A Study of the Mechanism of Hydrophilic Interaction Chromatography and its Application in the Analysis of Medical Drugs and Metabolites

A Thesis presented to

The Institute of Pharmacy and Biomedical Science

University of Strathclyde

In Partial Fulfilment

Of the Requirements for the Degree

Doctor of Philosophy

By

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2017

Acknowledgment

In this wee page I would like to express my sincere gratitude and appreciation to everyone who supported me during my journey toward this achievement. My greatest thanks go to my God for blessing me with a continuous gaudiness and patience to accomplish this study. I have wholeheartedly enjoyed the challenge of examining and researching controversial issues with the support and gaudiness of my supervisors Dr. David Watson, Dr. Oliver Sutcliffe, and Dr. Darren Edwards, who were always available to help which add a great quality to my research. A special thanks is also extended to Dr. Melvin Euerby, from the HiChrom Ltd, for providing insight and expertise that greatly assisted the research.

I dedicate my success to my beloved parents, my dad Yousef Santali and my mum Nadia Fallath, who gave me the chance, trust and heart prayers to achieve my dream. Thank you for being understanding and patient all the way through the duration of my study in the UK. In this few lines, words can't express my endless love and gratitude for you.

An extended love and deep thanks to my lovely sisters, Enas and Ebtehal and my brothers, Mohammad, Abdullah, and Sultan for their best wishes

and support. My thanks and love to my amazing granny, Aisha Hafez, my aunties, Hanan and Manal, and uncle Hafez for their constant encouragement during the entire process.

I am sincerely grateful to my friends in Glasgow, those who share me the hard time as well as the good one with their truthful love and continuous support. They make me feel home, and been a great part of my success. I heartily express my sincere thanks to Ruwida, Faten, Roua, Jonans, Riza, Banan, Jehan, Jamila , Alicia, Hanan, Salha, Afnan, Weam, Wala B., Manal.B, Zain.Z, Dema, Ikram, Gazwah, Oumima & Safa, Maryam, Lubna, Safa & Ayatyt, Nadia, Muna A., Nora, Rawnak, Amany, Tagreed, Ola, Smaher, Hewi, auntie Saeda (Maryam's mum) & auntie Aisha (Faten's mum) & Ali Gniber's family. The friendships we developed will last a lifetime.

I would also like to thank my external examiner Dr. Tony Edge, for the enjoyable discussion during my VIVA and for the stimulating feedback, which improved the quality of my PhD thesis.

Appreciation is extended to Dr. Stuart Jones, From HPLC Academy, for his inspiration which redirect my writing of the thesis toward the best. Thank you for your precious words which made me confidant with the materials.

Very special thanks go again to Dr. Darren Edwards, my internal examiner, who was abundantly helpful all the way throughout corrections and offered invaluable assistance, support and guidance.

On a personal level, I'm immensely grateful to my best friend Dr. Cornel Gavata who was supportive and always inspiring throughout my study in the UK to achieve the best. I will never forget those special people in Bath St. accommodation who accommodate me with care & love and supported me to finish my corrections, especially Angela Kennedy & Lim Putthicheat.

Very special thanks to my PT, Stephen Brodie, for sharing the space and time with all care, support and pearl of wisdom. Thank you for your inspiration and the good time we share. The friendships we developed will last a lifetime

From my entire journey I learned that: life will knock you down but it's your choice whether or not to get up, and I was luckily blessed with the right surrounding which support me to stand up again better, confident and stronger.

Finally, I would like to acknowledge the government of Saudi Arabia for the financial and academic support.

Achievements:

1- Poster presentation at HPLC 2013 congress, Netherland, Amsterdam- **June 2013**.
2- Training Course in "HPLC in Analytical GMP Laboratories", Spain, Barcelona- **May 2014**.
3- Poster presentation at ISC 2014, 30th International symposium on Chromatography, Austria, Salzburg- **September 2014**.
4- Training Course in "Compliance in Analytical Laboratories", Copenhagen, Denmark -**October 2014**.
5- HPLC Method Development Training course with **Phenomenex**, Scotland, Edinburgh- **November 2014**.
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10- Certified Quality Control Manager from the European compliance academy- **2015**.
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Abstract

Hydrophilic interaction chromatography (HILIC) has been the subject of a few excellent reviews in recent years and most focused on different factors which impact on the separation of compounds in HILIC mode including column temperature, mobile phase composition, pH, buffer type and concentration. However, there have not been as many studies focusing on the retention mechanism and selectivity in hydrophilic chromatography. In the light of recent development in HILIC stationary phases and their applications, the need to understand the mechanisms that govern the separation in HILIC, which is not purely due to partitioning, and the contribution of stationary surface became a subject of study. This thesis explored the possible mechanisms which might be involved in HILIC chromatography by comparing the retention of test probes on bare silica gel and on a type-C silica hydride phase. In addition, a comprehensive retention and selectivity study of some commercially available hydride-based stationary phases was carried out in the analysis of hydrophobic acids and bases in hydrophilic interaction chromatography (HILIC). The applications of HILIC technique were also addressed in the analysis of plasma and urinary metabolites, impurity profiling and in the separation of '*Legal high'* regioisomers.

An interest in the HILIC retention mechanism was mainly inspired by the need to understand how to develop the optimal HILIC conditions and the

selection of a stationary phase. The retention of a series of positively charged probes on silica gel column was concluded that for this class of compounds both ion-exchange and hydrophilic partitioning were involved in retention. Ion exchange interactions were minimised by increase the ionic strength of the modifier in the mobile phase. The strength of the overall HILIC mechanism was increased with silica gel surface area. The unique properties of silica-hydride phases also assessed and are still not explained in terms of how the retention mechanism of this type of stationary phase is different from the other separation materials used in chromatography. The study confirmed the usefulness of HILIC in many analytical aspects. However, the mixed-interaction mechanisms which operate on different stationary phases for different compounds are still far from understood and more investigation is required.

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List of Abbreviations

2MMC	2-methylmethcathinone
3MMC	3- methylmethcathinone
4MMC	4- methylmethcathinone
Å	Angstrom
AmAct	Ammonium Acetate
AmFrmt	Ammonium Formate
ACN	Acetonitrile
API	Active Pharmaceutical Ingredient
A_S	Peak symmetry
BTM	Benzyltrimethyl ammonium chlride
BTE	Benzytriethyl ammonium acetate
BDM	Benzyldimethylhexyl ammonium chloride
C18	Octadecyl
C4	Butyl
C8	Octyl
CD	Cyclodextrin
CN	Cyanopropyl silica
DPH	Diphenhydramine hydrochloride
DXH	Dextromethorphan

Continue List of Abbreviations

DHS	Dihydorostreptomycin
EP	European Pharmacopeia
ESI	Electrospray Ionization
ESI-MS	Electrospray Ionization-Mass Spectrometry
Exp.	Experimental
FA	Formic acid
FDA	Food & Drug Administration
GC–MS	Gas Chromatography Mass Spectrometry
h	Hours
HCl	Hydrochloric Acid
HILIC	Hydrophilic Interaction Liquid Chromatography
HPLC	High Pressure Liquid Chromatography
KV	Kilo Volt
LC-MS	Liquid Chromatography-Mass Spectrometry
m/z	Mass-to-Charge Ratio
mM	Milli-molar
µm	Micro meter
MS	Mass Spectrometry
Min.	Minutes
Mol.wt	Molecular weight

Continue List of Abbreviations

--	Not Detected
NMR	Nuclear Magnetic Resonance
nm	Nanometre
NPLC	Normal Phase Liquid Chromatography
ppm	Part per million
RPLC	Reversed Phase Liquid Chromatography
RSD	Relative Standard deviation
STR	Streptomycin
SST	System suitability test
SP	Stationary Phase
SFC	Super critical-fluid chromatography
TMAA	Tetramethylammonium acetate
TFA	Trifluoroacetic acid
T_0/ T_M	Retention of un-retained compound
R_t/t_R	Retention Time
UDC	Cholesterol phase
UPLC	Ultra-pressure liquid chromatography
UV	Ultraviolet Detector
ZIC-HILIC	Zwitterion Stationary Phase
V_0	Dead volume

Chapter 1

Introduction to Hydrophilic Chromatography

1.1 Chromatography

Chromatography is an analytical technique based on the separation of molecules due to differences in their structure and/or properties. The term chromatography was coined in the early 1900s to describe a separation technique based on the relative interaction of a solute with two phases [1]. In the chromatographic system the components are separated by distribution between two phases, the stationary phase which is immobilised on the support particles and the mobile phase that flows through the packed bed or column in a definite direction. The mobile phase could be a liquid, gas or a supercritical fluid. Liquid mobile phase containing organic solvent or a mixture of two or more solvents that is capable of dissolving the solute or mixture and carrying it through the stationary phase, while helium or nitrogen gas is usually used in Gas chromatography and carbon dioxide for Supercritical-Fluid chromatography [2]. The retention factor is the affinity of a component towards the column at given mobile phase composition, temperature and column type. The molecules in the sample will have different affinities and interactions with the stationary support, leading to separation of molecules. Sample components that display strong interactions with the stationary phase will move more slowly through the column than components with weaker interactions. Different compounds can be

separated from each other as they move through the column. Most common separation techniques are partitioning, adsorption, ion exchange and size exclusion chromatography. Chromatographic separations can be carried out using a variety of stationary phases and the selection of separation method depends on the analyte's nature, for example volatile compounds and gases employ gas chromatography, ionic compounds employ ion exchange chromatography while liquid chromatography is ideal for non-volatile compounds while in the separation of compounds according to their molecular weight (size exclusion chromatography) is used [3]. High-performance liquid chromatography (HPLC) is a quantitative and/or qualitative type of liquid chromatography used to separate and quantify compounds that have been dissolved in solution. For example, HPLC can be used to determine the amount of a component in a mixture. In HPLC (liquid chromatography), the solutes in a sample solution will interact with the stationary phase in various degrees, the differences in the interaction with the column will cause the separation. The mechanism of separation is mostly caused by two fundamentally different retention modes in chromatography; partitioning and/or adsorption whereas in size exclusion chromatography ideally there is no interaction between the analytes and the stationary phase but the separation occurs depending on the accessible pores to a molecule ***i.e.,*** the smaller the molecule, the longer time it will take to elute from the column [4]. It should be noted that

chromatography is a separation technique and can only be used for detection when coupled with a suitable detector.

1.2 Chromatographic Performance

The successful use of chromatography requires an understanding of how a separation is affected by experimental conditions. The modern set up of HPLC system is outlined in **Figure 1-1;** it comprises an eluent (mobile phase) or a mixture of eluents and a high pressure pump that drives the eluent through the system. The sample to be separated is introduced by an injector where a pre-filled sample loop is brought online by turning the injector from the load to the inject position. The column where the separation is performed is placed in the direction of the injector. As different substances emerge from the column, ideally different signals in different retention times for each substance are recorded by a detector. There is a wide variety of detectors available on the market for use in liquid chromatography, from simple absorbance detectors to tandem mass spectrometers [4] and choosing a detector is dependent on the property of samples.

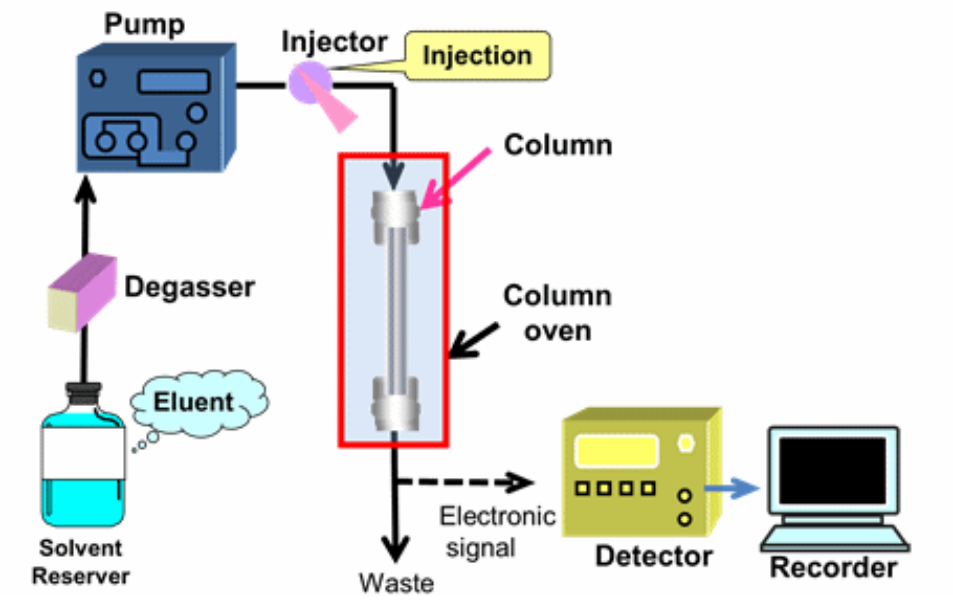


Figure 1-1: Schematic representation of a HPLC set-up.

In liquid chromatography, the separation between different compounds based on the difference in their retention on the stationary phase will determine the quality of the analysis. The analytes elute from the stationary phase surface as the mobile phase (eluent) flows through the column. The main mechanisms which are usually responsible for the retention in liquid chromatography is a direct interaction or binding between analyte and stationary phase or/and a partitioning of analytes between the bulk eluent and a water-rich stationary phase. Here the retention time is determined by the affinity of the analyte towards a particular phase; in other words, the stronger the interaction offered by the stationary phase compared to the eluent, the longer the retention time will be. These mechanisms are the usual causes of retention, however

there are other mechanisms/ forms of liquid chromatography that do not behave as liquid chromatography [5]. The time the analyte will spend in the stationary phase is called retention time (t_R) while un-retained compound that has no affinity for stationary phase will elute in solvent front and is referred to as t_0 or t_M, **Figure 1-2**. The relative retention factor, K, in [**Equation 1.1**] can be also used to describe the retention and it displays the ratio of the time a solute spends in the stationary and mobile phases [4].

$$K = \frac{t_R - t_0}{t_0} \qquad \textbf{Eq. 1.1}$$

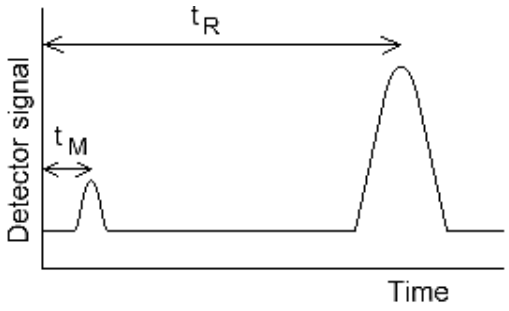


Figure 1-2: The retention time (t_R) of an analyte and un-retained compound (t_M).

In order to compare columns and analytes in different HPLC set-ups the retention factor and the number of theoretical plates of a column is used for column efficiency. A column with a high number of theoretical plates will have a narrower peak at a given retention time than a column with a lower N number and it can be calculated from **Equation 2.1**:

$$N = \alpha \left[\left(\frac{t_R}{W}\right)\right]^2 \qquad \textbf{Eq. 2.1}$$

Where w is the peak width (in the same units as t_R) at either the base-line (a=16) or at half peak height (a=5.54). The more plates per meter, the more efficient the column; in other words, it is determined by how narrow the peaks are in proportion to their retention time [4]. The term N is an indirect measure of peak width for a peak at a specific retention time. It is hypothetical concept to understand the process in a column as described in **Figure 1-3**, the plate model assumed that the chromatographic column contains a large number of separate layers, called *theoretical plates*. Separate equilibrations of the sample between the stationary and mobile phases occur in these "plates". The analyte moves down the column by transfer of equilibrated mobile phase from one plate to the next. For column comparison purposes, the number of theoretical plates per meter (N/m) is often used [4].

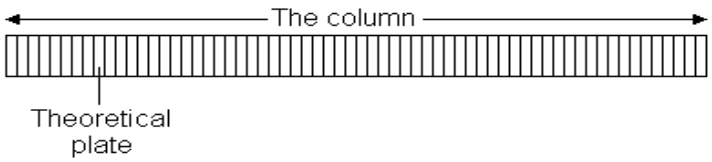


Figure 1-3: Model of theoritecal plates.

The number of plates is also an important parameter in the distillation tower; which is one of the most common liquid-liquid separation processes

in petrochemical industry. The plate in the distillation tower refers to the number of trays, or stages in the column that is dependent on the desired purity or difficulty of separation. The number of stages also determined the height of the column [6].

An accurate estimation of the void volume (V_0) is important for accurate determination of theoretical descriptions and retention factor. The void volume of well packed column is equivalent or equal to approximately 60% of the total column volume [7] and it is possible to estimate the value of V_0 from **Equation 3.1**:

$$V_0 = t_0 \times f \qquad \textbf{Eq. 3.1}$$

Where;

t_0 is the un-retained peak time

f is the column flow rate

In the ideal world, all chromatographic peaks would be symmetrical or Gaussian, however, due to the effect of instrument dead volume, adsorptive effects of the stationary phase and the quality of the column packing, peaks may show tailing or fronting behaviour. Tailing describes a peak whose tail portion distance (B) in the chromatogram is wider than distance (A) as illustrated in the **Figure 1-4** ,however, when (A) part is drawn out while (B) is steepened this will produce a fronting peak. The

ratio of A/B can be used to measure the asymmetry of the peak; the closer this value to 1 the better the peak shape is [7].

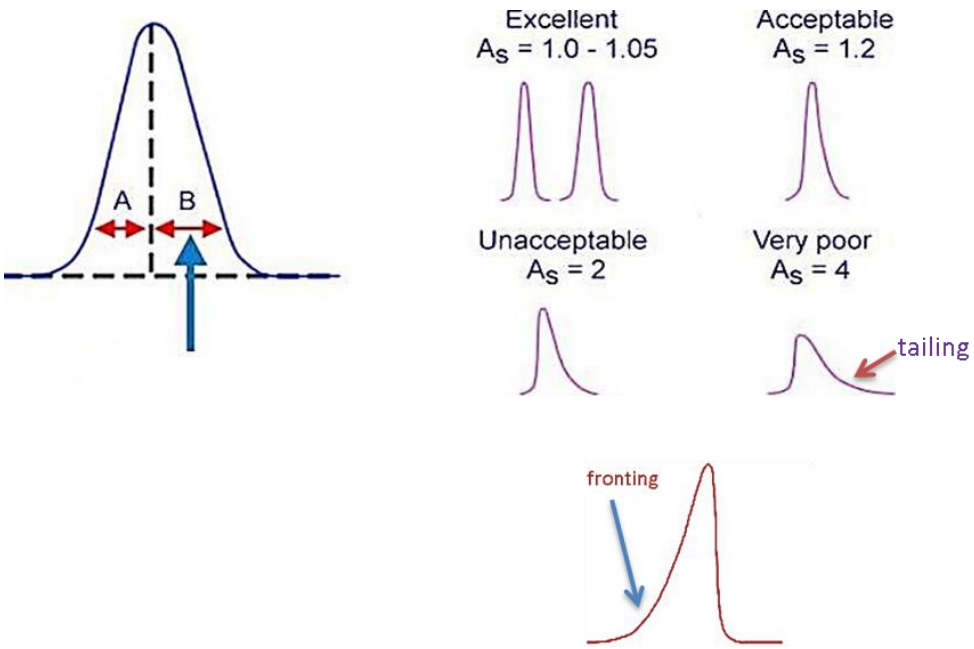


Figure 1-4: The asymmetry of peak shape.

1.2.1 Band Broadening and Column Efficiency

Column efficiency is affected by the amount of band broadening that occurs as the sample passes through the column. The analyte takes a certain amount of time to equilibrate between the stationary and mobile phase. If the velocity of the mobile phase is high, and the analyte has a strong affinity for the stationary phase, then the analyte in the mobile phase will move ahead of the analyte in the stationary phase. The result of

this effect is band broadening which will produce a poor separation and since the aim of chromatography is to resolve the peaks of compounds in a mixture, the most important equation to chromatographer is the resolution (**Equation 4.1**) between two peaks 1 and 2. The higher the resolution is the less the overlap between two peaks.

$$R = 1.18\left(\frac{t_{R2} - t_{R1}}{W_{h1} + W_{h2}}\right) = \frac{\sqrt{N}_2}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{K_2}{K_2 + 1}\right) \quad \textbf{Eq.4.1}$$

Efficiency Selectivity Retention

Where;

t_{R1} is the retention time of the first peak.

t_{R2} the retention time of the second peak.

