QUANTITATIVE STRUCTURE-RETENTION RELATIONSHIPS FOR SOLID-PHASE EXTRACTION ON MODIFIED SILICA

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Doctor of Philosophy

by

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To my beloved parents, Jim and Elvira, who have given much support and understanding throughout all my University days, and to my wonderful boyfriend, John, who has shared the best and the worst with me –

this is for you with all my love.

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ABBREVIATIONS

Å	Angstroms
AUFS	Adsorption Units at Full-Scale deflection
°C	degrees centigrade
cm	centimetre
g	gramme
m	metre
M	molar
mg	milligramme
min	minute
ml	millilitre
mol	mole
mV	millivolt
nm	nanometre
rpm	revolutions per minute
s	second
µg	microgramme
µl	microlitre
µm	micrometre
µmol/m	micromoles per square metre
v/v	ratio of volume to volume

SUMMARY

The work presented in this thesis was conducted in the field of analytical chemistry and was intended to improve the process of methodology development in the related fields of analytical toxicology and therapeutic drug monitoring. Most pharmaceutical and toxicological analyses require a sample clean-up step before quantitative detection of a drug by chromatography or spectroscopy. Solid phase extraction (SPE) techniques have recently assumed considerable importance for this purpose and many of these procedures utilise chemically modified silica as adsorbents, in which organic substituents have been introduced on to the silica surface. A diverse range of polar and non-polar substituents has been used to provide adsorbents for straight-phase, reversed-phase and ion exchange systems.

Bonded silica is used as a stationary phase in high performance liquid chromatography (HPLC) and although much research has been directed towards understanding the solute retention process in HPLC, as yet no attempt has been made to apply this theory to SPE. In this study, the physical and chemical parameters determining the retention of analytes on SPE sorbents were examined to elucidate the underlying mechanisms of the SPE process on different sorbents. A mathematical model of the process could then be constructed to predict the retention of novel analytes

based solely on their physical and chemical characteristics. These predictive rules are known as Quantitative Structure-Retention Relationships (QSRR). The substances of interest were ß-adrenoreceptor antagonist drugs related to propranolol.

In the first three chapters of the thesis, background material is given concerning silica stationary phases in liquid chromatography (LC), their synthesis and properties, the role of the mobile phase in (LC) systems and the interactions which take place between analytes and the mobile and stationary phases. Thereafter, QSRR are reviewed and parameters used to characterise analytes are introduced: these fall into two categories - dispersive parameters (e.g. molecular volume) and inductive parameters (e.g. dipole moment). Finally, particular problems arising in the SPE of basic substances are reviewed.

The problem was approached in a series of steps as follows:

(i) Forty three substituted benzene test compounds were selected as simple model solutes containing one or more common functional groups. Physical and chemical data were collected for each solute, including shape and size descriptors, polarity terms and hydrogen-bond donor/acceptor ability. Five non-polar Bond Elut® sorbents were selected: ethyl-, octyl-, octadecyl-, phenyl- and cyclohexyl-silica. Eight aqueous methanol mobile phases were used as eluents containing four different

percentages of methanol (20, 30, 40 and 50%), each duplicated at pH 5 and pH 7.

(ii) Although SPE methods normally use small cartridges, these were impractical for collecting sufficient data for significant statistical analysis. The bonded silica sorbents were packed into HPLC columns and retention capacity factors (k') were measured with a continuous flow system for each test solute in each chromatographic system. The following criteria for "digital" SPE chromatography were used: if the log k' value of a solute was greater than 1.7, it was assumed than the solute would be indefinitely retained in SPE under those chromatographic conditions ("off"). Log k' values of less than 1.7 indicated that the solute would be eluted ("on").

(iii) A second series of test solutes consisting of propranolol and fourteen synthetic analogue compounds were subsequently examined using octyl-, phenyl- and cyclohexyl-silica and 30:70 methanol:water with 0.3M tri-n-butylamine as an organoamine modifier to suppress analyte retention by active silanol groups. The effect of plasma protein solution and fresh plasma on retention behaviour (matrix effect) was also studied.

(iv) A database was compiled containing k' values and information relating to both the solutes and the chromatographic systems. The data was then used for statistical analysis. Independent solute parameters were first established by factor analysis then the logarithmic

capacity factors, log k', were correlated with these parameters by multiple linear regression analysis. A series of equations were obtained by this method which could be used to predict retention behaviour of solutes.

From the physicochemical parameters selected by multiple linear regression analysis, the following conclusions were drawn:

(i) The partition coefficient, log P, and the ionisation-corrected partition coefficient, log D, were dominant in the regression equations derived for the substituted benzene solutes. An inductive parameter, the number of hydrogen-bond donor groups, was also significant. With the n-alkyl bonded silica sorbents, solute retention increased with log P or log D. This was a reflection of the hydrophobic contribution to retention by the non-polar sorbent ligands. The hydrogen-bond donor term reflected the ability of the mobile phase components, methanol and water, to decrease retention by hydrogen-bonding to solutes i.e. log k' decreased as the hydrogen-bonding term increases.

(ii) Increased hydrophobic retention by octadecylsilica enhanced the hydrogen-bond donor contribution to retention of substituted benzene solutes, and the coefficient of this term was greater than for ethyl- and octyl-silica.

(iii) The volume of an acidic benzene solute was another retention-determining parameter on octadecylsilica. Such solutes resided in the most mobile part of the stationary

phase where they could be enveloped by the flexible bonded chains if their size was appropriate. No volume parameter was observed in the regression equations for the shorter n-alkyl chains.

(iv) Phenylsilica was shape-selective towards substituted benzene solutes as indicated by two additional terms, volume and connectivity.

(v) Phenylsilica appeared to undergo a phase transition at 40% methanol in water which increased the hydrophobic surface area of the bonded ligands.

(vi) Cyclohexylsilica could not be modelled successfully with substituted benzene solutes.

(vii) The ß-blocker test compounds were retained indefinitely on octyl-, phenyl- and cyclohexyl-silica through silanol interactions, unless tri-n-butylamine was added to suppress retention. Pretreating the sorbent with either plasma protein solution or fresh plasma also masked silanol behaviour. Use of tri-n-butylamine as well as pretreatment with the biological matrix aided fast elution of the solutes.

(viii) The three selected bonded phases for ß-blocker probes were all shape-selective, although octylsilica was not a suitable sorbent for modelling the retention behaviour of these solutes as the correlation between log k' and the selected physicochemical parameters was poor. Excellent correlations were achieved with the cyclic sorbents.

The retention prediction equations derived for phenyl- and cyclohexyl-silica with the ß-blocker compounds could be used to either predict log k' for a particular chromographic system, or more useful for SPE, a suitable eluent or sorbent could be selected by setting log k' at 1.8 for retention of a solute, and at 1.6 for elution, thereby allowing prediction of suitable systems for method development.

CHAPTER ONE

THE CHROMATOGRAPHIC SYSTEM

Introduction and Aims

Most pharmaceutical and toxicological analyses require a sample clean-up step before quantitative detection of a drug by such methods as high-performance liquid chromatography (HPLC), gas chromatography (GC) or mass spectrometry (MS). As the quantity of analyte in the sample is often less than a few microgrammes the extraction method must remove the majority of matrix and interfering components as well as preconcentrate the analyte.

The principle behind the different techniques available is basically the same - to retain the analyte(s) of interest allowing interfering compounds to be removed with an appropriate solvent. An important class of analyte in toxicology is drug substances, which are often organic and therefore soluble in organic solvents such as methanol or acetonitrile which allow concentration by evaporation. The phase which retains the analyte can be a water-immiscible solvent e.g. ethyl acetate as in the popular liquid-liquid extraction (LLE), a solid phase such as XAD-2 which is a polystyrene medium capable of removing proteins, fats and lipids, porous polymer beads for urine extraction or inorganic porous materials which depend on adsorption of the drug. The latter group includes diatomaceous earth, magnesium silicate, alumina and

charcoal, but the most widely used sorbent is silica because it can be easily modified with non-polar, polar and ion-exchange substituents to produce highly stable, non-swelling stationary phases. Termed "bonded phases", they allow a much wider range of extraction capabilities than other adsorbents due to the different retention mechanisms of the attached group i.e. hydrogen-bonding (polar), Van der Waals (non-polar) and ionic interactions.

Bonded phases have been routinely used as HPLC column packings for well over ten years and recently many novel solid-phase extraction (SPE) techniques have been developed which utilise the modified silicas for sample clean-up. A small quantity of bonded material (100-500mg) is packed into a polypropylene cartridge with a solvent capacity of 1-5ml. Application of positive pressure or a vacuum of 10-15 mm Hg draws the sample or solvent through the sorbent bed, normally chosen to retain the analyte, allowing the matrix to be washed off. Because the volumes of sample and elution solvent(s) required are of the order of 1ml, a fast and highly efficient method of extraction and preconcentration is available.

SPE has a number of advantages over conventional LLE; the use of a bonded silica phase and a solvent eliminates emulsion formation between the two immiscible solvents, common in LLE, which results in loss of analyte; the large volume of organic solvent utilised by LLE is often hundreds of millilitres compared to the few millilitres used in SPE; single-step extraction compared to the multiple

back-extractions often required by LLE; the ability of SPE to allow separation of a drug and its metabolites which cannot often be achieved successfully by LLE; successful automation. Hence SPE is more efficient than LLE in terms of increased amount of analyte extracted from the endogenous material and reduced analysis time and cost [1].

Many SPE methods have been developed for a wide range of analytes of toxicological and pharmaceutical interest [2-8] and although SPE is easy to use in terms of extraction technique, the fundamental principles are not fully understood. The bonded phases used for SPE are very similar to those developed for liquid chromatography (LC) and therefore LC theory can be applied in understanding the retention/extraction mechanism in SPE. A large amount of research has been directed to bonded phase theory over the past ten to fifteen years yet only a basic understanding of the retention/extraction mechanism of SPE has been gained. It is well established that as well as a primary interaction between the bonded moiety, be it non-polar, polar or ion-exchange, and the solute/solvent, there exists a secondary weak polar or cationic-exchange interaction attributed to unmodified silica surface hydroxyls. The ability to predict solute/solvent/sorbent interactions would improve development of new extraction procedures for SPE, especially for basic solutes which are retained by the excess surface silanols present on bonded phases. The secondary interaction is undesirable if its influence on the analyte cannot be predicted, but if it can be controlled an

extra degree of specificity is available to improve extraction. The role of free silanols is still highly controversial in terms of theory and more discussion will be given in the following section.

Aims

The following research work was intended to provide a further insight into the bonded phase retention mechanism by elucidating the physical and chemical characteristics of a solute which are most significant in influencing retention behaviour. Initial experiments to study the effect of different bonded sorbent substituents and mobile phase components on the retention times of a number of substituted benzene compounds were undertaken by HPLC. Quantitative structure-retention relationships (QSRR) were derived by statistical linear regression analysis to relate retention behaviour to solute descriptors including physical parameters (for example dielectric constant, melting and boiling points) and molecular parameters (for example dipole moment, molecular volume). Retention times were then collected for the ß-adrenergic receptor antagonistic drug propranolol and several of its analogues. Remodelling the equations for the ß-blocker compounds enabled a relationship to be established between their physicochemical properties and retention behaviour. The effect of blood proteins on the QSRR of the test compounds was also studied.

1.1 The Bonded Phase

Stationary phases used in gas and liquid chromatography must be thermally and hydrolytically stable, respectively, to withstand the chromatographic environments. Carbon, alumina and silica [9,10] have all been used as stationary phase sorbents, but in their unmodified forms they are neither very reproducible in performance nor highly selective [11].

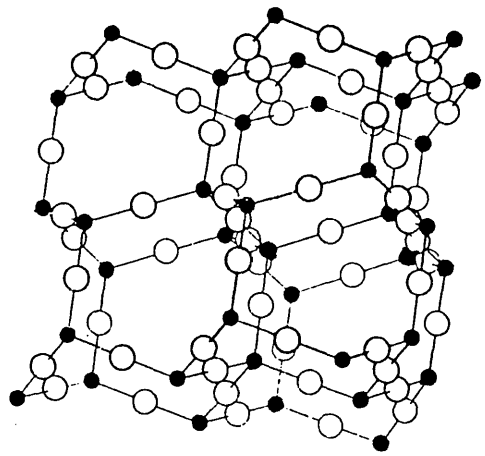
In the late 1960's Aue and Hasting [12] prepared surface-bonded silicones by reacting activated silica with mono- and di-methylchlorosilanes. Use of alkoxy- and organo-silanes gave bonded phases a new dimension by allowing non-polar, polar and ion-exchange groups to be bonded to the silica through surface hydroxyl groups. These phases offered a much wider range of selectivity capabilities due to the unique character of each one.

The following sections describe the types of bonded phase in more detail by discussing those aspects which influence their behaviour such as the properties of the silica substrate and their mode of synthesis.

1.1.1 Silica

Silica is an amorphous silicon-oxygen polymer with a large specific surface area (50-400 m^2/g) due to its high porosity (figure 1). Exposed silicon atoms at the surface are readily hydroxylated to form acidic silanols which can assume a number of configurations (figure 2). Steady-state luminescence spectroscopy studies have shown that the

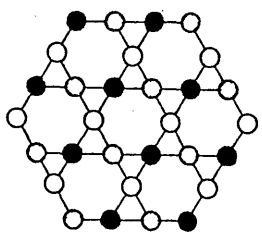
(a)



Crystalline silica

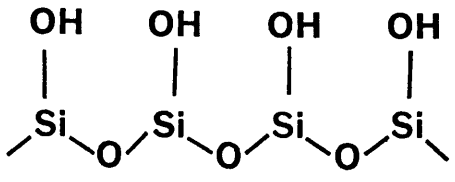
•-Si ○-O

(b)

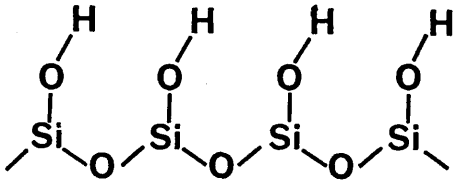


Silicon atoms in silica

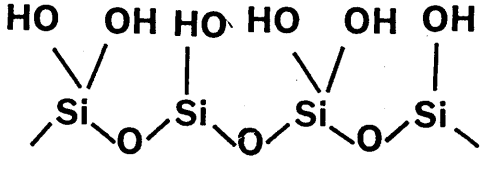
Figure 1 The structure of polymeric silica. (Wells AF. "Structural Inorganic Chemistry" 4th Ed.: Oxford University Press (1975))



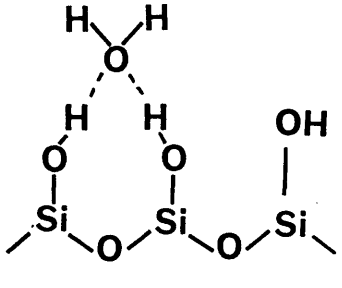
Isolated or
free silanols



Vicinal



Geminal



Physiadsorbed
water

Figure 2 Conformations of silanol groups at the silica surface.

silanols are not evenly distributed and that the surface is heterogeneous [13]. It is through the silanol groups that organic substituents can be attached to the silica. To optimise this reaction and the resulting phase's efficiency, it is necessary to know certain physical and chemical parameters of the silica substrate such as the size and shape of the particles, specific surface area, pore size and silanol concentration. Small changes in these characteristics will result in changes in the bonding density and bonded layer thickness.

The shape of the silica particles determines the ease with which a liquid will flow through the sorbent bed. Large, irregular particles of approximately 40 μm are preferred for SPE to reduce resistance of solvent passage through the cartridge (figure 3). As the solvents are pumped through the stationary phase under much higher pressures in HPLC, spherical particles sizes of the order of 5-10 μm are used to make the phases to increase the number of theoretical plates and consequently the efficiency of separation. Very small silica particles, termed fines, must be kept to a minimum as they block the frits used to hold the bonded phase in the column or cartridge, increasing the pressure of the system, and may even break through if they are smaller than the pores of the frit (~2 μm).

Surface area is an important influencing factor for the degree of modifier coverage. The maximum number of accessible unassociated surface silanols must be initially present to ensure optimum surface bonding although other

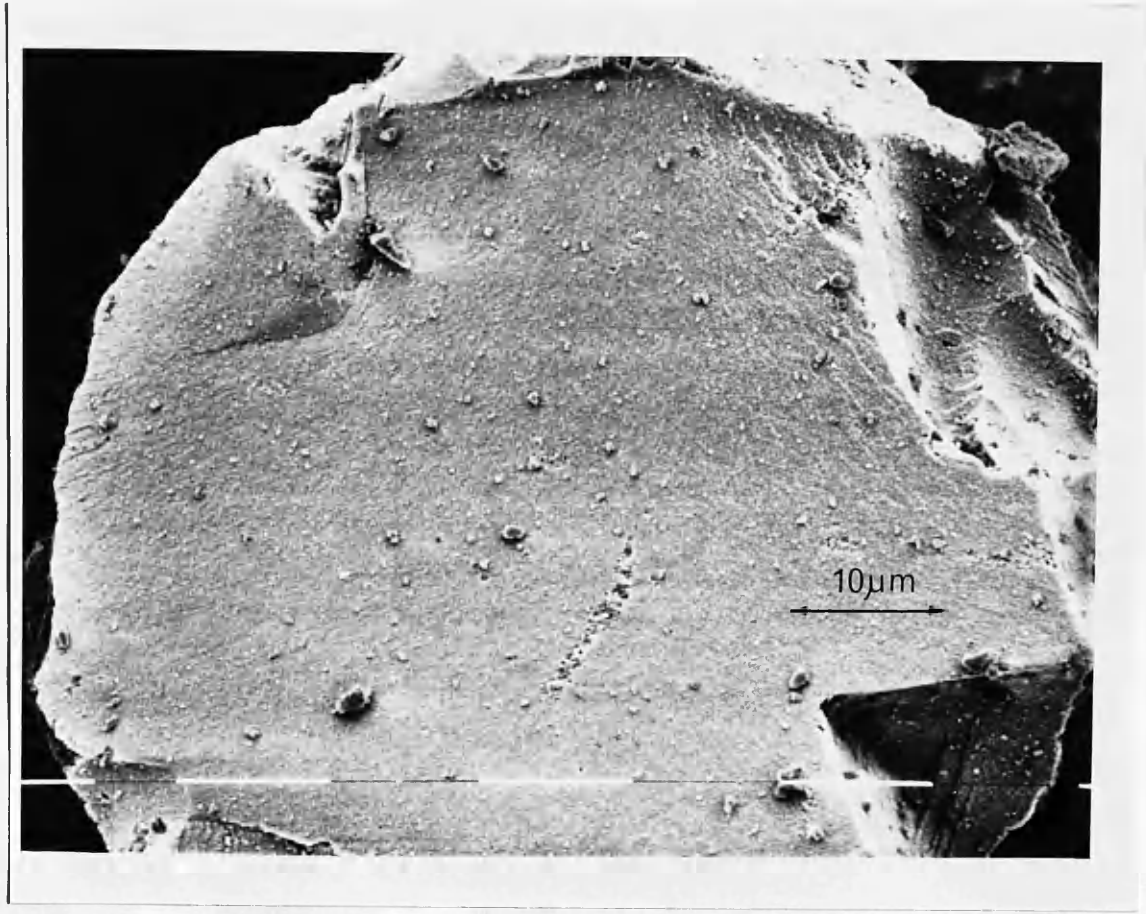


Figure 3 Scanning electron micrograph of a Bond Elut silica particle.

determining factors include pore size [14-16], a fully hydroxylated surface [17] and length of modifier chain [18], all of which will be discussed later on. A well-used method for measuring surface area is the Brunaur, Emmet and Teller (BET) method based on the extent of sorption of nitrogen or similar gas (at its melting point) into the porous silica substrate. The specific surface area, S_{BET}, includes that within the pores and is calculated by

$$S_{(BET)}(m^2/g) = X_m.A_m.N.10^{-18} \qquad \text{Equation 1}$$

where X_m is the specific monolayer capacity (calculated from the gas's isotherm between two specified relative pressures), A_m is the cross-sectional area of the gas used e.g. nitrogen or helium, and N is Avogadro's constant.

The porosity of the substrate is defined by the specific volume. The volume (V_p) is calculated from the following equation:-

$$V_p(cm^3/g) = \frac{1}{\rho_{Hg}} - \frac{1}{\rho_{He}} \qquad \text{Equation 2}$$

ρ_{Hg} is the apparent density of mercury and ρ_{He} is the apparent density of helium [19]. By combining S_{BET} and V_p, the mean pore diameter, D, can be calculated

$$D(nm) = \frac{4V_p}{S_{BET}} . 10^3 \qquad \text{Equation 3}$$

A major drawback with the BET measurement is that the gas molecules can penetrate into much smaller pores than the larger, bulkier organosilanes thereby giving a greater area than is truly available for substitution. It is therefore wiser to quote mean pore diameter rather than volume for pore dimensions. This parameter also prevents volume errors from "ink-pot" pores with narrow openings which prevent access of modifier or solute [20,21]. Ideally pore diameters should be greater than 10-11nm for maximum efficiency; smaller pores prohibit entry and prevent longer alkyl chains from extending [14,20,21] while larger pores encourage lower bonding densities [16]. The average pore size used in manufacturing is 8-10nm , but this can only be used as a guide because polymeric phases which possess a large bonded-layer thickness require larger pore sizes than the monolayer monomeric phases [20,22].

The different surface silanol configurations have been under much scrutiny to understand which are the active bonding sites for chemical modification. Temperature studies coupled with Fourier transform-infrared spectroscopy (FT-IR) [23,24], GC [15] and adsorption isotherms [25] suggest that acidic isolated sites are the most reactive although more sensitive techniques such as diffuse reflectance-infrared spectroscopy (DRIFT-IR) [11] and silicon-29 cross polarisation magic angle spinning-nuclear magnetic resonance (^{29}Si CPMAS-NMR) [26,27] imply that both isolated and weakly hydrogen-bonded geminal sites are involved. Vicinal silanols may react to a lesser extent

because of steric constraints [11].

Silanols are present in the bonded phase even after extensive surface modification as steric restrictions prevent 100% reaction completion. Excess silanols are potential acidic, hydrogen-bonding sites capable of retaining basic and some acidic solutes. This behaviour will be discussed more fully in the following sections.

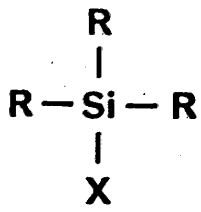
1.1.2 Chemical Modification

Bonded silica stationary phases for SPE and LC can be synthesised by similar surface-modifying reactions. They are superior to unmodified silica as they provide a more homogeneous chromatographic environment with better efficiency and enhanced selectivity. The groups bonded to the sorbent are normally organic and the modified silicas are categorised into three groups relative to the primary interaction exhibited by the attached organo-ligand: non-polar e.g. n-alkyl chains, polar e.g. cyanopropyl, or ion-exchange e.g. benzene sulphonic acid. Each of these classes has a different solute retention ability which will be discussed more fully in Section 1.1.3.

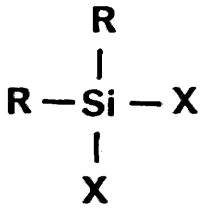
Surface-modifying reactions are performed with organosilanes either as a gas or as a liquid. Organoalkoxysilanes are preferred for synthesising polar phases while organochlorosilanes are often used to prepare the non-polar phases [16,28]. The characteristics of the resulting phase will be dependent on the functionality of the organosilane, which may possess a single bonding site as

with monofunctional reactants, or multiple bonding sites for polyfunctional compounds as shown in figure 4. It is only physically possible for monofunctional modifiers to form a monolayer on the surface *via* a 1:1 reaction with an accessible active site, but careful control of reaction conditions is necessary to maintain a monolayer when using bi- or tri-functional organosilanes. Phases synthesised from multifunctional organosilanes will possess multiple anchorage sites, although even with trifunctional substituents the maximum number of bonding sites never reaches three because of steric hinderance [29]. As these reactants have more than one hydroxyl or chloro group, the organosilane molecules will bind to each other to build a polymeric network. The two structures of the bonded phase each have their advantages and disadvantages which need to be weighed up when choosing a phase for a specific separation. Monomeric phases are preferred by research workers as they have a more defined structure referred to as 'brush' layers. They are easier than polymeric phases to synthesise reproducibly and possess better separation mass transfer. However, for commercial purposes polymeric phases are easier to manufacture as the synthesis does not need to be free of water - in fact water promotes the reaction. Polymeric coverage appears to enhance selectivity because of the increased amount of modifying ligand exposed to the solute [22].

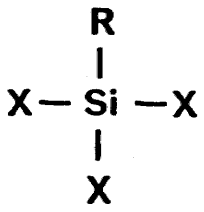
Thermal pretreatment of the silica is necessary before bonding takes place to ensure the optimum number of



Monofunctional



Bifunctional



Trifunctional

X- Cl, OCH_3, OC_2H_5

R- Non-polar, polar or ion-exchange group

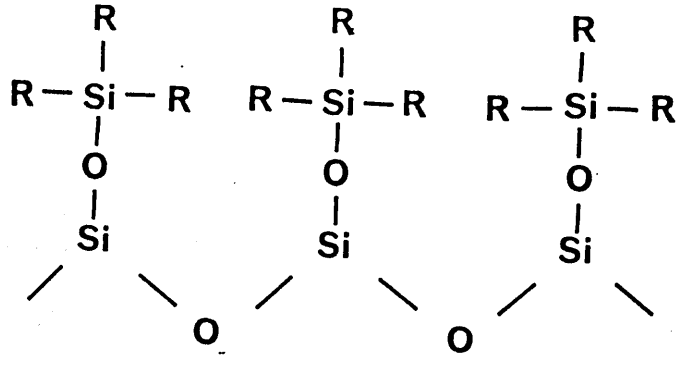
Figure 4 Structures of the three most commonly used organosilanes in the synthesis of modified silica.

reactive silanols, often quoted as 7-8 $\mu mol/m^2$ [19,17]. Heating the silica to around 130 °C will remove surface-bound water which promotes condensation polymerisation of the organosilanes and reduces the reactivity of the silanol groups. It is recommended that the sorbent is heated to a much higher temperature to remove as much physisorbed water as possible from the microporous structure. Van der Venne *et al.* [30] recommended a temperature of 600 °C after studying how thermal pretreatment affected pore structure, resulting carbon content and unreacted silanol concentration while another group found that temperatures up to 850 °C did not further affect the substrate structure [26]. At such temperatures the surface was dehydroxylated, creating siloxane groups such as $\equiv Si-O-Si\equiv$, which upon rehydroxylation with boiling water over a period of a few days provided a more homogenous cross-section of reactive silanols. Kohler's group observed that the rehydroxylated surface silanol concentrations did not exceed 6.5 $\mu mol/m^2$ with water alone [31] and showed that treating the silica with nitric acid before boiling in water increased the concentration to around 7 $\mu mol/m^2$, which is nearer the assumed value of the original silica before treatment. However, because of the extreme conditions involved such thorough pretreatment is often impractical in terms of time and in most cases it is sufficient to heat the silica to 130-140 °C where negligible surface water is present. It is critical that water is removed from the reaction vessel if a monomeric

phase is to be synthesised. All glassware must be thoroughly dried and is often treated with trimethylchlorosilane (TMCS) to block any possible reactive sites to which the organosilanes will attach themselves. If organochlorosilanes are used, dry pyridine is often added to remove the hydrochloric acid produced which helps the reaction to proceed. Multifunctional silanes have more than one possible binding site as depicted in figure 5(b). If excess water is present during the synthesis, a polymeric organic layer will result.

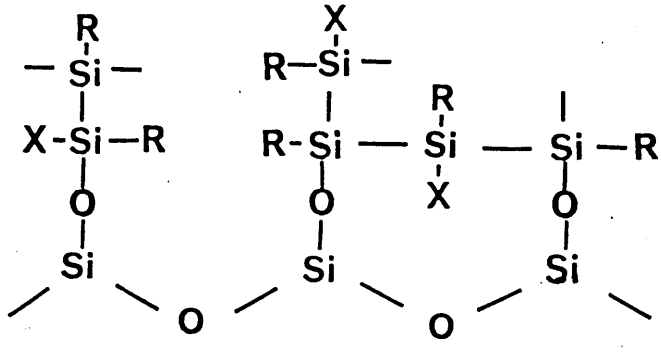
Undesirable reactive silanols are always present after modification even after the most careful work-up. Some sites are inaccessible due to steric hindrance of the large bulky organosilanes or because the silanols are situated in narrow pores. Water will hydrolyse ≡Si-Cl and ≡Si-OR sites forming more silanols. This gives all bonded phases unwanted potential sites for hydrogen-bonding derived retention of some acidic compounds such as benzoic acids and of basic solutes. This is especially troublesome in n-alkyl-modified phases as all the polar sites should be removed to create a totally non-polar environment. An effective method, though not totally successful, to reduce the number of excess silanols is by chemical treatment after synthesis. This is termed end-capping. Using a similar organosilane to the bonded organosilane moiety, such as TMCS, is effective as the chemical environment will not be drastically altered and a silanol-reactive, small molecule of this type is able to penetrate the polymeric network.

(a)



Monomeric

(b)



Polymeric

Figure 5 Structure of (a) monomeric and (b) polymeric bonded silica.

However, as Lochmuller and Marshall pointed out [32], the production of hydrochloric acid is detrimental to the bonded surface as it will attack siloxane bonds causing them to open up and form more acidic silanols. TMCS can be used to end-cap phases which will be used to separate acidic or neutral compounds, but these treated phases are not suitable for basic or benzoic acid solutes. They suggested using hexamethyldisilazane as the reaction by-product, ammonia, will further reduce surface polarity. One disadvantage is that the surface will have a greater chemical heterogeneity because of the different species present.

Trimethylphosphine has also been proposed as the phosphine produced is harmless with regard to the phase, and with careful handling the toxic gas can be removed safely [33]. Another approach involves treating the silica after rehydroxylation with an end-capping compound such as trimethylmethoxysilane or TMCS before modification. By doing this the most reactive silanol clusters will be blocked and the remaining silanols are free to bond with the appropriate organosilane [11,34]. It must be remembered that chemical pretreatment will result in long-chain alkyl phases with reduced carbon content because a significant amount of short chains will already occupy a number of active sites.

Sorbent parameters and the synthetic method used will be reflected in the overall performance and characteristics of the stationary phase and this will be reviewed in the subsequent section.

1.1.3. Characteristics of the Bonded Phase

Commercially manufactured bonded phases with the same nominal substituents exhibit significantly different solute selectivity capabilities [35,36]. Such diversities exist because of subtle variations in physical and chemical properties among silica substrates. The overall efficiency of the modified silica as a solid-phase extraction sorbent is sensitive to these differences and changes in separation performance are observed even between batches from the same manufacturer [37]. Character differences are also a result of the synthetic method used, which affects the extent of surface coverage and amount of carbon in the bonded layer. Most commercial bonded phases are polymeric as the reaction conditions are less stringent than those for monomeric phase. Researchers, however, prefer monomeric phases for studying the behaviour of bonded phases as they are easier to characterise because polymeric phases made from bi- and tri-functional reagents possess unreacted chloro or methoxy groups which are easily hydrolysed in aqueous solvent [38]. Polymeric layers therefore change in the chromatographic environment.

Fundamental differences exist between monomeric and polymeric phases. True monomerics are attached to the silica by only one ether link via a surface silanol. Non-polar monomeric n-alkyl bonded chains can be visualised in a "brush-like" conformation [39] which under certain temperature and solvent conditions collapse to form randomly distributed "droplets" [40]. Using carbon-13 CPMAS NMR to

study molecular movement of monomeric octyl- and octadecyl-silicas, Sindorf and Maciel found that motion is negligible near the silica anchorage point, but increases along the length of the chain towards the unattached end [41]. Octyl chains possess maximum movement around the seventh and eighth carbon-carbon bond whereas octadecyl chains reach maximum mobility about the eighth and ninth carbons, possibly because of restricted freedom imposed by the radius of movement around the end of the long chains. Polymeric phases are more motion-restricted due to multiple bonding between the modifier and substrate and intermolecular interaction between the modifying ligands [42]. Such phases are much harder to quantify as they possess both monomeric and complex polymeric character [43]. A typical polymeric layer is depicted in figure 6. Character differences between polymeric and monomeric phases become less obvious when particular n-alkyl chain lengths are used in their synthesis. Polymeric phases have been noted as exhibiting monomeric character when chain lengths are small while monomeric phases with greater than thirty carbons in the functional substituent show similar behaviour to polymeric phases with eighteen carbons in the ligand [44].

Solute retention is a function of the bonded phase structure although several research groups disagree over the phase property responsible. Sentell and Dorsey [45] conducted studies with phenyl and methyl compounds on monomeric phases and reported that selectivity relies on the degree of alkyl chain ordering. Two other research groups

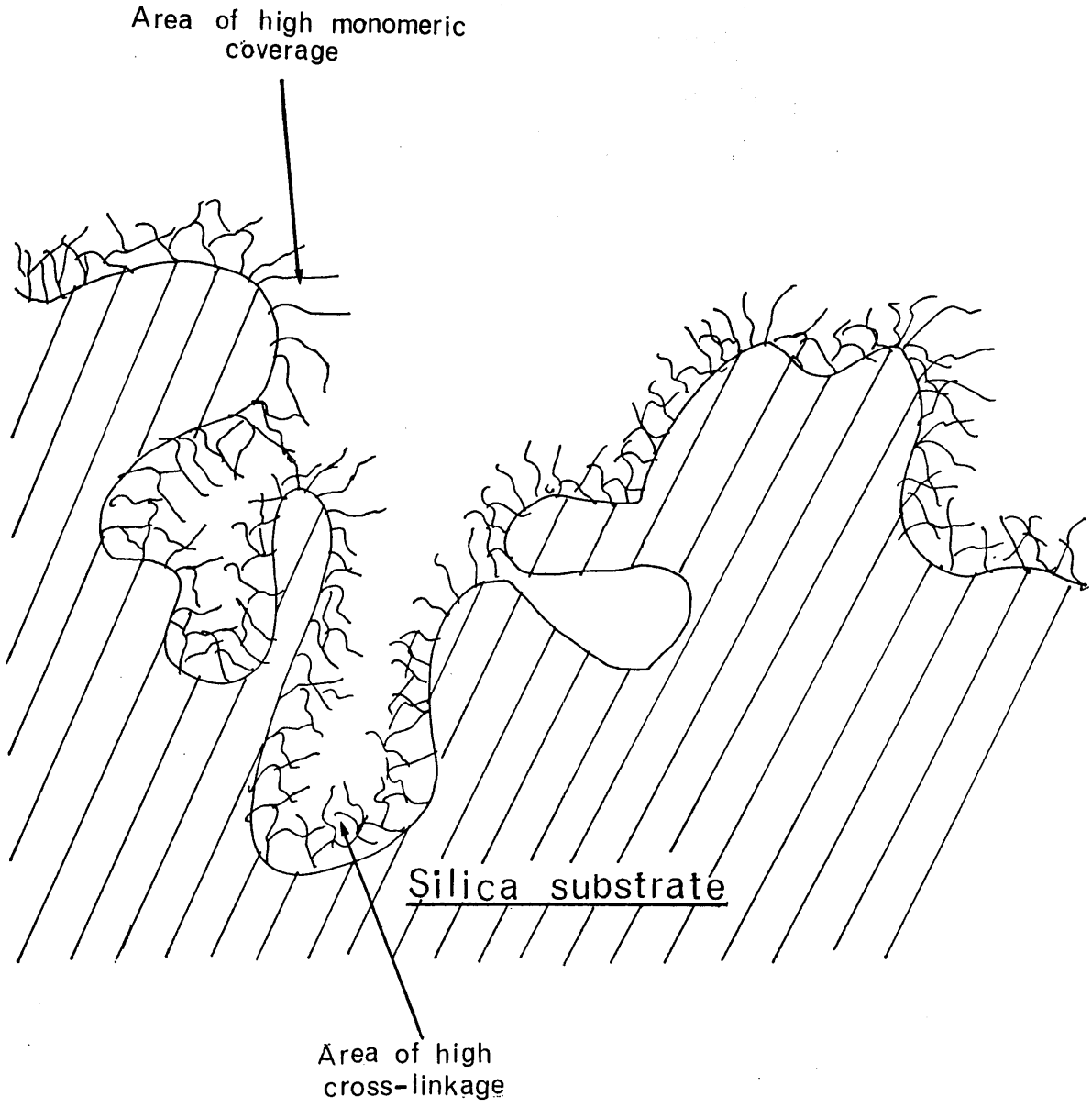


Figure 6 Cross-section of a polymeric bonded silica phase.

found selectivity to be dependent on extent of surface coverage [46,47]. Sander and Wise proposed that overall phase thickness is the determining factor from work that highlighted selectivity differences between monomeric and polymeric n-alkyl phases through changes in bonded layer thickness [44]. They found that the thickness of the bonded phase determines selectivity; monolayer thickness depends only on chain length whereas selectivity by polymeric layers is related to both chain length and degree of polymerisation. Lochmuller et al. [40] reported that alkyl chain lengths of more than twelve carbons are selective towards benzene solutes as the chains can envelope the solute molecules thereby enhancing non-polar interactions. Substituted polyaromatic hydrocarbons (PAH) used in the study were too large for such enclosure and some of their surface area was not exposed to the alkyl chains of the lengths used. Other groups have also noted that large, hydrophobic solutes such as PAH need to be enveloped by the n-alkyl chain for optimum retention and selectivity [45,48]. There is, however, general agreement that polymeric phases exhibit better selectivity than monomerics [43], particularly for PAH solutes [22,44,49]. A study on the effect of percentage carbon loading and length of n-alkyl chain on the retention behaviour of polar phenols and non-polar PAH solutes was undertaken by Hennion, Picard and Caude [50]. Both series of compounds were found to reach maximum retention at 15% carbon (w/w) octadecylsilica, but differences were apparent when the chain length was altered.

PAH experienced an exponential increase in retention as the number of carbons in the ligand increased while phenol solutes gave linear plots for retention capacity ratios versus length of chain. Selectivity for both groups improved as the non-polar character of the phase increased with chain length. This work highlights how differences between batches can alter phase retention character. If n-alkyl chains are less than four carbons in length, more than one retention mechanism will prevail *via* accessible silanol and non-polar interactions, giving rise to unexpected retention behaviour [47]. Such differences can be utilised to separate a series of non-polar solutes with subtle variations in polar character; polar groups are capable of hydrogen-bonding to silanols thereby increasing their retention times to varying extents.

