.82	0.63
Clomipramine	>20	>20	3.08	1.46	0.98
Desmethyl-clomipramine	>20	>20	2.64	1.28	0.88
Amitriptyline	>20	>20	2.27	1.20	0.84
Desipramine	>20	5.41	1.73	0.96	0.71
Imipramine	>20	6.12	1.97	1.07	0.77
Pindolol	>20	0.60	0.53	0.43	0.42
Propranolol	>20	2.08	0.96	0.59	0.49
Xylazine	>20	1.04	0.80	0.58	0.55
Terbutaline	5.18	0.67	0.56	0.42	0.39
Norephedrine	5.29	0.65	0.61	0.42	0.41
N-Methyl ephedrine	5.39	0.69	0.62	0.46	0.46
Barbitone	14.75	0.77	0.67	0.54	0.48
Phenobarbitone	31.65	1.84	1.08	0.66	0.49
Quinalbarbitone	>20	3.56	2.00	1.08	0.62
Butobarbitone	10.20	2.00	1.26	0.76	0.49
Benzoic acid	6.88	0.61	0.40	0.36	0.15
Salicylic acid	5.14	0.53	0.43	0.32	0.12
Resorcinol	7.96	0.69	0.62	0.49	0.42
Furosemide	10.27	0.88	0.51	0.39	0.31
Phenolphthalein	9.35	2.27	1.61	0.77	0.52
Phenol	5.86	1.83	1.68	0.85	0.51

2.4.1.3 *Effect of Organic Component*

All compounds showed decreasing capacity values with increasing percentage acetonitrile (Table 2.9). These results are similar to those obtained with the SCX/C_{18} column.

2.5 CONCLUSION

The data obtained indicates that the primary ion-exchange mechanism is a significant force in determining the retention indices of the more strongly basic cationic analytes. All the basic compounds showed varying retention times with changing eluent pH, the effect being more pronounced for more strongly basic analytes. Acidic compounds were little influenced by pH changes, so the retention of strongly basic compounds could be altered independently of the acidic compounds. The cation-exchange mechanism could be influenced by changing ionic strength, and in this respect, potassium was found to be the strongest competing cation. The C_{18}/SCX column has particular application for the determination of the catecholamines, the capacity factors on this column were found to be substantially higher than those obtained on the C_{18} column. Therefore changes in pH are more pronounced on this column and hence separations may be carried out simply by varying pH alone. Changes in the ionic strength results in substantial changes in the capacity values compared with the values on a C_{18} column. Variation in the percent acetonitrile in the mobile phase effected both columns in a similar manner; increasing percentages of organic modifier resulting in decreases in capacity factors for all the test compounds. The C_{18}/SCX mixed-mode column is capable of separating both basic and neutral compounds simultaneously, and should therefore be particularly useful in

screening for drugs of abuse. This phase could also find application where it is necessary to determine a pharmaceutical product along with its degradation/process impurities which collectively may not be suitable for analysis by either reversed-phase or ion-exchange chromatography alone.

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CHAPTER THREE

SIMULTANEOUS DETERMINATION OF PROPRANOLOL AND FUROSEMIDE IN HUMAN PLASMA BY MIXED-MODE CHROMATOGRAPHY

3.1 INTRODUCTION

Propranolol (Figure 3.1) is a competitive antagonist at β_1 and β_2 receptors. It antagonizes catecholamine action at both β_1 and β_2 receptors. It functions by blockade of cardiac β_1-receptors and reduces heart rate and contractility. β_2 receptor blockade increases airway resistance and decreases catecholamine-induced glycogenolysis and peripheral vasodilation. It has found wide application in the treatment of cardiac arrhythmia, sinus tachycardia[1] and angina pectoris. It has also been suggested for use for a number of other conditions including dysfunctional labour[2] and anxiety.[3] It has been employed in the long-term treatment of hypertension, often in combination with a diuretic or vasodilator.

To date there have been a number of techniques which have been used for the determination of propranolol including GC,[4] GC-MS,[5,6] ion-exchange HPLC[7] and HPLC.[8,9] Singh-Rekhi *et al.*[10] determined propranolol from human plasma/serum using pronethalol hydrochloride as the internal standard. The plasma was first mixed with acetonitrile and centrifuged, the supernatant was evaporated to dryness under nitrogen, and the residue was reconstituted in methanol. The analysis was carried out using a Hypersil CN column (25 cm x 4.6 mm) with a mobile phase of acetonitrile/1 % acetic acid, pH 3.6 with 0.2 % TEA (36:65). The calibration graphs were linear in the range 5 - 200 ng/ml and the recoveries from serum and plasma were 60.98 % and 81.35 %, respectively.

Cosbey *et al.*[11] extracted propranolol and a number of basic components from whole blood by solid-phase extraction. This method involved the use of two cartridges in series; namely superclean ENVIcarb and Bondelut PRS. The blood was first applied to the ENVIcarb, and any interferents present were removed by washing with water. The analytes of interest were then eluted with methanolic TFA and xylene/methanol (4:1). The eluates were then applied to the Bondelut

PRS cartridge and washed further with methanol and ethyl acetate. Elution was achieved with ethyl acetate/methanol/NH_3 (48:1:1) and recovery values for propranolol were in the region of 100 %. This method was found suitable for automated sample preparations.

Hubert *et al.*[12] isolated a number of β-blockers, including propranolol, from plasma using Bond Elut extraction cartridges. After pre-conditioning and washing, the analytes were desorbed with methanol or acetonitrile. The eluate was diluted with 0.05 M phosphate buffer, pH 3, and analyzed on a LiChrospher 100 RP-18 column with a mobile phase of 0.05 M phosphate buffer, pH 3.0/methanol or acetonitrile containing 0.5 % 2-aminoheptane. Calibration graphs were found to be linear in the region 5 - 500 ng/ml with a detection limit of 1.3 ng/ml; the recovery was found to be 93 %.

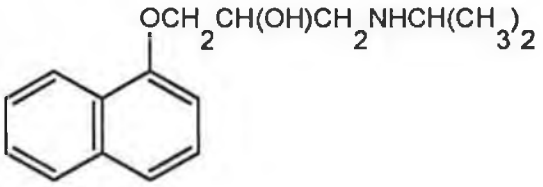
Furosemide (Figure 3.1) is considered to be a short-acting loop diuretic. It exerts its major effect by inhibiting sodium readsorption in the proximal convoluted tubule and the loop of Henle. It has a high capacity for NaCl readsorption in this segment and agents active at this site markedly increase water and electrolyte excretion and are referred to as "high ceiling" diuretics. Its major uses are in acute or chronic renal failure, congestive heart failure and liver cirrhosis.[13,14] It has also found use for treatment of hypertension, in particular in individuals with diminished renal function.

The principal methods for the determination of furosemide have been based mainly on HPLC[15,16] and GC-MS. Vree *et al.*[17] determined furosemide and its acyl glucuronide from human plasma and urine by HPLC with fluorescence detection. The components were analyzed on a Spherisorb ODS column using gradient elution. The initial mobile phase was acetonitrile: 0.05 % orthophosphoric acid, pH 2.1 (1:19) and these varied to a final mobile phase with a ratio of 41:59. Detection limits of 5 ng/ml were achieved for furosemide with a recovery value of 91.5 %.

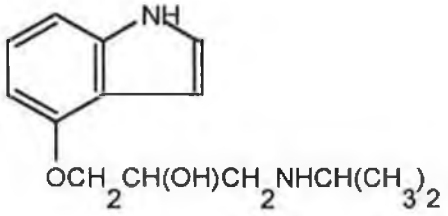
Bonet-Domingo *et al.*[18] determined a number of diuretics from urine samples using a SDS/propanol micellar eluent. This HPLC method used a C_{18} (12 cm x 4.6 mm) column and employed a phosphate buffer (10 mM), pH 4.5, 42 mM SDS with 4 % propanol. It was found suitable for resolution of a number of compounds including furosemide.

Farthing *et al.*[19] determined furosemide by a solid-phase extraction method using a Varian AASP advanced automatic sample processor. The column packing material used was a C_2 ethyl sorbent. This was activated with acetonitrile and KH_2PO_4. Analysis was carried out using a Nucleosil C_{18} column with a phosphate buffer (pH 3): acetonitrile (70:30) mobile phase. Calibration curves were found to be linear in the range 25 - 1000 ng/ml with recoveries in the region 80 - 85 %. Cline-Love *et al.*[20] developed a micellar liquid chromatographic technique which allowed determination of propranolol and furosemide in urine by micellar chromatography using a Hypersil C_{18} column and Brij 35 as the surfactant. The micellar mobile phase was optimized by varying the pH and concentration of Brij 35; depending on these conditions, either propranolol or furosemide could be determined; lower pH and lower surfactant levels allowed the determination of furosemide, while the addition of TEA in small amounts, as well as higher pH and higher surfactant concentration, were necessary for the determination of propranolol. To date no other method has been reported that allows the simultaneous determination of furosemide and propranolol.

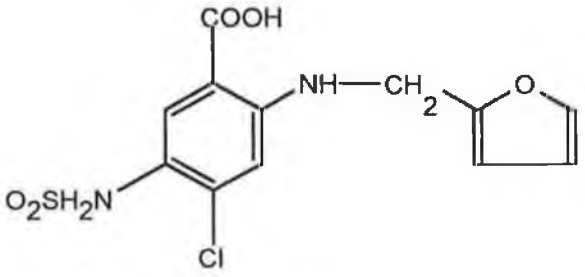
The aim of this work was to investigate the use of a mixed-mode stationary phase for the simultaneous determination of propranolol and furosemide in biological fluids. The development of the chromatography and the extraction procedures from first principles is also described. Two sample clean-up procedures were examined: liquid/liquid extraction and column-switching. Each of these methods was validated and a comparison of the two methods was carried out.



Propranolol



Pindolol



Furosemide

Figure 3.1: ***Chemical structures of propranolol, furosemide and pindolol (the internal standard).***

3.2 EXPERIMENTAL

3.2.1 *Reagents and solvents*

Propranolol, pindolol and furosemide were obtained from Sigma Chemical Co. (Dorset, U.K.). HPLC grade acetonitrile, diethyl ether, hexane and water were obtained from Labscan Analytical Sciences, (Dublin, Ireland). Analar grade sodium acetate, acetic acid and sodium hydroxide were obtained from Merck, (Darmstadt, Germany). Deionized water was obtained using an Elgastat spectrum water purification unit. A small pooled human plasma sample was obtained by drawing blood into evacuated tubes containing heparin as anticoagulant. These were then centrifuged at 3000 g for 5 min and the upper plasma layer was gently removed and stored at -18 °C until required for assay purpose.

3.2.2 *Standards*

Stock solutions were prepared by dissolving the appropriate amount of analyte in methanol (100 %) to yield a solution of concentration 1 mg/ml. A set of calibration standards were prepared by dilution of the stock solution with deionized water. The furosemide and propranolol standards covered the range 25 - 200 ng/ml and 50 - 400 ng/ml, respectively. A stock solution of pindolol (the internal standard) was made up to a concentration of 100 μg/ml in methanol. A working solution of 20 μg/ml was prepared by dilution of the stock solution with deionized water.

3.2.3 *Plasma Standards*

Aliquots of blank plasma were spiked with stock solutions to produce the required concentration of the two drug components and the internal standard.

3.2.4 *Instrumentation and Operating Conditions*

Furosemide, propranolol and pindolol were separated on a C_{18}/SCX (5 μm) reversed-phase/cation exchange column (250 mm x 4.6 mm i.d.) supplied by Shandon Scientific Ltd. (Cheshire, U.K.). The mobile phase was 100 mM sodium acetate (pH 4.0) -acetonitrile (67:33) delivered at a flow rate of 1.0 ml/min by a Waters 510 HPLC pump (Waters Associates, Milford, MA, USA). Injections were made using a Rheodyne (Cotati, CA, USA) injection valve fitted with a 20 μl loop. The analytes were detected by ultraviolet absorption at 230 nm using a Waters Model 486 spectrophotometric UV detector (Waters, Milford, MA, USA). The resulting chromatograms were recorded on an integrator (Waters 746 Data Module). For the purpose of column-switching a Waters 501 pump and an extraction column were connected to the analytical assembly via a ten-port switching-valve (Figure 3.2). The extraction column (10 mm x 1.5 mm i.d.) was packed with Hypersil C_{18} (30 μm) material. For column-switching purposes 100 μl of each standard was centrifuged for 30 s and 50 μl was introduced into the chromatographic system *via* a loop. Under the described chromatographic conditions the mean retention times for the elution of furosemide, pindolol and propranolol were 5.70, 7.30 and 12.50 min, respectively.

3.2.5 *Procedures*

3.2.5.1 *Extraction method 1 (liquid/ liquid extraction)*

As furosemide is acidic and propranolol is basic, a single extraction step will not permit the simultaneous recovery of the two compounds. Therefore it is neccessary to carry out a double extraction; this involves first extracting the furosemide from acidified plasma and then extracting the propranolol and pindolol from alkaline plasma. The extraction procedure was based on a method by Kerremans *et al.*[21] Furosemide was extracted by adding 25 μl of 2.0 M acetic acid to 125 μl of spiked plasma, and vortex-mixing each tube for 30 seconds. After the addition of 1 ml diethyl ether:hexane (65:35), the drug was extracted by vortex mixing for 150 seconds. Following centrifugation at 1000 g for 15 min at 4 °C, the upper organic layer (800 μl) was transferred into a clean polypropylene Eppendorf tube (Figure 3.2). The plasma was then subjected to further extraction to remove basic components following a procedure based on a method by Pritchard *et al.*[22] In this procedure, the basic components were extracted by adding 50 μl 1 M NaOH to the plasma and vortex mixing for 60 s. After addition of 1 ml of diethyl ether to all tubes, they were then centrifuged at 1000 g for 15 min at 4 °C. The upper organic layer (800 μl) was removed and added to the previously extracted acidic layer. The combined layers were evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 100 μl of mobile phase and a 20 μl aliquot was injected for chromatography.

EXTRACTION TECHNIQUES

ACIDIC LIQUID/LIQUID EXTRACTION

Plasma standards (125 µl) + 2.0 M acetic acid (25 µl)
+ 1 ml diethyl ether:hexane (65:35) + vortex for 150 s
Centrifugation for 15 min, 1000 g at 4 °C
800 µl organic layer removed to an Eppendorf tube

↓

BASIC LIQUID/LIQUID EXTRACTION

125 µl of resubmitted plasma + 50 µl of 1 M NaOH
+ 1 ml diethyl ether + vortex for 150 s
Centrifugation for 15 min, 1000 g at 4 °C
800 µl organic layer added to previous layer

↓

Combined layers evaporated to dryness under a gentle stream of nitrogen. Reconstituted in 100 µl of mobile phase
20 µl injected onto chromatographic system

Figure 3.2: ***Flow chart for liquid/liquid extraction.***

3.2.5.2 *Extraction method 2 (Column-switching)*

The spiked plasma sample was introduced via the injector port and swept onto the extraction column by water. The drug components were selectively retained by the packing material in the extraction column, while the endogenous plasma components were eluted to waste. Upon switching the valve, the mobile phase was diverted in a backflush mode via the extraction column, where it desorbed the drugs and swept them onto the analytical column for separation. The ten port valve used in this part of the study is shown in Figure 3.3 while

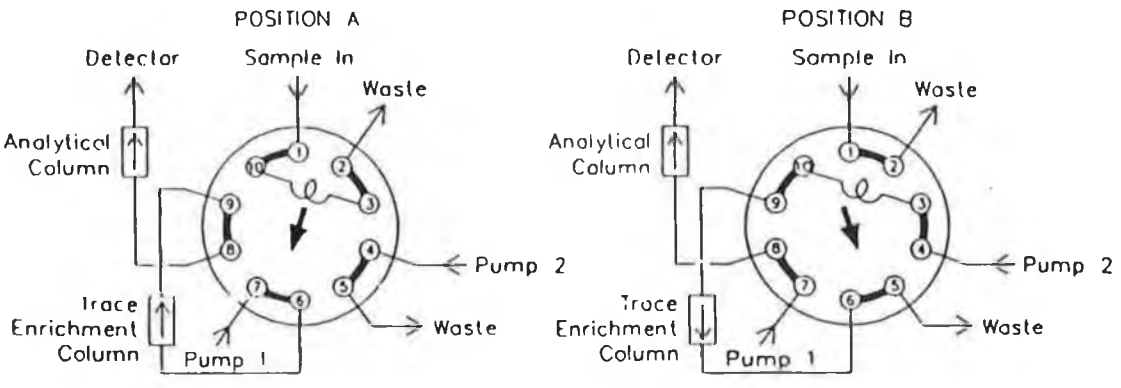


Figure 3.3: ***Ten port injection valve: Position A, the sample is loaded into the injection loop while the precolumn is been washed with mobile phase. On switching the valve to position B, the loop is flushed with the eluent from pump 2, allowing endogenous components to be eluted to waste. On returning to position A, the sample is eluted of the pre-column and onto the analytical column.***

3.2.6 *Calibration and calculation*

Evaluation of the assay was carried out by the construction of a five-point calibration graph covering the concentration range 50 - 300 ng/ml (furosemide) and 50 - 500 ng/ml (propranolol) in plasma for the liquid/liquid extraction method. The concentration ranges for the column-switching method for furosemide and propranolol were 25 - 200 ng/ml and 50 - 400 ng/ml, respectively. The slope and the intercept of the calibration graphs were determined through linear regression of the drug to the internal standard peak-height ratio versus drug concentration. Individual peak-height ratios were then interpolated on the calibration graphs to determine values of concentration found as compared to concentration added.

3.3 RESULTS AND DISCUSSION

3.3.1 *Development of chromatography*

It is often necessary in drug management programmes to co-administer a number of components together, in order to overcome the side effects of the individual components. Since propranolol and furosemide are occasionally co-administered, the aim of this work was to develop a suitable method for the simultaneous determination of furosemide and propranolol in plasma. The initial step was to select the mobile phase conditions and selection was guided by previous work carried out in Chapter 2.

The development of a chromatographic method for the simultaneous determination of furosemide and propranolol began with selection of a suitable mobile phase. It is necessary for the mobile phase to elute both the acidic and basic components in a short period of time from a SCX/C_{18} stationary phase and

the mobile phase selected initially contained sodium acetate and acetonitrile. Sodium acetate was selected as it allowed furosemide to elute separately from the void volume, this fact being important in the analysis of plasma samples whose endogenous components elute with or close to the void volume. On increasing the concentration of sodium acetate from 0.01 M to 0.1 M, the retention time of propranolol decreased. The next parameter examined was the percentage of buffer, this value being increased up to a maximum of 65 - 70%. Above this value, the retention time of propranolol increased considerably, therefore, a value of 67.0 % was selected. A number of pH values were examined, but lower values gave longer retention for furosemide. Acetonitrile was chosen over methanol since it gave shorter retention times for the basic compounds, as it is important to keep the analysis time as short as possible and prevent peak tailing of the later eluting peaks. The final mobile phase selected was 0.1 M sodium acetate (pH 4.0) : acetonitrile (67:33).

The wavelength of the detector was determined by scanning solutions of each of the drug components in the buffer selected over the range 200 - 400 nm. The maximum absorbance for furosemide, propranolol and pindolol was 230.5 nm, 232.5 nm and 223.3 nm, respectively. The value selected was 230 nm.

The final step was to select a suitable internal standard. A number of compounds were investigated, including pindolol and terbutaline; the later however eluted too closely to furosemide to be suitable as an internal standard. The remaining compounds interfered with either furosemide or propranolol and hence pindolol was selected as its retention was intermediate between those of furosemide and propranolol.

3.3.2 *Development of an extraction procedure*

A method was set up which enabled the simultaneous extraction of furosemide, propranolol and pindolol. The extraction was carried out on spiked plasma samples. As it is not possible to extract acidic and basic components in a single extraction, it was necessary to implement a double extraction. First, furosemide was extracted from acidified plasma, and then propranolol was extracted from alkaline plasma; the two extracts were then combined. A technique was developed based on a method by Lindstrom *et al.*[23] in which furosemide was extracted from acidified plasma with diethyl ether. The pH of spiked plasma was adjusted by the addition of hydrochloric acid, and the furosemide was then extracted with diethyl ether by vortex mixing. Following centrifugation the organic layer was removed and dried under a stream of nitrogen. The extraction solvent was modified by the addition of hexane which improved the recoveries obtained. Also the replacement of hydrochloric acid by acetic acid[21] resulted in increased recoveries. The extraction of propranolol was based on a method by Pritchard *et al.*[22] which allowed determination of propranolol from alkaline plasma. The solvent used for extraction was diethyl ether. Following centrifugation, the organic layer was removed, added to the previous extract, and dried under a stream of nitrogen. Figure 3.4 indicates chromatograms of samples following extraction by this method.