W_{h1} the peak width at half height of peak 1 and $\mathbf{W_{h2}}$ is the width of the second peak at half height (both widths in unit of time).

K_2 and N_2 is the retention factor and the plate number of the second peak.

The factor that has the biggest and direct effect on resolution is the column selectivity (α), which represents the separation power of particular adsorbent to the mixture of this particular component. This might governed by the chemistry of the column and mobile phase. Thus, selectivity will impact the efficiency of the column [5]. Selectivity can be expressed as in **Equation 5.1**:

$$\alpha = \frac{K_1}{K_2} \qquad \textbf{Eq. 5.1}$$

Where; K is the retention factor of compounds 1 and 2.

The efficiency of the column can be affected by the plate height (H) as shown in **Equation 6.1** when L is the length of the column:

$$N = \frac{L}{H} \qquad \textbf{Eq. 6.1}$$

The plate height, H, describes the extent of peak dispersion, caused by mixing the analyte with the mobile phase. According to **Equation 6.1**, the smaller the value of H the higher the efficiency will be and one approach to reduce the plate height is to reduce the mobile phase velocity [5] also to minimise the column diameter which is in direct relation to plate height, **Equation 7.1**:

$$H_p = 2\lambda\, d_\rho \qquad \textbf{Eq. 7.1}$$

Where;

H_p is the Height equivalent to Theoretical Plate

d_p is the average particle diameter

λ is constant, independent factor specific to the quality of the column packing, which is almost close to 1

1.2.2 **The Van Deemter Equation**

An adequate separation between components is highly required in chromatography so each compound can be collected in its purest form. In order to achieve the desired separation between compounds, peaks should be narrow as possible. Ideal separation can be achieved when the resolution between two peaks ≥1.5. The interference of poor peak shape will reduce this value. A loss in peak efficiency can be observed as a wider analyte band, and therefore, the three terms A, B and C of the van Deemter equation (**Equation 8.1**) can be viewed as factors that contribute to band broadening [4].

$$HETP = A + \frac{B}{\mu} + (C_s + C_m).\mu \qquad \textbf{Eq. 8.1}$$

- HETP = height equivalent to a theoretical plate, a measure of the resolving power of the column [m].
- A = Eddy-diffusion parameter caused by un-ideal packing [m], particle size and particle size distribution.
- B = diffusion coefficient of the eluting particles in the longitudinal direction, resulting in dispersion [$m^2\ s^{-1}$].
- C = mass transfer coefficient of the analyte between mobile (m) and stationary phase (s).
- μ = Linear velocity [$m\ s^{-1}$].

To control the band broadening effects, the van Deemter parameters (**Equation 8.1**) should be minimized. The A term, **Figure 1-5**, is determined by a phenomenon called Eddy Diffusion. It describes the diffusion of molecules through column particles and some paths will be longer than others. The particles that find the shortest path around the column will be eluted more quickly than those that travel a longer way which leads to the broadening of the band. The flow rate of the mobile phase has a negligible effect on the A term but the column packing, particle size and particle size distribution could affect the diffusion as it determines the movement of the solute.

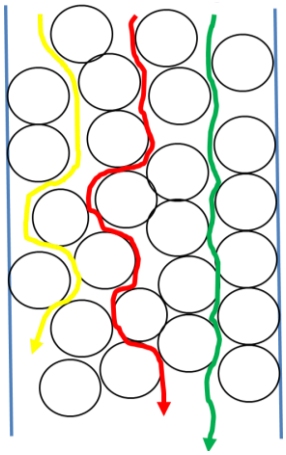


Figure 1-5: Different pathways by analyte's molecules cause term A.

The B term in **Figure 1-6** is called the longitudinal diffusion and relates to the movement of an analyte molecule outward from the center to the edges of its band. At very low flow rates, the normal diffusion rates in the mobile phase become appreciable with respect to the flow rate. As the

solute passes through the column, the random diffusion is superimposed upon its normal movement, which results in band broadening. The term B has a much larger effect at low mobile phase velocity (flow), thus higher column velocity will limit this distribution keeping the band tighter.

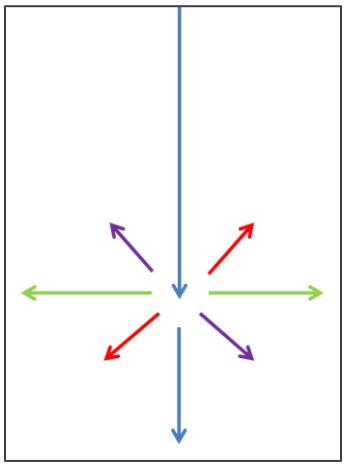


Figure 1-6: Diffusion of solutes caused by the B term.

The packing material of a stationary phase is porous to allow a very large surface area for separation to occur. However, the solvent enters inside the pores of the stationary phase and it remains stagnant relative to the normal flow through the column. This will give arise to term C, **Figure 1-7**, also referred to as the resistance to mass transfer. As the analyte moves down the column in the bulk mobile phase, it takes a certain amount of time for the analyte to equilibrate between the stationary and mobile phase. It has to travel through the stagnant mobile phase contained in pores before it can interact with the surface; a process that takes a finite amount of time. This gives a concentration gradient between the bulk and

the surface. Effectively it takes a finite amount of time for the analyte to partition into the stationary phase and back out, all the while the mobile phase is moving. This delay in returning to the mobile phase flow broadens the band of material passing down the column. If the velocity of the mobile phase is high, and the analyte has a strong affinity for the stationary phase, then the analyte in the mobile phase will move ahead of the analyte in the stationary phase causing more band broadening. Therefore, it is necessary to minimise equilibration time for the analyte molecules between the phases. This can be achieved by reducing the particle size (diameter) of the packing material to make the pores as shallow as possible.

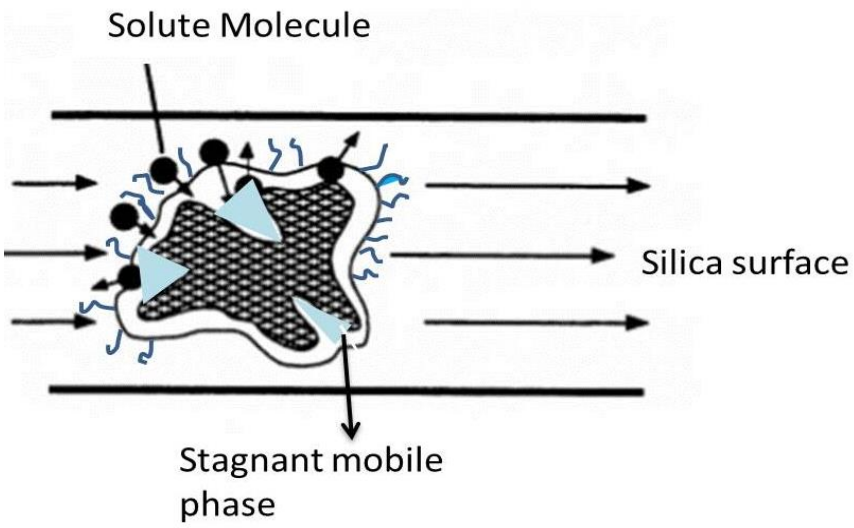


Figure 1-7: Term “C “the diffusion of analyte into and out of the stagnant mobile phase in the silica pore.

As the efficiency of the column is dependent on the flow linear velocity, the next section will discuss the effects of flow velocity in terms of the broadening processes. At low flow rates, the diffusion rate of the solute approaches the flow velocity, which results in poor efficiency. Whilst at higher flow rates the contributions from the other factors of band broadening become significant. **Figure 1-8** displays the first plot by van Deemter; it shows that at high and low flow rates the efficiency will be poor, however in between an optimum flow corresponds to a maximum efficiency [8].

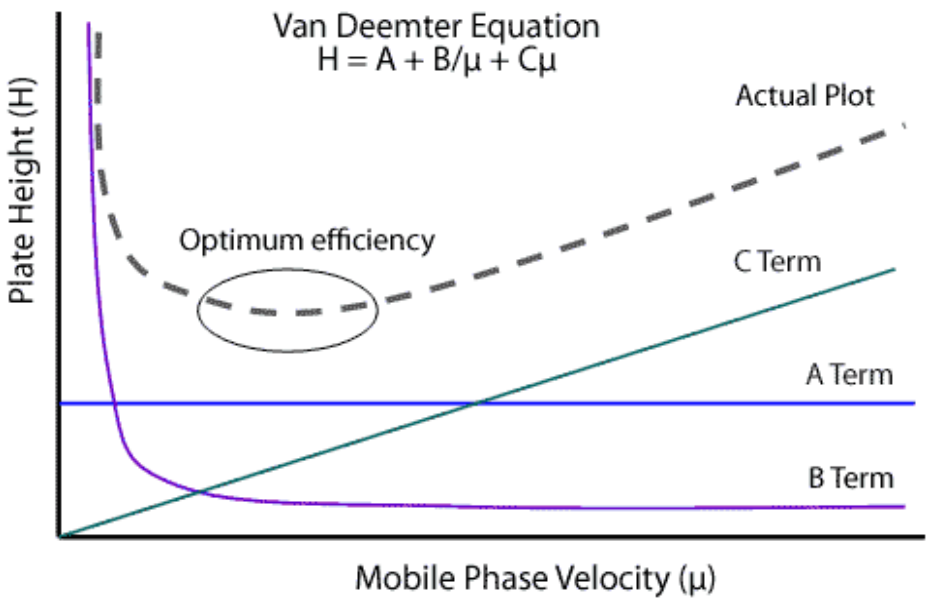


Figure 1-8: The van Deemter Equation describes the relationship between column flow rate and peak efficiency, referred to band broadning.

1.3 Columns Packing Material and Particles Size

Silica gel and its derivatised forms are the most commonly used column packing materials in HPLC. Therefore, the quality criteria of silica gel for a chromatographic column merits attention. The distribution of the packing materials at the top and bottom of the column has influence on the column efficiency. A broader mass distribution yields to a decrease in column efficiency, suggesting that the packing material in HPLC is potentially useful for column quality control. In addition, the uniformity of particle size is the key parameter for column efficiency of the liquid chromatography [5].

The so-called Type B silica has low metal content thus due to its purity it has become the standard and has been the basis of the most commonly used material for silica-based analytical HPLC packing materials since the early 1990s [3]. The high content of metals in silica gel (Type A) causes unwanted interactions with basic compounds. These metals will increase the acidity of residual silanols by withdrawing electrons from the adjacent silanos, producing undesirable tailing with basic solutes and thus, poor separation [5].

The particle size of any stationary phase is another important factor to consider in terms of band broadening. It has been reported that smaller column particles offer advantages to both efficiency and resolution [9]. A reduction in particle size improves both inter and intra-particle mass transfer. In a porous particle, solutes transfer from the moving mobile

phase outside of the particles into the stagnant mobile phase within the pores in order to interact with the stationary phase. Following this interaction, the solute molecule must diffuse out of the particle and continue its journey down the column. Such a mass transfer occurs many thousands or even millions of times during the differential separation processes while the solute is eluted from the column [8]. The use of smaller particles shortens the path length of this diffusion process, improves mass transfer, and provides better efficiency. Manufacturers can now produce small diameter particles with small particle size down to 1.5 µm average diameter, although 3–3.5 µm and 5 µm particles are still the standard size [8]. Although smaller particles will improve the efficiency, it will also result in back pressure, **Equation 9.1**.

$$\Delta \boldsymbol{P} = \frac{\mu . \boldsymbol{f} . \boldsymbol{L}}{\theta . d_{p^2}} \qquad \textbf{Eq. 9.1}$$

ΔP =pressure, μ= fluid viscosity,

L = column length, f=flow arte,

θ =particle diameter, d_p= particle size

1.4 Separation Techniques

1.4.1 Normal phases Liquid chromatography (NPLC)

Normal-phase liquid chromatography (NPLC) is a technique that uses columns packed with polar stationary phases combined with nonpolar or moderately-polar mobile phases to separate the components of mixtures. Some examples of the stationary phases used in NPLC are unmodified silica gel or silica gel, which has diol, cyano-alkyl, amide-alkyl or amino-alkyl groups bounded to it. The rate at which individual solutes migrate through NPLC columns is primarily a function of their polarity. Less polar solutes move the fastest and therefore exit the column and are detected first, followed by solutes of increasing polarity, which move more slowly. The composition of the mobile phase in NPLC is usually an organic solvent mixture composed of a very non-polar solvent mixed with a more polar solvent such as hexane/isopropanol or hexane/ethyl acetate. However, polarity can sometimes play a secondary role relative to a solute's ability to experience a specific interaction with active sites on the stationary phase surface. The uniqueness of these specific solute–stationary phase interactions gives NPLC advantages over the more widely practised reversed-phase liquid chromatography (RPLC) technique.

However, NPLC has a number of pros that limit its application such as the low solubility of polar analytes in the non-polar mobile phase in addition it is difficult to ionise compounds in a non-polar mobile phase which interfere

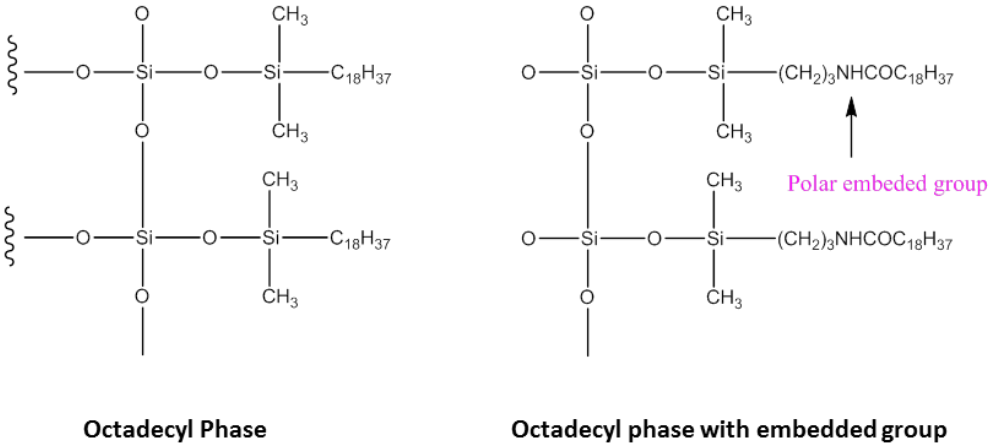
with an electrospray mass spectrometer. Tailing, fronting and shifting of retention time is one of the disadvantages which can occur as a result of the slow equilibration with the stationary phase that gives a non-linear isotherm in addition to the stability issues which relate to some of the polar stationary phases. The major problem with NPLC relates to controlling the amount of water in the mobile phase. For example, the solubility of water in hexane is nearly 0.2 % under LC conditions. Any changes in the water content of the mobile phase can cause changes in peak shape and retention. Also, in sensitive cases, a change in the humidity of the laboratory can change the chromatography. Another drawback is that it is difficult to apply NPLC technique to aqueous samples because most environmental and biological compounds are water-soluble and samples can contain considerable amounts of water [8, 9]. However, NPLC is preferred for samples with limited solubility in water and it is useful technique to separate compounds that differ in the number or character of functional groups in particular for isomer separation. In 1970, reversed phases appeared which had the opposite polarity to NPLC and therefore offer a different separation mechanism [8, 9].

1.4.2 Reversed phase liquid chromatography (RPLC)

Reversed phase chromatography is an adsorptive process, which relies on a partitioning mechanism to effect separation, in which the solute molecules

partition between the mobile phase and the stationary phase. The distribution of the solute between the two phases depends on the chemistry of stationary phase, the hydrophobicity of the solute and the composition of the mobile phase. RPLC typically utilizes nonpolar stationary phases and aqueous-based polar mobile phases, the elution order of solutes in a mixture is related to their hydrophobicity; the more hydrophobic the solute is, the strong affinity it will have towards the non-polar stationary phase while polar solutes move faster and appear before less polar solutes. RPLC is useful for separating mixtures in which components differ in molecular weight and/or water solubility. Initially, experimental conditions are designed to favour adsorption of the solute from the mobile phase onto the stationary phase. Subsequently, the mobile phase composition is modified to favour desorption of the solute from the stationary phase back into the mobile phase. Reversed phase columns include octadecyl (C18), octyl (C8) and butyl (C4) where alkyl chains are bound to silica. In order to achieve retention for the low and intermediate polar compounds, varying mixtures of water and organic solvent can be used. However, this approach was not useful for highly polar compounds and some of problems have appeared. For example, poor wetting and shrinking of the ligand on the stationary phases in the presence of high amount of water. Consequently, a serious change on the surface properties might occur, which led to non-reproducible results and poor efficiency. To overcome the wettability problems of the reversed

phases, polar endcapping or embedded groups were used to reduce the collapsing factor of the non-polar stationary phase in the presence of 100% of aqueous mobile phase, **Figure 1-9**. However, the retention efficiency of intermediate and highly polar compounds did not show much improvement [9].



Octadecyl Phase **Octadecyl phase with embedded group**

Figure 1-9: Octadecyl phases with and without polar embedded groups.

The retention of highly polar compounds was difficult on RP stationary phases as polar analytes have a strong affinity for the aqueous mobile phase and will elute in the void volume. To retain charged analytes and avoid void-volume elution for polar charged compounds, ion-pair reagents such as alkyl sulfonates for cations or tetrabutylammonium for anions were used which can offer an ion-exchange mechanism with reversed phase chromatography. Although this technique is economical and it produces

desirable separation, it is not compatible with mass spectrometry because of the presence of an in-volatile modifier. In addition, different ion pairings result in a complex retention mechanism where the slow equilibration time of the stationary phase with ion pair reagent may lead to a gradual drift in retention times. To improve the retention of neutral and highly polar compounds in RP columns, a derivatisation method can be used which involves addition of derivatisation regents to convert one or more polar functional groups to a non-polar group in the analyte thus producing hydrophobic properties to promote retention. However, side products may appear if more than one polar group is derivatised and some polar groups do not react with the derivatisation reagent therefore derivatisation could be time consuming and not always quantitative [9].

1.4.3 Hydrophilic interaction liquid chromatography (HILIC)

Hydrophilic interaction liquid chromatography (HILIC) is a technique that has become increasingly popular for the separation of hydrophilic and ionisable compounds, which are difficult to separate by reversed-phase chromatography (RP) due to their low retention. HILIC occurs on a polar stationary phase such as a bare silica or a polar bonded phase when it is used together with an eluent that contains at least 2.5% water and >60% of an organic solvent such as acetonitrile (ACN) [10]. The acronym HILIC was first introduced by Alpert in 1990 [11], the number of publications on HILIC

has noticeably increased since 2003, as outlined in the comprehensive review by Hemström and Irgum [12]. Like NP-LC, HILIC employs traditional polar stationary phases such as silica, amino or cyano, but the mobile phase used is similar to those employed in the RP-LC mode. This technique can be successfully used to analyse charged substances, as in ion chromatography (IC) [13 - 16].

In HILIC a high organic content mobile phase is employed, it shows better sensitivity in mass spectrometry and also good retention for polar ionic compounds. Hydrophilic interaction liquid chromatography has established itself as the separation mode of choice for uncharged highly hydrophilic and amphiphilic, (molecules having both hydrophilic and hydrophobic parts), compounds that are too polar to be well retained in RP-LC but have insufficient charge to allow effective electrostatic retention in ion-exchange chromatography. Meanwhile, HILIC separation is attracting a lot of interest since it solves many previously difficult separation problems, such as the separation of small organic acids, basic drugs, and many other neutral and charged substances. It has been successfully applied to the analysis of carbohydrates [17, 18], peptides [11, 13, 19, 20], polar pharmaceuticals [14, 16, 21] and contaminants in food [22].