Quoting percent carbon loading (P_c) for comparison of different bonded phase samples is not recommended as it does not allow for surface area differences. More widely accepted is the combination of P_c with other experimentally-determined sorbent characteristics to calculate the degree of surface coverage, N, in micromoles of modifier per metre^2 of silica.

$$N\ (\mu mol/m^2) = \frac{P_C}{[1200\ n_C - P_C(M-1)]S} .10^6 \quad \text{Equation 5 [51]}$$

where n_c is the number of carbon atoms in the substituting molecule, M is the substituent's molecular weight and S is

the surface area of the unmodified silica (m^2/g). Spectroscopic methods have also been used to measure surface coverage. Sindorf and Maciel employed silicon-29 CPMAS NMR to estimate extent of coverage for both monomeric and polymeric phases [52]. Spectral intensity differences between the unmodified silica and the bonded phase related to the extent of modification and δ-value shifts indicated degree of polymerisation. Silica-bonded charge-transfer groups have been quantitatively examined by photoacoustic spectroscopy [53].

The degree of modification determines the motional freedom of a bonded phase. Clark and Lal studied coverage effects on the behaviour of monomeric phases [54]. They observed that as monomeric coverage increases, the self-associated, collapsed chains uncoil by repulsion. The thickness of the bonded layer therefore alters with coverage. It has been suggested that the depth of the layer influences solute selectivity [44] which would account for selectivity variations among phases prepared by different workers and manufacturers. Polymeric layers become motion-restricted when coverage is high [39] with extent of bonding dependent on silica pore size [20]. If the pore diameter is too small, coverage is less than optimum as organosilane access is restricted. Such phases resemble monomeric phases in behaviour [22,43]. However, large pores encourage greater polymerisation and the overall pore size is decreased [21]. Sander and Wise found that a 300Å pore diameter was required for a good polymeric coverage of

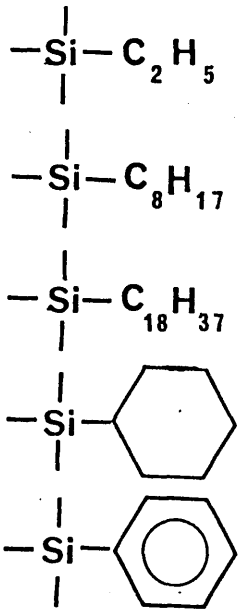
5 $\mu mol/m^2$. They suggested that monomeric coverage should be independent of pore size as long as the pores are large enough to allow chain extension e.g. 21Å for octadecylsilica [20].

Dynamic alterations of the bonded chains can be induced by changes in temperature. Such transitions alter the environment to which a solute is exposed during chromatography [55]. Gas chromatography studies on changes in solute retention times (ln k') have confirmed that densely bonded n-alkyl phases experience a motional transition over a narrow temperature range dependent on the length of the organic chain [55-57]. As the temperature increases, solute retention increases until the onset temperature, T_o. This transition is represented by a sinusoidal drop in the linear plot of ln k' versus 1/T. Below T_o, the chains self-associate and the solute is eluted relatively quickly. When sufficient thermal energy is put into the system, the chains reorientate and become more mobile. When T_o is reached, the phase assumes a more stable conformation. The chains retain some degree of conformation when thermal energy is removed. Solute and solvent molecules in liquid chromatography are exposed to a different bonded phase environment when the temperature is equal to or greater than T_o, which may increase polar solute retention through exposed silanols. The chains cannot relax back to their original relaxed state when the thermal energy is removed from the system after reordering [56]. T_o varies with chain length and solvent [58].

Incremental T_o changes of +10 ° per methylene group were observed for alkyl chains between eight and ten carbon atoms in length [56]. At room temperature and in aqueous, nonsolvating conditions, octadecylsilica is in a collapsed state and solutes are retained through entrapment and partitioning rather than adsorption.

A range of functional groups can be bonded to silica as shown in figure 7. The different primary interactions exhibited by the functional groups will dominate the character of the bonded silica and bonded phases are normally chosen for a particular extraction on this basis. All of the bonded phases exhibit secondary weak cationic exchange or non-polar capabilities to varying degrees through non-bonded, exposed silanols or n-alkyl chains (for example, from end-capping reagents or chain extensions to prevent substituted polar groups from interacting with exposed surface silanols) respectively. Many organic analytes in toxicology are ionisable and at the appropriate pH (designated by the pKa value(s) of the analyte) will become relatively non-polar, making octyl- and octadecyl-silicas popular choices for SPE and HPLC. Such phases are rather non-selective and therefore suitable for general drug screens which remove most organic and other non-polar compounds allowing polar endogenous material to be washed away with aqueous solvent. Unless the analyte has to be eluted in a solvent suitable for subsequent derivatisation or quantitative analysis, the bonded phase is normally chosen to retain the analyte(s) non-selectively.

Non-Polar



Ethyl

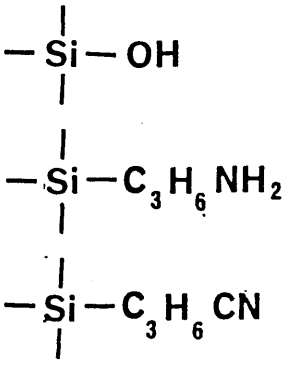
Octyl

Octadecyl

Cyclohexyl

Phenyl

Polar

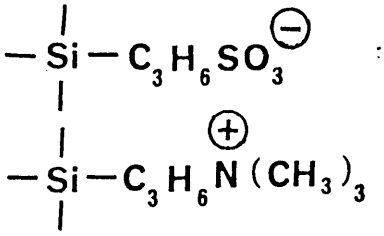


Silica

Aminopropyl

Cyanopropyl

Ion-Exchange



Sulphonylpropyl

Trimethylaminopropyl

Figure 7 Examples of the variety of functional groups which can be bonded to a silica sorbent.

The retention/elution behaviour of the analyte(s) is then controlled by the solvents chosen. Selectivity effects of the mobile phase solvents will be discussed in the following section. Careful choice of bonded phase, solvents and the use of both primary and secondary interactions make HPLC and SPE highly versatile separation and extraction techniques respectively.

1.2 The Mobile Phase

Liquid chromatography is used to separate components in a liquid sample by distribution between two phases. The phases may be two immiscible solvents e.g. water and a lipophilic organic solvent as used in LLC, or a solid phase and a liquid phase as in thin layer chromatography (TLC), SPE and HPLC which employ solid supports such as silica and alumina. Separation by LLC is described as partition chromatography because the sample components will disperse in either one of the two solvents depending on the ability of each liquid to solvate the compounds. The mechanism by which components are separated in TLC, HPLC and SPE was originally believed to be an adsorption process and the method is so named. In chromatography the sorbent is termed the stationary phase because it is immobilised in the system while the mobile phase, i.e. the solvents, move through the stationary phase by, for example, applied pressure, gravity flow or by capillary action. The sample is introduced as a solution to the chromatographic system. In HPLC the sample is injected into the continously flowing mobile phase

whereas in SPE and TLC the sample is applied directly onto the stationary phase and moves through the system with the mobile phase under pressure or by capillary action respectively. The components in the sample pass through the system at different rates determined by their distribution between the two phases. Components which are attracted to the stationary phase are retained and subsequently eluted by the mobile phase in a time related to the degree of stationary phase-solute interaction. Retained solutes may require many column volumes of mobile phase to elute them and consequently their retention times, i.e. the time taken for a molecule to move through the system, are long. Sometimes the solute may be adsorbed so strongly that the mobile phase may have to be changed for elution to take place.

Unfortunately the retention mechanisms in adsorption chromatography are not as clear-cut as the name suggests. Both adsorption and partition processes are possible giving rise to much controversy over the principal interactions responsible for retention. An overview of proposed theories is given in section 1.3. Regardless of the mechanism by which the solute is retained, the degree of retention and rate of elution are dominated by the choice of mobile phase [48,59]. The role which the eluent plays depends on the mode of separation. Both routine HPLC and TLC demand a solvent system capable of eluting the solutes within a reasonable time while, ideally, separating them so that the analytes are detected as a series of single peaks or spots.

On the other hand, because SPE is used to extract analytes from a matrix, three eluents are required - one to enhance retention of the analytes by the stationary phase, thereby immobilising the molecules during sample application, one to wash away endogenous material while keeping the analytes on the sorbent and another strong enough to elute the retained components in a single step (figure 8). The stationary and mobile phases in SPE should promote "digital chromatography" [60], that is, the analyte is either fully retained (stationary, "off") or eluted (mobile, "on").

1.2.1. Effect of Solvent on the Bonded Phase

As mentioned in section 1.1.3, the sorbent is usually chosen before the mobile phase unless special requirements dictate otherwise. Single solvents and combinations of solvents with varying elution strengths allow a wide scope in the choice of an appropriate mobile phase. Mobile phase selection will depend upon the primary interactions exhibited by the solvent components as well as those from the solute and stationary phase. Enhancement of attraction between analyte and stationary phase is desired in SPE during the loading stage so the sample application solvents must encourage solute-sorbent interaction. Polar phases including unsubstituted silica, diol- and aminopropyl-silica will attract solutes capable of hydrogen-bonding and dipole-dipole interactions. A non-polar solvent such as hexane will therefore facilitate retention on polar phases. Likewise non-polar, n-alkyl-bonded silica will show greatest

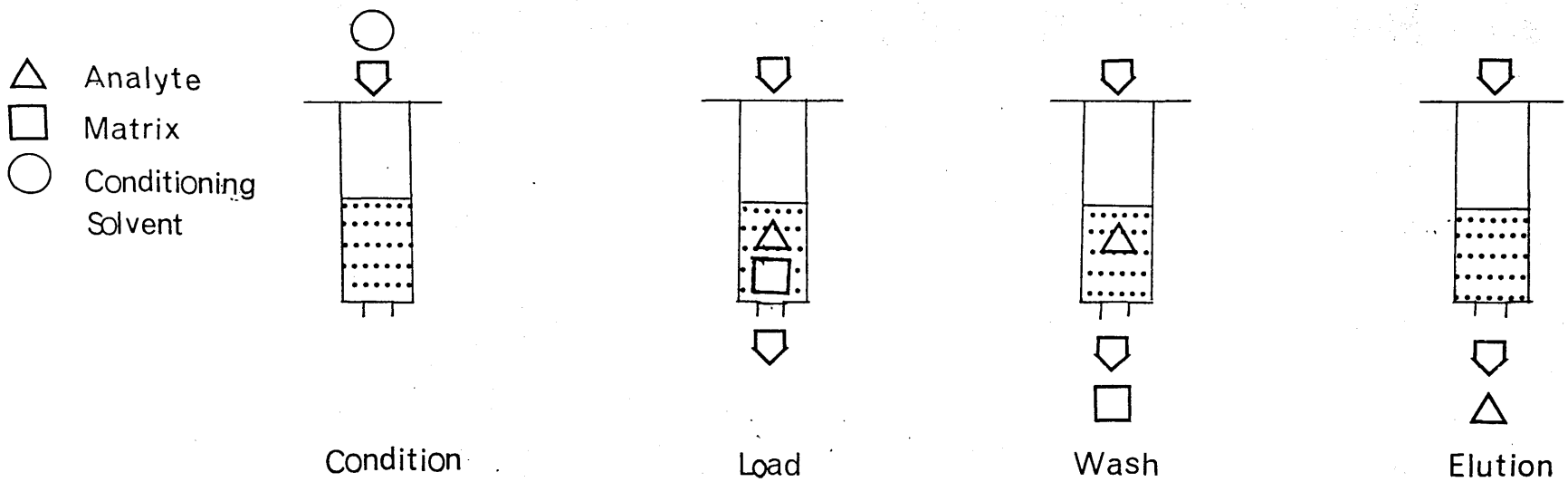


Figure 8 The four stages of a solid-phase extraction.

affinity for solutes which exhibit Van der Waals or dispersion forces. Suitable solvents are polar e.g. methanol and water. Conversely, solvents capable of breaking the solute-sorbent bonds are used as eluting mobile phases.

The most popular solvents employed in reversed-phase HPLC (where the modified sorbent is less polar than the mobile phase) and SPE (using non-polar modified sorbent) are polar, specifically methanol, acetonitrile, tetrahydrofuran (THF) and water. The organic solvents (termed "organic modifiers") are miscible with water to give aqueous binary phases suitable for most reversed-phase HPLC and SPE applications. Another important property of the organic modifiers is their ability to solvate the non-polar bonded chains. This "conditioning" effect is necessary to solvate the bonded chains so that they are in their fully extended or swollen conformation to maximise mobile phase component incorporation and solute retention [61]. When the bonded phase is dry or exposed to a non-wetting solvent such as water, the hydrocarbon chains remain disordered and collapsed [58,62] and HPLC peaks show strong fronting, suggesting minimal solute contact with the shrunken bonded phase [48]. Intercalation of polar organic solvent molecules between the chains encourages chain extension because the solvent can distribute throughout the bonded phase network, overcoming interchain attraction. Monomeric chains extend when conditioned whereas polymeric phases swell [42,46]. As long as the bonded phase is prevented

from drying out totally, it will remain solvated when different mobile phases are used. Although THF and acetonitrile are stronger solvating agents than methanol in terms of the amount of energy required for solvation [61,63], methanol is the preferred conditioning solvent as it tends to form a monolayer 0.6nm thick near the silica surface [48]. Acetonitrile and THF are adsorbed more strongly as they possess greater hydrophobic capacity and as a result create an undesirable multilayer to which solutes may be adsorbed in preference to the stationary phase [64,65]. Methanol also increases the motility of the organised, extended chains through hydrogen-bonding [61,64]. It must be remembered that the conditioning solvents are actually bonding to a layer of adsorbed water on the silica surface and not to the sorbent directly [49]. When aqueous phases are used after the bonded phase has been conditioned with an organic solvent, the highly polar water molecules will compete with and replace some of the solvating molecules [48]. A simple diagrammatic representation of the solvation of a non-polar sorbent is given in figure 9. If the percentage of organic modifier in the aqueous phase is low, the chains will collapse a little [66] and if a totally aqueous eluent is present, the chains cannot remain extended [62]. The chains then collapse and entrap solvent molecules [66,67]. Zwier conducted carbon-13 studies on octadecyl- and octyl-bonded silica and the structure of the solvated stationary phase [63]. He observed that the stationary phase contained immobilised

• Water

△ Organic Modifier

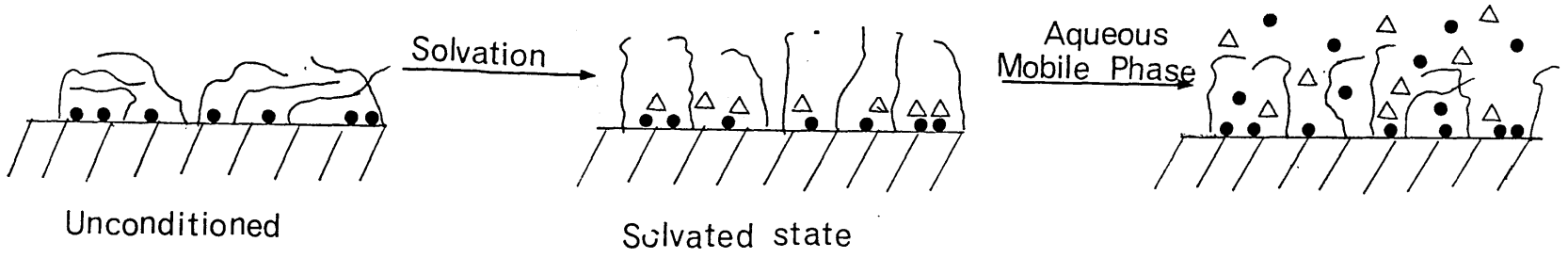


Figure 9 Behaviour of alkyl chains in an unconditioned and a conditioned environment.

solvent molecules besides the bonded chains. The distance from the surface which could then be classed as the stationary phase actually extended past the length of fully uncoiled octadecyl chains (2.5nm). When an 80:30 methanol:water mobile phase was used, the stationary layer was 4.3nm thick.

1.3. Behaviour of a Solute in the Chromatographic System

The properties and characteristics of both the stationary phase and the mobile phase in chromatography will affect the behaviour of a solute. The time a solute takes to pass through a liquid chromatographic system will reflect its preference for one of the phases - a short retention time (t_r) indicates preferred interaction with the mobile phase, a long retention time indicates preferred interaction with the stationary phase. In HPLC, the capacity factor, k', is normally quoted instead of t_r and is calculated by

$$k' = \frac{t_r - t_o}{t_o} \qquad \text{Equation 6}$$

where t_o is the time an unretained solute, such as a salt or radiolabelled mobile phase component, takes to pass through the column and corrects for delays caused by stationary phase packing faults and dead volume in the plumbing.

The principles behind reversed-phase LC solute retention have not yet been fully described by any one theory. It is now generally accepted that solute retention

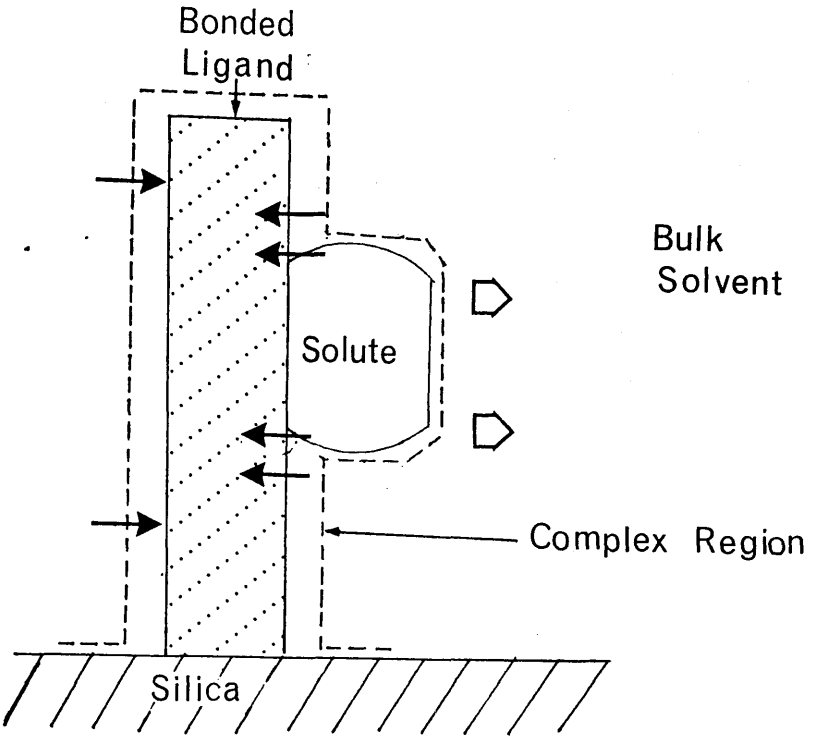
in a reversed-phase system does not occur by adsorption to the ends of the bonded chains alone as originally thought because the model does not allow for disordered, flexible n-alkyl chains, mobile phase effects or competition between the solute and the solvent layer. For a true partitioning process the bonded phase must behave as another liquid phase. Since the chains are anchored at one end, rendering them motionally restricted, and the bonded layer may only be a monolayer thick, partitioning cannot satisfactorily describe solute retention either.

In the late 1960's, Sinanoglu [68] proposed a theory to describe hydrophobic effects in biological systems. They described the process in terms of the so-called "solvophobic effect". Horvath et al. applied the solvophobic theory to reversed-phase chromatography [69,70]. They included properties of the bulk solvent such as surface tension and dielectric constant, and solute properties such as surface area and dipole moment as well as assuming that a complex is formed between the solute and non-polar chains. The free energy changes associated with the solvophobic effect arise firstly from putting a solute into the mobile phase and creating a cavity amidst the solvent molecules and secondly from interactions between the solute and solvent molecules. The following relationship describes solute retention:

$$\ln k' = \phi + \frac{1}{RT}\left[\Delta A(N\gamma + a) + NA_s\gamma(X^e - 1) + W\frac{\Delta z}{\xi}\right] + \ln\frac{RT}{P_oV} \qquad \text{Equation 7 [70]}$$

where ϕ is the phase ratio, ΔA is the sum of surface areas for the solute, the hydrocarbon substituent and the complex, N is Avogadro's number, γ is the bulk solvent surface tension (Nm^{-2}), A_s is the surface area of a solvent molecule (m^2), X^e adjusts macroscopic surface tension to molecular dimensions, a and W are solvent-dependent parameters, ΔZ is the sum of charge distribution and molecular size of the solute, the hydrocarbon ligand and the complex, ξ is the bulk solvent dielectric constant and R, T, P_o and V have their usual meanings. All the terms, except the phase ratio, are measurable physical properties of the solute and solvent. Figure 10 illustrates the solvophobic effect in a reversed-phase system.

Recently Dill redefined partitioning by considering the severe conformational constraints imposed on bonded ligands [71]. Termed an "interfacial phase", the stationary phase has a high surface:volume ratio with greatest rotational freedom away from the substrate. The conformation of the interfacial phase is dependent on three factors - (i) geometry, density and length of the ligands; (ii) a high degree of disorder; (iii) exclusion of poor solvents from the interfacial region which is assumed only to contain solute and chains. Solvent interactions within the interfacial boundary were ignored on the basis that their incorporation requires an unfavourably high degree of organisation. However, attractions between a solute and silanol groups were included. Evidence supporting this model includes a linear relationship between ln k' and log



Attraction between solute and hydrocarbon ligand facilitated by decrease in molecular surface area upon complex formation

Attraction between solute and solvent through polar interactions

Figure 10 The solvophobic effect on the partitioning of a solute molecule between an alkyl chain and an aqueous bulk mobile phase.

P, the solute's hydrophobicity (partitioning) coefficient, with a slope equal to 1 as predicted for a partitioning mechanism, a reduction in retention as surface density of substituents increases, and independence of solute selectivity from bonded chain organisation. Dill did not favour the solvophobic effect because it neglects creation of a cavity by solute molecules within the stationary phase. Although, for the reasons pointed out by Dill, the solvophobic effect is not definitive as a reversed-phase retention model, it is generally accepted as a satisfactory model to describe reversed-phase retention.

Jaroniec and Martire combined solute and solvent distribution models to define solute retention by a mixed mechanism of displacement (adsorption) and partitioning [72]. This thermodynamic approach included adsorbent heterogeniety and specific solute-solvent and solvent-solvent interactions. Consideration of solvent molecule displacement from the surface solvation layer by a solute is an important factor in this model as the solute could be retained through such an interaction. If a polar solvent is used as an organic modifier, a layer of organic modifier will form a solvation layer as already discussed in Section 1.2.1. A solute may compete with an adsorbed solvent molecule for a retention site by either displacement, association, or both (figure 11) [48,49,65]. Solvent molecules weakly held in a monolayer, either because they do not hydrogen bond or are highly polar, but present in low concentration, may be displaced by a well-retained

(a)

Displacement of weakly polar solvent or low concentration polar solvent molecule by a solute

(b)

Adsorption at an incomplete bilayer

(c)

Adsorption of a solute to a monolayer of hydrogen-bonded solvent

(d)

Mixed interaction at the completed bilayer

Figure 11 Competition between a solute molecule (X) and solvent molecules in (a) a displacement mechanism, (b)-(c) an adsorption mechanism and (d) a mixed interaction mechanism.

solute molecule (k'>10) (figure 11(a)). If an incomplete bilayer is formed (figure 11(b)) or if the monolayer is created with strongly bound solvent molecules and a solute with k'<10 interacts with it (figure 11(c)) [73], adsorption will occur. Both adsorption and displacement are possible if a complete bilayer of solvent is formed (figure 11(d)). The solute will displace weakly held, secondary layer solvent molecules and adsorb to the newly exposed primary layer.

The size and shape of a solute play important roles in determining the extent of retention. Solutes which are able to penetrate between chains, i.e. planar and linear molecules, will maximise the area of nonpolar surface available to interact by Van der Waals forces and are therefore retained to a greater degree than those which cannot intercalate as far into the stationary phase [46,74]. Berendsen and De Galan studied the effect of n-alkyl bonded chain lengths on the retention of various aromatic compounds [48]. They observed a critical chain length, independent of mobile phase composition, necessary to achieve maximum interaction between the bonded phase and the solute. Solutes with large nonpolar surface areas needed longer n-alkyl ligands in order to be encompassed for maximum effect. Consequently their retention times were greater than those of small solutes which could be enveloped by much shorter chains [75]. The depth of stationary phase into which a solute can penetrate must also be considered. The polarity change in going from the surface of the silica

sorbent to the unattached ends of the alkyl ligands is not constant, as depicted in figure 12(a). Schunk examined the different regions of the stationary phase using four different solutes - benzene, anisole, phenol and aniline [76]. He found that polar compounds such as phenol and anisole preferred to reside near the end of the alkyl chains with their polar substituent exposed to the polar bulk mobile phase. Benzene, being nonpolar, moved further into the stationary phase to the region of least polarity. Aniline was attracted even further down between the chains to the sorbent surface where the region of greatest polarity exists due to exposed reactive silanols and the solvated layer of methanol and water.

Under certain solvent pH conditions polar solutes, silica-surface bonded polar groups and unmodified, exposed silanols will be ionised. When the solvent pH is equal to the pKa value of an ionisable compound, the molecules will be 50% ionised and 50% unionised. If the solvent pH is increased by at least two pH units above the pKa of an acidic solute, or if the solvent pH is decreased by more than two pH units below the pKa of a basic solute, then the solute will be ionised and capable of interacting with other ionic species. Within the pH range range of pKa+2 for acids and pKa-2 for bases, the degree of ionisation varies dramatically. The use of a buffered mobile phase is therefore essential to ensure that an HPLC separation or bonded phase extraction can be repeated on different occasions under exactly the same conditions if the same

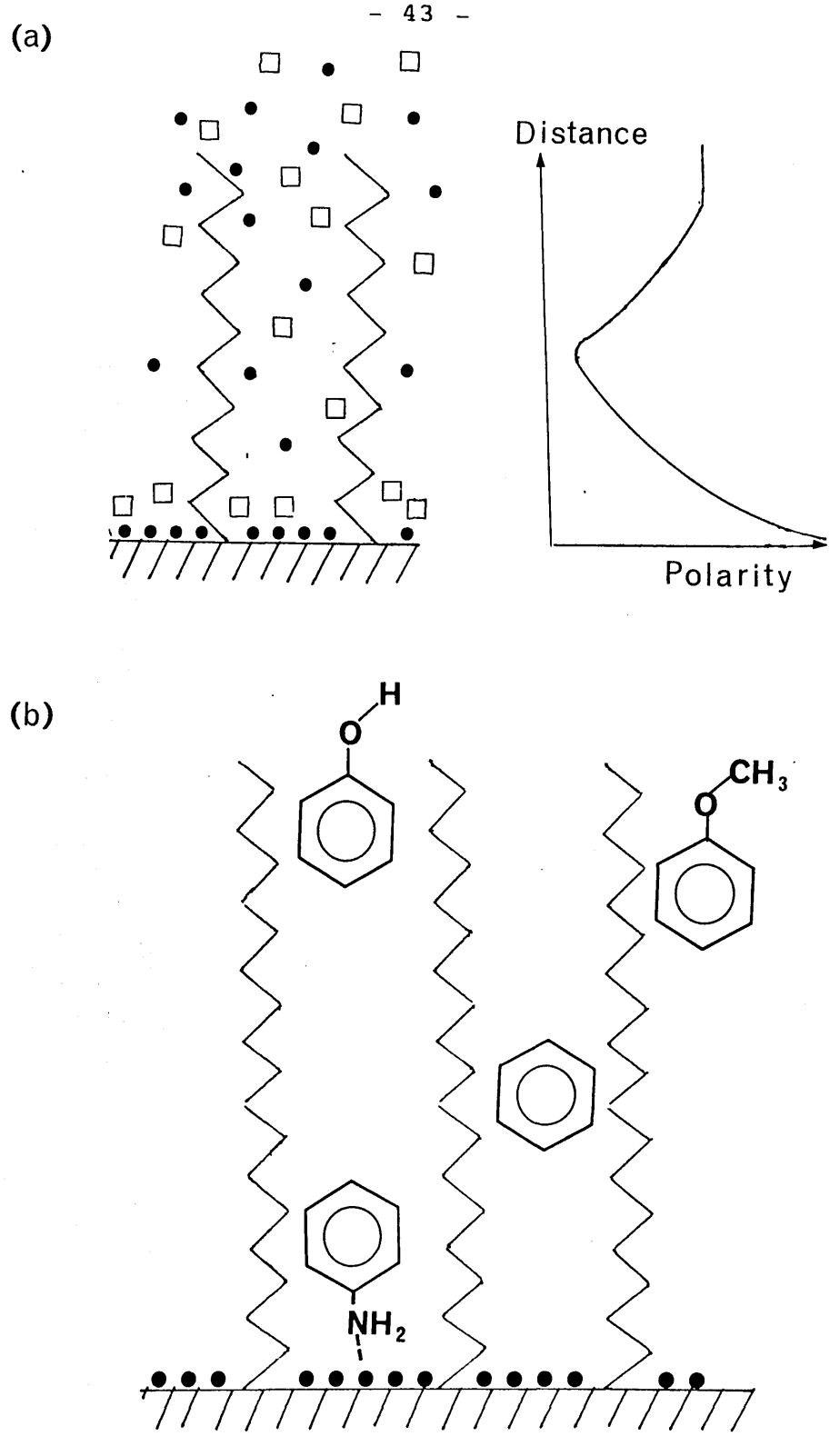


Figure 12 (a) Changes in the polarity of the stationary phase and (b) effect of such changes on the positioning of solute molecules within the stationary phase layer.

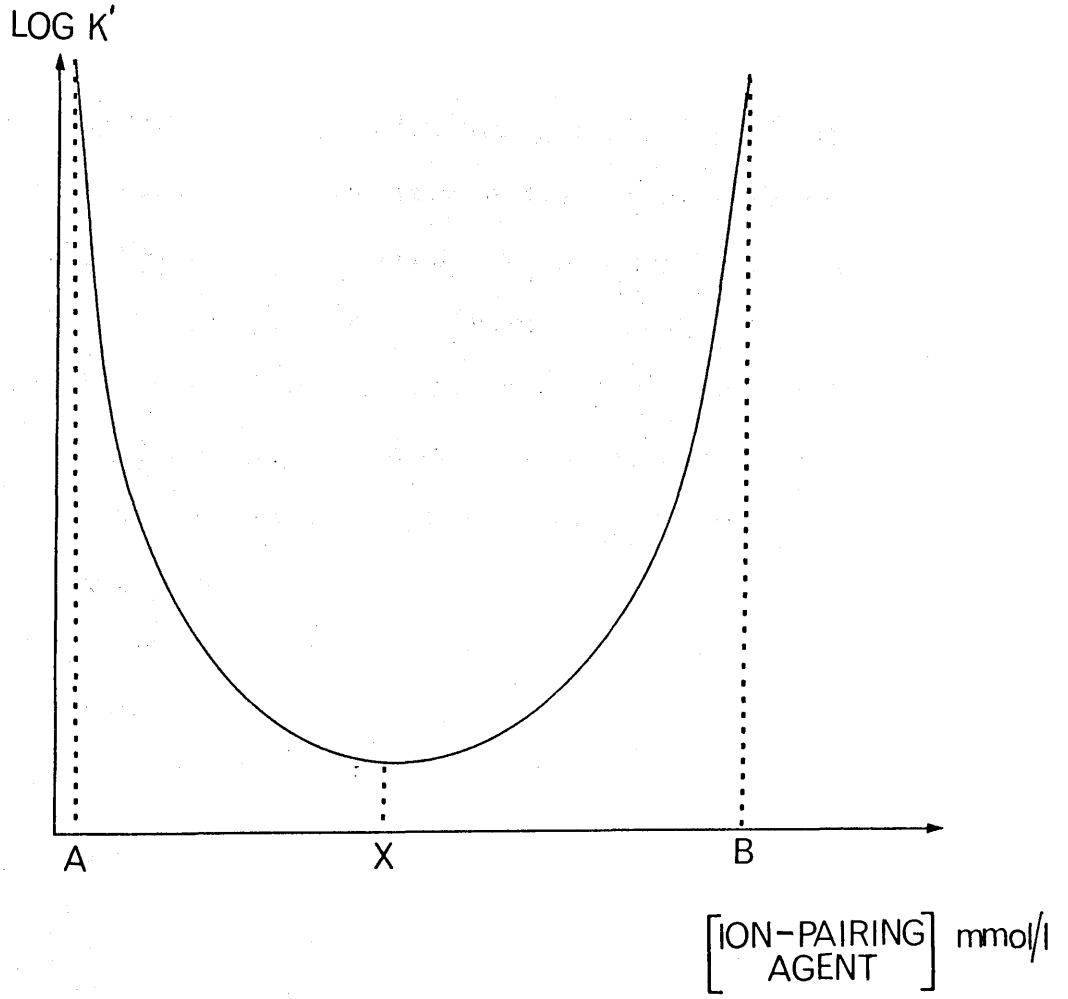
batch of bonded silica is used.

Predicting solute behaviour in a reversed-phase chromatographic environment is complicated by the existence of unreacted, exposed hydroxyl groups at the silica surface. When silanols participate in the retention process either through hyrogen-bonding when neutral or by weak cationic-exchange interactions when ionised (pH>4), strong acids and bases will be retained. Under these circumstances retention is no longer due to the solvophobic effect alone and the retention mechanism becomes a mixed mode of polar and hydrophobic interactions. The mobile phase pH range recommended by silica bonded phase manufacturers to avoid degradation of the bonded phase is between pH2 and pH9, but within these limits many organic bases and acids, including silica-surface silanols, are ionised. Although an ionic analyte is readily solvated by an aqueous mobile phase and should therefore be eluted quickly from a reversed-phase system, it is instead preferentially attracted by the competitive silanol sites. The retention time of the solute is thereby greatly increased. In a multi-analyte SPE, such a strong silanol-solute interaction prevents a single-step elution of all the analytes while in HPLC the silanol-retained analyte is recorded as a peak with low resolution and a long tailing slope. One way in which to overcome silanol effects in reversed-phase chromatography is by ion-pairing. A water-soluble salt in the mobile phase interacts with an ionised analyte to form a complex which is more soluble in the organic modifier present in the mobile

phase than the free analyte ion:-

$$A^+(aq) + B^-(aq) \rightleftharpoons AB(org) \qquad \text{Equation 7}$$

where A^+ and B^- are either ionised analyte or mobile phase salt species and AB is the hydrophobic complex formed through ion-pairing (a more detailed treatment of ion-pair chromatography theory is given in reference 77). Popular reversed-mobile phase ion-pairing additives include alkylsulphonates for basic analytes, alkylammonium counter-ions for carboxylic acid analytes, and chlorates for amines [78,79]. Addition of a salt to the mobile phase reduces analyte interactions with the silanols. Figure 13 shows the changes in retention of an ionised analyte as the concentration of salt increases. The plot is typically U-shaped. Initially retention decreases as the concentration of salt increases and ionised silanol sites are preferentially occupied by the salt ions rather than the ionised analyte until a minimum in analyte retention is observed at xmmol/l of salt. When the salt concentration exceeds the optimum amount, the ionised analyte starts to form a complex with the oppositely charged salt ion. Hydrophobic interactions between the complex and nonpolar bonded phase increase retention [80]. Analyte retention is now by solvophobic interactions. Tetraalkylammonium counter-ions are often used in the chromatography of basic drugs possessing an amine group. The counter-ions do not complex with the analyte, but instead occupy silanol sites,



Region A-X	Decreasing analyte retention by silanols which are preferentially occupied by ion-pairing agent
X mmol l	Optimum ion-pairing agent concentration to achieve minimum analyte retention by silanols
Region X-B	Increasing analyte retention by solvophobic effects as a hydrophobic complex forms between the analyte and ion-pairing agent

Figure 13 Changes in the retention time of an analyte through ion-pairing with a salt.

effectively masking solute-silanol interactions. The analytes are no longer retained by the silanols and are readily eluted [81].

A solute in a chromatographic enviroment will experience a range of interactive forces with the stationary and mobile phases as discussed in this section. The extent of solute-solvent-sorbent interactions is primarily influenced by the physical and chemical properties of the solute. The following chapter discusses the use of such properties in deriving correlations between a solute's retention time and its physicochemical descriptors in order to predict solute behaviour in reversed-phase liquid chromatography.

CHAPTER TWO

QUANTITATIVE STRUCTURE-RETENTION RELATIONSHIPS

Introduction

Medicinal chemists have successfully predicted the biological activity of drugs and other compounds by using quantitative structure-activity relationships (QSAR) in which the physical and chemical (physicochemical) properties of the molecule are related to its bioactivity [82,83]. A drug molecule *in vivo* partitions between lipid-containing tissue e.g. brain, heart, kidneys, and aqueous extracellular fluid e.g. blood, urine. The degree of partitioning is dependent on the affinity of the drug molecules for either a hydrophobic or hydrophilic environment. Parent drugs and their metabolites which are hydrophobic will tend to remain in the lipid membrane whereas hydrophilic compounds reside mainly in aqueous areas and readily pass into the blood or urine.

Reversed-phase liquid chromatography has been widely used to derive QSAR because the non-polar stationary phase and polar aqueous mobile phase are good models for *in vivo* systems [84,85]. Relationships between the physicochemical properties of a drug and its chromatographic retention can be used to predict drug transport and disposition in the body as well as the chemical interactions which occur between the drug molecule and its biological environment. This information aids prediction of the effectiveness of a

drug and also helps in the development and modification of a compound to maximise its bioactive potential. An example of HPLC applied to QSAR can be demonstrated by work conducted by Rittich, Polster and Kralik on the relationship between mono- and bi-functional phenols and their fungicidal activities [86]. They wished to show that the reversed-phase HPLC retention times of the substituted phenols were related to their fungicidal activities, C, by the hydrophobic descriptor, log P. Log P is called the partition coefficient and indicates the preference of a solute for either a hydrophobic or hydrophilic environment. Log P can be represented by the Hansch parameter, π , if, as in this case, the test solutes have a common parent structure. π represents the hydrophobicity of a substituent group on the parent structure and was found in this study to correlate well with log(1/C) (correlation coefficient, $r^2 > 0.9$).