It was necessary to determine which type of packing material in the pre-column would prove most suitable in terms of recovery of drug components and sample clean-up. This selection was determined by injection of an aqueous standard on all materials and determining the recovery compared to aqueous standards injected directly onto the analytical column. The concentration column used was packed with either C_{18}, CN or phenyl packing materials. This column was fitted between the pump and the detector. Of the three columns evaluated in

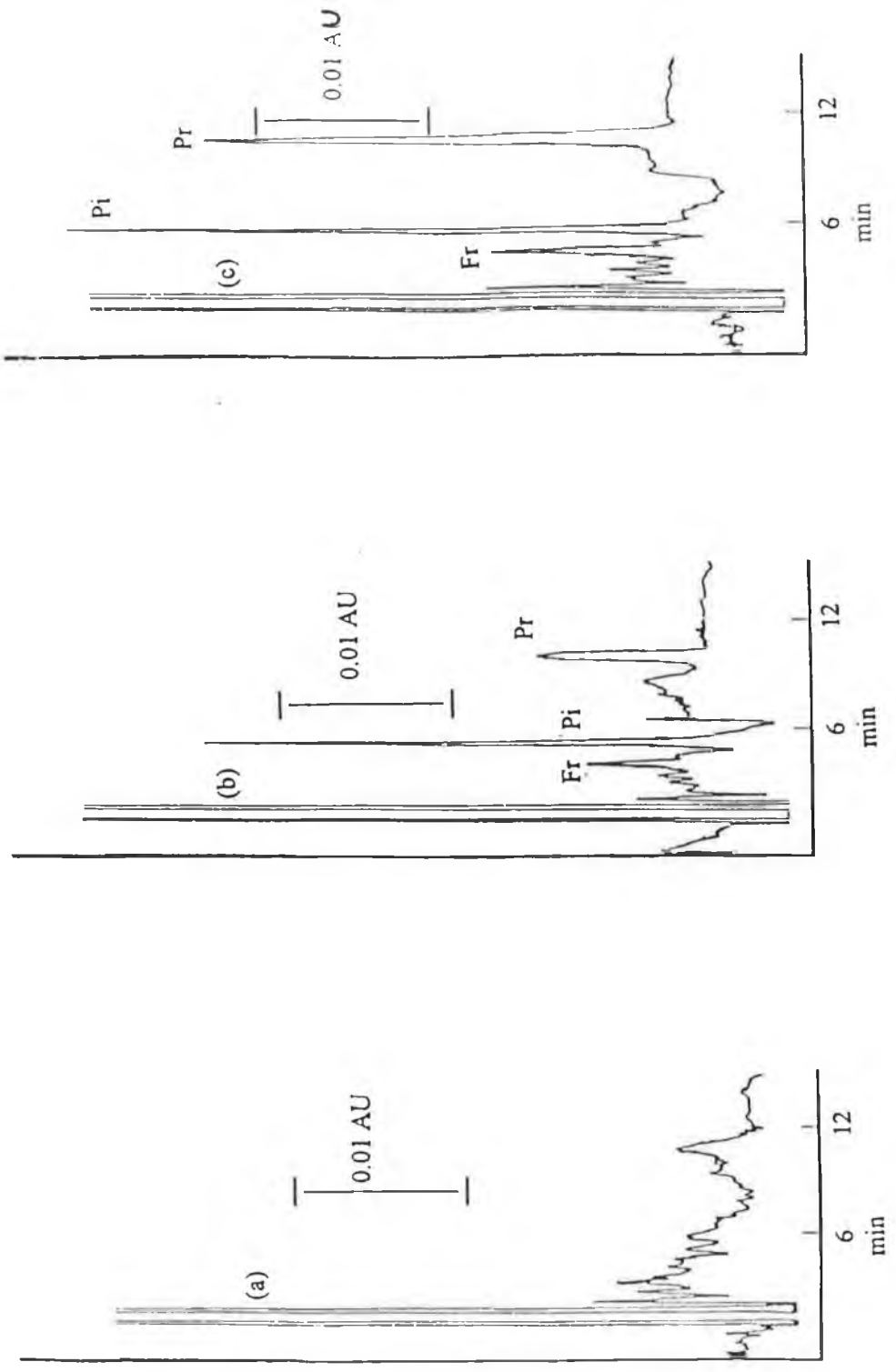


Figure 3.4: ***Chromatograms showing (a) blank plasma, (b) the lowest concentration and (c) the highest concentration following extraction by liquid/liquid extraction. Compounds were separated on a Hypersil C_{18}/SCX column using a mobile phase of 100 mM sodium acetate (pH 4)-acetonitrile (67:33 v/v) delivered at a flow rate of 1.0 ml/min.***

terms of drug recovery, C_{18} was the one which gave the best results, as both the CN and phenyl materials showed recoveries only in the region of 30 - 50 % for the basic compounds. Due to the build up of plasma on the pre-column it needed to be replaced on a regular basis. The rinse time was also varied: a range of values were examined and these included 1.0 - 5.0 min. It was found that times above 2 min, the recovery of furosemide decreased to a large extent; to values of below 50 % at 5 min. A rinse time of 1 min was selected as this allowed the greatest recovery while allowing the removal of endogenous components. Figure 3.5 shows chromatograms following extraction by this method of a) blank plasma, b) the lowest concentration and c) the highest concentration of furosemide and propranolol.

3.3.3 *Assay Validation*

Each of the methods developed for the simultaneous determination of propranolol and furosemide was validated over the concentration ranges shown in Tables 3.1 - 3.4 and 3.7 - 3.10.

3.3.3.1 *Limit of Detection*

The limit of detection was found to be 15 ng/ml for furosemide and 30 ng/ml for propranolol.

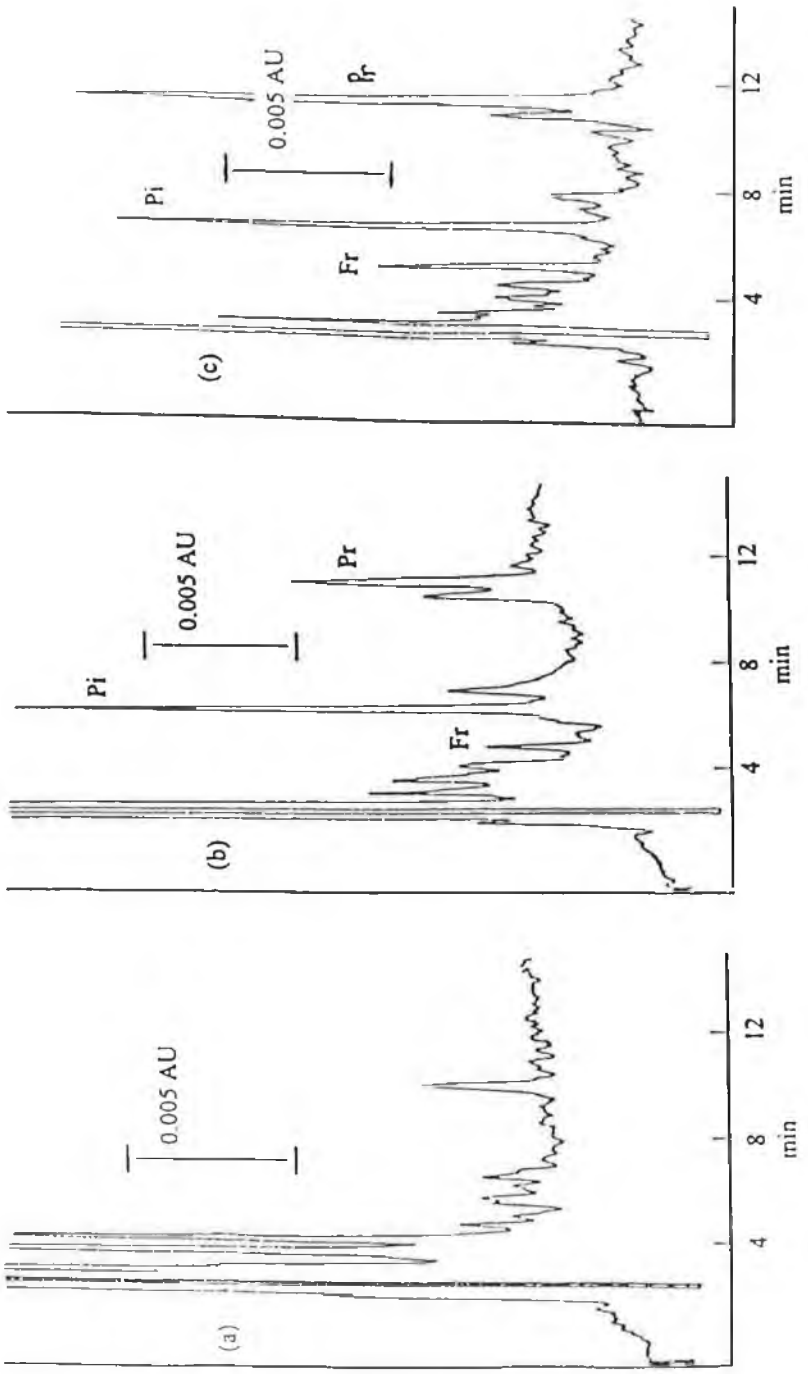


Figure 3.5: *Chromatograms showing (a) blank plasma, (b) the lowest concentration and (c) the highest concentration following extraction by column switching. Compounds were separated on a Hypersil C_{18}/SCX column using a mobile phase of 100 mM sodium acetate (pH 4)-acetonitrile (67:33 v/v) delivered at a flow rate of 1.0 ml/min.*

3.3.3.2 *Precision*

The data presented in Tables 3.1 - 3.4 demonstrate the inter- and intra-assay variation in the liquid/liquid extraction method. The data presented in Tables 3.7 - 3.10 demonstrate the inter- and intra- assay variation in the column-switching method. Inter-assay variation was assessed singly in four replicate runs. Intra-assay variability was determined in quadruplicate over the same concentration range. The precision of the method (as expressed by mean coefficient of variation) was determined for analyte to internal standard peak-height ratios when interpolated as unknowns on the regression lines. For inter-assay variation, peak-height ratios were interpolated on the four regression lines generated from the four replicate runs. For intra-assay variation, peak-height ratios were interpolated on a single regression line generated from the quadruplicate run. The mean coefficients of variation for each method are given in Tables 3.1 - 3.4 and 3.7 - 3.10.

3.3.3.4 *Linearity and Accuracy*

Linearity is defined by the correlation coefficient of the regression line, and accuracy is defined by the percentage difference between "added'' and "found" concentrations for inter-assay values. These are presented in Tables 3.5 - 3.6 and 3.11 - 3.12. The correlation coefficient of the regression line for the mean intra-assay value was better than 0.995 or better in all cases.

3.3.3.5 *Recovery*

Recovery of furosemide/propranolol from plasma was measured by calculating the percentage difference between the peak heights of extracted

standards and those of the authentic (unextracted) standards in the relevant concentration range. Using this method, the mean recovery by the liquid/liquid extraction method for furosemide from plasma was found to be 90.75 % and for propranolol to be 90.08 % (Table 3.5 and 3.6). The mean recovery by the column-switching extraction method for furosemide from plasma was found to be 76.32 % and for propranolol to be 90.80 % (Table 3.11 and 3.12). Figure 3.6 is a graphical representation of a comparison for the recoveries of the drug components by the two methods.

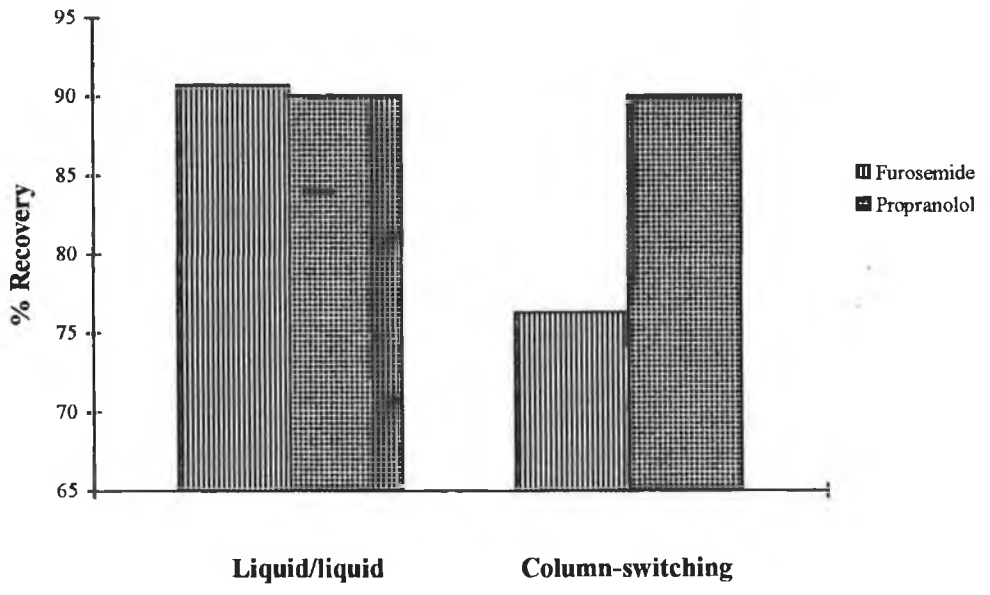


Figure 3.6: ***Bar chart showing the recovery of furosemide and propranolol from plasma by both liquid/liquid extraction and column-switching.***

3.3.3.6 *Selectivity*

A number of drugs were investigated as potential interferants in the method. These included nitrazepam, clonazepam and quinalbarbitone. None of these compounds were found to interfere.

TABLE 3.1

Precision and Linearity-INTRA-ASSAY from liquid/liquid extraction

Amount Propranolol added ng/ml	Peak height Ratio	Amount found ng/ml
50	0.15	53.65
100	0.26	106.03
150	0.31	129.84
300	0.73	329.84
500	1.09	501.27
50	0.15	53.65
100	0.22	86.99
150	0.31	129.84
300	0.72	325.08
500	1.07	491.75
50	0.16	58.41
100	0.24	96.51
150	0.33	139.37
300	0.68	306.03
500	1.05	482.22
50	0.14	48.89
100	0.27	110.80
150	0.33	139.37
300	0.69	310.80
500	1.08	496.51

	Concentration ng/ml				
	50.00	100.00	150.00	300.00	500.00
Mean Amount Found ng/ml	53.65	100.08	134.61	317.94	492.94
Standard Deviation	3.89	10.56	5.50	11.33	8.13
Coefficient of variation %	7.25	10.56	4.09	3.56	1.65
Difference between added and found	+3.65	+0.08	-15.40	+17.94	-7.06

TABLE 3.2

Precision and Linearity-INTER-ASSAY from liquid/liquid extraction

Amount Propranolol added ng/ml	Peak height Ratio	Amount found ng/ml
50	0.15	53.75
100	0.26	106.63
150	0.34	145.09
300	0.69	313.36
500	1.08	500.86
50	0.14	48.94
100	0.24	97.01
150	0.31	130.67
300	0.73	332.59
500	1.09	491.25
50	0.17	63.36
100	0.24	97.01
150	0.30	125.86
300	0.65	294.13
500	1.06	486.44
50	0.16	58.55
100	0.24	97.01
150	0.33	140.28
300	0.68	308.55
500	1.05	496.05

	Concentration ng/ml				
	50.00	100.00	150.00	300.00	500.00
Mean Amount Found ng/ml	56.15	99.42	135.48	312.16	493.65
Standard Deviation	6.20	4.81	8.78	15.88	6.20
Coefficient of variation %	11.04	4.84	6.48	5.09	1.26
Difference between added and found	+6.15	-0.58	-14.52	+12.16	-6.35

TABLE 3.3

Precision and Linearity-INTRA-ASSAY from liquid/liquid extraction

Amount Furosemide added ng/ml	Peak height Ratio	Amount found ng/ml
50	0.18	62.84
100	0.20	78.47
150	0.29	148.78
250	0.43	258.16
300	0.52	328.47
50	0.16	47.22
100	0.19	70.66
150	0.28	140.97
250	0.41	245.63
300	0.46	281.59
50	0.17	55.03
100	0.23	101.91
150	0.32	172.22
250	0.41	245.63
300	0.46	281.59
50	0.16	47.22
100	0.24	109.72
150	0.33	180.03
250	0.40	234.72
300	0.51	320.66

	Concentration ng/ml				
	50.00	100.00	150.00	250.00	300.00
Mean Amount Found ng/ml	53.08	90.19	160.50	246.04	303.08
Standard Deviation	7.48	18.60	18.60	3.89	8.25
Coefficient of variation %	14.09	20.62	11.59	3.89	8.25
Difference between added and found	+3.08	-9.81	+10.52	-3.96	+3.08

TABLE 3.4
Precision and Linearity-INTER-ASSAY from liquid/liquid extraction

Amount Furosemide added ng/ml	Peak height Ratio	Amount found ng/ml
50	0.19	62.05
100	0.23	94.57
150	0.31	159.61
250	0.41	240.91
300	0.47	289.69
50	0.19	62.05
100	0.22	86.44
150	0.25	110.83
250	0.43	257.17
300	0.51	322.21
50	0.17	45.79
100	0.22	86.44
150	0.29	143.35
250	0.38	216.52
300	0.46	281.56
50	0.19	62.05
100	0.23	94.57
150	0.33	175.87
250	0.41	240.91
300	0.46	281.56

	Concentration ng/ml				
	50.00	100.00	150.00	250.00	300.00
Mean amount Found ng/ml	57.99	90.51	147.42	238.88	293.76
Standard Deviation	8.13	4.69	27.76	16.76	19.35
Coefficient of variation %	14.02	5.18	18.84	7.02	6.59
Difference between added and found	+7.99	+9.50	-2.59	-7.87	-6.25

TABLE 3.5

Results for recovery of propranolol by liquid/liquid extraction

Propranolol peak height (mm)			
Concentration ng/ml	Authentic standards	Extracted standards	Recovery %
50	17	15	88.24
100	21	18	85.71
150	33	31	93.94
300	60	55	91.67
500	98	89	90.82

Mean recovery (± standard deviation) = 90.08 ± 3.18 %

TABLE 3.6

Results for recovery of furosemide by liquid/liquid extraction

Furosemide peak height (mm)			
Concentration ng/ml	Authentic standards	Extracted standards	Recovery %
50	12	11	91.67
100	14	13	92.86
150	17	15	88.24
250	24	22	91.67
300	28	25	89.29

Mean recovery (± standard deviation) = 90.75 ± 1.91 %

TABLE 3.7

Precision and Linearity-INTRA-ASSAY from column-switching

Amount Propranolol added ng/ml	Peak height Ratio	Amount found ng/ml
50	0.26	58.56
100	0.32	106.17
200	0.46	217.29
300	0.59	320.46
400	0.69	399.83
50	0.25	50.62
100	0.29	82.37
200	0.47	225.22
300	0.54	280.78
400	0.73	431.57
50	0.25	50.02
100	0.33	114.11
200	0.42	185.54
300	0.53	272.84
400	0.72	423.63
50	0.23	34.75
100	0.31	98.24
200	0.41	177.60
300	0.57	304.59
400	0.70	407.76

	Concentration ng/ml				
	50.00	100.00	200.00	300.00	400.00
Mean Amount Found ng/ml	48.64	100.22	201.41	294.67	415.70
Standard Deviation	9.99	13.55	23.37	21.86	14.49
Coefficient of variation %	20.54	13.52	11.60	7.42	3.49
Difference between added and found	-1.36	+0.22	+1.41	+5.33	+15.70

TABLE 3.8

Precision and Linearity-INTER-ASSAY from column-switching

Amount Propranolol added ng/ml	Peak height Ratio	Amount found ng/ml
50	0.23	35.68
100	0.30	90.36
200	0.45	207.55
300	0.58	309.12
400	0.70	402.87
50	0.25	51.30
100	0.29	82.55
200	0.44	199.74
300	0.54	277.87
400	0.69	395.05
50	0.25	51.30
100	0.33	113.80
200	0.48	231.00
300	0.55	285.68
400	0.67	379.43
50	0.24	43.49
100	0.34	121.62
200	0.44	199.74
300	0.57	301.30
400	0.73	426.30

	Concentration ng/ml				
	50.00	100.00	200.00	300.00	400.00
Mean Amount Found ng/ml	45.44	102.08	209.51	293.49	400.91
Standard Deviation	7.48	18.60	14.79	14.26	19.53
Coefficient of variation %	16.46	18.22	7.06	4.86	4.87
Difference between added and found	-4.56	+2.08	+9.51	-6.51	+0.91

TABLE 3.9

Precision and Linearity-INTRA-ASSAY from column-switching

Amount Furosemide added ng/ml	Peak height Ratio	Amount found ng/ml
25	0.13	32.40
50	0.16	61.81
100	0.19	91.23
150	0.26	159.85
200	0.30	199.07
25	0.13	32.40
50	0.14	42.21
100	0.20	101.03
150	0.27	169.66
200	0.30	199.07
25	0.12	22.60
50	0.16	61.81
100	0.23	130.44
150	0.26	159.85
200	0.31	208.60
25	0.12	22.60
50	0.14	42.21
100	0.19	91.23
150	0.24	140.25
200	0.30	199.07

	Concentration ng/ml				
	25.00	50.00	100.00	150.00	200.00
Mean Amount Found ng/ml	27.50	52.01	103.48	157.40	201.52
Standard Deviation	5.66	11.32	18.55	12.33	4.90
Coefficient of variation %	20.58	21.77	17.93	7.83	2.43
Difference between added and found	+2.50	+2.01	+3.48	+7.40	+1.52

TABLE 3.10

Precision and Linearity-INTER-ASSAY from column-switching

Amount Furosemide added ng/ml	Peak height Ratio	Amount found ng/ml
25	0.13	31.45
50	0.14	41.98
100	0.19	94.61
150	0.23	136.72
200	0.30	210.40
25	0.12	20.93
50	0.17	73.56
100	0.20	105.14
150	0.21	168.24
200	0.29	199.87
25	0.12	20.93
50	0.15	52.51
100	0.18	84.08
150	0.23	136.72
200	0.27	178.82
25	0.12	20.93
50	0.16	63.03
100	0.19	94.61
150	0.25	157.77
200	0.29	199.87

	Concentration ng/ml				
	25.00	50.00	100.00	150.00	200.00
Mean amount Found ng/ml	23.56	57.77	94.61	149.88	197.24
Standard Deviation	5.26	13.59	8.60	15.79	13.25
Coefficient of variation %	22.33	23.52	9.08	10.54	6.72
Difference between added and found	-1.44	+7.77	-5.39	-0.12	-2.76

TABLE 3.11

Results for recovery of propranolol by column-switching

Propranolol peak height (mm)			
Concentration ng/ml	Authentic standards	Extracted standards	Recovery %
50	22	19	86.36
100	28	23	82.14
200	38	36	94.74
300	55	52	94.55
400	79	76	96.20

Mean recovery (± standard deviation) = 90.80 ± 6.19

TABLE 3.12

Results for recovery of furosemide by column-switching

Furosemide peak height (mm)			
Concentration ng/ml	Authentic standards	Extracted standards	Recovery %
25	21	16	76.19
50	29	22	75.86
100	33	25	75.76
150	42	30	73.81
200	45	36	80.00

Mean recovery (± standard deviation) = 76.32 ± 2.26

3.4 CONCLUSION

This study shows that a mixed-mode C_{18}/SCX column can find application in the simultaneous determination of strongly basic and acidic compounds. The HPLC method described is capable of simultaneously determining propranolol and furosemide following extraction from human plasma. It was found that they could be adequately separated on a mixed-mode column in less than 15 minutes. Pindolol was the internal standard used. Liquid/liquid extraction and column-switching techniques were developed for the determination of propranolol and furosemide from plasma samples. Each of the methods was validated in terms of coefficient of variation and recovery, linearity and accuracy. The column-switching method has the advantage of being less tedious and time consuming than the liquid/liquid extraction method, though the recoveries obtained were much lower than those obtained for the liquid/liquid extraction method. The low recovery values obtained for furosemide by the column-switching method than liquid/liquid extraction may be due to the fact that furosemide has little affinity for the C_{18} material. If a different material in the pre-column, e.g. mixed-mode or a material more suitable for the determination of furosemide were used, higher recoveries could be obtained.