1.5 The Mechanism of separation in Hydrophilic Interaction Liquid Chromatography (HILIC)

Although HILIC has been widely applied, the mechanisms of retention for this chromatographic mode are still debated. The most accepted concept stated that retention is caused by surface adsorption [12, 23, 24], hydrophilic partitioning between the mobile phase and the water-rich layer that is established on the surface of stationary phase when high organic content is employed in the mobile phase [12, 23, 24] and/or electrostatic interactions with charges on the stationary phase [23] as described in **Figure 1-10**. Indeed, the presence of a water-rich adsorbed layer on the surface of HILIC stationary phases when acetonitrile-rich eluents (roughly 80-90% v/v) are employed has been demonstrated by means of different analytical techniques, such as the analysis of the elution times of water insoluble samples [25]. To understand the HILIC retention and separation, the polarity and ionisation of both analyte and stationary phase need to be taken into consideration. A retention mechanism exclusively based on partition cannot explain many experimental findings such as the different in selectivity of HILIC phases that have been observed in HILIC separations.

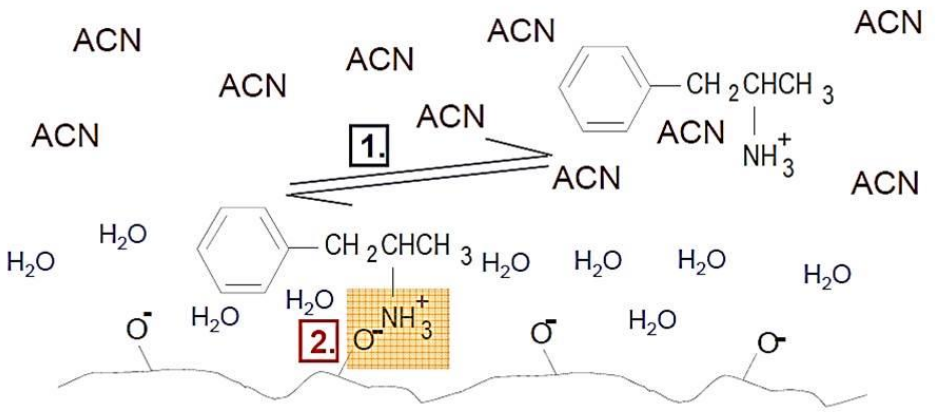


Figure 1-10: The concept of HILIC Mechanism, [1] partitioning in and out of adsorbed water layer and [2] ion exchange interaction with silanols.

1.5.1 Contribution of Ionic interactions and Adsorption to Partitioning in HILIC

As the polarity of solutes is the main factor that impacts on HILIC retention, there have been many attempts made to correlate the retention with physical descriptors of this property. The partition coefficient or log P values represent the log of a solute when it is distributed between an aqueous phase and *n*-octanol, which can be written as:

$$LogP = log\left(\frac{C_0}{C_w}\right) \qquad \textbf{Eq. 10.1}$$

Where C_0 is the concentration of the compound in octanol, and C_w is the concentration of the compound in water. Strictly, log P refers to the distribution of the non-ionised form of compounds. However, the distribution coefficient "Log D" refers to the equilibrium concentration

ratio of a given compound in its ionised and unionised forms between octanol and water and this can be used as an alternative for log P. The use for log D value requires knowledge of the pKa of the compound to calculate its ionisation at a particular pH.

Kadar *et al* [26] attempted to predict the suitability of some mono- or polyprotic acids and bases for HILIC analysis over the pH range of 0–14 using log D values that were produced by the ACD (Advanced Chemistry Development) calculation program. As the majority of active pharmaceutical ingredients are basic amines that will be protonated under acidic conditions, pH 3 was chosen to test the hypothesis that a relationship exists between the analyte's retention factor, k, and its log D. A partially immobilised water layer was assumed to exist on the phase at pH 3. Because of the large concentrations of ACN which are utilised in typical HILIC separations, possible differences in the pH values in water (w/w pH) and in the aqueous-organic mixture (w/s pH) could be observed, however, due to the limited data related to pK_a values in aqueous–organic mixtures, the authors decided to use the pH and pK_a in water. In this work, a bare silica column was used to assess the retention factor of 30 pharmaceutical compounds. The mobile phase consisted of 85%, 90%, or 95% ACN with 10 mM ammonium formate buffer at pH 3.0.

A non-linear correlation was obtained for log k versus log D at 85%, 90%, and 95% ACN. It was initially assumed that partition is the only retention mechanism on the bare silica column. However, the results indicated secondary interactions in addition to partitioning. If electrostatic interactions are involved between negatively charged silanols and positively charged basic compounds, it would be expected that basic probes would have longer retention than expected from a pure partitioning mechanism. Conversely, charged acidic compounds will exhibit less retention due to electrostatic repulsion thus would retain less than expected from a pure partitioning mechanism. Indeed, the study showed that the predicted retention factor, k, from log D values of several compounds at pH 3.0 was significantly less than the experimentally measured retention. Although an accurate prediction of k could not be made due to these secondary interactions, the authors concluded that there is a direct correlation between a compound's HILIC retention and its distribution ratio. This work was based on Alpert's theory [11] which explained HILIC separations as being due to a partitioning mechanism. However, the conclusion of this particular study suggested a contribution of secondary interactions to the retention in HILIC mode.

The retention characteristics of small polar solutes that were problematic in the RP chromatography including salicylic acid and its derivatives and different nucleosides were studied by Guo and Gaiki [16] in hydrophilic chromatography (HILIC) using polar silica-based stationary phases (amide,

amino, silica, and sulfobetaine, a zwitterionic phase contains a quaternary amine and sulfonic acid groups). The mobile phase consisted of ACN–water (85:15 v/v) with 20 mM ammonium acetate buffer. The acids were strongly retained on the amino column possibly due to the ionic interactions between positively charged groups on the amino surface and negatively charged acids while the neutral amide column provided a weak retention. The elution order on the bare silica column was similar to that on the amino column whereas the zwitterionic column showed the reverse of that on the amino column. Different selectivity and retentivity was also observed for the nucleosides on the tested columns under the same conditions indicating that specific interactions between the solutes and the surface functional groups is operating under a pure partition mode which confirms that the mechanism of separation in HILIC is complex and that the stationary phase gives a considerable electrostatic contribution to retention [16]. This finding was also demonstrated by other studies [26-30] which showed that the presence of functional groups on the surface contribute somehow to the selectivity in HILIC, supporting the hypothesis of a multimodal retention process in HILIC.

The cause of retention is thought to be either partitioning of solutes, between the water layer on the stationary surface and the bulk mobile phase, or adsorption via processes such as hydrogen bonding, dipole–dipole interactions or electrostatic forces between ionised silanols on silica materials,(or charged ligands on other types of columns), and ionised

solutes. In 1941, Martin and Synge confirmed the mechanism to be partitioning on a bare silica stationary phase by measuring the partition coefficient log P of amino acid derivatives, acetylproline and acetylphenylalanine, at low water content in the mobile phase (3.5:10) water/chloroform [31].

In 2008, McCally and Neue [25] confirmed the formation of water rich layer on the stationary phase surface on a bare silica stationary phase by observing the retention behaviour of un-retained benzene and toluene probes which are void volume markers in HILIC using a mobile phase containing a low amount of water. **Figure 1-11** shows a decrease in retention of these solutes as the water concentration in an ACN–water mobile phase was increased from 0% to about 30% v/v due to the low solubility of the hydrophobic test probes in water. However, when the water fraction increased above 30% a significant increase of retention can be seen for the hydrophobic compounds. The authors explained that this behaviour is due to the increase in the thickness of water layer as the water content in the mobile phase increased. After the water content in the mobile phase exceeds 30%, the difference in the polarity between the mobile phase and the stationary water layer has decreased to a point that allows benzene to partition into the water layer and adsorb on the surface of the silica via hydrophobic interaction with the hydrophobic siloxane bonds [25].

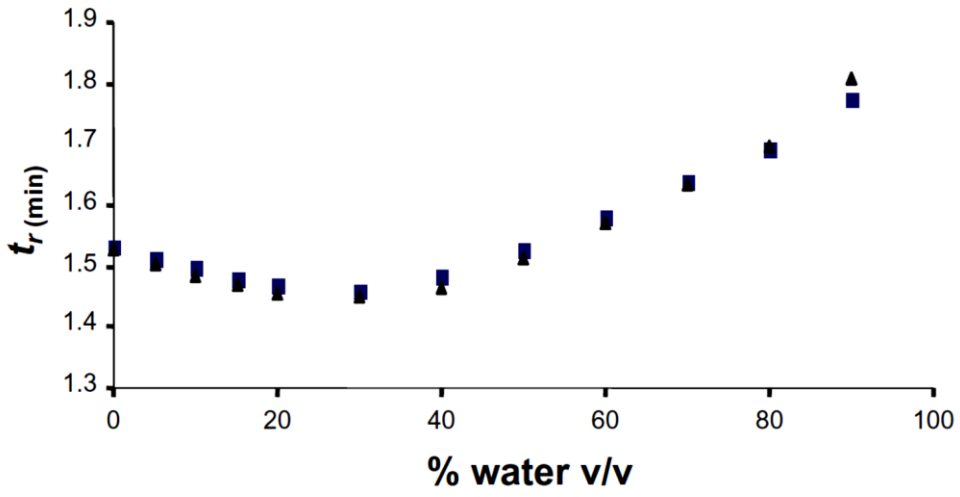


Figure 1-11: Retention time on bare silica phase for benzene (■) and toluene (▲) as a function of water content of aqueous ACN mobile phase [25].

In 2010, McCalley *et al.* [32] studied the retention mechanism of different strong basic pK_a > 9, strong acidic pK_a < 2 and neutral compounds on a silica, diol, amide, diol, sulfobetaine-zwitterionic and mixed mode stationary phases. The feature of the mixed mode phase is that it exhibits both hydrophilic and reversed-phase characteristics due to the presence of long carbon chain with a diol group. The study utilised either 85% ACN or 95% ACN in a mobile phase containing 5 mM ammonium formate at pH 3.0 to ionise the test probes while supressing the ionisation of silanol groups on the silica surface which will block the electrostatic interactions with charged analytes. In general, acidic probes exhibited weak retention on all tested columns; however amide, zwitterionic and especially diol phases showed a reasonable retention for acids. Despite the fact that the ionisation of solutes should increase their polarity and thus their retention by the strong partitioning into the aqueous layer, acidic probes showed a

weak retention and eluted near the void volume on the bare silica column which was the most retentive for basic solutes over other phases, suggesting an electrostatic interaction in addition to HILIC. At higher aqueous buffer concentrations (>15%), selectivity differences were observed on the tested mixed-mode columns where benzylamine retained longer than procainamide. However, on the bare silica column a reversed order of elution was observed, and co-elution on the amide column. At 5% water content in the mobile phase, the zwitterion column eluted the hydrophilic procainamide considerably before benzylamine. In all tested columns, the hydrophilic bases eluted after the hydrophobic bases nortriptyline and diphenhydramine as expected in HILIC, apart from the mixed mode phase, which strongly retained the hydrophobic base, nortriptyline, more than the hydrophilic ones at higher water content of 15%. The behaviour in the mixed mode phase can be attributed to some RP interactions with the hydrophobic alkyl chains in this phase when the water concentration is increased. Whereas the high retentivity of un-modified silica phase for basic compounds under HILIC could be due to the very large surface area of the silica column, 400 m^2/g, which will increase the volume of water associated with the stationary phase and/or the ionisation of silanol groups which can attract the ionised bases electrostatically and cause a strong retention on the silica surface while the repulsion will occur with acidic probes which might explain the poor retention of acidic solutes. The appreciable retention for all solutes on the

neutral phase diol was explained by the author, presumably due to the low acidity of starting material silica and/or the sufficient coverage of silanols by the bonded layer. It was concluded that the mechanism of separation in HILIC is complex, and the nature of the stationary phase gives a considerable contribution to retention as observed in the study that different columns offered unique selectivity even with the same mobile phase, and partitioning alone cannot satisfactorily explain the chromatographic behaviour of analytes under HILIC conditions.

As mainly bonded stationary phases based on silica gel materials, ion exchange can give a variable contribution to the retention of ionised solutes on different phases. Therefore, McCalley *et al.* [32] explored the retention behaviour of acidic, basic and neutral solutes at different ammonium formate buffer concentrations from 2-10 mM in the mobile phase at constant organic content of 90% ACN at pH 3.0. The analysis was run on amide, diol, mixed mode diol, silica and zwitterionic stationary phases. It was observed that as the buffer concentration increases, the retention of bases on negatively charged column sites is reduced, while the repulsion of acidic solutes from the same sites is reduced, thus the retention of acidic solutes is increased. **Figure 1-12** displays log k plots of the retention *vs.* the counter ion concentration in the mobile phase. The plot showed curved lines for the ionisable basic compounds with all tested stationary phases but a straight line for the neutral compounds. This observation confirmed that ion exchange interaction is of considerable

importance in the separation mechanism of ionisable compounds in HILIC [32].

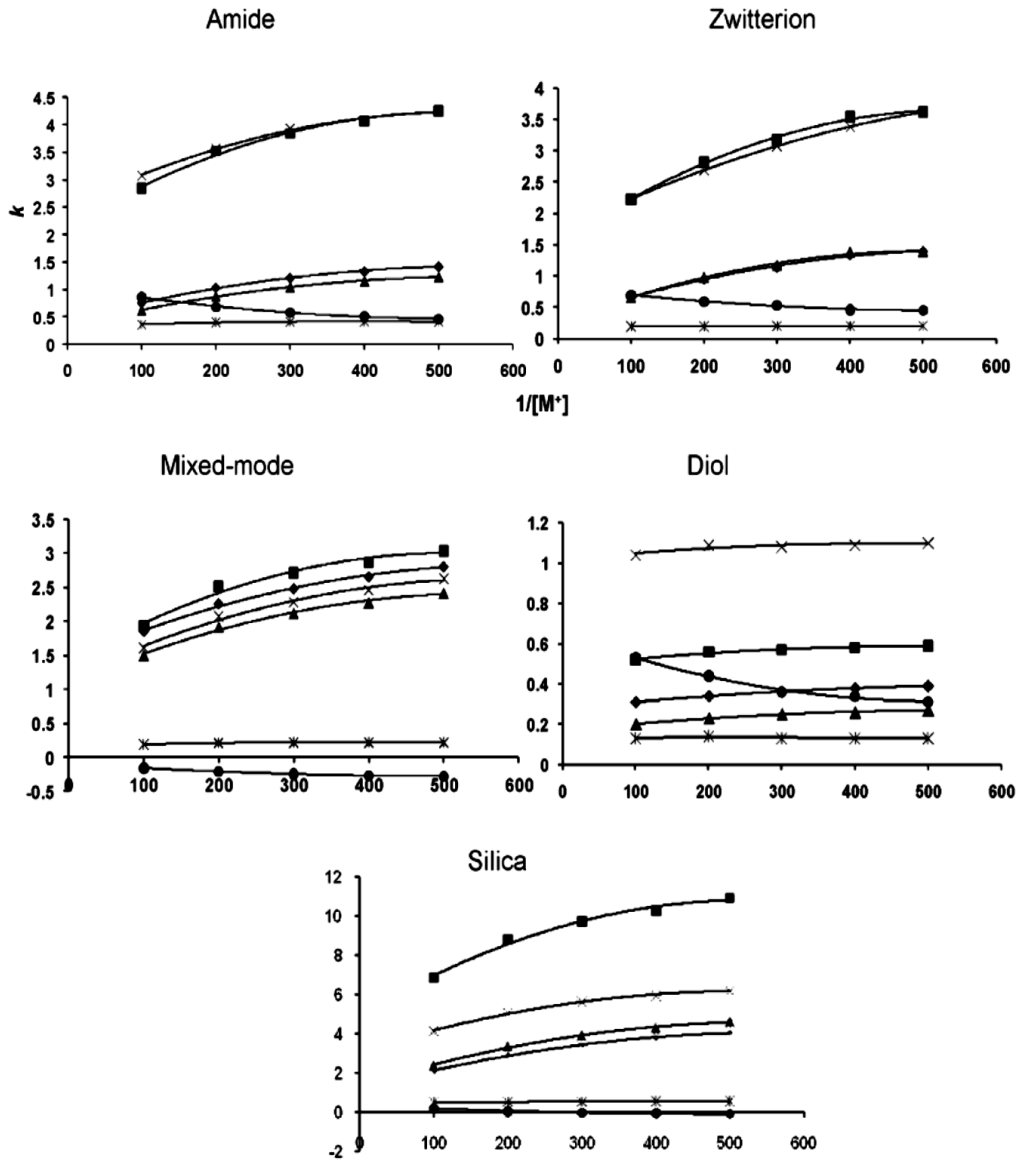


Figure 1-12: Plots of retention factor vs. 1/ [counter-ion concentration] for 5 different HILIC columns. Solute identities: ♦ = nortriptyline, ■ = procainamide, ▲= diphenhydramine, × = benzylamine, * =caffeine, ● = p-xylenesulfonic acid [32]. Mobile phase ACN–water (90:10, v/v) containing ammonium formate (concentration varied) at pH 3.0.

At very low ion concentration in the mobile phase the contribution of ion exchange was high for all except the diol column. In the case of zwitterionic stationary phases, the cation exchange might be due to the sulfonic acid groups on the surface of the phase. Amide and diol phases are neutral phases where the cationic exchange mechanism is presumably due to the ionisation of remaining Si-OH groups on the surface of bonding stationary phase ligand with diol having some shielding of the ionised silanols on the phase surface, or that the phase is bonded on a silica of low acidity [32].

The HILIC mechanism on silica hydride based stationary phases was also investigated by Pesek and co-workers [33]. The behaviour of polar and hydrophobic bases was investigated on a diamond hydride-based stationary phase under normal and reversed phase conditions. The organic content in the water/ ACN mobile phase ranged from 10-50% v/v for the RP study and 70-90% v/v for the HILIC study with 0.1% formic acid in the mobile phase in both conditions. The degree of interaction between the basic analyte and the stationary phase was also investigated by observing the difference in retention behaviour of test probes when the additive was changed from formic acid to acetic acid. A typical HILIC mechanism for the polar compounds was observed on the surface of the diamond hydride stationary phase, the retention increased when the organic (ACN) in the mobile phase was increased. Generally, the degree of ionisation of polar bases will decrease in mobile phases of high organic content due to ionisation suppression. Thus, the electrostatic interaction with the

stationary surface will decrease and therefore a weak retention is expected. However, **Figure 1-13** shows a strong retention for pyridoxine, a weak organic base, as the percentage of ACN increases although under high organic content the weak base will be almost completely unionised, which confirms the partitioning mechanism in HILIC. Surprisingly, the polar base, pyridoxine, exhibited some retention in RP condition with highly polar mobile phase while it was expected to elute at void volume, indicating weak electrostatic interactions between the bases and the hydride stationary phase at high aqueous mobile phase. It is also important to consider the fact that in the aqueous HILIC layer near the surface of the silica gel the ionisation of basic compounds may not be suppressed to a great extent.

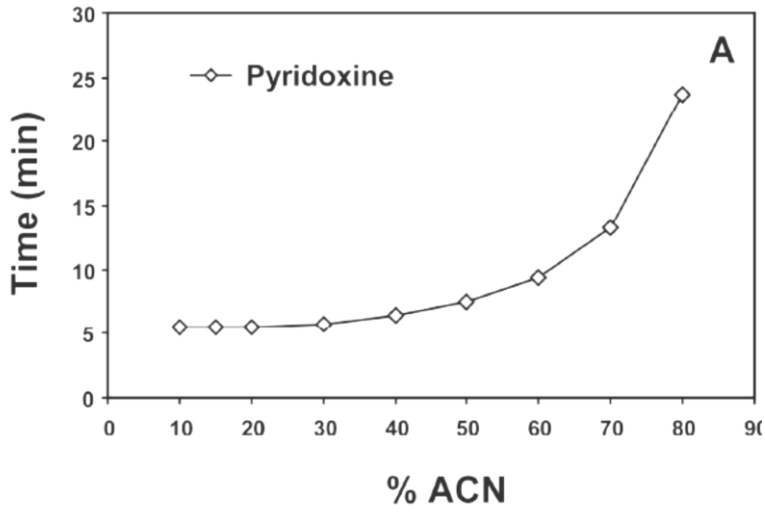


Figure 1-13: Effects of ACN concentration on the retention of the weak polar base pyridoxine [33].

Negative zeta-potential values of the solvated hydride surface were obtained with both acetic acid and formic acid as buffer additives, over the entire acetonitrile concentration range (0 - 100%, v/v) for the diamond hydride column. The obtained zeta-potential value at 50% acetonitrile/water with 0.1% v/v formic acid was (-7.60±1.60 mV) indicating that the silica hydride surface was negatively charged under these conditions which confirms the existence of adsorption mechanism. In contrast to the hydrophobic bases, nortriptyline and diphenhydramine, which showed a "U-shape" retention dependencies **Figure 1-14**, indicative the presence of both reversed-phase and normal-phase retention characteristics [33].