Quantitative relationships between solute structure and chromatographic retention (QSRR) are analogous to QSAR. QSRR are useful in three senses: (a) prediction of the retention capacity factor, k', (b) measurement of physicochemical parameters, and (c) understanding the retention process. Chen and Horvath [87] applied the fundamental principle of QSAR, linear free energy relationships (LFER), to QSRR. The LFER are a less rigorous thermodynamic treatment of the energy changes associated with the chromatographic system than classical thermodynamics and are conceptually easier to interpret

because they are expressed in chemical and physical parameters relating to the solute, solvent and sorbent. Consider the chromatographic retention capacity factor, k'. It is related to the thermodynamic equilibrium constant, K, by

$$k' = K\Phi \qquad \text{Equation 8}$$

where Φ is the phase ratio (the volume of stationary phase to the volume of mobile phase). The associated total free energy, $\Delta G°_T$, associated with solute-sorbent and solute-solvent interactions is given by

$$\Delta G°_T = -RT \ln K = -RT(\ln k' - \ln \Phi) \qquad \text{Equation 9}$$

Therefore

$$\text{Log } k' = \frac{-\Delta G°_T}{2.3\ RT} + \log \Phi \qquad \text{Equation 10}$$

where R is the gas constant and T is the absolute temperature. $\Delta G°_T$ can be sub-divided into two contributory free energies - that from the stationary phase, $\Delta G°_O$, and that from the mobile phase, $\Delta G°_W$. Thus

$$\text{Log } k' = \frac{-(\Delta G°_O + \Delta G°_W)}{2.3\ RT} + \log \Phi \qquad \text{Equation 11}$$

Free energy changes associated with the stationary phase are

assumed to be negligible, more from the point of view that the stationary phase is difficult to quantify in terms of associated energy rather than that the stationary phase does not contribute to the retention process. If the same mobile and stationary phases are used to determine log k', Φ becomes a constant. Ultimately,

$$\text{Log } k' = \frac{-\Delta G^{\circ}_{w}}{2.3RT} \qquad \text{Equation 12}$$

In general terms, LFER assume that the free energy in a biological or chromatographic system is a linear sum of energetic contributions, namely hydrophobic, electronic and steric interactions. Kamlet et al. [88] have shown that solubility parameters can be expressed in these easily characterised LFER terms:

$$\text{Log } k' = \alpha A + \beta B + \gamma C \qquad \text{Equation 13}$$

where A is the hydrophobic term, B is the electronic term, C is the steric term, and α, β and γ are constants.

Chromatographic retention can therefore be described by a few physical and chemical solute properties which relate to the interactions a solute is involved in with the bonded phase and the mobile phase in chromatography. The general interactions described by LFER can be separated into two distinct groups; dispersive and inductive forces [89,90]. Dispersive interactions can occur between

non-polar molecules or between non-polar and polar molecules. They are defined by "bulk" parameters such as molar volume, molecular weight and the number of carbon atoms either in the whole molecule or in a substituted side-chain. Linear correlations between log k' and bulk properties for homologous (cogeneric) series of solutes with equal polarity imply that the dispersive forces are additive [90]. However, different families of homologous solutes appear as a series of parallel lines on a graph of log k' versus (bulk parameter) through differences in electronic and steric properties exhibited by the different families [91]. Electronic and steric interactions are termed inductive and, unlike dispersive forces, multiple polar/steric effects do not follow linear relationships and generally they cannot be added together e.g. dipole moments [92 pp.108]. Inductive parameters include dipole moment, hydrogen bonding ability and topological indices. These parameters are a measure of solute polarity. Retention behaviour of non-cogeneric solutes has been successfully correlated with a mixture of hydrophobic, electronic and steric parameters [93,94].

A number of physicochemical properties have been proposed as good descriptors for QSRR, and although a large number of research workers have achieved good correlations between retention and such parameters, no one relationship has been accepted as definitive of the chromatographic process. Some of the more important and significant parameters which have been used as physicochemical

descriptors are discussed below.

2.1 Dispersive Parameters

Dispersive interactions are one of three types of Van der Waals forces and are called London interactions. They occur between molecules both with and without dipole moments, and are the most common interaction between two or more molecules. London interactions are long range and originate from the electric field generated by the electron cloud which surrounds the molecule. Because they are scalar, dispersive forces are additive.

Bulk parameters that define shape, volume and size of alkyl chains have been shown to correlate well with retention data for homologous series of alkanes, alcohols and alkylbenzenes in reversed-phase chromatography [95,96]. Members of a cogeneric series with equal polarity should not be distinguishable by inductive forces which reflect polar properties. This allows selectivity amongst such groups of solutes to be based purely on differences between their bulk parameters [97]. Such relationships are important in reversed-phase systems because the non-polar part(s) of a molecule will determine the solubility of the solute in the aqueous mobile phase; the more non-polar character a solute exhibits, the less soluble it will become in an aqueous medium.

2.1.1 Number of Carbon Atoms

A parameter which has been used extensively to

predict retention times in gas chromatography is the number of carbon atoms in the alkyl chain of cogeneric hydrocarbons (n_c) which ultimately gave rise to the Kovats retention index [92, pp.50]:

$$I_i(T) = 100 \times \frac{\log t'_{ri} - \log t'_{rc}}{\log t'_{r(c+1)} - \log t'_{rc}} + 100c \qquad \text{Equation 14}$$

where $I_i(T)$ is the Kovats GC retention index of a solute, i, at temperature, T; t'_{rc} is the corrected retention time of a homologous standard with c carbon atoms; $t'_{r(c+1)}$ is the corrected retention time of a second homologous standard with (c+1) carbon atoms and t'_{ri} is the corrected retention time of solute, i. Analogous retention indices for LC has been proposed by several other groups [98-101]. Jandera observed a linear correlation between log k' and log (n_c) when no polarity effects were operational between a pair of aromatic solutes [101]. Another group found excellent correlation between log k' and n_c for hydrocarbons and PAH when the two series were considered separately [102]. Such observations allow the free energy involved in transferring a non-polar solute from the mobile phase to the stationary phase to be calculated in terms of each methylene group increment added to the substituent. The enthalpy involved will be constant for n-alkyl chain substituents and has been quoted as 300 kcal/mol per methylene group [103]. However, non-linearity can occur when either the number of carbon atoms in the n-alkyl chain

of a cogeneric series of polar compounds is either less than 2 or greater than 8, possibly due to changes in polarity group shielding as the alkyl chain increases in length [104].

2.1.2 Volume and Surface Area Parameters

Both volume and surface area relate to solute solubility which is one of the most important properties affecting reversed-phase liquid chromatography. Solubility is an indicator of the ease with which a solute can make a cavity within the structural network of the bulk mobile phase in order to be solvated. This largely depends on the size of the solute when no inductive forces are in play; a large non-polar solute requires more energy than a small non-polar solute to be solvated by a polar mobile phase. It may be energetically more favourable for a large non-polar molecule to interact with the non-polar bonded phase through dispersive interactions compared to breaking polar bonds between mobile phase components [105] and this will be observed as an increase in the retention time of the solute. The partition coefficient, log P, which represents the hydrophobicity of a solute, is strongly related to solubility, although other electronic factors are also involved (Section 2.3) [106,107].

The increase in retention of alkyl-substituted molecules as the non-polar side chain increases in length, shown by log k' *versus* n_C plots, is a reflection of the increase in surface area [108]. The incremental surface area contribution made by the addition of a methylene group

to the overall surface area of an n-alkane was used by Mockel et al. in the prediction of retention times of polar-substituted n-alkanes [91]. The enthalpy effects which are associated with the transfer of a solute from the mobile phase to the stationary phase can be measured from Van't Hoff plots of ln k' versus (1/T) where retention is related to enthalpy, $-\Delta H$, by the expression

$$\ln k' = \frac{-\Delta H}{2.3RT} + \frac{\Delta S}{2.3RT} + \ln\Phi \qquad \text{Equation 15}$$

[97,109]

The slope of a Van't Hoff plot gives $-\Delta H$. The change in enthalpy between homologues of alkylphenols is linear [110], but it has been shown that $-\Delta H$ is not constant between homologous alkylbenzene compounds because of π-electron energy effects [103]. The length of the chain bonded to the silica sorbent does not appear to influence $-\Delta H$ and either octyl- or octadecyl-silica is suitable for such studies [111].

Volume and surface area are conformation dependent, one example being shown by work on six ethers [106]. Measurement of these properties therefore needs to be accurate. An early modelling technique called the Corey-Pauling-Koltzman (CPK) method used a scaled representation of the solute. The surface area was calculated from the maximum number of relatively-sized water molecules which could be physically packed around a styrofoam model of the molecule in its lowest energy form

[92, p.88]. Bondi and Van der Waals group increments and bond lengths, which are to be found in the literature, lend themselves more readily to computer-assisted measurement of size parameters [112]. The most common method of generating computer-calculated parameters is by Complete Neglect of Differentail Orbitals (CNDO/2). Volume is calculated by CNDO/2 by sampling points within a box enclosing the molecule which is input in its lowest energy conformation. The box is sampled at a density of 8000 points/$Å^3$ if box is less than 50$Å^3$, 1000 points/$Å^3$ if between 50$Å^3$ and 125$Å^3$, and 125 points/$Å^3$ if greater than 125$Å^3$. Parameters calculated by the CNDO/2 method are highly dependant on geometry and the length and angle of bonds.

Other parameters such as molar refractivity, polarisability, melting and boiling points, and molecular weight are all highly correlated to volume and surface area. It is therefore wise to use only one of these descriptors in multi-parameter QSRR to represent size.

2.1.3 Parameters Relating to Shape

There are not many solutes whose retention times in reversed-phase LC appear to be related to their shape apart from polyaromatic hydrocarbons. Polyaromatic hydrocarbons (PAH) form a special class of aromatic molecules which are rigid and planar. They have received special attention in QSAR and QSRR because they are toxic by-products of the combustion of fuels and synthetic foams. The retention behaviour of methyl-substituted PAH [99,113] and various PAH

which are found in coal fractions retention behaviour is shape-dependent [114], but simple descriptors such as the Verloop parameter are not sufficient. The Verloop parameter is a ratio of the length-to-breadth of a molecule (L/B). It is a simplified form of the Principal Elliptical Axes which serve to define the shape of a molecule in three dimensions by x, y and z coordinates. Other simple descriptors include the F-parameter created by Jinno and Kawasaki as a shape parameter for PAH [114] where

F = (number of double bonds) + (number of primary carbon atoms) + (number of secondary carbon atoms) - 0.5 Equation 16

(0.5 is deducted if a non-aromatic ring is included)

Rohrbaugh and Jurs introduced a "shadow index" to define the shape of PAH. It is based on the three-dimensional structure of the solute in the x, y and z planes. The shadow area defines any two of these planes together [113]. This parameter appears to be more significant for unsubstituted PAH than for substituted PAH on a polymeric octadecylsilica phase.

Unfortunately none of these three examples of simple shape descriptors correlate well with retention behaviour by themselves. They are often used in conjunction with a topological index, specifically the connectivity index, χ, developed by Kier and Hall in the mid-1970's from the Randic branching index [115]. A topological index defines the position, and often the type, of atoms in a molecule as a

mathematical graph. This graph is represented by a numerical index. The simplest connectivity index, termed the "zero order' index ($^0\chi$), is an index relating to the number of atoms in a molecule. It is calculated by

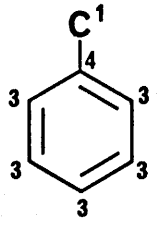
$$^0\chi = \sum(\delta_i)^{-0.5} \qquad \text{Equation 17}$$

A more useful connectivity index is the "first order" index, $^1\chi^v$. The postscript, v, indicates that the index is related to the valency of each atom in a molecule, but is often omitted because the index is assumed to be determined by this method anyway. Each atom in the molecule (except hydrogen atoms) is assigned a "δ-value" indicating the number of bonds between the atom and adjacent carbon atoms or heteroatoms. Figure 14 illustrates the calculation with toluene and methyl-4-hydroxybenzoate. The index is calculated by

$$^1\chi = \sum(\delta_i \delta_j)^{-0.5} \qquad \text{Equation 18}$$

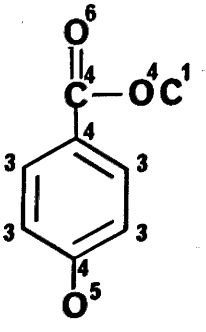
where i and j are adjacent atoms. Heteroatoms have valency-related δ-values, e.g. a nitrogen atom in a nitrile group has a δ-value of 5, but in a secondary amine the δ-value is 4. δ-values for heteroatoms can be found in Reference 115, pp.17.

The first order connectivity index is not strictly a descriptor of dispersive forces alone because it includes heteroatoms which participate in the retention process through inductive interactions. Electronic and steric



$$^1\chi = \frac{4}{\sqrt{(3.3)}} + \frac{2}{\sqrt{(4.3)}} + \frac{1}{\sqrt{(4.1)}}$$

$$= 2.411$$



$$^1\chi = \frac{2}{\sqrt{(3.3)}} + \frac{4}{\sqrt{(3.4)}} + \frac{2}{\sqrt{(4.4)}} + \frac{1}{\sqrt{(4.1)}} + \frac{1}{\sqrt{(4.6)}} + \frac{1}{\sqrt{(4.5)}}$$

$$= 3.249$$

Note 1 Hydrogen atoms have been omitted

Note 2 Numbers beside atoms indicate their respective δ-values.

Figure 14 Examples of calculation of the first-order connectivity index.

parameters are therefore needed to describe polar interactions more fully and commonly-used inductive parameters are discussed below.

2.2 Inductive Parameters

Inductive interactions arise from another kind of Van der Waals force, the Debye interaction. A molecule which has an unequally distributed electron cloud is said to possess a "dipole moment". The dipole moment may be permanent if atoms in the molecule have unequal electronegativities, such as oxygen and nitrogen, or the dipole moment may be induced in a non-polar molecule by a neighbouring molecule with a permanent dipole (figure 15). The energy associated with an inductive interaction is ten times stronger than a dispersive interaction. Polarisability is a measure of the ease with which the electron cloud of a molecule can be distorted. It is actually a dispersive force because it is additive (scalar) and is highly correlated to volume.

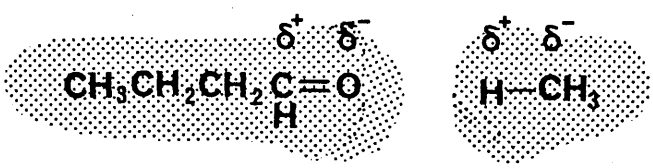
2.2.1 Dipole Moment and Δ

The very fact that inductive forces are not scalar makes them hard to quantify for chromatographic interactions. The electronic environment of the mobile phase in a chromatography system is continuously changing while that of the stationary phase is both random and non-uniform through incorporation of mobile phase components and the heterogenic nature of the surface. Interactions of

(a)

Distortion of electron cloud around butanal which has a permenent dipole moment.

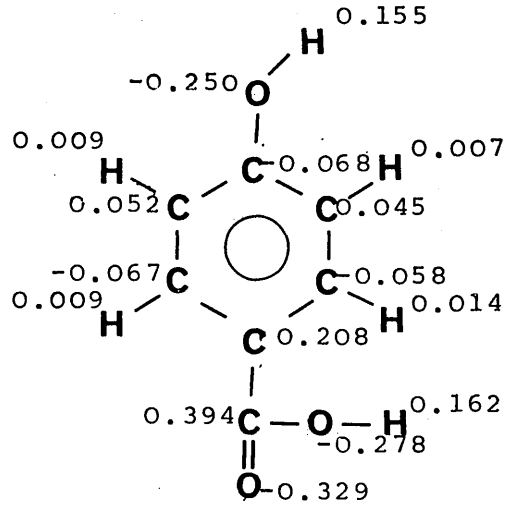
(b)



Induced distortion of electron cloud around methane (to create an induced dipole) by a molecule with a permanent dipole moment.

Figure 15 Permanent and induced dipole moments.

an inductive nature are highly orientation-dependent and it is very difficult at the present time to predict the position and orientation of a solute within such a random system. Dipole moments themselves have had relatively little success as polarity descriptors in QSRR, except for GC retention prediction [116]. This is mainly due to the fact that the dipole moment is a measurement over the whole molecule. As polar interactions are orientation-dependent, a more useful measurement would relate to the part(s) of a molecule most liable to interact by polar forces. Such a parameter, termed delta (Δ), was introduced by Kaliszan, Osmialowski and co-workers as a measure of the electron excess charge density distribution [90,117]. It is calculated from the difference between the two atoms in a molecule with the greatest electron excess and the greatest electron deficiency (figure 16). The charges on the individual atoms are generated by CNDO/2 calculations. The group found that Δ along with another CNDO/2-generated term, E_T, for a diverse set of substituted benzene compounds could be satisfactorily used to predict log k'. Replacement of Δ with the dipole moment greatly reduced the correlation [90]. E_T is, however, a very general descriptor of dispersive interactions, encompassing a number of individually- determined bulk parameters and was only used in this example to show that chromatographic retention on a polymeric octadecylsilica phase is related to both bulk and inductive forces.



$$\Delta = 0.394-(-0.329)$$
$$= \underline{0.723}$$

Figure 16 Example of the calculation of the excess electronic charge distribution parameter, Δ.

2.2.2 Ionisation Effects

An ionisable species can be classed as an acid, a base or an amphoteric molecule. An acid is a molecule which has a tendency to lose a proton and a base is a molecule which has a tendency to accept a proton. Amphoteric species such as amino acids have both a basic group and an acidic group. The strength of an acid or a base can be determined from the equilibrium constant, K, associated with the interaction of the species with water:

$$K = \frac{[H_3O^+].[A^- \text{ or } B^+]}{[H_2O].[HA \text{ or } HB]} \qquad \text{Equation 19}$$

where HA represents the acid species, HB the basic species and [] the concentration or activity of the species. As most measurements are made in dilute solution, $[H_2O]$ is constant. The equilibrium constant now becomes the acidic dissociation (or ionisation) constant, K_a. Taking the negative logarithm of K_a, the following expression is obtained:

$$pK_a = pH + \log ([A]/[B]) \qquad \text{Equation 20}$$

where A and B are the acid and basic species respectively. pK_a indicates the pH at which the solute will be 50% ionised and 50% unionised, i.e. the concentrations (activities) of the acidic and basic species are equal. The degree of ionisation of a solute changes rapidly within the

difference of a couple of pH units from the pK_a value. As a rule, an acidic solute will be at least 99% ionised when $pH > (pK_a+2)$ and for a basic solute when $pH < (pK_a-2)$. The extent of ionisation is an important factor in determining the aqueous solubility of an ionisable solute in reversed-phase liquid chromatography. As a solute becomes ionised to a greater extent, it will show a preference for the aqueous mobile phase with respect to a relatively non-polar stationary phase. This effect is very important when considering the partition coefficient of a solute in such a system (Section 2.3).

A substituent on a parent molecule may alter the overall pK_a of the solute. The new pK_a can be calculated using the Hammett constant, σ [118]

$$pK_a \text{ (substituted molecule)} = pK_a^\circ + \sum \sigma \qquad \text{Equation 21}$$

where $^\circ$ indicates the parent molecule, e.g benzoic acid, aniline. Different substituents have different σ-values, a table of which can be found in Reference 118. The σ-value of hydrogen is taken to be zero and is the reference substituent. A positive σ-value relative to hydrogen indicates that the substituent is acid-weakening (electron-donating) while a negative σ-value indicates that the substituent is acid-strengthening (electron-withdrawing). Hammett constants are usually only quoted for substituents at the meta- and para-positions. Substituents in the ortho-position have too varied an effect on the pK_a

of the parent molecule through hydrogen-bonding, steric effects and short-distance electric field effects to make them of any use in the simple prediction of solute pK_a from the above equation.

Ionisation effects represented by σ are not suitable polarity descriptors when considered by themselves. Most often they are used in conjunction with other parameters, such as the partition coefficient, which are affected by the ionisation behaviour of the solutes with a range of different functional groups [94,119,120]

2.2.3 Hydrogen Bonding

In reversed-phase liquid chromatography where the mobile phase normally consists of water and an organic modifier, the hydrogen-bonding ability of a solute becomes relevant. A hydrogen atom in a molecule which is attached to an electronegative element, such as oxygen or a halogen, will create a highly polarised bond. The polarised group is known as a "hydrogen-bond donor" (HD). When in contact with an atom which has a lone pair of electrons, e.g. nitrogen, carboxylic or alcoholic oxygen, known as a "hydrogen-bond acceptor" (HA), a weak hydrogen bond is formed. Water self-associates through hydrogen bonding and this explains its high boiling point. Methanol is a popular choice of organic modifier because it exhibits both HD and HA effects. Thus a wide range of solutes can form hydrogen bonds with this organic solvent and, in reversed-phase liquid chromatography, the solute retention times should be

reduced because of the favourable hydrogen-bonding interaction with the mobile phase components.

Jinno and Kawasaki [94], and Masuda et al. [96] have demonstrated that the numbers of HA and HD groups are relevant when used as a descriptors in multi-parameter correlation equations. The solvatochromic terms, α and β, are more quantitative descriptors of HA and HD ability respectively, and indicate to a better degree the strength of the hydrogen-bonding ability of a solute than just the number of HA and HD groups [112,121,122].

As will be realised by now, descriptors which represent inductive forces are not a good choice for single parameter relationships with retention. Yet when used in conjunction with bulk parameters, a more complete picture of the reversed-phase retention process can be built up.

One very important parameter still to be discussed is the partition coefficient, log P. As will be shown, log P is a reflection of many of the dispersive and inductive parameters which have already been discussed in the preceeding two sections.

2.3 The Partition Coefficient, Log P

In a biological system a solute, such as a drug, partitions between hydrophobic (lipophilic) tissue and an aqueous liquid. The preference shown by the solute for either phase will be related to the physical and chemical properties of the molecule as already mentioned. The lipophilic nature of a solute is represented by the

logarithm of the partitioning coefficient, P, which is expressed by

$$P = \frac{\text{(Concentration of solute in the lipophilic phase)}}{\text{(Concentration of solute in the hydrophilic phase)}} \qquad \text{Equation 22}$$

The greater the lipophilicity of a solute, the greater will be the concentation of solute in the lipophilic environment and P will be greater than one.

In order to model the biological environment for QSAR, an accurate representation of the interactions which would be exerted by both the hydrophobic and hydrophilic compartments was necessary. Conventional liquid-liquid chromatography was originally considered the best method for determining the partitioning coefficient. This method is known as the "shake-flask" method. Although a range of immiscible organic/aqueous systems have been tried, the most popular system is water and n-octanol. The n-octanol/water system has been adopted as the standard reference for partitioning behaviour and many compounds have been measured in this system [123].

If the solute is reasonably water-soluble, the sample is prepared in octanol-saturated buffer and the partitioning coefficient, P, is calculated by

$$P = \frac{A_B - A_A}{A_A} \times \frac{V_{aq}}{V_{org}} \qquad \text{Equation 23}$$

where A_B is the amount of solute present in the

octanol-saturated aqueous sample before partitioning between the two phases, A_A is the amount of solute in the aqueous phase after partitioning, V_{aq} is the volume of aqueous sample used and V_{org} is the volume of buffer-saturated octanol added to the sample.

Log P only applies to unionised solutes. If the solute is ionised, the partition coefficient is lower than would be expected because more of the solute will reside in the aqueous layer. The partition coefficient can be corrected for ionisation effects and is then known as the dissociation coefficient, log D, if such a correction is used.

<u>For basic compounds</u>-

$$\text{Log } D = \text{Log } P - \log \left[1 + 10\uparrow(pK_a - pH)\right] \qquad \text{Equation 24}$$

<u>For acidic compounds</u>-

$$\text{Log } D = \text{Log } P - \log \left[1 + 10\uparrow(pH - pK_a)\right] \qquad \text{Equation 25}$$

It is important to note whether the lipophilicity of a solute was measured in the ionised or unionised form as a difference of two pH units between pK_a and pH would decrease log P by 2.01. Care should be taken when using literature values in this respect.

Log P and log D correlate linearly with log k' with a slope equal to 1 if the chromatographic system is identical to the <u>n</u>-octanol/water system. However, a linear relationship is not normally observed because of three restricting factors; (a) the silica-bonded alkyl chains do

not act as a real organic liquid because the number of degrees of freedom is reduced through attachment to the sorbent, (b) the silica sorbent is a heterogeneous surface with active, exposed silanols acting as weak cation-exchange sites which will attract bases and strong acids, and (c) methanol is present in the mobile phase. The presence of methanol affects the chromatographic partitioning of solutes with polar substituents, e.g. phenols [119,124], which hydrogen-bond to the polar organic modifier thereby reducing both their retention time and partition coefficient. There are several alternatives to overcome such problems including coating of the stationary phase with n-octanol [125], using an organic amine and n-octanol in a phosphate buffer to reduce silanol effects [126] and using the Collander equation to account for the presence of methanol. The Collander equation has the form

$$\text{Log } P = \text{Log } k' + \text{Log } K \qquad \text{Equation 26}$$

where K is a constant relating to the equilibrium between the mobile phase and the stationary phase in the chromatographic system. Extrapolation of the plot (log k' versus % organic modifier) to 0% organic solvent is a popular way of reducing the error caused by the presence of the non-aqueous solvent and is often used as a means of determining log P. It is a more efficient and faster method of determining the partition coefficient than the shake-flask method. However, there are three limiting

factors on measuring hydrophobicity by high performance liquid chromatography;

(a) the physicochemical state of the solute.

Ionisation, as already mentioned, can reduce or increase retention through either increased attraction of the solute for the aqueous mobile phase, interaction with silanols or intramolecular interaction between polar functional groups which will make the molecule appear more hydrophobic [127,128].

(b) the percentage of organic modifier in the mobile phase.

The amount of methanol in the mobile phase is important as below 10% and above 50% (v/v) methanol in water, the relationship between log k' and (% methanol) is no longer linear [106,124]. One reason is that high percentages of methanol increase the pH of the mobile phase by approximately two units which may ionise a solute which would not normally be expected to ionise in a particular system [129].

(c) the solvent system used to measure hydrophobicity.

It must also be remembered that octanol and methanol have different characteristics such as chain length and degree of polarity. Although log k' *versus* (amount of methanol) is linear (within a certain range), log k' has a quadratic relationship with acetonitrile [130,131]. At low % acetonitrile, the organic modifier is easily incorporated into the water network, but in greater quantities, acetonitrile is not so readily accepted into the normal

structure and the properties of the mobile phase change [95].

Relating the capacity factor to concentration of methanol (φ)

$$\log k' = a\varphi + \log k'_w \qquad \text{Equation 27}$$

where $\log k'_w$ is the extrapolated capacity factor at 0% methanol and a is a constant. Such a relationship has been noted for caffeine and its metabolites [132] and benzodiazepines on a phenyl phase [133]. Opperhuizen et al. used a solubility parameter, S, in place of the constant in equation 27, and found that the following relationship which held for alkyl and chlorinated hydrocarbons:

$$\log k' = \log k'_w - S\varphi \qquad \text{Equation 28}$$

[134]

The addition of a salt to alter the ionic strength of the solvent system in either LLE or HPLC will also affect partitioning through 'salting-out' effects which can reduce the affinity of a solute for the hydrophobic phase and thereby increasing log P, as demonstrated for carboxylic acids with phosphate buffers [135].

In summary, the problems which arise between log P_{LLE} and log P_{HPLC} are: additional phase components in HPLC; immobility of the bonded phase in HPLC; different hydrogen-bonding properties exerted by methanol and silanols as compared to octanol in LLE.

Attempts to predict retention from the influence of different solute substituents on solute hydrophobicity have been successful by employing Hansch parameters, π, where

$$\pi = \log P_{HX} - \log P_{H} \qquad \text{Equation 29}$$

where $\log P_H$ is the n-octanol/water partition coefficient of a parent compound and $\log P_{HX}$ is the partition coefficient of the substituted parent compound. π is a free energy constant because it is derived from an equilibrium. Electron-withdrawing functional groups such as -OH increase solubility of the solute in water through hydrogen-bonding as do substituents with oxygen and nitrogen lone pairs [119]. The Hansch parameter has been widely applied as a measure of the contribution of a functional group to partitioning behaviour, including anti-rheumatics [136], substituted benzene and phenol compounds [94] and pyridines [128]. Yamamoto *et al.* replaced π with a solubility parameter which could be used for a homologous series [109], but solubility parameters break down for polar solutes thereby reducing the applicability of the solubility parameter [137].

A similar substituent parameter was introduced by Rekker [138]. Called the 'fragmental hydrophobic constant', f, it was intended to replace π as f corrects for folding of long alkyl chains.

$$\text{Log } P = \sum a_n f_n \qquad \text{Equation 30}$$

where a is the incidence of a given fragment in the molecule and f is the fragmental substituent constant. Rekker's fragmental constant has been applied to the retention prediction of aromatic acids [139], but D'Amboise and Hanai found that solutes with small values of log P_{Rek}, e.g. n-alcohols, did not correlate well with alkylbenzenes and polyaromatic hydrocarbons [140].

Solvatochromism is a phenomenon that relates changes in the spectrum, e.g. intensity, shape and position of peaks in the spectrum of a molecule to changes in its environment. Solvatochromic parameters include π^* which is an index of solvent polarisability, i.e. the ability of a solvent to stabilise a charge or dipole by virtue of its dielectric effect, α which represents the ability of a solvent or solute to accept a proton (H-bond acceptor) and β which represents the ability of a solvent or solute to donate a proton (H-bond donor) [121]. Kamlet, Taft and fellow workers have applied the above solvatochromic parameters to solubility:

Solubility, S, = cavity term + polarity term + hydrogen- bonding term

i.e.

$$S = a + b(V/100) - c\pi^* - d\beta - e\alpha \quad \text{Equation 31}$$

where V is the molar volume and a, b, c, d and e are constants [88]. Leahy applied these equations to partitioning and retention prediction, using a

computed-generated Van der Waals volume, V_{vdv}, instead of molar volume as V is actually correlated with π^*, β and α [121,122]. He reported that such equations in the above form could be applied to a diverse set of substituted benzene solutes except those which possessed a basic centre.

CHAPTER THREE

PROBLEMS ASSOCIATED WITH THE ANALYSIS OF PROPRANOLOL

Propranolol belongs to a group of drugs classed as ß-adrenoreceptor antagonists which also includes atenolol, timolol, practolol and corwin. Known commonly as "ß-blockers", they are orally administered in the treatment of coronary disease and hypertension. Propranolol works by preventing adrenaline and noradrenaline from activating $beta_1$ receptors which are predominant in the heart. When activated, the $beta_1$ receptors increase heart rate and cause other symptoms associated with anxiety. ß-blockers reduce the oxygen demand made on the heart under duress by reducing the heart rate which in turn improves coronary flow. Propranolol is a lipophilic drug (log P=3.56) so that it readily crosses the blood-brain barrier and is normally administered as the hydrochloride salt to aid dissolution in the stomach. The therapeutic level is quoted as 0.05-1µg/ml [141]

Sensitive analytical techniques are required in forensic toxicology, clinical pharmacokinetic studies and drug testing in sport for the detection of ß-blockers and their metabolites in biological fluids. The most common methods are HPLC with ultra-violet and fluorescence detection, immunoassays or gas chromatography [142-144]. It can be seen from the structure of propranolol (Figure 17) that it is composed of a large hydrophobic centre

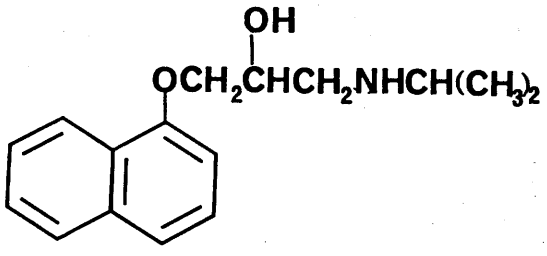


Figure 17 The structure of propranolol.

(naphthalene ring) and a polar side chain with a basic centre. The basic nature of propranolol, and indeed many other drugs, makes it difficult to extract and analyse the compounds efficiently with bonded silica sorbents because basic solutes are retained longer than expected. This is due to exposed active acidic silanol sites present at the silica surface which interact with basic centres through either ionic or hydrogen-bonding attractions. There are thought to be two different types of active silanols - one with a weak binding ability, but a high capacity, and the other strong binding ability, but low capacity. These have been observed for phenylsilica phases and are assumed to exist in most bonded silica LC systems [145,146]. In HPLC this phenomenon can be observed by the increased retention times of basic compounds and the tailing of detected peaks as the basic solute overloads the low capacity sites and passes through the column at different rates.

Often a mobile phase additive is employed to minimise the effect of silanol interaction with basic compounds. Common methods include lowering the pH of the eluent to approximately pH4 to suppress ionisation of the silanol groups [147-149] and addition of an ion-pairing agent which forms a neutral complex with the cationic solute [150-152]. Another widely used method in the analysis of basic drugs by reversed-phase liquid chromatography is the use of a competing amine in the mobile phase introduced by Wahlund *et al*. [153-155]. The amine should possess a reasonably bulky or long alkyl

substituent to block the maximum possible number of silanols. De Shutter and co-workers observed that total suppression of silanol participation with an organic amine as the sole additive reduced selectivity between quarternary ammonium drugs. Addition of a second additive, sodium 1-octyl-sulphonate, to the mobile phase restored selectivity while reducing peak tailing [156].

In solid-phase extraction, the main objective is to elute all compounds of interest as efficiently and cleanly as possible in the minimum number of steps and minimum amount of eluent. When selectivity between a parent compound and its metabolites, or between structurally-similar solutes, is not a stipulation, organoamines are widely used as eluent additives, the most popular amine additives being triethyl- and tripropyl-amine [155,157-159]. Work by Ruane and Wilson on the SPE of ß-blockers showed that when silanol activity is minimised by an organoamine, the retention behaviour of a basic drug in SPE still cannot be predicted by hydrophobic parameters log P and pK_a alone and that some additional factors must be responsible for the behaviour of the solutes [160].

It is well known that extraction methods developed on a particular manufacturer's bonded phase may not work on a phase with the same functional group(s) made by another manufacturer [152,158]. This is because the properties of bonded phases are sensitive to the different types of silica which can be used as the sorbent, and also the various synthetic methods used to prepare the phases. If

the extent of participation of the silanol groups could be predicted, then the selectivity from the active sites could be used to improve the efficiency of the purification process and to add an additional dimension of selectivity.

CHAPTER FOUR

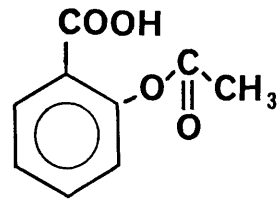
EXPERIMENTAL METHODS AND RESULTS

4.1 Experimental

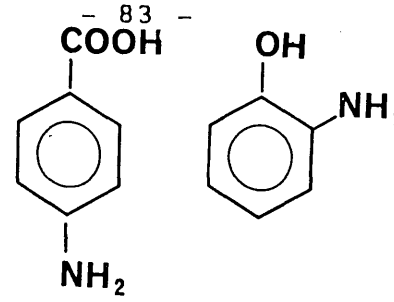
4.1.1 Test Compounds

A range of analytical-grade mono-, bi- and tri-functional benzene compounds was obtained from various chemical manufacturers (Figure 18). Each solute was dissolved in the mobile phase to be used except the benzene, toluene and cumene solutions which were prepared with 100% HPLC-grade methanol (BDH Chemical Co., Poole, UK). Solution concentrations were between 1-10mg/ml or 1-10µl/ml depending on whether the compound was in a solid or liquid form at room temperature.

Propranolol and fourteen of its analogues synthesised at ICI Pharmaceuticals (Alderley Park, Cheshire, UK) were selected as model compounds for the retention prediction of ß-blocking drugs (Figure 19). The ICI registry numbers of the compounds were M046004 (compound no.1), M115715 (compound no.2), M115716 (compound no.3), M045655 (compound no.4), M065318 (compound no.5), M109056 (compound no.6), M109055 (compound no.7), M047070 (compound no.8), M087086 (compound no.9), M081509 (compound no.10), M045520 (compound no.11), M049666 (compound no.12), M052092 (compound no.13), M052487 (compound no.14), M051932 (compound no.15). The identity and purity of each substance were confirmed by

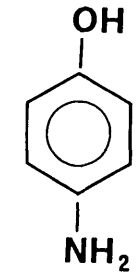


Acetyl Salicylic Acid

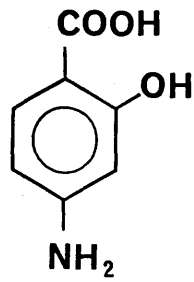


p-Amino Benzoic Acid

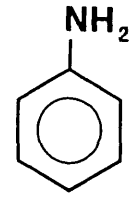
o-Amino Phenol



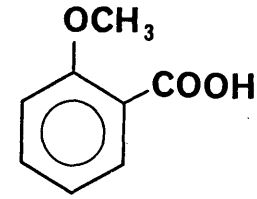
p-Amino Phenol



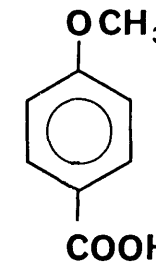
p-Amino Salicylic Acid



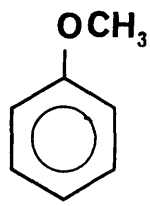
Aniline



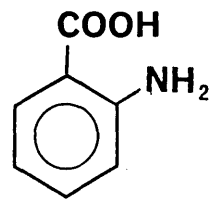
o-Anisic Acid



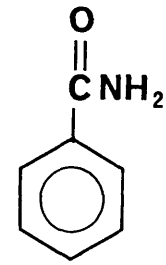
p-Anisic Acid



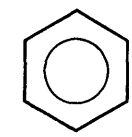
Anisole



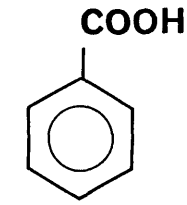
Anthranilic Acid



Benzamide

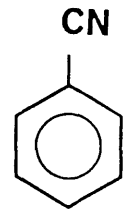


Benzene

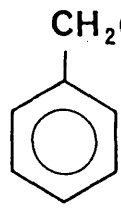


Benzoic Acid

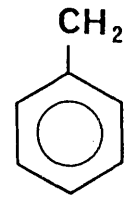
Figure 18 Structures of the benzene test solutes. cont./...