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CHAPTER FOUR

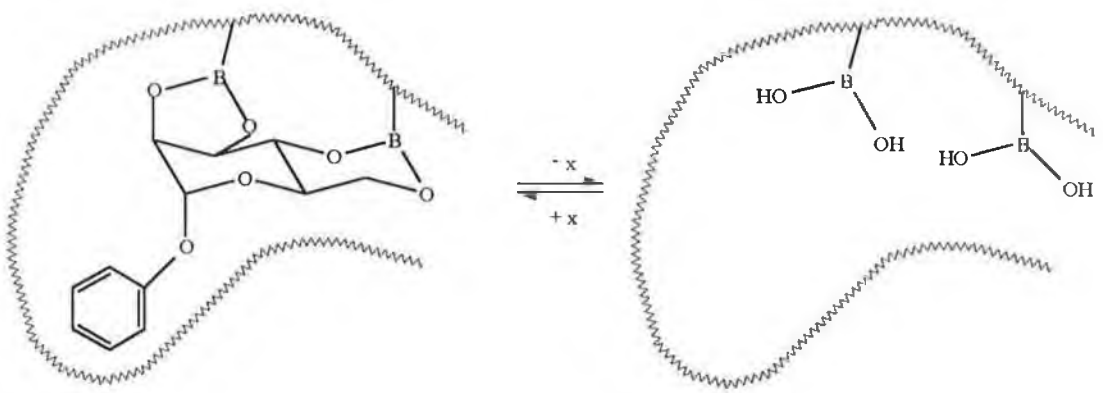
MOLECULAR IMPRINTING

4.1 INTRODUCTION

The area of molecular imprinting is an emerging technique for the preparation of polymers with highly selective recognition properties, and is only a recently developed concept, having been first developed by Wulff[1] in 1972. The basic idea involves the preparation of synthetic polymers with a predetermined selectivity; that is, polymers are prepared which attempt to mimic antibody combining sites. During the polymerization step the monomers link together around the target molecule; the "imprint" molecule being held in place by interactions with the monomers. The copolymerization of these monomers with cross-linkers in the presence of the imprint molecule results in a rigid structure with sites selective for the imprint molecule.

Two different types of approach to this technique have been developed: covalent and non-covalent molecular imprinting. Both techniques are similar, as in both cases the functional monomers are chosen to allow interactions with the functional groups of the imprint molecule. In both cases, the polymerization occurs in the presence of the print molecule while interacting with the complementary functionality found in the monomer. The resulting polymers have been found to have a good affinity for the original print molecule.

The first approach is the "covalent method" which has been used by both Wulff and Shea. With this method the imprint molecule is covalently coupled to a polymerizable molecule; i.e. an adduct composed of the print molecule and the monomer is synthesized and added to the polymerization mixture. This is then copolymerized with a cross-linker after which the imprint molecule is chemically cleaved from the highly cross-linked polymer. This method relies on a reversible reaction as shown in Figure 4.1. There are quite a number of applications of this method involving amino acids and amino acid derivatives,[2] aromatic ketones,[3] and natural and derivatised sugars.[1]



(a) (b)

Figure 4.1: ***Schematic representation of (a) a cavity obtained by polymerization with the covalently bound template. The template (phenyl-α-D-mannopyranoside [x]) can be removed with water or methanol to give (b). Addition of the original template causes the cavity to be reoccupied, giving (a) again. The binding of the template is by covalent bonds.***

The second approach, i.e. the "non-covalent method" has been pioneered by Mosbach *et al.*[4] Using this method, the imprint molecules are mixed with functional monomers and these are then capable of interacting non-covalently with the imprint molecules. The preparation of these polymers is discussed further in Section 4.2. The recognition sites are capable of selectively recognizing the imprint species, therefore the imprint species that interacts during the imprinting procedure is capable of interacting again during the rebinding with the polymer by non-covalent interactions. These interactions can be ionic or hydrophobic in nature, or involve hydrogen bonding. This method of molecular imprinting has allowed a number of molecularly imprinted polymers (MIPs) to be prepared against a large number of components including amino acid derivatives,[5,6] peptides,[7] diazepam and theophylline,[8] as well as a number of other drug compounds. This method has an advantage over the "covalent method" in that no covalent modification of

the print molecule is required. A variety of different binding interactions may exist, and hence this procedure would be regarded as a simple and general method for preparation of a molecularly imprinted polymer.

It is also possible to use a combination of both approaches, where the monomers and the imprint molecules are covalently coupled[9] during the polymerization, and the subsequent rebinding takes place by non-covalent interactions. The area of molecular imprinting is expanding rapidly and has found application in a number of areas including the preparation of tailor-made separation materials for use in HPLC, as enzyme mimics or catalytically active polymers in enzyme technology, and finally as sensors in biosenor-like configurations (where the polymers are used as substitutes for the biological materials that are normally employed).

4.1.1 *Preparation of molecularly imprinted polymers (non-covalent method)*

In the non-covalent approach, the functional monomers are mixed with the template molecules; the monomers are then capable of interacting with the imprint molecule. These functionalized monomers are allowed to "pre-arrange" around the print molecule by non-covalent interactions (i.e. electostatic, hydrophobic and hydrogen bonding). The monomers are polymerized with cross-linking monomers and the result is a highly cross-linked, rigid polymer. The imprint molecules are then extracted (using a Soxhlet extraction apparatus) and the polymer now has recognition sites which are complementary to the target molecule. The particles are ground to a size in the range of 25 - 45 μm (Figure 4.2).

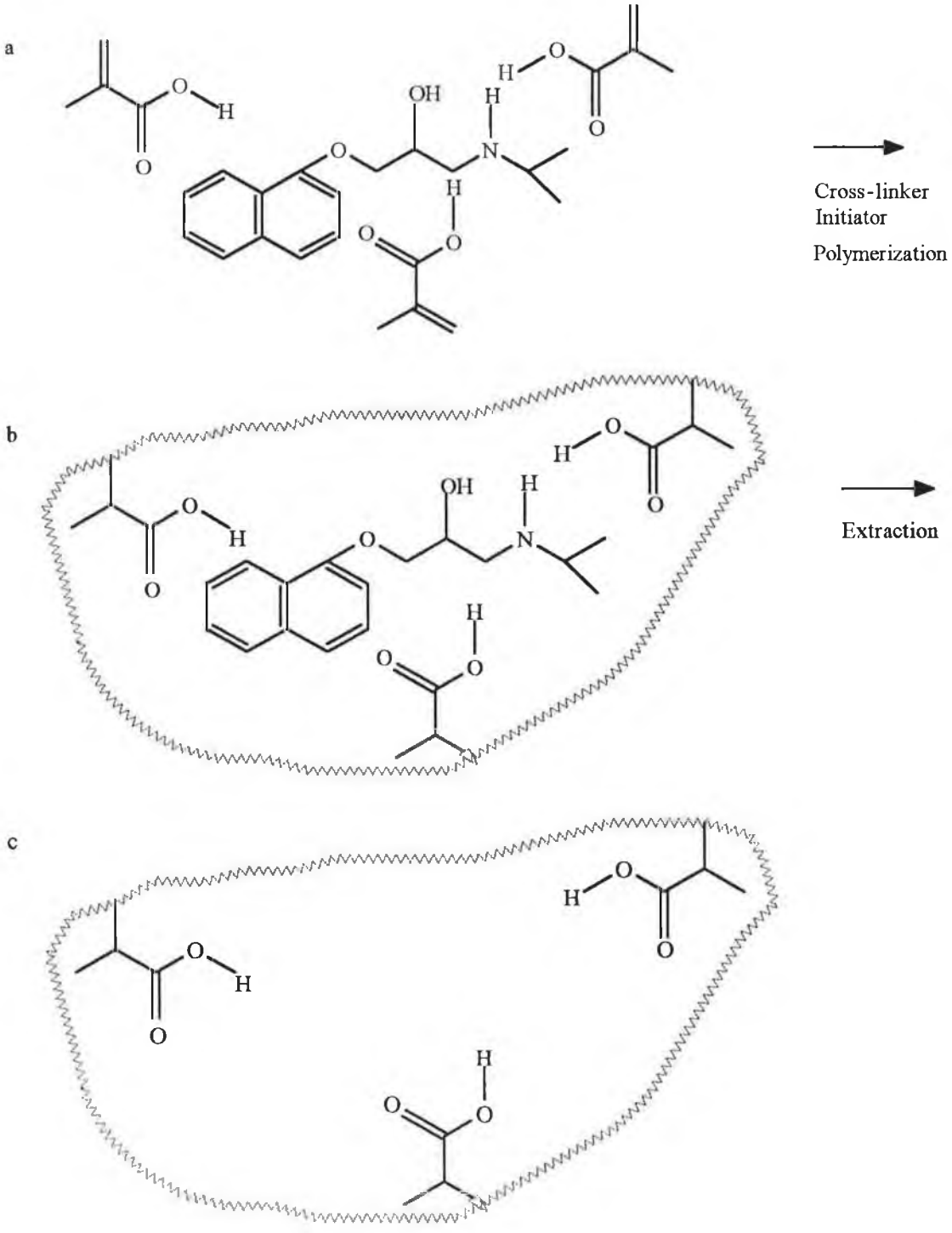
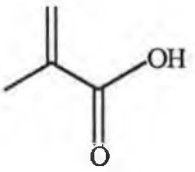


Figure 4.2: ***The preparation of molecular imprinted polymers. a) the functional monomer methacrylic acid is mixed with the print molecule propranolol and ethylene glycol dimethacrylate the cross-linking agent is added; the solvent used is chloroform, b) the polymerization reaction is started by addition of the initator [2,2'-azobis(2-methylpropionitrile), AIBN] and c) the extraction of the template.***

4.1.2 *Materials for molecular imprinting*

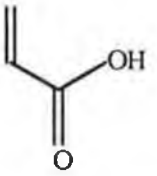
To date, a number of monomers have been used which include methacrylic acid **1**,[10] acrylic acid **2**,[10] methyl methacrylate **3**,[6] 4-vinylpyridine **4**,[11] 2-vinylpyridine **5**,[12] itaconic acid **6**,[7] and *p*-vinylbenzoic acid **7**.[13] Figure 4.3.a shows the functional monomers most commonly used in molecular imprinting. A number of cross-linking agents have also been used which include *N,N'*-methylenediacrylamide **8**,[6] ethylene glycol dimethacrylate **9**,[10] pentaerythritoltriacrylate (PETRA) **10**[14] and trimethylolpropane trimethyl acrylate (TRIM) **11** (Figure 4.3.b).

The functional monomers were chosen so as to facilitate specific interactions with the functional groups of the imprint molecules. Methacrylic acid (MAA) **1** is the most widely used functional monomer, whereas ethylene glycol dimethacrylate (EDMA) **6** is the the most commonly used cross-linker. The carboxylic acid of **1** forms ionic interactions with amino groups and hydrogen bonds with polar functions. The ionic interaction is stronger than the hydrogen bonding interaction, a fact which is reflected in better selectivities of polymers interacting with the imprint molecules *via* ionic bonds than of polymers interacting *via* hydrogen bonds. Other interactions found to contribute to the molecular imprinting process include dipole-dipole and hydrophobic interactions. However, the pentaerythritol derivative[15] polymers **10** and **11** have been found to show better load capacities, selectivities and resolving capabilities when used as stationary phases in LC. A variety of polymers have been prepared, encompassing styrene, silica and acrylic-based polymers. These are assumed to interact *via* ionic interactions with amines and *via* hydrogen bonds with amides, carbamates and carboxyls. The introduction of 4-vinylpyridine **4**[12,15] as a monomer in non-covalent molecular imprinting (MI) made the ionic interactions possible between the recognition



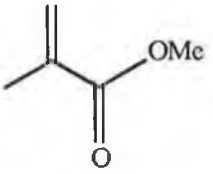
methacrylic acid

1



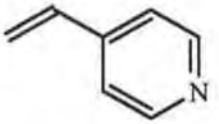
acrylic acid

2



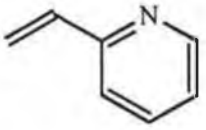
methyl methacrylate

3



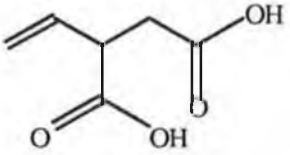
4-vinylpyridine

4



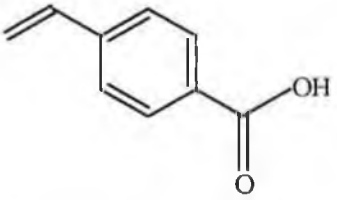
2-vinylpyridine

5



itaconic acid

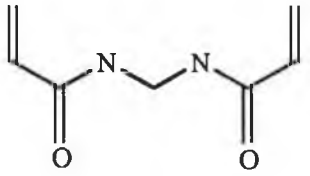
6



p-vinylbenzoic acid

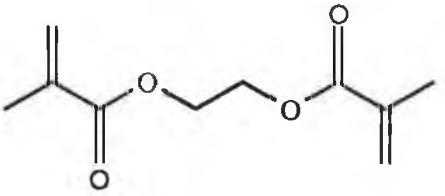
7

FIGURE 4.3.a: ***Monomers used in non-covalent molecular imprinting.***



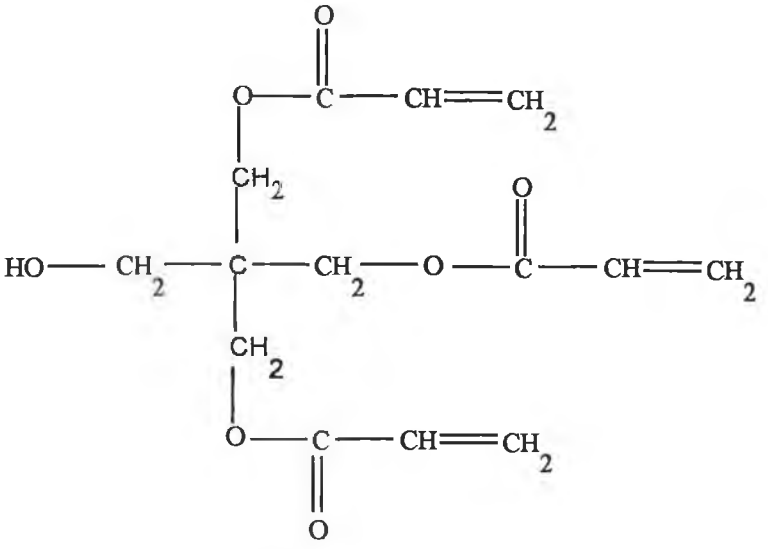
N,N'-methylenediacrylamide

8



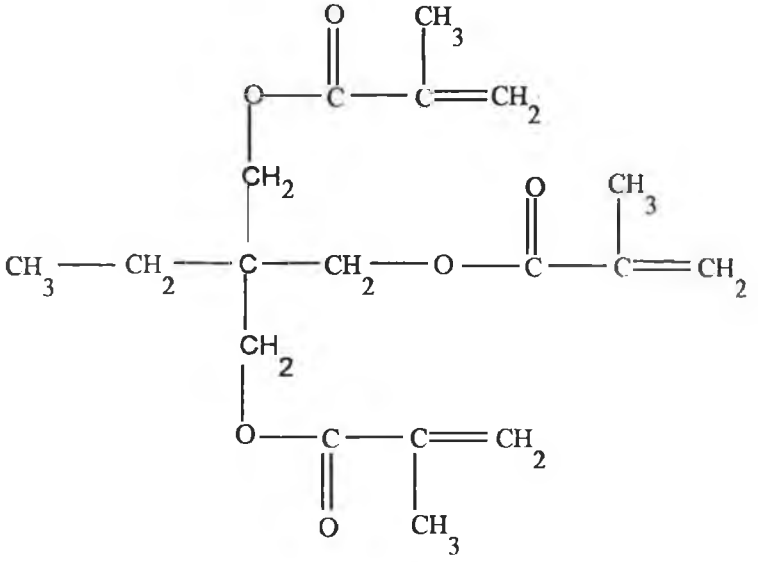
ethylene glycol dimethacrylate

9



pentaerythritol triacylate (PETRA)

10



trimethylolpropane trimethacrylate (TRIM)

11

FIGURE 4.3.b: ***Cross-linking monomers used in non-covalent molecular imprinting.***

sites of the polymers and the imprint species containing the carboxyl functionality. This had an advantage over other monomers in that it resulted in better selectivities for such imprint species compared to the selectivities that have been achieved with polymers prepared with methacrylic acid. A similar monomer, 2-vinylpyridine **5** was also found to achieve better selectivities compared to methacrylic acid.[12]

4.1.3 *Molecular imprints and their use in chiral separations*

One of the most common uses for molecularly imprinted polymers is for the determination of racemates.[16] There is a need for optically pure enantiomers, since stereoisomers can express varying pharmacological activity, and in some cases can even be used against different symptoms. However, it is possible for the two enantiomers to interact differently with the biological system and only one of these to have the desired effect.

The increasing demand for optically pure compounds has resulted in an interest in asymmetric synthesis and development of supports for efficient chiral separations. There is a need for analytical methods to be able to perform pharmacokinetic studies and examine final products of asymmetric synthesis. Chiral separation by LC is a widely studied area with three main approaches: 1) addition of chiral additive to the mobile phase, 2) derivatization with an optically active reagent; and 3) separation on a chiral stationary phase (all previously discussed in Chapter 1).

The field of chiral stationary phases (CSPs) is still growing and MIPs have found use in this area,[17] as an optically active compound (one enantiomer of a pair) can be imprinted and the resulting polymer can discriminate between the imprint and its antipode. The advantage of this method is that it allows the manufacture of specific tailor-made polymers for a given separation process.

Andersson *et al.*[18] prepared molecular imprints against *N*-protected amino acids to evaluate their ability to resolve the enantiomers of print

molecules in the chromatographic mode. Mixtures of two racemates, Cbz-aspartic acid and Cbz-glutamic acid were examined on polymers prepared against the L- form of both of these compounds. The print molecule was the most retained compound on the respective polymer preparation indicating that efficient substrate selectivity between compounds with very similar structures was possible.

Kempe *et al.*[12] examined the use of 4-vinylpyridine and 1-vinylimidazole as functional monomers for the preparation of novel molecularly imprinted polymer systems. They found that the polymer prepared with 4-vinylpyridine was found to be more efficient in racemic resolution than those prepared with 1-vinylimidazole. The ratio of monomer to template was optimized and found to be 12:1, as below this value it was not possible to satisfy all possible interaction points of the target molecule because of competition from the other species present in the mixture. A ratio of > 12:1 results in increased non-specific binding sites within the rigid polymer, thereby reducing the selectivity of the polymer. The introduction of a nitrogen-containing aromatic monomer into the field of molecular imprinting increases the number of compounds that can be used as template molecules, and this in turn enhances the general versatility of molecular imprinting.

O'Shannessy *et al.*[17] examined a number of polymerization parameters in order to determine the influence of these factors on the ability of the polymer to separate a racemic mixture of the print molecule. These parameters included variation of the solvent employed during polymerization, the reagents used for initiation of the polymerization, and the effect of molar ratio of functional monomer to print molecule. Polymers prepared in a less polar solvent, e.g. chloroform, were found to be superior to those prepared in more polar solvents, e.g. acetonitrile. Furthermore, polymers prepared at 0 °C using light-induced initiation were superior to those prepared at 60 °C using heat-induced initiation.[19] An increase in the molar ratio of functional monomer to print molecule improved separation, however, the second

component was found to tail considerably. They found the optimal ratio of functional monomer to print molecule to be 4:1.

4.1.4 *Preparation of monomers*

There are two main types of classification of optically active polymers; main chain and side chain chirality. Main chain chirality possess chiral centres in the polymer backbone while side chain possess chiral pendant groups. The use of polymers (MI) which are optically active might further enhance the enantioselectivity of these materials when used as chiral stationary phases by creating a chiral cavity for the imprint molecule to interact with.

