DUBLIN CITY UNIVERSITY

# Evaluation of drug-free plasma profiles by high-performance liquid chromatography following liquid-liquid extraction.

by

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November 1991

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**ABSTRACT**

The effect of varying the percentage of organic modifier and the pH of the aqueous component in the eluent was investigated on drug-free plasma profiles using reversed-phase HPLC. Two different organic modifiers were used (methanol and acetonitrile) on two columns with differing selectivities (C - 18 and CN). Thus three variables were involved at any time - the column type, the organic modifier and either the percentage organic modifier or the pH of the eluent. The samples were prepared by liquid-liquid extraction into a diethylether solvent. The resultant chromatograms were assessed in terms of the number of interfering peaks present above a certain value of peak height. Analysis of the results showed that the number of peaks depended strongly on the percentage of organic modifier and was generally inversely proportional to the pH. The effects were also significantly dependant on the type of column and organic modifier used. An additional 'salting out' procedure revealed that for particular salts, the number of interfering peaks was reduced.

## ACKNOWLEDGEMENTS

I would sincerely like to thank:
Dr. M. Kelly,
Peig, Teresa, Veronica and Damien
Kamal Sagar,
My Family, especially Christina
and above all, Jean Cahill and Cliona whose typing skills will never be forgotten.

## TABLE OF CONTENTS

To Declan, for being a great distraction when times were tough:

I, Ciara Mullan truthfully declare that the following work has been all my own. It has not or never will be submitted to any other institution or establishment.

Ciara Mullan

Ciara Mullan B.Sc.

# CHAPTER I

# THEORY OF CHROMATOGRAPHY

Chromatography is a physical method of separation in which the compounds to be separated are distributed between a mobile phase and a stationary phase. A sample introduced into a mobile phase is carried along through a column containing a distributed stationary phase. Species in the sample undergo repeated interactions (partitions) between the mobile phase and the stationary phase. If both phases are suitably chosen, the sample components are gradually separated into bands in the mobile phase. At the end of the process, separated components emerge in order of increasing interaction with the stationary phase whereby the least retarded component emerges first and the most strongly retarded component elutes last. Partition between the phases exploits differences in the physical and/or chemical properties of the components in the sample. Adjacent components (peaks) are separated when the later emerging peak is retarded sufficiently to avoid overlapping with the peak that emerges ahead of it.

The history of chromatography dates back to 1903 when the Russian botanist Tswett produced coloured bands by separating concentrated plant extract on a column of adsorbent material. This process he called chromatography. In the 1930's Kuhn and Lederer, Reichstein and Van Euw separated natural products using column chromatography. In 1941 Martin and Synge introduced liquid-liquid partition chromatography and also came up with the underlying principles of gas-liquid chromatography. They subsequently received the Nobel Prize for their work. The concept of the theoretical plate as a measure of chromatographic efficiency was another of their claims to fame.

In the 1950's Kirchner introduced the concept of thin layer chromatography which was made popular by Stahl at a later date. It wasn't until 1952 that the practical application of Gas Chromatography was first realised by James and Martin and for the next few decades the technique quickly developed into a far more sophisticated technique than liquid chromatography. Then, in the 1960's Giddings proved that the theory behind gas chromatography could be applied to liquid chromatography. From 1967 to 1969 Horvath, Lipsky, Preiss, Huber and Kirkland developed the practical application of liquid chromatography through the early stages. They introduced the concept of high pressure

systems (up to 5000 psi) to achieve rapid separation with better efficiency which is the basis for high performance liquid chromatography.

Improvements in the rates of mass transfer by choice of support materials was the next advance in HPLC technology. This was achieved by shortening the diffusion paths over which the solutes had to diffuse. Two approaches were investigated; either by creating impervious glass beads with the stationary phase, or more commonly, by producing particles which were totally porous but of smaller diameter, (<40$\mu$m).

The aforementioned solid liquid adsorption chromatography has been largely superceded by bonded phase chromatography which uses surface modified silica based stationary phases. Halasz et al. produced the first bonded phases by reacting silica with alcohols and amines. Since then, materials of greater hydrolytic stability have been manufactured by reacting silica with the alkylsilanes. Today, a wide range of bonded supports is available to suit a variety of analytical needs. HPLC technology is still advancing. Some of the most recent developments are: column switching which conveniently ensures automated clean-up of the sample in liquid chromatography, highspeed liquid chromatography provides a rapid throughput and "box car" liquid chromatography.

## 1.1 THEORY OF CHROMATOGRAPHY

The chromatographic behaviour of a solute can be described in numerous ways for column chromatography, the retention volume, $V_r$ (or corresponding retention time, $t_r$), and the partition ratio (or capacity factor), k', are the terms most frequently used. The retention volume is equal to the volume of mobile phase required to move a solute from one end of the column to the other. By varying the stationary - mobile phase combinations and various operating parameters, the degree of retention can be varied from nearly total retention to a state of free migration.

Retention behaviour indicates the distribution of a solute between the mobile and stationary phases. The retention volume, $V_r$, may be evaluated directly from the

corresponding retention time, $T_r$, by the equation.

$$V_r = F \times t_r \qquad 1.1.1$$

where F is the flow rate which is assumed to be constant. The average linear velocity, U, of the mobile phase,

$$U = \frac{L}{tm} \qquad 1.1.2$$

is measured by the migration of a nonretained solute, tm and L is the column length.

No material whatsoever can elute prior to tm. When converted to volume, Vm (or Vo) it is know as dead volume or dead space. It includes the effective volume contributions of the sample injector, any connecting tubing, the column itself and the detector, assuming negligible extra-column effects. The adjusted retention time, tr', or volume Vr' is given by

$$tr' = tr - tm \quad \text{or} \quad Vr' = Vr - Vm$$

When a solute enters a chromatographic system, it immediately distributes between the stationary and mobile phases. If the mobile phase flow is stopped at any time, the solute assumes an equilibrium distribution between the two phases. The concentration in each phase is given by the thermodynamic partition coefficient.

$$K = \frac{Cs}{Cm} \qquad 1.1.3$$

where Cs and Cm are the concentrations of solute in the stationary and mobile phases, respectively. When K = 1, the solute is equally distributed between the two phases. The partition coefficient determines the average velocity of each solute zone or more

specifically, the zone centre as the mobile phase moves down the column.

When the peak maximum of a symmetrical peak appears at the column exit, half of the solute has eluted in the retention volume, Vr, and half remains distributed between the volume of the mobile phase, Vm, and the volume of the stationary phase Vs, thus

$$VrCm = VmCm + VsCs \qquad 1.1.4$$

Combining the above equation with equation 1.1.3 and rearranging gives a fundamental equation in chromatography.

$$Vr - Vm = K\,Vs \qquad 1.1.5$$

This relates column dead volume and the product of the partition coefficient and the volume of the stationary phase to the retention volume of a solute.

The partition ratio or capacity factor k', relates the equilibrium distribution of the sample within the column to the thermodynamic properties of the column and to the temperature and is thus a very important consideration in column chromatography. In other words k' is a measure of the time spent in the stationary phase relative to the time spent in the mobile phase for a given set of operating parameters. Numerically it is defined as the ratio of the moles of a solute in the stationary phase to the moles in the mobile phase.

$$k' = \frac{ns}{nm} = \frac{CsVs}{CmVm} \qquad 1.1.6$$

$$k' = K\,\frac{Vs}{Vm} \qquad 1.1.7$$

Alternatively k' can be stated as the additional time a solute band takes to elute, as compared with an unretained solute (for which k' = 0), divided by the elution time of an unretained band:

$$k' = \frac{tr - tm}{tm} = \frac{Vr - Vm}{Vm} \qquad 1.1.8$$

This equation states how many dead volumes (or tm) are required to attain Vr (or tr).

A useful measure of retention is R where R = the fraction of solute molecules in the mobile phase.

$$R = \frac{nM}{nM + nS} = \frac{1}{1 + k'} \qquad \text{(dividing by nM)} \quad 1.1.9$$

The fraction of solute molecules in the stationary phase is given by

$$\frac{nS}{nM + nS} = \frac{k'}{1 + k'} \qquad 1.1.10$$

The relative retention, $\alpha$, of two solutes where solute 1 elutes before solute 2 is given by

$$\alpha = \frac{k_2'}{k_1'} = \frac{k_2}{k_1} \qquad 1.1.11$$

$$\alpha = \frac{Vr'_2}{Vr'_1} = \frac{tr'_2}{tr'_1} \qquad 1.1.12$$

## 1. (2) COLUMN EFFICIENCY AND RESOLUTION

K and k' are independent of the total solute concentration when the operating conditions are such that the partition between the stationary phase and the mobile phase is linear. The resultant profile of a solute band approaches a gaussian distribution curve after a number of partitions between the phases. However the solute band tends to broaden as it passes through the column resulting in a decrease at the peak maximum. Because of this broadening the resolution of adjacent solute bands is affected and this in turn affects chromatographic efficiency.

The efficiency of a chromatographic system is expressed as a dimensionless quantity known as the effective plate number, Neff.

$$\text{Neff} = 16 \left(\frac{tr'}{Wb}\right)^2 \qquad 1.2.1.$$

where Wb = width at the base of the peak

It reflects the number of times the solute partitions between the two phases during its passage through the column. Often it is easier to measure the width at half the peak height.

$$\text{Neff} = 5.54 \left(\frac{tr'}{W_{1/2}}\right)^2 \qquad 1.2.2.$$

where $W_{1/2}$ = width at half peak height.

Measurement of the peak width at half the peak height is less sensitive to peak asymmetry since tailing often shows up below the measurement location.

Plate number is an indication of how well a column has been packed. However it cannot adequately predict column performance under all conditions. It is used mainly as a

FIGURE 1.

Peak asymmetry factor: (a) symmetrical band and (b) band tailing present.

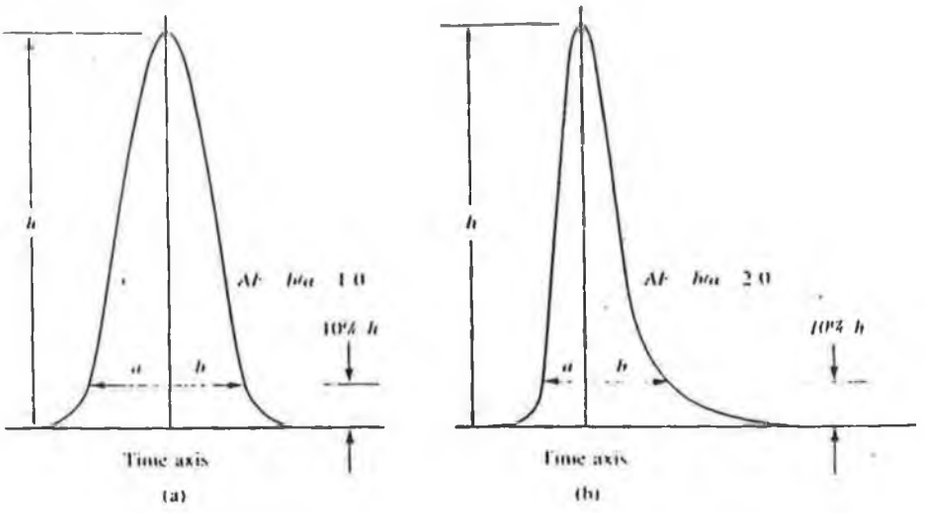
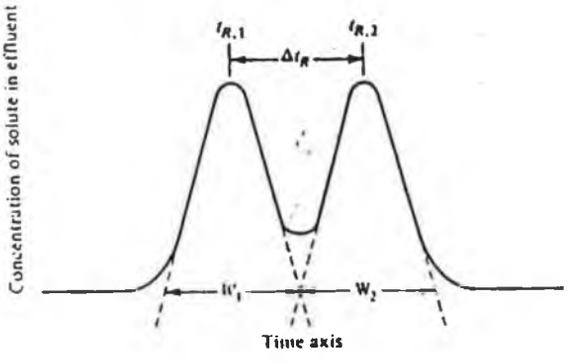



FIGURE 2

Definition of resolution.



measure of the kinetic contributions to band broadening and other contributions such as extra-column effects and thermodynamic factors (e.g. peak tailing) should also be considered.

Plate height, H, is the distance a solute moves while undergoing one partition:

$$H = \frac{L}{Neff} \qquad 1.2.3.$$

H can be useful for expressing column efficiency in units of length without specifying the length of the column. For an efficient column H has a small value.

**ASYMMETRY - TAILING AND FRONTING**

Symmetrical bands are normally observed only for samples that do not exceed some maximum size (usually 1 mg of sample per gram of stationary phase). If k' is higher at lower concentrations of solute the low concentration wing of the eluent peak moves more slowly than the high concentration wing. Thus, an initially symmetric band becomes skewed as it moves through the column and develops a sharp front and a long tail, hence the phenomenon of tailing. The opposite type of asymmetry is known as fronting. The solution in this case is to reduce the amount of sample until all the band shapes are symmetrical.

The peak asymmetry, AF, is given as the ratio of the peak half-widths at a given height - usually a line is drawn parallel to, and 10% distant from the base of the peak, (Figure 1). For example, it is acceptable for AF to be within the range of 0.95 - 1.15 for a peak of k' = 2. Asymmetrical peaks may result from (a) unsuitable combination of sample and column packing, (b) a poorly packed column, (c) extra-column effects such as injection problems. AF provides a quantitative arbitrary value for tailing, but also because visual perception of tailing tends to fail for sharper peaks. Thus it is not uncommon for the early eluting peaks in a chromatograph to "appear" less tailed than

the later peaks, even though the AF values may be nearly the same for both (1).

**RESOLUTION**

The degree of separation or the resolution of two adjacent bands is defined as the distance between band peaks (or centers) divided by the average bandwidth. If retention and bandwidth are measured in units of time, the resolution, Rs, is

$$Rs = \frac{tr_2 - tr_1}{0.5\,(W_1 + W_2)} \qquad 1.2.4$$

This process is depicted in Figure 2.

If inadequate, the resolution of adjacent peaks can be improved either by increasing the separation between the peaks, (which involves column sensitivity) or by narrowing individual peaks (which involves column efficiency).

Equation 1.2.4. defines resolution in a certain situation but fails to relate resolution to the conditions of separation or to suggest how to improve resolution. The following equation explicitly includes the terms involving the thermodynamics and kinetics of the chromatographic system

$$Rs = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'}{1 + k'}\right)\left(\frac{L}{H}\right)^{1/2} \qquad 1.2.5$$

Resolution (in 1.2.5) is a function of three separate factors.

(1) A column selectivity factor that varies with $\alpha$ .
(2) A rate of migration or capacity factor that varies with k'
(3) An efficiency factor that depends on L/H.

Each factor can be calculated from the chromatogram and adjusted more or less independently. The first two factors are essentially thermodynamic where as the efficiency factor is associated with the kinetic features. Thus by changing $\alpha$ and k' (by selecting different stationary and mobile phases or varying temperatures and pressure) and/or L/H, a reasonable resolution such as Rs = 1.5 should be achievable. This in known as baseline resolution.

**BAND BROADENING**

Various processes occur on a column during a chromatographic separation that contribute to band broadening or peak variance, $\sigma^2$. Plate height expresses in simple terms the extent of band broadening and the contributing factors. It is a function of thermodynamic and kinetic processes within the column. The three factors involved are due to:

(1) Flow irregularities that lead to convective mixing.
(2) Transverse and longitudinal diffusion in the mobile phase.
(3) A finite rate of equilibration of solute between the stationary and mobile phases ie; mass transfer.

These factors are incorporated in the van Deemter equation (1):

$$H = A + \frac{B}{u} + C\,(\text{stationary})u + C\,(\text{mobile})u \qquad 1.2.6$$

Equation 1.2.6 and it's components are displayed graphically in Figure 3. Average linear velocity of the mobile phase, $u$, is used because it can be directly related to the speed of analysis whereas the flow rate depends on the column cross section and the column volume occupied by packing material. The A term is due to "eddy diffusion" ie; it results from inhomogeneity of flow velocities and path lengths around packing particles. The B term arises because of the effect of longitudinal or axial, diffusion ie; random molecular motion within the mobile phase. The C (stationary) term results from

FIGURE 3
Typical $H/u$ (van Deemter) curve for a gas chromatographic column.

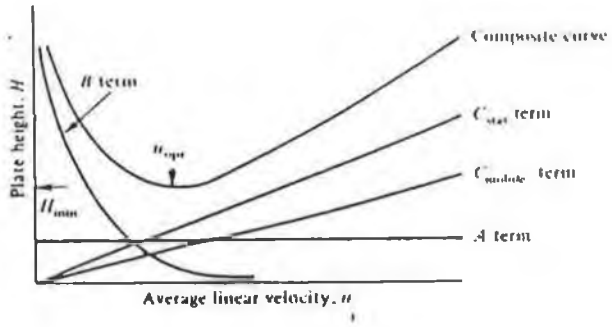
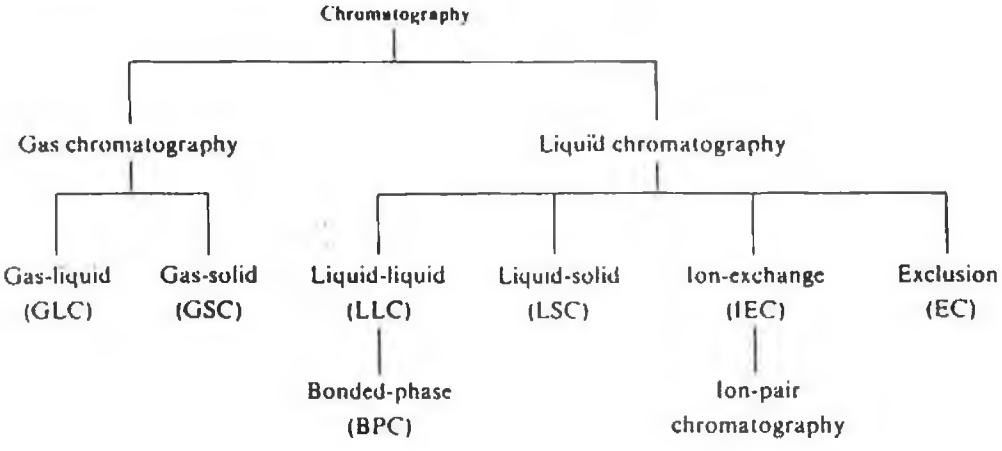



TABLE 1 CHROMATOGRAPHIC METHODS



resistance to mass transfer at the solute to stationary phase interface and the C (mobile) term represents radial mass transfer resistance between adjacent stream lines of mobile phase.

## CLASSIFICATION OF CHROMATOGRAPHIC METHODS

There are many variables involved in chromatography by which it is classified into several types. The mobile phase can be a gas or liquid, whereas the stationary phase can only be a liquid or solid. Liquid-liquid chromatography (LLC) is the name of the process where the separation involves a simple partitioning between two immiscible phases, one stationary and the other mobile. When physical surface forces are mainly involved in the retentive ability of the stationary phase the process is known as liquid-solid (or adsorption) chromatography (LSC). In Ion-exchange chromatography (IEC), ionic components of the sample are separated by selective exchange with counter ions of the stationary phase. The use of exclusion packings as the stationary phase brings about a classification of molecules based largely on molecular geometry and size. Exclusion chromatography (EC) is referred to as gel permeation chromatography or gel filtration chromatography depending on the type of mobile phase being used.