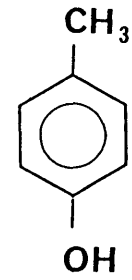
Benzonitrile



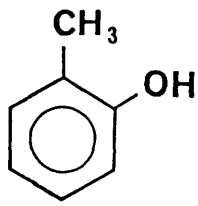
Benzyl Alcohol



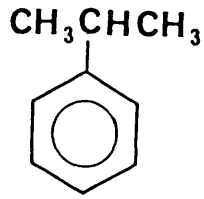
Benzylamine



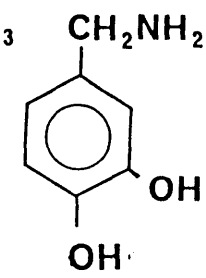
p-Cresol



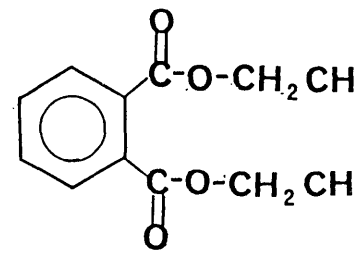
o-Cresol



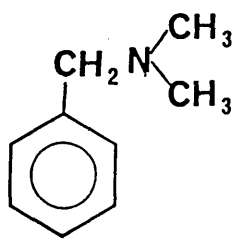
Cumene



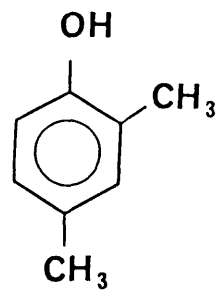
3,4-Dihydroxy Benzylamine



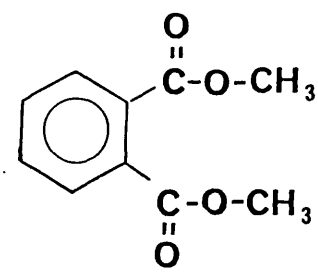
Diethylphthalate



N,N-Dimethyl Benzylamine

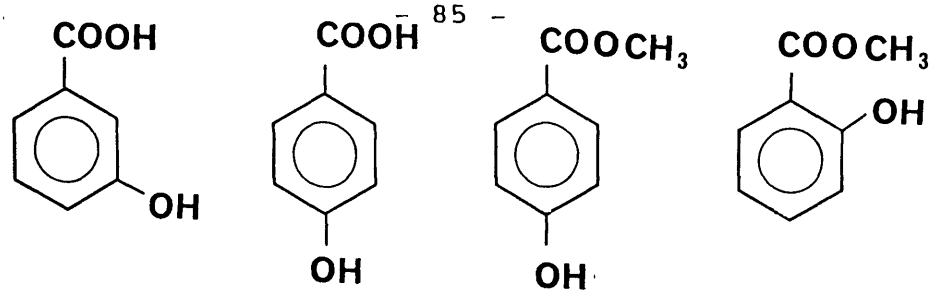


2,4-Dimethyl Phenol

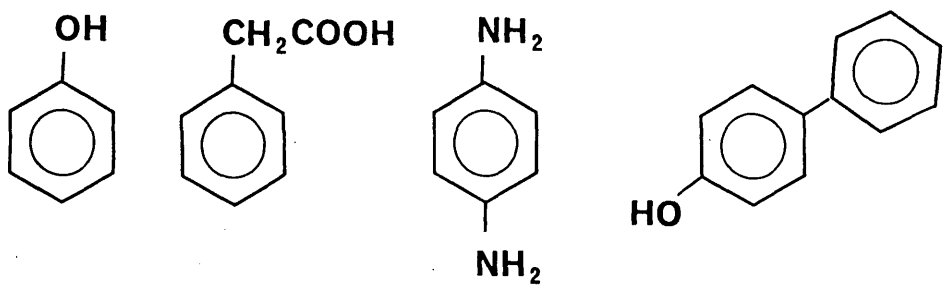


Dimethylphthalate

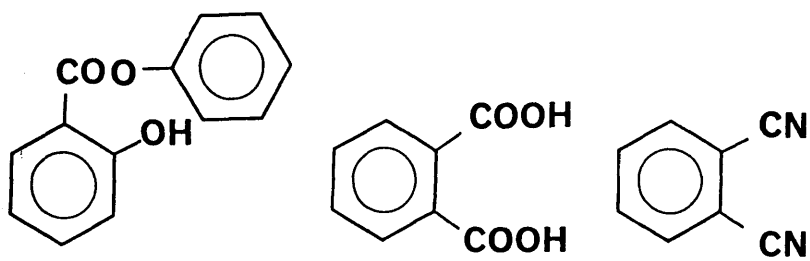
Figure 18 cont./...



m-Hydroxy Benzoic Acid | p-Hydroxy Benzoic Acid | Methyl-4-Hydroxy Benzoate | Methylsalicylate

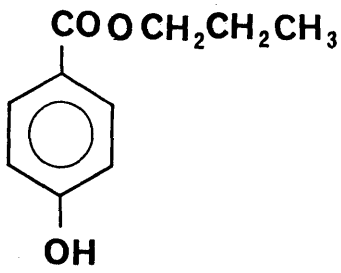


Phenol | Phenyl Acetic Acid | p- Phenylene Diamine | p-Phenylphenol

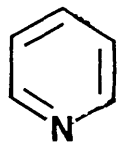


Phenylsalicylate | Phthalic Acid | Phthalodinitrile

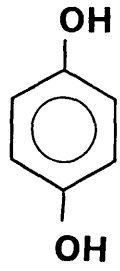
Figure 18 cont./...



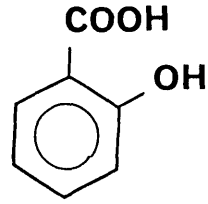
n-Propyl-p-hydroxy Benzoate



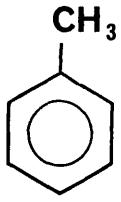
Pyridine



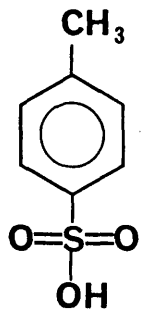
Quinol



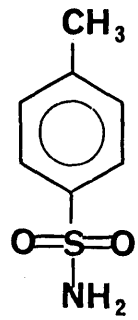
Salicylic Acid



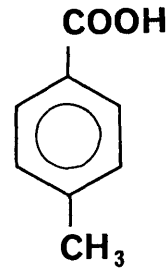
Toluene



Toluene-p-sulpnonic Acid



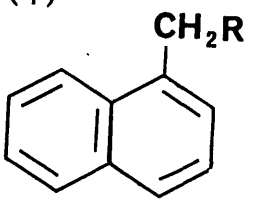
Toluene-p-sulphonamide



p-Toluic Acid

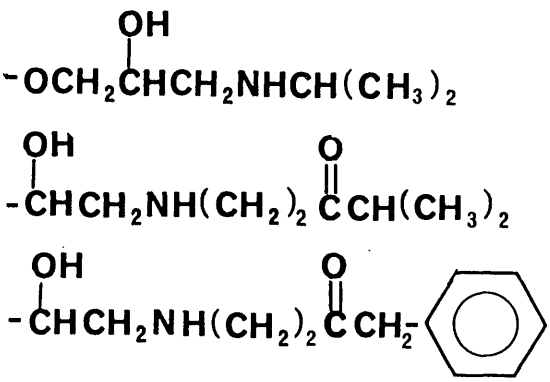
Figure 18

(1)



Compound Number:

R=

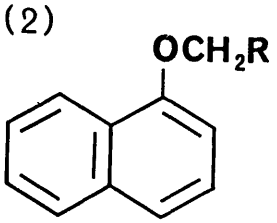


-1

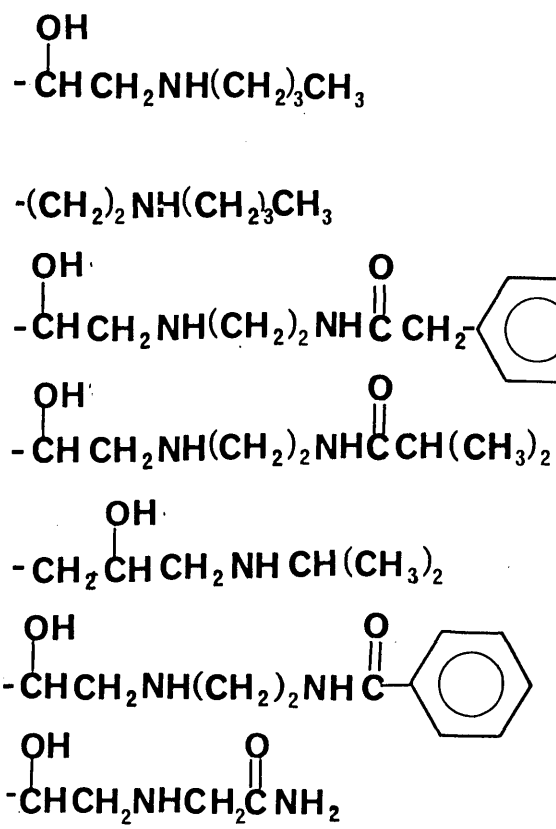
-2

-3

(2)



R=



-4

-5

-6

-7

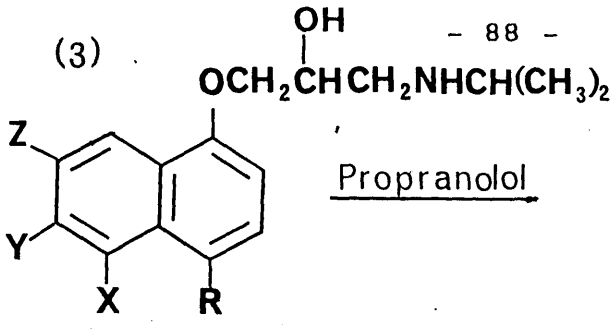
-8

-9

-10

Figure 19 Structures of propranolol and analogues used as test solutes. cont./...

(3)



Propranolol

-11

R= -OCH_3 -12

X, Y, Z = H

X= -OCH_3 -13

R, Y, Z = H

Y= -OCH_3 -14

R, X, Z = H

Y, Z= -OCH_3 -15

R, X = H

Figure 19

injecting a standard solution of the drug in methanol (1mg/ml) into a VG Analytical liquid chromatograph-mass spectrometer combination (model 70-250S) *via* a plasmaspray interface operating at a temperature of 240°C (VG Analytical Instruments, Manchester, UK). For all other HPLC experiments, standard solutions were prepared at a concentration of 10µg per ml of mobile phase.

4.1.2 Instrumentation

High performance liquid chromatographic retention times were measured on a Hewlett Packard 1081B liquid chromatograph fitted with a nitrogen- or compressed air-operated variable volume injector, autosampler tray and column compartment temperature regulator (Waldbronn, FRG). Mobile phases were pumped through the system at 1±0.1ml/min. Peaks were detected by a Kratos Spectroflow 757 variable wavelength UV detector (Kratos, NJ, USA) set at a sensitivity level of 0.1 AUF and a wavelength of 254nm for benzene solutes and 290nm for propranolol and its analogues. A potentiometric chart recorder was used to record the HPLC peaks and was also used in conjunction with an integrator (LDC 308, Shannon, Ireland) for both the effect of temperature on selected benzene solutes and ß-blocker retention behaviour studies.

4.1.3 Bonded Phase Columns

Five non-polar modified silica phases manufactured by Analytichem International Inc. (Harbour City, CA, USA) as

used in their Bond Elut® solid phase extraction cartridges were examined. The phases studied with the benzene compounds were ethylsilica (Lot No.032059), octylsilica (Lot No.0628234), octadecylsilica (Lot No.0729123), cyclohexylsilica (Lot No.103079) and phenylsilica (Lot No.080560). Octyl-, cyclohexyl- and phenyl-silica were chosen for the ß-blocker studies. The average particle size and pore diameter quoted by the manufacturer were 40µm and 60Å respectively. The percentages of carbon and hydrogen were measured for each of the phases by elemental microanalysis (Table 1).

The bonded phase packings were supplied in loose powder form and were packed by the tap-fill method, described by Snyder and Kirkland [162], into 100x4.5mm internal diameter stainless steel HPLC column tubes fitted with 15µm-pore bed support frits and female column-end fittings (Jones Chromatography, Mid Glamorgan, UK). Prior to packing, the HPLC columns and fittings were washed thoroughly with distilled water and methanol to remove any dirt or grease, then dried under nitrogen.

4.1.4 Mobile Phases

(a) Benzene Solute HPLC Studies

Eight aqueous methanol mobile phases were used for these chromatographic studies. Four different concentrations of HPLC-grade methanol were prepared with buffers dissolved in de-ionised, distilled water (MilliQ System, Waters, MA, USA). The concentrations used were 20%,

PHASE	% CARBON	% HYDROGEN
Ethylsilica	5.36	1.10
Octylsilica	11.75	2.12
Octadecylsilica	17.68	3.53
Cyclohexylsilica	8.46	1.41
Phenylsilica	10.15	0.96

Table 1 Elemental microanalyses of ethyl-, octyl-, octadecyl-, phenyl- and cyclohexyl-silica.

30%, 40% and 50% (v/v) methanol in aqueous buffer. Each concentration was duplicated and buffered to approximately pH7 or pH5 using buffer tablets (BDH Chemical Co.) or an acetic acid/sodium acetate buffer [161] respectively.

(b) ß-Blocker Solute HPLC Studies

A mobile phase containing 0.3M tri-n-butylamine acetate in methanol:water (30:70 v/v) was prepared by adding tri-n-butylamine (10.2ml) and glacial acetic acid (12ml) to methanol (150ml). De-ionised, distilled water (350ml) was then added and the solution mixed thoroughly. The pH of this mobile phase was 4.1.

When in use, a mobile phase would be prepared every 1-2 days. Prior to use the mobile phase was degassed with helium.

A Pye Unicam pH meter, model 291 (Cambridge, UK) fitted with a glass reference electrode was used to measure solution pH.

4.1.5 HPLC Measurements

Before retention measurements were made, each newly packed bonded phase was conditioned with 15ml methanol. Between 30 and 120ml of mobile phase was subsequently passed through the column to achieve equilibrium before the test solutes were injected into the system. All mobile phases used for the benzene solute studies were recycled. The column pressure did not exceed 90 bar. Distilled, deionised water was flushed through the system for 120 min between mobile phase changes.

Octyl-, cyclohexyl- and phenyl-silica were chosen for retention prediction studies with the propranolol test solutes. The mobile phase used for these studies was 30:70 (v/v) methanol:water buffered with 0.3M tributylamine/acetic acid. The effects of plasma protein solution and fresh plasma on the retention behaviour of selected propranolol analogues were also studied by HPLC with the three chosen phases. After solvation of the selected phase with methanol as described above, the silanol selectivity coefficient, α_{SiOH}, was determined with 5µl each of 1mg/ml N,N-diethyl-m-toluamide (N,N-DETA) (Aldrich Chemical Co., Dorset, UK) and 40mg/ml anthracene (Aldrich Chemical Co.) in 100% acetonitrile which was used as the mobile phase [163]. The void time, t_o, for the silanol concentration determination was measured with 5mg/ml sodium nitrite (BDH Chemical Co.) in 65:35 (v/v) acetonitrile:water.

$$\alpha_{SiOH} = \frac{k'(N,N\text{-DETA})}{k'(\text{Anthracene})} \qquad \text{Equation 32}$$

(k' was calculated according to equation 33)

After α_{SiOH} was measured, 10ml of 100% methanol then 30ml de-ionised, distilled water was passed through the system at 1ml/min before the following sequence was used to measure the retention times under different matrix conditions:

(i) Equilibration of column for 30 min with the buffered 30:70 methanol:water (v/v) mobile phase (pH4) described in section 4.1.4(b).

(ii) Measurement of retention times for the test solutes.

(iii) Removal of silanol-bound tributylamine with 20ml water acidified with glacial acetic acid to pH2.6.

(iv) α_{SiOH} remeasured.

(v) Injection of 1ml plasma protein solution. Removal of excess plasma with 10ml deionised, distilled water.

(vi) Equilibration of column with 20ml of 30:70 (v/v) methanol:water with no buffer added.

(vii) Measurement of retention times for the test solutes with only plasma effects.

(viii) Removal of silanol-bound plasma products with 20ml water acidified with glacial acetic acid to pH2.6.

(ix) Equilibration of column with the pH4-buffered aqueous methanol mobile phase for 30 min.

(x) Measurement of retention times for the test solutes with plasma and tri-n-butylamine effects.

Steps (iii) to (x) were repeated with fresh plasma instead of plasma protein solution. α_{SiOH} values are shown in Table 2.

The column compartment was maintained at a constant temperature of 25±0.2°C by the internal regulator for all HPLC determinations except the temperature studies where the column was subjected to temperatures of 25, 30 and 35°C. The column temperature was allowed to equilibrate overnight.

Approximatly 10μg of each test solute in 10μl of mobile phase was injected onto the column. Each chromatographic run was repeated at least three times and the retention time, t_r, taken as the average value. The void volume, t_o, was measured with either sodium nitrite for the benzene solute studies, or uracil for the ß-blocker studies. The solute capacity factor, k', was calculated by

$$\text{Log } k' = \frac{t_r - t_o}{t_o} \qquad \text{Equation 33}$$

4.1.6 Partition Coefficient Measurements of Propranolol and its analogues

The following method for partition coefficient (log P) and dissociation constant (log D) measurements was adapted from one used by the Physical Chemistry Division of ICI Pharmaceuticals (Alderley Park, Cheshire, UK).

Log P was measured at pH9.5 (de-ionised, distilled

PHASENAME	[a]SiOH		
	After Solvation with 100% methanol	After removal of tributylamine	After removal of plasma protein solution
Octylsilica	0.70	0.69	0.70
Cyclohexyl-silica	1.44	1.50	1.80
Phenylsilica	5.50	5.33	-[a]

[a]-Not measured

Table 2 Silanol concentrations of octyl-, cyclohexyl- and phenyl-silica after solvation, use of tributylamine and use of plasma protein solution.

water buffered with pH9.2 tablets (BDH Chemical Co.) and adjusted to pH9.5 with diethylamine), and the dissociation constant, log D, was measured at pH4.

Solutions of each test compound in octanol-saturated buffer (0.1mg/ml, 10ml) were transferred to separate clean, dry centrifuge tubes. Buffer-saturated octanol (100 µ l) was added to the tubes which were then capped and vortexed for 1 min. The tubes were mixed overnight on a rock/roll platform to equilibrate. The solutions were left to stand for 10 min before centrifuging for 15 min at 3700 rpm (Gallenkamp, UK). The aqueous layer was filtered and transferred to a fluorescence cuvette and the amount of drug measured by spectrofluorimetry with an emission wavelength of 342nm and an excitation wavelength of 290nm, both slitwidths being set at 2.5nm (Perkin Elmer, Model LS-5 luminescence spectrometer, Cambridge, UK). The partition coefficient was calculated by

$$P = \frac{A_a - A_b}{A_b} \times \frac{V_{aq}}{V_{oct}} \qquad \text{Equation 34}$$

where A_b and A_a are the degree of fluorescence before and after extraction respectively, V_{aq} is the volume of stock solution used (10ml) and V_{oct} is the volume of saturated octanol added to the aqueous solution (0.1ml). The dissociation constant, D, was calculated using the same equation as for P.

4.2 Computational Methods

4.2.1 Physical and Chemical Solute Properties

Physical and chemical properties taken from the literature are shown in Table 3. A molecular modelling system called VIKING (ICI Pharmaceuticals Division, Alderley Park, Cheshire, UK) was used in tandem with the molecular property calculation package, ICICAL (ICI Pharmaceuticals Division), to generate molecular volumes, dipole moments, polarisabilities, moments of polarisability, elliptical principal axes, momentums and atom charges for all solutes used. Molecular structures and properties were generated by the CNDO/2 (Complete Neglect of Differential Overlap) method using standard bond angles and bond lengths.

The following parameters were calculated using some of the properties shown in Table 3:

$$\text{Molar Refractivity, } R_m \ (cm^3mol^{-1}) = \frac{(\eta^2-1)}{(\eta^2+1)} \frac{M}{\rho} \qquad \text{Equation 35}$$

$$\text{Molar Volume, } V_m \ (cm^3mol^{-1}) = \frac{M}{\rho} \qquad \text{Equation 36}$$

$$\text{Submolecular polarity parameter, } \Delta = q_1 - q_2 \qquad \text{Equation 37}$$

[92]

where η is the refractive index, M is the molecular weight, ρ is the density, and q_1 and q_2 are the charges on two atoms which have the greatest charge difference in a molecule.

PHYSICOCHEMICAL SOLUTE PROPERTIES	SOURCE
(a)Taken From Literature	
Molecular Weight, MW Melting Point, mp (°C) Boiling Point, bp (°C) Density, ρ (gcm^{-3}) Refractive Index	[164]
Partition Coefficient, Log P Hammett Constant, σ (meta- and para-substituents) Number of Electron Acceptor Groups, HD Number of Electron Donor Groups, HA Hansch Parameter, π	[123]
First Order Molecular Connectivity, $^1\chi^v$	[115]
F-value	[94]
Ionisation Constant, pK_a	[118]
Hydrogen bond donor ability, ß	[112,121]
(b)Computed Parameters	
Volume, $Å^3$ Dipole Moment (μ) } for whole molecule Polarisability } and substituents Moment of Polarisability } Principle Elliptical Axes Momentum Atomic Charge	VIKING and ICICAL computer-aided molecular modelling systems.

Table 3 Source of solute physicochemical data.

4.2.2 Statistical Data Analysis

Solute physical and chemical properties were entered into a database compiled with Scientific Information Retrieval (SIR) software, version 2 (SIR Inc., IL, USA), on both Glasgow University's VME network (ICL 3980 mainframe computer) and an IBM PS/2, model 50 personal computer (PC). Data to be used in a particular statistical analysis was transferred from the database to the statistical package SPSSX PC+, version 2.1 (SPSS Inc., IL, USA) on the IBM PC.

Correlation analyses are presented in accordance with the guidelines recommended by Charton et al. [165].

4.3 Results

Chromatographic retention times were determined by HPLC for a range of mono-, bi- and tri-functional benzene solutes using five non-polar stationary phases and four aqueous methanol mobile phases, each duplicated and buffered to approximately pH5 and pH7. Various combinations of stationary phase, mobile phase organic modifier concentration and pH allowed retention times to be determined in forty different chromatographic systems. The logarithmic capacity factors (log k') values for each of the benzene test solutes are presented in Table 4. The retention times measured by hand and those measured by the integrator were precise to within ±0.1min. The solutes were eluted with the mobile phase in the order of highest methanol concentration (50%) to the lowest concentration (20%) to eliminate possible bonded phase character changes caused by irreversibly-bound solutes which do not elute at low methanol concentrations e.g. diethylphthalate and cumene. Very good peak shapes were achieved considering that the Bond Elut phases consisted of large, irregularly-shaped particles not intended for use as HPLC stationary phases. Three examples of typical HPLC peak shapes obtained for aniline are shown in figure 20. The number of theoretical plates, N, for each bonded phase used, measured with aniline, are shown in Table 5.

Statistical analysis was performed to test for the presence of linear correlations between log k' and the solute physicochemical properties. Appendix I contains the

LOG K' VALUES FOR THE ETHYL PHASE

SOLUTE NAME	20% MeOH pH 5.0	20% MeOH pH 7.0	30% MeOH pH 5.1	30% MeOH pH 7.0
Acetyl salicylic acid	b	1.41	0.29	1.21
p-Amino benzoic acid[c]	–	–	–	–
o-Amino phenol[c]	–	–	–	–
p-Amino phenol	b	b	0.14	b
p-Amino salicylic acid[c]	–	–	–	–
Aniline	c	c	0.31	0.49
o-Anisic acid[c]	–	–	–	–
p-Anisic acid[c]	–	–	–	–
Anisole	c	c	0.95	1.01
Anthranilic acid[c]	–	–	–	–
Benzamide	b	0.48	0.31	0.43
Benzene	c	c	b	b
Benzoic acid	0.22	b	0.15	-0.65
Benzonitrile	c	c	b	4.38
Benzyl alcohol	c	c	0.45	0.55
Benzylamine[c]	–	–	–	–
o-Cresol	c	c	0.77	0.82
p-Cresol	c	c	0.70	0.75
Cumene	c	c	d	d
3,4-Dihydroxy benzylamine				
Diethyl phthalate	c	c	d	d
N,N-Dimethyl benzylamine[c]	–	–	–	–
2,4-Dimethyl phenol	c	c	0.95	1.09
Dimethyl phthalate	c	c	b	b
m-Hydroxy benzoic acid	-0.18	-1.24	-1.03	a
p-Hydroxy benzoic acid	b	a	0.01	-1.21
Methyl-4-hydroxy benzoate	c	c	0.93	0.97
Methyl salicylate	c	c	1.26	1.31
Phenol	c	c	0.46	0.57
Phenyl acetic acid	0.19	b	0.13	-0.33
p-Phenylene diamine[c]	–	–	–	–
p-Phenyl phenol	c	c	d	d
Phenyl salicylate	c	c	b	b
Phthalic acid	a	-0.41	-0.60	-0.91
Phthalodinitrile	c	c	0.88	b
n-Propyl-p-hydroxy benzoate	c	c	c	1.63
Pyridine	c	c	0.52	0.74
Quinol	0.05	0.13	-0.02	0.12
Salicylic acid	-0.19	-1.41	-0.36	-0.79
Toluene	c	c	b	b
Toluene-p-sulphonamide	c	c	-0.48	-0.99
Toluene-p-sulphonic acid	-0.39	-1.72	-0.53	-0.99
p-Toluic acid[c]	–	–	–	–

a-negative or zero k' value b-no elution time obtained c-not used in this particular system d-k' value greater than 50 (log k' > 1.70)

Table 4 Logarithmic capacity factors of substituted benzene test solutes from high performance liquid chromatographic studies. cont./...

LOG K' VALUES FOR THE ETHYL PHASE (cont.)

SOLUTE NAME	40% MeOH		50% MeOH	
	pH 5.5	pH 7.2	pH 5.6	pH 6.9
Acetyl salicylic acid	-0.58	-0.47	-0.50	-0.61
p-Amino benzoic acid[c]	-	-	-	-
o-Amino phenol[c]	-	-	-	-
p-Amino phenol	-0.32	0.20	c	c
p-Amino salicylic acid[c]	-	-	-	-
Aniline	0.16	0.42	0.15	0.26
o-Anisic acid[c]	-	-	-	-
p-Anisic acid[c]	-	-	-	-
Anisole	0.75	1.00	0.58	0.67
Anthranilic acid[c]	-	-	-	-
Benzamide	0.11	0.38	0.05	0.14
Benzene[b]	-	-	-	-
Benzoic acid	-1.12	-0.66	-0.20	-0.26
Benzonitrile	0.64	0.85	0.45	0.55
Benzyl alcohol	0.29	0.52	0.21	b
Benzylamine[c]	-	-	-	-
o-Cresol	0.54	0.79	0.39	0.50
p-Cresol	0.55	0.79	0.39	0.51
Cumene	d	d	1.24	1.33
Diethyl phthalate	d	1.61	d	1.04
3,4-Dihydroxy benzylamine	-0.21	1.32	0.00	1.04
N,N-Dimethyl benzylamine[c]	-	-	-	-
2,4-Dimethyl phenol	0.84	1.05	0.56	0.69
Dimethyl phthalate[c]	-	-	-	-
m-Hydroxy benzoic acid	-0.53	-1.40	-0.56	-0.55
p-Hydroxy benzoic acid	-0.24	-1.70	a	-0.55
Methyl-4-hydroxy benzoate	0.69	0.91	0.41	0.53
Methyl salicylate	1.60	1.26	0.77	0.87
Phenol	0.30	0.54	0.21	0.31
Phenyl acetic acid	-0.08	-0.47	-0.19	c
p-Phenylene diamine	-0.23	0.34	-0.12	c
p-Phenyl phenol	1.32	1.58	0.91	1.03
Phenyl salicylate[b]	-	-	-	-
Phthalic acid	-0.81	a	-0.86	a
Phthalodinitrile	0.49	0.74	0.34	c
n-Propyl-p-hydroxy benzoate	0.79	1.53	0.83	c
Pyridine	0.36	0.62	0.28	0.41
Quinol	-0.18	0.09	-0.13	-0.12
Salicylic acid	-0.17	-0.66	-0.65	-0.68
Toluene[b]	-	-	-	-
Toluene-p-sulphonamide	0.24	0.49	0.11	0.19
Toluene-p-sulphonic acid	-0.91	-0.74	-0.86	-1.25
p-Toluic acid[c]	-	-	-	-

a-negative or zero k' value b-no elution time obtained c-not used in this particular system d-k' value greater than 50

Table 4 cont./...

LOG K' VALUES FOR THE OCTYL PHASE

SOLUTE NAME	20% MeOH pH 5.0	20% MeOH pH 7.0	30% MeOH pH 5.1	30% MeOH pH 7.0
Acetyl salicylic acid	c	c	-0.04	c
p-Amino benzoic acid[c]	-	-	-	-
o-Amino phenol[c]	-	-	-	-
p-Amino phenol[c]	-	-	-	-
p-Amino salicylic acid[c]	-	-	-	-
Aniline	c	c	0.48	b
o-Anisic acid[c]	-	-	-	-
p-Anisic acid[c]	-	-	-	-
Anisole	c	c	1.32	1.57
Anthranilic acid[c]	-	-	-	-
Benzamide	0.62	0.78	0.34	0.56
Benzene	c	c	b	b
Benzoic acid	-0.69	-0.23	0.12	0.08
Benzonitrile	c	c	1.05	1.22
Benzyl alcohol	c	c	0.72	0.89
Benzylamine[c]	-	-	-	-
o-Cresol	c	c	1.11	1.29
p-Cresol	c	c	1.12	1.25
Cumene	c	c	d	d
Diethyl phthalate	c	c	d	d
3,4-Dihydroxy benzylamine	-1.62	c	-0.68	0.88
N,N-Dimethyl benzylamine[c]	-	-	-	-
2,4-Dimethyl phenol	c	c	1.42	d
Dimethyl phthalate	c	c	1.30	1.61
m-Hydroxy benzoic acid	0.14	-0.73	-0.12	-0.43
p-Hydroxy benzoic acid	0.30	-0.60	0.07	-0.85
Methyl-4-hydroxy benzoate	c	c	1.09	1.29
Methyl salicylate	c	c	1.63	-
Phenol	c	c	0.73	0.91
Phenyl acetic acid[c]	-	-	-	-
p-Phenylene diamine[c]	-	-	-	-
p-Phenyl phenol	c	c	d	d
Phenyl salicylate	c	c	0.73	0.91
Phthalic acid	-0.08	a	-0.38	a
Phthalodinitrile[c]	-	-	-	-
n-Propyl-p-hydroxy benzoate[c]	-	-	-	-
Pyridine	c	c	0.45	0.76
Quinol	-0.01	0.22	-0.20	0.21
Salicylic acid	c	c	-0.07	-
Toluene	c	c	b	b
Toluene-p-sulphonamide	-	-	0.59	0.78
Toluene-p-sulphonic acid[c]	-	-	-	-
p-Toluic acid[c]	-	-	-	-

a-negative or zero k' value b-no elution time obtained c-not used in this particular system d-k' value greater than 50

Table 4 cont./...

LOG K' VALUES FOR THE OCTYL PHASE (cont.)

SOLUTE NAME	40% MeOH pH 5.5	40% MeOH pH 7.0	50% MeOH pH 5.6	50% MeOH pH 7.3
Acetyl salicylic acid	-0.22	c	-0.58	c
p-Amino benzoic acid[c]	-	-	-	-
o-Amino phenol[c]	-	-	-	-
p-Amino phenol[c]	-	-	-	-
p-Amino salicylic acid[c]	-	-	-	-
Aniline	0.32	0.62	0.12	0.32
o-Anisic acid[c]	-	-	-	-
p-Anisic acid[c]	-	-	-	-
Anisole	1.15	1.38	0.88	0.95
Anthranilic acid[c]	-	-	-	-
Benzamide	0.16	0.37	-0.55	0.11
Benzene[b]	-	-	-	-
Benzoic acid	0.25	-0.28	-0.03	-0.01
Benzonitrile	0.81	0.97	0.55	0.63
Benzyl alcohol	0.51	0.68	0.27	0.41
Benzylamine[c]	-	-	-	-
o-Cresol	0.90	1.10	0.62	0.72
p-Cresol	0.87	1.10	0.53	0.68
Cumene[b]	-	-	-	-
Diethyl phthalate	1.63	1.37	1.19	1.21
3,4-Dihydroxy benzylamine	-0.48	0.92	-0.58	0.88
N,N-Dimethyl benzylamine[c]	-	-	-	-
2,4-Dimethyl phenol	1.20	1.42	0.88	0.96
Dimethyl phthalate	0.98	1.37	0.65	0.72
m-Hydroxy benzoic acid	-0.30	-0.58	-0.64	-0.35
p-Hydroxy benzoic acid	-0.05	-0.65	-0.40	-0.43
Methyl-4-hydroxy benzoate	0.81	1.03	0.40	0.59
Methyl salicylate	1.43	1.75	1.08	1.28
Phenol	0.53	0.71	0.29	0.42
Phenyl acetic acid[c]	-	-	-	-
p-Phenylene diamine[c]	-	-	-	-
p-Phenyl phenol	1.68	d	1.25	1.38
Phenyl salicylate	0.54	0.78	0.30	0.43
Phthalic acid	-0.50	a	-0.93	a
Phthalodinitrile[c]	-	-	-	-
n-Propyl-p-hydroxy benzoate[c]	-	-	-	-
Pyridine	0.32	0.56	0.12	0.31
Quinol	-0.29	0.03	-0.56	0.18
Salicylic acid	-0.26	c	-0.55	c
Toluene	d	d	d	1.34
Toluene-p-sulphonamide	0.32	0.60	0.02	0.23
Toluene-p-sulphonic acid[c]	-	-	-	-
p-Toluic acid[c]	-	-	-	-

a-negative or zero k' value b-no elution time obtained c-not used in this particular system d-k' value greater than 50

Table 4 cont./...

LOG K' VALUES FOR THE OCTADECYL PHASE

SOLUTE NAME	20% MeOH pH 5.2	20% MeOH pH 7.4	30% MeOH pH 5.4	30% MeOH pH 7.5
Acetyl salicylic acid	0.38	0.48	0.07	0.22
p-Amino benzoic acid[c]	-	-	-	-
o-Amino phenol[c]	-	-	-	-
p-Amino phenol	-0.29	-0.16	-0.58	-0.11
p-Amino salicylic acid[c]	-	-	-	-
Aniline	c	c	0.48	b
o-Anisic acid[c]	-	-	-	-
p-Anisic acid[c]	-	-	-	-
Anisole	c	c	d	1.71
Anthranilic acid[c]	-	-	-	-
Benzamide	0.62	0.78	0.34	0.56
Benzene	c	c	b	b
Benzoic acid	0.69	-0.23	0.12	0.08
Benzonitrile	c	c	d	1.19
Benzyl alcohol	c	c	0.72	0.89
Benzylamine[c]	-	-	-	-
o-Cresol	c	c	1.11	1.29
p-Cresol	c	c	1.12	1.25
Cumene	c	c	d	d
Diethyl phthalate	c	c	b	b
3,4-Dihydroxy benzylamine	-0.57	-0.78	c	-0.74
N,N-Dimethyl benzylamine[c]	-	-	-	-
2,4-Dimethyl phenol	c	c	1.42	d
Dimethyl phthalate	c	c	b	b
m-Hydroxy benzoic acid	0.14	-0.73	-0.12	-0.43
p-Hydroxy benzoic acid	0.23	-0.89	-0.07	-1.00
Methyl-4-hydroxy benzoate	c	c	b	1.17
Methyl salicylate	c	c	b	b
Phenol	c	c	0.94	b
Phenyl acetic acid	0.95	0.40	b	0.22
p-Phenylene diamine	c	c	-0.98	-0.44
p-Phenyl phenol	c	c	d	1.68
Phenyl salicylate	c	c	b	0.85
Phthalic acid	0.05	a	-0.21	a
Phthalodinitrile	c	c	d	0.85
n-Propyl-p-hydroxy benzoate	c	c	b	0.85
Pyridine	c	c	0.48	0.68
Quinol	-0.10	0.06	-0.26	-0.08
Salicylic acid	0.22	0.46	-0.02	0.19
Toluene	c	c	b	b
Toluene-p-sulphonamide	c	c	-0.14	0.22
Toluene-p-sulphonic acid	0.23	0.47	-0.14	0.24
p-Toluic acid[c]	-	-	-	-

a-negative or zero k' value b-no elution time obtained c-not used in this particular system d-k' value greater than 50

Table 4 cont./...

LOG K' VALUES FOR THE OCTADECYL PHASE (cont.)

SOLUTE NAME	40% MeOH		50% MeOH	
	pH 5.5	pH 7.7	pH 5.7	pH 8.0
Acetyl salicylic acid	-0.21	-0.05	-0.68	-0.22
p-Amino benzoic acid[c]	-	-	-	-
o-Amino phenol[c]	-	-	-	-
p-Amino phenol	-0.80	-0.55	-0.68	-0.50
p-Amino salicylic acid[c]	-	-	-	-
Aniline	0.34	0.28	0.18	0.22
o-Anisic acid[c]	-	-	-	-
p-Anisic acid[c]	-	-	-	-
Anisole	1.32	d	1.05	1.57
Anthranilic acid[c]	-	-	-	-
Benzamide	0.08	0.14	-0.11	-0.03
Benzene[b]	-	-	-	-
Benzoic acid	b	-0.26	-0.02	-0.39
Benzonitrile	b	0.90	b	0.60
Benzyl alcohol	0.53	0.51	0.28	0.36
Benzylamine[c]	-	-	-	-
o-Cresol	0.90	b	0.65	0.69
p-Cresol	0.90	0.97	0.62	0.66
Cumene[d]	-	-	-	-
Diethyl phthalate	d	d	d	1.30
3,4-Dihydroxy benzylamine	c	c	c	-1.23
N,N-Dimethyl benzylamine[c]	-	-	-	-
2,4-Dimethyl phenol	1.25	d	0.96	1.00
Dimethyl phthalate	b	b	0.61	b
m-Hydroxy benzoic acid	-0.50	-0.98	-0.72	b
p-Hydroxy benzoic acid	-0.34	-1.28	-0.52	-1.11
Methyl-4-hydroxy benzoate	b	0.82	0.44	b
Methyl salicylate	d	1.64	1.25	1.29
Phenol	0.53	0.51	0.29	0.37
Phenyl acetic acid	0.25	-0.03	-0.03	-0.14
p-Phenylene diamine	-0.85	-0.98	-0.61	b
p-Phenyl phenol	d	1.61	1.36	1.44
Phenyl salicylate	0.54	0.51	0.33	0.38
Phthalic acid	-0.61	a	-1.03	a
Phthalodinitrile	0.45	0.43	0.20	0.13
n-Propyl-p-hydroxy benzoate	1.54	1.32	1.13	1.16
Pyridine	0.30	0.36	0.14	0.20
Quinol	-0.43	-0.34	-0.58	-0.48
Salicylic acid	-0.29	-0.11	-0.68	-0.25
Toluene[b]	-	-	-	-
Toluene-p-sulphonamide	-0.55	-0.31	-0.68	-0.26
Toluene-p-sulphonic acid	-0.43	-0.07	-0.88	-0.23
p-Toluic acid[b]	-	-	-	-

a-negative or zero k' value b-no elution time obtained c-not used in this particular system d-k' value greater than 50

Table 4 cont./...