Tamai *et al.*[20] synthesized optically active polymethacrylate derivatives possessing pendent chiral 1,1-binaphthalene moieties. Their ability to resolve racemates was measured by coating the polymers onto spherical macroporous silica gel and preparing HPLC columns. It was found that a polymer with the 1,1-binapthalene moiety farthest from the polymer backbone gave rise to substantially better enantiomeric separation.

Andersson *et al.*[21] have synthesized a new amino acid based cross-linker for the preparation of substrate-selective acrylic polymers. The idea was to prepare monomers possessing different functional groups which were found suitable for the preparation of highly cross linked macroporous polymers. This was achieved by modifying an amino acid (L-phenylalanine) by the introduction of acrylic groups. The polymers imprinted with L-phenylalanine ethyl ester were found to be chiral and these were found to show preference for L- specificity.

Molecular imprinting is dependant on various interactions. Therefore to exploit it to its full potential it is necessary to prepare new monomers possessing different functional groups to enable maximum interaction between the imprint molecules and the monomers during polymerization.

The work described here involves the preparation of molecularly imprinted polymers to 7-hydroxycoumarin (7-OHC). The amount of solvent used in the polymerization step was examined by spectroscopic studies to determine if it affected the selectivity of the MIP for 7-OHC. Further spectroscopic studies included examination of the load capacity of the MIP, the equilibration time and the selectivity of the MIP for 7-OHC over other members of the coumarin family. The polymer which showed the greatest selectivity for 7-OHC was further used as a solid-phase extraction material for the determination of 7-OHC from urine (Chapter 5).

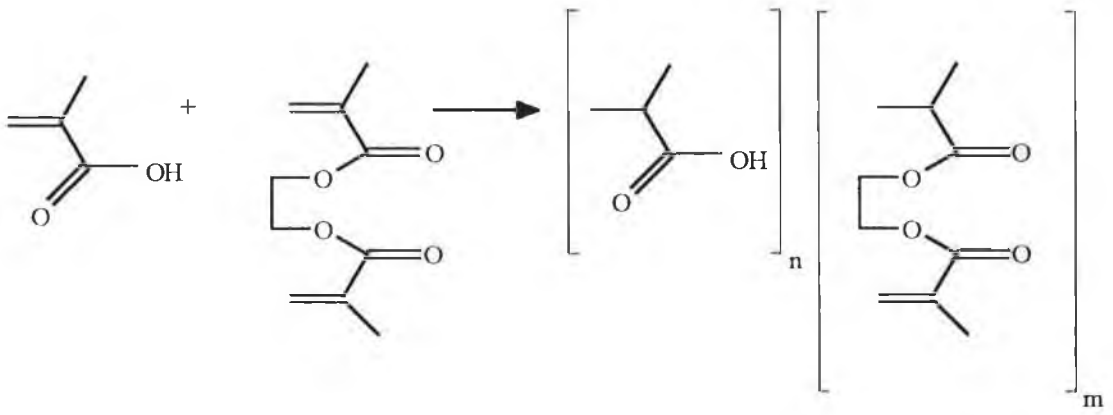
The next step was to prepare a monomer which incorporated a chiral centre in its side chain. This work was based on modification of a commonly used monomer, methacrylic acid, with a number of compounds which already contained a chiral centre. These compounds included menthol and the amino acids alanine, phenylalanine, leucine and valine. A monomer was prepared from a reaction involving alanine and acryloyl chloride. This was copolymerized with EDMA using *S*-propranolol as the imprint molecule. A second MIP was prepared from MAA and EDMA again using propranolol as the imprint molecule. Both these polymer were examined further by capillary electrophoresis (Chapter 6).

4.2 RESULTS AND DISCUSSION

4.2.1 *Polymerization of monomers, target compound 7-OHC*

The aim of the work was to prepare a molecularly imprinted polymer to 7-OHC by the "non-covalent" method. This was carried out based on the method of Vlatakis *et al.*[9] A solution of methacrylic acid **1** was added to ethylene glycol dimethacrylate **9**, chloroform and the target molecule 7-hydroxycoumarin. Polymerization was initiated using AIBN [2,2'-azo-bis (2 methylpropionitrile)], and as the polymer formed it was precipitated out of

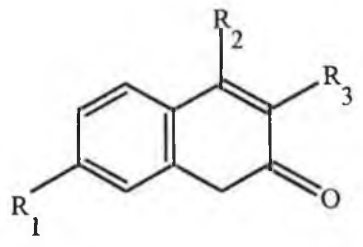
solution. Both methacrylic acid and EDMA have vinyl protons in the region 5.0 - 6.0 ppm; therefore on polymerization it was expected that there would be no protons observed in this region. This was indeed found: the ^{1}H-NMR spectrum showed a singlet at 1.60 ppm for the three protons of the methyl group from the methacrylic acid. The singlet at 0.97 ppm was due to the methyl group of the EDMA and the signal at 3.46 ppm was due to the methylene group on the EDMA. Also the signal at 3.2 ppm is due to the methylene group of the backbone of the polymer. The signal at 11.6 ppm is due to the hydroxyl group of the methacrylic acid.



1 **9** **12**

At this point it was decided to modify the polymerization process by varying the amounts of chloroform added; 15, 25 and 35 ml were added to the mix prior to polymerization while all other parameters remained unchanged.

The selectivity of these polymers for 7-OHC was examined by spectroscopic studies. A number of other spectroscopic studies were carried out on the polymer selected from the previous section: a) the 7-OHC MIP was examined for selectivity of 7-OHC over other drug components including a number of coumarins; b) the amount of time necessary for equilibration of the polymer with the test solution was investigated; and c) the capacity (uptake of 7-OHC) of the material was determined. The structures of the compounds used are shown in Figure 4.4.



	R_1	R_2	R_3
Coumarin	H	H	H
7-hydroxycoumarin	OH	H	H
7-hydroxy-4-methylcoumarin	OH	CH_3	H
7-diethylamino-4-methylcoumarin	$N(Et)_2$	CH_3	H
Coumarin-3-carboxylic acid	H	H	CO_2H

Figure 4.4: ***Structure of coumarin compounds.***

Figure 4.5 shows a typical spectroscopic study of solutions of 7-OHC, 7-OHC polymer after mixing with 7-OHC standard (10 μg/ml), and a solution of polymer after mixing with methanol (blank) for 60 min. It shows the uptake of 7-OHC by the 7-OHC polymer.

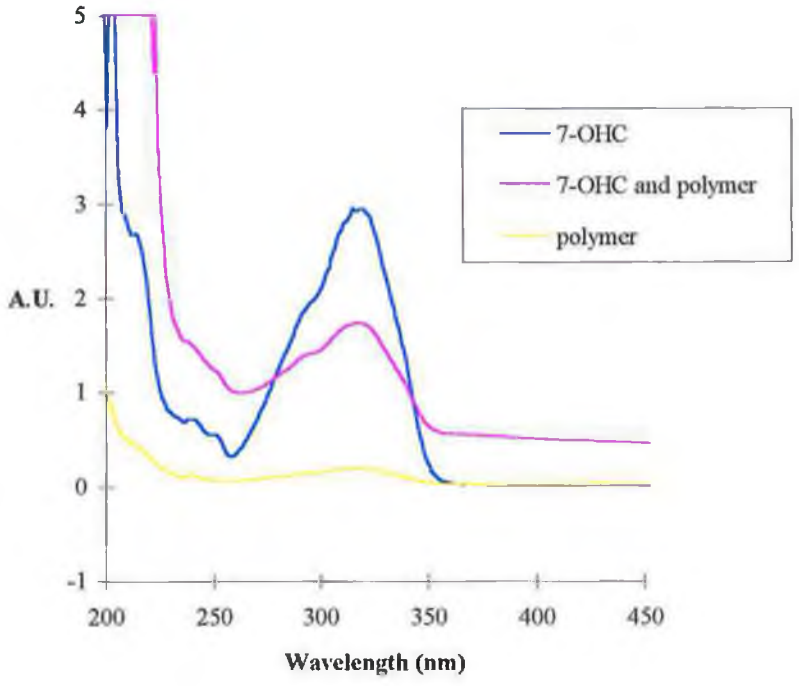


Figure 4.5: ***The uptake of 7-OHC polymer of 7-OHC solution after mixing for 60 min.***

4.2.2 *Spectroscopic studies: Selection of amount of solvent for polymerization*

The amount of solvent (chloroform) used in the polymerization was varied, the amounts investigated were 15, 25 and 35 ml. The polymers were prepared in the usual manner (Section 4.2.1). Known amounts (0.1 g) of polymer were stirred in 10 μg/ml solutions of 7- hydroxycoumarin for 60 min. The polymers were filtered, rinsed with methanol and dried and solutions (1 mg/ml of polymer) prepared in acetonitrile. These were then scanned in the region 200 - 450 nm. From the results obtained it would seem that the polymer prepared in 15 ml of chloroform was more selective for 7- OHC (Figure 4.6).

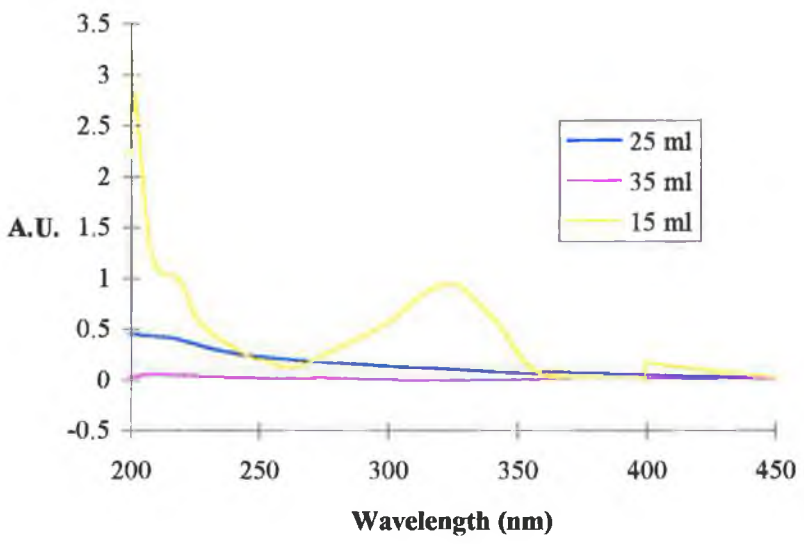


Figure 4.6: ***The selection of the amount of solvent for the polymerization reaction. UV scans carried out in the region 200 - 450 nm.***

4.2.3 *Spectroscopic studies: Selectivity*

The polymer was prepared in the usual manner using 15 ml of chloroform and with 7-OHC as the template. To determine the selectivity of the polymer, known amounts (0.10 g) of polymer were stirred in solutions of 7-OHC, coumarin, 7-diethylamino-4-methylcoumarin, amitriptyline and methanol (blank). The polymers were filtered, rinsed and dried (as in 4.2.2) and solutions prepared in acetonitrile (1 mg/ml polymer). These were then scanned in the region 200 - 450 nm. From the results obtained (Figure 4.7) it would seem that the polymer prepared against 7- hydroxycoumarin was relatively selective for this compound over the other drug compounds.

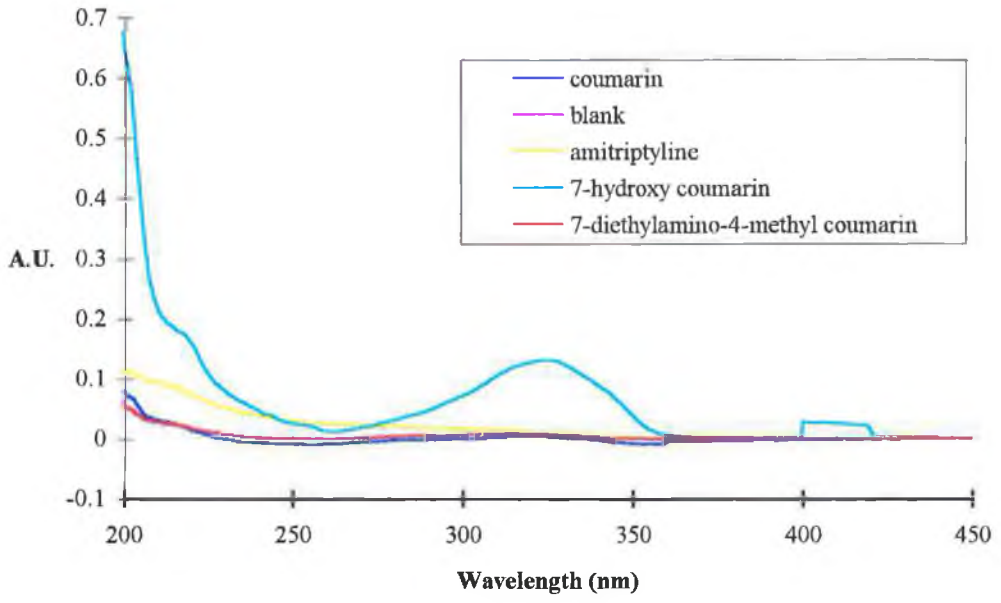


Figure 4.7: ***The selectivity of the polymer prepared to 7-hydroxycoumarin, using a number of other drug components. UV scans carried out in the region 200 - 450 nm.***

4.2.4 *Spectroscopic studies: Effect of Stir Time*

The next parameter for examination was the effect of stir time or equilibration time. This was the amount of time necessary for the polymer to interact (and as such allow the 7-OHC to be taken up by the polymer). Small amounts of the polymer were equilibrated with solutions of 7-hydroxycoumarin for 30, 60, 120 and 180 min. The polymers were filtered, rinsed and dried as before and were scanned in the region 200 - 450 nm. From the results obtained in Figure 4.8 variation was observed by varying the stir time between 30 and 180 min and hence it was decided to use equilibration times of 60 min for further studies.

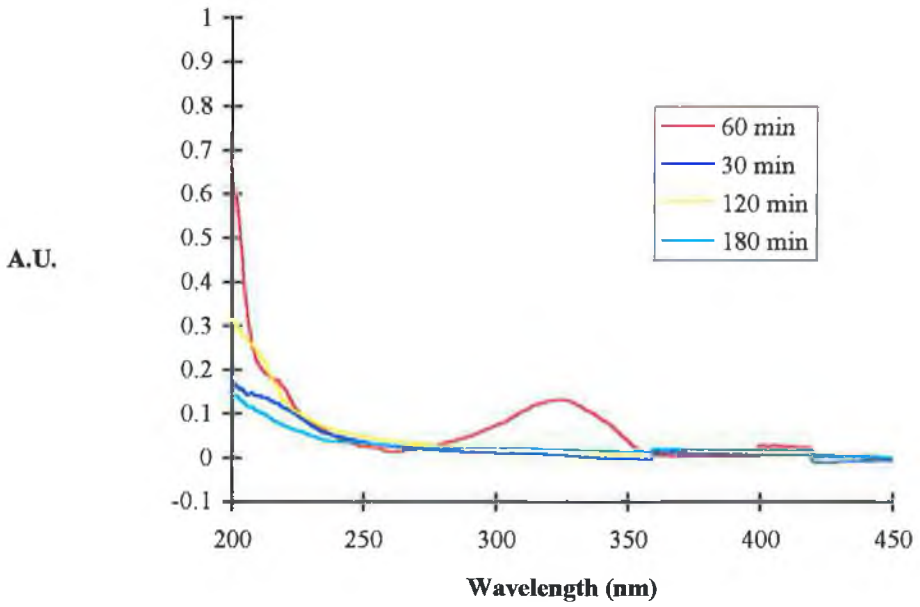


Figure 4.8: ***The effect of stir time required for the polymer prepared to 7-hydroxycoumarin to interact with the target compound. UV scans carried out in the region 200 - 450 nm.***

4.2.5 *Spectroscopic studies: Determination of capacity of material*

To determine the capacity of the selected polymer, solutions of various concentrations of 7-hydroxycoumarin (1 mg/ml, 100 μg/ml, 50 μg/ml 10 μg/ml and 1 μg/ml) were mixed with known amounts of the MIP. After mixing the polymer with the sample solutions for one hour, the polymer was filtered and rinsed and dried. Solutions were prepared from the dry polymers in acetonitrile and these were examined by UV spectroscopy. From the UV scans obtained, it would seem that the polymer was unable to absorb 7-OHC in large quantities from the highly concentrated solutions. It seems for the quantity of polymer examined it was more suitable for the lower concentrations. Hence, for the amount of material used (0.10 g polymer), the maximum solution permissible was a solution of 10 μg/ml.

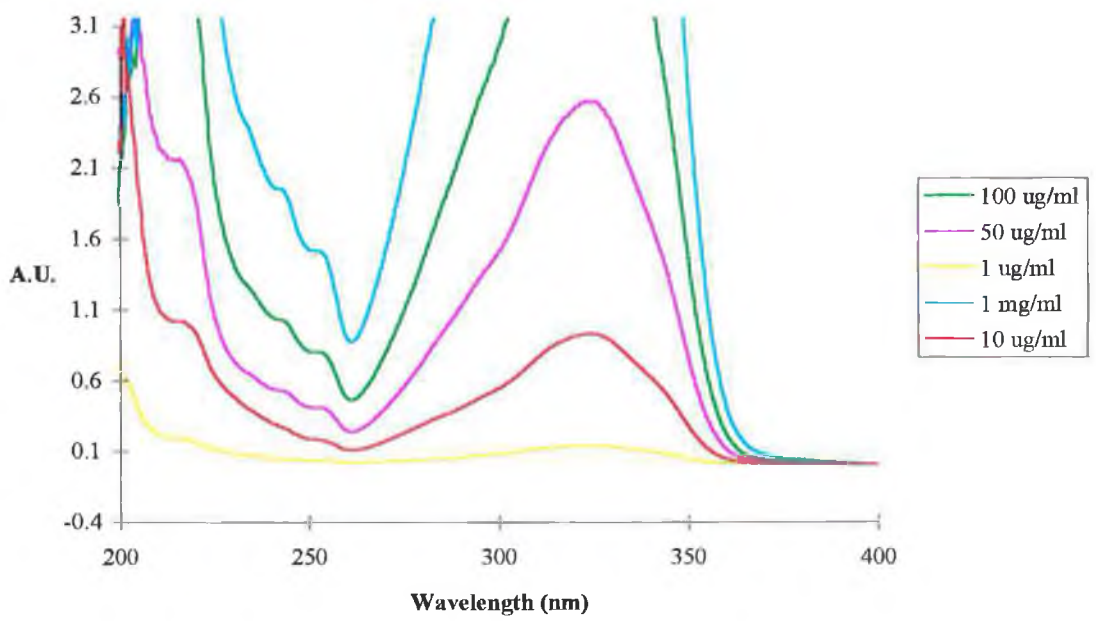
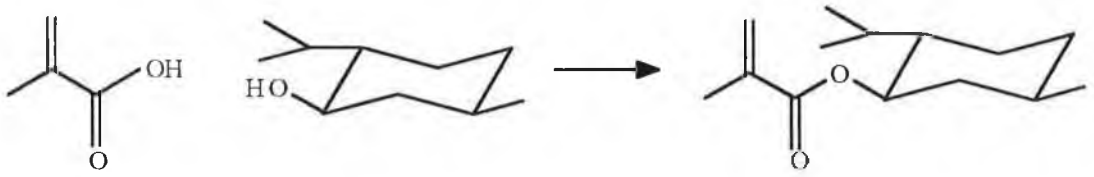


Figure 4.8: ***The capacity of the polymer prepared to 7-hydroxycoumarin using a range of concentrations. UV scans carried out in the region 200 - 400 nm.***

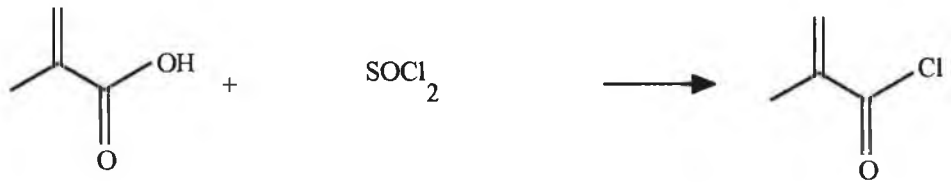
4.2.6 *Preparation of a chiral monomer*

The aim of this work was to prepare a monomer containing a chiral centre. Inital work involved esterification of methacrylic acid **1** with *l*-menthol **11** by the reaction scheme below. However from the results obtained it was impossible to isolate the chiral monomer in sufficient quantities for further use.

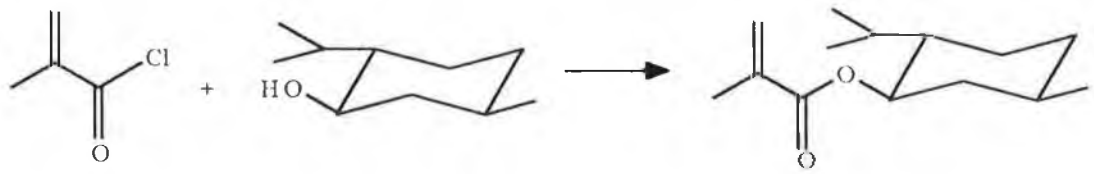


1 **11** **13**

The next scheme to be examined was the conversion of methacrylic acid to the corresponding methacryloyl chloride by reaction with thionyl chloride. The acyl chloride was then reacted with *l*-menthol. Unfortunately this product could not be isolated.