Gas chromatography is the process involving a gas as a mobile phase and is further divided into gas-solid chromatography (GSC) and gas-liquid chromatography (GLC). **TABLE 1** demonstrates the classification of chromatographic methods.

# CHAPTER 2

# DRUG ANALYSIS BY HPLC

## 2.1. DRUG ANALYSIS BY HPLC

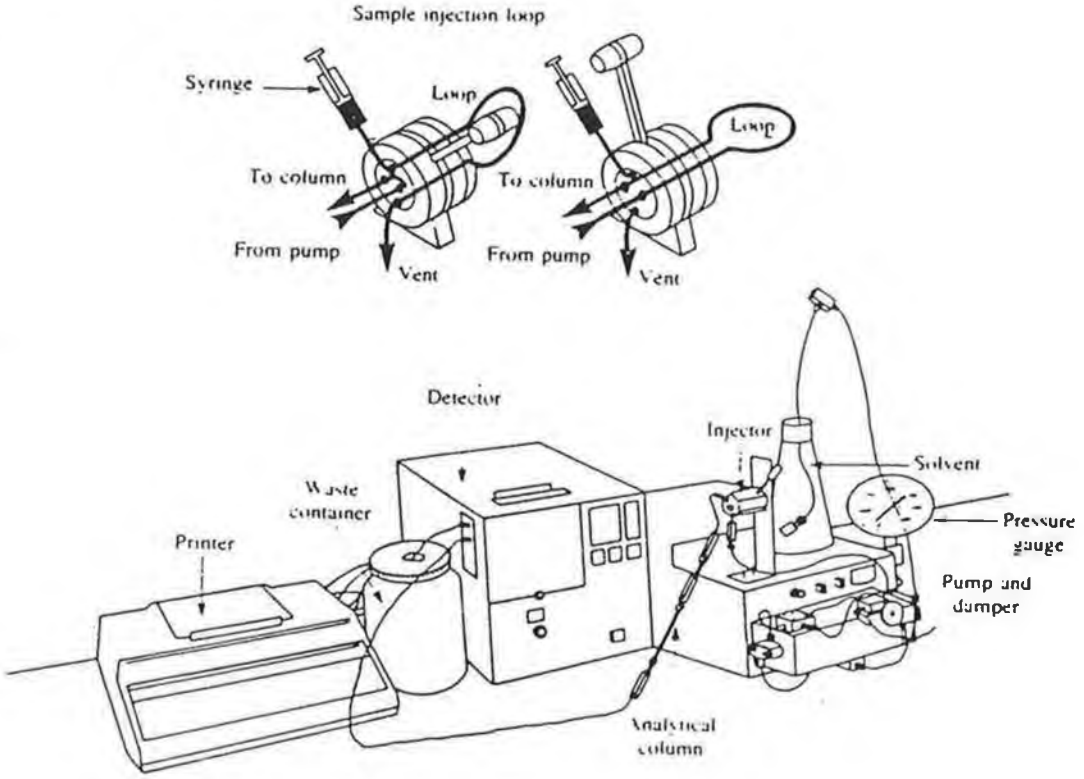
High performance liquid chromatography separates sample molecules as a result of specific interaction between solute molecules with both the stationary and mobile phases. HPLC has a much wider range of suitable analytes because it is not limited by sample volatility and thermal stability, unlike gas chromatography which is only capable of analysing 20% of known compounds (2). Another advantage of HPLC is increased selectivity compared to GC because the mobile phase interacts with solutes in HPLC as well as the stationary phase. In GC, the mobile phase is not involved in these solute interaction. HPLC is suitable for the separations of macromolecules and ionic species, labile natural products, polymeric materials and a wide variety of other high molecular-weight poly-functional groups. HPLC also remains the main chromatographic method for drug analysis (3).

### INSTRUMENTATION

A schematic diagram of a HPLC instrument is presented in Figures 4 and 5.
Typical Components:

(1) Solvent reservoir for the mobile phase.
(2) Pump: delivers the mobile phase to the column. A wide range of pressure and flow rates is useful for optimization. The pumping system should be pulse-free or else should have a pulse damper.
(3) Sampling valve (or loop): is used to inject the sample into the mobile phase just before it reaches the column.
(4) Guard column: preceeds the analytical column and is necessary to prevent contamination of the other column by small particles.
(5) Analytical column: packed with appropriate material for a particular separation.
(6) Pressure gauge: to measure column inlet pressure, this device is inserted in front of the column.

FIGURES 4,5 General instrumentation for HPLC.



(7) Detector: connected to a data handling device, e.g. chart recorder.

## 2.3 OPTIMIZATION

HPLC is capable of separating a variety of compound, even at ultra-trace levels. However it is not always easy to determine from the published literature, the specific conditions necessary for optimum separation (4 - 7). HPLC has two interactive phases and the many variables involved are linked by equations; but there are more unknowns than equations, hence the analytical problem has several degrees of freedom (tm, $\Delta$p, N), where tm is the migration time of a non-retained solute, $\Delta$P is the change in pressure and N is the number of theoretical plates. Therefore the process of optimization is based on trial and error.

Several sets of experimental conditions can be used. Optimization means finding the most practical way of achieving the best separation. Depending on the interest of the analyst whether it is short analysis time or lowest - cost equipment, certain trade-offs must be made. First, the appropriate HPLC system is chosen, thus fixing certain parameters such as k, N and $\alpha$. The compounds of interest usually need two to ten times longer to migrate through the column than the unretained solute. The mobile phase viscosity and the diffusion coefficients of the solutes in the mobile phase are also of concern. The column packing characteristics and type effect the column length and particle size too. The resolution requirements in pharmaceutical analysis by HPLC are frequently higher than those for other multicomponent separations where the components are present in similar proportion. Furthermore, it is often necessary to separate a large number of components most of which are present at very low levels and have unknown structures. This places extraordinary requirements in terms of selectivity of separation and resolution of minor components as they may not be detected because of variability of stationary phase, improper selectivity evaluations or poor resolution at the tail end of the major peak. The problem with determining, from published literature the specific condition for a given separation stems from a narrow focus provided by most researchers i.e. the publications tend to deal primarily with the separations of

components of interest to them. According to a study by S. Ahuja (8); even when all other conditions are optimum, variability in columns from manufacturer to manufacturer or from the same manufacturer can affect separations. It was also revealed that studies with selectal probes can help select reliable columns and assure the same detectability for the observed components. It is also important to work with Rs (resolution) values greater than 1.25 for close peak pairs, especially when the component of interest elutes at the tail end of the main peak. It is best to let the concentration of the minor component and the analysis time dictate the acceptable Rs value (8).

Analytical methods that can control impurities to ultratrace levels are available (9 - 11), however the level to which any impurity should be controlled is primarily determined by its pharmacologic and toxicologic effects.

This project involves **optimization** of a reversed-phase HPLC system for separating drug-free plasma extracts in a way that compares effects of varying (a) percentage of organic modifier (b) pH of aqueous solution in the mobile phase on two different columns. The application of solvent optimization to the development of isocratic reversed-phase liquid chromatography has been reported in several publications. M. Gazdag et al. adopted two different approaches to solvent optimization for controlling band spacing for the maximum resolution of samples, i.e. solvent strength and solvent type (12). To improve the separation selectivity further, the combination of these two approaches was examined as a (global) optimum mobile phase composition requires the optimization of the solvent strength by varying the percentage of organic component and of the solvent selectivity of methanol, acetonitrile, tetrahydrofuran and water. It was found that the combination of "solvent strength" and "solvent type" optimization provided a markedly better separation than either procedure alone. The advantages and limitations of combined "solvent strength" - "solvent type" optimization were summarized as follows (12):

(a) It provides a markedly better separation
(b) A global optimum can be found with a significant ability to effect changes in band spacing.

(c) The optimization procedure includes "solvent strength" optimization; when the samples do not require "solvent type" optimization, the experiments can be finished when the local optimum has been obtained. Several examples of HPLC method development based on "solvent strength" optimization were illustrated by Snyder et al. (13) which is very practical approach.

(d) Large numbers of experimental runs are required to obtain the global optimum. The number of experiments can possibly be reduced. However there is a risk to failure to recognise the global optimum.

Among the methods applicable to the optimization of reversed-phase systems two approaches can be distinguished. The first type is the "iterative lattice method" developed by Schoenmakers et al. (14) in which the sample resolution is expressed as a function of the composition of the mobile phase prepared from two isoelutropic mixtures from e.g., methanol - water, acetonitrile - water etc. This procedure is a typical "solvent-type" optimization introduced originally by Glajch and Kirkland (15) based on the almost constant elutropic strength of the mobile phase during an experiment. However, such an approach often requires a large number of experimental runs.

An alternative approach introduce by Quarry et al. (16) for optimizing band spacing, is based on the variation of the solvent strength (organic solvent concentration in the mobile phase). Although this procedure is less powerful, it is simpler and faster than the interactive lattice method, requiring fewer experimental runs and can lead to significant changes in band spacing for many samples (13).

In this project, the iterative lattice method is used in addition to solvent strength variation the solvent is varied. The other variables are pH of the aqueous buffer solution and the type of column.

**THE EFFECTS OF VARYING THE MOBILE PHASE**

Smith and Rabuor (17) carried out a study where the application of methanol - aqueous

ethylenediamine - ammonium nitrate eluents was investigated for the HPLC separation of basic drugs on a silica stationary phase. These eluents were shown to be more reproducible than previously studied systems based on methanol - aqueous ammonia - ammonium nitrate eluents. The effects of different eluent pH and buffer concentrations were examined.

Basic drugs frequently cause problems when analysed by reversed-phase HPLC. A number of alternative methods have therefore been proposed for their separation and one of the most successful approaches has been the use of the silica columns with high pH buffered eluents containing a high proportion of methanol. This method was originally proposed by Jane (18) who used an ammonium nitrate buffer and has subsequently been studied by Law and co-workers (19, 20). In a recent study, Schmid and Wolf (21) have examined a similar high-methanolic system and looked at the effect of sodium acetate buffer and ammonia concentration by using
the tricyclic drugs. In all these systems the separation is effectively based on the ion-exchange properties of the silica stationary phase.

According to a paper by Flanagan and Jane (22) the use of silica columns together with non-aqueous eluents provides a stable yet flexible system for the high-performance liquid chromatographic analysis of basic drugs. Their results showed that at constant ionic strength, eluent pH influenced retention via ionisation of surface silanols and protonation of basic analytes, pKa values indicating the pH of maximum retention. At constant pH, retention was proportional to the reciprocal of the eluent ionic strength for fully protonated analytes and quarternary ammonium compounds. The addition of water up to 10% (v/v) had little effect on retention if the protonation of the analytes is unaffected. Thus, it was likely that retention was mediated primarily via cation exchange with surface silanols. However additional factors must have played a part with compounds such as morphine which gave tailing peaks at acidic or neutral eluent pH's. This paper however deals with the separation of basic drugs whose retention on the silica column/non-aqueous ionic eluent system was mediated primarily via cation exchange with the surface silanols. The present study involved a reversed - phase high performance liquid

chromatographic separation.

Reversed - phase chromatography uses a hydrophobic bonded packing, usually with an octadecyl (C-18) or octyl (C-8) functional group and a polar mobile phase, often a partially or fully aqueous mobile phase. Polar substances prefer the mobile phase and elute first. As the hydrophobic character of the solute increases, retention increases. Generally, the lower the polarity of the mobile phase, the higher its eluent strength. The elution order is the reverse of that for normal - phase. Methanol and acetonitrile are popular solvents because they have low viscosity and are readily available with excellent purity. Eluents intermediate in strength between these solvents and water are usually obtained by preparing mixtures. Since the optimum composition of the mobile phase must generally be found by trial and error, it is convenient to start with a 1:1 water/methanol mixture. If the sample components elute at or near the transit time of a non retained solute, tm, a lower concentration of methanol is indicated. Changing to acetonitrile, dioxane, or mixtures of 1, 4-dioxane/methanol or acetonitrile/2-propanol can often improve selectivity. In reversed-phase chromatography, solvent gradients are generated by a continuous decrease in the polarity of the eluent during the separation - for example, by gradually increasing the organic solvent content in water/methanol or water/acetonitrile mixtures.

The analysis of basic compounds and quaternary ammonium compounds by reversed-phase HPLC, with aqueous mobile phases on chemically modified silica gel is often hindered by the occurrence of badly tailing peaks (23). Poorly reversible interaction of the solute with residual free silanol groups and/or complexation with metal ions in the stationary phase may be the cause of this undesirable behaviour. A comparison was made by Lingeman et al. between the use of aluminium oxide and non-modified silica gel as cation-exchange materials for the separation of basic drugs (amines) with aqueous solvent mixtures. The retention behaviour of the amines was studied and appeared to be controlled predominantly by the pH and the concentration and nature of the modifier. It was found that the selectivity of ion exchange chromatography on aluminium oxide or silica gel can be improved by the addition of organic solvents to the aqueous mobile

phase. In the aluminium oxide system the addition of a co-solvent was necessary to achieve resolution of the different solutes whereas in the silica gel system resolution could be achieved even with water-rich eluents. A more general treatment of the effect of co-solvent in the aluminium oxide system has been provided by Laurent et al. (24).

The problem of poorly shaped peaks in the reversed-phase HPLC analysis of basic drugs, may be solved by the addition of a deactivating agent, such as an aliphatic amine, to the eluent (25, 26). This is because these badly shaped peaks are often caused by unwanted interactions with the stationary phase. An alternative approach has been the aforementioned use of silica gel as an ion-exchange material with eluents containing high proportions of methanol and either perchloric acid (27, 28) or high pH buffers (29 - 32). After the report by Jane (22) which stated that retention was mainly controlled by analyte pKa and stereochemistry, Bidlingmeyer et al. (33) suggested that the silica column also showed marked evidence of hydrophobicity and that the order of retention of related compounds often resembled that of reversed-phase HPLC. They attributed this effect to the presence of siloxane groupings. To test these findings, Law (20) examined 69 monobasic aryl alkylamines using an aqueous methanol eluent at pH 9.1. He showed that there was a linear relationship between the retention times of the amines and the reciprocal of the ionic strength and concluded that cation exchange was the predominant mechanism of retention and the separation was primarily controlled by the eluent pH. Although there were deviations from the expected linearity, these were small, and the proposed hydrophobic mechanism was ruled out. Non-polar compounds were effectively unretained.

In recent studies, Cox and Stout (34) have looked in detail at the retention of ionic compounds on silica using "pseudo reversed phase" conditions. They used a limited set of test compounds and concentrated on the pH ranges 2.1 - 7.0 and 15 - 75% methanol. They also observed a minimum capacity factor at approx. 50% organic modifier and found a linear relationship between reciprocal ionic strength and capacity factors. However the curve showed a positive intercept suggesting that a second retention mechanism was effective in addition to the ion-exchange mode. This extra effect

depended on the method of preparation of the silica.

Extensive studied of the importance of the use of appropriate mobile phase solvents have been carried out (35 - 39). The importance of the effect of solvent compositions in which analytes are dissolved has received much less attention than that of mobile phase composition but it is gradually gaining recognition. For example, unexpected peaks produced as a result of an inappropriate choice of sample solvent were called ghost peaks (40) or system peaks (41). Williams et al. (42) found peak broadening of analytes when the percentage of organic solvent in the sample solution was increased. Peak distortion, including peak splitting has also been observed by other workers (43). Chiba and Singh (44) stated that the resolution of two compounds they tested was strongly influenced by the percentage of organic solvent, pH, and buffer strength of sample solutions. Perlam and Kirshbaum observed that the extent of the effect of sample solvents was dependant on the compound to be analyzed (45). Recently, Hoffman et al.reported on the distortion and multiplication of peaks when the sample solution was significantly stronger than the mobile phase (46). It is clear from the above that further studies are required in order to understand fully the complicated effects associated with solvent composition in order to perform efficient reversed-phase HPLC analyses.

Chiba and Vukmanic studied the effects of organic solvents in sample solutions on chromatographic peak profiles, assessed by retention time, peak height and peak width for methanol and acetonitrile in reversed-phase HPLC (39). Two benzimidazole carbamate degradation products of the fungicide benomy were studied: methyl-2-benzimidazole carbamate (MBC) and 3-butyl-2, 4-dioxo (1, 2 -a)-s-triazino-benzimidazole (STB). The overall effect was more noticeable with STB than with MBC. Peak splitting was observed only with STB when larger volumes of solvents were injected. Peak broadening was observed even with 10$\mu$l injections in some instances. In general, as the percentage of organic solvents was increased in sample solvents, greater deterioration of the peak profiles was observed. This, however was not always so with STB.

Smith and Westlake studied the effect of operating conditions on separations using an

organic buffer (47). They separated basic drugs by HPLC on silica using a methanol-aqueous pH - 10 buffer eluent from 3-(cyclohexylamino)-1-propane-sulphonic acid and sodium 3-(cyclohexylamino)-2-hydroxy-1-propanesulphonate. The buffer could be reproducibly prepared. They concluded that this new method was indeed susceptible to changes in the operating conditions; increases in the operating temperature or the ionic strength of the eluent or a decrease in the proportion of the buffer caused the relative capacity factors to increase whilst increasing pH of the eluent or the proportion of methanol caused the relative capacity factors to decrease.

Antia et al. derived a three parameter equation to express the dependance of the logarithmic retention factor, K, on the volume fraction of the retention modulator, $\Psi$, in a binary eluent such as the organic modifier used in reversed-phase chromatography eluents (47). It is based on the competitive binary adsorption isotherm of the eluate and the modulator generated by employing the ideal adsorbed solution (IAS) method.
All the previous references to studies of the effect of varying the mobile phase composition and/or strength apply to drug analysis by HPLC. The only study before that examined the effects of a variation in mobile phase composition and pH on profiles of drug-free plasma was carried out by Kelly and Smyth but since the present project evolved directly from that previous study, its relevance is great. Thus, a more extensive discussion of it will be included in later chapters.

## COLUMN TO COLUMN REPRODUCIBILITY

Given the columns available today, the HPLC user is challenged to develop mobile phase conditions that not only achieve the separation required but also minimize column-to-column differences.

In the studies by Smith et al. on the development of robust and reliable methods for the analysis of basic drugs by HPLC, the reproducibility of the experimental conditions (29) and the stationary phase (31) has been determined and the conclusions have been tested in national (30) and international collaborative studies (32).

Within a single laboratory, good reproducibility should be obtained under controlled conditions, but in interlaboratory studies, the variations were much larger and it appeared that their method was very sensitive to changes in the operating conditions. Two areas of particular concern were the column temperature and the differences in concentrations of the ammonia stock solutions used to prepare the buffer-ammonia-ammonium nitrate. Although the pH appeared to remain unaltered, significant changes in the ionic strength would result from changes in the ammonia concentration. It was concluded that this eluent lacked reproducibility for the analysis of basic drugs and they went on to try to examine alternative buffer systems. In an initial study ethylenediamine was examined (17) and found to be better than ammonia though solid buffer components were preferable. The later study describes separation carried out using buffers prepared from non-volatile organic sulphonic acid amine buffer components and the effects of changes in the operating conditions on the selectivity and retention.