LOG K' VALUES FOR THE CYCLOHEXYL PHASE

SOLUTE NAME	20% MeOH		30% MeOH	
	pH 5.2	pH 7.2	pH 5.3	pH 7.6
Acetyl salicylic acid	a	-0.41	-0.56	-0.23
p-Amino benzoic acid	0.02	c	-0.26	c
o-Amino phenol	-0.19	c	0.10	c
p-Amino phenol	-0.80	-0.55	-0.68	-0.50
p-Amino salicylic acid	-0.68	c	-1.22	c
Aniline	0.34	0.78	0.22	0.58
o-Anisic acid	-0.19	c	-0.34	c
p-Anisic acid	0.50	c	0.10	c
Anisole	b	1.53	0.84	0.67
Anthranilic acid	0.04	c	-0.06	c
Benzamide	0.36	0.80	0.19	b
Benzene	b	b	0.73	1.19
Benzoic acid	-0.15	-0.51	-0.29	-0.15
Benzonitrile	0.82	1.37	0.69	1.15
Benzyl alcohol	0.37	0.94	0.25	0.78
Benzylamine	0.51	c	0.44	c
o-Cresol	0.64	b	0.47	b
p-Cresol	0.68	1.29	0.51	b
Cumene[b]	-	-	-	-
Diethyl phthalate	d	d	1.64	d
3,4-Dihydroxy benzylamine[c]	-	-	-	-
N,N-Dimethyl benzylamine	0.75	c	0.64	c
2,4-Dimethyl phenol	d	1.66	0.74	b
Dimethyl phthalate	1.18	d	0.90	1.59
m-Hydroxy benzoic acid	-0.48	-0.64	-0.50	-0.37
p-Hydroxy benzoic acid	-0.17	-1.11	-0.29	-0.50
Methyl-4-hydroxy benzoate	0.87	1.56	0.59	1.30
Methyl salicylate	1.36	b	1.14	1.72
Phenol	0.02	0.91	0.21	0.75
Phenyl acetic acid	-0.02	-0.08	-0.24	0.22
p-Phenylene diamine	0.75	0.77	0.22	0.91
p-Phenyl phenol[d]	-	-	-	-
Phenyl salicylate	b	0.93	0.25	b
Phthalic acid	-0.52	a	-0.74	a
Phthalodinitrile	0.75	1.30	0.53	1.02
n-Propyl-p-hydroxy benzoate	d	d	1.27	d
Pyridine	0.74	1.06	0.59	0.94
Quinol	-0.07	0.36	-0.17	0.25
Salicylic acid	-0.48	-0.34	0.31	-0.26
Toluene	1.21	1.78	1.12	d
Toluene-p-sulphonamide	0.48	1.05	0.24	0.71
Toluene-p-sulphonic acid	-0.75	-0.51	-0.82	-0.67
p-Toluic acid	0.50	c	0.25	c

a-negative or zero k' value b-no elution time obtained c-not used in this particular system d-k' value greater than 50

Table 4 cont./...

LOG K' VALUES FOR THE CYCLOHEXYL PHASE (cont.)

SOLUTE NAME	40% MeOH		50% MeOH	
	pH 5.4	pH 7.0	pH 5.8	pH 7.9
Acetyl salicylic acid	-0.78	-0.03	-1.20	-1.66
p-Amino benzoic acid	-0.15	-0.67	-0.44	c
o-Amino phenol	0.04	0.29	-0.17	c
p-Amino phenol	0.08	0.16	-0.17	0.04
p-Amino salicylic acid	-0.70	-0.32	-0.48	c
Aniline	-0.05	0.59	-0.11	0.32
o-Anisic acid	-0.40	-0.14	-0.72	c
p-Anisic acid	-0.03	0.21	-0.48	c
Anisole	0.88	1.51	0.20	0.74
Anthranilic acid	-0.12	0.25	a	c
Benzamide	-0.48	0.74	0.37	0.23
Benzene	0.58	0.97	0.20	0.68
Benzoic acid	-0.30	-0.13	-0.66	a
Benzonitrile	0.47	1.19	a	0.60
Benzyl alcohol	0.14	-0.20	-0.06	0.40
Benzylamine	0.47	0.41	-0.17	c
o-Cresol	0.34	1.12	-0.01	0.55
p-Cresol	0.33	1.15	-0.01	0.54
Cumene	d	d	0.79	1.50
Diethyl phthalate	1.07	d	0.90	d
3,4-Dihydroxy benzylamine[c]	-	-	-	-
N,N-Dimethyl benzylamine	0.63	1.28	0.56	d
2,4-Dimethyl phenol	0.56	1.36	0.13	0.72
Dimethyl phthalate	0.60	1.54	0.03	b
m-Hydroxy benzoic acid	-0.52	-0.48	-1.02	-1.06
p-Hydroxy benzoic acid	-0.36	-0.62	-0.69	-1.36
Methyl-4-hydroxy benzoate	0.38	1.53	0.01	0.56
Methyl salicylate	0.86	0.97	0.33	0.96
Phenol	0.16	-0.92	-0.11	0.40
Phenyl acetic acid	-0.30	0.06	-0.60	-0.88
p-Phenylene diamine	0.20	b	0.05	0.13
p-Phenyl phenol	0.99	1.31	0.33	1.07
Phenyl salicylate	0.19	0.66	b	b
Phthalic acid	-0.78	a	a	-0.96
Phthalodinitrile	0.31	1.21	0.00	0.42
n-Propyl-p-hydroxy benzoate	0.88	d	0.28	1.06
Pyridine	0.45	1.28	0.13	0.42
Quinol	-0.18	0.05	-0.34	0.01
Salicylic acid	-0.70	0.24	-1.56	-1.19
Toluene	0.89	1.48	0.42	0.97
Toluene-p-sulphonamide	-0.05	b	-0.21	0.28
Toluene-p-sulphonic acid	-0.78	0.32	b	a
p-Toluic acid	0.04	0.35	-0.46	c

a-negative or zero k' value, b-no elution time obtained c-not used in this particular system d-k' value greater than 50

Table 4 cont./...

LOG K' VALUES FOR THE PHENYL PHASE

SOLUTE NAME	20% MeOH pH 5.5	20% MeOH pH 7.2	30% MeOH pH 5.6	30% MeOH pH 7.3
Acetyl salicylic acid	-0.78	-0.24	-1.20	-0.31
p-Amino benzoic acid	-0.15	-0.76	-0.44	-
o-Amino phenol	0.04	0.11	-0.17	-
p-Amino phenol	0.08	0.00	-0.17	-0.08
p-Amino salicylic acid	-0.70	-0.61	-0.48	-1.30
Aniline	-0.05	0.41	-0.11	0.33
o-Anisic acid	-0.40	-0.57	-0.72	-0.64
p-Anisic acid	-0.03	-0.14	-0.48	-0.29
Anisole	0.88	1.25	0.20	1.01
Anthranilic acid	-0.12	-0.55	-	-0.50
Benzamide	-0.48	0.45	0.37	0.32
Benzene	0.58	0.91	0.20	0.76
Benzoic acid	-0.30	-0.45	-0.66	-0.59
Benzonitrile	0.47	1.07	-	0.91
Benzyl alcohol	0.14	0.42	-0.06	0.32
Benzylamine	0.47	0.47	-0.17	0.41
o-Cresol	0.34	0.70	-0.01	0.58
p-Cresol	0.33	0.68	-0.01	0.57
Cumene	-	-	0.79	-
Diethyl phthalate	1.07	-	0.90	-
3,4-Dihydroxy benzylamine	c	-0.16	c	c
N,N-Dimethyl benzylamine	0.63	1.29	0.56	1.21
2,4-Dimethyl phenol	0.56	1.02	0.13	0.84
Dimethyl phthalate	0.60	1.51	0.03	1.20
m-Hydroxy benzoic acid	-0.52	-0.72	-1.02	-0.80
p-Hydroxy benzoic acid	-0.36	-0.86	-0.69	-0.85
Methyl-4-hydroxy benzoate	0.38	0.96	0.01	0.71
Methyl salicylate	0.86	-	0.33	1.33
Phenol	0.16	0.36	-0.11	0.30
Phenyl acetic acid	-0.30	-0.33	-0.60	-0.46
p-Phenylene diamine	0.20	0.25	0.05	-
p-Phenyl phenol	0.99	-	0.33	1.64
Phenyl salicylate	-	0.66	-	-
Phthalic acid	-0.78	a	a	-1.07
Phthalodinitrile	0.31	1.25	0.00	1.04
n-Propyl-p-hydroxy benzoate	0.88	-	0.28	1.36
Pyridine	0.45	0.92	0.13	0.71
Quinol	-0.18	-0.14	-0.34	-0.24
Salicylic acid	-0.70	-0.22	-1.56	-0.45
Toluene	0.89	1.30	0.42	-
Toluene-p-sulphonamide	-0.05	0.63	-0.21	b
Toluene-p-sulphonic acid	-0.78	-0.13	-	-0.25
p-Toluic acid	0.04	-0.09	-0.46	-0.21

a-negative or zero k' value b-no elution time obtained c-not used in this particular system d-k' value greater than 50

Table 4 cont./...

LOG K' VALUES FOR THE PHENYL PHASE (cont.)

SOLUTE NAME	40% MeOH pH 5.6	40% MeOH pH 7.6	50% MeOH pH 5.8	50% MeOH pH 7.8
Acetyl salicylic acid	-1.06	-0.21	-1.74	-0.49
p-Amino benzoic acid	-0.46	-1.13	-0.52	a
o-Amino phenol	-0.14	-0.03	-0.16	-0.11
p-Amino phenol	-0.14	-0.17	-0.19	-0.26
p-Amino salicylic acid	-1.54	-0.69	b	-1.19
Aniline	-0.05	0.41	-0.11	0.33
o-Anisic acid	-0.61	-0.69	-0.71	-0.54
p-Anisic acid	-0.24	-0.78	-0.60	-0.45
Anisole	0.58	0.58	0.37	0.26
Anthranilic acid	-0.38	-0.66	b	-0.79
Benzamide	-0.05	0.15	-0.15	-0.01
Benzene	0.42	0.94	0.26	0.50
Benzoic acid	-0.63	-0.63	-0.77	-0.59
Benzonitrile	0.41	0.73	0.26	0.43
Benzyl alcohol	0.03	0.18	-0.10	0.13
Benzylamine	0.26	0.39	0.27	0.35
o-Cresol	0.19	0.43	0.01	0.30
p-Cresol	0.18	0.44	0.02	0.28
Cumene	b	d	b	1.12
Diethyl phthalate	1.11	1.36	0.70	0.97
3,4-Dihydroxy benzylamine	0.05	c	0.61	c
N,N-Dimethyl benzylamine	0.53	1.24	0.10	0.41
2,4-Dimethyl phenol	0.36	0.63	0.14	0.48
Dimethyl phthalate	0.62	0.36	b	0.65
m-Hydroxy benzoic acid	-1.17	-1.23	-1.68	-0.71
p-Hydroxy benzoic acid	-0.78	-1.53	-0.84	-1.49
Methyl-4-hydroxy benzoate	0.27	0.50	0.06	-0.03
Methyl salicylate	0.83	0.79	b	1.03
Phenol	0.00	0.18	-0.12	0.12
Phenyl acetic acid	-0.56	-0.21	-0.67	-0.42
p-Phenylene diamine	0.05	0.01	0.05	-0.13
p-Phenyl phenol	0.93	1.20	0.59	0.84
Phenyl salicylate[c]	-	-	-	-
Phthalic acid	-0.78	a	a	-1.07
Phthalodinitrile	0.54	0.93	0.35	0.52
n-Propyl-p-hydroxy benzoate	0.70	1.00	0.40	0.68
Pyridine	0.45	0.62	0.24	0.30
Quinol	-0.37	-0.39	-0.43	-0.32
Salicylic acid	-1.14	-0.58	-1.59	-0.41
Toluene	0.68	0.81	0.47	0.70
Toluene-p-sulphonamide	0.00	0.12	-0.11	0.29
Toluene-p-sulphonic acid	-1.41	-0.53	-2.59	-0.32
p-Toluic acid	-0.24	-0.39	-0.53	-0.45

a-negative or zero k' value b-no elution time obtained c-not used in this particular system d-k' value greater than 50

Table 4

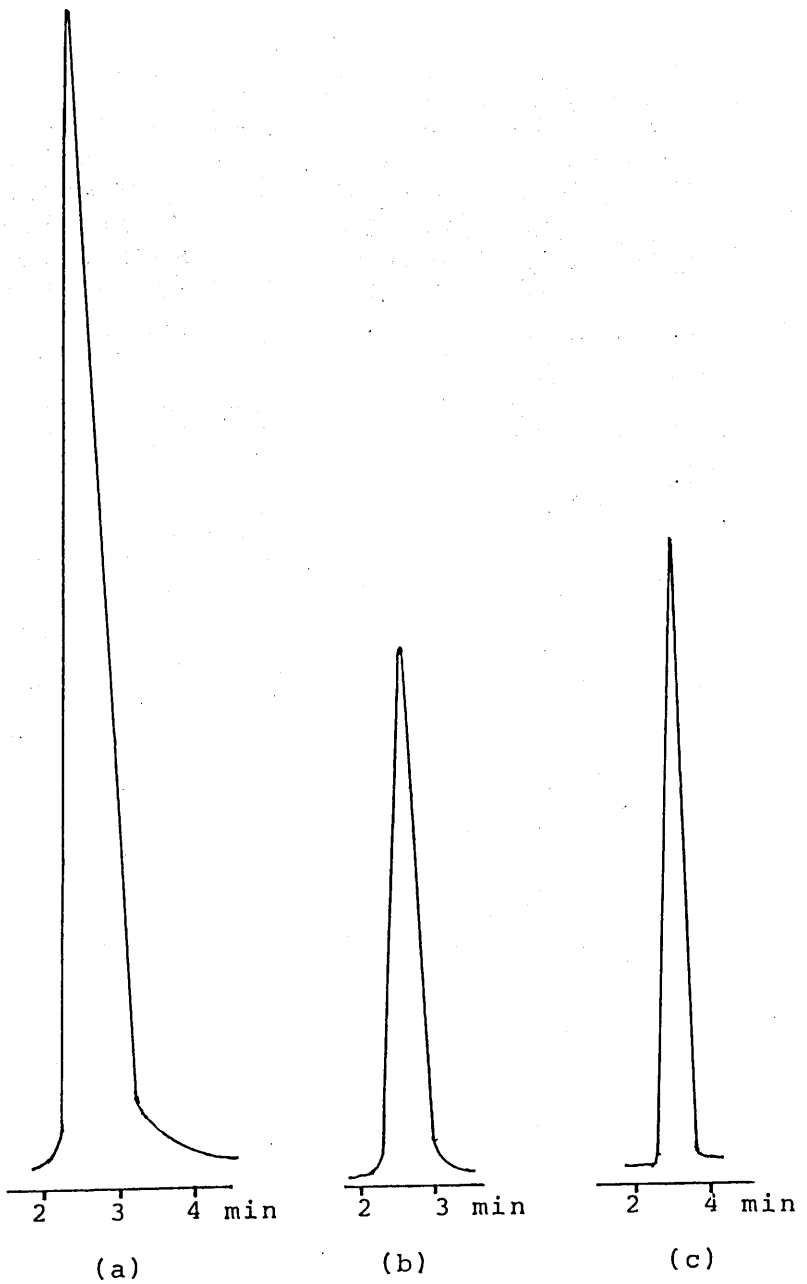


Figure 20 Examples of aniline peak shapes when eluted with 50:50 (v/v) methanol:water (pH7) from (a)octylsilica, full-scale deflection (FSD)= 10mV, (b)cyclohexylsilica, FSD=20mV, (c)phenylsilica, FSD=50mV.

PHASE	Number of Theoretical Plates, N
Ethylsilica	138.50
Octylsilica	157.60
Octadecylsilica	97.73
Cyclohexylsilica	216.41
Phenylsilica	152.70

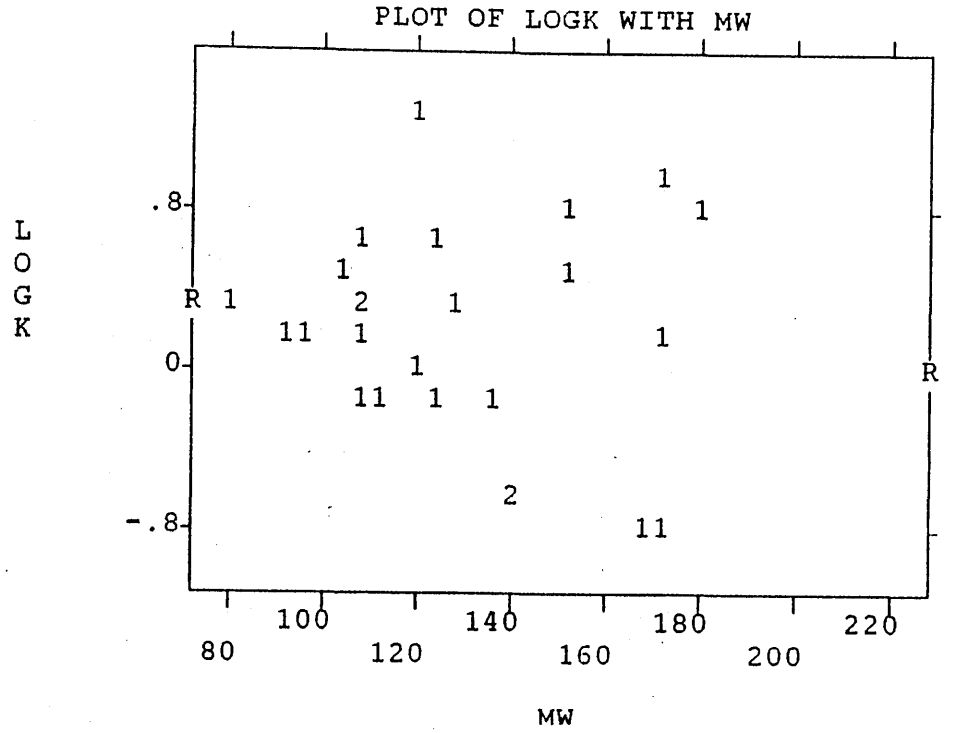
Table 5 The number of theoretical plates for each of the five chosen Bond Elut phases.

solute physicochemical properties. In order to establish that no single solute physicochemical property was highly correlated with log k' (correlation coefficient, R^2, > 0.8), initial statistics involved simple linear plots of log k' against (i) molecular weight (ii) connectivity (iii) Hansch parameter and (iv) log P for all of the chromatographic systems. From the R^2 values, it was concluded that more than one variable would be necessary in most circumstances to predict the reversed-phase retention behaviour of the benzene solutes. Figure 21 shows a typical correlation analysis printout.

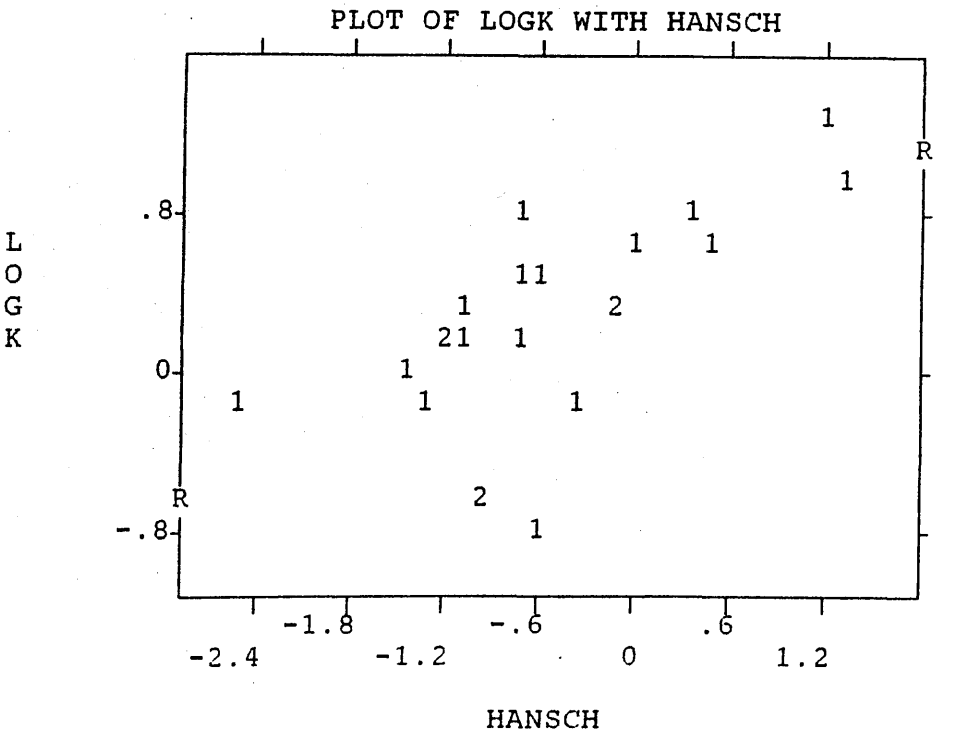
The physical and chemical properties collected for each solute were chosen so that they would cover the major interactions expected to influence the behaviour of a solute in reversed-phase LC; dipole/charge effects, size and shape, hydrophobicity and hydrogen bond donor/acceptor ability. However, some interactions can be described by more than one physicochemical descriptor e.g. volume, molecular weight and polarisability representing size; log P and the Hansch parameter both representing hydrophobicity. By performing factor analysis on the solute descriptors, groups of related parameters could be determined (figure 22). Subsequently, the following parameters were chosen from factor analysis as independent variables for multiple linear regression analysis:

Volume - molecular size
Δ - polarity/dipole effects

(cont ...)

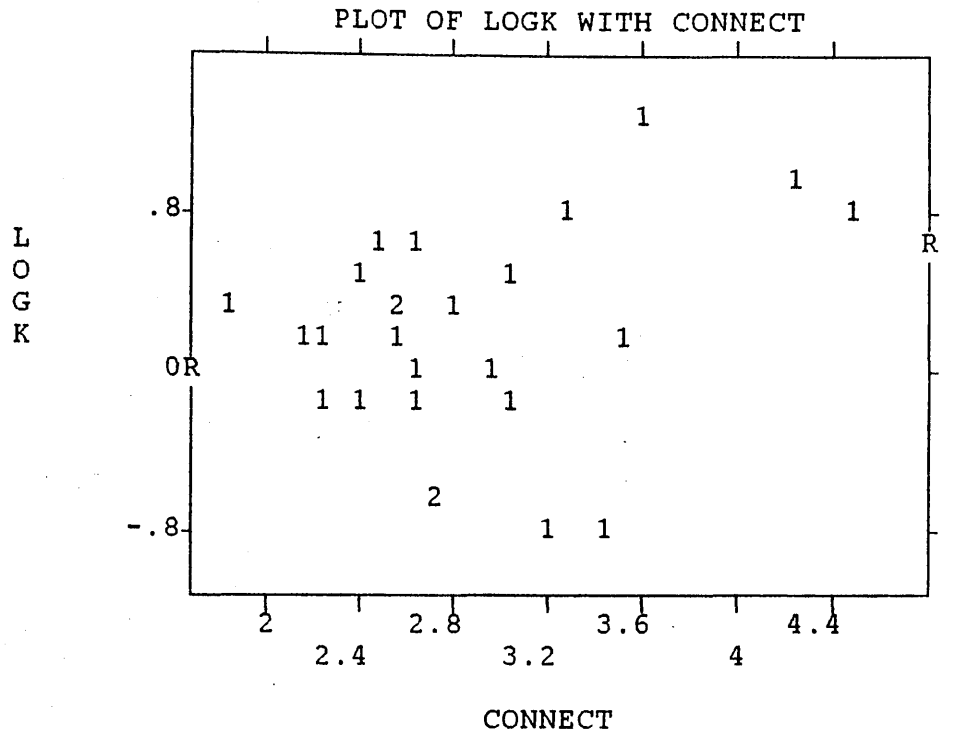


$R^2 = 0.017$ S.E. = 0.542

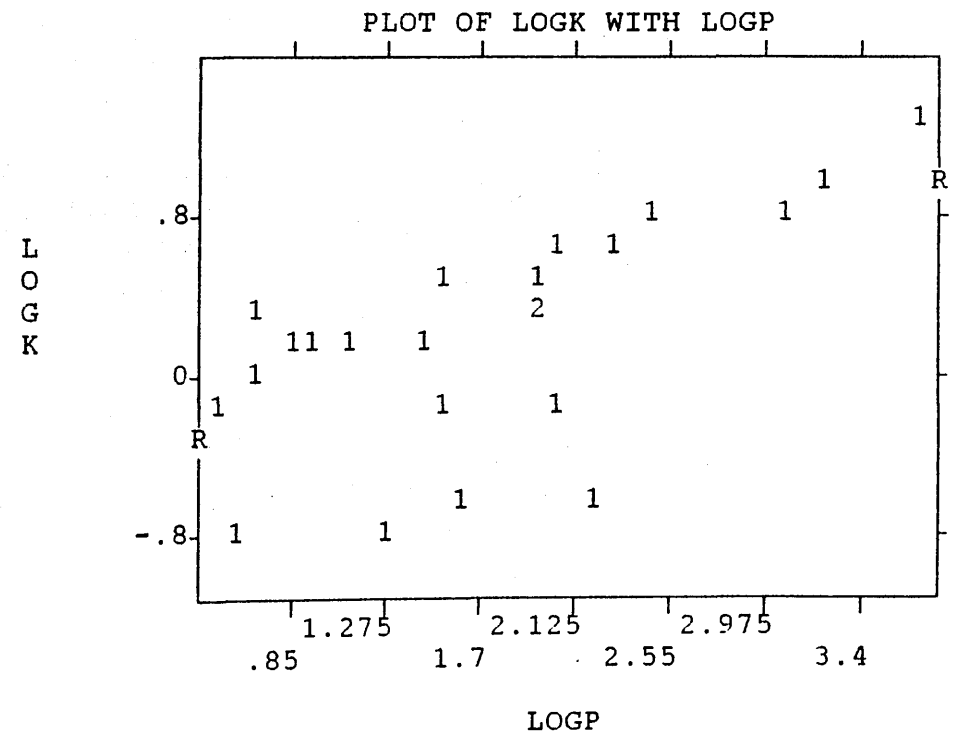


$R^2 = 0.408$ S.E. = 0.407

Figure 21 Correlation analysis between log k' and selected single variables. 50% methanol, ethylsilica phase. cont./...



$R^2 = 0.069$ S.E. = 0.517



$R^2 = 0.401$ S.E. = 0.439

Figure 21

	FACTOR 1	FACTOR 2	FACTOR 3	FACTOR 4	FACTOR 5	FACTOR 6
POLAR	.92300	.12717	.16120	.25670	.01727	.03885
VOLUME	.89944	.09010	.18896	.30175	.05517	.14443
F	.89068	-.28625	.01915	.21958	.21134	.03572
CONNECT	.88606	.23773	.14745	-.13370	-.03750	.05539
PZ	.87288	.14848	.24037	-.11049	.07127	.25191
VM	.85364	-.11764	.39916	.44173	.05970	.16239
RM	.69154	-.12421	.40742	.42239	.57577	-.16873
PX	.66875	-.18269	-.01733	-.20328	.36145	-.43418
HANSCH	.66528	-.53216	-.43008	.21445	-.11740	-.18555
LOGP	.65173	-.56031	-.34524	-.18197	-.16034	.04698
MW	.63958	.33603	.27216	.36671	-.12712	.02847
MOMPOL	.40366	.19416	-.07964	-.32628	.01241	.35150
HAADDHD	.16118	.91958	.22206	-.06551	.08526	.08190
HDONOR	-.08587	.89298	.02062	-.36606	-.02309	-.07421
DENSITY	.17418	.88863	.14547	.07334	-.26423	.15487
MP	-.15757	.86605	.06156	.05185	-.20751	-.03369
HACCEPT	.35344	.72885	.39322	.20439	.24753	.21958
PKA1	.20007	-.63409	.29319	-.28205	.55740	-.06007
BETA	.40423	.61899	.51041	.35297	.14415	.19975
DIPMOM2	.15963	.07287	1.06142	.05270	-.15252	-.28628
DIPOLE	.33389	.17359	.79525	.13401	-.09999	.27886
DIMOMSUM	.31402	.26558	.79001	-.08555	-.02629	.33864
BP	-.16172	.33549	.69316	.36344	-.04340	.37405
PY	.25572	.04900	.04676	.92120	.04118	.09701
HASUBHD	.46823	-.18169	.31566	.57777	.13699	.42322
REFINDEX	.03119	-.07701	-.36088	.11681	.97913	-.11696
DIPMOM1	.13527	.06179	.21516	.15851	-.14876	.89556

Figure 22 Results of factor analysis.

Connectivity	- molecular shape
Beta(ß)	- degree of hydrogen-bonding ability
Log P, Log D	- partition and ionisation-corrected partition coefficients respectively
HA, HD, HA-HD	- number of hydrogen bond acceptors and donors, and their difference respectively

Stepwise multiple linear regression (MLR) analysis was performed on the experimental data using log k' as the dependent variable and the selected physicochemical properties as independent variables. Two examples of output from SPSSX/PC for MLR analysis are shown in figure 23 (a glossary of statistical terms used can be found in Appendix II). As small retention times (log k' < -0.50) were difficult to measure accurately, these cases were omitted from the regressional analysis. Also omitted were retention times for the substituted benzene solutes measured on octyl-, octadecyl- and ethyl-silica at 20% methanol because of the small number of solute retention measurements. The log k' values determined at the two pH values of each mobile phase were not treated separately except when the regression was not satisfactory (R^2 < 0.7).

MLR analysis was divided into three stages. First, MLR was performed on neutral solute data only. Next, acidic compound data was added to the neutral compounds to observe any polarity/ionisation effects on the correlation. Log P was corrected for ionisation effects to log D as discussed in Section 2.3. Finally, basic compounds were also added to give a complete relationship

```
SPSS/PC+ The Statistical Package for IBM PC

GET FILE='Q:\OLDIMAGE\ANITA\MUM1.DAT'.
SELECT IF (PHASNAME EQ 'C8 ' & PERCENT EQ 30 & COMPOUND EQ 1 & LOGK GE -0.50)
IF (COMPPOL EQ 1) LOGD=LOGP-LG10(1+10**(PH-PKA1)).
IF (COMPPOL EQ 2) LOGD=LOGP.
IF (COMPPOL EQ 3) LOGD=LOGP-LG10(1+10**(PKA1-PH)).
REG VAR=VOLUME CONNECT DELTAQ BETA LOGD LOGK HACCEPT HDONOR HASUBHD
The raw data or transformation pass is proceeding
     38 cases are written to the uncompressed active file.
 /DEP=LOGK
 /MET=STEP
 /RES=HIS(SRE)
 /SCAT=(*SRE,*PRE) (LOGK,*PRE).

        * * * *  M U L T I P L E  R E G R E S S I O N  * * * *

Listwise Deletion of Missing Data

Equation Number 1    Dependent Variable..   LOGK

Beginning Block Number  1.  Method:  Stepwise

Variable(s) Entered on Step Number
   2..     HDONOR


Multiple R              .95967
R Square                .92096
Adjusted R Square       .91409
Standard Error          .17754

Analysis of Variance
                    DF        Sum of Squares        Mean Square
Regression           2              8.44743             4.22371
Residual            23               .72496              .03152

F =     134.00148        Signif F =  .0000

------------------ Variables in the Equation ------------------

Variable                B         SE B        Beta           T  Sig T

LOGD                .49015       .04870      .73717      10.064  .0000
HDONOR             -.30395       .07070     -.31492      -4.299  .0003
(Constant)          .47124       .12673                   3.718  .0011

------------- Variables not in the Equation -------------

Variable     Beta In  Partial  Min Toler         T  Sig T

VOLUME         .06503   .23120     .64010     1.115  .2771
CONNECT        .06062   .21469     .63513     1.031  .3137
DELTAQ         .06767   .20230     .48787      .969  .3431
BETA           .01586   .04263     .54013      .200  .8432
HACCEPT   -4.260E-03  -.01120     .43330     -.053  .9586
HASUBHD        .01837   .06078     .57490      .286  .7779
```

2

Figure 23 Two typical outputs of multiple linear regression analysis from SPSSX-PC. cont./...

Residuals Statistics:

	Min	Max	Mean	Std Dev	N
*PRED	-2.1904	1.5594	.5582	.8900	33
*ZPRED	-5.0524	1.3983	-.3239	1.5311	33
*SEPRED	.0360	.2334	.0697	.0394	33
*ADJPRED	-2.1904	1.5594	.5543	.9012	33
*RESID	-.4997	2.7804	.1542	.6289	33
*ZRESID	-2.8144	15.6607	.8685	3.5423	33
*SRESID	-2.7472	9.4809	.5867	2.5382	33
*DRESID	-.4997	2.7804	.1581	.6385	33
*SDRESID	-2.7472	9.4809	.5993	2.5676	33
*MAHAL	.0654	15.5080	2.8638	3.3364	33
*COOK D	.0000	11.0106	.5892	2.0286	33
*LEVER	.0026	.5965	.1125	.1289	33

Total Cases = 38

3

Histogram - Studentized Residual

```
NExp N      (* = 1 Cases,     . : = Normal Curve)
2   .00   Out **
0   .00  4.50
0   .00  4.00
0   .02  3.50
2   .08  3.00 **
0   .31  2.50
0   .92  2.00 .
2  2.16  1.50 *:
5  3.99  1.00 ***:*
4  5.76   .50 **** .
7  6.51   .00 ******:
2  5.76  -.50 **   .
5  3.99 -1.00 ***:*
1  2.16 -1.50 *.
2   .92 -2.00 :*
1   .31 -2.50 *
0   .08 -3.00
0   .02 -3.50
0   .00 -4.00
0   .00 -4.50
0   .00   Out
```

4

Standardized Scatterplot[5]

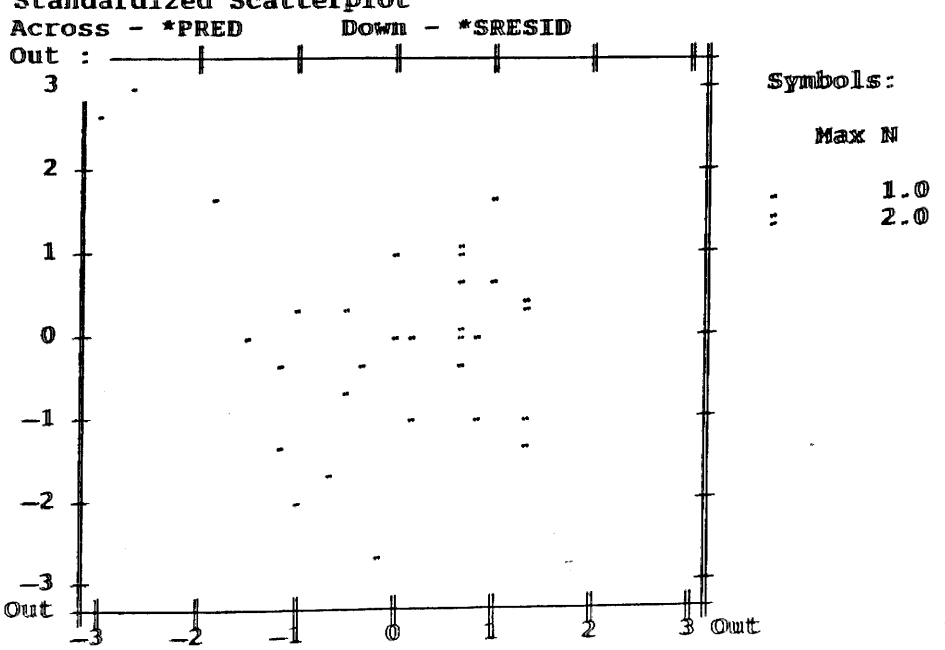
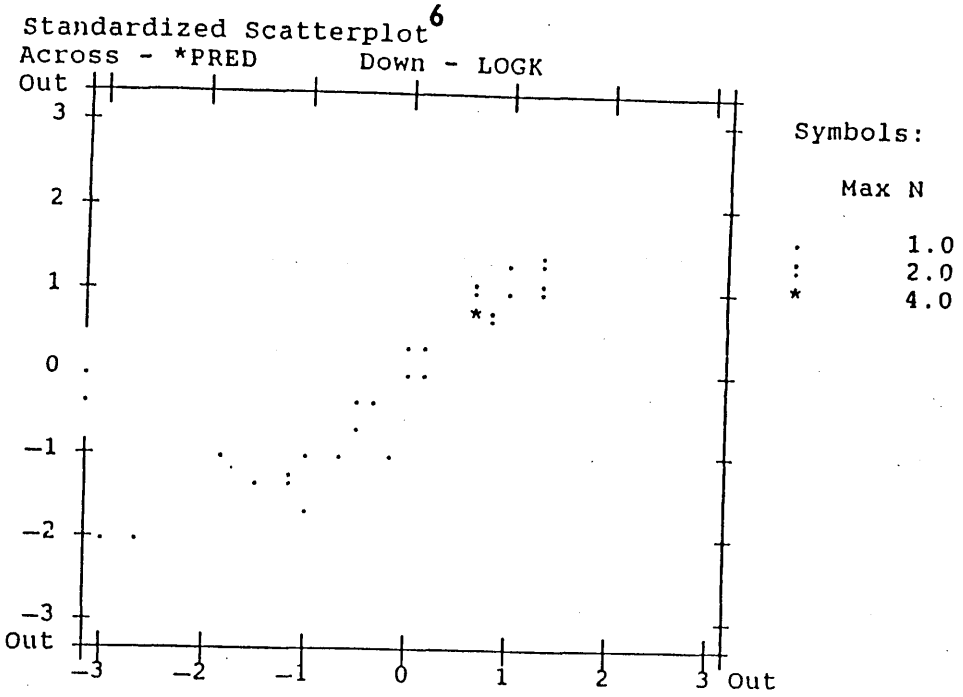


Figure 23 cont./...



Note 1: Variables selected from database under specified conditions.

Note 2: Statistics relating to the equation derived by multiple linear regression analysis. Please refer to Appendix II for definition of terms.