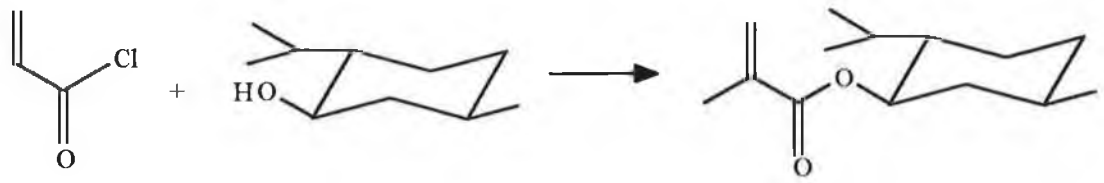


2 **14**



14 **11** **13**

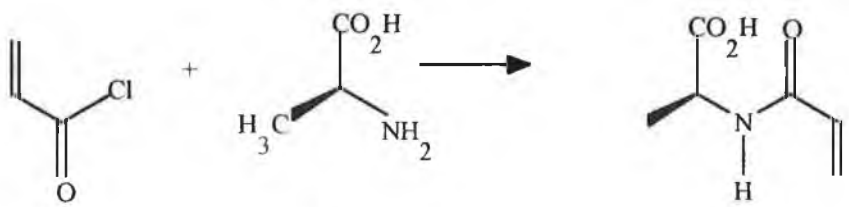
The next stage involved reacting the acryoyl chloride (commercially available) with menthol in an attempt to produce compound **15**. Again it was unsuitable as the final product could not be obtained.



14 **11** **15**

The next stage involved the attempted synthesis of *N*-acyl amino acids by reacting either methacryoyl chloride or acyloyl chloride with the following amino acids; alanine, phenylalanine, leucine and valine by a method described by Iwakura *et al.* [22] However, only the reaction of alanine and acryloyl chloride yielded an analytically pure compound which could be used in further polymerization reactions.

4.2.7 *Synthesis of N-acryloyl-alanine*



14 **16** **17**

A portion of L-alanine **16** (L-α-aminopropionic acid) was added to a solution of sodium hydroxide. Acryoyl chloride **14** was added dropwise to this solution at 4 °C. When the reaction was complete, the mixture was neutralized with concentrated HCl. A white precipitate was formed and this

was filtered and dried. Repeated recrystallization from ethanol yielded an analytically pure sample which was submitted for ^{1}H-NMR analysis. This data confirmed that the compound **17** had been prepared due to the presence of vinyl protons which were observed in the region 5.0 - 6.0 ppm.

4.2.8 *Polymerization of N-acryloyl-alanine*

A polymer was prepared using the prepared monomer *N*-acryloyl alanine **17** (replacing methacrylic acid used in the previous polymerization). The target molecule was *S*-propranolol. The resulting polymer was submitted for ^{1}H-NMR analysis and the absence of the vinyl protons was again observed in the region 5.0 - 6.0 ppm. This polymer is used in further work in Chapter 6.

4.2.9 *Polymerization of monomers in the presence of S-propranolol*

As before (4.3.1) the polymerization was carried out using methacrylic acid **1** and ethylene glycol dimethacrylate, chloroform and the target molecule *S*-propranolol. The polymer formed was found to have no vinyl protons in the region 5.0 - 6.0 ppm (Used for further work in Chapter 6).

4.3 EXPERIMENTAL

^{1}H-NMR spectra were measured at 400 MHz using a Bruker AM 400 spectrometer. ^{13}C -NMR spectra were measured at 100 MHz. Infrared spectra were recorded on a Perkin-Elmer, 2000FT-IR infrared spectrophotometer. Ultra-violet absorption spectra were recorded on a UV-3100, Shimadzu UV-VIS-NIR spectrophotometer. Thin layer chromatography (TLC) was carried out on a silica gel TLC plates (layer thickness 0.20 mm, Riedel-de-Haen). Flash chromatography columns was performed according to Still *et al.*[23] with silica 32 - 63 μm, 230 - 400 mesh. Chloroform was purified by passing through an alumina column. A short path distillation (Kugelröhre) of methacrylic acid was carried out prior to its use. All polymerizations were carried out under nitrogen.

4.3.1 *Polymerization of methacrylic acid in the presence of the target molecule 7- hydroxycoumarin*

The polymerization was carried out by mixing (0.45 g, 5.2 mmol) methacrylic acid, (0.21 g, 1.4 mmol), 7- hydroxycoumarin ethylene glycol dimethacrylate (EDMA) (4.68 g, 23.5 mmol) and azo-iso butyronitrile (AIBN) (0.06 g, 0.37 mmol). Chloroform (15 ml) was added and the mixture was degassed under vacuum and then sonicated for 5 min. It was then sparged with nitrogen for 5 min. The polymerization was carried out by heating the mixture to 60 °C for 12 hours under nitrogen. As the polymer was formed it precipitated out of solution. This was filtered and then ground in a pestle and mortar to give particles in the range 45 - 65 μm. The material was then placed in a Soxhlet apparatus and the 7-hydroxycoumarin was extracted using methanol:acetic acid (9:1 % v/v). Repeated sedimentation of the polymer removed any fines present and the polymer was dried overnight under vacuum.

IR (Nujol Mull): 3310, 2984, 1743, 1471, 1385, 1296, 1261, 955, 722 cm^{-1}.

^{1}H-NMR (DMSO-d_6):δ 1.6 (1H, s), 2.33 (2H, d), 3.2 (1H, q).

4.3.2 *Synthesis of N-acryoyl alanine*

The synthesis was based on a method by Iwakura *et al.*[22] L-alanine (11.24 g, 125 mmol) was added to a round bottom flask and 50 ml of water was added. Sodium hydroxide (10.0 g, 1000 mmol) was added to the solution. When the alanine had dissolved in the solution acryoyl chloride (11.31 g, 125 mmol) was added dropwise with stirring at 0 - 10 °C. When the reaction was complete, the mixture was neutralized with concentrated HCl. The precipitate thus formed was washed with ethanol. The desired product was then obtained from the ethanol extracts and three recrystallizations from ethanol gave an analytically pure sample.

IR (Nujol Mull): 3307, 3079, 1733, 1600, 1295, 1222, 1172, 1067, 975, 816, 681, 660 cm^{-1}.

^{1}H-NMR (DMSO-d_6):δ 1.5 (3H,d), 4.2 (1H, q), 5.6 (1H, dd), 6.1 (1H, dd), 6.3 (1H, dd), 8.4 (1H, d).

^{13}C-NMR (DMSO-d_6): δ 17.5, 47.8, 125.6, 131.5, 164.2, 174.3.

4.3.3 *Polymerization of acryloyl alanine in the presence of the target molecule S-propranolol*

The polymerization was repeated using *S*-propranolol as the target molecule (0.112 g, 1.4 mmol) and the monomer (0.22 g, 1.7 mmol) prepared in section 4.3.2. The weights of the remaining reactants were reduced, the weights used being ethylene glycol dimethacrylate (1.588 g, 7.8 mM) and AIBN (0.02 g, 1.2 mM). The amount of solvent was also reduced (5 ml) and

the mixture was degassed under vacuum and then sonicated for 5 min. The polymerization and extraction was carried out as before (section 4.2.12).

IR (Nujol Mull): 2969, 2845, 1700, 1652, 1557, 1471, 1378, 1260, 1160, 961, 723 cm^{-1}.

^{1}H-NMR (DMSO-d_6):δ 1.2 (1H, d), 1.6 (6H, m), 2.3 (1H, d).

4.3.4 *Polymerization of methacrylic acid in the presence of the target molecule S-propranolol*

The polymerization was repeated using *S*-propranolol as the target molecule (0.112 g, 1.4 mmol). The weights of the remaining reactants were reduced, the weights used been (0.15 g, 1.7 mmol) methacrylic acid, ethylene glycol dimethacrylate (1.588 g, 7.8 mmol) and AIBN (0.02 g, 1.2 mmol). The amount of solvent was also reduced (5 ml) and the mixture was degassed under vacuum and then sonicated for 5 min. The polymerization and extraction was carried out as before (section 4.2.12).

4.4 CONCLUSIONS

The method of "non-covalent" imprinting was found to be a relatively simple straightforward procedure for the preparation of a MIP to 7-OHC. By modification of the amount of solvent used in the reaction the selectivity of the polymer could be altered. This was determined by spectroscopic studies which allowed the polymers to be examined by a quick and simple method, the lower amounts of solvent produced more selective MIPs. Further spectroscopic studies examined the stir time (60 min was found to be suitable), the selectivity (the polymer was found to be selective for 7-OHC over other coumarins) and the capacity of the material (10 μg/ml was found to be the maximum for the amount of polymer examined).

Although the number of monomers is quite large, only a small number of these have found use/or indeed have been used in molecular imprinting. A number of groups have shown that by using monomers other than methacrylic acid and cross-linking monomers other than EDMA, better selectivities and load capacities are obtained from the polymers prepared from these materials. It has also been shown that the addition of further points of interaction increase selectivity. Therefore, by use of a monomer other than methacrylic acid, or indeed by incorporating further functional groups and chiral centres, the selectivity could be increased further. A monomer was prepared from alanine and acryloyl chloride and this was then polymerized using *S*-propranolol as the imprint molecule. The possibility of making stereospecific polymers by the technique of molecular imprinting will be extended further and will find increasing application in the determination of enantiomers. Therefore, there is a need for methods of preparation of monomers, in particular on a large scale, by simple methods.

It is also necessary to examine the methods of preparation of polymers from these monomers and determine whether there is indeed enhanced selectivity. A number of parameters to be varied include variation in the monomer to template ratio, and the use of photo-initiation instead of thermal initiation. There is great potential to prepare a large amount of material suitable for drug analysis, in particular enantiomeric analysis, and indeed provide a cheap alternative to speciality chiral stationary phases.

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CHAPTER FIVE

USE OF A MOLECULARLY IMPRINTED POLYMER AS A SOLID-PHASE EXTRACTION MATERIAL

5.1 INTRODUCTION

Molecularly imprinted polymers (as previously mentioned in Chapter four) are prepared by polymerization of the functional monomer and cross-linking monomer in the presence of the target molecule. These MIPs have found a number of applications, e.g. as stationary phase and solid-phase extraction materials, for the analysis of numerous drug components, in particular for enantioselective separations.

Sellergren *et al.*[1] prepared a molecularly imprinted polymer using methacrylic acid as the functional monomer to a *L*-phenylalanine derivative by the "non-covalent method". This material was packed into a column and a racemic mix gave a maximum separation factor (α) of 3.5. They found that the selectivity was governed by the number and nature of interactions between the substrate and the polymer stationary phase, as well as the shape and rigidity of the substituents of the print molecule. The capacity of the material was 1.5 mg of racemate to 1 g of polymer. Substrates other than the respective template molecule were found to be poorly resolved in most cases.

Sellergren[2] prepared chiral stationary phases and examined the selectivity and sample load capacity. Using either the D- or L- form of a particular amino acid derivative, the MIP was found to show high enantioselectivity in the chromatographic mode. However, broad peaks were obtained and slow mass transfer was found; this was due to a higher number of interactions between the solute and the MIP stationary phase. However, a polymer prepared at low temperature showed a more rapid mass transfer and higher selectivity. It was also found to achieve more rapid mass transfer and higher selectivity by increasing the column temperature.

Moradian *et al.*[3] prepared a MIP to L-*p*-aminophenylalanine anilide, and packed it into a column. This was then used for the chromatographic analysis of

a racemic mixture, with separation factors (α) of above 8 being obtained. This was due to the multiple points of attachment between the print molecule and the polymer. However, it was necesssary to thermostat the column to 85 °C to elute the enantiomers. A high degree of selectivity was obtained by the polymer for the template molecule.

A chiral stationary phase was prepared by Fischer *et al.*[4] which allowed the direct enantioseparation of β-adrenergic blockers. The print molecule used was (*S*)- (-) timolol. They also examined the use of a different monomer, itaconic acid (ITA). Both polymers achieved baseline separation of a racemic mix of timolol with R_s values between 1.9 and 2.0 being obtained for ITA and MAA, respectively. ITA was found to give sharper peaks and a higher degree of selectivity. The advantage of molecular imprinted stationary phases over many other stationary phases is the predictable elution order of the enantiomers, the print molecule being eluted last. The load capacity of a particular MIP prepared by this method from MAA was found to be about 500 μg of a racemic amino acid derivative per 1 g of dry polymer.

Imprints have been made against the bronchodilator theophylline and the tranquilizer diazepam.[5] Cross-reactivity profiles of these MIPs were practically identical to those reported for monoclonal antibodies against these drugs. The anti-theophylline MIPs were used for the determination of theophylline concentration in patient serum samples, the results obtained were comparable to those obtained using an immunoassay technique.

In medical/environmental fields it is often necessary to analyze target molecules in the presence of complex mixtures, and in some cases carry out some pretreatment steps. In fact, preconcentration may be necessary to permit analysis using the "end method" of determination. It may also be necessary to remove the analyte from a complex mixture which may include a number of interferents. The

most commonly used methods which allow such sample preparation (refer to chapter1) include liquid/liquid extraction and solid-phase extraction (SPE).

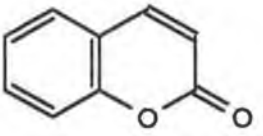
SPE[6] allows the analyte to be sorbed onto the solid-phase while any interfering components are eluted to waste. Solid-phase materials normally used are sorbents that are hydrophobic which allows uncharged analytes to be adsorbed. Hydrophilic analytes cause more problems as regards enrichment and clean-up as they cause disturbances in subsequent chromatographic analysis. Affinity chromatography, with immobilized biomolecules exhibiting high affinity and specificity toward their substrates, have also been used for this purpose. However, there are problems associated with this technique, in that poor stability exists and results obtained are often irreproducible.

Molecularly imprinted polymers have found application as solid-phase extraction materials, and this has been demonstrated by Sellergren.[7] He prepared a SPE material to a drug used in the treatment of AIDS-related pneumonia; i.e. pentamidine (PAM). This MIP was found suitable for removal of PAM from dilute solutions of urine. He found that at a physiological concentration (30 mM) this gave an enrichment factor of 54 using a PAM-selective polymer, whereas the enrichment factor on a benzamidine (BAM) imprinted reference polymer was only 14. The high selectivity of the polymer allowed the drug to be detected directly in the desorption step, thus eliminating the need for a subsequent chromatographic analysis. The preparation of the solid-phase extraction columns involved the polymerization of the monomers in a sealed glass tube heated to 60 °C for 24 hr in an oven. The glass tubes were cut and fitted with column end fittings and connected to a HPLC system. This method allowed a selective enrichment while allowing the analysis of low concentrations. This technique may offer a solution for the determination of metabolites or adducts of close structural similarity.

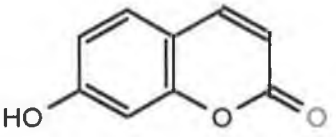
The aim of this work was to find an application for the MIP previously prepared in chapter 4. The MIP prepared to 7-hydroxycoumarin (7-OHC) was packed into a cartridge and used as a solid-phase extraction phase. The extraction procedure using the solid-phase extraction cartridge is described from first principles. The method was validated for the extraction of 7-OHC from urine samples in the range 10 - 50 μg/ml. The selectivity of this polymer prepared to 7-OHC over other members of the coumarin family was also examined (Figure 5.1).

To date, there have been a number of methods for the determination of 7-OHC by TLC,[8] HPLC[9] and CZE. Coumarin is a naturally occurring compound in a number of plants and has found use in the treatment of a number of diseases including brucellosis, cancer, burns and rheumatic disease.[10] It has been shown that approximately 63 % of the total dose of coumarin administered to patients was recovered as the 7-OHC derivative[11] within 24 hr. Tan *et al.*[12] developed a method for the extraction of coumarin and 7-OHC from whole blood, using spectrofluorimetric detection, where the mean recovery was found to be 94.8 %. This method involved repeated extraction of coumarin and 7-OHC with diethyl ether and combination of the extracts. Although this method was found to be sensitive, it had a number of drawbacks including the need for two extractions, and the use of large sample volumes.

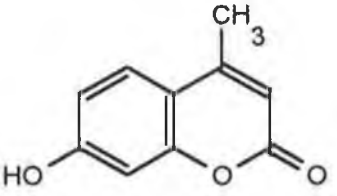
Egan *et al.*[13] used a spectrofluorimetric method for the determination of 7-OHC from urine and plasma using extracted and unextracted samples. As 7-OHC is conjugated to glucuronide, it had to be treated with β-glucuronidase for 30 min at 37 °C to release it as 7-OHC, allowing free, total and conjugated 7-OHC to be determined. Extraction was carried out using diethyl ether, the sample being then dried down and reconstituted in an aliquot of phosphate buffered saline (PBS), pH 10.0, and transferred to a 96 well microtitre plate (20 μl aliquots). The fluorescence was determined at 370 nm (excitation) and 450 nm (emission) and



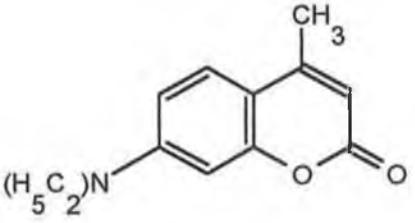
coumarin



7-hydroxycoumarin



7-hydroxy-4-methylcoumarin



7-diethylamino-4-methylcoumarin

Figure 5 1. ***Chemical structures of coumarin, 7-hydroxycoumarin, 7-hydroxy-4-methylcoumarin and 7-diethylamino-4-methylcoumarin.***

was found to be linear in the range 0. 5 - 10 and 10 - 100 μg/ml. The percentage of the dose recovered as 7-OHC over a 24 hr period was 92 - 98 %.

Bogan *et al.*[14] developed a method for the determination of free and total 7-OHC from urine and serum by capillary electrophoresis, utilizing UV detection at 210 nm. The assay was found to be linear in the range 0 - 50 μg/ml with a limit of quantitation of 1 μg/ml. Urine from two volunteers, who had been administered coumarin, was analyzed by CE and HPLC, and the methods were compared and contrasted. The CE method gave rise to shorter analysis times 1.5 min. compared to 12 min. for HPLC. However, no statistical difference between results was observed. Therefore, it was decided to use CE as the method of determination of 7-OHC based on the previously mentioned method.

Capillary zone electrophoresis (CZE) was first developed by Jorgenson *et al.*[15,16] in the early 1980's and it is a rapidly developing separation technique with high resolving power and separation speed. However, only ionic or charged solutes can be separated by this method, as its separation occurs by differential migration of analytes in an electric field. The capillary used is a narrow-bore with an internal diameter in the region of 25 - 75 μm, which is usually filled only with buffer.

The sampling procedure occurs by initially placing both ends of the capillary in the buffer reservoirs. These reservoirs also contain the electrodes which make the electrical contact between the capillary and the high voltage power supply. The sample is loaded onto the capillary by either one of two methods: an electric field or by an external pressure. The capillary is then replaced in the buffer reservoir and the electric field is applied and the separation is carried out.

A fundamental part of any CE separation is the electroosmotic, or electroendosmotic flow (EOF). This constitutes the bulk flow of liquid in the capillary and is due to the surface charge on the interior capillary wall; resulting

from the effect of the applied electric field on the solution double-layer at the wall. The fused silica has numerous silanol groups which exist in an anionic form (SiO^-). Counter-ions build up near the surface to maintain charge balance and form a double-layer thus creating a potential (zeta potential) difference close to the capillary wall. When a voltage is applied across the capillary, the cations become attracted to the cathode. These cations are solvated and as they move towards the cathode they drag the bulk solution towards the cathode also. The zeta potential is dependent on the surface charge on the capillary, and since this charge is pH dependent, variations in the pH results in changes in the EOF. Under alkaline conditions the silanol groups are mainly deprotonated and the EOF is large, whereas at low pH it is quite small. The zeta potantial is also affected by the ionic strength of the buffer; increases in ionic strength result in decreases in zeta potential, and hence the EOF decreases.

Separation in electrophoresis is dependent on the differences in the analyte velocity in an electric field. The velocity of the ion[15] is described by

$$v = \mu_e E \qquad = \mu_e (V/L) \qquad 5.1$$

where v = ion velocity, μ_e = electrophoretic mobility, E = applied electric field, V = applied voltage, L = total capillary length.

The time required for an analyte to migrate to the point of detection is called the migration time. The migration time and a number of other experimental parameters are used in the calculation of the apparent mobility of the analyte:

$$\mu_a = IL/(tV) \qquad 5.2$$

$$\text{where } \mu_a = \mu_e + \mu_{eof} \qquad 5.3$$

where I = effective capillary length (length to the detector), t = the migration time of the analyte and μ_{eof} = the apparent electric osmotic flow.

In the presence of electric osmotic flow (EOF), the measured mobility is called the apparent mobility, μ_a. The effective mobility, μ_e, can be extracted from the apparent mobility by independently measuring the EOF using a neutral marker that moves at a velocity equal to the EOF, e.g. acetone, methanol, DMSO and mesityl oxide.

5.2 EXPERIMENTAL

5.2.1 *Reagents and solvents*

HPLC grade methanol and diethyl ether were obtained from Labscan Analytical Sciences, (Dublin, Ireland). Analar grade sodium hydroxide and phosphoric acid were supplied by BDH (Poole, UK). Analytical grade dipotassium hydrogen phosphate and potassium dihydrogen phosphate were used as buffer salts and were obtained from Merck (Darmstadt, Germany). The electrolyte solution used was 0.025 M phosphate buffer, pH 7.5, which was prepared daily by dissolving 0.02 M K_2HPO_4 and 0.005 M KH_2PO_4 in deionized water. A Britton-Robinson (BR) buffer solution was prepared containing 11.48 ml acetic acid (99.7 %), 12.44 g boric acid and 13.5 ml phosphoric acid (85%) per litre. The pH was adjusted using 2.0 M sodium hydroxide. Extraction columns were prepared in prewashed syringe barrels (2 ml) and qualitative filter paper discs (No. 1, Whatman, Maidstone, UK) were used as frits. 7-OHC and the other coumarin compounds were obtained from Sigma (St. Louis, MO, USA).