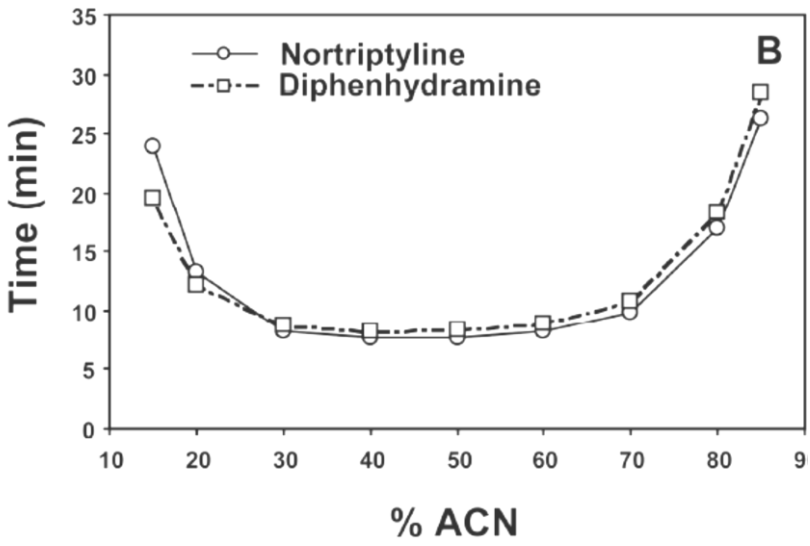


Figure 1-14: Effects of ACN concentration on the retention of hydrophobic bases (nortriptyline and diphenhydramine) [33].

Overall, the mechanism of separation in HILIC is complex and many studies to determine the relative importance of the partitioning and adsorption retention mechanisms when the ionic contribution is held constant were inconclusive, and it appears that both mechanisms may contribute in HILIC mode. The differences in selectivity between various columns indicate that the stationary phase cannot function merely as an inert support for a water layer into which the solutes partition from the bulk mobile phase. Bicker and co-workers [29] reported the same observations and concluded that there were three major retention mechanisms on bare silica, or columns bonded with a neutral ligand: (1) HILIC-type partitioning, (2) HILIC-type weak adsorption such as hydrogen bonding between solutes and the bonded ligands or the silanols (which could be influenced by the experimental conditions), and (3) strong electrostatic forces for ionised solutes, which could be repulsion or attraction. In principle, the multi- or mixed mechanism of separations seems to be common under HILIC conditions and are associated with useful selectivity effects.

1.6 Type of HILIC columns

Approximately in the same time when Alpert introduced the HILIC technique, Huber *et al*. noted that the correct selection of a suitable adsorbent is an essential step for the success of so-called “solvent-generated” liquid-liquid chromatography, the category to which basic HILIC

mechanism can be formed [34, 35]. The family of HILIC stationary phases with various support materials and surface chemistry has continuously enlarged to suit specific separation problems. A wide variety of column chemistries are available for HILIC separations, which have been summarised in recent reviews and the selectivity differences have been demonstrated between different phases [34, 36]. In practice, any stationary phase that has enough polar functionality to form water rich layer on its surface could be used for HILIC applications. Suitable polar functionalities include silanols on unmodified silica, neutral hydrophilic moieties e.g. diol, hydroxyl, cyano, amide as well as anionic e.g., amino, cationic e.g., carboxylate and zwitterionic e.g., sulfoalkylbetaine. However, aminopropylsiloxane-bonded silica columns have been widely used for HILIC applications in the analysis of sugars and other carbohydrates, but due to the formation of glycosylamines, by reaction of the amino groups bonded to the stationary phase with reducing sugars (formation of Shiff's bases), this results in deactivation of the column and loss of the sugar analytes with an undesirable effect on quantitative analysis. Consequently, the majority of HILIC applications which have been reported are for bare silica [37, 38], **Figure 1-15**.

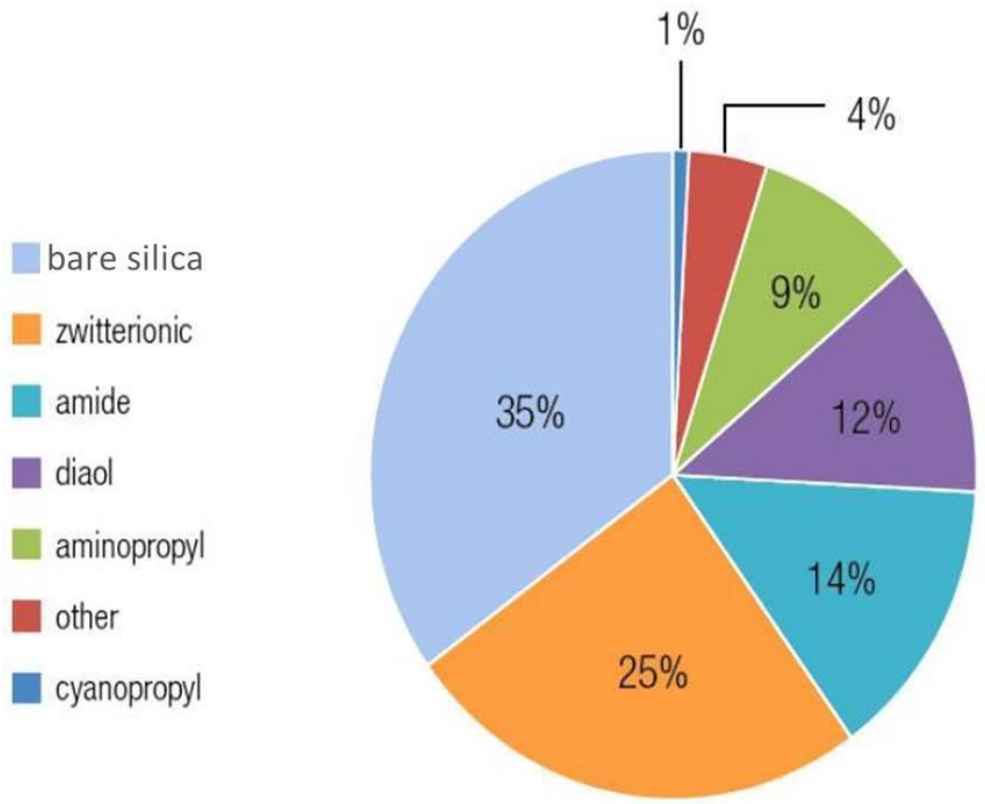


Figure 1-15: HILIC phases *Vs*. % of applications based on Scifinder Scholar 2007 search of the Chemical Abstracts database 2003-2012.

1.6.1 **Bare silica gel**

Silica gel is an amorphous silicic acid polymer represented as $SiO_2 \bullet H_2O$. The structure consists of a network of siloxane bonds (Si–O–Si) and terminal silanol groups (Si–OH). Silanols can exist in three known forms: free silanol, vicinal (hydrogen bonded with a neighbouring silanol group), and germinal**,** **Figure 1-16**. On the surface of silica gel are found silanol groups (Si-OH), of which there are Silanol groups generally exhibit weak acidity, though it can vary in strength depending on the individual state of the group. The classic

use of silica gel columns is for normal phase chromatography as it classified as a polar phase. In pharmaceutical analysis, most HILIC applications still use un-modified bare-silica as a stationary phase because of its high stability in comparison to the chemically bonded silica phases which in some cases are subject to bleeding and therefore bare silica phase becomes the preferable choice. Basic analytes strongly retain in bare-silica columns due to hydrogen bonding and/or electrostatic (ion-exchange) interactions with the silanol groups.

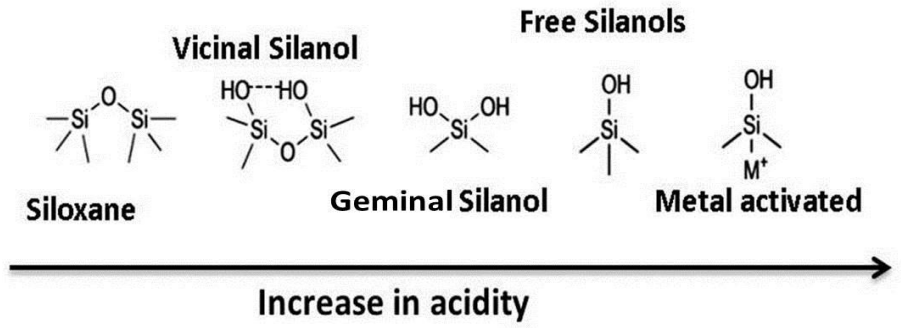


Figure 1-16: Types of silanol groups.

One of the earliest types of silica is type **A** silica gel which is prepared by precipitation from alkali silicate solutions, typically sodium silicate. It is highly acidic because of metal contamination which stems from the monomer used; this activates the surface of silanol groups, increasing their acidity. Also metal ions can form complexes with some chelating solutes, allowing analytes to form several bonds to a single metal ion. These effects can cause strong retention and/or asymmetric peaks [5]. In 1990s, an

alternative method was used to produce silica gel by transferring the gel into a volatile organosilane such as tetraethoxysilane which could be distilled leaving heavy metals behind. This pure form is then hydrolysed to create a silica *sol* which is purified and is referred as type **B** silica gel. In general, silica is stable within a pH range between 2- 8, in which at higher pH dissolution may occur and at lower pH it may hydrolysed which limits column life-time. Therefore, different approaches were developed to stabilize the silica, either by using high density coatings, or using hybrid materials. At the present time, type **B** silica is preferably used in the preparation of modern column packings, because it is more stable at intermediate and higher pH values (11-12), also it contains very low amounts of metals in addition to its symmetrical peaks and better separations [39, 40]. At higher pH values, silanol groups are ionised and cation exchange plays an important role in retention, especially for positively charged basic compounds [41].

In order to overcome the problems caused by the silanol groups on the silica surface, silicon hydride or what is known commercially as Type **C** silica was produced. It is prepared by the conversion of Si-OH groups into the non-polar Si-H groups in the presence of triethoxy-silane (TES) and an aqueous HCl catalyst. This new type of silica gel is less polar than the ordinary silica gel and it was reported to replace more than 95% of silanols from the silica surface [42 - 45]. Different studies have shown the power of

this type of phase in the separation of acids and bases in the HILIC mode when buffered mobile phases contain more than 50–70% organic solvent (usually acetonitrile) are used as well the stability of this phase was reported [46, 47].

There are many types of bare silica columns which have been produced by different manufacturers such as Hypersil and Kromasil. Although it is the same type of phase, it has been noted that columns from different providers could produce differences in selectivity and surface properties [48]. This is possibly due to the differences in the preparation or the degree of silica purity which used to process the column. The separation mechanism on the bare silica column depends on the properties of the analyte and the mobile phase. The retention of basic analytes on a bare silica surface is due to the attraction force between the negatively charged silanols on the surface and the positively charged bases. Similarly, a strong retention for bases could be observed under HILIC conditions on silica gel phase due to the contribution of the partitioning mechanism beside the electrostatic force. In HILIC, the retention of un-ionised acids could be achieved on silica gel surface as partitioning is the predominant mechanism for the retention. HILIC also showed an appreciable retention for ionised acids on silica gel column which in normal conditions would experience repulsion affects from the ionised silica surface. This could occur because the ionisation of solutes will increase their polarity and therefore the opportunity to partition into the (polar) water layer that established on the

silica surface when applying high organic content and thus the retention will be due to partitioning. The attractive and repulsive forces by the silica surface is summarised in **Figure 1-17** for a compound with acidic, basic and neutral groups.

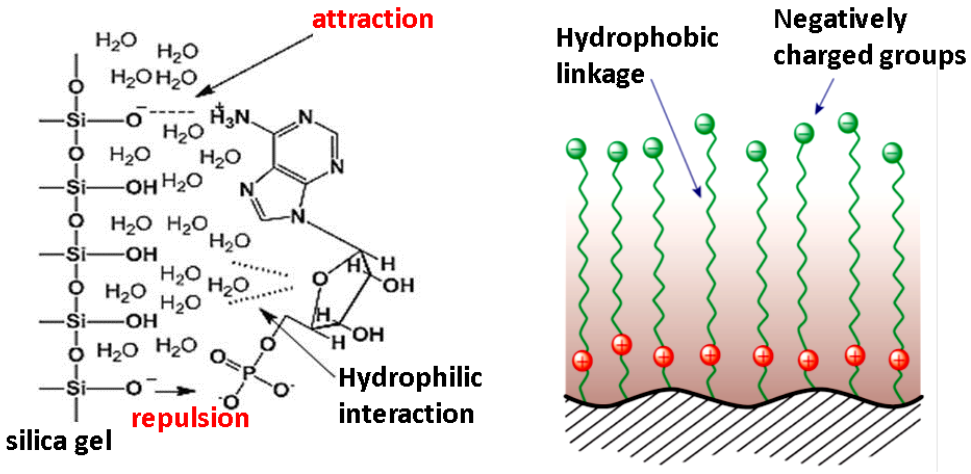


Figure 1-17: Types of interaction occurring between an analyte and a silica gel surface when a high organic content mobile phase is applied.

The absence of ligands on unmodified silica has the advantage of simplifying the interactions with the stationary phase. The application of silica gel under HILIC mode showed a clear improvement over NPL in the case of analysing biological extracts where the interesting compounds are polar and exist in a complex matrix. However, most polar compounds in biological matrixes are not soluble in organic solvents.

1.6.2 **Silicon Hydride Columns (Type C Silica)**

Silica-hydride materials represent a new type of HPLC stationary phase based on high-purity silica, whereby the surface of this new material is largely populated with the more non-polar silicon-hydride (Si–H) groups, instead of the polar silanol groups (Si–OH) that cover the surface of modified silica (**Figure 1-18**) to overcome the problem of the ionisation of the silanol groups. The presence of the hydride groups which chemically attached onto the surfaces of the silica particles produces many useful chromatographic qualities.

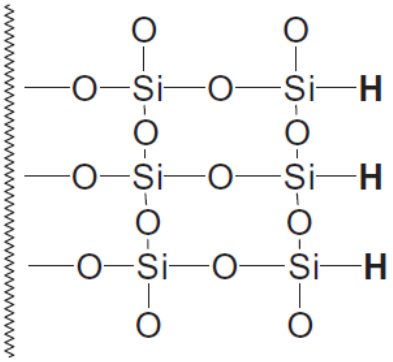


Figure 1-18: Surface configuration of silica hydride.

Figure 1-19 displays the process used to prepare this new type of silica which starts with a **silanization,** reaction between silica and triethoxysilane (TES), in the presence of hydrochloric acid as a catalyst [46, 49]. This reaction maybe followed by a further modification to attach alkyl groups to the surface via **hydrosilation** in which Si-H groups react with the

unsaturated groups to produce a surface which is stable for a long time in water or air.

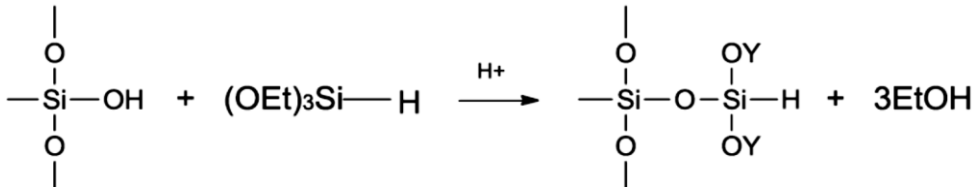


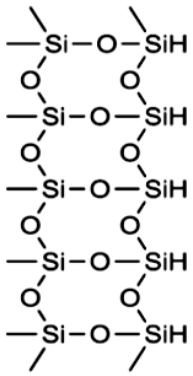
Figure 1-19: The hydrosilation reaction of the Silica gel with triethoxysilane (TES) resulting in a silicon hydride surface [49].

The resulting Si-H stationary phase is less polar than the silica gel therefore it is less attractive to water which can provide some new selectivity for HILIC of non-polar compounds and improve the reproducibility of elution [33]. The retention in hydride phases when there is a high amount of water in the mobile phase (RP mode) is due to increase the affinity of non-polar compounds toward the relatively non/less polar Si-H stationary phase. However, in HILIC mode the elution order is reversed because of the low content of water in the mobile phase, usually around 5-30% [47]. Therefore, the retention on hydride columns can be achieved in both HILIC and RP modes with different selectivity which offers this type of phase unique properties in comparison to the bare silica phase, which has very high hydrophilic properties and cannot retain non-polar compounds under RPLC conditions [50]. However, Bawazeer *et al*. [43] found that the silica hydride phase is more retentive for bases under HILIC conditions than the

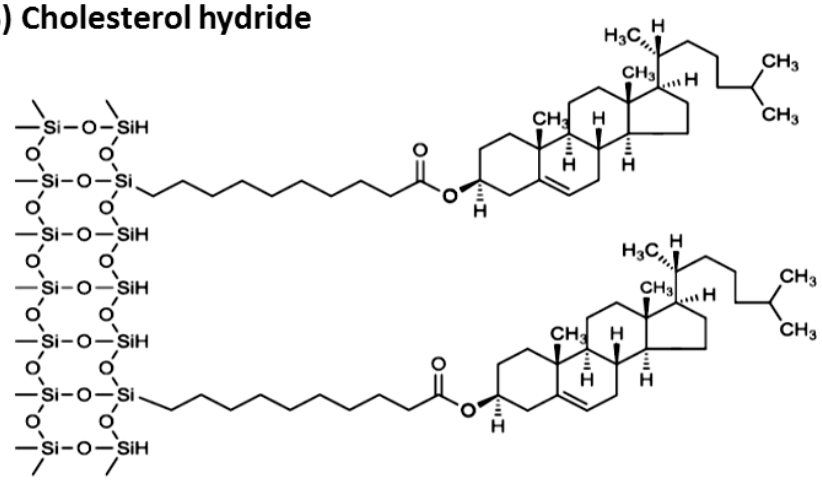
silica gel phase, which will be extensively ionised in the experimental pH. This observation was surprising as the provider of this phase had proposed that there is little or no potential for silanophilic activity [46] indicating that the type **C** silica had an exciting new aspect of chromatographic behaviour which needed to be explored in greater depth.

To enhance selectivity capabilities and enable the improvements in existing analytical protocols, different silicon hydride bonded stationary phases, **Figure 1-20,** have been designed for the demands of new analytical challenges to be met. For example, the UDC Cholesterol phase which is chemically linked to cholesterol and the phenyl hydride phase. An identical elution for cholesterol and bidentate C_{18} hydride phases was observed by Soukup and Jandera (2012) who compared the elution order in HILIC separation for some flavonoid compounds. However, the retention time increase with the polarity of the phases [51].

A) Silica hydride



B) Cholesterol hydride



C) Bidentate C18 hydride

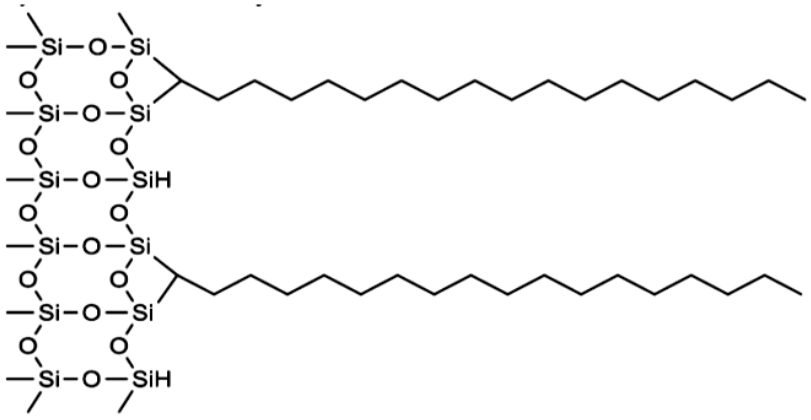


Figure 1-20: Chemical structures of **A**) Silicon hydride, **B**) Cholestrol hydride and **C**) Bidentate C18 hydride.