Note 3: Specialised treatment of the residual data between the predicted and experimental capacity factors. Please refer to the "SPSSX Advanced Statistics Guide" Norusis, MJ (editor), M^{C}Graw-Hill books (1985), pp.28-22.

This data was not used in interpreting the regression equations.

Note 4: Histogram of residual data. Ideally the data should follow a normal distribution curve.

Note 5: Scatterplot of studentised residual data versus predicted log k'. Ideally the data should be randomly scattered.

Note 6: Scatterplot of experimental capacity factors versus predicted capacity factors. Ideally the data should follow a linear plot, passing through the origin. Note that the axes for both this and the above scatterplot are not indictive of the true values.

Figure 23 cont./...

SELECT IF (PHASNAME EQ 'CH ' & PERCENT EQ 50 & LOGK GE -0.50 & COMPPOL EQ 2)

Variable(s) Entered on Step Number
1.. LOGP

Multiple R	.67378
R Square	.45398
Adjusted R Square	.43578
Standard Error	.31038

Analysis of Variance

	DF	Sum of Squares	Mean Square
Regression	1	2.40282	2.40282
Residual	30	2.89000	.09633

F = 24.94271 Signif F = .0000

---------- Variables in the Equation ----------

Variable	B	SE B	Beta	T	Sig T
LOGP	.29341	.05875	.67378	4.994	.0000
(Constant)	-.18151	.12662		-1.433	.1621

---------- Variables not in the Equation ----------

Variable	Beta In	Partial	Min Toler	T	Sig T
VOLUME	-.01641	-.01747	.61909	-.094	.9257
CONNECT	.01812	.01860	.57503	.100	.9209
DELTAQ	-.20207	-.27140	.98502	-1.519	.1397
HDONOR	-.25986	-.27863	.62773	-1.562	.1291
HACCEPT	-.19747	-.24219	.82133	-1.344	.1893
HASUBHD	-.03789	-.05021	.95889	-.271	.7885
BETA	-.07910	-.09197	.73811	-.497	.6227

End Block Number 1 PIN = .050 Limits reached.

Residuals Statistics:

	Min	Max	Mean	Std Dev	N
*PRED	-.1698	.8924	.3909	.2744	33
*ZPRED	-2.0050	1.8100	.0089	.9856	33
*SEPRED	.0549	.1245	.0742	.0210	33
*ADJPRED	-.2100	.9086	.3881	.2747	33
*RESID	-.4399	.6076	-.0133	.3101	33
*ZRESID	-1.4172	1.9577	-.0429	.9992	33
*SRESID	-1.4419	2.1073	-.0379	1.0326	33
*DRESID	-.4686	.7040	-.0105	.3321	33
*SDRESID	-1.4695	2.2447	-.0368	1.0459	33
*MAHAL	.0000	4.0201	.9419	1.1259	33
*COOK D	.0006	.3523	.0381	.0617	33
*LEVER	.0000	.1297	.0304	.0363	33

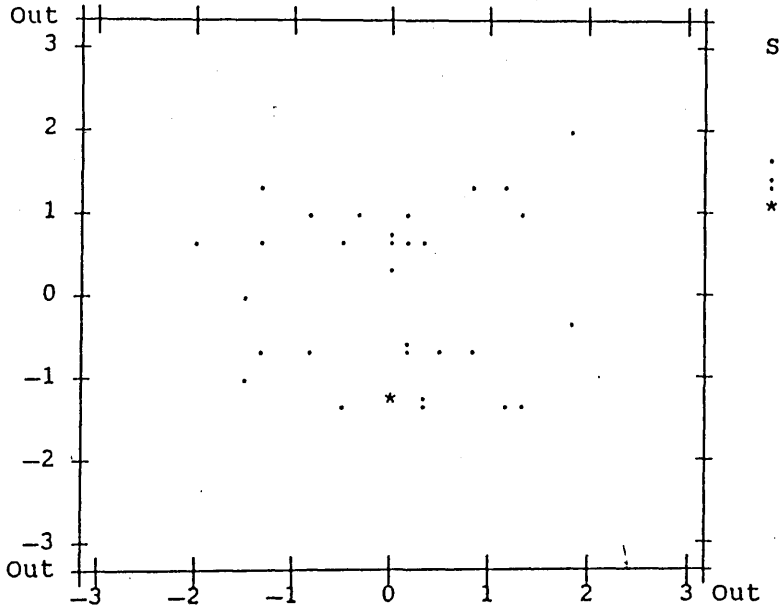
Total Cases = 36

Figure 23 cont./...

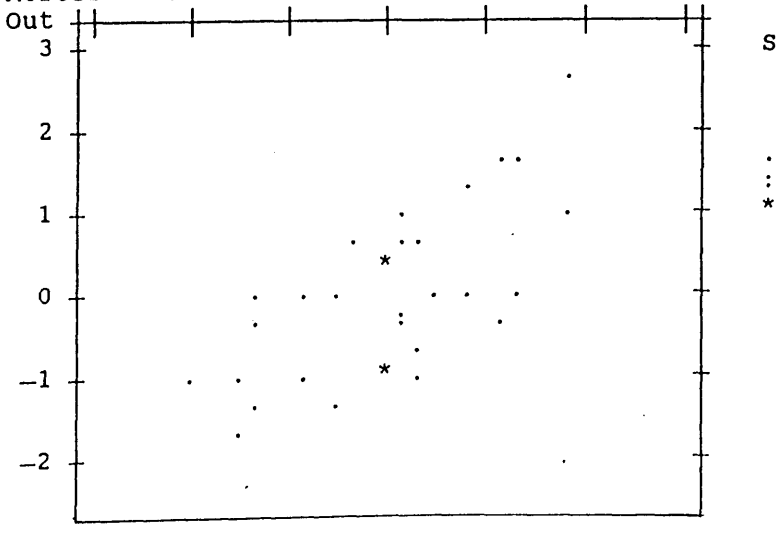
Histogram - Studentized Residual

```
NExp N       (* = 1 Cases,     . : = Normal Curve)
0   .03    Out
0   .05   3.00
0   .13   2.67
0   .29   2.33
1   .60   2.00 :
0  1.10   1.67 .
3  1.81   1.33 *:*
4  2.66   1.00 **:*
7  3.50    .67 ***:***
1  4.13    .33 *  .
1  4.37    .00 *  .
1  4.13   -.33 *  .
6  3.50   -.67 ***:**
1  2.66  -1.00 * .
8  1.81  -1.33 *:******
0  1.10  -1.67 .
0   .60  -2.00 .
0   .29  -2.33
0   .13  -2.67
0   .05  -3.00
0   .03    Out
```

Standardized Scatterplot
Across - *PRED Down - *SRESID



Standardized Scatterplot
Across - *PRED Down - LOGK



between the benzene test compound retention times and the solute properties (Table 6).

Once correlation equations were developed for the substituted benzene model compounds, retention prediction of the propranolol analogues was undertaken. Three bonded phases were chosen for this study; octyl-, cyclohexyl- and phenyl-silica. Being basic in nature and therefore attracted to active silica-surface silanol groups, the analogues could not be eluted from these three stationary phases within the required k' value with aqueous methanol alone. Addition of a silanol-site competitor, tri-n-butylamine, to the aqueous methanol eluent allowed the solutes to be eluted from the sorbent with a k' value of less than 50, which was taken throughout these studies as an upper retention limit. A 30:70 (v/v) methanol:water mobile phase with 0.3M tributylamine buffer was found to elute the solutes efficiently in terms of retention times and peak shape. Capacity factors for the propranolol analogues are given in Table 7. The effect of a biological matrix on the retention behaviour of ß-blocker drugs was also investigated. Plasma protein solution and fresh plasma were injected on to the stationary phase column before eluting the model compounds with the above mobile phase. Table 8 contains log k' values for this study and plots of log k' against structure are presented in figure 24. Typical peak shapes are presented in figure 25.

The retention data for the ß-blocker analogues was treated statistically in the same way as the benzene test

(a)Correlation Equations for Each Phase and Percentage Methanol in Mobile Phase

Ethylsilica

30% Methanol	Neutrals only	Log k' = 0.581LogP + 1.033ß - 0.132HD - 0.010(HD-HA) - 0.570 R^2=0.981 S.E.=0.059 N=22 F=278.391
	Acids & Neutrals	Log k' = 0.617LogD + 1.083ß -0.478Δ - 0.123HD - 0.469 R^2=0.971 S.E.=0.087 N=26 F=202.385
	Acids, Neutrals & Bases	Log k' = 0.562LogD - 0.205 R^2=0.917 S.E.=0.142 N=33 F=289.030
40% Methanol	Neutrals only	Log k' = 0.472LogP - 0.145 R^2=0.817 S.E.=0.200 N=28 F=121.523
	Acids & Neutrals	Log k' = 0.326LogD - 0.200HD + 0.001V - 0.274 R^2=0.860 S.E.=0.199 N=32 F=62.270
	Acids, Neutrals & Bases	Log k' = 0.326LogD - 0.201HD + 0.493V - 0.262 R^2=0.860 S.E.=0.195 N=36 F=66.410
50% Methanol	Neutrals only	Log k' = 0.367LogP - 0.119HD - 0.095 R^2=0.953 S.E.=0.080 N=26 F=256.372
	Acids & Neutrals	Log k' = 0.368LogD - 0.119HD - 0.097 R^2=0.964 S.E.=0.078 N=29 F=351.948
	Acids, Neutrals & Bases	Log k' = 0.361LogD - 0.121HD - 0.076 R^2=0.961 S.E.=0.088 N=33 F=343.850

Octylsilica

30% Methanol	Neutrals only	Log k' = 0.620LogP - 0.219HD + 0.203 R^2=0.910 S.E.=0.140 N=22 F=97.769
	Acids & Neutrals	Log k' = 0.513LogD - 0.311HD + 0.441 R^2=0.923 S.E.=0.188 N=31 F=151.153
	Acids, Neutrals & Bases	Log k' = 0.416LogD - 0.623HD + 0.769 R^2=0.686 S.E.=0.339 N=33 F=28.316
40% Methanol	Neutrals only	Log k'= 0.646LogP - 0.168HD - 0.068 R^2=0.900 S.E.=0.164 N=25 F=96.688
	Acids & Neutrals	Log k' = 0.566LogD - 0.202HD + 0.112 R^2=0.920 S.E.=0.174 N=32 F=150.275

Table 6 Multiple linear regression equations between log k' and physicochemical properties of the substituted benzene test solutes.

cont./...

	Acids, Neutrals & Acids	Log k' = 0.562LogD - 0.206HD + 0.126 R^2=0.917 S.E.=0.172 N=36 F=155.306
50% Methanol	Neutrals only	Log k' = 0.530LogP - 0.145HD - 0.197 R^2=0.895 S.E.=0.132 N=25 F=95.030
	Acids & Neutrals	Log k' = 0.422LogD - 0.107 R^2=0.819 S.E.=0.222 N=30 F=113.978
	Acids, Neutrals & Bases	Log k' = 0.497LogD - 0.409HD + 0.045 R^2=0.807 S.E.=0.225 N=34 F=57.563
Octadecylsilica		
30% Methanol	Neutrals only	Log k' = 0.579LogP - 0.388HD + 0.445 R^2=0.944 S.E.=0.155 N=16 F=127.878
	Acids & Neutrals	Log k' =-2.522B - 0.376HD + 0.010V + 0.113LogD - 1.170 R^2=0.879 S.E.=0.234 N=26 F=39.088
	Acids, Neutrals & Bases	Log k' =-2.370B + 0.011 V - 0.359HD + 0.129LogD + 0.893 R^2=0.870 S.E.=0.227 N=29 F=41.248
40% Methanol	Neutrals only	Log k' = 0.609LogP - 0.356HD + 0.071 R^2=0.940 S.E.=0.151 N=19 F=141.301
	Acids & Neutrals	Log k' = 0.213LogD - 0.367HD + 0.013V - 1.438B + 0.019 R^2=0.825 S.E.=0.292 N=26 F=29.358
	Acids, Neutrals & Bases	Log k' = 0.214LogD - 0.364HD + 0.012V - 1.424B - 0.025 R^2=0.828 S.E.=0.279 N=28 F=32.332
50% Methanol	Neutrals only	Log k' = 0.566LogP - 0.222HD - 0.192 R^2=0.934 S.E.=0.144 N=24 F=156.553
	Acids & Neutrals	Log k' = 0.198LogD + 0.013V - 1.342B - 0.634 R^2=0.813 S.E.=0.254 N=29 F=35.797
	Acids, Neutrals & Bases	Log k' = 0.195LogD + 0.001V - 1.373B + 1.106 Δ - 0.289HD - 0.328 R^2=0.894 S.E.=0.186 N=29 F=45.026
Phenylsilica		
20% Methanol	Neutrals only	Log k' = 0.204LogP - 0.405HD + 0.011V - 0.369 R^2=0.953 S.E.=0.100 N=15 F=88.700
	Acids & Neutrals	Log k' = 0.241LogD - 0.318HD + 0.578 R^2=0.884 S.E.=0.183 N=20 F=65.812

Table 6 cont./...

	Acids, Neutrals & Bases	Log k' = 0.241LogD - 0.318HD + 0.575 R^2=0.884 S.E.=0.178 N=22 F=69.580
30% Methanol	Neutrals only	Log k'= 0.302LogP + 0.357 χ - 0.223HD - 0.709 R^2=0.847 S.E.=0.188 N=27 F=45.247
	Acids & Neutrals	Log k' = 0.268LogD - 0.001V - 0.186HD - 0.710 R^2=0.811 S.E.=0.238 N=35 F=45.448
	Acids, Neutrals & Bases	Log k' = 0.243LogD - 0.294HD + 0.001V - 0.621 R^2=0.775 S.E.=0.263 N=38 F=39.941
40% Methanol	Neutrals only	Log k' = 0.244LogP - 0.218HD + 0.162χ - 0.286 R^2=0.833 S.E.=0.167 N=15 F=55.992
	Acids & Neutrals	Log k' = 0.201LogD - 0.236HD + 0.001V - 0.368 R^2=0.780 S.E.=0.210 N=41 F=45.883
	Acids, Neutrals & Bases	Log k' = 0.174LogD - 0.204HD + 0.001V - 0.552 Δ - 0.500 R^2=0.795 S.E.=0.202 N=44 F=40.739
50% Methanol	Neutrals only	Log k'= 0.246LogP + 0.157χ - 0.735Δ - 0.709 R^2=0.853 S.E.=0.136 N=33 F=62.860
	Acids & Neutrals	Log k' = 0.136LogD - 0.105HD + 0.001V - 0.736 R^2=0.860 S.E.=0.147 N=37 F=56.055
	Acids, Neutrals & Acids	Log k' = 0.136LogD - 0.105HD + 0.001V - 0.732Δ - 0.453 R^2=0.856 S.E.=0.146 N=41 F=57.259
Cyclohexylsilica		
20% Methanol	Neutrals only	Log k' = 0.540LogP + 0.043 R^2=0.558 S.E.=0.382 N=24 F=28.754
	Acids & Neutrals	Log k' = 0.180LogD - 0.532HD + 1.008 R^2=0.607 S.E.=0.422 N=33 F=24.156
	Acids, Neutrals & Bases	Log k' = -0.716HD + 1.410 R^2=0.533 S.E.=0.445 N=44 F=38.615
30% Methanol	Neutrals only	Log k'= 0.330LogP + 0.036 (HA-HD) + 0.051 R^2=0.628 S.E.=0.287 N=26 F=20.451

Table 6 cont./...

	Acids & Neutrals	Log k' = 0.199LogD + 0.0302(HA-HD) - 0.229HD - 0.710 R^2=0.672 S.E.=0.331 N=36 F=23.490
	Acids, Neutrals & Bases	Log k' = -0.471HD + 0.033(HA-HD) + 0.892 R^2=0.490 S.E.=0.400 N=46 F=18.273
40% Methanol	Neutrals only	Log k' = 0.453LogP - 0.144 R^2=0.479 S.E.=0.396 N=33 F=28.613
	Acids & Neutrals	Log k' = 0.170LogD - 0.250HD + 0.568 R^2=0.490 S.E.=0.416 N=45 F=20.185
	Acids, Neutrals & Bases	Log k' = 0.293LogD - 0.346HD + 0.339 R^2=0.500 S.E.=0.409 N=50 F=22.893
50% Methanol	Neutrals only	Log k'= 0.293LogP - 0.182 R^2=0.436 S.E.=0.310 N=33 F=24.943
	Acids & Neutrals	Log k' = 0.235LogD - 0.084 R^2=0.504 S.E.=0.334 N=37 F=36.524
	Acids, Neutrals & Acids	Log k' = 0.195LogD - 0.250HD + 0.163 R^2=0.445 S.E.=0.347 N=41 F=15.856

(b) Correlation Equations for Each Phase Including Different Percentages of Methanol in the Mobile Phase

Ethyl Silica

Log k' = 0.315 LogD - 0.016(% MeOH) - 0.194 HD + 0.004V + 0.416

R^2=0.745 S.E.=0.243 N=106 F=69.778 $\sigma = \pm 0.243$

Octylsilica

Log k' = 0.424 LogD - 0.016(% MeOH) - 0.291 HD + 0.004V + 0.592

R^2=0.872 S.E.=0.202 N=112 F=150.539 $\sigma = \pm 0.202$

Octadecylsilica

Log k' = 0.177 LogD - 0.361 HD + 0.011V - 0.017(% MeOH) - 2.338B + 0.926

R^2=0.841 S.E.=0.240 N=100 F=0.240 $\sigma = \pm 0.240$

Cyclohexylsilica

Log k' = 0.155 LogD - 0.419HD - 0.015(% MeOH) + 0.018V -0.393 χ - 0.749 Δ + 0.239 HA + 0.385

R^2=0.614 S.E.=0.354 N=172 F=35.754 $\sigma = \pm 0.354$

Phenylsilica

Log k' = 0.183 LogD - 0.016(% MeOH) - 0.204 HD + 0.0128V - 0.501 Δ - 0.156 χ + 0.108

R^2=0.818 S.E.=0.205 N=148 F=101.350 $\sigma = \pm 0.205$

Table 6

COMPOUND NUMBER	CAPACITY FACTOR, LOG K'		
	Octylsilica	Cyclohexylsilica	Phenylsilica
1	0.29	0.34	0.34
2	0.49	0.50	0.35
3	1.20	0.98	0.86
4	0.84	0.75	0.65
5	0.70	0.67	0.67
6	1.35	1.19	1.09
7	0.78	0.72	0.57
8	0.63	0.60	0.56
9	1.40	-	1.04
10	0.37	0.19	0.01
11	0.41	0.46	0.40
12	0.67	0.62	0.65
13	0.61	0.62	0.67
14	0.47	0.50	0.55
15	0.14	0.29	0.39

Table 7 Logarithmic capacity factors of the β-blocker test solutes from high performance liquid chromatography. 30% methanol(v/v), 0.3M tri-n-butylamine, pH4.

PHASENAME	COMPOUND NUMBER	CAPACITY FACTOR, LOG K'	
		With Plasma Protein Solution+30:70(v/v) Methanol:water	With Fresh Plasma +30:70(v/v) Methanol:water
Octylsilica	4	-[a]	0.39
	6	-	0.60
	7	-	-0.06
	8	-	0.15
	10	-	-0.35
	11	-	-0.11
	12	-	-0.09
	15	-	-0.31
Cyclohexyl-silica	4	-[b]	-[b]
	6	-[b]	-[b]
	7	-[b]	0.99
	8	1.03	0.83
	10	0.31	0.20
	11	0.70	0.53
	12	0.93	0.75
	15	0.64	0.49
Phenylsilica	4	-[c]	0.67
	6	-	1.16
	7	-	0.62
	8	-	0.63
	10	-	0.07
	11	-	0.41
	12	-	0.73
	15	-	0.53

[a]-No elution within k' < 50 under these conditions [b]-Peak too broad to be measured [c]-Not measured under these conditions

Table 8 Logarithmic capacity factors for the study of fresh plasma and plasma protein solution on the retention behaviour of selected β-blocker test solutes. cont./...

PHASENAME	COMPOUND NUMBER	CAPACITY FACTOR, LOG K' With 30:70(v/v) methanol:water + 0.3M TBA	With Plasma Protein Solution+Eluent	With Fresh Plasma+Eluent
Octylsilica	4	1.10	-0.22	0.39
	6	1.03	0.15	0.60
	7	1.03	-0.32	-0.21
	8	0.90	-0.41	-0.18
	10	0.48	-0.76	-0.10
	11	0.72	-0.57	-0.26
	12	0.90	-0.46	-0.20
	15	0.37	-0.87	-0.59
Cyclohexyl-silica	4	0.82	0.54	0.65
	6	1.27	0.96	1.08
	7	0.80	0.47	0.59
	8	0.68	0.38	0.51
	10	0.25	-0.16	0.06
	11	0.49	0.20	0.35
	12	0.68	0.41	0.54
	15	0.35	0.06	0.20
Phenylsilica	4	0.56	0.50	0.58
	6	1.18	0.96	1.01
	7	0.49	0.49	0.54
	8	0.47	0.48	0.54
	10	-0.09	0.05	0.03
	11	0.31	0.33	0.37
	12	0.56	0.59	0.63
	15	0.26	0.31	0.35

Table 8

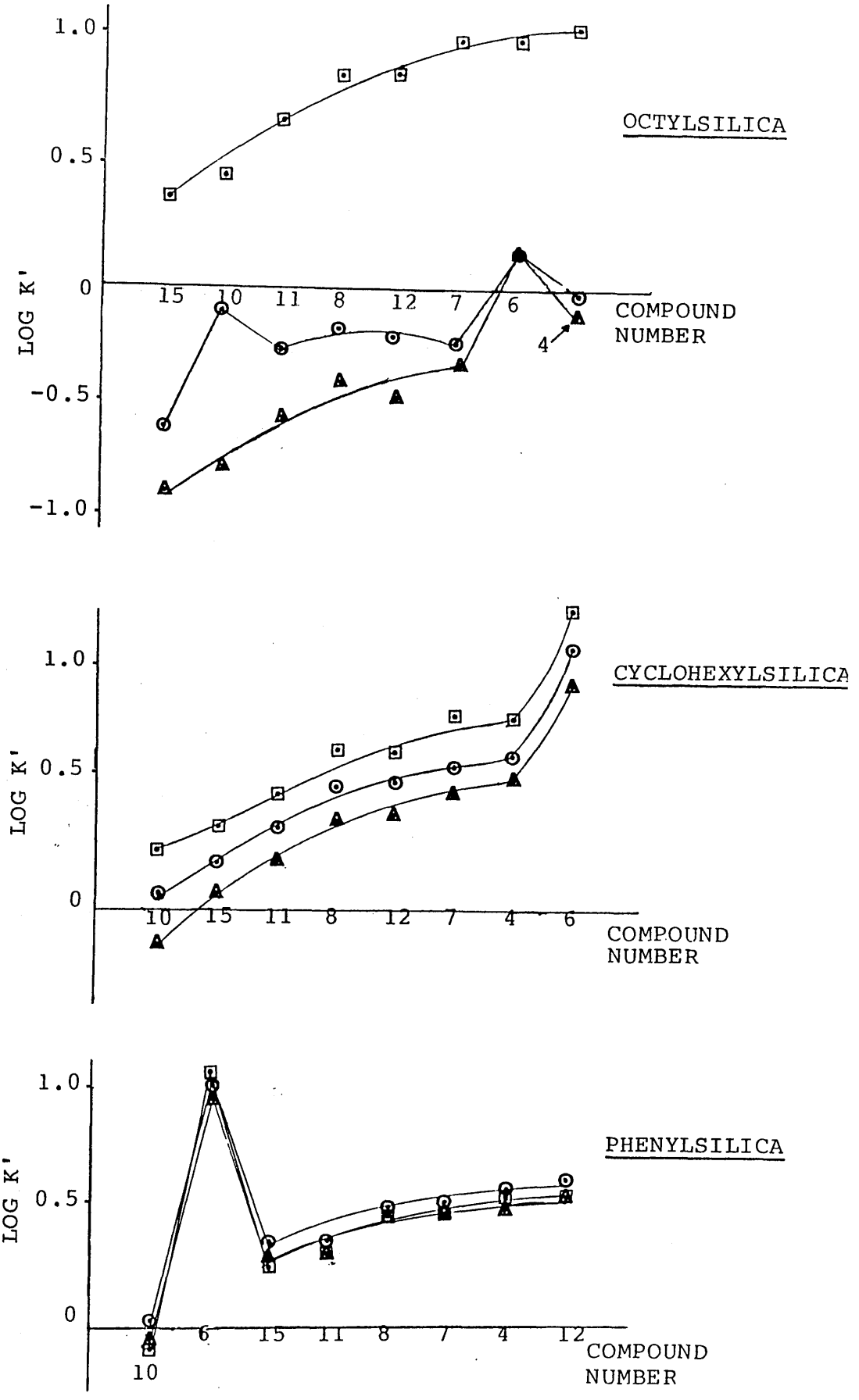


Figure24 Effect of □ pH4-buffered 30:70 methanol:water (v/v) eluent only, O whole plasma + eluent, Δ plasma protein solution + eluent on the retention behaviour of propranolol analogues.

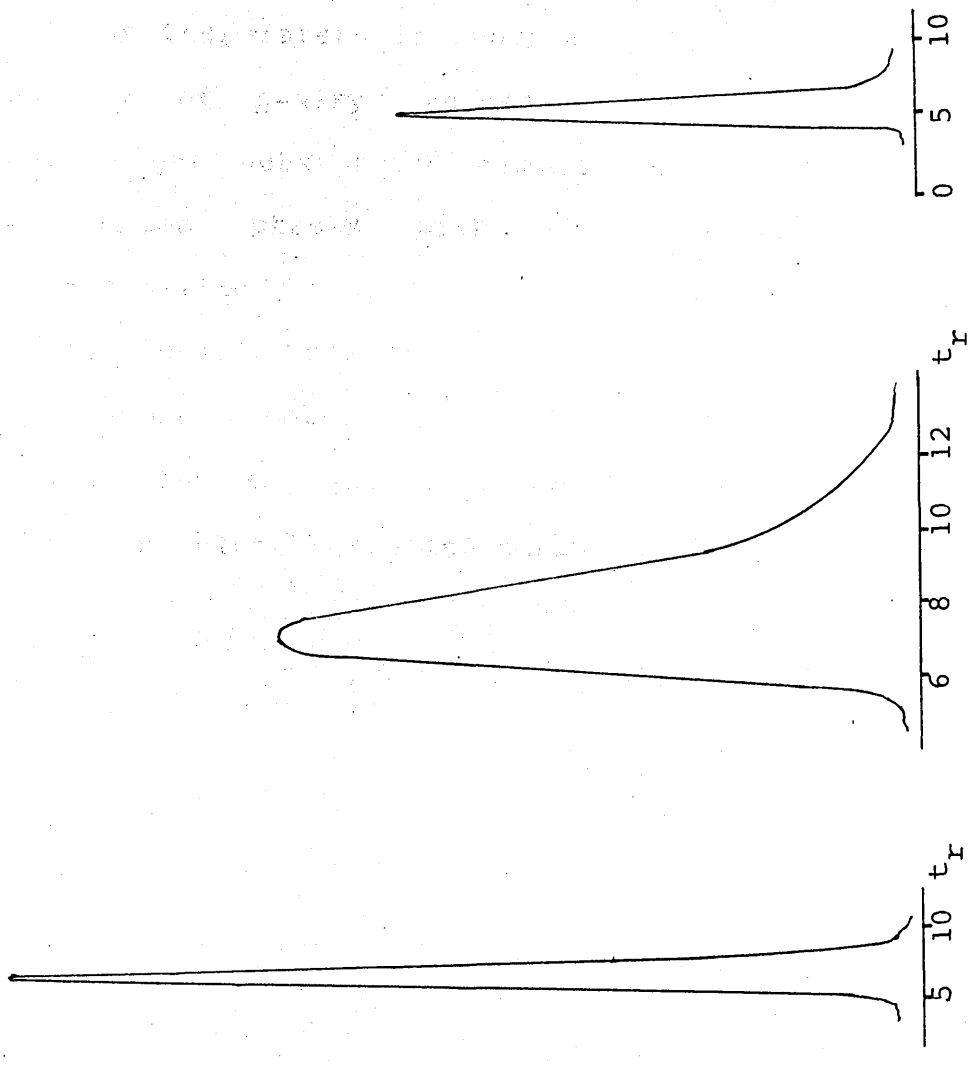


Figure25 Examples of propranolol peak shapes. Elution from (a) cyclohexyl-, (b) octyl- and (c) phenyl-silica with 30:70 (v/v) methanol:water + 0.3M tributylamine buffer. Full scale deflection = 10mV, flowrate = 1ml/min.

compounds. Correlation equations were constructed for the three chosen phases with and without the addition of matrix components. The same independent variables used in the MLR analysis of the benzene solutes were correlated with the log k' values of the propranolol analogues. Correlations are presented in Table 9.

As temperature is known to affect the conformational behaviour of n-alkyl bonded chains and thus retention times, eight substituted benzene probes were eluted from the bonded phases with the longest chain length (octadecylsilica) and the shortest chain length (ethylsilica). Retention times were determined in 50:50 (v/v) methanol:water and at 25, 30 and 35°C (Table 10). Plots of log k' against temperature were made to observe changes in solute retention behaviour (Figure 26).

Octylsilica	
Eluent Only	Log k' = 0.349χ - 2.015 R^2=0.632 S.E.=0.238 N=23 F=25.039 $\sigma = \pm$ 0.24
Eluent + Plasma Protein Solution	Log k' = 0.322χ - 0.027(HA-HD) - 2.426 R^2=0.934 S.E.=0.082 N=8 F=50.156 $\sigma = \pm$ 0.08
Eluent + Fresh Plasma	No correlataion.
Cyclohexylsilica	
Eluent Only	Log k' = 0.769 χ - 0.186HD - 0.012V - 1.428 R^2=0.870 S.E.=0.095 N=23 F=30.030 $\sigma = \pm$ 0.10
Eluent + Plasma Protein Solution	Log k' = 0.404χ - 0.206HA - 1.907 R^2=0.985 S.E.=0.045 N=8 F=191.828 $\sigma = \pm$ 0.05
Eluent + Fresh Plasma	Log k' = 0.369χ - 0.182HA - 1.580 R^2=0.955 S.E.=0.066 N=8 F=74.905 $\sigma = \pm$ 0.07
Phenylsilica	
Eluent Only	Log k' = 0.373 χ - 0.286HD - 1.675 R^2=0.800 S.E.=0.130 N=23 F=26.987 $\sigma = \pm$ 0.13
Eluent + Plasma Protein Solution	Log k' = 0.713χ - 0.400HD - 0.010V - 1.358 R^2=0.992 S.E.=0.025 N=8 F=299.685 $\sigma = \pm$ 0.03
Eluent + Fresh Plasma	Log k' = 0.324χ - 0.160HA - 1.317 R^2=0.886 S.E.=0.094 N=8 F=28.328 $\sigma = \pm$ 0.09

Table 9 Multiple linear regression equations between log k' and physicochemical properties of propranolol and selected solutes.

PHASENAME	TEST SOLUTE	LOG K'		
		25°C	30°C	35°C
Ethyl				
	Salicylic acid	-1.10	-1.15	-1.22
	Benzoic acid	-1.10	-1.15	-1.22
	Phenol	-0.46	-0.47	-0.48
	Aniline	-0.43	-0.46	-0.46
	Benzene	-0.05	-0.08	-0.10
	2,4-Dimethylphenol	-0.15	-0.19	-0.21
	p-Phenylphenol	_[a]	_[a]	_[a]
	n-Propyl-p-hydroxybenzoate	-0.18	-0.24	-0.28
Octadecyl				
	Salicylic acid	-0.57	-0.55	-0.65
	Benzoic acid	-0.70	-0.66	-0.75
	Phenol	0.05	0.14	0.09
	Aniline	0.08	0.06	0.02
	Benzene	1.04	1.01	1.00
	2,4-Dimethylphenol	0.79	0.79	0.73
	p-Phenylphenol	1.26	1.20	1.14
	n-Propyl-p-hydroxybenzoate	_[a]	_[a]	_[a]

[a]-not eluted within k' < 50

Table 10 Logarithmic capacity factors of selected benzene test solutes for the study of temperature on high performance liquid chromatographic retention.

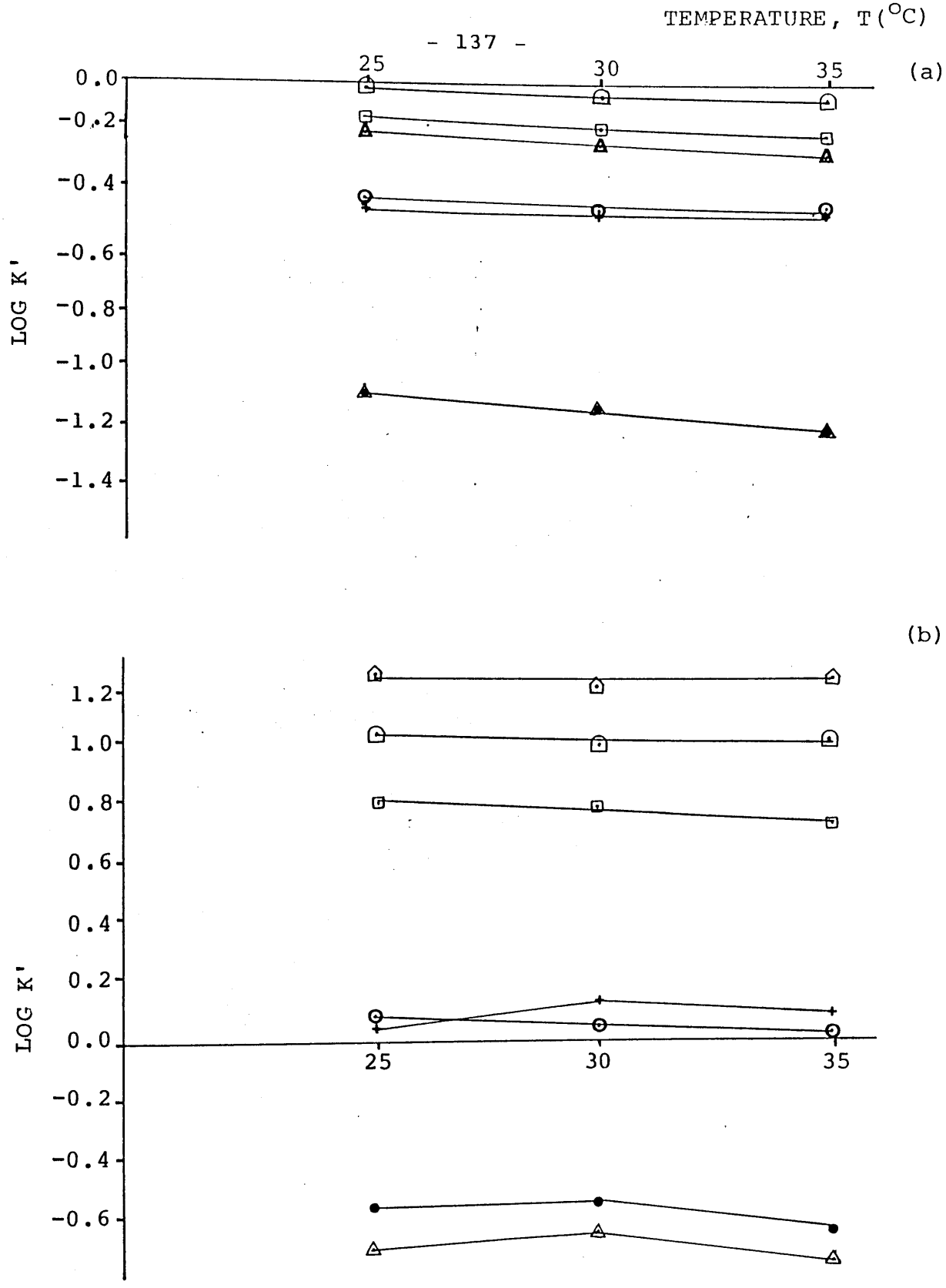


Figure 26 Effect of temperature on the retention of •salicylic acid, △benzoic acid, + phenol, Oaniline, ⌂benzene, □2,4-dimethylphenol, ⌂p-phenylphenol, Δn-propyl-p-hydroxy-benzoate. (a) ethylsilica (b) octadecyl-silica, 50:50 methanol:water, pH7.9 (1.0ml/min flowrate)

CHAPTER FIVE

DISCUSSION

The chromatographic environment is a complex system incorporating the mobile phase, the stationary phase and the solute. The contributions from each of these components cannot be separated because of the random nature imposed by both the bulk mobile phase and the bonded sorbent. The heterogeneous nature of the system has therefore resulted in a lack of parameters for these two phases with which to describe their contributions to solute retention. As a consequence and for simplicity, the stationary phase and the mobile phase need to be assumed as homogeneous in studies relating to solute retention prediction. This leaves only the solute with readily-generated, reliable physicochemical data, and the most common approach to understanding the chromatographic retention process involves observation of changes in retention of solutes as either the sorbent or mobile phase is changed. Interpretation of the retention process can then be made from the properties of the solute which appear to determine retention and elution behaviour. By keeping both the mobile phase and the sorbent constant, and by adding to or changing the substituents on a solute, the contribution of a functional group to retention and elution can be examined.

This study was undertaken to apply solute retention

prediction to the solid-phase extraction of ß-blocker drugs in order to evaluate the usefulness of prediction rules in solid-phase extraction method development. The ability to predict the conditions required for retention/elution would aid the development of new solid-phase extraction techniques and improve existing methods, resulting in reduced expense and time to the development toxicologist.

5.1 The Experimental Approach

The selection of model benzene solutes was based primarily on the functional groups commonly encountered in ß-adrenergic receptor drugs, in particular propranolol. Mono-, bi- and tri-functional benzene compounds were chosen because (a) they encompassed a wide variety of suitable substituents, (b) the compounds were easily obtainable and (c) the solutes had a common benzene nucleus in their structure which served as a convenient chromophore for the detection of the solutes by UV spectrometry. Forty three solutes were chosen with acidic, basic and neutral substituents including carboxylic, amino, phenolic, methoxy, nitrile and alkyl groups. Multifunctional benzene solutes permitted the influence of neighbouring substituents and their relative positions around the ring to be explored. Several small series of solutes were used, such as phthalic acid, dimethylphthalate and diethylphthalate; also ortho-, meta- and para-hydroxy benzoic acid.

The pH of the aqueous methanolic mobile phases used in the HPLC studies were chosen as approximately 5 and 7, the pH values to which biological samples might be buffered for SPE.