5.2.2 *Standards*

Stock solutions were prepared by dissolving the appropriate amount of analyte in methanol (100 %) to yield a solution of concentration 1 mg/ml. A set of calibration standards were prepared by dilution of the stock solution with deionized water, covering the range 10 - 50 μg/ml.

5.2.3 *Urine Standards*

Blank urine was obtained from a volunteer who had not been administered coumarin or 7-hydroxycoumarin. Aliquots of this blank urine were spiked with standard solutions of 7-OHC and vortex mixed to give the required concentration in the range 10 - 50 μg/ml.

5.2.4 *Instrumentation and Operating Conditions*

The capillary used was a 27 cm x 50 μm I.D. fused-silica column (Beckman Instruments), with a capillary-to-detector distance of 20.0 cm. The capillary was prepared by rinsing with 0.1 M sodium hydroxide for 1 min and then with buffer solution for 1.2 min. This was based on a method by Bogan *et al.*[14] The sample was applied to the capillary by a 3-s pressurized injection (0.5 p.s.i.) and separation was achieved with an applied voltage of 20 kV (rise time 0.2 min) at 25 °C. Typical running current was 100 μA. The resultant electropherogram was monitored at 210 nm with a photodiode array detector using Beckman System Gold software. The migration time for 7-hydroxycoumarin was 0.92 min.

5.2.5 Procedures

5.2.5.1 *Column preparation and extraction procedure*

The columns were prepared by a method described by Boyd.[17] which involved the use of syringe barrels. Two filter paper discs were placed in a 2 ml syringe barrel and 0.40 g of polymer was added to the barrel and two more filter discs were placed on top. The columns were preconditioned by flushing with 3 ml of methanol, 2 ml of water and 1 ml of buffer solution. The column was compressed with a syringe plunger. Sample (250 μl) was applied to the top of the column. The column was washed with 2 ml of deionized water and allowed to dry for 30 min. The drug component was eluted with 3 ml of methanol. This eluate was evaporated to dryness under a stream of nitrogen at 60 °C, and reconstituted in 250 μl of phosphate buffer (0.025 M).

5.2.6 Calibration and Calculation

Evaluation of the assay was carried out by the construction of a five-point calibration graph covering the concentration range 10 - 50 μg/ml. The slope and the intercept of the calibration graphs was determined through the linear regression of the drug height versus drug concentration graphs. Individual peak heights were interpolated on the calibration graphs to determine values of concentration found as compared to concentration added.

5.3 RESULTS AND DISCUSSION

5.3.1 *Development of extraction procedure*

The aim of this work was to use the MIP prepared to 7-hydroxycoumarin as a solid-phase extraction material. MIP stationary phases function by interactions between the polymer and the target compound (hydrogen bonding and ionic interactions); therefore the MIP prepared to 7-OHC should show recognition for the original 'template' molecule and hopefully some selectivity for it over other similar structure compounds, i.e. members of the coumarin family.

A method was set up which allowed the extraction of 7-hydroxycoumarin from urine. The columns were prepared as described in section 5.2.1. To optimize the extraction a number of parameters were varied, and these included the pH of the buffer used prior to the application of the sample, the wash step and the elution step. Each of the parameters was examined in triplicate. The columns were pre-conditioned with methanol and then water.

The pH of the buffer was the first parameter varied; this was carried out by applying 1 ml of buffer of appropriate pH to the pre-conditioned cartridges. The pH was varied using Britton-Robinson buffer in the range 3 - 10 pH units. From the data obtained the recoveries were found to vary, at very low pH, (3 - 4) values of c.a. 40 % were obtained; while at high pH, values in the region 0 - 5 % were obtained. Values between these two ranges gave the best recoveries with pH 6 giving values in the region 80 - 96 %.

The next parameter to be studied was the wash step. This is important at this stage to remove any endogenous components without eluting 7-OHC. A number of wash procedures were examined including 100 % H_2O and 50:50 H_2O/

methanol. The washings were dried down to determine if any 7-OHC had been eluted at this stage. Based on the results obtained, it was decided to use water as it gave cleaner extracts at a later stage and allowed 7-OHC to remain on the column while the use of 50:50 methanol:water caused small amounts of 7-OHC to elute. The amount of wash was also varied in the region 1 - 5 ml, with 2 ml being found the most suitable.

The final step was to select a suitable elution solvent and a number of solvents were examined, including methanol, diethyl ether, hexane:diethyl ether (50:50) and ethyl acetate. Methanol gave rise to the best recoveries, with the amount of solvent used being varied in the region 1 - 5 ml, with 3 ml of methanol being found to be suitable for elution of 7-OHC. Poor recoveries were obtained with the other solvents; hexane, (50:50) diethyl ether/hexane showed recoveries in the region 0 - 5 %. Diethyl ether gave values of 30 - 40 % while ethyl acetate extracts produced a large number of interfering peaks. An increase in the volume of these solvents did not improve the recoveries.

5.3.2 *Assay validation*

The method was validated for the determination of 7-hydroxycoumarin by extraction using a MIP-SPE over the concentration range 10 - 50 μg/ml. Electropherograms showing blank and spiked (50 μg/ml) urine samples from the validation study are shown in Figure 5.1.

5.3.2.1 *Limit of Detection*

The LOD was formed to be found to be 5 μg/ml for 7-hydroxycoumarin.

5.3.2.2 *Precision*

The precision is expressed as the mean coefficient of variation, determined from peak height of the analyte when interpolated as unknowns on the regression line. The data presented in Table 5.1 and 5.2 demonstrated the inter- and intra-assay variations for the method. Inter-assay was determined from four replicate runs, from four separate days, while the intra assay was determined in quadruplicate over the same concentration range.

5.3.2.3 *Linearity and accuracy*

This is defined by the correlation coefficient of the regression line and accuracy is defined by the percentage difference between "added" and "found" concentration for Inter- assay values presented in Table 5.1. A correlation coefficient of 0.994 or better was obtained.

5.3.2.4 *Recovery*

The recovery of 7-hydroxycoumarin from urine was measured by calculating the percentage difference between the peak heights of extracted standards and those of authentic standards in the relevent concentration range. The mean recovery was found to be 90.36 $\pm$ 7.37 %.

5.3.2.5 *Selectivity*

A number of other coumarins and drug compounds were investigated as potential interferents. A number of the coumarins, coumarin-3-carboxylic acid and 7-diethylamino-4-methylcoumarin showed some cross reactivity for the polymer.

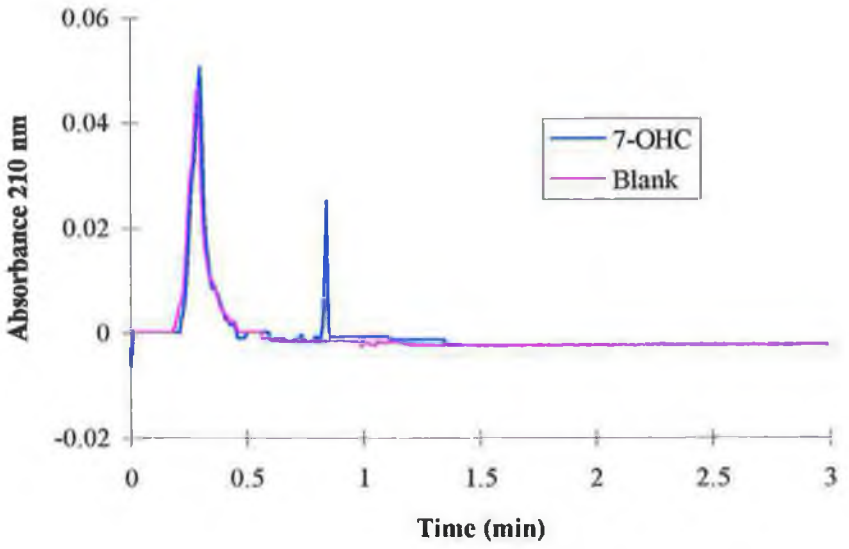


Figure 5.1: ***Electropherograms of 7-hydroxycoumarin extracted from urine and blank urine. Background electrolyte consists of 0.025 M phosphate buffer, pH 7.5, Temperature 25 ℃, Voltage 20 kV.***

TABLE 5.1
Precision and Linearity-INTER-ASSAY

Amount 7-OHC added µg/ml	Peak height	Amount found µg/ml
10	0.00353	11.97
20	0.00569	19.17
30	0.00876	29.40
40	0.01200	40.20
50	0.01476	49.40
10	0.00347	11.77
20	0.00594	20.00
30	0.00873	29.30
40	0.01171	39.23
50	0.01556	52.07
10	0.00325	11.03
20	0.00553	18.63
30	0.00876	29.40
40	0.01221	40.90
50	0.01506	50.40
10	0.00303	10.43
20	0.00534	18.00
30	0.00839	28.17
40	0.01178	39.47
50	0.01523	50.97

	Concentration ng/ml				
	10.00	20.00	30.00	40.00	50.00
Mean Amount Found ng/ml	11.30	18.95	29.07	39.95	50.71
Standard Deviation	0.71	0.85	0.60	0.76	1.11
Coefficient of variation %	6.28	4.49	2.06	1.90	2.19
Difference between added and found	+1.33	-1.05	-0.93	-0.05	+0.71

TABLE 5.2
Precision and Linearity-INTRA-ASSAY

Amount 7-OHC added µg/ml	Peak height	Amount found µg/ml
10	0.00286	11.20
20	0.00547	19.90
30	0.00811	27.20
40	0.01172	40.73
50	0.01497	51.57
10	0.00353	10.77
20	0.00569	17.97
30	0.00876	28.20
40	0.01200	39.00
50	0.01476	48.20
10	0.00329	10.63
20	0.00544	17.80
30	0.00910	30.00
40	0.01198	39.60
50	0.01461	48.37
10	0.00322	11.07
20	0.00589	19.97
30	0.00912	30.73
40	0.01201	40.37
50	0.01566	52.53

	Concentration ng/ml				
	10.00	20.00	30.00	40.00	50.00
Mean Amount Found ng/ml	10.29	18.91	29.03	39.93	50.17
Standard Deviation	0.27	1.19	1.62	0.78	2.21
Coefficient of variation %	2.62	6.29	5.58	1.95	4.41
Difference between added and found	+0.92	-1.09	-0.97	-0.07	+0.17

TABLE 5.3

Results for recovery of 7-hydroxycoumarin

	7-hydroxycoumarin peak height		
Concentration ug/ml	Authentic standards	Extracted standards	Recovery %
10	0.00357	0.00329	92.16
20	0.00703	0.00589	77.38
30	0.00972	0.00910	93.62
40	0.01288	0.01198	93.01
50	0.01528	0.01461	95.62

Mean recovery ($\pm$ standard deviation) = 90.36 $\pm$ 7.37 %.

5.4 CONCLUSION

The area of solid-phase extraction as a method of sample preparation is increasing in size and in many cases replacing liquid/liquid extraction. The number of phases available for this technique is increasing, and these include ion-exchange materials, polymers and mixed functional phases and this in turn expands the range of components which can be extracted by this technique.

Although polymers have already found use in this area, the use of an MIP allows a polymer material to be prepared to a specific component. This would be particularly useful if the component is unsuitable for solid-phase extraction by any of the materials previously mentioned. This could also be particularly useful when a component has to be removed from its metabolites or compounds with very similar structures.

This study shows that a MIP prepared to a 7-hydroxycoumarin allowed it to be selectively retained, while allowing endogenous components to be removed. It was capable of extracting 7-OHC in the range 10 - 50 μg/ml. Previous methods reported extraction in this range, but, it was necessary to use large amounts of solvent and samples. This method overcomes the need for such large amounts of solvent. The method was validated in terms of coefficient of variation and recovery, linearity and accuracy.

The presented analytical scheme can thus in favourable cases enrich and clean-up a sample to a level that allows direct analyte determination upon desorption. It should prove to be a suitable alternative to the LLE techniques previously described. Possible further work would include the use of other monomers/cross-linking monomers to try and increase the selctivity of the polymer and examination of the ratio of monomer to template, again to improve selectivity.

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CHAPTER SIX

USE OF A MOLECULARLY IMPRINTED POLYMER AS A CHIRAL SELECTOR IN CAPILLARY ELECTROPHORESIS

6.1 INTRODUCTION

As previously mentioned in chapter 5, capillary electrophoresis allows the separation of ionic or charged analytes, based on the difference in their electrophoretic mobilities. Neutral analytes are not suitable for separation by this technique; however, the development of electrokinetic chromatography[1] (EKC) has solved these problems. EKC is a branch of CE which uses the technique of CZE in combination with the chromatographic separation principles. This involves using a homogenous solution that contains an ionic "carrier", and has found application in the analysis of neutral analytes. This technique also offers greatly improved selectivity for the separation of ionic compounds.[2]

6.1.1 *Micellar electrokinetic chromatography (MEKC)*

Micellar electrokinetic chromatography (MEKC) is a mode of EKC in which surfactants (micelles) are added to the buffer solution.[3] The charged molecules or charged molecular aggregates are employed in EKC as a separation carrier or a "pseudo-stationary phase", which corresponds to the stationary phase in conventional chromatography. The separation carrier is transported by electrophoresis at a different velocity from the surrounding buffer solution. The analyte distributes itself between the carrier and the surrounding medium. These surfactants are used in concentrations higher than the critical micelle concentration (CMC), and this particular mode has found application among the various EKC modes. MEKC has also found use for the separation of ionic solutes as well as the neutral or non-ionic solutes. It has, therefore, been found suitable in the determination of pharmaceuticals, including cationic, anionic and neutral species.[4] Apart from the surfactant, other parameters can be further manipulated and these include buffer cation, ionic strength, current and temperature.

6.1.2 *Mechanism of MEKC*

The separation principle of MEKC is based on the differential partition of the analyte between the micelle and the surrounding aqueous phase, as in micellar chromatography (Figure 6.1). An ionic surfactant (sodium dodecyl sulphate, SDS) is added to an operating buffer solution in MEKC, and these surfactant molecules form micelles or aggregates above the CMC. The hydrophobic end groups orient toward the centre and the charged groups point outward towards the surface; the formation of these micelles allows the separation of electrically neutral or non-ionic solutes. The system is composed of two phases; the micellar phase and the aqueous phase. When an anionic surfactant is employed, the micelle migrates toward the positive electrode by electrophoresis. However, the strong electroosmotic flow (EOF) transports the buffer solution towards the negative electrode due to the negative charge on the surface of the fused-silica capillary. The velocity of EOF is usually faster than that of the electrophoretic migration of the micelle under the neutral or alkaline conditions, resulting in a fast-moving aqueous phase and a slow-moving micellar phase.

When a non-ionic solute is injected into the solution, a fraction of it is incorporated into the micelle and therefore migrates at the same velocity as that of the micelle. The remaining fraction migrates at the velocity of EOF. The surfaces of SDS micelles have a large negative charge, giving them a large electrophoretic mobility toward the anode. However, most buffers exhibit a strong electroosmotic flow towards the cathode. The magnitude of electroosmotic flow is slightly greater than that of the micelle migration, resulting in a fast moving aqueous phase and a slow moving micellar phase. Therefore an electrically neutral solute can be separated by the difference in the distribution coefficients between the micellar phase and the surrounding aqueous phase.

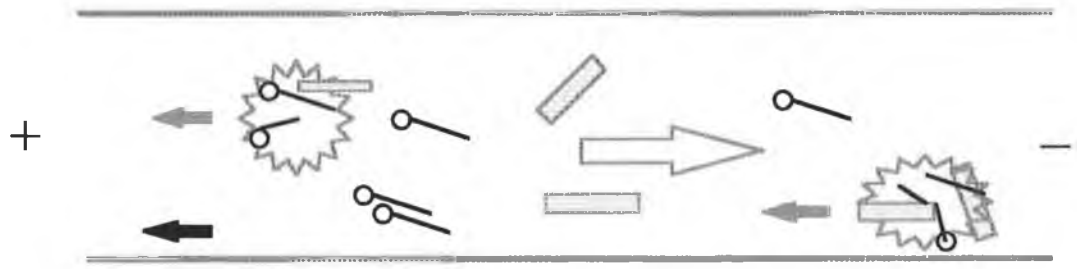


Figure 6.1: ***Separation principle of micellar electrokinetic chromatography (MEKC). Surfactant = ○—, Analyte = ▭, Electroosmotic Flow = ⇨ and Electrophoresis = ⬅.***

MEKC can provide enhanced selectivity for the separation of ionic solutes as well as the separation of electrically neutral or non-ionic solutes through the ionic interaction between the solute and the micelle. The migration velocity in MEKC usually depends on the hydrophobicity of the solute. The more hydrophobic the analyte, the stronger it interacts with the micelle and hence the slower it migrates compared to the hydrophilic analytes. Another useful application of MEKC is optical resolution of a chiral compound with a mixed micelle or with a chiral micelle.

6.1.3 *Application of MEKC (chiral separations)*

As previously mentioned in Chapter one, drug enantiomers interact quite differently with biological components. These components include enzymes, receptors and plasma proteins, and these can lead to differences in absorption, distribution, metabolism and elimination of the drug compound; therefore, many drug enantiomers show different pharmacological effects. Hence it is necessary to have methods that are able to discriminate between the two enantiomers; a number of methods utilize HPLC and CZE.

In CZE, chiral selectivity can be obtained by either adding the chiral selector to the running buffer or by immobilization of the chiral selector in the capillary. Enantiomeric separation by MEKC utilizes chiral surfactants which form chiral micelles. Most analytes are adsorbed onto the surface of the micelle or interact with the polar groups of the surfactants. Therefore, surfactants with chiral polar groups have found use for chiral discrimination. A number of chiral surfactants are available, but to date only a few of them have found use for enantiomeric separations by MEKC; e.g. sodium *N*-dodecanoyl-L-valinate[5] and bile salts.[6] There are a number of chiral selectors, and these are mainly inclusion complexes, which act by spatially enclosing the guest molecule, or at least part of it. In EKC, the most commonly used inclusion compounds are cyclodextrins[7] and its derivatives,[8] or crown ethers[9] (previously described in Chapter 1).

6.1.4 *Enantiomeric separation by cyclodextrin-modified MEKC (CD-MEKC)*

Cyclodextrin-modified micellar electrokinetic chromatography[10] (CD-MEKC), is a branch of MEKC in which the CD is added to the micellar solution. It was developed for the separation of electrically neutral, highly hydrophobic compounds (Figure 6.2). The separation of these substances is normally difficult by electrophoretic techniques; however, by this method a water insoluble hydrophobic analyte is partitioned between the micelle and CD. When the analyte is incorporated into the micelle, it migrates with the micellar velocity. When it is included in the cavity it migrates with the electroosmotic velocity, separation being achieved by differential partition of the analyte between the CD and the micelle.

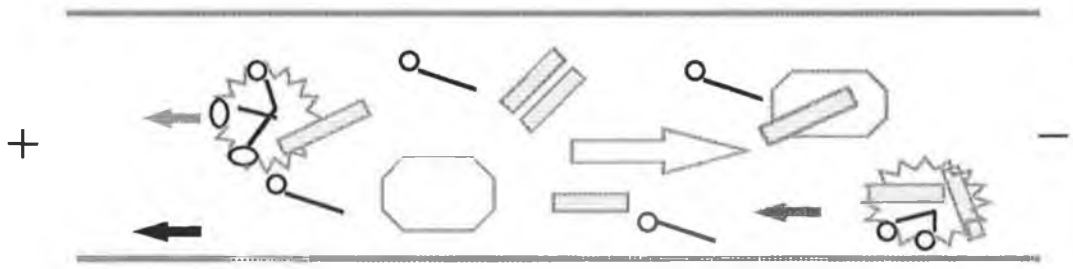


Figure 6.2: ***Schematic of the separation principle of cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC). Surfactant =***  ***Analyte =***  ***Electroosmotic Flow =***  ***Electrophoresis =***  ***and cyclodextrin =*** 

This mode has been found to be suitable for the separation of polycyclic aromatic hydrocarbons[11] and a number of other compounds including β-blockers.[12]

Cyclodextrins are commonly used as chiral selectors in CZE and there are a number of them available. They absorb very little in the UV region and are stable over a wide pH range and are non-toxic. They are reasonably soluble and this solubility can be increased further by substitution. The use of cyclodextrins dissolved in buffer solutions has been found to be particularly effective for separating hydrophobic compounds. Since the CD does not interact with the micelle, a CD in the micellar solution should behave as another phase in comparison with the micelle, and migrate at an identical velocity to that of the bulk solution, as the CD itself is electrically neutral. Thus the analyte may be partitioned between three phases, the micelle, the CD and the aqueous phase. The micelle migrates at a different velocity from that of the CD or the aqueous phase. In CD-MEKC, β or γ-CD's and sodium dodecyl sulphate are normally used. It should be mentioned that CD-MEKC is a different technique from CD-EKC. In CD-MEKC a neutral CD is added to

the micellar solution, while in CD-EKC an anionic CD derivative without the micelle is employed. CD-MEKC is also very effective for enantiomeric separation because of the chirality of the CD itself.

The technique of CD-EKC has been used by Guttman *et al.*[13] to examine the use of hydroxypropyl-β-cyclodextrin as a chiral selector for the separation of racemic propranolol. The effects of a number of parameters were examined including buffer pH, concentration of chiral selector, applied electric field and temperature on the chiral separation. These parameters were optimized to obtain the maximum separation of the enantiomers with minimal analysis time; $R_s = 1.75$ in less than 5min.