In another study on the long term reproducibility of capacity factors and retention indices, Smith and Burr determined the reproducibility of retention measurements over a two-year period as part of a compilation of a database of substituent parameters for the prediction of retention indices in reversed-phase HPLC (48). Among their findings was the fact that although the capacity factors on each column were reasonably consistent, there were significant differences between the columns even though they were all packed with the same batch of Spherisorb ODS2. Despite the precautions taken to ensure that the experimental conditions remained as constant as possible, there is still considerable variation in the results of up to 18% from the mean values. In conclusion, they found that the variation in the capacity factors over a prolonged period, even under closely controlled conditions, is considerable and is emphasised by variations in the measurement of column void volume.

Smith et al, determined the batch to batch reproducibility of the stationary phase on a silica column with a methanol ammonium nitrate eluent (49). They discovered that not only should each lab standardize on the same brand of packing material (in interlaboratory studies), but standardization should also involve only a single batch of

packing material (ideally). This may be possible for labs within one organization e.g. U.K. forensic science labs, but places severe limitations on the generation of retention databases for more general use.

So despite controlling as many factors as is feasible, experimental variation cannot be totally eliminated. This project is concerned with measuring the effects that occur when a certain condition is varied e.g. varying the percentage organic modifier in the eluent. All the other operating conditions must be carefully controlled, so that they can be assumed to be constant.

## TECHNICAL CONSIDERATIONS IN HPLC DRUG ANALYSIS

### REVERSED PHASE COLUMN:

This mode of chromatographic separation is more popular than reversed phase HPLC for many reasons which have been reviewed. Advantages of reversed-phase include resistance to contamination, a water-based mobile phase, ease of equilibration and broad applicability. The first choice of reversed-phase column is usually "$C_{18}$" or "$C_8$" ie; columns containing porous silica particles surface-bonded with $C_8H_{17}$ or $C_{18}H_{37}$ alkyl chains. However, the difference between these columns in terms of chromatographic properties is very insignificant according to a review by Giese (51). Selectivity differences are less likely between $C_8$ and $C_{18}$ than between either of these alkyl phases and other more polar bonded phases such as cyano and phenyl, which can also be used in the reversed-phase mode by incorporating adequate water in the mobile phase. This project compares drug-free plasma profiles on two different columns $C_{18}$ and cyano (CN).

### COLUMN EFFICIENCY

The theory behind column efficiency has already been mentioned, however, there are a few technical considerations concerning column efficiency. The column length and the particle size are important factors influencing separation efficiency.

In this regard, laboratories are increasingly using columns 15cm long, packed with porous particles that are normally 5 m in diameter. A recent development, high speed liquid chromatography, involves using very short columns (e.g. 3 to 8cm) with a conventional internal diameter, packed with 3 m particles and run at a very high flow rate (2 to 5ml/min). Together with a fast response-detector system, analysis times can be significantly shortened. The increased back pressure, however can lead to faster wear of the pumping system and the increased likelihood of column contamination or plugging from extraneous particulate matter owing to the close spacing of the particles in the packing.

Column efficiency is usually high when the column internal diameter is between 3 and 6mm. These dimensions allow for ease of packing, limited elution volume of the mobile phase and moderate pressure drop (51). Thus, most columns have internal diameters within this range.

The stability of a column is a factor which keeps improving; mechanically they are capable of withstanding more and more pressure. Contamination from impure samples is still a problem but with proper sample cleanup and injection techniques, a column can be used for more than 2,000 analyses. It's important to maximize column lifetime by using a suitable guard column. Nevertheless, more work is needed on regenerating columns contaminated by impure samples such as the usefulness of strong injections between each sample (52).

**THE MOBILE PHASE**

The flexibility and ease of control of the mobile phase makes it a useful tool for efficient separations. In this project two organic modifiers or co-solvents are compared. These are methanol and acetonitrile which are the two most common co-solvents. The advantages of acetonitrile are: it is a less-polar organic solvent which typically provides an adequate solvent strength at a lower concentration than methanol. Acetonitrile also yields a mobile phase with water that has a lower viscosity, which makes the column

more efficient and allows a lower pumping pressure. Selectivity differences between these two solvents also can arise largely because acetonitrile is a hydrogen bond acceptor which methanol is a hydrogen bond donor (51). Methanol has an advantage in that it is cheaper.

Degassing the mobile phase in HPLC is very important. Two reasons for this are to avoid formation of gas bubbles in the chromatographic system which would be most likely to occur in the pump and the flow cell. Secondly, to overcome interference of dissolved gas, especially dissolved oxygen, with the performance of the detector. For example dissolved oxygen can absorb light at a low wavelength, thus limiting the sensitivity of the analysis. In a paper comparing methods for removing oxygen from methanol based on the residual absorbance of the methanol at a low wavelength, the order of decreasing effectiveness for oxygen removal was reflux > helium sparging > vacuum > ultrasonication (53). The temperature of the mobile phase is also an important consideration. It is controlled by increasing the temperature of the column and capillary tubing which lies before it. The higher the mobile phase temperature, the more soluble the drug within it is likely to be and hence the retention of the drug will be diminished. Generally, a higher mobile phase temperature means improved efficiency since viscosity is inversely proportional to temperature ie; the mobile phase becomes less viscous with increasing temperature allowing quicker molecular diffusion. A disadvantage of increasing the mobile phase temperature is decreased column stability. More information on temperature contribution to resolution in reversed-phase LC is available (54).

**DETECTION**

The two most commonly used detection systems for drug analysis by HPLC, are ultraviolet and to a lesser extent, fluorescence. Both are convenient and sensitive for therapeutic drug monitoring. The only drugs not detectable by ultraviolet are those with low absorptivities even at low wavelengths or those with a very low sample concentration. Fluorescence is not widely used since not many drugs fluoresce, but when applicable, it

is very sensitive and more selective than ultraviolet detection. There are two types of ultraviolet detector: fixed wavelength and variable wavelength. Although the fixed type is cheaper and more sensitive at the wavelengths available, the variable wavelength UV detector is now the first choice. These detectors have become extremely sensitive and their ability to select particular wavelengths, whether very low or relatively high (e.g. from 190nm to 300nm) can provide additional sensitivity and specificity over fixed wavelength detectors.

In spite of generally poorer detection possibilities than in gas chromatography the number of applications of HPLC in many areas is increasing steadily. On-line and off-line pre-column derivitization techniques in connection with sensitive, selective detectors are the most efficient approaches to improving the sensitivity of determination and the detection limits (55), but they are often not available or not readily applicable for some less reactive compounds or very dilute samples. Consequently, less sensitive UV detection should still be used in many applications. Both the precision of the integrated peak areas and the limits of detection improve with increasing signal-to-noise ratio (56). The detection limits are usually defined as the concentration of the sample solute that gives a signal-to-noise ratio of two (57) or three (58). The signal-to-noise ratio can be improved either by enhancing the detector signal or by limiting the noise originating from the detector and the pumps of the instrument. The baseline noise is affected by fluctuations in the flow-rate, pressure and temperature (56) and depends on the design of the detector; signal processing may also contribute to noise (59, 60).
The useful signal i.e., the height or the integrated area of a chromatographic peak, can be enhanced by using a sensitive detector with optimized parameter settings, an efficient chromatographic column with a small dead volume, a chromatographic system allowing a low retention and simultaneously a sufficient separation selectivity and sample volumes as large as possible without deteriorating the column efficiency. The flow rate of the mobile phase also affects the heights and areas of the peaks, which usually increase with decreasing flow rate (61).

Computerized processing of the digitized detector signal offers a possibility of "bunching"

and averaging several successive signal readings to be stored in the computer memory. In addition, diode array spectrophotometric detectors make it possible to "bunch" simultaneous signals from several photodiodes in a spectral segment of a preselected width. This method of data processing can also affect the reproducibility, sensitivity and detection limits.

The signal-to-noise ratio can also be enhanced by using a filter to reduce the noise amplitude, but this approach is limited by the requirement to retain accurate peak shapes (59). Fourier transformation of the chromatographic signal can be utilized for improving the signal-to-noise ratio by discriminating and cutting off the high-frequency noise from the useful signal (62). Smoothing procedures have been suggested that allow one also to filter the noise with frequencies close to that of the signal (63).

Jandera and Prokes (61) investigated the practical feasibility of applying computerized accumulation of chromatograms from repeated high speed runs to enhance the sensitivity and to decrease detection limits in the HPLC analyses using UV detection. They also compared this approach with other possibilities such as the on-line sample enrichment technique using column switching and other techniques. For the investigation, a relatively simple reversed-phase separation of a mixture of chlorobenzenes was used. They reported that the computerized accumulation of subsequent repeated chromatograms in high speed HPLC made it possible to increase the signal-to-noise ratio and to decrease the minimum detectable concentrations approximately in proportion to the square root of the number of accumulations with good accuracy of quantification. A compromise between the time of analysis and the detection limits would have to be made. It was concluded that it was important to use a detector designed to yield a high signal-to-noise ratio to obtain low detection limits using the accumulation approach. The UV detector, set to the wavelength of the absorption maximum should use a narrow spectral band width setting and a signal acquisition frequency of 10 - 20 signal readings during the elution of a chromatographic peak.

It was found also that on-line sample enrichment techniques based on solid phase

extraction resulted in detection limits at least one order of magnitude lower than those achievable using computerized accumulations of repeated chromatograms, but the latter approach would possibly be a useful option for analyses where no adequate sorbent was available or where the sample enrichment techniques were too tedious.

# CHAPTER 3

# SAMPLE PREPARATION AND CLEAN-UP FOR CHROMATOGRAPHIC ANALYSIS

## SAMPLE PREPARATION AND CLEAN-UP FOR CHROMATOGRAPHIC ANALYSIS

One of the aims of this project is to determine how efficiently liquid-liquid extraction will "clean up" drug-free plasma profiles using reversed-phase HPLC. It is therefore important to review the relevant literature on sample preparation and sample clean-up for chromatographic analysis.

After a sample has been received, it must be prepared for analysis; the sample preparation stage is intended to improve the specificity of the assay by removing the majority of the matrix whilst concentrating on the analyte. The specificity of any assay is derived partly from the analysis but also from the initial clean-up process. After the analysis, the response is quantified and the analyte concentration or amount is calculated as the final result together with any analytical observations. Until recently, advances in analytical techniques and computer control of instruments were not matched by improved sample preparation. This meant that sample clean-up could become the rate limiting step for a laboratory.

### PLASMA CLEAN-UP

Of all biological fluids, blood is of the greatest analytical interest since it is the most important transport medium in the human body and blood levels of most therapeutic and diagnostic substances correlate with their function (64). Because of better handling, the use of plasma or serum as a biological matrix is preferable over blood.

Plasma and serum is produced by removing the cellular components of blood by centrifugation or natural clotting. This step must be regarded as a pre-analysis purification. There are, however, some cases when drug analysis in blood is preferable to plasma. These are:

1) When the partition between corpuscular components and plasma depends on conditions which cannot easily be controlled and/or when the drug is preferentially bound to blood cells.

2) When the sample volume is small.
3) When blood samples are dehiscent and decomposed making the production of plasma or serum impossible (e.g. in forensic medicine).
4) When the drug develops its effect in the blood cells (e.g. chlorquine).
5) When blood levels reflect the therapeutic and toxic effects better than plasma levels (65).

For development of an adequate sample preparation method the factors to be considered are both the chemical properties of the constituents in question and the biological matrix. The substance of interest must be extracted from its biological matrix prior to analysis. Proteins and other macromolecules may interfere with detection and columns may get plugged or quickly inactivated. The ideal extraction procedure should be reproducible with little loss of the material of analytical interest. It should be rapid and allow several samples to be analyzed in a short period of time and it should be inexpensive. A schematic flow diagram of steps used for liquid-liquid sample clean-up of basic and acidic drugs is presented in Figure 6.

For development of an extraction procedure, pKa, partition coefficients in organic solvents and binding to blood components should be available. The distribution of blood components influence the choice of an adequate matrix. Basic drugs often have a large volume of distribution and are detectable in blood only in low concentrations, especially when administered at low doses (1mg/kg body weight) (66). Most acidic and amphoteric drugs can be quantitatively determined in serum, plasma or urine. They usually remain in the intravasal compartment and have a high affinity to plasma proteins.

The difference between serum and plasma should be explained at this point. Serum is produced by natural clotting of fibrogen with a removal of hemo - and lipoproteins, which bears the risk of losing drugs into the clot. Plasma, on the other hand, which must be anticoagulated, is rich in lipids and lipoproteins, since these compounds cannot be removed by centrifugation. Blood (freshly drawn) however, with its corpuscular components, lipids, lipoproteins and proteins requires a purification step before

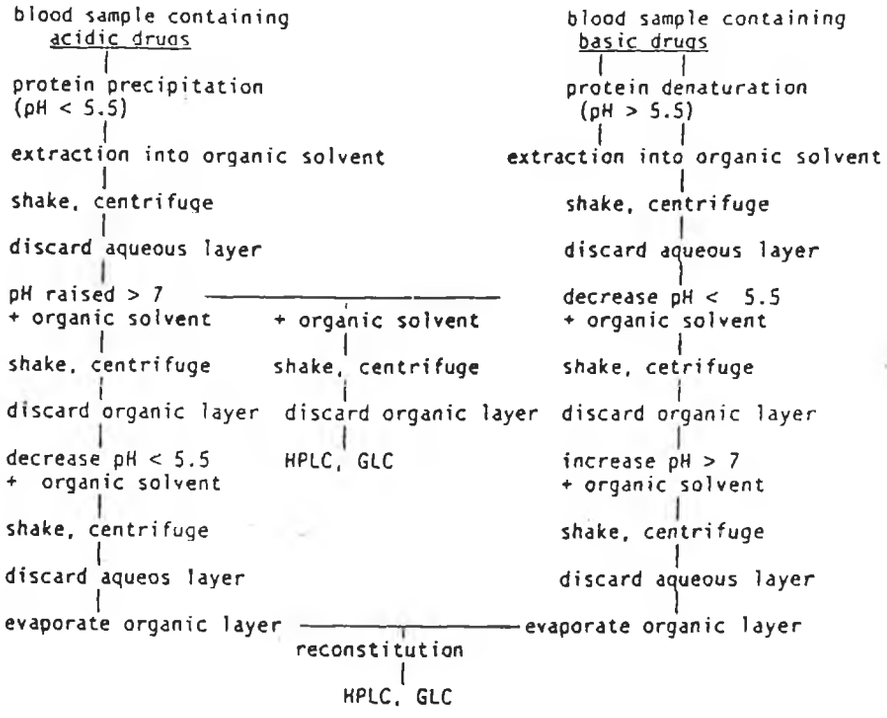



Fig. 6 Liquid-liquid clean-up procedures for blood sample analysis.

Fig. 6 is a schematic flow-diagram of steps used for liquid-liquid sample clean-up of basic and acidic drugs. The diagram demonstrates the way of blood sample preparation for GC and HPLC analysis. For reversed phase chromatography the aqueous back-extracts can be directly injected into the HPLC system The side columns of the diagram show purification steps by basic or acidic back-extraction, the middle column the removal of interfering materials with an organic solvent, inmiscible with the aqueous layer containing the compounds of interest, in which these materials are insoluble.

extraction, usually hemolysis and protein precipitation. Similar extraction procedures are used for blood, serum and plasma.

In most cases of blood sample preparation, anti-coagulants like ethylendiamine tetracetate (EDTA) and acid-citrate dextrose (ACD) are added to the samples. Both of these anti coagulants contain ultra-violet - absorbing impurities, which can interfere with the material to be detected (67). The anticoagulant can also influence the accuracy of the measurement (68). The completeness of protein removal plays a major part in the effectiveness of the sample preparation. Thus a step of protein removal is essential for all extraction procedures from blood, plasma and serum (64).

However, although it is almost mandatory to remove all inert proteins, the components of analytical interest should be recovered. Hemolysis without protein precipitation is achieved by: freezing (- 20 degrees °C) and subsequent thawing of the sample or by ultra-sound or osmotic shock. The following deproteination techniques can be used: (69)

(1) Change in pH by adding a strong acid to the sample.
(2) Change in the ionic strength by addition of salts.
(3) Change in temperature by heating the sample and denaturing the proteins.
(4) Change in dielectric constant by addition of organic solvents.
(5) Filtration and ultra-filtration.

During or after protein precipitation the pH required for the extraction procedure has to be adjusted.

**OPERATIONS OF SAMPLE PREPARATION**

Sample preparation can be considered as a number of unit operations each of which are capable of a specific task (70). These techniques are the fundamental building blocks for any clean-up scheme as they can be taken and matched to the particular analytical challenge. One must realize that one particular technique should not exist in isolation,

but should be used together with other appropriate techniques required by the analytical method. A list of some typical unit operations useful for sample preparations is shown in Table 2. These operations can be classified into four groups (as in table).

1) **Stabilization and release of analyte from the matrix:**
These operations are either to cleave a molecule into a more convenient form to assay, to release an analyte by breakdown of the biological matrix or to stabilize the analyte to avoid artefact formation by undesirable reactions or enzymatic degradation. Molecular cleavage: A common pathway of metabolism, for either endogenous or exogenous compounds, is conjugation, where a polar moiety, e.g. glucuronic acid, glutathione or sulphate, is covalently bonded to the compound. The main aim of conjugation is to make the compound more polar to aid excretion from the body. Thus the aim of the techniques in this group are to cleave the conjugate and release the original compound for assay.

Breakdown of the biological matrix. Where the analyte is bound to a compound of the matrix, enzymes can be used to break down the components of the matrix and release e.g., protein bound compounds. Proteases, such as pepsin (71) and subtilysin (72), can be used to provide a relatively controlled breakdown of plasma and blood.

2) **Removal of endogneous material:** Since a biological matrix may be solid or particulate e.g. muscle, milk, tissue, faeces or blood, it therefore consists of many components: macromolecules such as proteins, carbohydrates and lipids. The operations in group 2 are responsible for removing the majority of the biological material from the sample matrix prior to analysis.

3) **Liquid Handling Procedures:** These techniques may appear to be routine and unimportant but they tend to bridge the techniques in the other groups.