Being basic in nature, ß-blocker drugs are difficult to extract because of unpredictable retention through silanol interactions. Propranolol and several synthetically-modified analogues were chosen to represent this group of drugs in the modelling of retention behaviour. Fourteen analogues of propranolol were selected with functional group variations in the side chain and around the naphthalene ring. This enabled the influence of groups adjacent to the basic centre involved in the silanol interaction to be examined, as well as the effects of introducing substituents onto the dominantly lipophilic fused ring system of naphthalene. These test compounds also allowed the influence of size to be studied.

In this present work it was also considered important to study matrix effects on retention and elution. Biological fluids contain proteins and lipids which may change the properties of the sorbent if they interact with the bonded chains or exposed silanol groups. These components are normally present in much greater concentrations than the drugs and their metabolites and the matrix may readily saturate much of the available stationary phase surface. As the retention of ß-blocker drugs may be changed by such alterations to the bonded phase, the ß-blocker test solutes were prepared as

aqueous solutions, and also in fresh plasma and plasma protein solution to investigate this phenomenon.

Bonded silica extraction cartridges under the trade name of Bond Elut were chosen because they are among the most widely used cartridges for the solid-phase extraction of drugs from biological matrices. Like the majority of other commercial bonded phase sorbents, Bond Elut® is polymeric to enhance selectivity [22].

Non-polar phases were selected for testing as reversed-phase extractions are widely employed in toxicology, including the more frequently encountered octyl- and octadecyl-silicas as well as the lesser used ethyl-, cyclohexyl- and phenyl-silicas. As little research has been conducted on the latter two phases, the present study is particularly informative with respect to the retention behaviour and selectivity to be expected.

A large number of retention data was required for significant statistical analyses to be performed. Extraction of the test solutes with the Bond Elut® cartridges would have been slow and the analysis of fractions collected during these steps would only indicate if a solute was eluted or retained indefinitely under each particular set of conditions. Thus a continuous flow system similar to HPLC was used to provide the data needed for statistical analysis in the shortest time, but more importantly to enable relative retention times/volumes of solute series to be studied.

The bonded silica phases were supplied loose and

packed into short (10cm) HPLC columns to imitate SPE cartridges. Packing into the stainless steel columns by the slurry method did not yield as many theoretical plates as columns packed by the dry tap-fill method described in reference 162 (Table 11), so HPLC columns were subsequently packed with the sorbents by the latter method. This unexpected result is undoubtedly due to the size and irregular shape of the particles compared to the much smaller, spherical packings used in HPLC.

Degradation of the material by hydrolysis of the Si-O-C ether bond with the aqueous mobile phases did not appear to be a problem. For example, the number of theoretical plates, N, measured with aniline for a column filled with the octyl phase immediately after packing was 157.6. After two weeks of continuous use (average time in which to collect retention data for the solutes with four out of a total of eight mobile phases), N had dropped by just 4.2 to 153.4. Thus it was concluded that loss of silica-bonded ligand was negligible over the time-span normally required for the retention measurements.

Methanol was chosen as an appropriate organic modifier for the aqueous mobile phases not only because it is the most widely used organic modifier in reversed-phase HPLC, but because of its properties as compared to acetonitrile (ACN) and tetrahydrofuran (THF) which are also commonly used. Methanol possesses both hydrogen-bond donor and acceptor abilities unlike ACN and THF which are only hydrogen-bond acceptors. Thus methanol exhibits similar

PACKING METHOD	NUMBER OF THEORETICAL PLATES, N
Slurry packing with methanol	116
Dry tap-fill	222

Table 11 Comparison of the number of theoretical plates for a column packed with Bond Elut octylsilica by the slurry method and the dry tap-fill method.

SOLVENT	POLARISABILITY VOLUME ($\times 10^{24} cm^3$)	DIELECTRIC CONSTANT, ξ
Methanol	3.23	30
Water	1.48	80

Table 12 Some properties of methanol and water

polarisation properties to water (Table 12). Methanol also does not drastically alter the solvation sphere of water molecules associated with a solvated solute and/or silica-bonded ligand as it is approximately the same size and shape as a water molecule and can therefore readily replace a water molecule without disrupting the sheath of solvent. Use of methanol therefore minimises changes to the bulk mobile phase structure as the percentage of organic modifier is changed, and, unlike ACN and THF, methanol forms only a single monolayer with the water bound to the silica surface of the sorbent. Adsorption of the solute by a thick layer of adsorbed solvent is thereby reduced.

Methanol is often used to "condition" reversed-phase SPE sorbents before sample application as it readily solvates the non-polar ligands attached to the silica. This encourages polymeric bonded phases to swell and lowers the energy needed for solvation by the bulk aqueous mobile phase used as eluent. Because methanol has a lower dielectric constant than water (Table 12), it is easier for a solute to form a cavity within the methanol network. This is observed by reduced solute retention times as the percentage of methanol in the mobile phase increases, e.g. with octadecylsilica and 30% (v/v) methanol buffered to pH 7, the log k' values of acetylsalicylic acid, anisole and toluene-p-sulphonamide are respectively 0.22, 1.71 and 0.22, but at an increased percentage of methanol (50% v/v, pH7), the solute retention times are reduced to -0.22, 1.57

and -0.26 respectively. Note that the retention times of the two polar solutes are greatly reduced by an increase in percentage organic modifier compared to the neutral anisole.

This pattern is a reflection of the positions that the solutes adopt within the stationary phase network as already depicted in figure 12, p.43. Polar solutes, except bases, tend to reside at the unattached end of the bonded ligands to enhance their hydrogen-bonding or ionisation interactions with the polar bulk mobile phase. Therefore such polar solutes are influenced to a greater extent by changes in mobile phase composition than solutes such as anisole which prefer to reside deeper in the stationary phase where more non-polar interaction with the hydrophobic ligands is favoured. The concentration range of organic modifier was chosen as 20-50% methanol because of the adverse changes to mobile phase pH at methanol concentrations above 50% (v/v) [106,124] and also because of the difficulty of eluting hydrophobic solutes within a reasonable time with less than 20% (v/v) methanol.

A second mobile phase additive was necessary for the retention studies of propranolol and analogues to nullify ion-exchange retention through exposed silanol groups. Tri-n-butylamine (0.3M) was used as the modifying amine to block such potential retention sites and maximise reversed-phase behaviour of the basic compounds. The optimum concentration of tri-n-butylamine was selected as the concentration at which the peak shape and height of propranolol was no longer improved by additional amounts of

modifier, and the composition of the aqueous methanolic mobile phase was taken as the concentration at which the retention time of propranolol was no longer reduced upon further addition of methanol to the mobile phase (30% (v/v) methanol).

The dynamics of the stationary phase have been noted as changing when the system is subjected to varying temperature [55]. At a specific temperature, T_o, the stationary phase reaches a stable conformation [166] and T_o values vary for different lengths of bonded chains [56]. A study of the effect of column temperature on the retention of selected substituted benzene solutes was conducted on the test phase with the shortest chain length (ethylsilica) and one with a longer ligand length (octadecylsilica). Solutes were chosen to represent the three main families of compounds: polar (acids and bases), non-polar and phenols, and were eluted with 50% (v/v) methanol at pH7. The lowest temperature at which the column could be regulated within the HPLC column compartment was 25° so the behaviour of the eight test solutes was examined at temperatures of 25°C, 30°C and 35°C. From the plots of log k' versus temperature in Figure 26 (p.137), the following conclusions could be drawn:

(a) Effect of temperature on the ethylsilica phase

With the short-chain ethyl phase, the solutes were eluted at each temperature in the order expected from their general physical properties, i.e. benzene, being non-polar, was retained longer than phenol which in turn was eluted

more slowly than the more polar salicylic acid. When the temperature increased, the solutes were eluted slightly faster than at the previous temperature as the silica-bonded chains increased in mobility and the solutes were exposed to more of the bulk mobile phase, in which the solutes became more soluble with increasing temperature. The unattached ends of the chain became more mobile than the rest of the ligand as the temperature rose, and consequently solutes which reside in the more mobile region, i.e polar solutes such as salicylic acid and benzoic acid, experienced less restriction imposed by the hydrocarbon ligands and also less energy was required to create a cavity in the mobile phase. Thus they showed a greater reduction in retention times than the less polar solutes as the temperature rose. All of the plots for this phase were linear implying that either the stationary phase had not reached its T_o temperature or, more likely, that it was already in its most stable conformation as T_o for ethylsilica is less than 25°C because the bonded chains are short [58].

(b) Effect of temperature on the octadecylsilica phase

Again the test solutes were eluted in the order expected at each temperature. However, it can be seen from Figure 26 that a break in linearity occured for the polar acidic solutes including phenol. This, again is related to the position of the solutes within the stationary phase layer. At 30°C, the long silica-bonded hydrocarbon ligands undergo a conformational transition towards the end

of the chains. Thus the retention of solutes within this region will be affected as shown in Figure 27. Solutes which partition further into the stationary phase layer do not experience any drastic change in their environment, except slightly increased freedom, and therefore follow linear behaviour as temperature increases. Hence it can be concluded that octadecylsilica does not reach its most stable conformation until the column temperature is above 30°C. This must be kept in mind when comparing retention data performed on octadecylsilica below 30°C and where the column temperatures used are different. Experimental studies carried out under different chromatographic conditions for retention data comparison should be performed at a constant temperature to eliminate such uncertainty.

Interestingly, aniline, which is a polar solute and would therefore be expected to reside near the end of the stationary phase near the bulk mobile phase, does not show non-linear behaviour like phenol which is similar in polarity. This illustrates that aniline is pulled further into the stationary phase network by ionic interaction with silanol groups and is in a less chaotic environment, as observed by Schunk [76].

The adaptation of SPE to HPLC requires appropriate criteria to be chosen when the terms "retention" and "elution" are used in the 'digital' chromatography concept applied to SPE. If k' was greater than 50, the solute was taken to be irreversibly retained under those particular

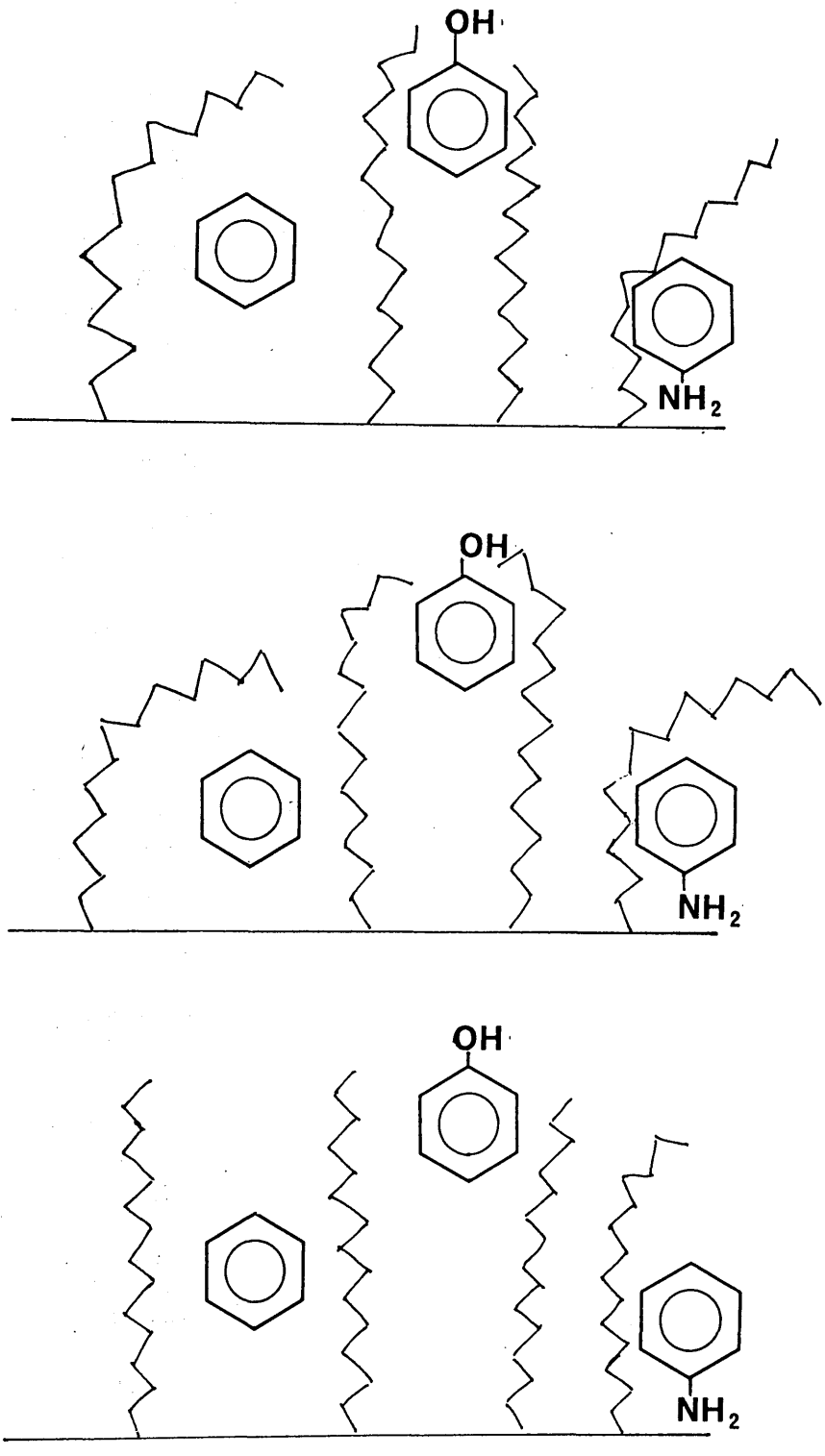


Figure 27 Effect of sorbent chain mobility on solutes in the chromatographic system.

chromatographic conditions ("off" in SPE). If k' was less than 50, it was presumed to be eluted with that particular mobile phase from the bonded phase used ("on" in SPE). This concept will be discussed in more detail further on with respect to the precision of the prediction equations. It is important to realise that in the practical application of QSRR theory to cartridge-SPE methods the correlations between predicted and actual retention behaviour are of a lower degree than those needed for QSRR studies of the theory of HPLC.

Solutes eluted with logarithmic retention capacities of less than -0.50 were not used in the regression analysis as such times could not be measured to a sufficiently high degree of precision.

5.2 Selection of Physicochemical Descriptors

The chemical and physical properties chosen to represent the test solutes had to be carefully selected in order to cover the two major interactions which prevail in liquid chromatographic retention; dispersive and inductive forces. Table 13 lists the parameters collected for each solute. A number of descriptors are clearly intercorrelated with other properties, e.g. both the partition coefficient, log P, and the Hansch parameter represent the hydrophobic potential of a solute; both volume and polarisability reflect solute size. If highly intercorrelated independent physicochemical variables are used together in the same regression equation, the

LIST OF PHYSICOCHEMICAL PROPERTIES DETERMINED FOR THE TEST SOLUTES

Molecular weight, MW
Melting point, mp
Boiling point, bp
Density
Refractive index
Partition coefiicient, log P
F-parameter
Hansch parameter, π
No. of hydrogen-bond acceptors, HA
No. of hydrogen-bond donors, HD
HA-HD
HA+HD
Ionisation constant, pKa
First order connectivity index, χ
Molar refractivity, Rm
Molar volume, Vm
Excess electronic charge distribution coefficient, Δ
Hammett constant, σ
Momentum, Mx, My and Mz
Volume of whole molecule, V
Dipole moment of whole molecule, DM
Summation of dipole moments for substituted groups
Principal elliptical axes, Px, Py and Pz
Polarisability of whole molecule
Summation of polarisabilities for substituted groups
Moment of polarisability of whole molecule
Summation of moments of polarisability for substituted groups
Hydrogen-bond acceptor ability, β

Figure 13 List of parameters collected for the test solutes.

correlation coefficient, R^2, is falsely increased due to the intercorrelation. Therefore only one significant variable describing possibly a number of other similar descriptors has to be selected. In this study, such selection was performed by factor analysis which grouped together parameters relating to size and shape, hydrogen-bond acceptor/donor ability and polarity (Table 14). From this the following parameters were selected as independent variables for multiple linear regression (MLR) analysis: V, χ, log P, HA, HD, HA-HD, Δ & β.

If a solute possessed two or more ionisable substituents, the substituent which was thought to exert the greatest influence on the ionisation behaviour of the molecule as a whole was selected. For example, with p-hydroxybenzoic acid, the pK_a taken to represent the whole molecule was that of the carboxylic acid substituent (4.67), in preference to the pK_a of the weaker phenolic substituent (9.37).

Momentum and principal elliptical axes could not be used in MLR because they are dependent on the orientation of the molecule. Momentum would have been a good parameter to describe the "tumbling" motion of a solute which would be useful when considering how a molecule rotates about its centre of gravity. The principal elliptical axes would also have been useful for representing the planarity and elongation of a substituted molecule, particularly when the bonded phase may only allow a particular shape of molecule to "slot in" between ligands. Limitations of the VIKING

CHEMICAL/PHYSICAL PROPERTY	PHYSICOCHEMICAL DESCRIPTORS WHICH ARE RELATED
Size and Shape	Polarisability V F π Vm Rm χ Log P MW Moment of Polarisability
Hydrogen-bond acceptor/donor ability	HA+HD HD HA ß
Polarity	Total dipole moment Fragmental dipole moment Δ

Figure 14 Physicochemical parameters selected by factor analysis.

program on the selection of standard orientational axes meant that these variables had to be excluded.

No other appropriate descriptors are presently available, especially relating to the mobile phase and the stationary phase. Those used include the most recently developed parameters published in the field of QSRR. However, as will be discussed in suggestions for further work, new parameters need to be developed to satisfactorily predict the effects of inductive parameters and geometric factors on chromatographic retention.

5.3 Approach to Computational Methods

5.3.1 Molecular Modelling

The solute physicochemical parameters had to be accurately measured to reduce errors in the prediction equations. A number of properties such as molecular weight, density, log P (substituted benzene solutes only) and χ were found in the literature as already shown (Table 3, p.99). However, particular properties of all solutes, such as volume and dipole moment, had to determined by a molecular modelling system. Structures to be measured were constructed by VIKING software (ICI Pharmaceuticals). A calculation package called ICICAL then accessed the structures from VIKING and performed the required calculations by the CNDO/2 method. The structures were input in their lowest energy form, e.g. salicylic acid was constructed with the substituents orientated so as to

maximise intramolecular hydrogen bonding. The potential energy of phthalic acid, dimethyl- and diethyl-phthalate were highly orientation-dependent and were assigned zero dipole moments if drawn in the wrong conformation. This resulted in no VIKING/ICICAL-generated atomic charge data for phthalic acid because the orientation of the lowest energy form could not be represented accurately enough.

5.3.2 Statistical Analysis

With the large amount of experimental and physicochemical data generated for statistical analysis, it was necessary to file the data in a hierarchical-structured database. SIR was chosen as the database package for this reason. This allowed each test solute to be treated as an individual case. Associated with each case were three records; one containing the physical and chemical parameters for the solute, another containing details of the chromatographic systems in which that solute's retention times were measured, and a third with the appropriate experimental retention data.

A compatible statistical analysis package, SPSSX/PC, was use to retrieve selected cases and records from SIR and perform a variety of statistical manipulations on the data. Simple linear correlation analysis was initially performed to confirm that no single variable alone correlated significantly with log k' (dependent variable). A correlation coefficient of less than 0.8 was accepted as indicating that the single variable correlation was not

significant. Once it was established that more than one variable would be necessary to correlate log k' with solute physicochemical properties (independent variables) in the majority of situations, factor analysis was performed to reduce the number of independent variables and to highlight independent variables which were highly intercorrelated. Although work by Leahy [112,122] shows that log P is highly correlated to volume, solute polarity and ß, indicating that only one of these variables should be used as an independent variable, omission of log P or log D from the regressional analysis variable list resulted in very poor correlations, for example:

Octadecylsilica 40% methanol

With log D	R^2 = 0.828
Without log D	R^2 = 0.489

Thus the eight physicochemical descriptors described in the previous section were selected as independent variables.

If multiple linear regression generated a prediction equation where the predicted dependent variable, log k'_{pred}, was ideally correlated with the experimental value, log k'_{expt}, the correlation coefficient would be either +1 if log k'_{pred} increases in value as log k'_{expt} increases or -1 if log k'_{pred} decreases with increasing log k'_{expt}. If R^2=0, then there is no linear correlation between the dependent variable and any independent variables although it may be possible that a non-linear correlation exists. A correlation coefficient

of greater than 0.9 is considered very good, although in this work a correlation coefficient of better than 0.8 was accepted to indicate a good linear correlation.

Generally MLR equations take the form of

$$\text{Dependent} = \sum_{n}^{n}\left[A_n.(\text{Independent})_n\right] + C \qquad \text{Equation 38}$$

where A is the constant associated with a particular independent variable and C is the intercept of the slope with the y-axis. The following statistics relating to the regression equation were deemed important for data interpretion: R^2, standard error of the correlation (S.E.), and mean square residual, σ^2. These were used to verify the "goodness-of-fit" of the prediction equation.

If a regression relationship did not appear to be linear from either the scatterplot of log k'_{pred} versus log k'_{expt} or residuals versus log k'_{pred}, no attempt was made to derive non-linear relationships through lack of sufficient data for meaningful curve-fitting.

5.4 Discussion of Experimental and Statistical Results

5.4.1 Substituted Benzene Test Compounds

An initial review of the experimental data alone shows that a number of the test compounds follow expected retention patterns according to their general physical properties. For instance, polar solutes such as benzoic acid and acetylsalicylic acid tend to elute faster than

more non-polar solutes such as benzonitrile which in turn generally elute faster than even more non-polar solutes such as p-phenylphenol. Retention times also tend both to decrease as the percentage of methanol in the mobile phase increases and to increase as the number of carbons in the bonded chain increases. Unfortunately many of these trends are not linearly related to a single parameter and therefore cannot be defined by simple concepts like log P or polarity, although, as would be expected in reversed-phase systems, the hydrophobic parameters log P and log D feature prominently in all of the regression equations. Some deviations do exist, for example o-hydroxybenzoic acid (salicylic acid) elutes more slowly than m- and p-hydroxybenzoic acid. Answers to such questions can only be found when the underlying process of retention relating to the structure of a solute has been determined.

In order to build up a complete picture of the retention process, the test solutes were divided into 3 groups relating to their ionisation behaviour. Solutes with $6< pK_a <8$ or those which are weak acids or bases were classed as neutral, solutes with $pK_a<6$ were classed as acidic and solutes with $pK_a>8$ were classed as basic. This allowed the effect of solute ionisation behaviour to be studied as this property will affect hydrophobicity. The behaviour of each group of compounds under the different experimental chromatographic conditions is discussed separately below.

Solute retention on cyclohexylsilica could not be predicted by the physicochemical parameters selected for the substituted benzene solutes. Although the lack of correlation was disappointing, it was not unexpected as it was thought that the phenyl or cyclohexyl phases would be orientation-dependent because of the geometry of the ligands. More encouraging correlations were achieved with the ß-blocker analogues and the characteristics of this phase will be discussed more fully in Section 5.4.2

A short-hand notation of the chromatographic conditions will be used in the following text. Ethyl-, octyl-, octadecyl-, phenyl- and cyclohexylsilica will be abbreviated to C2, C8, C18, PH and CH respectively. The number after the phase name will represent the percentage of methanol and the pH of the mobile phase respectively, e.g. log $k'_{CH,50,7.9}$ relates to the logarithm of the retention capacity of a solute in a system with a cyclohexylsilica phase, eluted with 50% methanol in water at pH 7.9.

5.4.1.1 Neutral Substituted Benzene Solutes

Compounds which are not ionised under a particular set of chromatographic conditions should follow straightforward reversed-phase retention/elution behaviour relating to both their lipophilic tendencies and an inductive term. This is indeed observed with this sub-set of solutes (Table 6, p.125). In the chromatographic systems where the bonded phase is an n-alkyl chain (except

ethylsilica (C2) at 30% methanol), the log k' value can be predicted from a hydrophobic term (log P) and an inductive term (HD) as has already been shown by others [94,96,157]. As log P is a positive variable, log k' will increase as hydrophobicity becomes more pronounced:

Benzonitrile	log P=1.56 $\log k'_{C8,30,5.5}=0.81$
p-Phenylphenol	log P=3.20 $\log k'_{C8,30,5.5}=1.68$

On the other hand, HD is a negative term, that is, log k' decreases as HD increases. This can be illustrated with the following:

Phenol	HD=1 $\log k'_{C8,30,5.5}=0.53$.
Quinol	HD=2 $\log k'_{C8,30,5.5}=-0.29$

HD is a reflection of a solute's ability to hydrogen-bond with the polar components of the mobile phase [59] so solutes with hydrogen-bonding potential are eluted faster than those which do not:

p-Cresol	HD=1 $\log k'_{C8,50,7.3}=0.68$
Toluene	HD=0 $\log k'_{C8,50,7.3}=1.34$

The effect of extending the length of an alkyl silica-bonded chain is to increase the constant relating to log P. The lipophilic contribution of the C8 phase is

greater than that of the C2 phase as shown by reduced constants for log P in the regression equations for the latter, although C18 appears to exert the same lipophilic interaction on non-polar solutes as C8 which indicates that lipophilic interaction reaches a maximum once the solute can be enveloped by an n-alkyl chain [40,45,48]. This emphasises the importance of quoting bonded phase coverage per m^2 rather than as percentage carbon by weight because although C18 certainly has a greater carbon content, the number of ligands may be less than for C8 due to the bulkiness of the octadecyl groups. Therefore a less hydrophobic environment is created and hydrophobic effects are not as large as expected [110].

The significance of the hydrogen-bonding term in the regression equation increases with the sorbent chain length, as observed by Miyake et al. [127]. This is due to the reduced C2 and C8 retention times of solutes which are only moderately hydrophobic and possess a hydrogen-bond donor group. As the solutes tend to be eluted quickly from these two phases through reduced hydrophobic interaction, they are not differentiated according to their hydrogen-bond donor capabilities. However, C18 increases the retention of these solutes by more pronounced hydrophobic attraction and thus the solutes are exposed for longer to intercalated solvent molecules. This results in a greater contribution of HD to retention.

The elution times of solutes from C2, C8, C18 and PH phases decrease as the percentage of methanol increases,

although this is not obvious from the relative contributions of hydrophobicity, hydrogen-bonding ability and size factors between different systems. What is observed is a compensation i.e. log P may increase and the effect of HD may become more pronounced as the amount of organic modifier increases, but the residual constant in the regression equation becomes more negative, which results in a net lowering of log k'. Neutral compound retention will decrease as more methanol is added to the eluent. Methanol makes the n-alkyl stationary phase swell and consequently as the amount of methanol increases, solutes that partition down into the bonded ligands are then exposed to more of the bulk mobile phase which results in a lower degree of retention.

The retention mechanism of the PH phase appears to be different to that of n-alkyl bonded phases. A shape/size factor is included in addition to log P and HD, implying that retention by this aromatic phase is shape-related. The connectivity index, χ , appears to contribute as much to retention as the hydrophobic term and therefore is important. Retention increases as the size of a solute increases because the term is positive. Thus, the more hydrophobic and bulkier the solute, the longer it will be retained by the bonded phase:

Solute	χ	Log P	Log k'
Phenol	2.13	1.46	0.30
n-Propyl-p-hydroxy-benzoate	4.45	3.04	1.36
p-Phenyl phenol	4.21	4.20	1.64

p-Phenylphenol could also be retained by $\pi-\pi$ interactions between the aromatic rings, but as no parameters were available to measure π-energy effects, this phenomenon could not be investigated further.

Neutral compounds should not be ionised under the conditions used in this work and therefore should not show appreciably different retention behaviour under different pH conditions. Yet this is observed, for example,

Benzylalcohol

$\log k'_{PH,40,5.6}=0.03$
$\log k'_{PH,40,7.6}=0.18$

This must be due to other factors besides ionisation, especially in this case as benzylalcohol is such a weak acid ($pK_a=15.40$), and one of these must be protonation of the alcohol, analogous to the formation of H_3O^+. It may be expected that hydrogen-bond acceptor ability would become important in this situation, but this is not observed under the conditions used in this work. Another influencing factor may be the different buffering salts used which were sodium acetate/acetic acid for pH5 and

phosphate in the commercial buffer tablets used for pH7. If the buffer ions interact with the bonded sorbent through ion-exchange, the character of the surface may alter. Also, slight differences in the percentage of methanol in the mobile phase resulting from measurement errors during eluent preparation could explain these observations.

Compounds which oxidise quickly when exposed to air change their retention behaviour after oxidation has occurred. One example which highlights this problem is quinol. Although care was taken to prepare the solution freshly when required, experimental retention was longer than predicted:

Retention of Quinol

Bonded Phase	Eluent Conditions	log k'_{expt}	log k'_{pred}
C8	30% methanol, pH7	0.21	0.11
PH	50% methanol, pH5	-0.32	-0.52

In summary, the retention behaviour trends exhibited by the neutral solutes reflect reversed-phase behaviour as expected. By using these observations as a reference point, the effect of ionisation on elution can be studied by examining changes in the retention mechanism with acidic test probes.

5.4.1.2 Acidic Solute Retention Behaviour

Hydrophobicity is a major influence on solute

retention in reversed-phase systems as shown by the behaviour of neutral solutes in the previous section. Changes in the properties of a solute which ultimately alter its hydrophobic character will therefore affect retention behaviour. Solutes in their ionised form become more soluble in the aqueous eluent and therefore their hydrophobicity appears smaller than predicted resulting in over-estimation of retention by prediction equations. Correcting log P to log D should compensate for partial or total ionisation of compounds under particular conditions. For acidic solutes:

$$\text{Log D} = \log P - \log\left[1+10^{\uparrow(pH-pK_a)}\right] \qquad \text{Equation 25}$$

and log D was used instead of log P in MLR analysis. This correction also takes into account the differences in degree of ionisation at different values of mobile phase pH. Data for the acidic compounds were added to that for the neutral compounds and new retention prediction equations were constructed (Table 6, p.125).

The R^2 values of the equations do not change significantly after addition of the acidic solutes to the data set and are still better then 0.8. This implies that ionisation corrections have successfully removed possible prediction errors arising from ionisation effects on lipophilicity. Log D remains highly significant for C2, C8 and PH even though its contribution is reduced in these cases. For the phases containing short n-alkyl chains the

number of hydrogen-bond donor groups becomes more significant as would be expected because the acidic solutes will have more hydrogen-bonding character than the neutral compounds. The connectivity index in regression equations for the PH phase is replaced by volume indicating that the size of an ionic solute is more important than the shape. Although it would appear that the contribution to retention from volume is less significant than that from the connectivity index, it must be noted that the volume parameter is two orders of magnitude greater than the connectivity index e.g. for benzoic acid, χ is 2.615 and volume is 104.337. Therefore, volume is just as significant for acidic solutes as connectivity appears to be for neutral compounds.

The retention mechanism for acidic solutes appears different on C18 to that for the neutral compounds on the same phase. The hydrophobicity term decreases significantly and HD remains approximately constant, but volume (V) and ß are added to the equations. Log k' increases with volume (V), but decreases as ß increases. Both V and ß are more dominant than log D. Although ß represents hydrogen-bond donor ability like HD, it is more representative of relative HD ability than just the number of donor groups. The inclusion of a volume term reflects the residence of acidic solutes near the flexible, unattached end of the stationary phase layer. As the unfixed parts of the silica-bonded chains have more freedom to rotate and coil around solute components than the middle

of the bonded phase where the non-polar solutes tend to reside, the size of solute will become a determining factor for retention on a C18 phase.

The percentage of organic modifier in the mobile phase does not appear to affect the retention behaviour of the majority of acidic solutes in any predictable way. No strong trends are observed as the amount of organic modifier increases which is expected for acidic solutes, which are exposed to more of the bulk solvent than neutral solutes, because as noted in Section 5.1, methanol exhibits similar hydrogen-bonding properties to water and thus a change in composition of the bulk mobile phase should not significantly change acidic solute solvation. An exception is C18 where the contribution of lipophilicity to retention is reduced as the percentage of methanol increases, as illustrated by acetylsalicylic acid:

Behaviour of Acetylsalicylic acid on Octadecylsilica, pH5

20% Methanol	log k'=0.38	log D=-0.37
30% Methanol	log k'=0.07	log D=-0.56
40% Methanol	log k'=-0.21	log D=-0.66
50% Methannol	log k'=-0.68	log D=-0.86

The phenyl phase appears to behave in an unusual fashion with regard to the retention behaviour of acidic and neutral solutes. Acids such as m- and p-hydroxybenzoic acids follow the expected pattern of decreasing retention with increasing percentage of methanol:

Acidic Solute Behaviour on Phenylsilica, pH 5

Solute Name	% Methanol	log k'
m-Hydroxybenzoic acid	20	-0.52
	30	-1.02
	40	-1.17
	50	-1.68
p-Hydroxybenzoic acid	20	-0.36
	30	-0.69
	40	-0.78
	50	-0.84

However, neutral solutes decrease in retention from 20 to 30% methanol, but retention increases at 40% and is lowered again at 50%:

Acidic Solute Behaviour on Phenylsilica, pH 5

Solute Name	% Methanol	log k'
Anisole	20	0.88
	30	0.20
	40	0.58
	50	0.37
Benzene	20	0.58
	30	0.20
	40	0.42
	50	0.26

One theory is that the phenyl ligands attached to silica undergo a phase transition in aqueous methanol not unlike that observed for n-alkyl chains when a critical temperture, T_o, is reached. When the amount of methanol in the system is below a critical concentration, the phenyl rings will have a lower potential energy by interacting

amongst themselves rather than extending fully into the bulk mobile phase, and as a consequence this conformation prevents neutral solutes from partitioning between them. At the critical concentration of methanol, which appears to be 40% methanol in water, the phenyl rings re-orientate and are fully solvated, creating spaces between adjacent rings into which neutral solutes can penetrate. This would explain the increase in retention times at 40% methanol as π-π interactions between the benzene solutes and the phenyl bonded rings are enhanced.

The anomolous behaviour of the homologous series of o-, m- and p-hydroxybenzoic acids was highlighted earlier. On C2 and C8 under both pH conditions the three acids were eluted within much the same time. However, on C18 and PH at pH 7, the m- and p-hydroxy acids were eluted more rapidly then o-hydroxybenzoic acid. This is due to an intramolecular interaction between the carboxylic acid group and the adjacent phenolic group in the ortho acid. As a consequence, the solute assumes a less polar configuration and effectively adopts a fused ring configuration thereby reducing hydrogen-bond donor ability, but increasing volume and hydrophobicity. Similar behaviour has been reported by Minick et al. [127]. As retention by both C18 and PH phases is dependent on size and hydrogen-bond donor ability, it becomes clear why the ortho-acid experiences increased retention. Such an anomaly does not occur for o- and p-cresol because the two substituents are a methyl and a phenolic group which do not

participate in intramolecular interactions.

5.4.1.3 Basic Solute Retention Behaviour

After correction of log P to log D using Equation 24 (p.70), addition of basic compound data to the that of neutral and basic solutes for MLR did not reduce R^2 for the three phases C2, C8 and PH, except at 30% methanol However, with the exception of C8, these equations were still significant (R^2 >0.8).

The retention times for aniline were very similar across the different sorbents at each composition of mobile phase. This suggests that aniline must be positioned within the stationary phase where the length or shape of the non-polar ligand does not affect retention i.e. near the silica surface in the region of least mobility of the bonded ligands. This behaviour is also displayed by phthalodinitrile which, incidentally, is also retained longer than aniline as a result of increased silanol attraction:

Solute	Chromatographic System	log k'_{expt}
Aniline	C18, 40%, 5.5	0.34
	C2, 30%, 5.1	0.31
Phthalodinitrile	C18, 40%, 5.5	0.45
	C2, 30%, 5.5	0.88

The predicted log k' values for aniline under the above conditions are:

Phase	% Methanol & pH	Log k'_{pred}
C18	40% Methanol, pH5.5	0.32±0.28
C2	30% Methanol, pH5.5	0.30±0.21

The variance is calculated by $\sqrt{\sigma^2}$. It can therefore be assumed that the variables selected by MLR reflect the retention mechanism in a non-polar reversed-phase system using methanol as the organic modifier.

5.4.2 General Prediction Equations for Substituted Benzene Solutes

General prediction equations were derived for each Bond Elut phase for all mobile phase compositions and with all substituted benzene test probes. These are shown in Table 6, p.128. Prediction of retention on cyclohexylsilica with substituted benzene solutes was not successful (R^2 = 0.614). It was concluded that the retention mechanisms on this phase can not be adequately investigated with the test compounds chosen as they do not seem to reflect the properties that determine retention by cyclohexylsilica. As shown further on, this phase was modelled better with the ß-blocker test solutes because they possess a relatively long side-chain which can intercalate between the ligands on the sorbent. Aliphatic model compounds may therefore be a better choice for modelling retention behaviour on CH.

The contribution from the percentage methanol in the bulk eluent reflects how an increase in the concentration

of organic modifier results in a decrease in log k' (negative relationship). The percentage of methanol was entered into the database as 20, 30, 40 or 50% and, as noted above for the volume coefficient, the coefficient of the mobile phase composition term in the MLR equation should be adjusted accordingly to allow direct comparison of its significance with that of the coefficients of the other independent variables. The coefficient for %MeOH does not change between the different bonded phases indicating that the effect exerted by the organic modifier on retention is constant for all non-polar phases.

In a previous study, Zwier found that the compositions of the eluent which is incorporated into the octyl- and octadecyl-silica stationary phases are different from the bulk mobile phase composition [63]. Less water than expected is found in the stationary layer and the relative percentage of methanol here is greater than in the bulk eluent (Table 15). The percentages of methanol in the prediction equations for C8 and C18 were replaced with those from Zwier's work. The equations were essentially

Phasename	% Methanol in bulk mobile phase	% Methanol in stationary phase layer
Octylsilica		
	20	23
	30	32
	40	40
	50	48
Octadecylsilica		
	20	50
	30	60
	40	65
	50	69

Table 15 Percentages of methanol in the bulk mobile phase and in the stationary phase layer. From reference 63.

the same in both cases with no change in R^2. Although both ways of representing the amount of methanol in the system are satisfactory, it was decided that the percentage of methanol in the bulk mobile phase would be better in this instance because it is more readily available.

The hydrophobic contribution increases from C2 to C8, but is reduced for C18 and PH. However, the reduction in hydrophobic contribution for these two phases is compensated by an increase in the size contribution from solute volume which is almost the same for PH and C18.