Palmarsdottir *et al.*[14] also used this technique when examining the use of a number of cyclodextrins in the determination of a number of racemic compounds including *rac*-terbutaline monosulphate, *rac*-bambuterol, *rac*-terbutaline, *rac*-brompheniramine, *rac*-propranolol and *rac*-ephedrine. They examined how resolution, migration time and efficiency could be affected by the type, degree of substitution and concentration of the cyclodextrin. The cyclodextrins examined included α-cyclodextrin, β-cyclodextrin, dimethyl-β-cyclodextrin and hydroxypropyl-β-cyclodextrin. They also examined the applied voltage and pH of electrolyte and found that the most effective parameters for optimizing were pH and cyclodextrin type and concentration. The chemically modified β-CDs proved to be more powerful chiral selectors than β-CD for the compounds examined.

Fanali[15] also examined the effects of type of shape and amount of cyclodextrins added to the background electrolyte on the migration time and the resolution of the enantiomers studied (terbutaline and propranolol). A phosphate buffer, pH 2.5, containing 5 mM heptakis(2,6-di-*O*-methyl)-β-cyclodextrin or 15 mM β-cyclodextrin gave good resolution for racemic terbutaline. The best resolution for propranolol was obtained using 50 mM phosphate buffer (pH 2.5) - 4 M urea - 40 mM β-cyclodextrin in 30 % (v/v)

methanol. Modified CDs have been shown to give a greater possibility of resolution for compounds which cannot be resolved using the native CD.

St. Pierre *et al.*[16] also used native and derivatized CDs as CE buffer additives while monitoring the effects of pH and cyclodextrin concentration on the enantiomers of chlorpheniramine, terbutaline, atropine, metoprolol and propranolol. They used three different β-cyclodextrin (BCD) modifiers (BCD, 2-hydroxypropyl BCD and methyl BCD). Enantioselectivity was obtained for propranolol, chlorpheniramine and terbutaline.

Separation of a number of dansyl DL-amino acids by MEKC with and without cyclodextrins was examined by Miyashita *et al.*[17] using a borate buffer. The didansyl derivatives migrated slower than the other amino-acids. They also found that the addition of SDS to the buffer solutions containing either β-CD or γ-CD resulted in the chiral separation of DNS amino acids.

Schutzner *et al.*[18] examined the use of diastereomeric derivatives using polyvinylpyrrolidone as a buffer additive in capillary zone electrophoresis and it offered hydrophobic as well as hydrophilic interactions to the analytes. The test substances were converted to their diastereomers and separated using CZE. The network formed by the linear polymer affected the mobility of the diastereomers to a different extent and hence enhanced the selectivity of the system.

Wang *et al.*[19] carried out MEKC using a polymerized chiral micelle for the separation of (±) -1,1'-bi-2-naphthol and D,L-laudanosine. A comparison study of separation was carried out on the micelle polymer and the corresponding non-polymerized surfactant under the same separation conditions. The polymerized micelle was found to demonstrate chiral recognition. The rigidity of the polymerized micelle was found to improve the mass transfer rate and this was in turn found to reduce peak broadening.

Another approach for improving the selectivity of enantiomeric separation when CDs are used as chiral additives is the addition of non-chiral compounds, e.g. organic solvents[15,20] or urea[13] or the use of polymers,[21] in the

chemical environment. The formation of cyclodextrin complexes with different guest molecules is not well understood, and there is as yet no way to predetermine if a certain type of cyclodextrin can be used for the separation of certain enantiomeric pairs of compounds. Therefore the enantioseparation still remains a system of trial and error. The use of molecularly imprinted polymers could overcome this problem, as the MIP would be prepared selectively to one of the enantiomers and this one would have a greater affinity for the MIP and hence be retained longer; therefore, the elution order can be easily predicted.

Chiral resolution of various racemic amino acid derivatives, peptides, other organic acids, and some drug components including timolol[22] and naproxen,[23] have been studied on non-covalent molecularly imprinted stationary phases. For instance, Kempe *et al.*[24] prepared a MIP to the L-enantiomer of an amino acid derivative, and this was found by HPLC to be more retarded than its corresponding D-enantiomer. O'Shannessy *et al.*[25] prepared an MIP to L-phenylalanine, and this was capable of efficient enantiomeric resolution of the racemic mixture of the original print molecule. The functional monomer used was methacrylic acid and the MIP was capable of resolving enantiomers of compounds with structural similarities to the original print molecule.

An MIP had previously been prepared to *S*-propranolol[26] by photo-initiation and used in a radioligand binding assay. This MIP was utilized in an aqueous based assay and showed high substrate selectivity for propranolol in the presence of structurally similar β-blockers. Furthermore, in a toluene-based assay a cross-reactivity of 1 % was obtained for the *R*- enantiomer, which was found to give better results than those obtained by biological antibodies.

For the determination of the enantiomers of propranolol, it was decided to use a micellar system using low concentrations of 2 polymers prepared to *S*-propranolol as additives to the buffer. The two polymers were prepared as

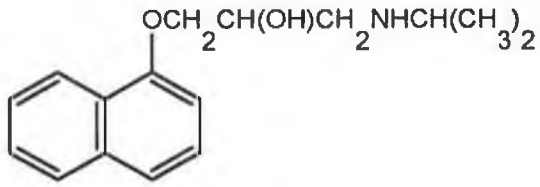
described in chapter four and will be described as Polymer A (prepared from methacrylic acid) and Polymer B (prepared from acryloyl alanine). The test compounds included (±) propranolol, (±) norephedrine and (±) phenylethylamine, the structures of which are depicted in Figure 6.3.

6.2 EXPERIMENTAL

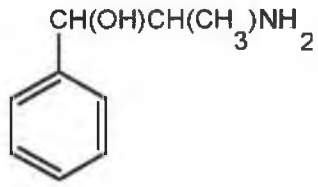
6.2.1 *Reagents and solvents*

A number of drug components were used in this study, which included (±) propranolol, (±) norephedrine and (±) phenylethylamine, and these were obtained from Sigma (St. Louis, MO, USA). HPLC grade methanol and acetonitrile were obtained from Labscan Analytical Sciences (Dublin, Ireland). Analar grade sodium hydroxide and phosphoric acid were supplied by BDH (Poole, UK). Analytical grade dipotassium hydrogen phosphate and potassium dihydrogen phosphate were used as buffer salts and were obtained from Merck (Darmstadt, Germany). Phosphate buffers of pH 3.0 - 4.5 were prepared by mixing appropriate concentrations of phosphoric acid and sodium dihydrogen phosphate solutions. Phosphate buffers of pH 5.0 - 8.0 were prepared by mixing appropriate concentrations of sodium dihydrogen phosphate and disodium hydrogen phosphate solutions.

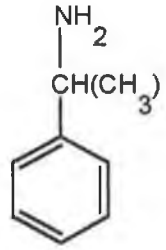
Sodium dodecyl sulphate (SDS) was purchased from Analar ® Biochemical. Standards were prepared from a 1 mg/ml stock solution in methanol and prepared in deionized water. The electrolyte solution used was 0.005 M phosphate buffer (pH 7.0), which was prepared daily. The solutions were filtered through a 0.45 μm filter and the polymer was added directly to these solutions without further filtration. The polymers were prepared to *S*-propranolol as described in sections 4.3.8 and 4.3.9. All water was purified using a Milli-Q-system.



propranolol



norephedrine



phenylethylamine

Figure 6.3: ***Structures of test compounds: propranolol, norephedrine and phenylethylamine.***

6.2.2 *Apparatus*

Electrophoretic experiments were carried out on a P/ACE 5500 system (Beckman, High Wycombe, UK), thermostatted at 25 °C. The fused-silica capillary tube had an internal diameter of 100 μm, a total length of 47 cm and a length of 40 cm from inlet to detector. A voltage of 15 kV was used for the separation, and the resulting electropherogram was monitored at 210 nm. The samples were loaded by a 3-s pressure injection and the observed current varied between 30 and 100 μA. The capillary was prepared by rinsing with 0.1 M sodium hydroxide for 1 min followed by buffer solution for 1.2 min. Each buffer was allowed to equilibrate on the capillary for 20 min prior to use. Electropherograms were recorded using Beckman System Gold software.

6.2.3 *Calculations*

The resolution for the system without micelles was determined from Equation 6.1:

$$R_s = 2(x_2 - x_1)/(w_1 + w_2) \qquad 6.1$$

where x_1 and x_2 are the migration times of components 1 and 2, and w_1 and w_2 are the peak widths of components 1 and 2.

If SDS is employed as the surfactant, the electrophoretic migration of the anionic micelle is in the direction of the anode. As a result, the overall migration velocity is slower compared to the bulk flow of solvent. Since analytes can partition into and out of the micelle, when an analyte is associated with a micelle its overall migration velocity is retarded. When an uncharged analyte resides in the bulk phase, its migration velocity is that of the EOF. Therefore analytes which have a greater affinity for the micelle have slower

migration velocities compared to analytes which spend most of their time in the bulk phase; however, in situations where a micellar buffer[2] is used, the above equation changes to:

$$R_s=N^{0.5}/4\ (\alpha-1/\alpha)\ (k'_2/1+k'_2)\ [(1-t_o/t_{mc})/(1+t_o/t_{mc}k'_1)] \qquad 6.2$$

$\alpha = k_2'/k_1'$, N = plate number, t_o = migration time of neutral component and t_{mc} = migration time of the micelle.

Capacity factors[2,27] were calculated from

$$k' = t_r-t_o/[t_o(1-t_r/t_{mc})] \qquad 6.3$$

6.3 RESULTS AND DISCUSSION

All separation parameters were calculated for both enantiomers. All samples were dissolved in water and the sample concentration used for the analysis was 50 μg/ml, this was to ensure that adequate peak heights could be obtained. Methanol was used as the neutral marker for measurement of electroosmotic flow (EOF), and quinine hydrochloride was used for measurement of the micelle flow. In order to bring about chiral separation, a difference in electrophoretic mobility between the complexed and the free enantiomers is necessary. To see if this difference was present under the various experimental conditions in this study, the migration times (t_m) of each analyte were compared while varying a number of parameters, including pH, ionic strength, polymer type, concentration of polymer, and addition of SDS.

According to Rawjee and Vigh,[28] when performing chiral analysis of enantiomeric drugs containing one asymmetric centre and having only one charged functional group in the molecule (acidic or basic), the separation buffer pH and the chiral selector concentration are the two most important parameters defining chiral selectivity. Using R, S- propranolol, R, S-

norephedrine and R, S -phenylethylamine as test compounds; the initial buffer conditions were selected by varying pH and ionic strength; once established, the effects of chiral selector and chiral selector concentration were then investigated in terms of resolution of the enantiomers.

6.3.1 *Effect of pH*

The initial step involved the selection of a buffer which would allow the elution of the sample components within a short period of time, and give rise to suitable peak shape. The buffer found suitable for this was 0.005 M phosphate buffer, pH 7.0. The average migration times from duplicate injections of each compound are shown in Table 6.1. From this data, a pH of 7.0 was selected as it gave reasonable migration times with the best corresponding peak shape. Changes in migration time were observed for all compounds on changing pH. High values were obtained at low pH for all compounds and low values were obtained at high pH. This was due to the reduction in electroosmotic flow which is normally observed at low pH. Therefore as the pH increases, the EOF increases and this results in shorter retention times.

Table 6.1: ***Effect of pH on average migration times of the test compounds with a background electrolyte of 0.005 M, phosphate buffer; voltage 15 kV; temperature 25 °C.***

pH	R,S-propranolol	R,S-norephedrine	R,S-phenylethylamine
3.0	6.23	5.37	4.71
4.0	3.66	3.28	3.01
5.0	2.71	2.60	2.44
6.0	2.71	2.75	2.61
7.0	2.28	2.16	2.06
8.0	2.27	2.21	2.03

6.3.2 *Effect of ionic strength of phosphate buffer*

It was observed that the changes in ionic strength affected the migration times; increases in ionic strength resulted in increases in migration times. However, at these higher concentrations, the electropherograms obtained were of poor baseline shape and the peak shapes were ill-defined. The currents produced were also extremely high, and would be unsuitable for use, as further additions of additives to the background electrolyte (BGE) would result in further increases in the current.

Table 6.2: ***Effect of ionic strength on migration values of R,S-propranolol, R,S- norephedrine and R,S- phenylethylamine in the background electrolyte; phosphate buffer pH 7.0; voltage 15 kV; temperature 25 ℃.***

Ionic Strength	R,S-propranolol	R,S-norephedrine	R,S-phenylethylamine
0.005 M	2.28	2.16	2.06
0.010 M	2.46	2.33	2.22
0.020 M	2.61	2.48	2.61
0.050 M	2.91	2.85	2.74

6.3.3 *Effect of variation in the concentration of MIP*

The effect of concentration of chiral selector on enantiomeric resolution was investigated next, maintaining the pH of the running buffer at the previously defined value of pH 7.0 and the ionic strength at 0.005 M; all other separation parameters were kept constant as described above. Two polymers were examined; Polymer A was prepared from methacrylic acid and Polymer

B was prepared from the monomer (acryloylalanine). The migration times were observed to increase as the amount of polymer added increased.

The MIP interacts with the analytes in a number of ways, including formation of hydrogen bonds and ionic interactions. Hence, by interacting with the MIP, the analyte moves more slowly than the free analyte because of its increased mass-to-charge ratio. It is also possible that the analyte has sites for specific hydrogen bonding interactions with the -OH group of the polymer, as well as Van der Waals interactions between the methyl groups on the polymer and hydrophobic groups of the analyte. By varying the types of functional groups used as side chains in the polymer, various types of analyte interactions with the MIPs can be studied. Tables 6.3.a and 6.3.b present the migration times for the enantiomers of the three compounds and the calculated R_s values for Polymer A and B, respectively. Figure 6.4 shows the relationship between the resolution of the compound wrt the % polymer in the CE separation using Polymer A, while Figure 6.5 shows the same plot for Polymer B, with the optimum % for Polymer B being found to be 0.05 %. Although some selectivity is observed for Polymer A at 0.03 %, the resolution value obtained is only 0.330, which in terms of resolution values is quite low. In contrast, for polymer B the resolution obtained at 0.05 % is 1.48, which is acceptable for separation of two components (Figure 6.6). The modified polymer proved to be a more powerful chiral selector than the polymer prepared from methacrylic acid for the components studied. This is possibly due to the extra side chain on the polymer backbone which incorporates a chiral centre. The addition of this group probably allows a more favourable interaction with the template enantiomer over its antipode. Further increases in polymer concentration resulted in decreases in the migration times, and the solubility of the polymer at higher concentrations also decreased.

Table 6.3.a: *Migration times and resolution values of the R- and S-enantiomers of propranolol, norephedrine and phenylethylamine using Polymer A as a chiral additive in the background electrolyte; 0.005 M, phosphate buffer, pH 7.0; voltage 15 kV; temperature 25 ℃.*

% polymer A	propranolol			norephedrine			phenylethylamine		
	R-	S-	R_s	R-	S-	R_s	R-	S-	R_s
0.00	2.287	2.287	0.000	2.162	2.162	0.000	2.063	2.063	0.000
0.01	2.470	2.469	0.010	2.456	2.434	0.023	2.113	2.118	0.043
0.02	2.254	2.253	0.010	2.145	2.121	0.025	1.841	1.849	0.045
0.03	3.136	3.162	0.330	2.941	2.945	0.051	2.706	2.721	0.054
0.05	2.519	2.504	0.088	2.670	2.690	0.130	2.065	2.088	0.065
0.06	2.259	2.251	0.070	2.251	2.235	0.100	2.047	2.049	0.010

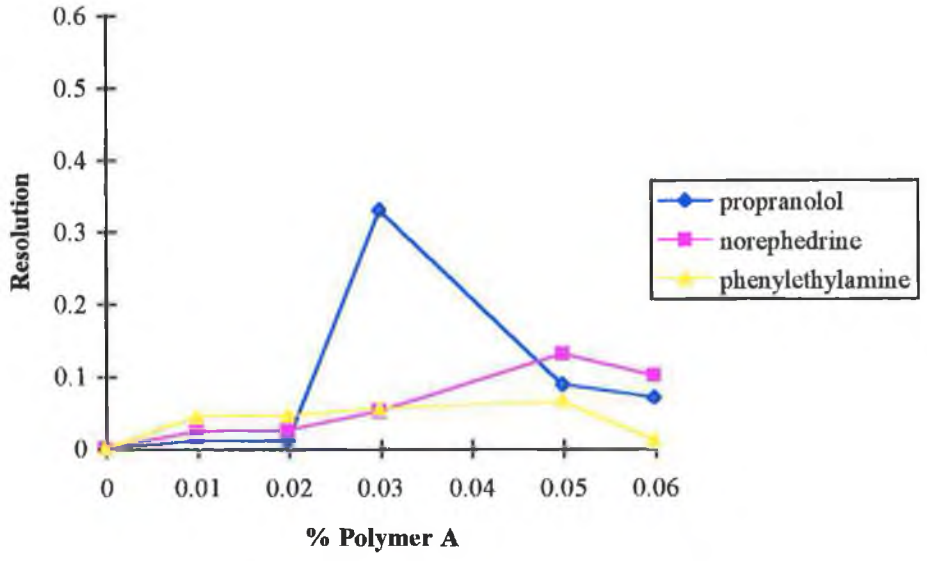


Figure 6.4: *Effect of addition of increasing concentrations of polymer A to the running buffer; Conditions: 0.005 M phosphate buffer, pH 7.0; temperature 25 ℃; voltage 15 kV.*

Table 6.3.b: *Migration times and resolution values of R- and S-enantiomers of propranolol, norephedrine and phenylethylamine using Polymer B as a chiral additive in the background electrolyte; 0.005 M, phosphate buffer, pH 7.0; voltage 15 kV; temperature 25 ^{0}C.*

% Polymer B	propranolol			norephedrine			phenylethylamine		
	R-	S-	R_s	R-	S-	R_s	R-	S-	R_s
0.00	2.287	2.287	0.000	2.162	2.162	0.000	2.063	2.063	0.000
0.01	2.298	2.304	0.046	2.280	2.220	0.240	1.830	1.850	0.210
0.02	2.390	2.470	0.029	2.320	2.490	0.640	1.636	1.648	0.110
0.03	3.850	3.891	0.020	3.052	2.925	0.300	2.137	2.149	0.070
0.04	2.143	2.208	0.030	2.084	2.091	0.080	1.984	2.027	0.660
0.05	2.453	3.039	1.480	2.341	2.376	0.200	2.217	2.229	0.080
0.06	2.437	2.418	0.120	2.263	2.271	0.060	2.286	2.299	0.010

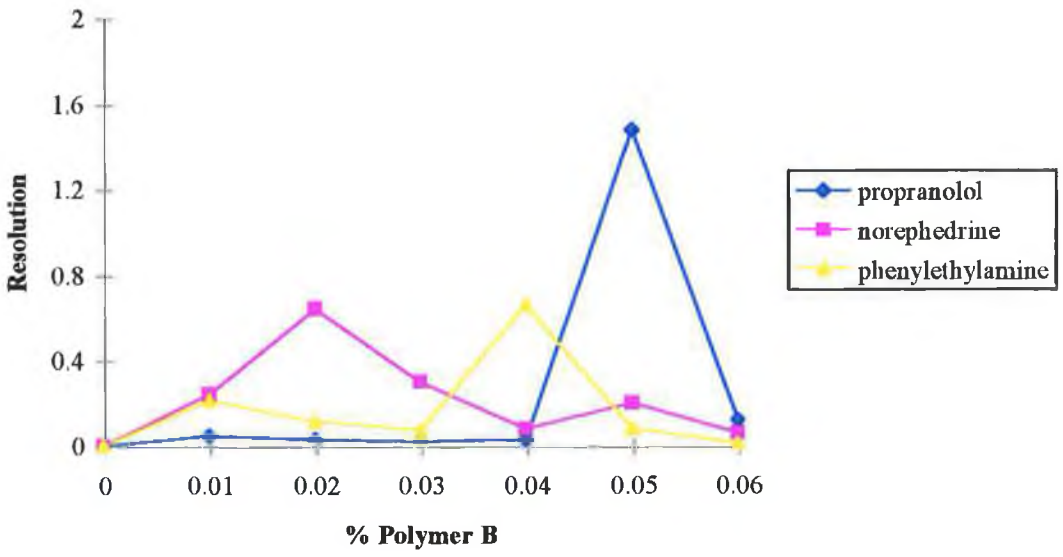


Figure 6.5: *Effect of addition of increasing concentration of polymer B to the running buffer; Conditions: 0.005 M phosphate buffer, pH 7.0; temperature 25 ℃, voltage 15 kV.*

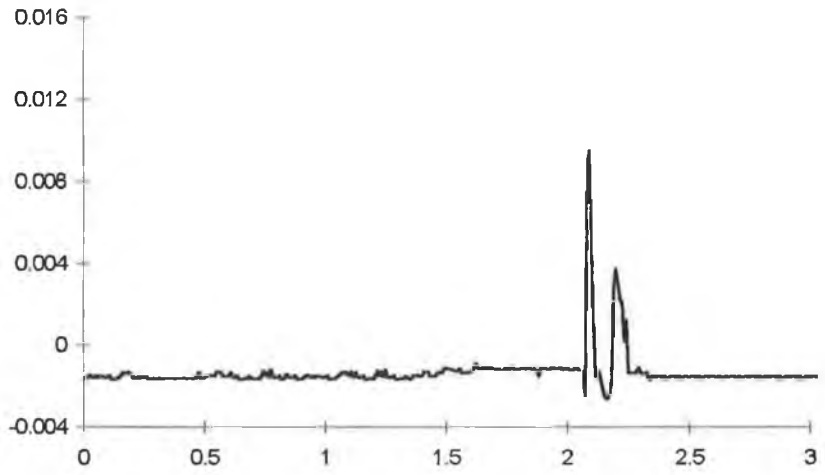


Figure 6.6: ***Separation of R-, S- propranolol using Polymer B as a chiral additive in the background electrolyte. Conditions: 0.005 M, phosphate buffer, pH 7.0; voltage 15 kV; temperature 25 ℃.***

6.3.4 *Effect of pH on the resolution of the test analytes*

For ionizable compounds, the most straight-forward way to affect mobility of the free enantiomer is by variation of the pH of the electrolyte solution. Another possibility would be to increase the concentration of the electrolyte solution (ionic strength) which affects the effective electrophoretic mobility of charged molecules. The effect of pH on the resolution and migration values was examined (Tables 6.4.a and 6.4.b for Polymers A and B respectively). All other variables were maintained at constant values (0.005 M phosphate buffer, Polymer A 0.03 % and Polymer B 0.05 %). The pH range examined was 5.0 - 8.0 since below values of 5.0 the migration values became very long and the peaks obtained were extremely broad. As seen in Figures 6.7 and 6.8, the resolution shows a maximum at pH 7.0 for propranolol (R_s = 0.33) for polymer A and (R_s = 1.48) for Polymer B.