1.6.3 HILIC columns based on silica gel with neutral surface ligands

The development of bonded phases is purposed to shield the residual silanols on the silica surface so they are less available for solute interactions. However, even when utilizing the best bonding technology (primary bonding plus end-capping) between 30 – 50% of the silanols on the silica gel surface are not bonded with either the primary or secondary moieties. Thus, the acting stationary phase is a combination of three components: bonded ligands, residual silanols, and preferentially adsorbed solvent molecules. The organic component of the mobile phase interacts with the hydrophobic ligands on the surface of the stationary phase and water adsorbs on the residual silanols [52]. The most common HILIC ligand phases which based on silica gel are discussed below.

1.6.3.1 Diol silica

Diol-bonded phases, **Figure 1-21**, have an intermediate polarity media between C_{18} and bare silica column. They are prepared by the end-capping of silanol groups by reacting with trimethylsilyl group to avoid the irreversible absorption of polar analytes onto the stationary phase. Despite being bonded to a diol containing ligand, diol silica phases are very close to bare silica in their degree of polarity but may have different selectivity. Diol silica is neutral phase although the surfaces might have

some remaining silanol groups even with end-capping [53]. The main reason for developing the diol silica stationary phase was to overcome the multi adsorption mechanism properties of the bare silica stationary phase and to offer different separation selectivity. Due to the high polarity of the diol stationary phase, it is suitable to use in HILIC mode and it can form hydrogen bonds which are essential to establish the hydration layer around the surface of the phase [53]. Although the separation conditions in HILIC are similar for both diol and silica phases in respect to the high organic content in the mobile phase, unlike the bare silica column diol phases can be used to separate non-polar compounds by a reversed phase mechanism which employs a higher aqueous content in the mobile phase because of the hydrophobic alkyl chain functionality that can interact with the non-polar analytes [53].

Example of a successful separation on a diol phase was reported by Liu [53] who analysed different ethoxylated alcohol surfactants that contain OH and different alkyl chain groups. It was found that in the presence of a high amount of water in the mobile phase, the separation was based on the alkyl chain lengths as in the reversed phase technique while the HILIC mechanism occurred at lower amount of water and the retention depended on the number of OH groups present in the chemical structure of the analytes. Diol phases also showed the ability to determine the mutarotation, the anomeric conversion of one anomer to the other, of some monosaccharides (sugar) in HILIC mode by recording the anomers

signals separately, not in a mixture, and thus it was possible to study the kinetics of anomeric conversion of sugars directly applying an isocratic analysis in short retention time *ca*. 10 min [54]. Despite the advantages of diol stationary phases over the bare silica phase, the bonded phases have shown stability issues under low pH which change the properties of the surface. To overcome this problem with acidic conditions, a cross-linked diol column, **Figure 1-21**, was developed and shows an increase in the stability against hydrolysis, stronger hydrophobic interaction and improved peak shape and resolution compared to the normal diol silica. The cross-linked diol stationary phase is commercially known as Luna HILIC and due to the presence of oxyethylene and hydroxyl groups within the cross-linked diol stationary phase; it combines in some ways the properties of both polyethylene glycol phase (PEG) and the diol [55].

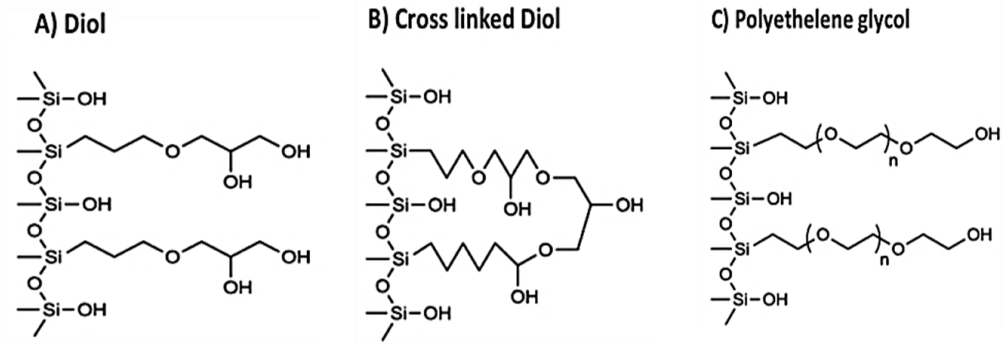


Figure 1-21: Some examples of diol stationary phases.

1.6.3.2 Aminopropyl silica

In amino columns the silica is bound to aminopropyl chains so that the surface of the stationary phase carries a primary amine group which is ionised at low pH with a positive charge. The aminopropyl stationary phase, **Figure 1-22**, is very suitable to use under HILIC conditions because it has a very polar surface and the largest use for amino columns is still in the field of sugar analysis. Although since many of the methods were developed before the terminology was coined it is not always noted as being a HILIC method. It has opposite interaction effect to bare silica for acidic and basic compounds thus acids with a negative charge are strongly attracted to the ionised aminopropyl groups via an anion exchange mechanism however positively-charge basic compounds will exhibit a repulsion effect with the ionised aminopropyl surface and low retention will observed. To reduce the strong retention of acidic compounds, it is advisable to increase the ionic strength of the buffer in mobile phase which will compete with the acids to interact with the ionised sites on the aminopropyl surface [13].

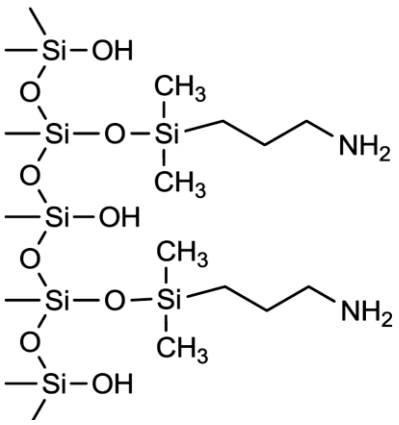


Figure 1-22: Amino propyl surface without end-capping.

Aminopropyl columns need a long equilibration time to stabilise when converting from one buffer to another. This issue was reported by Valette *et al.* [56] who found that changing mobile phase buffer from citrate to acetate at 100 mM required a several hundred column volumes in order to obtain a stable baseline. This bonded phase has shown stability issues when the aqueous eluents are used due to the hydrolysis between the silica and the aminopropyl silane ligand [57]. Consequently, aminopropyl can be lost from the silica surface and which causes peak shape deterioration. In order to overcome this stability issue, an amino packing based on a polymer instead of silica was produced and showed improvement in stability as reported by Person *et al*. [58] in the analysis of taurine and methionine [58]. The primary amino group in the aminopropyl phase could form Schiff's bases with aldehydes as described in **Figure 1-23**, and therefore may change the chemical structure of the surface [59].

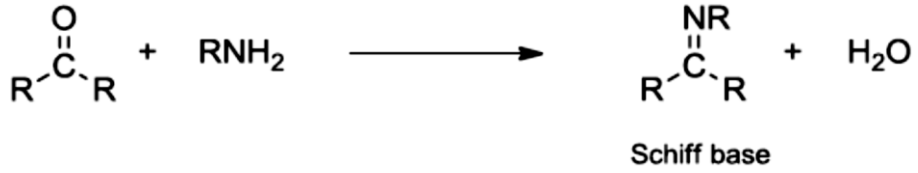


Figure 1-23: Chemical reaction of a primary amine and aldehyde group to produce a Schiff's base.

The secondary and tertiary amine stationary phases cannot form Schiff's bases with carbonyl compounds which may improve column life time. Temperature is typically used to reduce the number of anomeric forms of the sugar molecule. Aromatic amines bonded on silica stationary phases have also been used under HILIC conditions for the separation of polar compounds, and they reported to produce short elution times but symmetrical peaks [34]. In contrast to diol columns, aminopropyl column can eliminate the formation of the two peaks for anomeric compounds and combine them which provide better peak shapes [34].

1.6.3.3 Amide silica

Amide stationary phases have an amide containing ligand linked to the silica gel surface via a short alkyl spacer. Amide stationary phases differ from amino in the fact that amide phases do not have basic properties therefore ion exchange is not involved in the separation unless residual silanols play a part in the retention.

Amide columns show a better stability as there is no irreversible sample adsorption [12]. The surface of amide stationary phase is neutral thus there is no need to use an ionic modifier in the mobile phase and it is compatible with an evaporative light scattering detector (ELSD) since both together showed interesting results in the separation of mono- and oligosaccharides, sugar derivatives, amino acids and peptides under HILIC condition [60]. The carbamoyl-silica HILIC TSK-gel Amide-80, **Figure 1-24**, has been developed especially for HILIC.

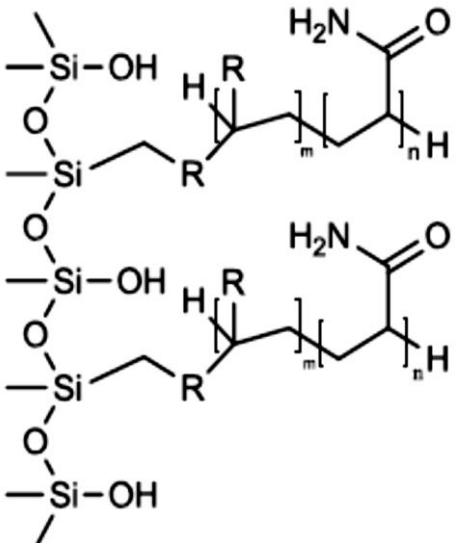


Figure 1-24: The surface chemistry of amide silica (TSK-Gel Amide-80) without end-capping.

1.6.3.4 Poly (succinimide)-bonded silica

After the development of the aminopropyl column, various attempts were made to bond different functional groups onto the aminopropyl phase. Alpert [61] suggested the reaction of the aminopropyl silica with polysuccinimide to provide a reactive bonded surface where further ligands could be attached. The first reaction was used to generate polysuccinimide silica then, from this point, further modification could be carried out to provide many new types of the silica based stationary phases as described in **Figure 1-25**. Polysuccinimide based phases have been applied in the separation of various highly polar compounds such as peptides, proteins, nucleic acid constituents, oligosaccharides, carbohydrates, *etc*. [34].

Polyaspartic acid phase is an example of a new phase generation based on aminopropyl which is prepared by the hydrolysis of poly (succinimide) silica. This phase possesses strong cation-exchange properties and shows mixed-mode HILIC/cation-exchange mechanism. The power of this phase was shown in the separation of hydrophilic peptides with selectivity complementary to the reversed-phase LC [62]. The alkali hydrolysis of the poly (succinimide) silica surface with 2-aminoethanol will form poly (2-hydroxyethyl aspartamide) silica surface. The strong cation exchanger, poly (2-sulfoethyl aspartamide) silica, could be prepared from the reaction with 2-aminoethylsulfonic acid. **Figure 1-25** displays the formation of these phases. Under HILIC conditions, these phases exhibited mixed modes of

separation including varying degrees of ion exchange and partitioning mechanisms. In the separation of the polar compounds, the poly (2-hydroxyethyl aspartamide) HILIC phase showed a loss of performance resulting in a lower efficiency when it was compared with more recent HILIC columns such as ZIC-HILIC [63]. An additional issue was seen with long term stability [60] and column bleeding for the poly (sulfoethyl) aspartamide phase [64].

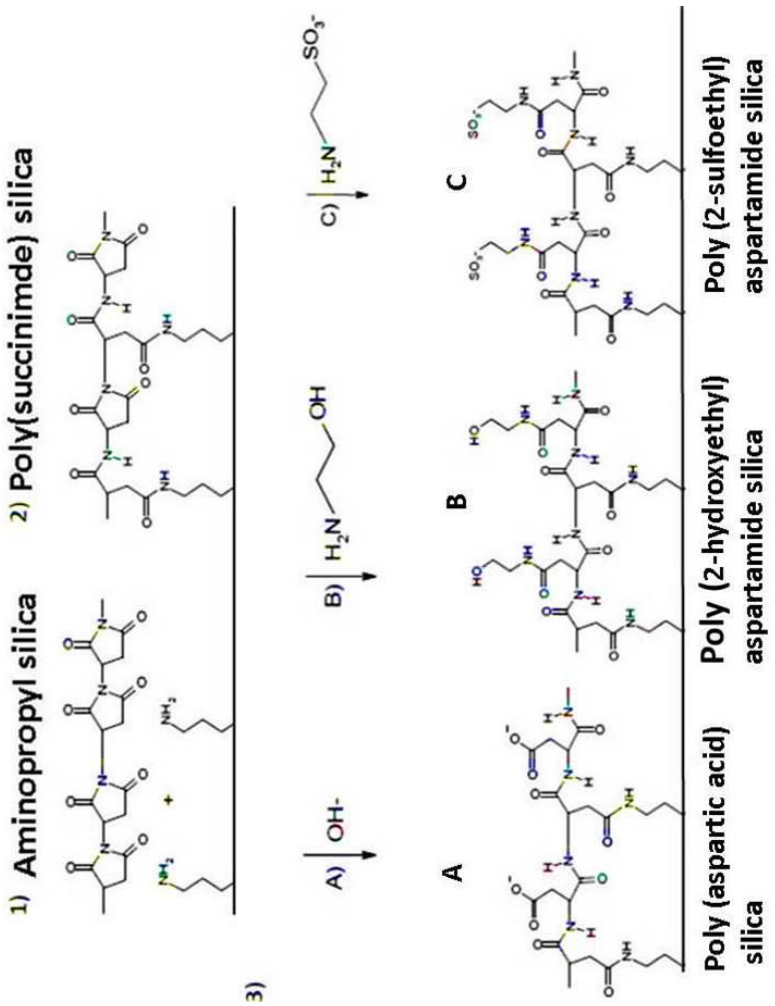


Figure 1-25: Surface chemistry of poly (succinimide) silica and its conversion to **A**) poly (aspartic acid) silica, **B**) poly (2-hydroxyethyl) aspartamide silica, and **C**) poly (2-sulfoethyl) aspartamide silica.

1.6.3.5 Cyclodextrin based columns

Cyclodextrins or CDs, **Figure 1-26**, are cyclic oligosaccharides consisting of six α-cyclodextrin, seven β-cyclodextrin, eight γ-cyclodextrin or more glucopyranose units linked by α-(1,4) bonds to form toroid ring structures that are narrow at one end and have a wide entrance at the other end. The outside surface of the ring is rich of polar hydroxyl groups (OH) but relatively hydrophobic cavities. Due to the numerous OH functionalities which can form hydration layer between the water and the external part of the toroid, cyclodextrins can act as HILIC stationary phases [65].

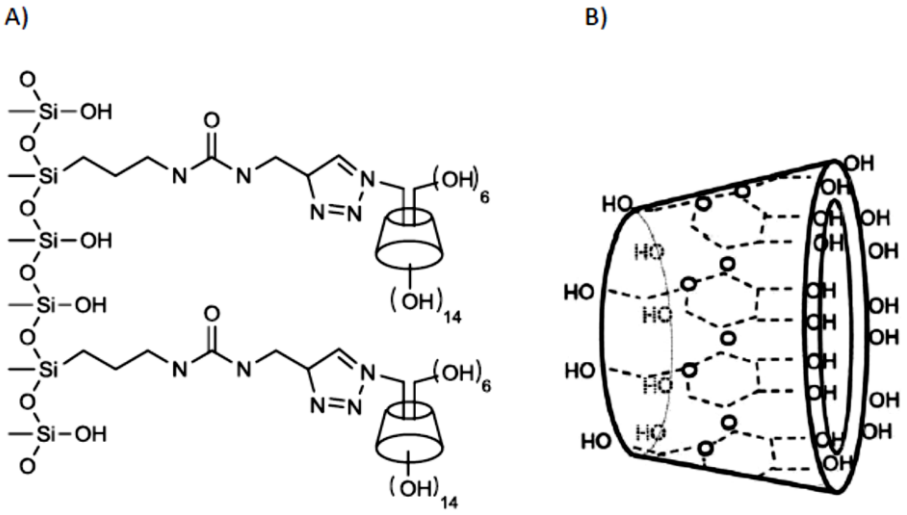


Figure 1-26: Chemistry surface of A) cyclodextrin phase and B) a toroid structure showing the interaction of a polar analyte with the column surface.

Because cyclodextrins are produced from optically active sugars, their cavities have chiral properties and therefore they can be used as chiral selectors in many aspects of analytical separation science [65]. Most of analyte retention occurs outside of the cyclodextrin in the water rich layer rather than inside the cavity [66]. As the number of the monosaccharide groups increases, the polarity of the CDs increases which leads to more retention for polar compounds [66]. Cyclodextrin phases have been widely used with liquid chromatography in the separation and detection of chiral compounds. Risley *et al.* [67] utilised a cycoldextrin phase for the separation of hydrophilic chiral compounds in normal phase and HILIC conditions and the results showed that HILIC mode was more promising and improved the enantiomer separation in comparison to the normal phase mode.

1.6.3.6 Cyanopropyl silica

Cyanopropyl silica (CN) is a polar stationary phase which contains cyano groups attached to a silica support via an alkyl propyl group, **Figure 1-27**. The ability of cyanopropyl-silica bonded phases to form hydrogen bonds is low and therefore low retention of polar compounds was reported under HILIC mode. For this reason, the application of cyano columns in HILIC mode is limited [34]. A CN column was used in the analysis of some compounds such as denaturants in alcohol [64] and also for peptides;

however no retention was observed under HILIC mode using a high organic solvent in the mobile phase [65] which could be due to the low affinity of the hydrophilic compounds toward cyano surface.

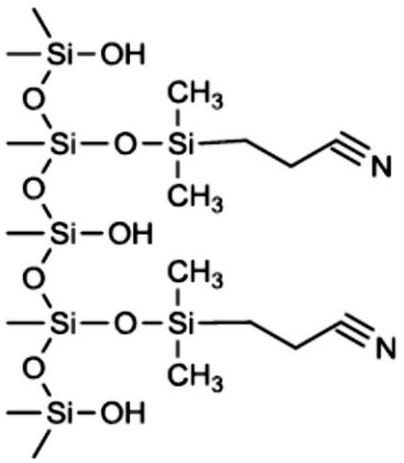


Figure 1-27: Chemical structure of cyanopropyl silica stationary phase.

This column has been successfully used in normal and reversed phase chromatography. However, under HILIC condition CN phases have shown a major stability issue because the CN ligand is not stable with high organic content in the mobile phase, thus the CN columns may experience bond loss when an intermediate polarity solvent is used [68].

1.6.3.7 Sulfoalkylbetaine silica

Sulfoalkylbetaine silica also called ZIC-HILIC is a neutral zwitterionic stationary phase where the silica surface contains strongly basic quaternary ammonium groups linked to strongly acidic sulfonic acid groups via a short alkyl spacer. The positive and the negative charged groups exist in a ratio of 1:1 therefore the ion-exchange interaction of the zwitterionic stationary phase is weak for the anionic and cationic compounds. Sulfoalkylbetaine zwitterions are potent osmolytes with a strong ability of binding water to the surfaces which should reduce ion exchange interactions in HILIC separations [69], however the study by Guo *et al*. found that both partitioning and adsorption played a role in the retention on the tested sulfo-alkylbetaine modified silica HILIC column [16]. Also it was observed that the zwitterionic stationary phase was the least affected by changing the pH while amino phase showed significant retention variations due to the effect of ion-exchange in different pHs [16].

A water-rich layer is established on the surface of ZIC-HILIC phase when an aqueous-organic mobile phase is applied and is known as bulk water. The separation is achieved by partitioning mechanism of the solutes between the eluent and the bulk water on the stationary surface. This process is exothermic and can be affected by factors such as acidity or basicity of the solutes, the dipole interactions and hydrogen bonding [70]. The electrostatic forces of each charge in the zwitterionic stationary phases are

partly counterbalanced by the proximity of an ion with opposite charge. Therefore, in HILIC mode partitioning mechanism will govern the retention of polar compounds, in other words; the retention of the compounds will increase with their hydrophilicity which will allow strong partition on the polar water-rich layer on the zwitterionic surface [71]. **Figure 1-28** displays a new development of two types of zwitterionic phases; the polymer based ZIC-pHILIC and the silica based ZIC-cHILIC phosphorylcholine column. The two zwitterionic HILIC materials (ZIC-pHILIC and ZIC-cHILIC) differ in the charge order and the type of the negatively charged group which might affect the selectivity and the retention order of peptides and other analytes during separation. For example, the two zwitterionic HILIC columns have shown ability to separate both cationic and anionic compounds of carboxylic and amino acids in plant tissues; however, due to the differences in surface properties of the two columns the analytes exhibited variations in the elution order [72].