The hydrogen-bond donor term coefficient increases from C2 to C8 to C18 and the ß term is introduced for C18 for the reasons given in Section 5.4.1.1 and 5.4.1.2.

The volume of a substituted benzene molecule is more important than the shape according to the equation for phenylsilica which was observed when acidic and basic solutes were included in the regression analysis with neutral test solutes.

The σ values quoted for each equation indicate the variance of the predicted log k' value.

Finally, the percentage carbon in the five non-polar bonded phases (determined by elemental microanalysis) was used as a sorbent descriptor in the regression equations, keeping the percentage of methanol in the mobile phase constant. The correlation coefficients were less than 0.723. From this, it can be concluded that percentage carbon is not a suitable parameter for sorbent characterisation and that surface coverage may provide better correlation.

5.4.2 Propranolol and Fourteen Analogues

Initial attempts to elute the ß-blocker model compounds from the chosen phases, C8, CH and PH, without a secondary mobile phase additive to block silanol activity resulted in indefinite retention of the test compounds, even with 100% methanol. Using the optimised mobile phase, which contained 30:70 (v/v) methanol:water + 0.3M tri-n-butylamine at pH4, the model compounds were eluted well within the log k' limit set at 1.7 (k'=50) (Table 7, p.129). From previous work carried out by other groups, it was expected that phenylsilica would be a more selective towards aromatic solutes than octylsilica because the aromatic, non-polar phase is shape-sensitive with respect to retention [43,46,49]. Although very little work has been conducted with the cyclohexylsilica, it was thought that it would display similar retention mechanisms to phenylsilica, that is, retention would be dominated by the shape of the solute.

The regression equations for data determined with the eluent alone in Table 9, p.135 show this to be the case. The connectivity index is selected for all phases, including the n-alkyl chain. Polyaromatic solutes are not good probes to use to observe the retention mechanism of octylsilica with the physicochemical parameters available, as shown by the lack of correlation (R^2=0.632). This correlation coefficient is similar to that determined for the cyclohexylsilica phase with the substituted benzene solutes. The regression equations for PH and CH, though,

are good (R^2=0.800 and 0.870 respectively). Size and shape appear to dominate selectivity by the CH phase more than the PH when no matrix effects are present. This may be caused by the stereochemistry of the cyclohexyl rings which require correct solute geometry for the molecules to fit between the rings. In all three equations, the log k'_{pred} values are over-estimated and a large negative constant is needed to correct for this.

Matrix effects on the retention of the ß-blockers were studied with fresh plasma and plasma protein solution (PPS), the difference between them being that fibrinogen and γ-globulins are removed from PPS along with several Factors leaving only Factors 5 and 6 in this solution. The effects of the different components in each solution could be seen when only 30:70 (v/v) methanol:water was used as the eluent (no TBA) and 1ml of plasma was pre-injected onto the columns before the test solutes were applied (log k' values given in Table 8, pp.130-131). If the components of the matrices did not affect the general behaviour of the silanol group interaction, the test solutes would be retained indefinitely as they were with no TBA. However, the ß-blocker compounds were eluted with k'<50 from all three sorbents when fresh plasma was used, and from CH when PPS was employed. This must be due to components in the plasma matrices which are capable of masking silanol activity, such as the polar components of fibrinogen. The capacity factors for the solutes from CH after pretreatment with PPS were increased:

Cyclohexylsilica

β-Blocker Analogue	Pretreatment with PPS?	TBA?	log k'
12	No	Yes	0.68
12	Yes	No	0.93
15	No	Yes	0.35
15	Yes	No	0.64

With fresh plasma pretreatment, the rate of solute elution when compared to that achieved with 30% methanol, no TBA, was faster for all solutes from C8, and slower for all solutes from CH and PH:

Sorbent	β-Blocker Analogue	Pretreatment with Fresh Plasma?	TBA?	log k'
C8	12	No	Yes	0.90
	12	Yes	No	-0.09
	15	No	Yes	0.37
	15	Yes	No	-0.31
CH	12	No	Yes	0.68
	12	Yes	No	0.75
	15	No	Yes	0.35
	15	Yes	No	0.49
PH	12	No	Yes	0.56
	12	Yes	No	0.73
	15	No	Yes	0.26
	15	Yes	No	0.53

The model compounds all possess a long side-chain with a basic nitrogen centre (Figure 19, pp.87-88). The molecules will orientate in the chromatographic systems so as to enhance silanol interactions, which will be more

energetically favourable than the partitioning of a large non-polar fused ring between the short bonded ligands of the three sorbents used. Both plasma solutions contain protein components which have predominantly hydrophobic properties and therefore the ability to saturate the non-polar phases through hydrophobic interaction. The matrix products will physically block silanol interaction to a certain degree, but some active sites are still accessible to the side-chains of the solutes. The solutes are eluted faster because the sorbent is saturated by the plasma matrix which has both hydrophobic and hydrophilic character.

Addition of TBA to the eluent when the two plasma solutions are again pre-injected results in much faster elution of the solutes as depicted in Figure 24, p.132 with PPS producing the fastest elution times. Now the majority of silanol sites are deactivated by both adsorbed hydrophobic proteins and tri-n-butylamine resulting in the much faster elution of the solutes through repulsion between the hydrophobic compartments of the stationary phase and the polar side-chains on the ß-blocker model compounds. Molecules such as analogue 15 with hydrogen-bonding groups on the second ring, opposite to the side chain, are eluted much faster than solutes such as analogue 12 with a second ring substituent on the same ring as the side-chain. This is because the polar substituents of analogue 15 are in the correct orientation to enhance hydrogen-bonding to the components of the bulk mobile phase:

Log k' for CH after pretreatment with PPS	
Analogue 12	log k'=0.41
Analogue 15	log k'=0.06

The elution patterns are the same for the TBA-modified eluent alone and under the different conditions created by the addition of a matrix, for both CH and PH, but on C8 the retention mechanism appears to change when plasma matrix products are present. This could be due to the comparatively long length of the non-polar sorbent ligands. When no matrix effects are in operation, the side chains of the solutes can easily penetrate in between the octyl chains to reach the silanol sites without a large hydrophobic repulsion by plasma proteins. The naphthyl rings are exposed to the non-polar sorbent ligands and retention is enhanced by hydrophobic attraction.

Compound 6 is a very bulky molecule with a phenyl group at the end of the side-chain attached to the naphthalene ring (Figure 19, p.87) and from the plots in Figure 24, appears to be retained longer than might be expected. Two reasons are possible. PH may increase retention through $\pi - \pi$ interaction while the CH bonded ligands allow the phenyl substituent of the solute to slot in between thereby increasing the hydrophobicity of the environment that the group is exposed to. Another explaination is that instead of being repulsed by the hydrophobic proteins when the matrix components are present, the solute is actually retained by increased non-polar interaction.

The regression equations derived for the elution of the test solutes with aqueous methanol + TBA alone, and also with different plasma matrices, indicate quite clearly that the shape of the solute is a major contributor to the retention mechanism. This emphasises the importance of the length of the side-chain and its bulkiness. Hydrogen-bonding effects also appear significant as a solute will be more readily solvated by the bulk mobile phase if it possesses hydrogen-bond donor groups.

Pretreatment with the plasma matrices greatly improves the correlation of log k' with χ and hydrogen-bonding ability on C8 (R^2=0.934) and CH (R^2=0.985), and PH (R^2=0.992) when a volume term is added.

CHAPTER SIX

CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

This study confirms that substituted benzene solutes and ß-blocker model compounds follow retention rules which would be expected with the non-polar sorbents chosen for investigation. Temperature studies on ethyl- and octadecyl-silica showed that the ionisation properties and polarity of a solute will determine the depth and the position that the solutes take up in the stationary layer. Acidic solutes were observed to occupy the stationary layer nearest to the bulk mobile phase to enhance polar interaction with the polar components of the eluent, methanol and water. They were therefore more susceptible to mobility changes at the unattached end of the bonded ligands which is reflected by the addition of a volume term to the prediction equation for acidic solutes on octadecylsilica. Octadecyl chains are more flexible than ethyl and octyl chains, and hence the size of a solute will determine whether the octadecyl chains can envelope the molecule to enhance hydrophobic interaction. Neutral solutes partition further into the stationary layer in order to maximise hydrophobic bonding and therefore will not be affected by mobility effects caused by changes in temperature. Basic solutes should behave like acidic solutes, but are found to interact by ion-exchange with

exposed, active silanol groups present on the silica surface. Like the neutral solutes, the retention times of bases did not appear to be affected by temperature suggesting that they, too, occupied positions well below the surface of the bonded phase.

With these observations in mind, the prediction equations generated by multiple linear regression to correlate log k' with solute physicochemical properties can be interpreted with similar conclusions. Using substituted benzene solutes as probes, good prediction equations were initially established for the n-alkyl bonded phases and phenylsilica. All equations contained a hydrophobic term, either log P or log D, and a hydrogen-bond donor term, HD. Log k' increased with hydrophobicity and decreased as HD increased, as expected in reversed-phase chromatography. With the long-chain octadecylsilica phase, the HD contribution was large. This is because phases containing the shorter n-alkyl chains did not retain polar solutes long enough for hydrogen-bond donor effects to be significant in determining retention behaviour.

Phenylsilica proved to be interesting with neutral substituted test compounds which highlighted a phase transition dependent on the percentage of methanol present. A break in the log k' values at 40% methanol, similar to that observed at the critical phase transition temperature, T_o, suggests that phenylsilica undergoes a physical and spatial rearrangement. As the retention times of the neutral probes increased from 30 to 40% methanol, it

can be concluded that the phenyl rings rearrange so as to allow intercalation of solutes between them thus increasing hydrophobic interaction and, consequently, retention.

The regression equations developed for cyclohexylsilica with the benzene probes were poor. When the ß-blocker test compound data was used, the correlation improved dramatically and a shape term, represented by χ, was included. This indicates that either the benzene probes themselves, or the physicochemical parameters available at this moment in time, are not suitable for such a phase. As it seems that the side-chain on propranolol and its analogues provides a good probe for cyclohexylsilica, aliphatic compounds may serve as good test solutes for further investigative work on cyclohexylsilica.

Like cyclohexylsilica, the retention mechanism for phenylsilica was reflected well in the retention behaviour of the ß-blocker solutes. The size of a molecule, probably relating to the length and bulkiness of the naphthalene side chain, was an important factor. Octylsilica was not modelled well with these probes and/or physicochemical parameters.

Without a secondary mobile phase additive such as an organoamine, the ß-blocker solutes were retained by octyl-, cyclohexyl- and phenyl-silica indefinitely. Tri-n-butylamine was a good competing agent and reduced retention times dramatically when present in the eluent.

Similar masking behaviour could be achieved by pre-coating the sorbent with plasma, as shown by the elution of the solutes within the log k' limit of 1.7. The combined effect of plasma pre-coating and addition of TBA to the eluent resulted in fast elution of the ß-blocker solutes and excellent retention prediction.

The high correlation coefficients (R^2>0.800) and low variance (σ<±0.10) obtained for the phenyl- and cyclohexyl-silica phases indicate that total masking of silanol interactions provides a predictable chromatographic system in which the retention behaviour of propranolol and related compounds can be predicted to a high level of significance.

The prediction equation can be used either to predict log k' in a particular chromatographic system or, more useful for solid-phase extraction, to predict the percentage of methanol which would allow either retention (log k' >1.8) or elution (log k' < 1.6) of a solute from a particular bonded phase.

Although the retention volumes of the test compounds used in these studies could be predicted accurately with the derived prediction equations, practical application to SPE in the form of, for example, a computer simulation and prediction of optimum extraction conditions, can only be made once more parameters are available for the bonded phase and the eluent. At present, the heterogeneity of the two phases has prevented descriptors from being applicable for all bonded silica phases. Parameters are still

required to predict the contribution of silanols to retention of basic solutes, which may be related to the properties of the silica used as a substrate in silica bonded phases as well as to the ligand bonding density and clustering of active silanol sites. The mobile phase also lacks descriptors because it is a continuously-changing environment, so obviously some means of predicting the random behaviour of the components needs to be developed. A better solute parameter for inductive interactions is also needed. The excess electronic charge distribution coefficient, Δ, is good in that it indicates the part of the molecule which is most liable to take part in ionic and polar interactions, but requires further development to incorporate a geometrical or vector component relating to the physical distribution of the electronic charge excess on the analyte molecule.

A new type of bonded phase has recently become avaiable commercially. Bonded alumina, with either octadecyl or methyl substituents, provides new and interesting sorbents, ideally suited to the extraction of basic compounds as the alumina surface is basic. However, acidic solutes will be retained on this phase in the same way that basic compounds are retained by acidic silanol groups in modified silica. Thus SPE phases with the selectivity of silica bonded phases, but with no unpredictable retention behaviour have yet to be developed.

Abbreviations used in the following Appendix

MW	Molecular weight
MP	Melting point (°C)
BP	Boiling point (°C)
Ref. Index	Refractive index
Log P	Octanol/water partition coefficient
HA, HD	Number of hydrogen bond acceptors and donors respectively
χ	First order molecular connectivity index
Δ	Quantum chemical excess electronic charge distribution parameter
$M_{x,y,z}$	Moments of inertia in planes x, y and z
$P_{x,y,z}$	Principal elliptical axes in planes x,y and z

TEST COMPOUND PHYSICOCHEMICAL PARAMETERS

Benzene Test Solutes	MW	MP	BP	Density	Ref. Index	Log P
PHTHALODINITRILE	128.13	141.0				
TOLUENE-p-SULPHONIC ACID	172.20	104.0				1.25
TOLUENE-p-SULPHONAMIDE	171.21	138.5				0.85
PHENYLACETIC ACID	136.15	77.0	265.5	1.2280		1.41
ANILINE	93.13	251.0		1.0217	1.5863	0.90
BENZENE	78.11	5.5	80.1	0.8786	1.5011	2.13
p-PHENYLENE DIAMINE	108.14	140.0	267.0			
QUINOL	110.11	173.0	285.0	1.3280		0.55
2,4-DIMETHYLPHENOL	122.70	27.0	210.0	0.9650	1.5420	2.30
CUMENE	120.19	-96.0	152.4	0.8618	1.4915	3.66
ANISOLE	108.14	-37.5	155.0	0.9961	1.5179	2.11
BENZOIC ACID	122.12	122.4	249.0	1.2659	1.0749	1.87
ANTHRANILIC ACID	137.14	146.0		1.4120		1.21
SALICYLIC ACID	138.12	159.0	211.0	1.4430	1.5650	2.23
ACETYL SALICYLIC ACID	180.16	135.0				1.46
METHYL SALICYLATE	152.15	-8.0	223.3	1.1738	1.5369	2.46
PHENYLSALICYLATE	214.22	43.0	173.0	1.2614		
O-ANISIC ACID	152.15	101.0	200.0			
M-HYDROXYBENZOIC ACID	138.12	201.5		1.4730		1.50
P-AMINO BENZOIC ACID	137.14	188.0		1.3740		0.46
P-AMINO SALICYLIC ACID	153.14	150.0				0.87
p-HYDROXY BENZOIC ACID	138.12	201.5		1.4680		1.58
METHYL-4-HYDROXYBENZOATE	152.15	131.0	270.0			1.96
n-PROPYL p-HYDROXYBENZOATE	180.20	96.2		1.0630	1.5050	3.04
P-ANISIC ACID	152.15	185.0	275.0			1.96
P-TOLUIC ACID	136.15	182.0				2.27
BENZAMIDE	121.14	132.5	290.0	1.0792	1.3410	0.64
BENZONITRILE	103.12	-13.0	190.7	1.0102	1.5289	1.56
p-PHENYLPHENOL	170.21	165.0	305.0			3.20
PHENOL	94.11	43.0	181.8	1.0722	1.5509	1.46
O-AMINO PHENOL	109.13	174.0		1.3280		0.62
p-AMINOPHENOL	0.13	0.0	0.0			0.04
PHTHALIC ACID	166.13	210.0		1.5930		0.60
DIETHYLPHTHALATE	222.24		298.0	1.1175	1.5000	
DIMETHYLPHTHALATE	194.19	0.0	283.8	1.1905	1.5138	2.22
PYRIDINE	79.10	-42.0	115.5	0.9819	1.5095	0.66
TOLUENE	92.14	-95.0	110.6	0.8669	1.4961	2.69
O-CRESOL	108.14	30.9	191.0	1.0273	1.5361	1.96
P-CRESOL	108.14	34.8	201.9	1.0178	1.5312	1.94
BENZYLAMINE	107.16		185.0	0.9813	1.5401	1.09
3,4-DIHYDROXYBENZYLAMINE						
N,N-DIMETHYLBENZYLAMINE	135.21	-60.0	185.3	0.9286	1.5153	1.79
BENZYLALCOHOL	108.14	-15.3	205.4	1.0419	1.5396	1.10

Appendix I Physicochemical properties of substituted benzene solutes and propranolol analogues used as test compounds. cont./...

Benzene Test Solutes	F	π	HA	HD	HA+HD	HA-HD	pKa1	pKa2
PHTHALODINITRILE	3	-1.14	2	0	2	2	6.22	
TOLUENE-p-SULPHONIC ACID	3						-1.34	
TOLUENE-p-SULPHONAMIDE	4	-1.26	2	2	4	0	10.14	
PHENYLACETIC ACID							4.30	
ANILINE	3	-1.23	1	1	2	0	4.58	
BENZENE	3	0.00	0	0	0	0		
p-PHENYLENE DIAMINE	3	-2.46	2	2	4		2.97	
QUINOL	3	-1.34	2	2	4	0	9.91	11.56
2,4-DIMETHYLPHENOL	5	0.45	1	1	2	0	9.94	9.34
CUMENE	4	1.16	0	0	0	0		
ANISOLE	4	-0.02	1	0	1	0		
BENZOIC ACID	3	-0.32	1	1	2		4.20	
ANTHRANILIC ACID	4	-1.55	2	2	4	0	1.89	4.87
SALICYLIC ACID	3	-0.99	2	2	4	0	2.97	13.65
ACETYL SALICYLIC ACID	5	-0.96	2	1	3	1	3.38	
METHYL SALICYLATE	4	-0.68	2	1	3	1	9.87	
PHENYLSALICYLATE	6		2	1	3	1		
O-ANISIC ACID	4	-0.34	2	1	3	1	3.90	
M-HYDROXYBENZOIC ACID	3	-0.99	2	2	4	0	4.08	9.98
P-AMINO BENZOIC ACID	4	-1.55	2	2	4	0	2.41	4.85
P-AMINO SALICYLIC ACID	4	-2.22	3	3	6	0	2.05	3.66
p-HYDROXY BENZOIC ACID	3	-0.99	2	2	4	0	4.67	9.37
METHYL-4-HYDROXYBENZOATE	4	-0.68	2	1	3	1	9.07	
n-PROPYL p-HYDROXYBENZOATE	5	0.40	2	1	3	1		
P-ANISIC ACID	4	-0.34	2	1	3	1	4.48	
P-TOLUIC ACID	4	0.24	1	1	2	0	4.37	
BENZAMIDE	3	-1.49	1	1	2	0		
BENZONITRILE	3	-0.57	1	0	1	1		
p-PHENYLPHENOL	6	1.29	1	1	2	0	9.96	
PHENOL	3	-0.67	1	1	2	0	9.92	
O-AMINO PHENOL	3	-1.90	2	2	4	0	4.66	
p-AMINOPHENOL	3	-1.90	2	2	4	0	11.91	4.31
PHTHALIC ACID	3	-0.64	2	2	4	0	2.76	4.92
DIETHYLPHTHALATE	7	1.02	2	0	2	2		
DIMETHYLPHTHALATE	5	-0.02	2	0	2	2		
PYRIDINE	3						5.23	
TOLUENE	4	0.56	0	0	0	0		
O-CRESOL	4	-0.11	1	1	2	0	10.21	
P-CRESOL	4	-0.11	1	1	2	0	10.23	
BENZYLAMINE	4						9.33	
3,4-DIHYDROXYBENZYLAMINE	4						9.43	
N,N-DIMETHYLBENZYLAMINE	4						9.03	
BENZYLALCOHOL	4	-1.03	1	1	1	0	15.40	

Appendix I cont./...

Benzene Test Solutes	χ^{-188}	R_M	Vm	Δ	σ
PHTHALODINITRILE	2.775			0.278	
TOLUENE-p-SULPHONIC ACID	3.401			0.687	
TOLUENE-p-SULPHONAMIDE	3.481			0.700	
PHENYLACETIC ACID	3.046			0.722	
ANILINE	2.199	39.31	91.15	0.344	
BENZENE	2.000	34.25	88.90	0.000	
p-PHENYLENE DIAMINE	2.399			0.320	
QUINOL	2.269		82.91	0.418	-0.37
2,4-DIMETHYLPHENOL	2.462	51.86	127.15	0.422	-0.30
CUMENE	3.614	52.96	139.46	0.065	
ANISOLE	2.661	42.85	108.56	0.344	
BENZOIC ACID	2.615	6.96	95.47	0.729	
ANTHRANILIC ACID	2.750		97.12	0.745	
SALICYLIC ACID	2.723	40.22	95.72	0.731	
ACETYL SALICYLIC ACID	3.617			0.749	
METHYL SALICYLATE	3.255	52.51	129.62	0.748	
PHENYLSALICYLATE	4.666		169.83	0.763	
O-ANISIC ACID	3.150				
M-HYDROXYBENZOIC ACID	2.723			0.724	0.37
P-AMINO BENZOIC ACID	2.330		99.81	0.724	
P-AMINO SALICYLIC ACID	2.550			0.624	
p-HYDROXY BENZOIC ACID	2.723			0.723	0.45
METHYL-4-HYDROXYBENZOATE	3.025			0.721	0.45
n-PROPYL p-HYDROXYBENZOATE	4.452	65.68	169.52	0.722	
P-ANISIC ACID	4.480			0.723	
P-TOLUIC ACID	2.960			0.716	
BENZAMIDE	2.653	32.02	112.25	0.730	
BENZONITRILE	2.384	40.91	102.08	0.266	
p-PHENYLPHENOL	4.206			0.429	-0.01
PHENOL	2.134	36.22	87.77	0.442	0.00
O-AMINO PHENOL	2.410		82.18	0.459	
p-AMINOPHENOL	2.334			0.420	-0.66
PHTHALIC ACID	3.183		104.29		
DIETHYLPHTHALATE	3.030	76.49	198.87	0.730	
DIMETHYLPHTHALATE	3.236	64.01	163.12	0.726	
PYRIDINE	1.850	31.42	80.56	0.000	
TOLUENE	2.411	40.64	106.29	0.065	
O-CRESOL	2.551	42.60	105.26	0.400	-0.10
P-CRESOL	2.551	42.71	106.25	0.435	-0.17
BENZYLAMINE	2.672	44.43	109.20	0.730	
3,4-DIHYDROXYBENZYLAMINE	2.947			0.388	
N,N-DIMETHYLBENZYLAMINE	3.470	57.26	145.61	0.246	
BENZYLALCOHOL	2.580	42.20	103.79	0.409	

Appendix I cont./...

Benzene Test Solutes	Mx	My	Mz	Volume
PHTHALODINITRILE	630.715	383.212	247.503	117.905
TOLUENE-p-SULPHONIC ACID	923.006	831.093	188.548	131.375
TOLUENE-p-SULPHONAMIDE	930.711	838.760	195.969	133.750
PHENYLACETIC ACID	713.567	578.589	138.171	119.500
ANILINE	283.640	193.815	90.941	90.125
BENZENE	178.441	89.222	89.219	81.873
p-PHENYLENE DIAMINE	428.795	338.372	92.679	100.737
QUINOL	431.509	340.786	90.722	93.649
2,4-DIMETHYLPHENOL	550.660	385.895	171.152	120.125
CUMENE	537.718	421.167	153.029	130.125
ANISOLE	408.316	307.880	103.628	103.875
BENZOIC ACID	538.586	407.401	131.185	104.337
ANTHRANILIC ACID	621.431	410.239	21.036	110.000
SALICYLIC ACID	610.263	406.198	210.836	109.249
ACETYL SALICYLIC ACID	1007.915	783.634	230.670	147.125
METHYL SALICYLATE	810.493	592.870	220.816	125.750
PHENYLSALICYLATE	2060.176	1753.240	306.935	180.625
O-ANISIC ACID	769.307	425.741	346.758	124.000
M-HYDROXYBENZOIC ACID	717.746	527.505	190.241	110.337
P-AMINO BENZOIC ACID	762.103	630.317	132.945	110.625
P-AMINO SALICYLIC ACID	850.038	640.695	210.504	115.625
p-HYDROXY BENZOIC ACID	766.653	634.763	131.890	110.385
METHYL-4-HYDROXYBENZOATE	1011.933	867.100	148.026	127.625
n-PROPYL p-HYDROXYBENZOATE	1788.418	1584.000	213.998	161.625
P-ANISIC ACID	1004.245	856.501	150.935	126.750
P-TOLUIC ACID	765.791	634.544	134.441	119.500
BENZAMIDE	538.098	407.486	130.612	109.250
BENZONITRILE	420.067	330.853	89.214	100.500
p-PHENYLPHENOL	1329.880	1328.834	179.183	158.000
PHENOL	283.584	193.668	89.916	87.875
O-AMINO PHENOL	376.624	225.381	152.394	97.313
p-AMINOPHENOL	430.099	339.548	91.701	97.345
PHTHALIC ACID	837.774	466.114	373.881	123.125
DIETHYLPHTHALATE	1757.527	1160.041	827.213	198.375
DIMETHYLPHTHALATE	1156.295	729.529	648.322	164.625
PYRIDINE	171.808	86.603	85.205	78.032
TOLUENE	289.248	200.028	92.414	97.125
O-CRESOL	383.034	227.642	158.585	104.250
P-CRESOL	435.293	345.352	93.135	105.125
BENZYLAMINE	433.760	332.603	105.503	107.750
3,4-DIHYDROXYBENZYLAMINE	735.519	576.927	188.894	119.375
N,N-DIMETHYLBENZYLAMINE	742.773	628.912	149.045	140.875
BENZYLALCOHOL	427.582	346.134	106.664	104.875

Appendix I cont./...

Benzene Test Solutes	Molecular-Dipole Moment(DM)	Substituent DM	Px	Py	Pz
PHTHALODINITRILE	2.965	3.070	0.000	1.544	1.838
TOLUENE-p-SULPHONIC ACID	3.556	2.708	0.487	1.267	2.441
TOLUENE-p-SULPHONAMIDE	4.452	3.633	0.549	1.314	2.451
PHENYLACETIC ACID	0.739	1.483	0.297	1.414	2.083
ANILINE	0.545	0.354	0.175	1.348	1.757
BENZENE	0.000	0.000	0.000	1.424	1.424
p-PHENYLENE DIAMINE	0.174	0.682	0.228	1.290	2.069
QUINOL	0.937	1.500	0.000	1.353	1.838
2,4-DIMETHYLPHENOL	0.297	0.879	0.408	1.545	2.110
CUMENE	0.160	0.081	0.633	1.393	2.103
ANISOLE	0.525	0.768	0.315	1.262	2.059
BENZOIC ACID	1.645	1.390	0.000	1.358	2.014
ANTHRANILIC ACID	2.045	1.927	0.171	1.536	1.952
SALICYLIC ACID	2.220	3.491	0.212	1.418	1.886
ACETYL SALICYLIC ACID	1.956	2.783	0.380	1.343	2.668
METHYL SALICYLATE	2.157	2.100	0.289	1.422	2.468
PHENYLSALICYLATE	2.524	2.332	0.001	1.533	3.326
O-ANISIC ACID	2.347	2.188	0.289	1.807	1.939
M-HYDROXYBENZOIC ACID	1.743	2.077	0.000	1.455	2.089
P-AMINO BENZOIC ACID	2.276	1.803	0.166	1.303	2.307
P-AMINO SALICYLIC ACID	1.514	2.708	0.161	1.355	2.312
p-HYDROXY BENZOIC ACID	1.485	2.100	0.000	1.318	2.207
METHYL-4-HYDROXYBENZOATE	1.293	2.004	0.289	1.248	2.714
n-PROPYL p-HYDROXYBENZOATE	1.165	2.093	0.436	1.239	3.416
P-ANISIC ACID	1.491	2.145	0.289	1.253	2.570
P-TOLUIC ACID	1.975	1.449	0.297	1.281	2.406
BENZAMIDE	2.342	2.508	0.000	1.357	2.073
BENZONITRILE	1.798	1.579	0.000	1.368	1.774
p-PHENYLPHENOL	0.471	1.064	1.028	1.042	2.850
PHENOL	0.509	0.741	0.000	1.371	1.653
O-AMINO PHENOL	0.962	1.084	0.177	1.434	1.776
p-AMINOPHENOL	0.858	1.097	0.175	1.318	1.953
PHTHALIC ACID	0.000	0.000	0.247	1.494	1.926
DIETHYLPHTHALATE	1.258	2.464	0.731	2.172	2.767
DIMETHYLPHTHALATE	1.359	2.452	0.740	2.056	2.266
PYRIDINE	0.542		0.000	1.270	1.467
TOLUENE	0.280	0.050	0.325	1.314	1.862
O-CRESOL	0.325	0.822	0.315	1.507	1.807
P-CRESOL	0.529	0.803	0.315	1.284	2.059
BENZYLAMINE	0.303	0.260	0.349	1.316	2.061
3,4-DIHYDROXYBENZYLAMINE	0.323	1.740	0.502	1.354	2.274
N,N-DIMETHYLBENZYLAMINE	0.144	0.224	0.595	1.317	2.394
BENZYLALCOHOL	0.583	0.609	0.441	1.284	2.028

Appendix I cont./...

Benzene Test Solutes	Molecular Polarisability (Polar)	Substituent Polar.	Molecular Moment of Polar.
PHTHALODINITRILE	14.036	4.476	5.631
TOLUENE-p-SULPHONIC ACID	17.060	7.757	3.366
TOLUENE-p-SULPHONAMIDE	17.487	8.121	5.704
PHENYLACETIC ACID	14.775	4.912	0.658
ANILINE	11.909	1.892	9.736
BENZENE	10.404		0.771
p-PHENYLENE DIAMINE	13.421	3.784	0.450
QUINOL	11.713	2.286	2.956
2,4-DIMETHYLPHENOL	14.723	5.587	7.477
CUMENE	15.941	5.915	8.538
ANISOLE	12.876	2.925	0.034
BENZOIC ACID	12.935	3.138	11.230
ANTHRANILIC ACID	14.463	5.030	14.650
SALICYLIC ACID	13.652	4.281	23.460
ACETYL SALICYLIC ACID	17.436	7.859	41.360
METHYL SALICYLATE	15.484	6.055	27.300
PHENYLSALICYLATE	23.223	13.716	1.837
O-ANISIC ACID	15.484		19.770
M-HYDROXYBENZOIC ACID	13.652	4.281	9.360
P-AMINO BENZOIC ACID	14.463	5.030	7.918
P-AMINO SALICYLIC ACID	15.182	6.173	9.195
p-HYDROXY BENZOIC ACID	13.652	4.281	8.653
METHYL-4-HYDROXYBENZOATE	13.167	6.055	5.405
n-PROPYL p-HYDROXYBENZOATE	19.157	9.677	10.860
P-ANISIC ACID	15.484	6.063	5.468
P-TOLUIC ACID	14.775	5.360	6.885
BENZAMIDE	13.377	3.509	0.060
BENZONITRILE	12.203	2.238	3.764
p-PHENYLPHENOL	20.671	11.170	16.570
PHENOL	11.030	1.143	0.053
O-AMINO PHENOL	12.552	5.030	2.156
p-AMINOPHENOL	12.552	3.035	13.150
PHTHALIC ACID	15.610	6.276	0.912
DIETHYLPHTHALATE	22.929	13.432	23.420
DIMETHYLPHTHALATE	19.264	9.824	15.280
PYRIDINE	9.471		4.349
TOLUENE	12.249	2.222	0.049
O-CRESOL	12.876	3.365	0.656
P-CRESOL	12.876	3.365	2.122
BENZYLAMINE	13.756	3.737	1.424
3,4-DIHYDROXYBENZYLAMINE	15.081	4.880	2.766
N,N-DIMETHYLBENZYLAMINE	17.449	7.429	29.390
BENZYLALCOHOL	12.876	2.925	4.069

Appendix I cont./...

Benzene Test Solutes	Substituent Moment of Polar.	β
PHTHALODINITRILE	0.000	0.66
TOLUENE-p-SULPHONIC ACID	3.819	
TOLUENE-p-SULPHONAMIDE	4.957	
PHENYLACETIC ACID	3.062	0.50
ANILINE	1.585	0.41
BENZENE		0.10
p-PHENYLENE DIAMINE	3.170	0.72
QUINOL	1.552	0.56
2,4-DIMETHYLPHENOL	5.852	0.35
CUMENE	7.867	0.12
ANISOLE	3.335	0.32
BENZOIC ACID	1.265	0.40
ANTHRANILIC ACID	2.850	0.71
SALICYLIC ACID	1.718	0.63
ACETYL SALICYLIC ACID	9.192	0.70
METHYL SALICYLATE	5.662	0.62
PHENYLSALICYLATE	12.202	0.63
O-ANISIC ACID		0.62
M-HYDROXYBENZOIC ACID	2.041	0.63
P-AMINO BENZOIC ACID	2.850	0.71
P-AMINO SALICYLIC ACID	3.626	0.94
p-HYDROXY BENZOIC ACID	2.041	0.63
METHYL-4-HYDROXYBENZOATE	5.662	0.62
n-PROPYL p-HYDROXYBENZOATE	15.425	0.64
P-ANISIC ACID	4.600	0.62
P-TOLUIC ACID	3.803	0.41
BENZAMIDE	2.340	0.67
BENZONITRILE	0.000	0.38
p-PHENYLPHENOL	8.655	0.43
PHENOL	0.776	0.33
O-AMINO PHENOL	2.850	0.62
p-AMINOPHENOL	2.361	0.62
PHTHALIC ACID	2.056	0.70
DIETHYLPHTHALATE	17.658	
DIMETHYLPHTHALATE	9.771	
PYRIDINE		0.64
TOLUENE	2.538	0.11
O-CRESOL	3.314	0.35
P-CRESOL	3.314	0.35
BENZYLAMINE	4.050	0.63
3,4-DIHYDROXYBENZYLAMINE	4.856	1.09
N,N-DIMETHYLBENZYLAMINE	10.444	0.57
BENZYLALCOHOL	2.988	0.55

Appendix I cont./...

Propranolol Analogues	MW	Log D	F	HA	HD	HA+HD	HA-HD	χ	Δ
M045520	243.35	3.13	8	3	2	5	1	6.797	0.398
M045655	273.37	3.31	6	3	2	5	1	7.414	0.398
M046004	273.37	2.37	8	3	2	5	1	7.254	0.401
M047070	273.37	1.22	8	3	2	5	1	7.297	0.431
M049666	289.37	3.00	9	4	2	6	2	7.463	0.398
M051932	307.39	2.44	1	5	2	7	3	7.457	0.398
M052092	289.37	3.28	9	4	2	6	2	7.463	0.398
M052487	289.37	3.37	9	4	2	6	2	7.457	0.412
M065318	243.35	3.52	6	2	1	3	1	6.839	0.394
M081509	274.32	1.22	8	5	3	8	2	6.811	0.646
M087086	364.45	4.07	8	5	3	8	2	8.846	0.654
M109055	330.43	4.04	8	5	3	8	2	8.458	0.733
M109056	378.47	3.93	8	5	3	8	2	9.632	0.752
M115715	314.43	2.34	8	3	4	7	1	8.208	0.652
M115716	362.47	3.16	8	4	3	7	1	9.382	0.753

Propranolol Analogues	Volume	Molecular Dipole Moment(DM)	Substituent DM	Molecular Polarisability (Polar)	Substituent Polar
M045520	242.750	1.429	1.476	30.460	14.336
M045655	265.875	1.395	1.409	32.309	16.173
M046004	263.000	1.036	1.009	32.309	16.173
M047070	263.875	2.896	0.971	32.309	16.173
M049666	267.375	1.531	1.277	32.980	14.336
M051932	292.625	1.403	1.313	35.510	14.336
M052092	267.075	0.972	1.325	32.980	14.336
M052487	267.000	1.809	1.368	32.980	14.336
M065318	240.625	0.754	0.844	29.800	13.635
M081509	241.125	2.541	2.876	30.202	14.162
M087086	330.875	2.596	2.917	41.656	25.529
M109055	306.375	2.716	3.200	37.179	21.099
M109056	344.125	2.896	3.447	43.103	26.982
M115715	296.750	2.003	1.989	36.898	20.762
M115716	334.250	2.936	3.109	42.433	26.282

Appendix I cont./...

Propranolol Analogues	Molecular Moment of Polar. (MP)	Substituent MP
M045520	37.500	34.185
M045655	45.220	45.629
M046004	43.920	42.982
M047070	43.160	43.057
M049666	43.700	34.185
M051932	52.100	34.185
M052092	46.400	34.185
M052487	45.600	34.185
M065318	39.200	36.365
M081509	31.940	25.805
M087086	52.080	56.086
M109055	56.590	63.899
M109056	59.610	67.614
M115715	53.730	56.984
M115716	56.880	60.357

GLOSSARY OF TERMS USED IN MULTIPLE LINEAR REGRESSION ANALYSIS

R^2	Correlation between the dependent variable (log k') and the selected independent variables (physicochemical parameters) in the regression equation. Ideally $\|R^2\|$ should be 1 for a true linear correlation and zero if the correlation is non-linear.
Adjusted R^2	Correction of R^2 relative to the number of cases used to create the regression equation.
Standard Error (SE)	Estimate of the standard deviation of the distibutions of the dependent variables from a normal distribution curve.
Residual	The error in prediction, in this instance the difference between the experimentally determined log k' and the predicted log k'.
Mean Square σ	Sum of squares for either the regression or residuals divided by their respective degrees of freedom, DF.
DF	Number of degrees of freedom for regression or residuals.
F-ratio	Ratio of the mean square of regression to the mean square of the residuals. Should be greater than zero and should lie above a certain value according to the number of degrees of freedom that the regression and the residuals possess.
B	Coefficient of an independent variable in the regression equation. A positive B-value implies a positive correlation with the dependent variable, i.e. the independent variable increases as the dependent variable increases, and a negative B-value implies the reverse, i.e. the independent variable decreases as the dependent variable increases.
Beta	If the magnitudes of the independent variables are different because of different units, then the coefficients of the independent variables, B, are standardised to allow direct comparisons of the contribution of independent variables to the regression equation.
T-test	Indicates if the residuals for log k'_{pred} are random and tests for non-linearity.

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