Table 6.4.a: *Migration times and resolution values of R- and S-enantiomers of propranolol, norephedrine and phenylethylamine using Polymer A as a chiral additive in the background electrolyte; 0.005 M, phosphate buffer, voltage 15 kV; temperature 25 ℃.*

pH Polymer A	propranolol			norephedrine			phenylethylamine		
	R-	S-	R_s	R-	S-	R_s	R-	S-	R_s
5.0	10.42	10.75	0.20	6.260	6.280	0.01	8.190	8.140	0.13
6.0	2.520	2.640	0.18	2.270	2.280	0.05	2.190	2.200	0.05
7.0	3.136	3.162	0.33	2.941	2.945	0.05	2.706	2.721	0.05
8.0	2.340	2.350	0.03	2.240	2.260	0.07	1.230	1.210	0.08

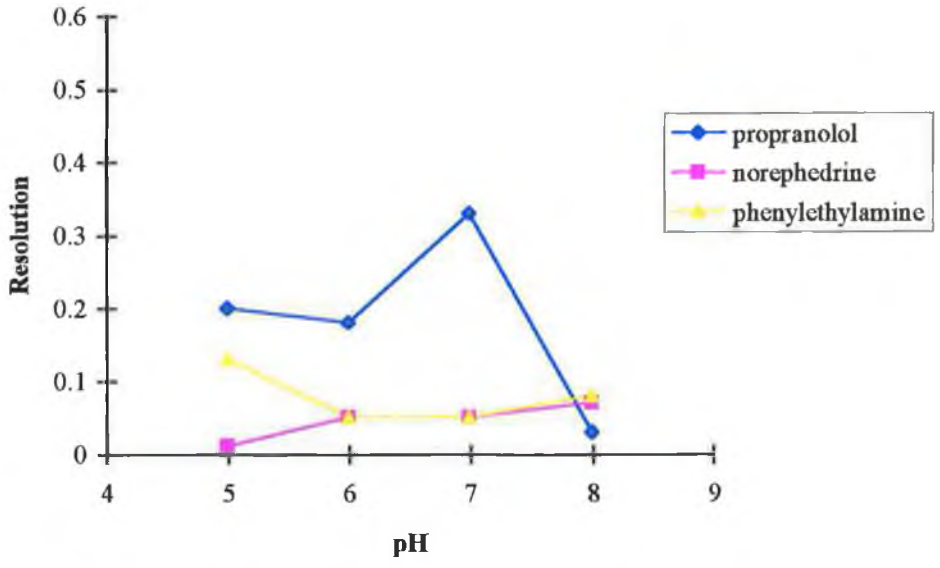


Figure 6.7: *Effect of change in pH of the running buffer on separation of R- and S- enantiomers of test compounds,; conditions: 0.005 M phosphate buffer, 0.03 % MIP A; temperature 25 ℃; voltage 15 kV.*

Table 6.4.b: *Migration values and resolution values of the R- and S-enantiomers of propranolol, norephedrine and phenylethylamine using Polymer B as a chiral additive in the background electrolyte; 0.005 M phosphate buffer, voltage 15 kV; temperature 25 ℃.*

pH / Polymer B	propranolol			norephedrine			phenylethylamine		
	R-	S-	R_s	R-	S-	R_s	R-	S-	R_s
5.0	11.60	11.34	0.16	5.342	5.366	0.09	6.695	7.010	0.34
6.0	7.120	7.220	0.21	4.420	4.450	0.20	5.250	5.260	0.19
7.0	2.455	3.039	1.48	2.341	2.376	0.20	2.217	2.229	0.08
8.0	2.985	2.955	0.17	1.551	1.556	0.01	1.194	1.390	0.02

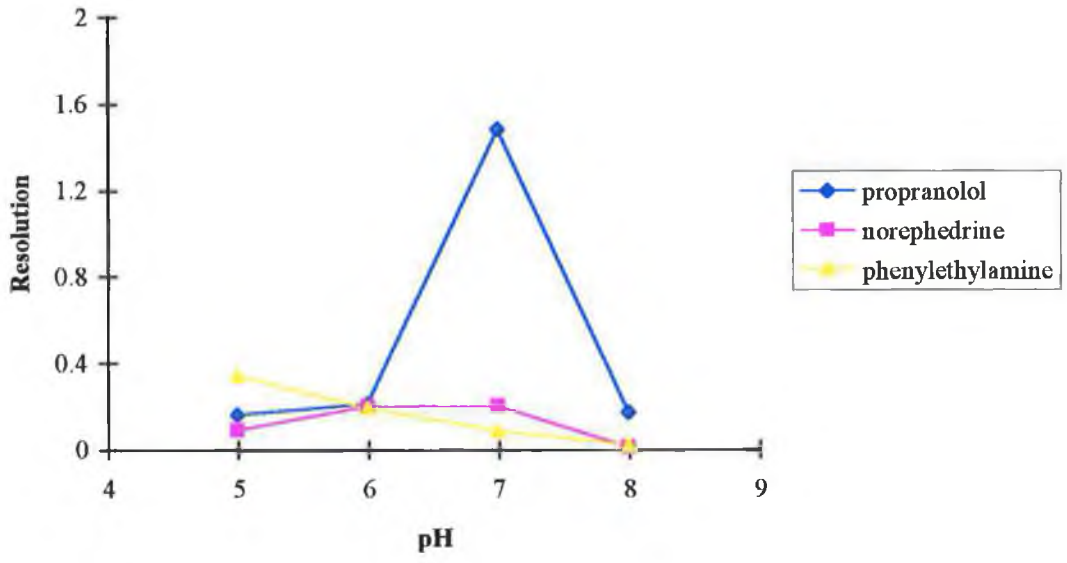


Figure 6.8: *Effect of change in pH of the running buffer on the separation of R- and S- enantiomers of the test compounds: conditions: 0.005 M phosphate buffer, 0.05 % MIP B; temperature 25 ℃; voltage 15 kV.*

6.3.5 *Effect of concentration of SDS*

In some separations it is necessary to add further additives to the background electrolyte to enhance the separation. The use of surfactants for this purpose is fairly commonplace, in particular the use of sodium dodecyl sulphate (SDS). This is added up to concentrations where micelle formation will occur and therefore, as such produce a 'pseudo-phase' for the analytes to interact with. The addition of SDS is effective for separating hydrophobic compounds, since the MIP will not interact with the micelle, and the MIP in the micellar solution should behave as another phase in comparison with the micelle. Thus the analyte may be partitioned between three phases: the micelle, the MIP and the aqueous phase. Separation can be achieved in the MIP-MEKC system because the micelle migrates at a different velocity from that of the MIP or the aqueous phase. It is a well known fact that MIPs can effect chiral recognition; thus the MIP-MEKC system can also be applied to optical resolution.

Based on work by Terabe *et al.*[9] they found it necessary to add a surfactant to obtain a separation while using cyclodextrins as their chiral additive. As Polymer A showed little enantioselectivity, it was decided to add a surfactant to the background electrolyte, therefore small amounts of the surfactant sodium dodecyl sulphate (SDS) was added to the buffer solution. This was also added to buffer solutions containing Polymer B. Three concentration levels were examined, i.e. 0.005 M, 0.01 M and 0.02 M. SDS forms micelles at a concentration of greater than 0.005 M SDS and hence exists above its critical micelle concentration (CMC) and acts as a pseudo-stationary phase. As SDS is anionic it can form ion-pairs with the protonated amine groups in the test compounds. Overall increases in SDS concentration resulted in increased migration times for the compounds examined. Additions above the SDS CMC did not show a large effect on the differentiation of migration times between enantiomers (Table 6.5.a and 6.5.b).

Table 6.5.a: *Migration times of the R- and S-enantiomers of propranolol, norephedrine and phenylethylamine using Polymer A (0.03 %) as a chiral additive in the background electrolyte with various concentrations of SDS; 0.005 M phosphate buffer pH 7.0; voltage 15 kV; temperature 25 ℃.*

SDS Polymer A	propranolol		norephedrine		phenylethylamine	
	R-	S-	R-	S-	R-	S-
0.005 M	3.101	3.121	2.540	2.590	2.780	2.760
0.010 M	3.176	3.184	2.843	2.852	2.963	2.968
0.020 M	3.255	3.249	3.143	3.152	3.342	3.350

Table 6.5.b: *Migration times of the R- and S-enantiomers of propranolol, norephedrine and phenylethylamine using Polymer B (0.05 %) as a chiral additive in the background electrolyte with various concentrations of SDS; 0.005 M, phosphate buffer pH 7.0: voltage 15 kV; temperature 25 ℃.*

SDS Polymer B	propranolol		norephedrine		phenylethylamine	
	R-	S-	R-	S-	R-	S-
0.005 M	2.790	2.800	2.145	2.150	2.087	2.095
0.010 M	3.000	3.050	2.390	2.380	2.470	2.472
0.020 M	3.234	3.246	2.573	2.585	2.843	2.856

6.3.6 *Effect of ionic strength*

Using a background electrolyte of phosphate buffer, pH 7.0 and 0.05 % Polymer B, the effect of ionic strength on the resolution and migration times of the enantiomers was examined. Increases in the ionic strength resulted in decreases in the migration times and for propranolol decreases in resolution (Figure 6.9).

Table 6.6: ***Migration values and resolution values of the R- and S-enantiomers of propranolol, norephedrine and phenylethylamine using polymer B as a chiral additive in the background electrolyte; 0.005 M, phosphate buffer pH 7.0, voltage 15 kV; temperature 25 ℃.***

Ionic Strength B	**propranolol**			**norephedrine**			**phenylethylamine**		
	R-	**S-**	**R_s**	**R-**	**S-**	**R_s**	**R-**	**S-**	**R_s**
0.005 M	3.392	2.980	1.50	2.304	2.315	0.060	2.322	2.325	0.300
0.010 M	2.470	2.469	0.09	2.456	2.434	0.098	2.118	2.113	0.150
0.020 M	2.254	2.253	0.01	2.145	2.131	0.145	1.841	1.842	0.002

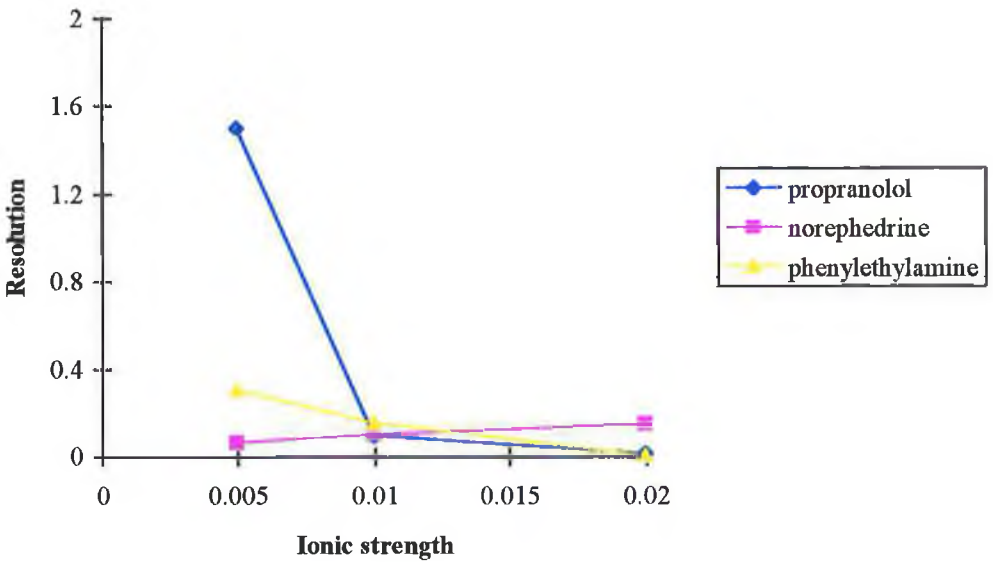


Figure 6.9: ***Effect of changes in the ionic strength of the running buffer; conditions: phosphate buffer, pH 7.0, 0.05 % Polymer B; temperature 25 °C; voltage 15 kV.***

6.3.7 *Effect of applied voltage on resolution*

Examination of the effect of the applied electric field on the chiral resolving power was studied next. The BGE used was 0.005 M, phosphate buffer, pH 7.0, 0.05 % Polymer B. Table 6.7 gives the results of the migration times and resolution values at a number of voltages. The maximum value selected was 15 kV, as above this value current problems occurred, probably due to the excessive Joule heat which was possibly not being removed from the capillary.

Table 6.7: ***Migration times and resolution values of the R- and S-enantiomers of propranolol, norephedrine and phenylethylamine using Polymer B as a chiral additive in the background electrolyte; 0.005 M phosphate buffer pH 7.0 , temperature 25 °C.***

voltage	propranolol			norephedrine			phenylethylamine		
	R-	S-	R_s	R-	S-	R_s	R-	S-	R_s
5.0 kV	16.32	16.54	0.12	15.87	15.92	0.21	18.11	18.21	0.17
10.0 kV	11.54	11.67	0.02	10.21	10.48	0.20	12.67	12.71	0.01
15.0 kV	3.392	2.980	1.50	2.304	2.315	0.06	2.322	2.325	0.30

6.3.8 *Effect of temperature on resolution*

The final parameter examined was the effect of temperature using the same conditions as above. At elevated temperature it was necessary to take into account the running buffer conductivity increase resulting in higher current, caused by the elevated temperature, thus increasing the Joule heat being developed. It was found that 25 °C was the most suitable temperature for this separation. Decreasing the temperature would result in significant increases in migration times.

Table 6.8: ***Migration times and resolution values of the R- and S-enantiomers of propranolol, norephedrine and phenylethylamine using Polymer B as a chiral additive in the background electrolyte; 0.005 M, phosphate buffer pH 7.0; voltage 15 kV.***

Temp.	propranolol			norephedrine			phenylethylamine		
	R	S-	R_s	R-	S-	R_s	R-	S-	R_s
25 ^{0}C	3.39	2.98	1.50	2.30	2.32	0.06	2.32	2.33	0.300
30 ^{0}C	5.23	5.24	0.005	4.87	5.01	0.088	4.92	5.02	0.040
35 ^{0}C	6.64	7.14	0.48	5.03	5.07	0.041	5.71	5.74	0.039

6.3.9 *Organic Modifier*

There was an attempted addition of acetonitrile to the background electrolyte. However, on allowing the BGE to stand for 1 hour the solutions became very cloudy, and hence were unsuitable for use. To date it has been used in a number of methods, and levels of up to 10 % organic modifier have been used.

6.3.10 *Blank Polymer*

Solutions of blank polymer (prepared without a template) were then tested and found unsuitable, as they were extremely insoluble in BGE and hence resulted in large background currents. O' Shannessy *et al.*[29] found that a polymer prepared without a template molecule resulted in the imprint molecule eluting at the same time as the non-interacting void marker, indicating that the presence of the print molecule in the pre-polymerization mixture is essential for subsequent recognition.

6.4 CONCLUSION

This work describes the use of a MIP prepared to *S*-propranolol to optimize enantiomeric separations in CE. Other parameters which were varied included pH, concentration of phosphate buffer, concentration of SDS, field strength and temperature. It was found that a running buffer which gave a resolution of 1.48 for the separation of propranolol was phosphate buffer 0.005 M, pH 7.0, MIP 0.05 %. This type of system has an advantage over HPLC systems in that only small amounts of chiral agents are required and the solvent requirements (if necessary) are typically of the order of 20 ml per day. MEKC can be more easily utilized to achieve optical resolution especially in terms of the preparation of columns and solutions and therefore the use of MEKC is expected to increase.

One disadvantage of using polymer particles is the lack of solubility in mainly aqueous background electrolytes, however, further modifications of the side chains of the monomer/polymer could increase its solubility. Also variations in the monomer/cross-linker ratio; and decreases in the amount of cross-linker would increase solubility. Indeed the use of cross-linkers other than EDMA could improve selectivity. In many cases, the opportunity for an increased number of interactions between the analytes and MIP moieties ultimately improves enantiomeric separation. Resolution of enantiomers is dependent on both the strength and selectivity of the interaction between the MIP and the solute enantiomers. Also the possibility of using other surfactants could be examined to determine if the resolution values could be expanded.

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CHAPTER SEVEN

CONCLUSIONS AND FUTURE WORK

7.1 CONCLUSIONS

In chapter two, a mixed-mode column was examined to determine its retention characteristics. Prior to the advent of prepared mixed-mode columns, secondary interaction mechanisms were deemed to interfere with the retention mechanisms (particularly silanol groups on C_{18} and their interaction with amine functionalities). However, it has been discovered that by mixing at least two phases, both the interactions may be manipulated in the determination of a sample containing a number of components. The retention parameters of a C_{18}/SCX column were examined and it was found that the SCX material was responsible for the interaction of the strongly basic components and hence retention could be modulated by increasing the ionic strength of the buffer or indeed by changes in the buffer cation. Therefore problems with long retention times that normally occur on C_{18} materials can be overcome by using a mixed-mode column where the choice of buffer can decrease these retention times.

Certain mixtures cannot be analyzed by either the same column, same buffer conditions, or by gradient elution. However, the use of mixed-mode materials allows mixtures to be analyzed simultaneously. This mixed-mode column is then used for the determination of an acidic component and a strongly basic component. Under normal C_{18} conditions, the acidic component would elute shortly after the void volume while the strongly basic component would be retained, resulting in extremely long retention times. However, the use of this phase allows their simultaneous determination, and the assay was validated for extraction of the components from plasma by liquid/liquid extraction and column switching. As C_{18}/SCX is capable of separating both basic and neutral/acidic compounds simultaneously, it has application in the determination of drugs of abuse. A further application would be in the determination of a pharmaceutical product along with its degradation/process impurities, which collectively may not

be suitable for analysis using either of the phases separately. Future work in this area could involve the mixing of another phase into the column or possibly the use of materials other than C_{18}, i.e. phenyl, C_8 and CN.

The next section involved the preparation of a polymer by a technique known as molecular imprinting to 7-hydroxycoumarin. A number of polymers were prepared by varying the amount of solvent used in the polymerization reaction, and it was found that decreasing the amount of solvent increased the selectivity of the polymer. This was investigated using UV spectroscopic studies of the polymers following equilibration with the target compound. Further work involved the examination of the polymer selected for selectivity of 7-OHC over other members of the coumarin family. The equilibration time and the load capacity of the polymer were also examined. The use of UV spectral studies allowed the polymers to be examined quickly prior to their use as solid-phase extraction materials ensuring that the most suitable polymer was used in the next stage.

Also examined were the use of chiral monomers/polymers as molecularly imprinted polymers. Although there are a number of monomers available, methacrylic acid is the most commonly used. It was decided to try and incorporate a chiral centre on the side chain. This was achieved by reacting acryloyl chloride with alanine. This monomer was then polymerized by a standard method using a molecular imprinting procedure using *S*-propranolol as the template molecule. A polymer was then prepared with methacrylic acid, again using *S*-propranolol. These polymers were then used as chiral additives in CE. Future work in the area of monomer/polymer preparation could involve development of improved simple methods for the preparation of chiral monomers/polymers on a large scale from low cost starting materials. Also, examination of other cross-linking agent monomers to prepare new copolymers to

try and increase selectivity even further could be attempted. In addition, the polymers could be prepared by photo initiation instead of the thermal method.

An application for these prepared MIPs was their use as solid-phase extraction materials. An assay was developed for the extraction of 7-OHC from urine and this was validated in the range 10 - 50 μg/ml. It was found to be a cost effective and easy way to prepare SPE cartridges for a specific component. These would find particular use where it is necessary to remove a specific component from its metabolites or from compounds with similar structures. It is also necessary to examine their use for extraction from plasma and other biological fluids.

Finally, quite a number of MIPs have been prepared for use in enantiomeric separation, and quite good resolutions have been obtained. The use of the polymer particles prepared previously were examined to determine if they were suitable for use as chiral additives. This was determined by adding small amounts of the polymers to the buffer used in the determination of the enantiomers by CZE. The second polymer (Polymer B) with its added chiral centre was found to exhibit better enantioselectivity than its methacrylic acid counterpart. It would seem that this polymer offered further points of interaction and a change of size in the cavity. Future work in this area would include preparation of polymers with different monomers, cross-linkers and copolymers of these. These can then be used for a series of test compounds and the points of interaction examined to determine which functionalities react best with the different monomers. The preparation of polymers with increased solubility should further increase their use in both CZE and HPLC as chiral additives.

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