TABLE 2

CLASSIFICATION OF SAMPLE PREPARATION UNIT OPERATIONS BY FUNCTION

| | | |
|---|---|---|
| *Group 1: Release of the analyte from the biological matrix* | | |
| Hydrolysis | Acid | |
| | Base | |
| | Enzyme | |
| | | Proteases |
| | | Lipases |
| | | β-Glucuronidase |
| | | Arylsulphatase |
| Sonication | | |
| *Group 2: Removal of endogenous compounds* | | |
| Liquid-liquid extraction | | |
| Liquid-solid extraction (solid-phase extraction) | | |
| High-performance liquid chromatography | | |
| Precipitation | Organic solvents | |
| | Inorganic acids and salts | |
| | Ammonium sulphate | |
| Ultrafiltration | | |
| Dialysis | | |
| Immunoextraction | | |
| Micellar liquid chromatography | | |
| Supercritical fluid extraction | | |
| Saponification | | |
| Lyophilization | | |
| *Group 3: Procedures for liquid handling* | | |
| Aspiration | | |
| Centrifugation | | |
| Dilution | | |
| Evaporation | | |
| Filtering | | |
| Freezing | | |
| Mixing | | |
| Pipetting | | |
| Salting-out | | |
| Separation | | |
| *Group 4: Enhancement of selectivity and sensitivity* | | |
| Pre-column derivatization | GC | |
| | HPLC | |
| Post-column derivatization | Enzyme reactors | |
| | Solid-phase reactors | |
| | Ion pair as a detector | |
| | Photochemical derivatization | |
| | Segmented flow reactors | |
| | Packed bed reactors | |
| Selective detection modes | Diode array detection | |
| | Electrochemical detection | |
| | Fluorescence detection | |
| | Sensors and biosensors | |

4) **Enhancement of Selectivity and Sensitivity:** These techniques enhance the assay sensitivity and specificity by either: Derivitization of an analyte, pre-column or post-column or by enhancing the detection system (61).

Unit operations provide the analyst with a very useful means of critically evaluating the sample preparation methods.

## TECHNIQUES FOR SAMPLE PREPARATION USING BIOLOGICAL MATRICES

The general methods used for the removal of endogenous material include dilution, precipitation, ultrafiltration, LLE (liquid-liquid extraction), LSE (liquid-solid extraction) and HPLC. The sample must firstly be liquidized (if it is a solid) before it can be used in any of the aforementioned techniques. In a review by Maickel (73), the procedures, advantages and disadvantages of homogenization methods are covered (i.e. the procedures etc. by which solids or semi-solids can be made liquid).

### DILUTION

Where the analyte is present in a sufficiently high concentration or there is a specific detection system, then dilution is a very simple and effective means of sample preparation. It may be useful in many situations e.g. to reduce the viscosity or ionic strength of the sample.

### PRECIPITATION

Removal of proteins and precipitation of other endogeneous material are simple and effective methods of sample preparation that can be used alone or with other methods of sample clean-up. The main reason for removing proteins from plasma is because they can precipitate when they enter the mobile phase and cause increases in back pressure or deterioration of the column in HPLC. It is a simple and quick method of sample clean-up. The main problem is loss of analyte. Ways of deproteination have been given earlier.

In a study of the evaluation of the relative efficacy of various techniques for deproteinizing plasma samples prior to high performance liquid chromatography, Blanchard (74) describes a number of useful methods of protein removal using the Lowry (75) method of protein determination which evaluates their efficacy with greater sensitivity. He reported on the volume of precipitating agents added to 0.5ml of plasma and determined the amount of protein remaining in the supernatant. The results of this study showed that TCA (trichloroacetic acid) and perchloric acid are very efficient at precipitating proteins; however the pH value of the supernatant is very low and the analyte must be stable for these methods to work. The four organic solvents tested in this work are less effective than the acids above; however they are commonly employed prior to an HPLC analysis and their relative effectiveness (acetonitrile > acetone > ethanol > methanol) is inversely related to their polarity (74). Dilution occurs during protein precipitation and if no further sample preparation is undertaken, it may result in a lower sensitivity of the assay method.

**ULTRAFILTRATION**

This technique involves removal of proteins and other large macromolecules without precipitation. This can be used to measure the amount of 'free' (or non-protein bound) analyte in plasma, but is also a sample clean-up method.

The procedure uses cone-shaped membranes that fit into centrifuge tubes; aliquots of the sample are placed in the tops of the cones and centrifuged gently. The membranes allow passage of molecules smaller than the exclusion limit that aren't bound to macromolecules. Blanchard proved that virtually all the protein in the plasma can be removed but a potential problem is the binding of the analyte to the membrane and control experiments should be undertaken to find the extent of this. An advantage of this technique over protein precipitation is its applicability to small sample volumes since no dilution occurs during filtration (76). A major advantage of this technique over dialysis is its speed, especially when hollow fibre membranes are used.

**DIALYSIS**

This is the separation of an analyte from its matrix by diffusion through a semi-permeable membrane rather than centrifugal force as with ultra-filtration. The membrane acts as a filter restricting the size of molecule that can pass through it thus holding back the larger molecules (macromolecules) and letting lower-molecular-mass molecules through. Diffusion is a slow process and is driven by a concentration gradient which stops acting when equilibrium is reached. The maximum recovery of analytes is thus 50%. Dialysis is only suitable for compounds which are relatively weakly protein bound.

**LIQUID-LIQUID EXTRACTION**

This method of sample preparation has been used for many years. It entails the direct extraction of the biological material with a water-immiscible solvent. The isolation of the analyte is achieved by partitioning it between an organic and an aqueous phase; The Nernst Distribution Law governs the equilibrium distribution between the two phases (77,78). The distribution ratio is influenced by the choice of extracting solvent, pH value of the aqueous phase and the ration of the volumes of organic to aqueous phases. The initial conditions of the extraction should be such that the analyte is preferentially distributed into the organic solvent.

This project investigates the effect of liquid-liquid extraction on drug-free plasma prior to HPLC analysis. Different percentages of organic modifier (and types of organic modifier) and different pH values of the aqueous mobile phase component are tried and their resultant chromatograms are compared in terms of clean-up effectiveness.

Low analyte recovery after liquid-liquid extraction, can be enhanced by successive extractions of the sample to produce acceptable recoveries (77,79), but in practice it is often the case that a large excess of extracting solvent can be used in order to save time and achieve the same result.

Blood sample preparation procedures using liquid-liquid extraction (LLE) can be divided into four steps (64).

1) Hemolysis and protein precipitation.
2) Extraction of the components of interest.
3) Purification and removal of interfering materials.
4) Volume reduction and reconstitution for chromatographic analysis.

The method chosen for extraction depends on the pKa of the material to be analysed. Acidic drugs are extractable at pH < 5.5 and basic drugs at pH > 5.5. In blood, one fraction of the drug is bound to plasma proteins and other blood components, and the other fraction is free. By deproteination and extraction the drug - protein bonds must be broken or the recovery may be decreased. A low recovery can also occur if (a) the compounds of interest are co-precipitated or (b) physically entrapped in the protein precipitate. Basic drugs can be extracted from blood without prior procedures by the use of appropriate buffer solutions with a pH ranging from 6 to 14. Usually the pH to be chosen is 3 units above the pKa value because then, more than 99% of the basic drug is in its ionized form and can be extracted into an organic solvent. Due to their ionic strengths, these buffer solutions cause protein denaturation with minimal loss of the drug (66).

Acidic drugs can be extracted after lowering the pH < 5.5. The low pH causes protein precipitation with the risk of co-precipitating the analytes. Four main techniques are used in liquid-liquid purification of blood:

1) Conversion of the drug to the ionized form by changing the pH allows the drug to be extracted into an aqueous phase. The organic layer is removed and discarded. In the second step the lipophilic, unionized form of the drug is reconstituted by changing the pH in the opposite direction and the drug can be back extracted into an organic solvent.

2) The drug is dissolved in an aqueous/organic solvent, e.g. water/acetonitrile, and the interfering compounds are removed by washing the sample with a lipophilic solvent, that is not miscible with the aqueous layer e.g. hexane. Compounds of interest dissolved in a lipophilic solvent can also be purified by washing with an aqueous solution.

Liquid-liquid extraction can be combined with solid-liquid purification steps:

1) The extracted sample is spread on a TLC plate. After development the circle of silica adsorbing the compounds of interest is scraped off the plate and the silica gel is extracted (80).

2) Interfering materials are removed by adsorption on a solid phase or after liquid-liquid extraction the compounds of interest are adsorbed on a solid phase and hence separated from interfering material.

Another form of liquid-liquid extraction is the use of silica material like Extrelut R (81 - 84). Though the extraction columns contain solid phase material, the basic principle is a liquid-liquid extraction. Extraction with diatomaceous earth obeys the same principle (85 - 87). Silica gels are porous carrier materials. Water molecules distribute on the surface of the silica gel and become the stationary phase. Compounds are dissolved in the water phase and are eluted from the columns by organic solvents, immiscible with water. Such columns can be used at a pH range from 1 - 13. After protein precipitation by acid or buffer, the aqueous blood sample is pulled by vacuum through the column (83, 84). Silica gel can also be used for sample purification by absorbing interfering materials from blood without absorbing the components to be eluted.

**CHOICE OF EXTRACTING SOLVENT**

The relative lipophilicity or hydrophobicity of the analyte will determine the choice of the solvent as the analyte must be soluble in it for the extraction to function. Before any

extractions were made in this project some time was spent choosing a suitable solvent for use in the rest of the project, by comparing extraction effectiveness and resulting chromatograms for various solvents.

Solvents suitable for extraction should have a low boiling point so they can be efficiently removed at the final stage of the extraction. They should also have a low viscosity coefficient so mixing with the sample matrix won't be a problem. The solvents for liquid-liquid extraction, can be arranged in order of polarity into what is known as the elutropic series (70) (Table 3).

Also to be considered, is the amount of water that can dissolve in the solvent since this can influence the amount of co-extracted interference in the final extract. In general, for extraction of biological samples the rule is: the less polar the solvent the more selective it is; so the solvent of choice is usually the least polar one in which the analyte is still soluble.

Selectivity is a major advantage of liquid-liquid extraction, and depending on the solvent choice, the analyte or drug of interest can be extracted from most of the endogeneous components. More so, this can be used to an even greater advantage where a non-specific spectrophotometric assay is employed. For example, if a drug is extensively metabolised, and the metabolites have the same chromophore as the parent compound, then interference may arise. However the drug may be selectively removed by using a lipophilic solvent to extract the sample, leaving the metabolites in the biological fluid.

On the other hand, a hydrophilic solvent may extract the drug as well as the metabolites to allow simultaneous determination of each analyte, if chromatography is used to give the separation.

Some of the problems with solvents are as follows; some manufacturers of solvents may have added small amounts of other compounds to their products to prevent oxidation or decomposition of their product. The presence of any other chemicals within a solvent

TABLE 3

PHYSICAL PROPERTIES OF SOME SOLVENTS FOR LIQUID-LIQUID EXTRACTION

| Solvent | Boiling point (°C) | Solvent polarity ($p'$) | Solubility in water (%) |
|---|---|---|---|
| *n*-Hexane | 69 | 0 | <0.001 |
| Carbon tetrachloride | 77 | 1.7 | 0.08 |
| Cyclohexane | 81 | 0 | 0.01 |
| Chloroform | 61 | 4.4 | 0.82 |
| Dichloromethane | 40 | 3.4 | 1.30 |
| 1,1-Dichloroethane | 57 | | 5.03 |
| Diethyl ether | 35 | | 6.04 |
| Ethyl acetate | 77 | 4.3 | 8.08 |

may alter its polarity and affect the specificity and recovery of a method. Another problem that occurs is solvent impurities. The extracting solvent may introduce impurities into an analysis in which case it may be necessary to re-distil or purify the solvent before it's used, eg. ethyl acetate (85).

**PRACTICAL ASPECTS OF LIQUID-LIQUID EXTRACTION**

As previously described, for successful extraction, the pH value of the aqueous phase must be adequately adjusted to ensure that the analyte is un-ionised to allow partition into the organic phase. It was shown with the extraction of a molecule of a carboxylic acid moiety that when the pH is below 3, the compound can be extracted into an organic solvent, whereas above pH 5 it remains in the aqueous phase (79).

It is important that rapid equilibrium of extraction is achieved and the organic solvent is usually present in excess since a large surface area is preferable. Mixing can be carried out by hand, by a mechanical shaker or by a vortex mixer. Formation of emulsions may occur, after mixing, which will cause problems since they are difficult to break and cannot always be separated by ultra-sonication or centrifugation. The solvent is removed by various methods - evaporation, freezing in liquid nitrogen, pipetting etc. Problems may arise in this area due to:

1) The conditions used to evaporate the solvent may lead to low recovery of the analyte.
2) If there is a safety hazard involved in handling the solvent since most of them are toxic and/or inflammable. The vapours therefore need efficient ventilation.
3) Removal of waste solvents can be expensive and troublesome.

**SALTING OUT**

Since solvent evaporation may be time consuming, salting out is a technique which may be used to separate or form a solid boundary between two phases (water and water-

soluble solvent eg. ethanol). Studies were reported which have shown the success of this method: in the analysis of histamine $H_2$ receptor antogonists cimetidine (86). and oxmetidine (87). Following extraction into an organic solvent and re-extraction into an acid phase, acetonitrile (200$\mu$l) is added to the acid followed by about 5g of solid potassium carbonate. The addition of the carbonate increases the ionic strength and polarity of the aqueous phase which forces the acetonitrile containing the analytes to form a discrete layer on the surface. This layer is recovered and injected directly into the chromatograph. In a study by Rustum, caldrazine was extracted from whole blood by adding 500$\mu$l of acetonitrile to 1.0ml of whole blood followed by salting-out of acetonitrile by the addition of potassium carbonate in excess (88). It was concluded that using the salting out extraction procedure with no evaporation or derivatization step increased the sensitivity, reproducibility and accuracy of the method significantly.

Liquid-liquid extraction is not suitable for all compounds; for example, highly polar molecules are not usually extracted by this method. The use of a suitable ion paring reagent, however, can extend LLE to molecules of this type (79). It is also possible to form complexes to extract polar molecules so that the polarity of the newly formed molecule is amenable to extraction.

Direct derivatization of drugs in untreated biological samples prior to gas chromatography has been studied and presented in a paper by Vessman et al. (89). This report has discussed derivatization in the sample matrix followed by extraction of the analyte. It also deals with two-phase reactions where derivatization takes place in the organic phase whilst extraction of the analyte is continuing from the aqueous sample. It could be useful to take the principles from this paper and apply them to HPLC analysis.

An attempt to overcome some of the drawbacks of LLE is the use of disposable columns - Chem Elut or Tox Elut (Analytichem International, Harbor City, CA, U.S.A.) columns, which have a diatomaceous earth support. The diluted sample to be extracted is poured through the cartridge and held on the support as a very thin film. The extracting solvent

is then passed through the column, and the high surface area of the film allows very efficient extraction of the analytes of interest. This approach avoids emulsion formation but not the evaporation of the solvent used in the extraction.

**SOLID PHASE EXTRACTION**

An alternative method of isolating the compound of interest is liquid-solid or solid-phase extraction. In this technique, the biological matrix is mixed with an adsorbent, which separates the solid phase and the analyte is then eluted with an appropriate solvent. The effectiveness of this approach depends on (1) the relative affinities of the analyte between the biological matrix and the adsorbent and (2) the relative ease of eluting the compound for subsequent analysis.

According to Whelpton et al. this technique should be seen as an important addition to sample preparation techniques and not as a replacement (90).

LSE is relatively easy to use since separating a liquid and a solid is considerably easier than separating two immiscible liquids. The general approach used today involves the solid adsorbent packed in a small column or cartridge and the flow through this packed bed gives the surface area necessary to ensure a high extraction and clean-up. As a result, most solid phase extraction schemes can have a higher throughput than a comparable liquid-liquid extraction because of the ease of handling the solid phase. In addition to this advantage, the potential for automation is easier compared with other methods of sample preparation.

Classical adsorbents such as carbon, celite and alumina have been used for many years. Although these adsorbents avoid emulsion formation, often the analytes were eluted from the solid phase by organic solvents which still had to be evaporated. Ion-exchange resin-loaded papers were introduced by Dole et al. (91). for early urine screening programmes for detecting misuse of drugs. However, their performance was unreliable. Recently more reliable products with various types of ion exchange resins incorporated into a

PTFE membrane have been introduced (92). These are packaged into a disposable filter through which the sample is passed and the analytes extracted prior to analysis or components of the matrix removed, eg. metallic cations to prevent decomposition of analytes in the remaining sample. The recent trend has been to use chemically modified silicas for sample preparation and they are available commercially from an increasing number of vendors.

The main advantages of LSE are selectivity due to a wide choice of bonded phases available, speed and being able to process a large number of samples efficiently in a relatively short time (93). LSE can cope with a wide range of analyte polarities. For example, Doyle et al. (94). extracted temalastine and eight of its metabolites including compounds with carboxylic acid and primary amine functions. An interesting use of LSE is for clean-up prior to NMR analysis for metabolite identification in urine (95, 96). A disadvantage of the technique is the need to centrifuge all thawed plasma samples prior to analysis to remove fibrins and other particulate matter which might block the flow of solvents through the sorbent bed.

Liquid-liquid extraction, in comparison is a major sample preparation technique which is very versatile and well documented. However because of the drawbacks, it is starting to be replaced by LSE and HPLC sample preparation for routine applications with high throughput. Although LLE can be used to assay large numbers of samples efficiently and can be adapted to batch-mode operation, it still tends to be labour intensive and slow due to the number of transfer steps involved.

**HPLC SAMPLE PREPARATION**

A liquid chromatograph is also capable of separation and clean-up so that it can either enhance any preparation scheme already taken on or it can perform both the extraction and quantification stages. It can be argued that elaborate purification procedures make the separating power of a chromatographic column redundant, although insufficient purification can lead to deterioration of the column efficiency (97). Thus a balance must

be achieved between sample clean-up by the column and external techniques. Where there is a strong element of selectivity and trace enrichment in the sample preparations step, fewer demands will be placed on the quality of the chromatography. On the other hand, the choice of a selective detection mode may simplify the preparation stage to a point where it is relatively easy to automate for greater throughput (97).

In on-line sample preparation by HPLC standard equipment plus a switching valve and timed events can be used to prepare and analyze biological samples. In a paper by Ulhein (98). there are three main approaches that can be used. These are as follows:-

1) Solvent programming: where the column and stationary phase are not changed. Also trace enrichment is the simplest method of HPLC sample preparation where the sample, either diluted or neat, is pumped onto a pre-column of the chromatograph.
2) Column Switching: where the mobile phase is constant but different columns are used to effect the separation. After direct plasma injection on a precolumn, the segment of interest is switched to the analytical column (99). The recovery and reproducibility in this technique are good, however this complicates the instrumentation. The details, advantages and disadvantages of this method have been reviewed recently (100, 101).
3) Column and solvent switching where one chromatograph provides a crude separation of the sample and a fraction containing the analytes is transferred to another chromatograph for the analytical separation. The approach consists essentially of two chromatographs with a short manually packed pre-column that has a low strength mobile phase by which the analytes are retained without precipitation of protein; simultaneously another pump delivers the final mobile phase through the analytical column, and the two columns are connected by a switching valve. Both systems are run in parallel and the eluent containing the analyte fraction is switched onto the analytical column for the final separation.

Boxcar chromatography is a further refinement of column switching. This involves the partial separation of a fraction containing the compounds of interest on a first column with diversion of the resulting fraction to a second column (102). The concept of this approach is that the second column will be filled with several samples at the same time, but the separation that is undertaken by the second column will be simpler than with a conventional extract.