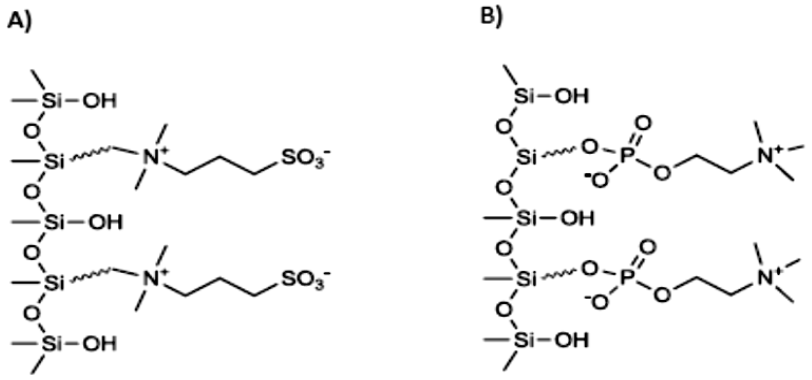


Figure 1-28: The chemical structure of **A**) ZIC-HILIC and **B**) ZIC-cHILIC stationary phases.

1.7 Factors affecting the separation in HILIC

Factors like the type of organic solvent, pH, salt type and salt concentration in the mobile phase and the temperature of the column can affect the separation by the HILIC mechanism which could change the elution order, retention time, separation efficiency and even the chemical properties of the analytes and the stationary phase. This section will discuss the most common factors that have been reported to affect the separation in HILIC mode.

1.7.1 Effects of Column Temperature on Selectivity in HILIC

Column temperature has long been recognized as an important parameter in HPLC separation, which acts significantly on analyte diffusivity, mobile phase viscosity and analyte transferring enthalpy between mobile and stationary phases. From kinetic consideration, column temperature has been also used for improvement of HPLC performance [73]. Fast analysis without loss of efficiency, can be performed at high temperature compared to room temperature. In HILIC separation, column temperature can affect analyte's retention and selectivity based on thermodynamic considerations. Many studies were conducted to compare the effect of retention factor on mobile phase polarity and column temperature. It was assumed that a given mobile phase became less polar when temperature is high, thus explaining the observed decrease of analyte retention factors in

RP HPLC [73 - 76]. The retention of a solute in HPLC as a function of a temperature can be, in theory, described by the van't Hoff, **Equation 11.1**:

$$\mathbf{Ln\ k = ln\ \beta - \Delta \frac{H^\circ}{RT} + \Delta \frac{S^\circ}{R}} \qquad \mathbf{Eq.\ 11.1}$$

Where H° is the standard enthalpy change associated with the transfer of the solute from the mobile to the stationary phases, S° the corresponding standard entropy change, R the molar gas constant, T the absolute temperature, and β the phase ratio of the column and k is the equilibrium constant. To an approximation, it is possible to substitute k for K, the retention factor (assuming phase volumes are independent of temperature).

It can be seen from the equation above that the change of retention factor with temperature results from the ΔH° term. Because the H° term is normally negative, the retention factor decreases with increase of temperature. The relationship between ln K vs. 1/T is expected to be linear. However there are many factors that can complicate this and lead to non-linear plots. As a general rule, an increase in temperature would increase the diffusion coefficient and give in narrower peaks by reducing mass transfer effects. Simultaneously, a shorter retention time would be produced with an elevated temperature. Dolan also gave a summary in his review article on temperature selectivity in RP HPLC: "While changes in

retention as a function of temperature are ubiquitous, selectivity changes for any given solute pair is more pronounced for ionised samples and samples with more polar substituents" [77]. The effect of temperature on selectivity has been published by Carr's group using original zirconia-based columns and it stated that the functional groups on analyte structure played a very important role for selectivity when the column temperature was changed [78 - 80]. The observations of these studies deduced that selectivity changes with temperature depend mainly on the nature of analytes and the differences in the interactions between the analyte and stationary phase. The majority of solutes will show decreased values of ln *k'* as the temperature is increased. The slopes of van't Hoff plots are often similar for compounds of the same functional groups while for solutes of different compound classes the plot can vary widely and have either a positive or a negative slope [81]. Thus, operating at an elevated temperature will not only allow achieving higher efficiency and faster flow rates, but the actual retention of the solutes is reduced. The application of high temperature to increase the speed of HPLC separation extends to ion chromatography and to inorganic analysis. Le *et al*. [82, 83] reported a 50% reduction in analysis time when a number of selenium and arsenic species including inorganic forms, organometallics, and compounds with amino acids and sugars were analysed at 70 °C.

Temperature has often been used in chromatographic separations as a means to control retention and to improve the peak shapes of polar and

non-polar compounds. Moreover, column temperature has an influence on many other factors such as the diffusion of the solute, viscosity of the mobile phase and thermodynamic energy required for the transfer of solutes between stationary and mobile phases. An increase in column temperature was reported to improve the peak shape due to an increase in the diffusion coefficients for analytes as well shortening the analysis time and in consequence, column temperature positively improves the HPLC performance [2].

In HILIC separation, a decrease in retention was reported at high temperatures which was possibly result of reducing the difference in cohesive energy, hydrogen bonding and other polar interactions between the mobile and the stationary phases but the variation of temperature has greater impact on the composition of the mobile phase than on the retention of analytes [84]. The correlation between the column temperature and retention factor is often described by the van't Hoff equation; if retention is through partitioning between the mobile phase and the immobilised layer of water on the stationary phase, then the relationship between ln k and 1/T is linear [85].

The effect of the temperature was investigated on different HILIC columns such as neutral, charged and zwitterionic phases. A decrease in the retention of urea, sucrose and glycine on the neutral diol was observed as the column temperature was increased. The negative slope when the van't

Hoff plot was applied indicating an increase in the solubility of the analyte in the mobile phase as the temperature increases [86].

However, a charged surface such as bare silica and amine stationary phases exhibited different observations when aspirin (acid) and cytosine (base) were analysed. Bare silica showed a negative slope for both analytes at different temperatures. In contrast, the van't Hoff plot of the retention data on amino column for the aspirin (the negatively charged compound) gave a positive slope [16]. The ionic interaction in amino column is responsible for the positive correlation between temperature and retention because the increase in temperature is believed to reduce the solvation strength between the analyte and the mobile phase which may produce more loosely solvated ions and results in more activity of the ions towards ion exchange interactions [87]. In the case of the ZIC-HILIC stationary phase, both analytes aspirin and cytosine produced a negative van't Hoff slope indicating that the thickness of the solvating water layer is possibly reduced at higher temperatures [16].

A recent study by Soukup and Jandera [51] documented the effects of temperature on phenolic acids with several different silica-hydride stationary phases (*i.e*. unmodified silica-hydride, C_{18}, cholesterol and diamond hydride materials). The study was conducted in highly organic and highly aqueous mobile-phase ranges and in both modes it was demonstrated that retention times and peak widths decreased with

increasing temperature. Linear van't Hoff plots (log k versus 1/T) were observed, in both mobile phases and the tested stationary phases proved a high stability under high temperature and it was reported to perform efficiently for extended periods of time between 80 – 100°C without significant loss of retention properties.

1.7.2 The Effect of the Mobile phase Composition

The function of mobile phase is to dissolve samples that need to be analysed and it is used as eluent to carry the sample through the HPLC system and therefore it plays a very important role in the separation mechanism. Therefore, a suitable composition of mobile phase should be adjusted. Depending on the choice of mobile phase composition, the stationary phases exhibit either reversed-phase or ANP chromatographic behaviour allowing the separation of compounds with broad range of polarity in a single run in either isocratic or gradient elution mode [8].

HILIC on polar stationary phases employs organic-rich mobile phases, usually containing 5–40% water or a buffer to fix the pH (usually volatile ammonium formate or ammonium acetate especially when the detection technique is MS). The pH of the buffer is usually chosen to ensure that the polar structure or ionised form of the compounds is obtained.

The organic solvents which can be used in the preparation of HILIC mobile phase can be protic (donate proton) or aprotic (cannot hydrogen bond) depending on the separation conditions. Protic solvents can accept and donate hydrogen bonds and they include methanol, ethanol and isopropanol, examples of the common aprotic solvents are tetrahydrofuran and acetonitrile that cannot form H-bonds. Protic solvents are distinguished from aprotic in that the former competes for the polar sites on the stationary phase and displaces the water-rich layer on the surface of the stationary phase. In this case the water on the surface will be replaced by the organic solvent and this makes the stationary phase more hydrophobic [5], and therefore reduces the retention of polar analytes on the stationary phase.

Acetonitrile is preferentially used as organic modifier for HILIC. Methanol and/or ethanol are rarely employed in place of acetonitrile in some cases [27, 88], due to the similarity of some solvents to water; both methanol and water are protic solvents, which compete to solvate the surface of silica or of other polar stationary phases and provide strong hydrogen bonding interactions with each other. This applies, to a lesser extent, for other alcohols – ethanol, 2-propanol, etc., but not for acetonitrile, which does not show proton–donor interactions and hence provides larger differences with respect to aqueous-rich liquid stationary phase that established on the surface of polar adsorbents. Several attempts to replace acetonitrile with a less toxic solvent were reported, but this effort has not

been very successful so far, except for a few applications, where acetonitrile can be replaced with tetrahydrofuran [34]. However, the use of these viscous alternative solvents reported to reduce the efficiency in separation [89]. Acetone has similar polarity to acetonitrile, but shows lower retention under HILIC conditions and significant selectivity differences; further it absorbs in the UV region and provides lower intensity MS signals, so that it is not recommended for direct replacement of acetonitrile [90].

The mobile phase composition in HILIC and RP chromatography is similar however they differ in the amount of the organic solvent mixed with water. Usually the mobile phase in HILIC is less polar than the stationary phase. The mechanism of separation on HILIC is mainly by partitioning of the analyte between the bulk mobile phase and the water rich-layer surrounding the stationary phase, the more polar the analyte the more it will be retained by the column thus it is very critical to choose a suitable organic solvent in order to improve the selectivity in HILIC separation.

The selection of the organic solvent has strong effect on the retention in HILIC mode. The elution strength of organic solvents in HILIC increases generally in the order of increasing solvent polarity and ability to participate in proton-donor/proton-acceptor interactions. Under HILIC conditions, acetonitrile is a weaker solvent that provides higher retention

than methanol and water is the strongest eluting solvent [91]. Solvent strength from weakest to strongest as follow:

Tetrahydrofuran < acetone < acetontrile < isopropanol < ethanol < methanol< water

Li and Huang [15] compared the retention of epirubicin and its analogues, which are weak bases and possess highly similar structures, on a bare silica stationary phase employing different organic modifiers in the mobile phase as illustrated in **Figure 1-29**. No separation obtained for the four analytes when methanol was applied; however an improvement in selectivity was observed in the elution profile of isopropanol which is more hydrophobic than methanol due to the presence of long alkyl group, therefore it competes less with the hydration layer on the stationary phase offering more opportunity to the analytes to retain longer on the polar stationary surface. A great separation was observed for the analytes when changing the organic composition of the mobile phase from protic to aprotic solvent, tetrahydrofuran, and the selectivity was improved further with acetonitrile which is weaker hydrogen bond acceptor than THF [15]. In summary acetonitrile is the most compatible solvent in HILIC separation and it is better serves the partitioning mechanism as well providing retention selectivity and sharper peaks.

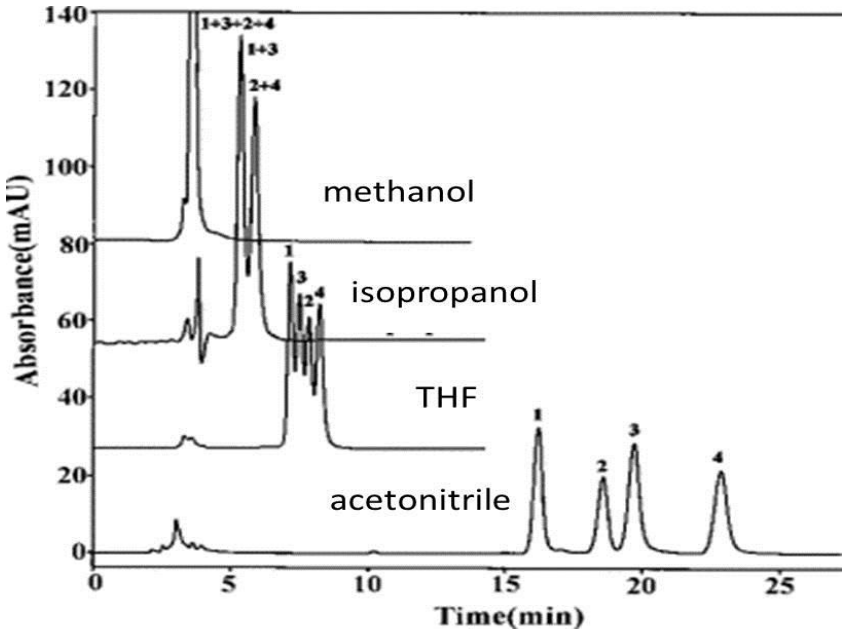


Figure 1-29: The effect of the different types of organic modifiers on the separation of epirubicin and its analogues ,mobile phase containing 20 mM sodium formate pH 2.9 and different organic solvents (10:90, v/v)) [15].

1.7.3 **The effect of pH**

Mobile phase pH is an important chromatographic factor since it can affect the charge state of both the stationary phase and polar solutes. The pH is a measure of the activity (usually approximated to the concentration) of hydrogen ions in a solution. It can control the degree of ionisation of acidic or basic groups within an analyte, and adjusting the pH of the mobile phase can solve the problem of a polar matrix. A significant electrostatic interaction can occur between a charged stationary phase and a charged solute due to the variation in pH and this can cause a marked affect in the retention of analytes as well as the selectivity in HILIC separation. In

general analytes are more polar in their ionised state than in their neutral forms therefore the charged analytes are strongly retained in HILIC by a partitioning mechanism. Further, pH affects the interaction between a charged molecule and a charged stationary phase through ion exchange which results in electrostatic attraction or repulsion. Theoretically, repulsion occurs when two similar charges are presented in the same domain of interaction; for example the negative charge of an acid interacts with negative charge of the stationary phase or the positive charge of a base interacts with the positive charge of a stationary phase which will cause repulsion and early elution and in some cases loss of peak shape.

It is well known that any ionising function is 50% un-protonated at a pH equal to its pK_a, approximately 90% un-protonated (or protonated) at a pH unit one unit above (or below) its pK_a, and approximately 99.9% un-protonated (or protonated) at a pH 2 units above (or below) its pK_a [41]. Basic compounds can be completely ionised at 2 pH units below their pK_a values and in their completely unionised states at 2 pH units above their pK_a values, acidic compounds behave oppositely. Thus the pH plays an important role in controlling the separation but the stability of silica surface should be considered because between pH values > 8 or < 2 silica can be destroyed or hydrolysed [3]. The polymer support used for the stationary phase of the ZIC-pHILIC column was an attempt to make the column stable at higher pH.

The retention of acidic analytes occurs in the negatively charged stationary phases via increasing the pH as the maximum hydrophilic interaction occurs when the analytes are ionised. However, the ionisation of the residual silanol groups will be promoted by increasing the pH when silica is used as a support and that can produce charge repulsion with negatively charged acids on one side. On the other side the hydration layer which forms on the silica gel surface can increase the retention of polar acid in the ionisation state. Therefore, the effect of increasing pH on the retention of acids is difficult to predict on silica column, however increasing the pH will overall increase the retention time of the acidic compounds [92].

The retention time of acetylsalicylic acid, aspirin (pK_a 3.5), on silica, amide and sulfobetaine phases at various pHs is shown in **Figure 1-30**. The mobile phase consists of ACN/10 mM ammonium formate (90:10). Aspirin shows a strong retention on silica phase at high pH of 4.8, although the surface silanol groups will be negatively charged and repulsion effects are expected for the ionised acids. However, due to increase the polarity of the ionised form of acids the hydrophilic interaction is more dominant than the electrostatic repulsion. This observation confirms the hydrophilic interaction with the surface at high organic-containing mobile phase. Also silica-based neutral phases can be negatively charged at high pH due to the presence of residual silanol groups on the silica surface as reported by Guo and Gaiki (2005) and Alpert (2008) who observed that amide possess

negative charges in a particular range of mobile phase pH [16, 93]. Consistent with this, both acidic and basic were found to behave similarly on the amide and silica phases when the mobile phase pH was varied from 3 and 6.5 [94]. Both the mobile phase pH ($^{s}_{s}pH$) and solute pKa ($^{s}_{s}pK_a$) are affected by the organic solvent (*i.e.,* acetonitrile) added to the mobile phase [95, 96]. Also the charge state of the solutes and stationary phase can be affected by the mobile phase pH resulting in significant variations on the retention and selectivity in HILIC analysis, since the charged stationary phase can have electrostatic interactions (attractive or repulsive) with the charged solutes [92]. In the retention of basic compounds, cytidine was strongly retained, 9.71 min., on the silica column at pH 6.5, when the Si-OH is extremely ionised due to the ionic interactions between the stationary phase and the analyte which have opposite charges in addition to the hydrophilic partitioning mechanism while the retention time is reduced at pH 3.3 when there is no ionic interaction from the silica surface [92].

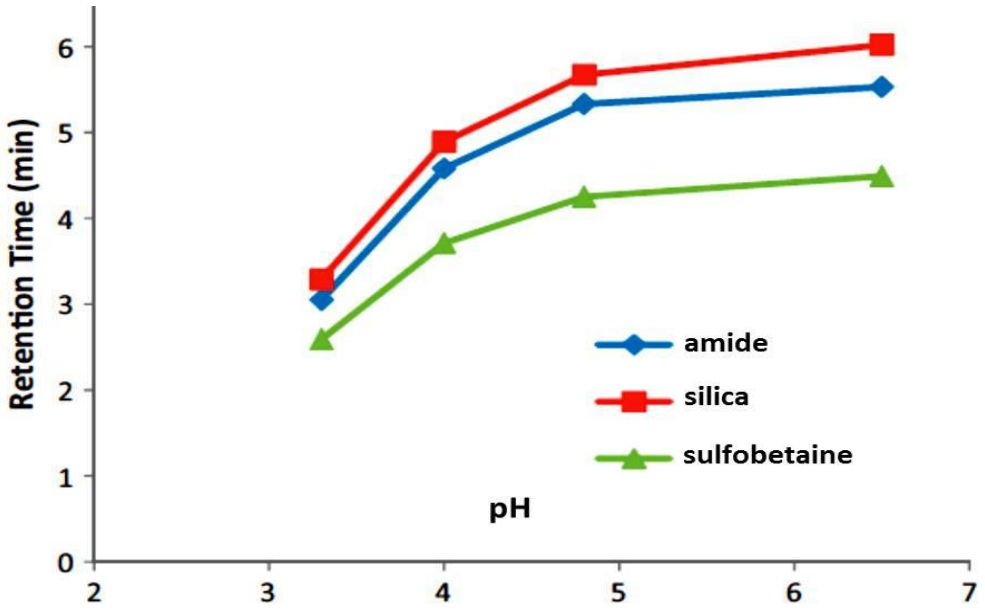


Figure 1-30: The effect of pH on the retention of aspirin on (♦) amide, (■) silica and (▲) sulfobetaine phases. Mobile phase contains 10 mM ammonium formate/ACN (10:90 v/v) at 30°C [16].

In the case of positively charged stationary phases such as the aminopropyl phase, the retention time increased significantly for acidic compounds as a result of both hydrophilic and ionic interactions with positively charged stationary phases. For example, **Figure 1-31** shows the retention of aspirin (pK_a 3.5) at pH 6.5 where it is strongly retained on an amino column due to the electrostatic attraction between the ionised acidic analyte and the charged amino groups while at pH 3.3 the retention is reduced as the analyte is partially ionised [16]. The basic analyte cytosine (pK_a 4.6) is also shown in **Figure 1-31** and it can be clearly seen that the retention behaviour of the base when the pH is below its pK_a value is much lower

than the retention when the pH rises above its pK_a. This behaviour could be explained as cytosine is positively charged in its ionisation form, thus it will experience charge repulsion from the positively charged amino group on the surface which will be also ionised at low pH. However, an increase the pH will reduce the ionisation of both the surface and the analyte; therefore will allow the hydrophilic interaction to take place on the stationary surface without charge repulsion which resulted in a significant increase in retention [16].