**AUTOMATION OF SAMPLE PREPARATION**

Of all the sample preparation techniques discussed so far, HPLC and LSE are the two major techniques that have the greatest potential for automation. Other methods such as protein precipitation and ultrafiltration are difficult to automate or may not be easily justified on the basis of a cost/benefit analysis.

Liquid-liquid extraction can be automated using robotics or continuous flow methods but these are not really as financially viable as automation involving LSE and HPLC which have seen many recent developments in this regard. To justify automation fully the laboratory must have a large throughput for the assay to be automated. The automation of an assay with only a few samples would not be cost effective. In a paper by Agasoster et al. a fully automated technique for HPLC of whole blood and plasma is described (103). Samples were automatically injected into a dialyser where proteins and blood cells were removed. The dialysates were then concentrated on a small column prior to analysis. The technique was used for the determination of oxytetracycline in whole blood and plasma.

In the future it is hoped that more techniques of sample preparation will be seen. Some new techniques are currently being tested and developed. Supercritical fluid extraction is one which offers promise as a sample preparation technique for biomedical analysis. SFE has been used for several years in the food industry for industrial-scale processing such as removal of caffeine from coffee beans (104). High-performance affinity chromatography is a technique used to purify biologically active molecules that combines

the specificity of affinity chromatography with the resolution and speed associated with HPLC. It is also a promising sample preparation technique where the analyte may be selectively extracted and eluted on-line using a 'designer' type column. Another potentially useful type of extraction scheme involves micelles which are used in micellar liquid chromatography (MLC) and a variety of analytical methods. Antibody-mediated extraction or immunoextraction has been used for the past few years for the selective extraction of endogeneous compounds such as leukotrienes and prostaglandins from biological samples. As immunoextraction offers a more selective method of extracting compounds, analytical chemists are now using it to extract drugs and other molecules. Finally, microwave processing is an 'up and coming' fast and clean procedure for the removal of proteins from a sample (105). Microwave processing can be used to accelerate diffusion processes, to speed up chemical reactions, to solubilize solid samples, to thaw frozen samples and to denature proteins. Microwave irradiation as a method of solubilization is a technique in which energy (heat) is used for the extraction of compounds from solid materials. Furthermore the technique is an excellent way of thawing frozen biological samples. Thus the future holds many hopeful new prospects in this field.

## CONCLUDING REMARKS

High-Performance Liquid Chromatography is one of the most important analytical techniques used in drug analysis. Its ability to simultaneously analyse for and discriminate closely related drugs and metobolites together with its high precision and accuracy and its easy applicability for new drug assays is well recognized today.

This topic has been reviewed extensively with regard to eluent optimization, reproducibility and technical considerations associated with HPLC drug analysis. The need for sample preparation and plasma clean-up has also been stated. The various techniques currently used together with practical aspects of liquid-liquid extraction have been described, supported by relevant journals and studies.

However, of all the literature used in this review, most of the information is associated with the removal of drugs from a biological matrix for subsequent analysis. Until now, only one other study has concentrated on the characterisation of stationary phases concerned solely with separating extracted plasma components (106). This study was carried out using on-line solid phase extraction and the idea of experimenting with a different extraction scheme (liquid-liquid extraction) evolved from that work.

This project aims to evaluate the effects of varying the type of column (C-18 or CN) and the mobile phase composition on drug-free plasma profiles after liquid-liquid extraction. In the mobile phase, the variables were the type of organic modifier (acetonitrile or methanol) and either the percentage organic modifier or the pH of the aqueous component.

# CHAPTER 4

## EXPERIMENTAL, RESULTS AND DISCUSSION

## EXPERIMENTAL

### Chemicals and Reagents

The solvents chloroform, hexane, diethyl ether, methanol, acetonitrile and ethyl acetate (HPLC grade) were supplied by Labscan Analytical Sciences (Dublin, Ireland). AnaLaR - grade sodium acetate and analytical grade acetic acid were obtained from BDH (Poole, U.K.) and Riedel de Haen (Hannover, Germany) respectively. The salts lithium chloride, sodium chloride, sodium carbonate, sodium sulphate and potassium carbonate were also supplied by BDH.

The plasma was received from the blood bank and deionised water was obtained regularly by purifying freshly distilled water using the Millipore (Millford, MA, USA) Milli - Q water purification system.

### Plasma Solutions

Frozen drug-free plasma was thawed at room temperature. A tray was filled with glass test tubes and 200µl of the thawed plasma was pipetted into each. The test tubes were then covered with parafilm and the tray was placed in a freezer. Test tubes were removed and thawed on a daily basis when required.

### Extraction Procedure

For each sample analysed, the following liquid-liquid extraction procedure was carried out beforehand. 800µl of the solvent (diethyl ether) was added to the thawed plasma (200µl) in a test tube. This mixture was then vortexed for one minute exactly and centrifuged for ten minutes. The organic layer was carefully removed and transferred into a small vial. The solvent was evaporated in a water bath by bubbling a stream of nitrogen through the sample. This "drying down" process was performed in a fume cupboard. When all the solvent had evaporated, the sample was reconstituted in

100μl of the mobile phase. The resulting solution was then vortexed for one minute. The sample was then ready for injection and 20μl was injected using a 25μl Hamilton microsyringe. Each sample was extracted and analysed in quadruplicate.

**Instrumentation and Operating Conditions**

To make up the mobile phase, stock solution of 0.025 M sodium acetate was prepared. This was done by dissolving the appropriate weight of sodium acetate in deionised water The pH of this solution was altered by addition of acetic acid (0.1M). The change in pH was measured using a standard glass electrode which was calibrated every day. The aqueous standards (pH4 and pH7) were prepared on a weekly basis. The mobile phase was then made by adding the correct percentage volume of methanol or acetonitrile to the aqueous component with adjusted pH. The solution was then filtered using a 0.45μm filter. Finally, the solution was degassed by sonication before use.

The plasma extracts were separated on a Spherisorb (Phase Separations, Clywd, U.K.) C-18 column (10μm) and a Spherisorb CN column (10μm). (Both columns were 250mm x 4.6 mm I.D.). The guard column was a Chrompak (Middelburg, Holland) which contained a stainless steel column dry-packed with Corasil (Waters, Milford, MA, U.S.A.) C-18 material (37 - 50μm) or Supelco (Bellaforte, PA, U.S.A.) CN material (24 - 40μm).

The mobile phase was delivered using a Waters Model 501 HPLC pump. The plasma samples were injected into a Rheodyne (Cotati, CA, U.S.A.) injection valve with a 20μl loop. The mobile phase flow rate was kept constant at 1.0ml/minute. The centrifuge was provided by BHG - HEMLE model z230.

The ultraviolet detector was Shimadzu ( Kyoto, Japan) model SPD - 6A with a sensitivity setting of 0.01 a.u.f.s. The chromatograms were recorded using a Philips (Eindhoven, the Netherlands) single pen recorder Model PM8251 chart recorder.

Before the extraction of the plasma, a range of extracting solvents were investigated. Instead of using plasma, 200$\mu$l of water was mixed with the solvent and after extraction, the samples were injected and their chromatograms compared to choose the extracting solvent containing fewest residual peaks.

Having chosen the most suitable extracting solvent, the cyano (CN) column was used first to separate the plasma extracts. The effect of changing the percentage of organic modifier (acetonitrile and subsequently methanol) was studied, keeping the pH value of the aqueous component constant at 6. The percentages of organic modifier were varied from 30% to 80%.

Then the effect of changing the pH of the aqueous component of the eluent, on the CN column, was studied. This time the percentage organic modifier was kept constant at 50%. The pH was varied from 3 to 7 and this was done using acetonitrile and subsequently methanol as the organic modifier.

The column was then replaced by a C-18 column and all analyses that were performed on the CN column were repeated on the C-18 column. The chromatograms resulting from each analysis were assessed in terms of peak height and the number of interfering peaks in the area of interest. The area of interest was from five minutes to twenty minutes (since this eliminated the large plasma peak which eluted before five mins). Graphs were constructed for comparison of the results.

In an additional part to this project the technique of salting-out was studied. Using five different salts (listed earlier) a saturated solution was made of each and a small amount (40 $\mu$l and subsequently 80 $\mu$l) of this solution was added to the plasma sample before the extracting solvent. This mixture was vortexed for one minute and the extraction procedure continued as before. Again the resulting chromatograms were assessed and compared.

**RESULTS TABLE 1**

Effect of varying the percentage of methanol in the mobile phase using a C-18 column.

| % METHANOL | MEAN NO. OF PEAKS |
|---|---|
| 30 | 1.66 |
| 40 | 2.0 |
| 50 | 2.33 |
| 60 | 4.66 |
| 70 | 6.33 |
| 80 | 7.33 |

**RESULTS TABLE 2**

Effect of varying the percentage of acetonitrile in the mobile phase using a C-18 column.

| % ACETONITRILE | MEAN NO. OF PEAKS |
|---|---|
| 30 | 4.33 |
| 40 | 3.66 |
| 50 | 3.66 |
| 60 | 5.0 |
| 70 | 7.33 |
| 80 | 9.0 |

**RESULTS TABLE 3**

Effect of varying the percentage of acetonitrile in the mobile phase using a CN column.

| % ACETONITRILE | MEAN NO. OF PEAKS |
|---|---|
| 30 | 1.33 |
| 40 | 2.33 |
| 50 | 2.33 |
| 60 | 1.0 |
| 70 | 0 |
| 80 | 0.75 |

**RESULTS TABLE 4**

Effects of varying the percentage of methanol in the mobile phase using a CN column.

| % METHANOL | MEAN NO. OF PEAKS |
|---|---|
| 30 | 1.0 |
| 40 | 1.25 |
| 50 | 1.5 |
| 60 | 1.33 |
| 70 | 2.0 |
| 80 | 1.0 |

**RESULTS TABLE 5**

Effects of varying the pH of the aqueous component of the mobile phase using methanol on a C-18 column.

| pH | MEAN NO. OF PEAKS |
|---|---|
| 3 | 1.33 |
| 4 | 1.66 |
| 5 | 1.33 |
| 6 | 2.33 |
| 7 | 3.0 |

**RESULTS TABLE 6**

Effects of varying the pH of the aqueous component of the mobile phase using acetonitrile on a C-18 column.

| pH | MEAN NO. OF PEAKS |
|---|---|
| 3 | 5.66 |
| 4 | 6.0 |
| 5 | 5.33 |
| 6 | 3.66 |
| 7 | 3.33 |

**RESULTS TABLE 7**

Effects of varying the pH on the aqueous component of the mobile phase using acetonitrile on a CN column

| pH | MEAN NO. OF PEAKS |
|---|---|
| 3 | 2.5 |
| 4 | 3.25 |
| 5 | 3.0 |
| 6 | 3.0 |
| 7 | 1.0 |

**RESULTS TABLE 8**

Effects of varying the pH of the aqueous component of the mobile phase using methanol on a CN column.

| pH | MEAN NO. OF PEAKS |
|---|---|
| 3 | 1.0 |
| 4 | 2.66 |
| 5 | 2.33 |
| 6 | 1.5 |
| 7 | 1.0 |

**RESULTS**

Results Tables 1, 2 show the number of peaks obtained with a variation in the percentage of methanol and acetonitrile on a C-18 column (respectively). The number of peaks obtained with the same variation of methanol and acetonitrile on a CN column are demonstrated in Results Tables 3 and 4 respectively.

The effect of pH variation, in the aqueous component of the mobile phase, on the number of peaks using methanol and acetonitrile is shown for a C-18 column in Results Tables 5 and 6. Also Results Tables 7 and 8 give the number of peaks obtained for the same pH variation on the CN column. These data are presented graphically in Figures 7 to 14.

The two columns used, C-18 and CN, were chosen for the following reasons: The most popular choice for reversed-phase separations are columns containing silica particles surface bonded with C-8 or C-18 alkyl chains. The difference between these two is inconclusively significant and hence a comparison between the two was not pursued. Selectivity differences are less likely between these two packing materials and other, more polar bonded phases such as cyano or phenyl which also can be used in the reversed-phase mode if adequate water is incorporated into the mobile phase (51). Hence the CN column was chosen as a contrast to C-18 because of its more polar bonded phase. The cyano column may be used in either normal-phase or reversed-phase modes and is known as an almost universal stationary support (107).

Both CN and C-18 columns were obtained from the same manufacturer to avoid any differences that might occur due to separate manufacturers.

To allow for a constructive and warranted comparison, methanol and acetonitrile were chosen as the organic modifiers since they differ in selectivity, solvent strength and also viscosity. They are both popular solvents and have relatively low viscosity and are readily available with excellent purity. The range used (30% - 80%) covers the proportions that

would be used in this type of analysis. Sodioum acetate was chosen as the buffer for the aqueous component of the mobile phase since it is a typical buffer which is widely used in biopharmaceutical applications. The variation in pH ranged from 3 - 7 which covers the range where most separations would be carried out.

The samples were analysed in quadruplicate ie; four extractions of blank plasma were analysed under the same operating conditions. This ensured that the accuracy of the result was more acceptable without much waste in the analysis time. A balance must be struck between reproducibility and time of analysis.

The criterion for peak measurement should be stated. The number of peaks eluting in the time between five and twenty minutes was counted and assessed. This allowed the exclusion of the early eluting plasma peak which was always present before the five minute mark. The peak heights were measured to give an estimation of how the sizes of the peaks differed. It would have been useful to incorporate the peak heights with the number of peaks in the overall assessment of the effects that occurred, but it appeared to be practical and an easy measure of interfering peaks to plot the number of peaks against the change in either pH or percentage organic modifier.

It was also important to distinguish between baseline deviations or 'blips' and small peaks which were valid. It was therefore decided to consider all peaks that had a peak height greater than 1mm on each chromatogram. This value may seem small but many definite peaks were small in height and on the condition that the baseline was very steady and unlikely to deviate this much, this value proved acceptable.

Finally several salts were used to determine the effect of "Salting Out" and to compare one salt to the next. The results of this endeavour is described in the discussion.

# PLOT OF PEAK NUMBER Vs % ORGANIC MODIFIER.

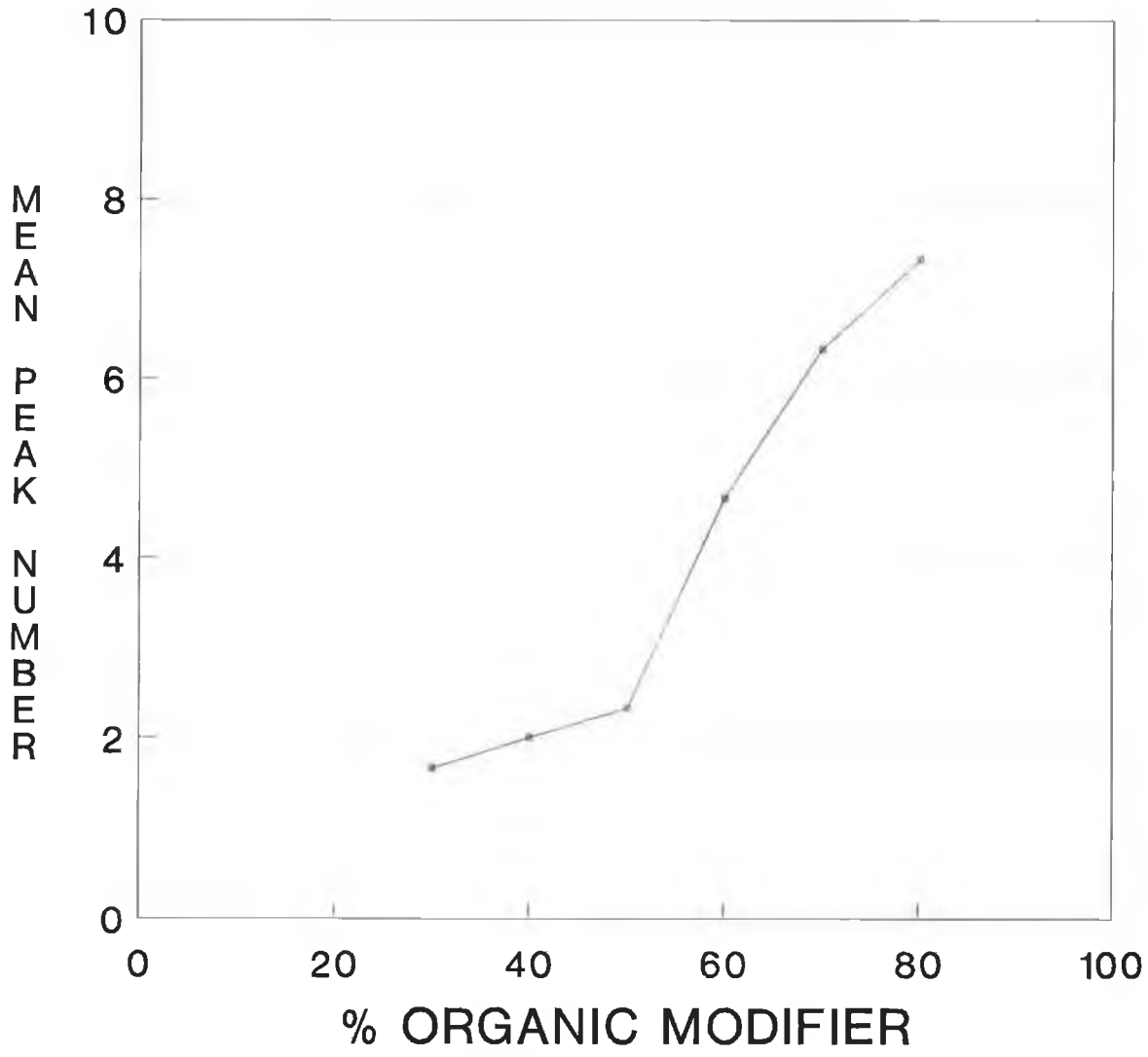



FIGURE 7

C-18 COLUMN, VARYING % MeOH,
MOBILE PHASE=.025 M ACETATE
BUFFER; pH=6

# PLOT OF PEAK NUMBER Vs % ORGANIC MODIFIER.

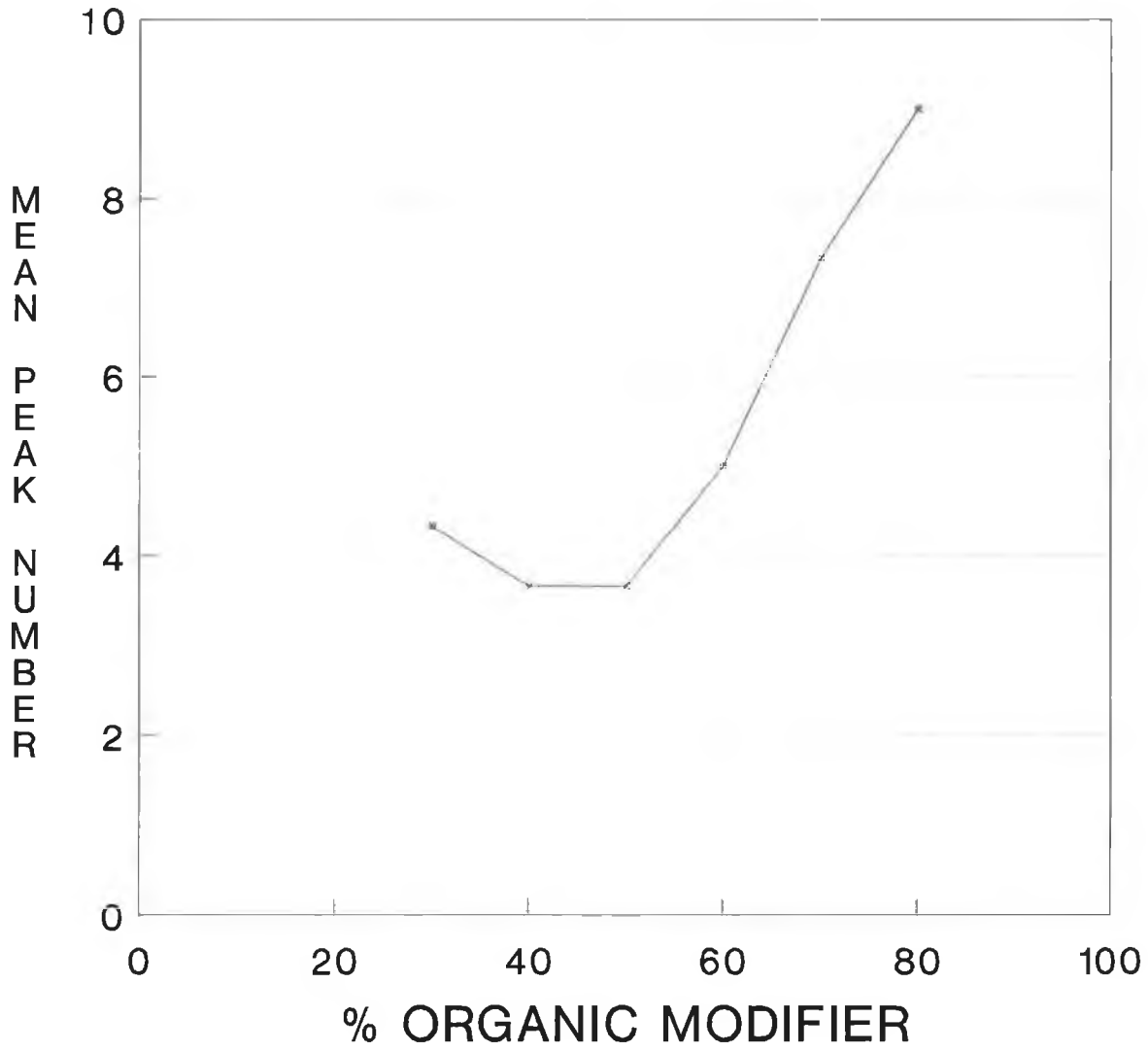



FIGURE 8

C-18 COLUMN,VARYING % ACN,
MOBILE PHASE=.025 M ACETATE
BUFFER; pH=6

# PLOT OF PEAK NUMBER Vs % ORGANIC MODIFIER.

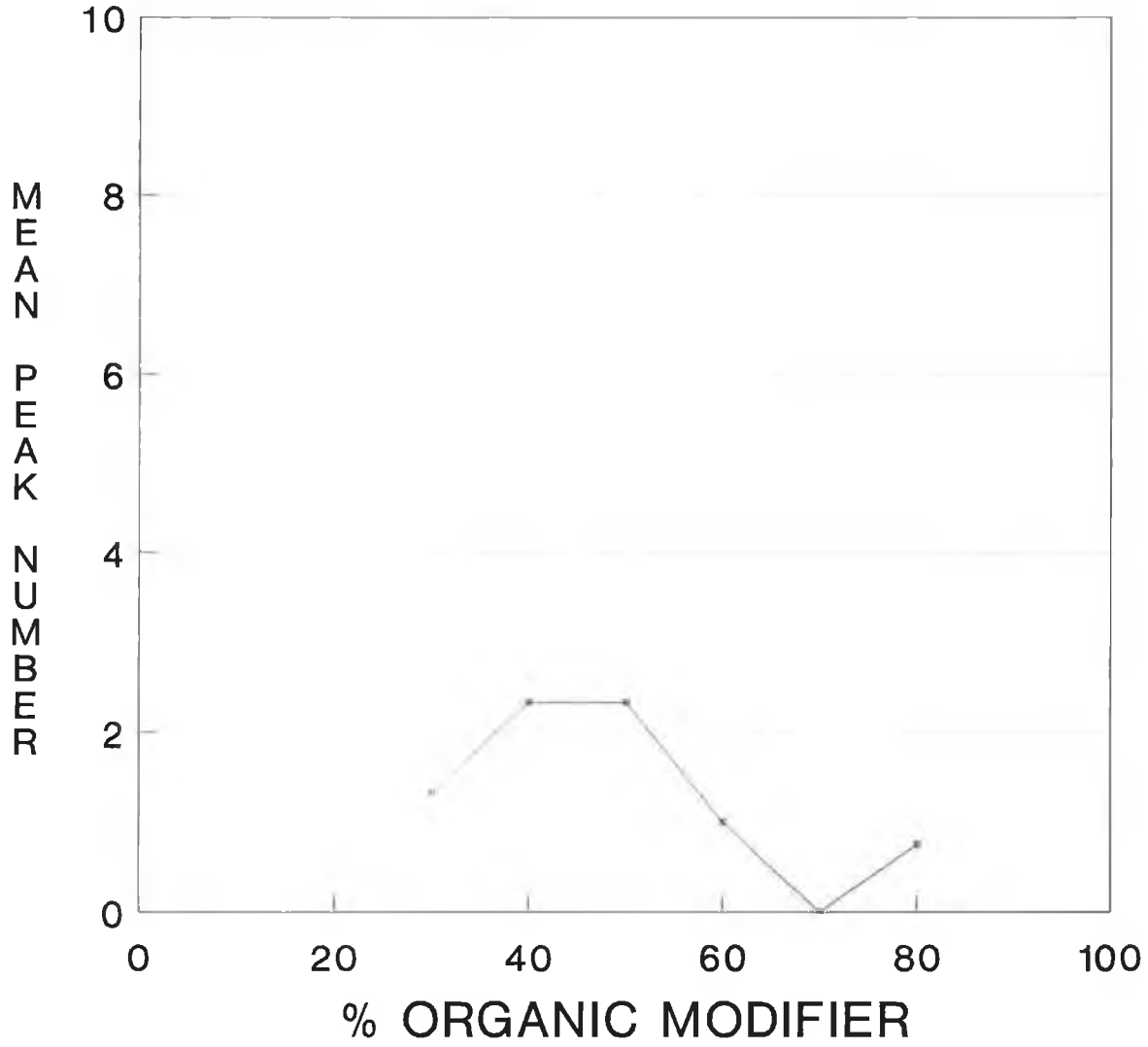



FIGURE 9

CN COLUMN,VARYING % ACN,
MOBILE PHASE=.025 M ACETATE
BUFFER; pH=6

# PLOT OF PEAK NUMBER Vs % ORGANIC MODIFIER.

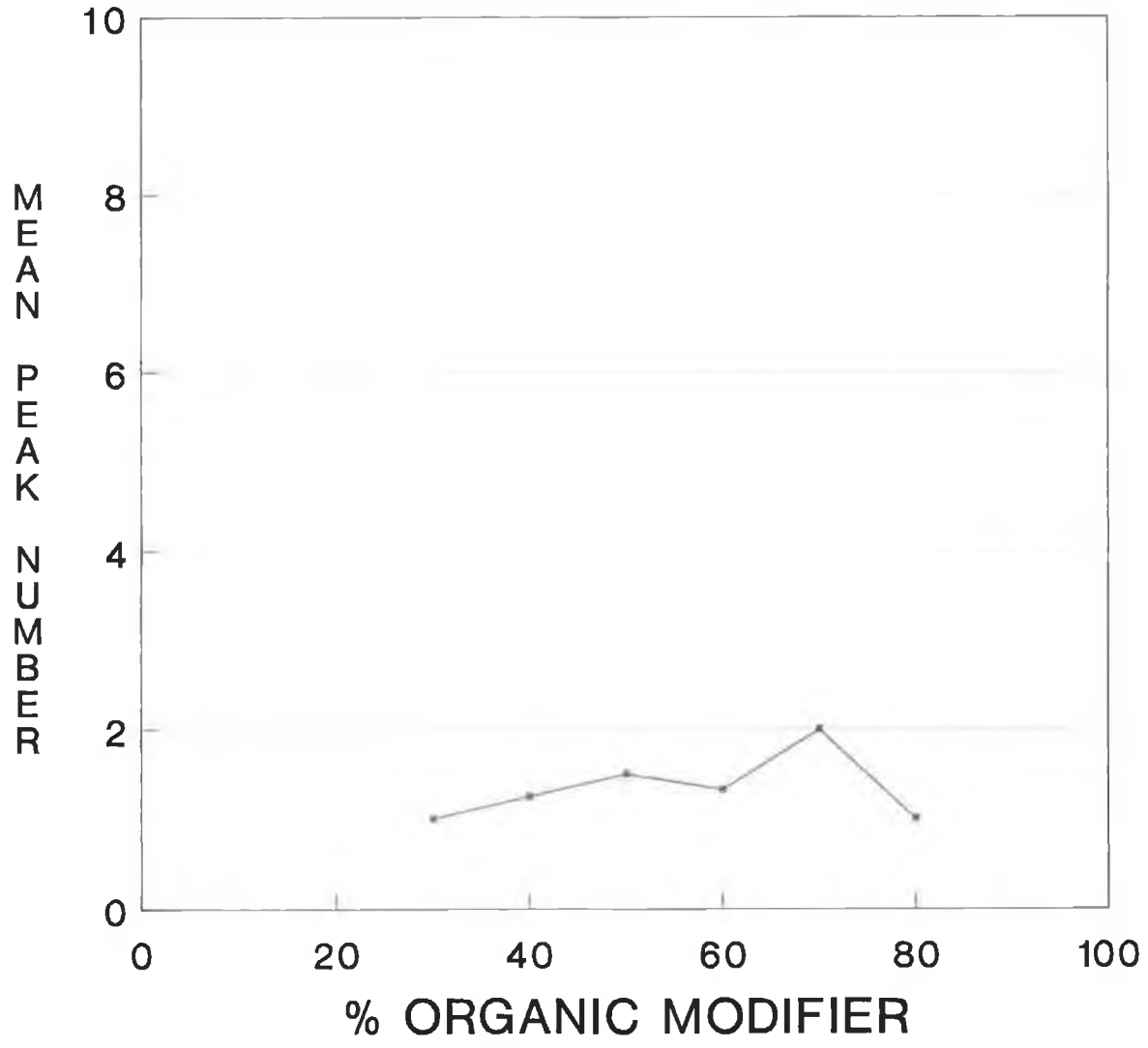



FIGURE 10

CN COLUMN,VARYING % MeOH,
MOBILE PHASE=.025 M ACETATE
BUFFER; pH=6

# PLOT OF PEAK NUMBER Vs pH VALUE

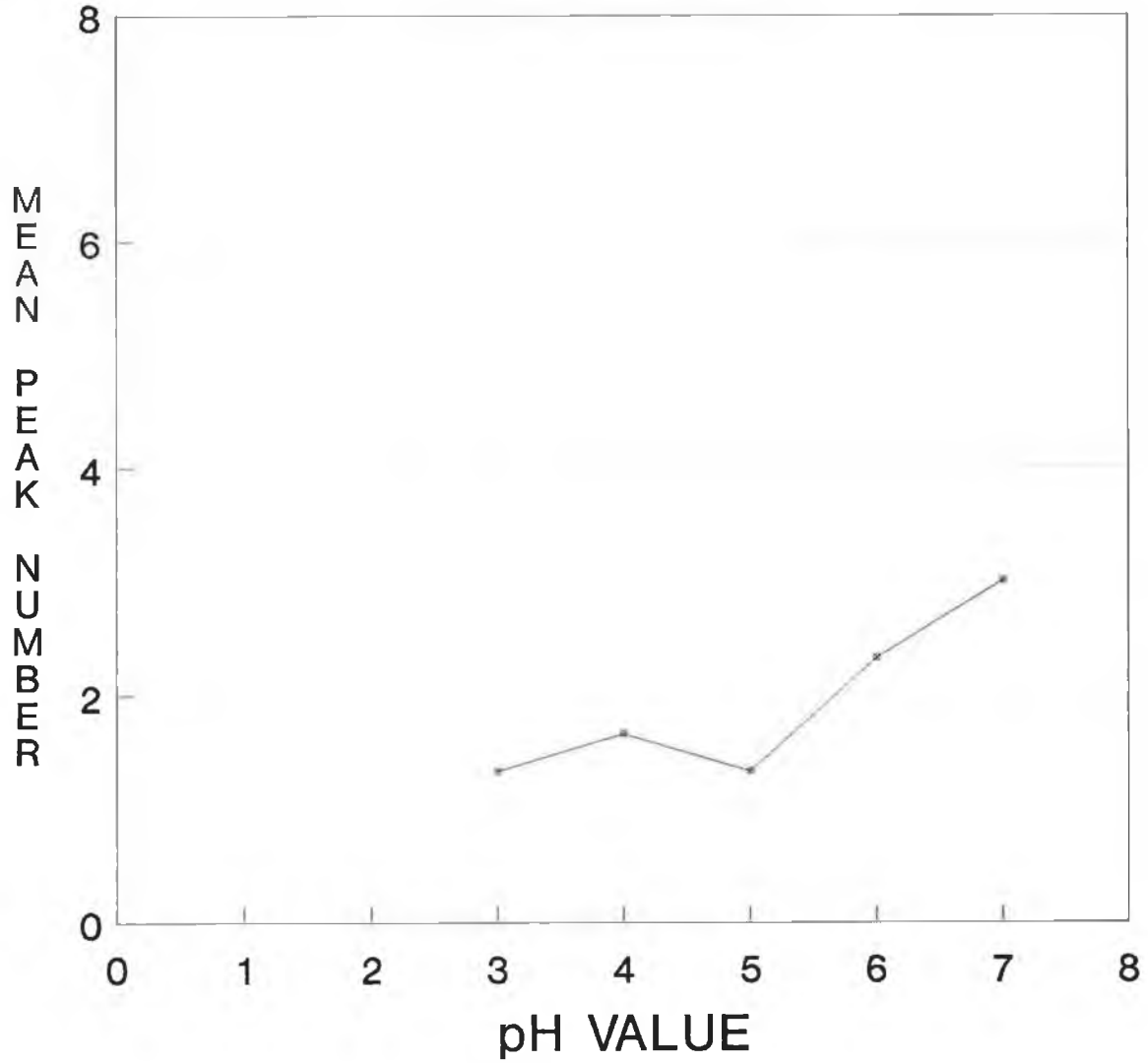



FIGURE 11

C-18 COLUMN, VARYING pH.
MOBILE PHASE=.025 M ACETATE BUFFER-METHANOL (1:1).

# PLOT OF PEAK NUMBER Vs pH VALUE

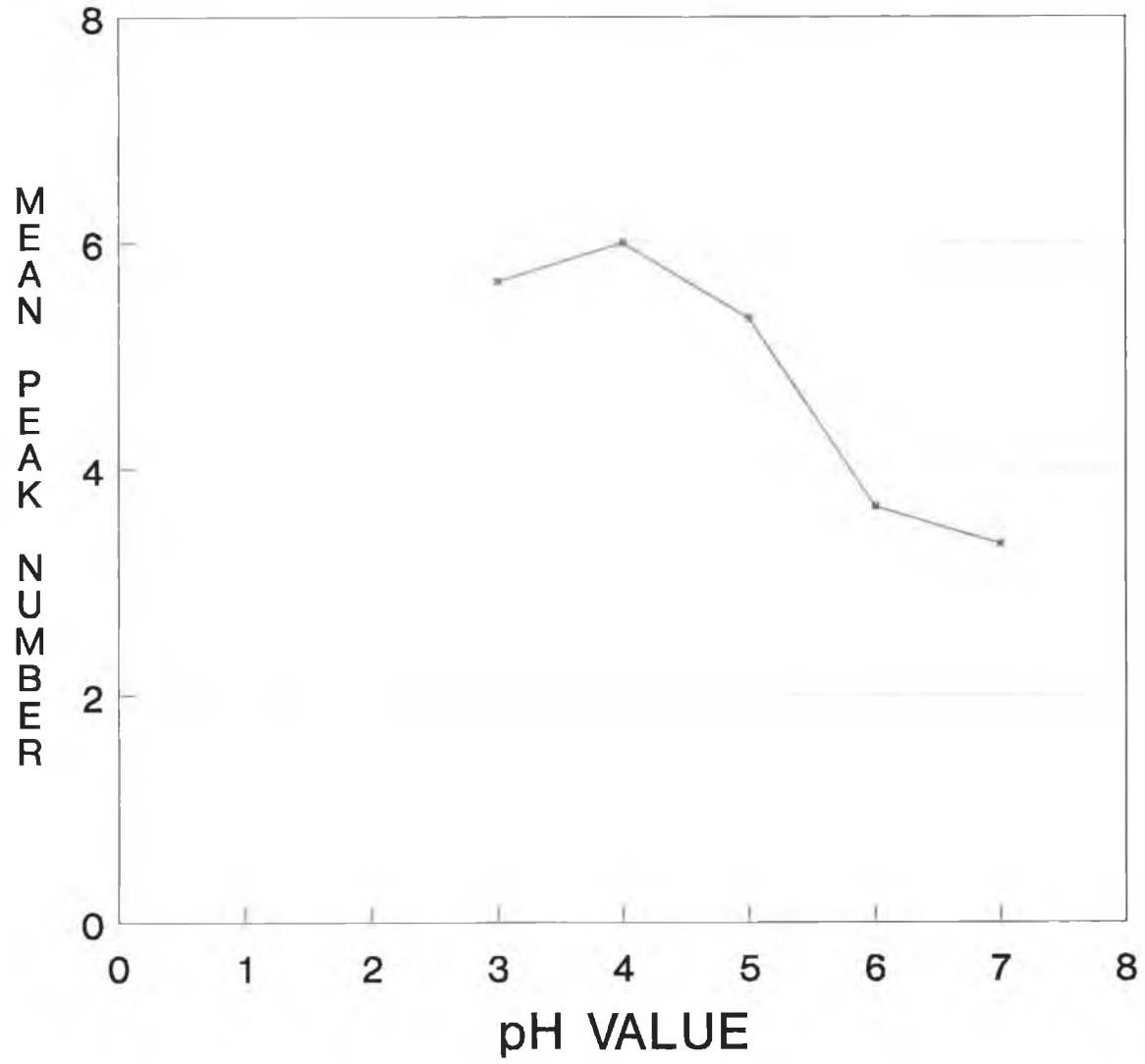



FIGURE 12

C-18 COLUMN, VARYING pH.
MOBILE PHASE=.025 M ACETATE BUFFER-ACTEONITRILE (1:1).