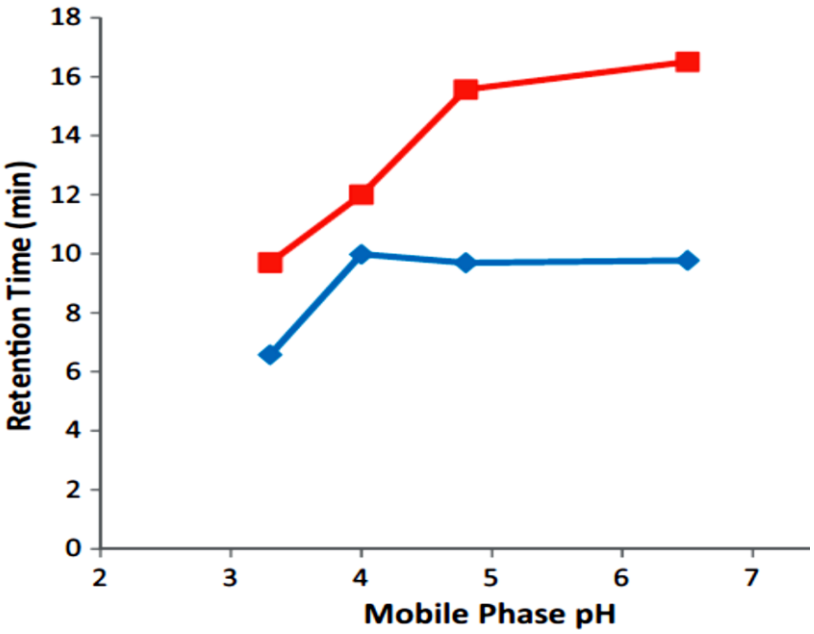


Figure 1-31: The effects of mobile phase pH on the retention time of aspirin (♦) and cytosine (■). Stationary phase: amino propyl, mobile phases 10 mM ammonium formate /ACN (10:90, v/v) [16].

Most of zwitterionic columns such as ZIC-HILIC share similar retention behaviour to silica gel since both have negative change group, however the charge effect of the ZIC-HILIC is small in comparison to the silica column [97]. Amide and diol are neutral silica-based support phases which might carry a negative charge because of the remaining un-covered silanol groups on the surface of the silica. For instance, increasing the pH of mobile phase from 3 to 6 showed a gradual increase in the retention of thiamine (positively charged) on a diol phase although the ligand of this phase is neutral but this observation supports the presence of ionic interactions of thiamine with the remaining silanol groups [98].

1.7.4 The effect of salt type and concentration

The function of buffer salts is to prevent the large changes in pH when acidic or basic material is added, to reduce peak tailing and /or retention of charged analytes [92]. The presence of buffer salts in the mobile phase can effectively reduce the electrostatic interactions (both attractive and repulsive) between charged solutes and stationary phases in HILIC mode. Organic soluble solutes such as ammonium salts of acetic and formic acids are recommended for HILIC. The volatility of these buffers is an advantage for the electrospray sources which are used in mass spectrometry. Generally, charged stationary phases, with a net positive or negative charge, require higher concentration of buffers than neutral or zwitterionic

phases. Many different substances have been used for buffering in HILIC; the most commonly used buffer is listed in **Table 1-1**. A buffer is most effective when used within ±1 pH unit of its pK_a, but may provide adequate buffering ±2 pH units from the pK_a [92].

Table 1-1: Common mobile phase buffer additives.

Buffering agent	**pK_a (25˚C)**	**pH range**
Phosphoric acid (pK_1)	2.1	1.1 – 3.1
Phosphoric acid (pK_2)	7.2	6.2 – 8.2
Phosphoric acid (pK_3)	12.3	11.3 – 13.3
Ammonia	9.2	8.2 – 10.2
Sulfonate	6.9	5.9 – 7.9
Acetate	4.8	3.8 – 5.8
Formate	3.8	2.8 – 4.8
Chloroacetate	2.9	1.9 - 3.9
Sulfonate	1.8	<1-2.8
Trifluoroacetic acid	0.5	<1.5

The selection of salt and concentration is a critical step that can make a significant difference in buffer-solubility which could change the selectivity of separation. Sometimes one can avoid precipitation of the buffer salt by using a very low concentration of buffer salt (*e.g.* 10 mM) in the aqueous

portion of the eluent. However, the resulting low buffer capacity can lead to slow equilibration, irreproducible retention, and poor peak shapes [99]. Furthermore, both retention and selectivity are functions of the salt concentration for ionisable solutes [100 - 103]. Thus, knowing the relationship between the buffer's solubility limit and volume fraction of organic co-solvent provides useful guidelines for the preparation of strong eluents to obtain a desirable separation. **Table 1-2** shows the solubility of some common buffers in acetonitrile. As shown, the solubility of the ammonium salts of phosphate is much higher than the potassium salts under comparable conditions also the acetate seems to be much more soluble in acetonitrile than phosphate. Buffer solubility decreases by a factor of 2 for a 10% increase in organic volume fraction in the range of 50–70% B [104].

Table 1-2: Solubility of various buffers in acetonitrile [104].

%B	**Ammonium acetate/ pH 5.0**	**Ammonium phosphate/ pH 3.0**	**Potassium phosphate/ pH 3.0**	**Ammonium phosphate/ pH 7.0**	**Potassium phosphate/ pH 7.0**
60	> 50 mM	>50 mM	>50 mM	50 mM	45 mM
70	> 50	>50	>50	25	20
80	> 50	35	20	5	0
90	25	5	0	0	0

The effect of changing buffer on the retention of aspirin (acidic probe) and cytosine (basic probe) was examined in different HILIC stationary phases includes amide, amino, silica and sulfobetaine [16]. Changing the buffer from ammonium acetate (pK_a 9.2, pH approximately 6.9) to ammonium bicarbonate (pK_a 10.3 [HCO3], 9.2 [NH_4^+], pH approximately 7.9) showed no significant difference in the elution times of cytosine (pK_a 4.5) in all tested phases due to the fact that cytosine is not ionised at the pH range of these buffers. However, aspirin exhibited a considerable decrease in the retention time on the negatively charged HILIC phases when the buffer changed from ammonium acetate pH 6.9 to bicarbonate pH 7.9. This might be due to the electrostatic interaction (repulsion) between the aspirin and the ionised surface at the experimental pH. Strong retention was observed for aspirin with ammonium acetate buffer on the positively charged amino column, **Figure 1-32**. This behaviour could be due to the ionisation state of the stationary phase which will be ionised (positively charged) at the acetate pH and thus the strong retention could be caused by a contribution of electrostatic interactions with the negatively charged aspirin. At the higher pH of the bicarbonate solution, the stationary phase surface will be no longer ionised and the retention mechanism will be solely due to partitioning.

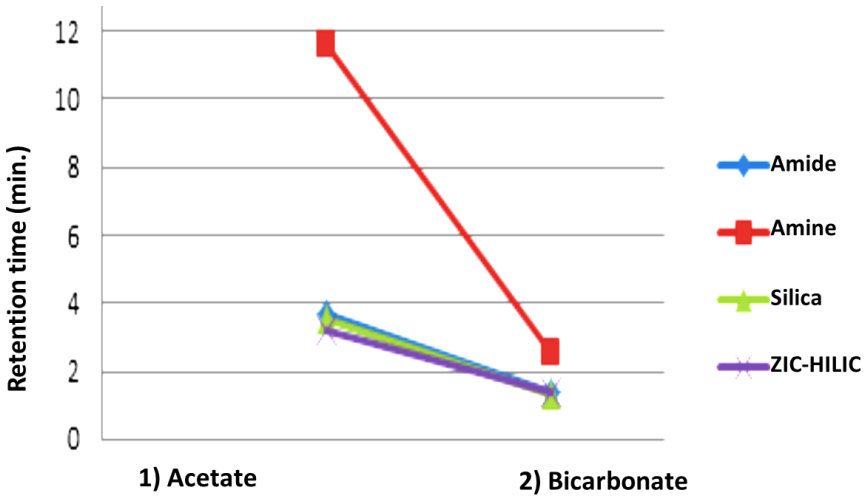


Figure 1-32: The effect of buffer type on the retention of aspirin with 10 mM **1**) ammonium acetate, pH 6.9 or **2**) ammonium bicarbonate, pH 7.9 and ACN (15:85, v/v) on different stationary phases [16].

The effect of buffer concentration is considerable in the case of electrostatic interactions. An increase the concentration of buffer can lead to increase in the retention of the acidic compounds as the electrostatic repulsion is reduced due to the presence of the buffer ions which may block the charge site on the stationary phase thus allowing more retention. In contrast, basic compounds will exhibit a reduction in their retention time as the buffer ions increase since the electrostatic attraction between the ionised bases and the stationary phase surface will be reduced by competition resulting in less retention. In HILIC, higher salt concentrations could drive more solvated salt ions into the water-rich liquid layer. This would result in an increase in volume or hydrophilicity of the liquid layer, leading to stronger retention of the solutes. This theory was confirmed by Guo *et al.* [16] who investigated the effect of different buffer

concentrations under HILIC condition on the retention time of aspirin on HILIC columns, including bare silica and aminopropyl stationary phases. A remarkable increase, from 7.2 to 20.2 minutes, in the retention time of aspirin was observed as the concentration of ammonium acetate buffer was decreased from 20 to 5 mM on the aminopropyl column. This increase in retention as discussed in previous sections due to the significant contribution of ion-exchange interaction on the amino phase to the retention of acids in HILIC. However, negligible increase was obtained in the retention of the acid on the bare silica due to the repulsion between the acid and the negative silanol groups when acetate concentrations were decreased. Alternatively, cytosine (pK_a 4.5) exhibited a small but significant increase in the retention when the concentration of buffer varied from 5 to 20 mM despite the fact that at pH 6.9 the ionisation of the stationary phase surface and the analyte was suppressed which indicated that increasing the salt concentration was promoting hydrophilic interactions [16].

1.7.5 Summary of factors affecting the HILIC mechanism

The concentration of acetonitrile is the most effective factor in promoting the HILIC mechanism as shown in **Figure 1-33**. The data is based on the retention of salicyluric acid on the amide, aspartamide, silica and sulfobetaine phases, which suggested hydrophilic interaction as the major mechanism for retention. In contrast, the acid was affected significantly by

the salt concentration on the amino phase where electrostatic interaction is a major factor in the retention [92].

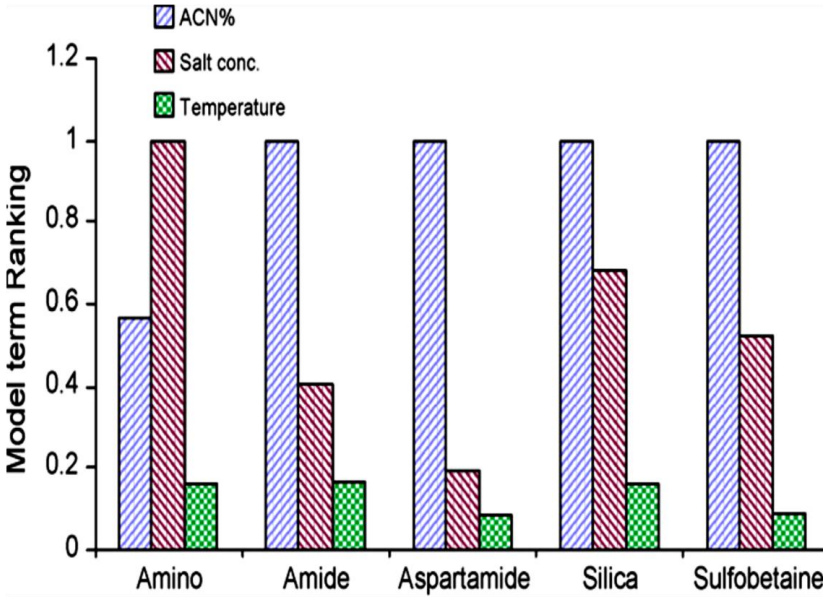


Figure 1-33: Effect of three factors on HILIC separation based on the retention of salicyluric acid on various polar stationary phases [92].

Salt concentration seems to be more significant than the column temperature in the retention of the acid on all tested phases. It could be due to electrostatic interactions between the acid and negative charges on the surface of these phases, although the effect of salt on the immobilised aqueous layer may play some role as well. Similar observation was reported by Quiming *et al*. who found that the retention of uric acids was affected by the salt concentration but less than by the column temperature on the diol phase due to the absence of electrostatic interactions between the neutral uric acids and the neutral diol phase [91].

1.8 Applications of HILIC in Pharmaceutical and Food Analysis

A general interest among chromatographers on HILIC separation has risen during the last decade. A significant number of organisations are now using the HILIC technique in different fields of science as summarised in **Figure 1-34**. Although the data states that more HILIC analysis is performed in the pharmaceutical industry, this is an absolute number and does not take into consideration the total number of analysis performed in each industry.

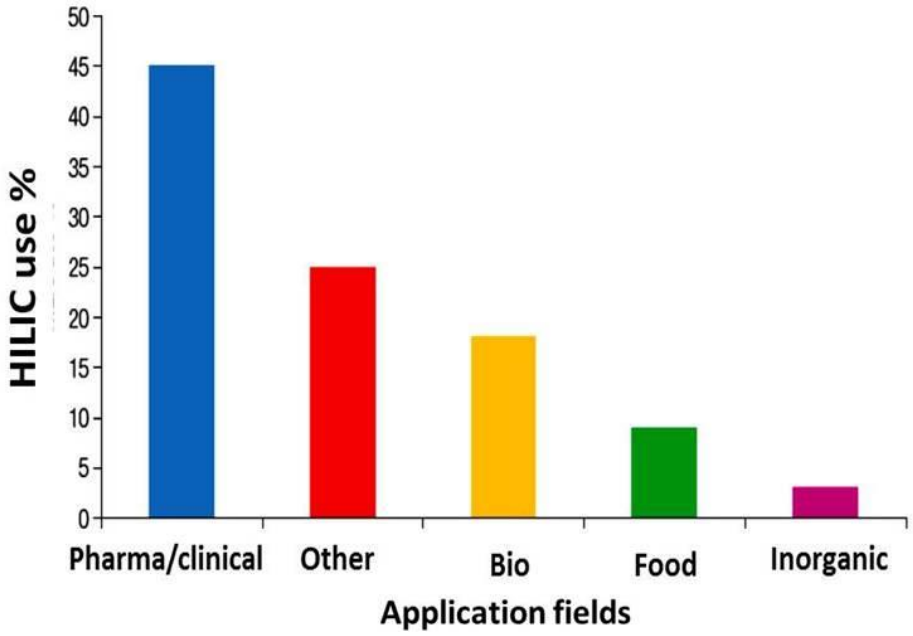


Figure 1-34: The distribution of HILIC applications.

Source: https://www.thermoscientific.com/content/dam/tfs/ATG/CMD/cmd-documents/bro/bro/chrom/lc/col/TG-21003-HILIC-Separations-TG21003-EN.pdf

The majority of HILIC applications are found in the pharmaceutical and clinical industries due to the wide variety of charges and polarity of pharmaceutically related molecules, thus analysing them using a simultaneous separation of the API and its respective counter-ion is a difficult challenge; however, HILIC is a powerful tool for the separation of hydrophobic and polar compounds even in complex mixtures [12, 26, 41]. This section will review some of the different molecules that have been successfully separated in HILIC.

1.8.1 **Analysis of Formulations**

HILIC method was applied to separate psedoephedrine hydrochloride (PSH), diphenhydramine hydrochloride (DPH) and dextromethorphan (DXH) in a cough-cold liquid formulation. The mobile phase was (95:5 (v/v)) methanol and aqueous ammonium acetate buffer. The three components were separated on a Supelcosil™ LC-Si silica column and the method was validated for quantitative determination [105]. A novel, fast and accurate HILIC technique was successfully separated and detected the active and inactive components of a mannitol injection. TSK-Gel Amide 80 column was employed with (75:25, v: v) ACN / 0.1% TFA in water using an isocratic elution. This study demonstrated the capability to separate gemcitabine, mannitol, and sodium cations. An evaporative light-scattering detector (ELSD) was applied for this study to provide a direct detection of inactive

excipients and inorganic salts lacking UV chromophores. All of the components were efficiently separated and the method was thoroughly validated for mannitol content to access the quantitative potential of the technique and the method was proved to be very robust [106].

1.8.2 **Analysis of Drug Impurities**

Drug impurities can be defined as trace amounts of compounds that can be produced from a degradation of the main API or arise as manufacturing impurities. The HILIC technique was reported to perform well for impurity detection and separation. A ZIC-HILIC column was used to develop a method for the analysis of iohexol and its impurities in order to achieve the desired separation of substances in a short analysis time. The experiment performed on HPLC with UV detection at 254 nm utilising a mobile phase consisting of ACN/ 72 mM ammonium acetate ((86.7:13.3) v/v), the pH was adjusted to 6.5 with glacial acetic acid. A successful separation was obtained for the critical peak pair (exo-and endo-iohexol) with minimal analysis time and the method was fully validated. Further, this study presented the advantage of QbD (Quality by Design) approach in HILIC method development [107]. The effectiveness of HILIC separation was demonstrated for the impurities in streptomycin (STR) and dihydrostreptomycin (DHS) which were investigated by hydrophilic interaction chromatography/electrospray ionization quadrupole ion

trap/time-of-flight mass spectrometry (HILIC/ESI-QIT/TOFMS). HILIC was performed on silica column for STR and DHS samples with isocratic elution using (30:70 v/v) 200 mM ammonium formate buffer (pH 4.5) and acetonitrile in the mobile phase [108].

A number of pyrimidines, purines and amides were used to examine the potential of HILIC retention. The study was attempted on silica and amino columns from three different manufacturers (Zorbax, Nucleosil, and YMC). The mobile phase consisted of (20:80, v/v) 25 mM potassium phosphate (pH 6.5) and acetonitrile. Separation of amides was achieved on amino and silica columns for oxamide, acetamide and formamide with varying elution order. Pyrimidines and purines, are polar compounds of significant biological and pharmaceutical interest, were separated on both silica and amino columns; however the most retention was observed on Zorbax column. In this study, both columns amino and silica showed the ability to separate amides including oxamide, acetamide and formamide under HILIC conditions; however the base-line resolution was only obtained on silica columns. Overall, HILIC using amino or silica columns offers an attractive, if not superior alternative to RP-HPLC for the determination of polar analytes in pharmaceutical matrices. The study concluded that HILIC mode can provide a high quality separation and acceptable baseline resolution for potential impurities in a drug substance [109].

Oxprenolol and its impurities were well separated on a cyanopropyl column by Al-Tannak *et al*. who used HILIC mode to analyse and identify the impurities using LC-MS [110]. A variety of HILIC columns were evaluated to separate six impurities in mildronate. Practically a ZIC-HILIC column and a bare silica gel column were compared and the study also investigated the effect of acetonitrile and buffer strength on retention times. Overall, the ZIC-HILIC column was the most fit for purpose. The method was fully validated for quantitative determination of the impurities [111]. In addition, a stability indicating method was developed for the quantitative determination of brimonidine tartrate and its degradants. The method was validated using a bare silica gel column at isocratic elusion with mobile phase consists of (92:8 v/v) acetonitrile/ammonium acetate (pH 7.0). The retention of brimonidine Increased with the increase in % of acetonitrile but any increase in the buffer strength or decrease in pH showed a decrease in retention time [112].

1.8.3 **Bioanalysis and Drug Metabolism**

Bioanalytical method validation is essential to ensure that an analytical method is fit for its intended purpose. An efficient novel chromatographic method for the determination of cidofovir, polar antiviral drug, in plasma was developed using a HILIC method. The chromatographic analysis was performed on a bare silica stationary phase using a mixture of acetonitrile:

ammonium hydrogen carbonate (pH 7.0; 20 mM) (72:28, v/v) in the mobile phase. This newly developed bioanalytical method was then fully validated according to the guidelines specified by the FDA (Food and Drug Administration) [113]. Many polar compounds including choline and acetylcholine showed a very good separation with HILIC in conjunction with bare silica stationary phase [114]. HILIC has also been applied to pharmacokinetic studies. For example nicotinic acid compounds were separated from their metabolites and detected in LC-MS using a bare silica column by Hsieh and Chen [115]. The development of HILIC methods was subject to the need to separate polar compounds that are difficult to separate using reversed phase chromatography. Neomycin, the aminoglycoside antibiotic, is a very polar compound with at least six amino groups in its structure. Oertel *et al.* [116] developed a HILIC method for the separation and analysis of neomycin in human plasma on a ZIC-HILIC column using mass spectrometry as the detection method.