# PLOT OF PEAK NUMBER Vs pH VALUE

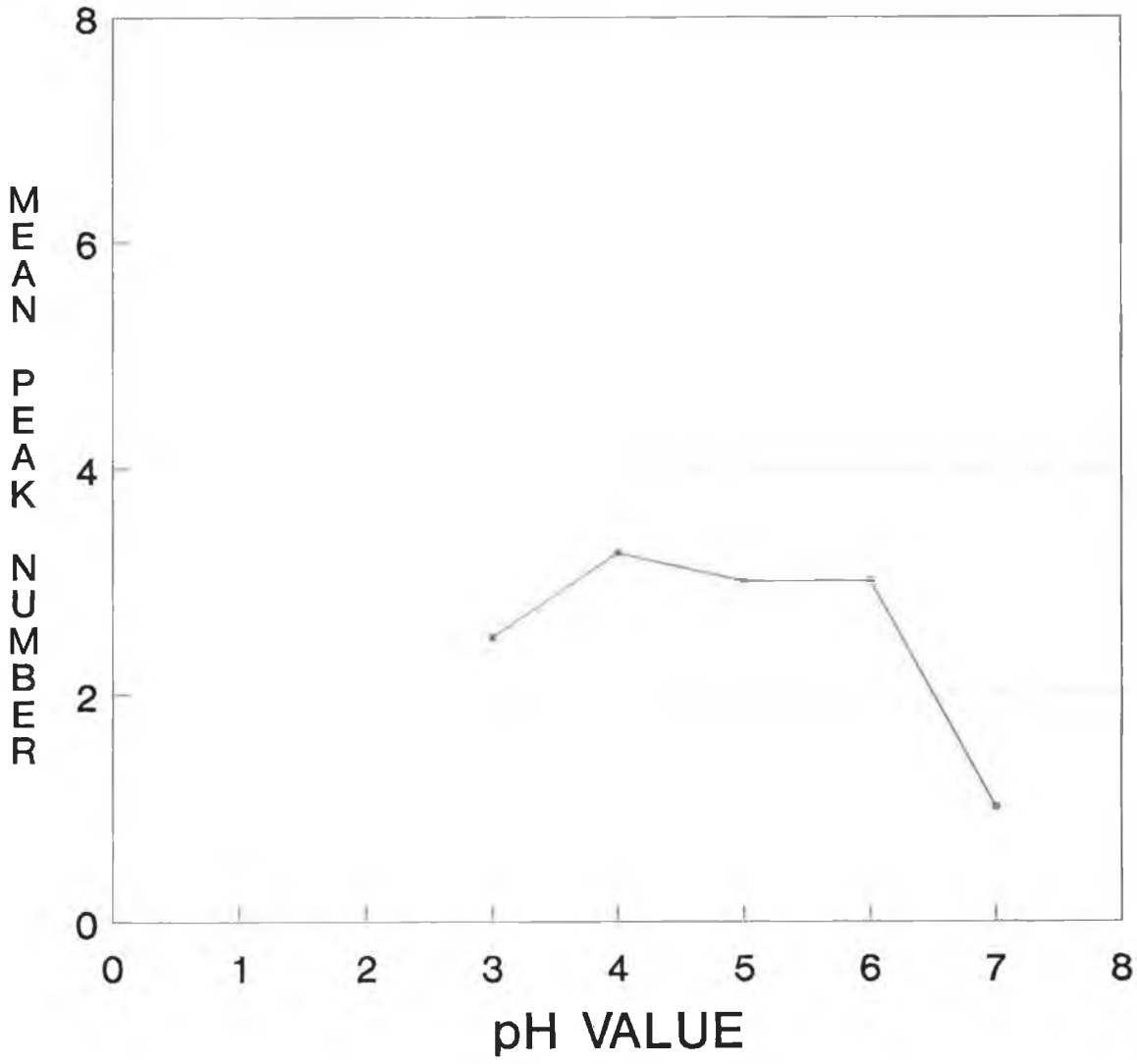



FIGURE 13

CN COLUMN, VARYING pH.
MOBILE PHASE=.025 M ACETATE BUFFER-ACETONITRILE (1:1).

# PLOT OF PEAK NUMBER Vs pH VALUE

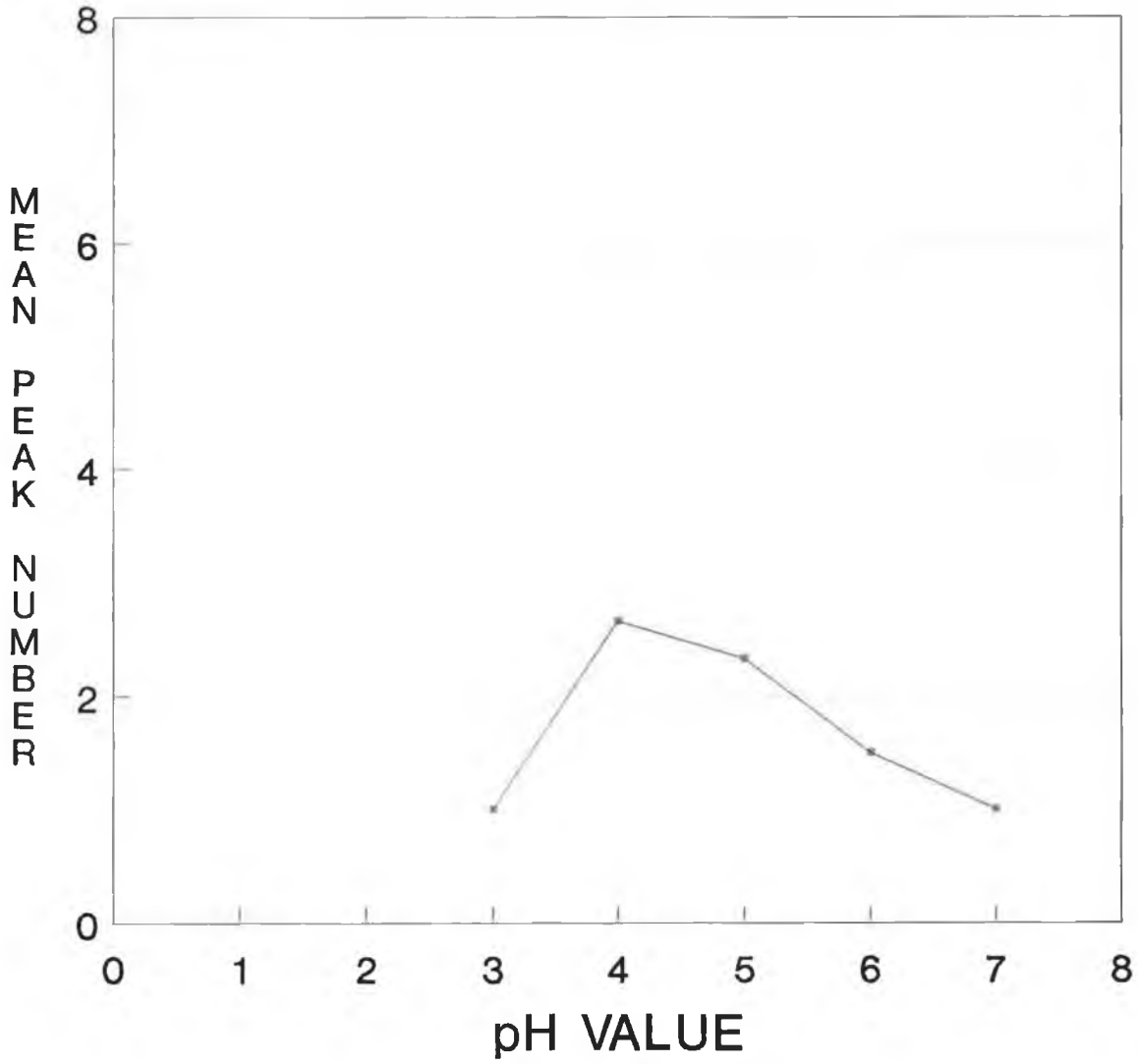
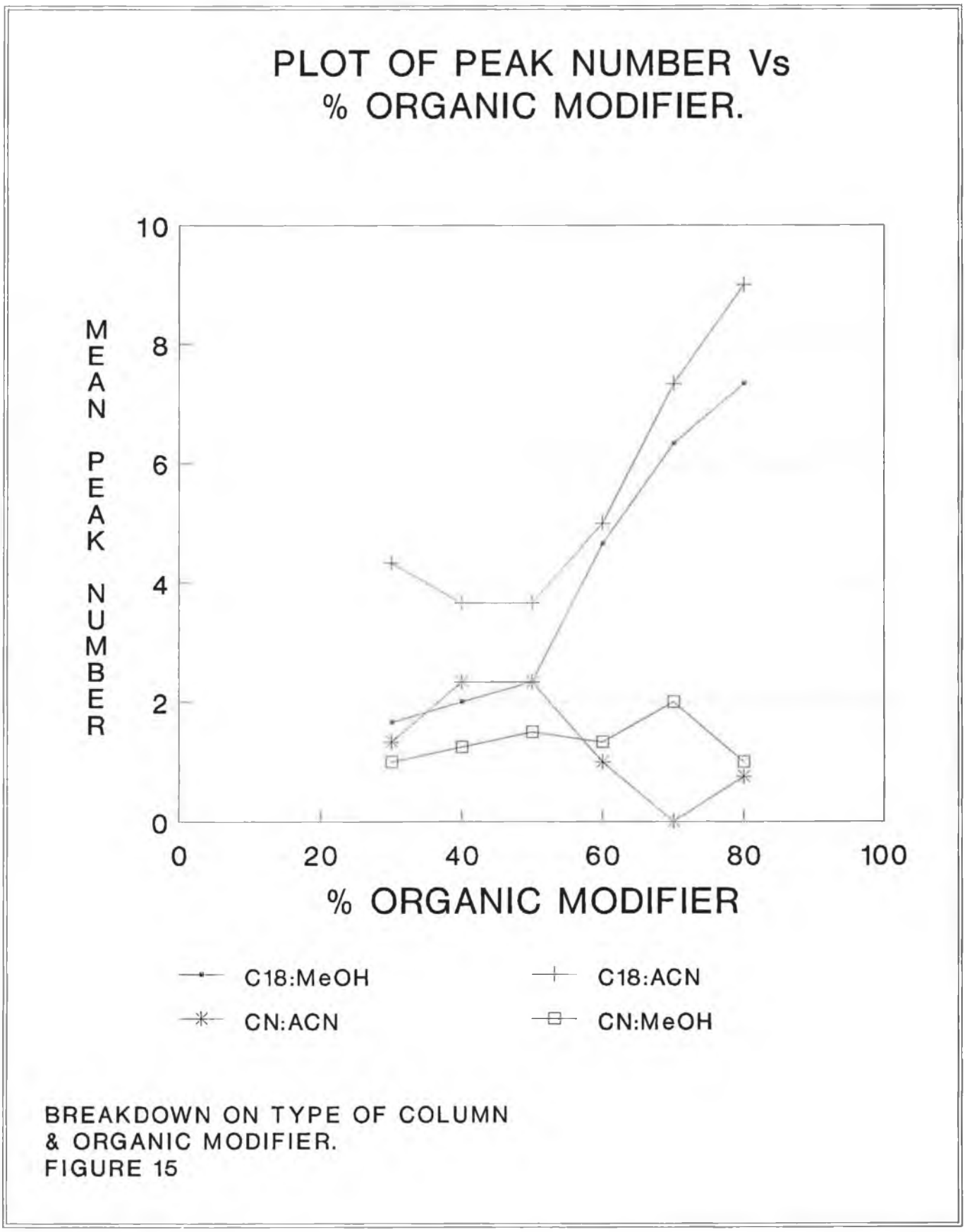
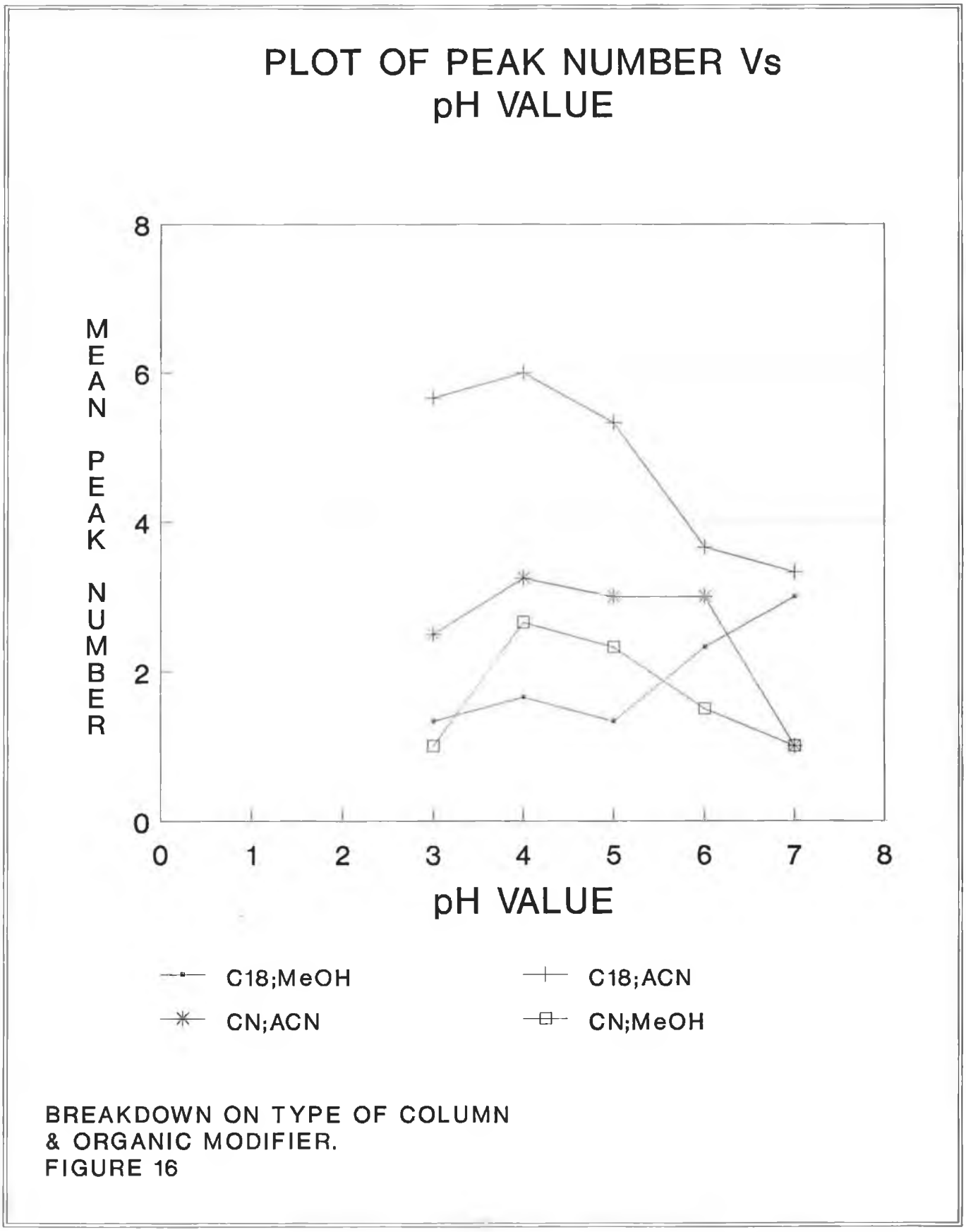



FIGURE 14

CN COLUMN, VARYING pH.
MOBILE PHASE=.025 M ACETATE BUFFER-METHANOL (1:1).




BREAKDOWN ON TYPE OF COLUMN
& ORGANIC MODIFIER.
FIGURE 15




BREAKDOWN ON TYPE OF COLUMN
& ORGANIC MODIFIER.
FIGURE 16

## DISCUSSION

The first experimental work in this project was the choice of a solvent which could adequately extract an aqueous solution. Of the four solvents tested, the following conclusions were drawn: Cholorform was unsuitable since it was heavier than the aqueous solution and thus fell to the bottom layer after the sample had been centrifuged. Having to remove the upper (aqueous) layer to get to the organic layer below would be very awkward for a large number of samples, so chloroform was ruled out. Hexane was also discredited since the resulting chromatograms consistently showed an unsteady baseline. The use of hexane would make it difficult to distinguish between actual peaks and noise on the baseline. Ethyl acetate was ruled out as a solvent because the baseline was uneven and also because the ethyl acetate may react with parafilm which was used at various stages. Diethyl ether seemed the most suitable of the four solvents and the chromatogram obtained from the water extract showed it to be reasonalby acceptable.

Looking at the results of the variation of the percentage organic modifier on the C-18 column (Figures 7 and 8) it would be expected that as the organic modifier is added to the aqueous component of the eluent, the plasma components should elute quicker since addition of the organic modifier to water decreases the polarity of the eluent thus making the eluent stronger. Water is the weakest eluent on a C-18 column (highly non-polar phase) so as the organic modifier is added to water the number of interfering peaks should be decreasing, since the eluent strength is increasing and thus, any plasma components should be eluting sooner with addition of the modifier. Also, since acetonitrile is a less polar solvent than methanol, one would expect that the addition of acetonitrile to water would produce a more marked reduction or change in the number of peaks obtained due to the more drastic change in eluting strength of the eluent. Methanol should strengthen the eluent as it is added to water, but not as much as acetonitrile should.

However, the graphs drawn represent a different trend in results. Figures 7 and 8 demonstrate that as the organic modifier was added, there was a general increase in the

number of peaks obtained in the chromatograms. The fact that these graphs were the opposite to what was expected could be explained by the unknown nature of the plasma solutes or components. If these plasma solutes or compounds contained in the plasma, were ionic in nature, the trend of increasing peak number with increasing organic modifier could be explained. As the percentage of methanol or acetonitrile is increased in the eluent, the proportion of the buffer ions in the aqueous component, decreases accordingly.

As the number of buffer ions in the eluent decreases, this means there will be less buffer ions competing against the ions present in the plasma extracts for sites on the column. Thus, as the percentage organic modifier increases, the number of competing ions is reduced and as a result, the compounds in the plasma are retained for longer on the column. Thus, there would be an increase in the number of interfering peaks obtained as the percentage of organic modifier increases, assuming the ionic nature of the plasma components. This would appear to be the case since a large increase in peak number was found for increasing acetonitrile and methanol (Figures 7 and 8). The effect of the above theory (in Figure 8) seemed to outweigh the expected decrease in peak number when the percentage acetonitrile increased from 50 to 80%.

Figures 9 and 10 show the effect of changing organic modifier percentage on a CN column. The new column (CN) contains the more polar cyano phase (compared to C-18), which means that water is a stronger eluent on this column. Thus, using the same percentage of organic modifier (eg. 30% acetonitrile) for the two columns should show less interfering peaks using the CN than for the C-18 column. This prediction is supported by the results since the number of peaks shown for the range of percentages of organic modifier (both methanol and acetonitrile) is significantly lower for the CN column. This is because as water is a stronger eluent on the CN column, it will follow that the plasma peaks are eluted quicker than on the C-18 column. Thus, the number of interfering peaks should be less. In Figure 9 as the acetonitrile proportion is increased, a decrease in the number of peaks does not occur until the percentage of ACN is increased beyond 50%. This could be accounted for since below 50%, addition

of acetonitrile did not make a significant increase in the solvent strength, but (in Figure 8) on C-18, since water is a weaker eluent, it did. The addition of methanol to the eluent on a CN column (Figure 10) demonstrated that very little change in the number of peaks was seen with increasing methanol. The explanation for this graph seems clear. Since the addition of an organic modifier to water on the CN column involves less change, compared to C-18, in solvent strength (due to the more polar CN phase), less peaks were expected for this column and less of a change in peak number. Also because methanol is a weaker eluting solvent than acetonitrile (ACN), the effect on the number of peaks by adding methanol should be less still. The effect of increasing percentage organic modifier on number of peaks should be most marked on the C-18 column using acetonitrile.

Comparing the results from column to column using the same organic modifier: methanol (Figures 7 and 10) and acetonitrile (Figures 8 and 9) leads to the following: Figures 7 and 10 show the effect of increasing methanol concentration in the eluent on the C-18 and CN columns, respectively. Methanol variation has a drastic effect on the number of peaks in Figure 7 demonstrating that 30% would be the optimum concentration in the eluent to achieve the best clean-up on the C-18 column. There is very little difference in contrast, varying methanol percentage on the CN column, proving that these separations certainly vary from column to column. This fact is reinforced by comparison of Figures 8 and 9 which involves comparing the variation of acetonitrile from one column to the other. The cyano column gives rise to a small range of variation in peak number and also a lower number of peaks in general. Figures 8 and 9 appear to be almost the exact opposite of each other (apart from the change from 70% acetonitrile to 80%) which indeed highlights the fact that analysis of samples, keeping all operating conditions constant, is dependant on the type of column.

In terms of the most effective clean-up, these graphs demonstrate that using a CN column over a C-18 column is probably the better choice, since the CN column yielded chromatograms where the number of interfering peaks was generally low. Methanol is also the preferred organic modifier over acetonitrile as it is associated with a cleaner

area of interest on the chromatogram. If a C-18 column is to be used, then between 40 and 50% acetonitrile or 30% of methanol should be used in the eluent. If a CN column is being used, then 30% or 80% methanol should be considered for the eluent and if acetonitrile is being used the cleanest extraction profile was found with 70% acetonitrile. The latter was the best overall combination which gave zero as the mean of the number of interfering peaks.

Selectivity differences occur in two columns because of the differences in polarity between C-18 and CN. Selectivity differences may arise between methanol and acetonitrile also mainly because acetonitrile is a hydrogen bond acceptor and methanol is a hydrogen bond donor (51). Figures 11 to 14 demonstrate the variation of the pH of the aqueous component in increments of 1.0 over the range 3.0 - 7.0. The organic: aqueous ratio of the eluent was maintained at 1:1 and both acetonitrile and methanol were used. The ionic strength of the aqueous component was 0.025M.

Generally, there was a decrease in the number of peaks as the pH value increased and a mean of the four graphs would show this. The reason for this relationship is that many of the components within the plasma extracts are acidic in nature and when these endogeneous acidic components are in a more basic mobile phase, they become ionised. When the components are ionised, their interaction with the stationary phase is lessened and they elute more rapidly off the column, with the result that the number of interfering peaks decreased as the pH is increased.

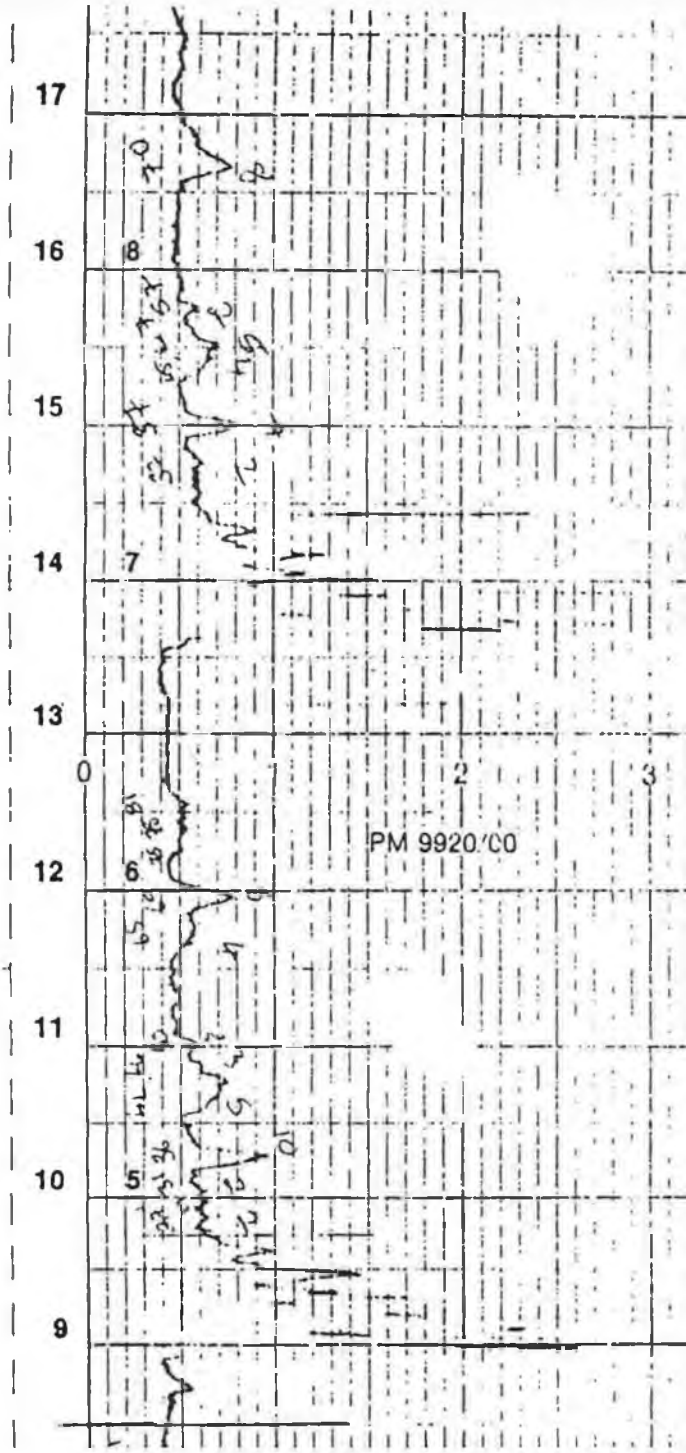
Comparing the two organic modifiers, acetonitrile and methanol, would indicate that on the C-18 column, using acetonitrile, there is a marked decrease from pH 4 to pH 7 (Figure 12). Whereas methanol brings about a slight but definite increase in the peak number as the pH increases (Figure 11). It was also observed that the number of peaks obtained using acetonitrile:water (1:1) was higher than the number of peaks involved using methanol:water (1:1) for the range of pH values. The latter eluent has less solvent strength than the former. Furthermore, on the CN column, the acetonitrile and methanol profiles for pH variation were quite similar (Figures 13 and 14); They both

show an increase in the number of peaks from pH 3 to pH 4 and from pH 4 to pH 7, they tend to decrease. Using methanol on the column resulted in a lower number of interfering peaks over the given pH range.

Comparison of the pH variation from column to column demonstrates that since the eluting power of CN (using for example the 1:1 acetonitrile:water eluent) is stronger than C-18, less peaks are seen over the pH range on the CN column (Figures 12 and 13). These two graphs show an overall decrease in the number of peaks with increasing pH. With methanol as the organic modifier, the difference from column to column is less marked, but still there are more peaks seen overall on the C-18 column than on the CN (Figures 11 and 14). It is interesting that for all four of these graphs representing pH variation, there is a definite increase in the number of peaks when the pH increases from 3 to 4. After this point the number of peaks decreases overall.

To achieve the cleanest separation of plasma extracts with a minimum amount of interfering peaks, methanol is preferred over acetonitrile and the CN column over C-18. The optimum pH value to use for the aqueous component would be 7, since the more basic the eluent, the more chance of ionising the acidic components present in the plasma extracts. Hence, the quicker these components elute, which reduces the number of interfering peaks and generally gives a cleaner area of interest for drug analysis in plasma. The exception according to the graphs is when methanol is being used on a C-18 column in which case a pH 3 is advantageous.

One important comment should be made with regard to evaluating the chromatograms. The peak height was measured but not used in the graphs. Only the number of interfering peaks was considered as a measure of the effect of varying the operating conditions. However looking at the chromatograms it was seen that there was a large variation in peak height and subsequently peak area over the range of results. It was interesting to find that this difference in peak size wasn't random and depended on which column and which organic modifier was used.


17
16
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11
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0
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PM 9920/00

FIG. 17 : CHROMATOGRAMS SHOWING OVERLAP OF PEAKS.
(70% ACN ON C-18)

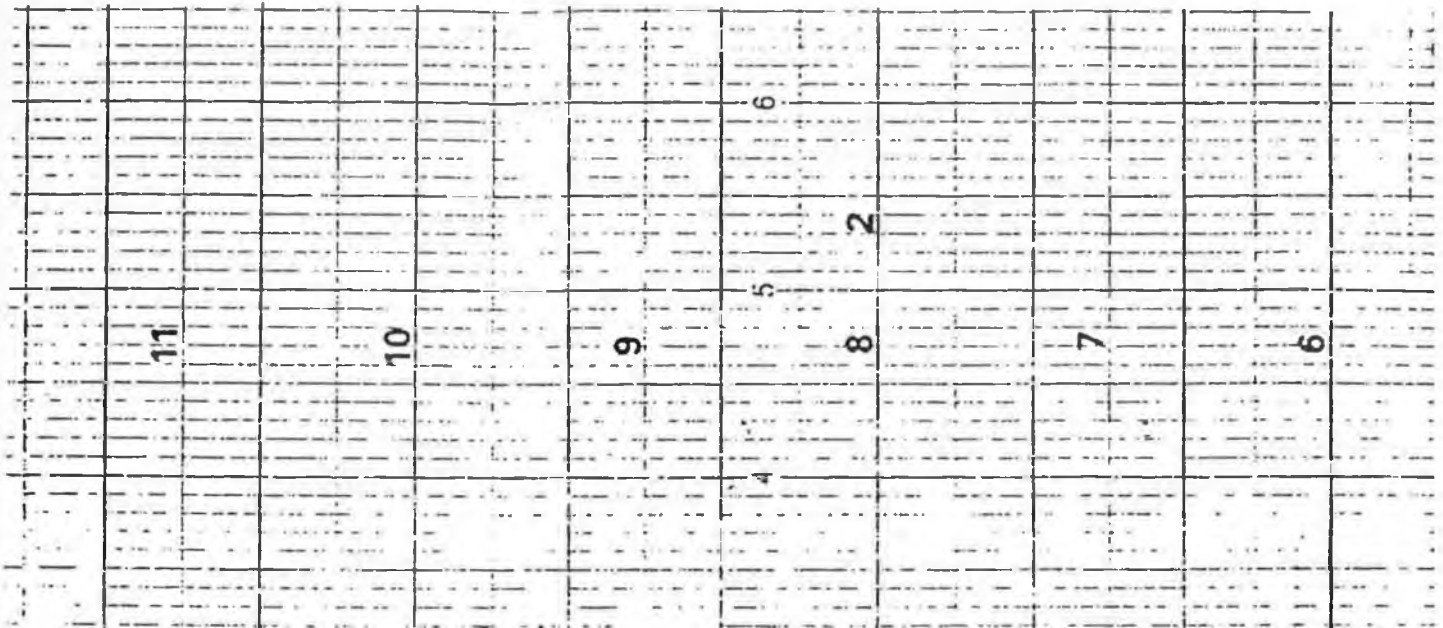

FIG 18: DIFFERENCE IN PEAK SIZE ON (a) CN AND (b) C-18

(a) CN COLUMN

PM 9920/00

(b) C-18 COLUMN

PM 9920/00

From the chromatograms, the CN column was associated with larger peaks in general than the C-18 column. Using acetonitrile these peaks were bigger than the peaks involving the use of methanol. Also the peaks on the CN column were more clearly defined than the C-18 peaks because they were bigger in area. On the C-18 column using methanol, the smallest peaks seemed to present themselves. It made counting the peaks slightly more difficult under these conditions. Using acetonitrile on the C-18 column the peaks were slightly bigger, on average, than those methanol and C-18. There were a lot more peaks (which were also smaller) counted on the C-18 column and a certain amount of these were overlapping which also could have lead to errors in counting them. An example of the overlap is shown in Figure 17 where 70% acetonitrile was used on the C-18 column. Because of this noticeable difference, the number of peaks is not the most accurate criterion for measuring the various effects in this project, since for example, three peaks on the CN column, using acetonitrile, may be as bad or worse than six peaks on the C-18 column using acetonitrile. This is because, if the peaks are much bigger (on CN), they may interfere more with an analysis, than a larger number of much smaller peaks (on C-18) would. An improvement to this project would be to work out an index which takes account of the peak size as well as the number of interfering peaks. Then a better representation of the effects of varying the percentage of organic modifier and the pH could be achieved.

Since the idea of this project arose out of a similar study to evaluate drug-free plasma profiles on HPLC following on-line solid phase extraction, it is necessary at this point to compare the two studies.

The paper by Kelly and Smyth (106). also varied the type of column (CN and C-18) and the eluent composition in terms of percentage organic modifier and pH of the aqueous component using acetonitrile and methanol. The ionic strength of the aqueous component was varied between 0.005 and 0.1 M acetate buffer, keeping the pH constant at 6.0 and the eluent constant with the ratio of 1:1 organic modifier to buffer. However, since this variation had the least effect on the number of peaks, it was excluded from the present study which had a time limitation. Using on-line solid phase extraction, the study

also showed that the percentage organic modifier exerted a profound effect on the blank plasma profiles. It also demonstrated that this effect depended on the type of organic modifier. Methanol variation resulted generally in less of a difference on the mean number of peaks than acetonitrile as in the present study.

On the CN column (after on-line solid phase extraction) the number of peaks decreased with increasing eluent strength and the opposite effect occurred on the C-18 column which showed an increase in the number of peaks with increasing eluent strength. This demonstrated how much the eluent strength effect differed from one column to the other. This difference was also seen in the present study although the way they differed was not identical.

The effect of varying the pH showed that a general decrease in the number of interfering peaks occurred with increasing pH. This agreed quite closely with the effect of pH variation in this project. Also the two studies showed that best results were obtained on the cyano column at high percentages of acetonitrile and on the C-18 column with low percentages of methanol. The two studies undoubtedly showed that the number of interfering peaks depended on:

1) the percentage of organic modifier,
2) the pH of the aqueous component,
3) the type of column and also the type of organic modifier used.

By using on-line column switching as an extraction technique, the effect of the mobile phase on the concentration column (as well as the analytical column) must be considered. Since the amount of plasma extracted depends on the analytical mobile phase, the idea of repeating the study of plasma profiles using off-line liquid-liquid extraction arose, as an interesting comparison. The off-line extraction technique had many disadvantages over the on-line solid phase extraction. There was the problem of the possible formation of artefacts. The off-line technique was more labour intensive and not as reproducible as on-line extraction. Column switching provided a rapid and

convenient analysis capable of working on a large number of samples.

Another slight problem which occurred in this project was an increase in back pressure which may have occurred because the plasma components started to plug the column or contaminate it. Although the guard column is present to protect the analytical column and it was repacked on a regular basis, it may not be enough if the plasma extracts are "dirty" or contain bulky components that might precipitate out on the column. This increase in back pressure was more evident using methanol since it has a higher viscosity than acetonitrile. This may be solved by regenerating the column using the appropriate liquids or by "back flushing" the column.

The final part of this project examined the effects of a salting-out extraction procedure which used a variety of salts and compared their effects. The salts used were: sodium carbonate, sodium sulphate, sodium chloride, lithium chloride and potassium carbonate. These plasma extracts were separated on the C-18 column using a mobile phase of 1:1 acetonitrile:buffer (pH 6). A saturated solution of each salt was made and 80$\mu$l was added to the plasma. The use of sodium sulphate, sodium chloride and lithium chloride resulted in very little reduction in the number of interfering peaks compared to the blank plasma extracted without adding a salt. However potassium carbonate and sodium carbonate both reduced the number of interfering peaks. Potassium carbonate eliminated one of the two main interfering peaks and sodium carbonate cleaned up the area of interest even more. The effect of using half this concentration of the salt was also examined and made no significant difference. Unfortunately the time available for this study became rather limited as more work and research into this area would have been interesting.

To improve the validity and accuracy of the results in this project the following suggestions could be considered:

1) Because the nature of the plasma components were largely unknown, it would have been useful to test the effect of eluent variation (in percentage organic

modifier and pH) on some known drugs, without the plasma matrix to examine the effects on known compounds and then to compare these effects with drug-free plasma profiles.

2) As previously suggested, an improved method of chromatogram assessment would take the peak area into account as well as the peak number. Perhaps an index system could be devised.

3) More time and concentration could be given to the "salting out" technique since this was only an extra endeavour suggested towards the end of the project

## CONCLUSION

The results of this study of drug-free plasma profiles in HPLC following liquid-liquid extraction can be summed up in the following way. The amount of interfering peaks in the chromatographic area of interest depend strongly on the concentration of organic modifier in the eluent. They also depend on the type of column used when varying this concentration. On a C-18 column, there is a general increase in the number of peaks as the organic modifier increases, whereas on a cyano column, there is a smaller variation in the number of peaks. The change in the percentage organic modifier also depends on the type of organic modifier. On a CN column, the peak number decreases with increasing concentration of acetonitrile and does not vary much with increasing methanol concentration. The number of interfering peaks is also affected by a variation in pH and in general, this number decreases with an increase in pH. Within this variation in pH, the results also vary with the type of organic modifier used and also with column type.

Using the results of this three factor analysis leads to the conclusion that the best percentage organic modifier to be used with a CN column is 70% acetonitrile and 30% methanol on a C-18 column. The optimum pH proved to be 7.0 on the CN column and 5.0 on the C-18 column.

The limitations of this project were that the size of the peaks were not accounted for as a measure of interfering with the area of interest on the chromatogram. Other limitations include time and the aforementioned idea of analysing known drugs in addition to drug-free plasma for comparison and a better understanding of the explanations behind the results.

This study was an 'offspring' of a similar study which differed in its technique of extraction (106). The off-line technique generally proved to be inferior to the on-line solid phase extraction in terms of speed, automation, applicability, convenience and artefact formation (which may have caused problems in liquid-liquid extraction). The

disadvantages of on-line solid phase extraction are batch to batch variation, limited mobile phase compatibility and the necessity for a second pump. However since very little research has been carried out with regard to drug-free plasma profiles and clean-up methods, this project has hopefully shed light on another possible extraction scheme for analysing plasma.

With the increasingly popular use of HPLC for pharmaceutical analysis using a plasma matrix, it is important to discover the best method of extraction and the optimum eluent composition for a particular column, in drug-free analysis.

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