A rapid LC-MS method of less than ten minutes analysis time was developed for cocaine and its metabolites in hair samples utilising a bare silica gel and HILIC mobile phase containing aqueous ammonium acetate buffer at pH 4.5. The organic solvent (acetonitrile) was varied between 82% and 40% B in a gradient elution. The method successfully retained the metabolite ecogonine-methyl-ester which is weakly retained on RP chromatography and the method fully validated [117]. HILIC was

successfully detected and separated cocaine from its metabolites in human tissue by gradient elution of an acetonitrile and 2 mM ammonium acetate buffer eluent [118]. A ZIC-HILIC column was used by Khreit *et al.* [99] and was able to detect 17 metabolites of methyl-methcathinone hydrochloride in rat hepatocytes. The HILIC column combined with a mass spectrometer was very useful in this type of study because most metabolic pathways produce compounds which are more polar than the parent compound [119].

1.8.4 **Other Applications of HILIC**

HILIC with mass spectrophotometry detection has shown great advantages in pharmaceutical development and drug discovery. Koh *et al.* [120] reported a successful HILIC method for the separation of the polar haemostatic agent, dencichine, from a traditional Chinese medicine. This was followed by a HILIC method for the determination of a neurotoxic agent from *Lathyrus sativus* seed, known as grass pea. Both methods were carried out using un-modified silica in HILIC mode and were fully validated [120]. HILIC was also a powerful tool in the analysis of polar compounds in food and drink analysis. Schlichtherle-Cerny *et al.* [121] reported a separation of many polar dipeptides and amino acids in parmesan cheese including glutamic acid and arginine utilising HILIC-MS on an aminopropyl stationary phase. Another example is HILIC detection method of carbohydrate rich drink for the separation of methionine and taurine. The

method was very simple and showed very good precision, accuracy, linearity and low limit of detection [122].

HILIC proved to be suitable for the analysis of microcystins. Dell'Aversano *et al.* developed a robust HILIC method that separated and detected many of the cyanobacterial toxins including saxitoxin, cylindrospermopsin and anatoxin- in algal samples with mass spectrometry detection and similar results were obtained from two different laboratories using different instrumentation which indicates that the method is robust [123]. The first application of HILIC technique of carbohydrates was useful in the analysis of oligosaccharides derived from glycoproteins on amide silica column [124]. The second application of HILIC after the carbohydrate analysis is the analysis of peptides and it was very effective due to the high polarity of these compounds. For example the separation of the phosphorylated peptides from Y-32P-ATP and inorganic 32P which were initially isolated by using solid phase extraction (SPE) and then HILIC separation was used on a poly (2-hydroxyethyl aspartamide) stationary phase [20]. HILIC showed good functionality in the analysis of proteins and protein digests that contain one or more polypeptides. Linder *et al.* [125] presented the potential utility of HILIC in the separation of post-translationally acetylated histones by using a mixed mode stationary phase including weak cation-exchange in HILIC mode.

In metabolomics, the application of RP chromatography is challenging due to the fact that many bio-fluids are mainly aqueous and thus likely to be rich of polar compounds that are poorly retained on RP stationary phases. HILIC chromatography was applied in the metabolomic profiling by Al-Barraty *et al.* [126] to study the effects of the xanthine oxidase inhibitor allopurinol on wild type Drosophila melanogaster. The study utilised a ZIC-HILIC stationary phase in combination with Fourier transform mass spectrometry. The results concluded that HILIC was a powerful technique to fulfil metabolomics purposes and detected metabolomics changes.

1.9 Instrumentation

1.9.1 High Performance Liquid Chromatography Instruments (HPLC)

High-performance liquid chromatography, **Figure 1-35**, is a technique in analytical chemistry designed to separate components in a mixture as well as quantifying them. The process starts with placing the sample on a tray for automatic injection into the column. Solvent is continually pumped through the column, and the separated compounds are quantified by a detector as they leave the column. The interaction between the analyte and the stationary phase depends on the chemical/physical characteristic of the analytes and the properties of the stationary and the mobile phase. The chemical properties of the analyte can determine the

most suitable detector to be used which includes the ultraviolet (UV) detector, fluorescence detector (FLD), refractive index detector (RI), evaporative light scattering detector (ELSD) or mass spectrometry (MS) detector. The resulting signal is plotted against time which is the chromatogram and it is used in quantification or/and identification purposes. The instrumentation is computer controlled which allows for data storage and further data interrogation, and the computer can also generate a final analysis report from the sample [81].

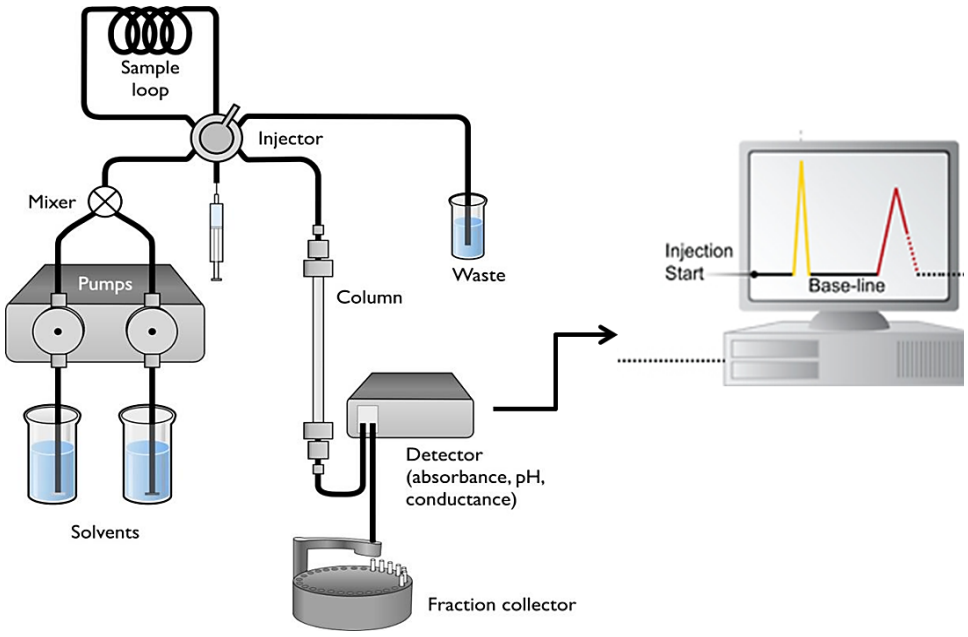


Figure 1-35: Schematic diagram of a HPLC system.

Apart from this automation of entire process, HPLC is characterised by the use of high pressure pumps for faster separation, re-useable and more

effective columns for enhanced separation, and a better control of the overall process for more precise and reproducible results. A UV detector is mostly used for compounds that have a chromophore because it is economical and easy to use. Refractive index or ELSD can be applied for compounds that are not detectable by the UV. For compounds that have a fluorophore, a fluorescence detector can be employed. The most powerful detector which can be used almost for all compounds is mass spectrometer thus LC-MS offers the highest sensitivity and selectivity and provide additional information such as molecular formula for analytes [3].

1.9.2 **Mass spectrometry (LC-MS)**

All general-purpose HPLC detectors, such as UV-absorption, fluorimetric, evaporative light-scattering, etc., can be used with HILIC separations. However, one of the main reasons for increasing interest in HILIC was its excellent suitability for direct coupling with MS or MS/MS detection.

Mass spectrometry is a technique that used to measure the characteristics of individual molecules, it converts the molecules into ions so that they can be moved about and manipulated by external electric and magnetic fields and detected in their ionised form. The main components of the mass spectrometer are ionisation source, ion separation device and detection device. The analyte firstly pass through the ionisation source which is

responsible for producing either positive or negative ions from the analyte. The ionised analyte is then transferred to the analyser which is held under high vacuum and which controls the movement of the ionized compounds. In the analyser, the separation process occurs based on the molecular weight to charge ratio of the ionized compounds and it is known as (m/z) which is the ratio of mass (m) to charge (z). Finally the identification of the separated ions is carried out and the signals transferred to an output [5], the process is explained in **Figure 1-36**. Animation video of how the mass spectrophotometry functions can be found in https://www.youtube.com/watch?v=NuIH9-6Fm6U.

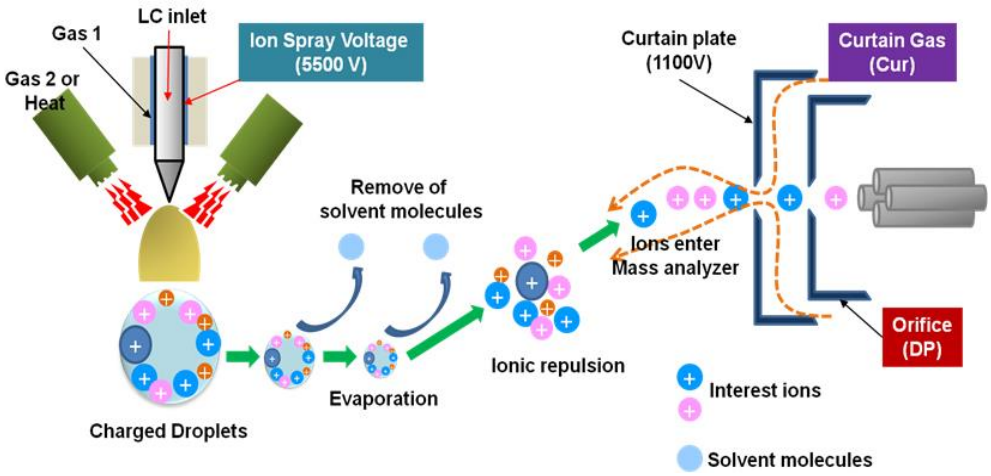


Figure 1-36: The process of Mass Spectrometry, (Cur) is counter flow of heated gas (O2 or N2), which shrinks the droplets and carries away the uncharged material the charged droplets are attracted toward the capillary orifice (DP). Online access [22/04/2017] http://emass.co.kr/y4/bbs/board.php?bo_table=edu_ms&wr_id=53[

When using RP-HPLC/MS with electrospray ionisation (ESI), low retention of polar samples on an alkyl silica column may result in poor resolution and unfavourable matrix effects. A highly aqueous mobile phase can also negatively affect the signal intensity and stability. However, the high organic content mobile phases that typically used for HILIC a separation is ideally suited the sensitive LC-MS analysis of water-soluble polar compound, because the organic solvent is much more volatile. Therefore, spraying conditions will improve and enhance the efficiency of de-solvation and ionisation in the ESI ion source, providing thus highly increased sensitivity over RP conditions [127 - 131]. Obviously, this is one of the major reasons for increasing interest in HILIC technique.

The ionisation process in the MS is influenced by several factors such as the analyte type, the buffer in the mobile phase and the type of the ion source mechanism. Some examples of the type of ion source used are chemical ionisation, electron ionisation and electrospray ionisation (ESI). The ionisation mode which is applied for the majority of biochemical compounds is ESI [9] shown in **Figure 1-37**.

Electrospray ionisation (ESI) is known as a soft ionisation method. **Figure 1-37** displays the spray of sample into the source via a fine capillary needle, which is held at a very high voltage to charge the compounds in the solvent. A Taylor cone is formed when the charged spray exits the tip of the

needle and ions of the opposite charge are pulled toward the capillary leaving the ions of the same charge within the spray. After that, a Columbic repulsion force occurs due to the high concentration of ions of the same charge within the droplets that make up by the spray can break up further. Nitrogen/ Oxygen gas flow and the heat assess this process to evaporate the solvent until single charged molecule is obtained [132].

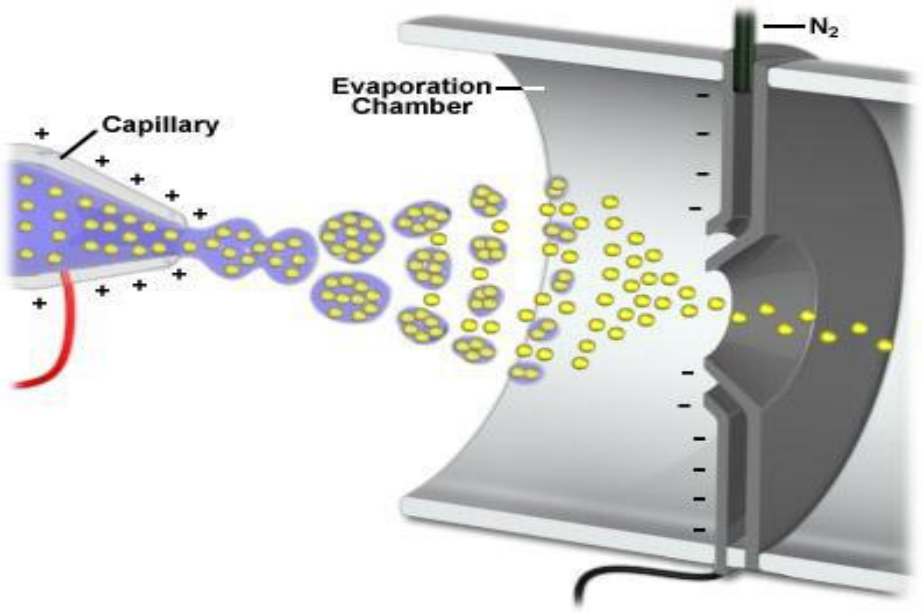


Figure 1-37: The process of electrospray ionisation (ESI).

1.10 Research Goals

The main interest of the work in this thesis is the hydrophilic interaction chromatography (HILIC) and the mechanism that causes the retention for either basic or acidic compounds. A better understanding of the mechanisms that govern the retention of solutes in HILIC chromatographic system could provide powerful advantage for achieving a desired separation and to control the analysis. Although there has been a dramatic increase in interest in the application of HILIC conditions and stationary phases, there is still not sufficient information regarding the retention in HILIC mechanism. Therefore, this project aims to explore the retention of different molecules under HILIC conditions and also evaluate HILIC retention on some commercially available hydride-based stationary phases. Since the mechanism of separation in HILIC is complex and that stationary phases give a considerable contribution to retention, understanding the mechanism of separation in HILIC mode might lead to better selection of optimal HILIC stationary phases and conditions. In this study HILIC was applied in the analysis of metabolites and impurity profiling in addition to the separation of regioisomers. The overall study focused on:

1- Better understanding of retention characteristics of analytes in HILIC to increase the scope of possible applications of liquid chromatography.

2- Comparing the chromatographic properties of silica gel and Type C silica- hydride modified silica gels.

3- Examining HILIC retention in commercially available *hydride-based* columns including Cogent Silica-C™, Cogent Phenyl Hydride™ and Cogent™ UCD-Cholesterol stationary phases.

4- Evaluate the contribution of stationary phase in the overall retention at high organic content in the mobile phase under HILIC conditions and assess the performance of a silica gel stationary phase in HILIC by applying the system suitability tests (SST) parameters.

5- Apply HILIC in the metabolomics analysis and the impurity profiling in addition to regioisomers separation of a drug of abuse.

Chapter 2

A comparison of silica C and silica gel in hydrophilic interaction mode: the effect of stationary phase surface area

2 A comparison of silica C and silica gel in hydrophilic interaction mode: the effect of stationary phase surface area

2.1 Introduction

The popularity of HILIC is based on its wide applicability and convenient operation. The advancement and applications of hydrophilic liquid chromatography were discussed in detail in the previous chapter. The demand to separate polar and non-polar compounds in a single analysis expanded the application of HILIC rapidly [24] particularly in metabolomic profiling where many biomolecules are polar and do not retain well on reversed phases. In HILIC the mobile phase is highly organic containing (>70% solvent, typically acetonitrile) a small percentage of aqueous solvent/buffer. Although the term HILIC suggests partitioning of substances into an adsorbed water layer on a polar stationary phase [1], the interactions involved are much more complicated than this. Thus it is necessary to re-evaluate the concept of HILIC as simply a form of partitioning chromatography [12, 24]. It has been stated that between 30-50% of the silanols on the silica gel surface remains un-bonded even after the end-capping process [56] as a consequence they can contribute to the HILIC process because HILIC stationary phases are based on silica gel [133]. Previous work showed that even alkyl columns would exhibit HILIC-like interactions [110] when high organic mobile phase was applied. The mechanism of HILIC even on the simplest phase silica gel remains poorly understood [12, 24]. Even in the case of bare silica gel there is a need for

better understanding of the retention mechanism in HILIC mode so that it can be fully exploited.

McCalley and Neue measured the thickness of the water layer on silica gel in HILIC mode using the hydrophobic probes benzene and toluene, which were excluded from the water filled pores of the two stationary phases studied. Using this method plus pycnometry they concluded that the % of the pore volume of a particular packing occupied by water under HILIC conditions varied between 13%, with acetonitrile/water (70:30) in the mobile phase, to 4% with acetonitrile/water (95:5) in the mobile phase [25]. Although they did not state it, from the results reported in their paper it would appear that the greater the surface area of a silica gel the larger would be the volume of the water pseudo-stationary phase. Their measurements were carried out with no pH modifier in the mobile phase and it is likely that with pH control the volume of adsorbed water might change with the degree of ionisation and consequent solvation of silanol groups. Gritti *et al.* used pyridine as a test probe in combination with frontal analysis and concluded that the maximum absorption excess of water in acetonitrile water mixtures occurred with 80% acetonitrile in the mobile phase [134]. Again no buffer modifier was used in that study. Overall the HILIC mechanism, even on a simple phase like silica gel, is complex and contributors to the effect include: ionic attraction and repulsion, the effect of % organic solvent on the pK_a values of weak acids

and weak bases and on the ionisation of buffer salts [4] and the effect of the concentration of buffer salts in the aqueous layer associated with the silica gel surface. Thus it would appear there is still much to explore with regard to the HILIC mechanism. A recent study used computer based simulation trying to understand the interaction between the mobile phase and the silica gel surface [135]. From the simulation it was observed that the layer water within *ca* 0.5 nm of the silica gel surface was not greatly affected by the composition of the bulk mobile phase, whereas if an absorption isotherm was plotted for water enrichment within 2 nm of the surface the maximum absorption excess for water occurred at around 5:95 water/acetonitrile. Thus, apart from pure water, there may be water enriched layers which may contribute to retention in HILIC mode. In the water enriched layers there is some acetonitrile content and this would be likely to affect the degree of ionisation of acidic and basic test probes and of buffer salts.

Due to the unique selectivity and versatility of chemically modified silica hydride stationary phases, there have been an increasing number of studies which utilised this type of phases in the past several years. The objective of hydride stationary phases with dual properties is to achieve separation of both polar and non-polar components in a single analysis with optimal performance. Due to an increasing demand in the fields of drug discovery, proteomics, and metabolomics to analyse numerous samples with a wide range of polarities, there is a need to understand the

separation mechanism in order to control factors and introduce better separations. In recent work Bawazeer *et al*., compared a 100 Å silica gel column and type C silica columns using a range of acidic basic and neutral test compounds [43] and found that the unmodified type C silica appeared to be more retentive than the silica gel column particularly for basic test compounds. The study were unable to completely explain the observations and it was apparent that neither the Si-H surface nor indeed the Si-OH surface were fully understood and there has not been a comprehensive exploration of the difference between the silanol bearing surface of silica gel and the type C silica surface. Thus the primary objective for the current study is to investigate the main factors governing retention on a silica C column in comparison with bare silica gel. The study explored the retention behaviour of three quaternary ammonium test probes, **Figure 2-1,** with different alkyl substitution in mobile phase containing tetramethylammonium acetate (TMAA) modifier at pH 6.0.

The quaternary ammonium system was used to maintain the ionisation of the test probes and the buffer ions constant at high organic content mobile phase, thus the only part which will be affected by the ionisation change is the silanol groups on the silica surface. The study also applied the method of McCalley and Neue to estimate the thickness of the water layer on the different stationary phases at 80 and 90% acetonitrile and went on to examine the behaviour of the columns with acidic and neutral probes.

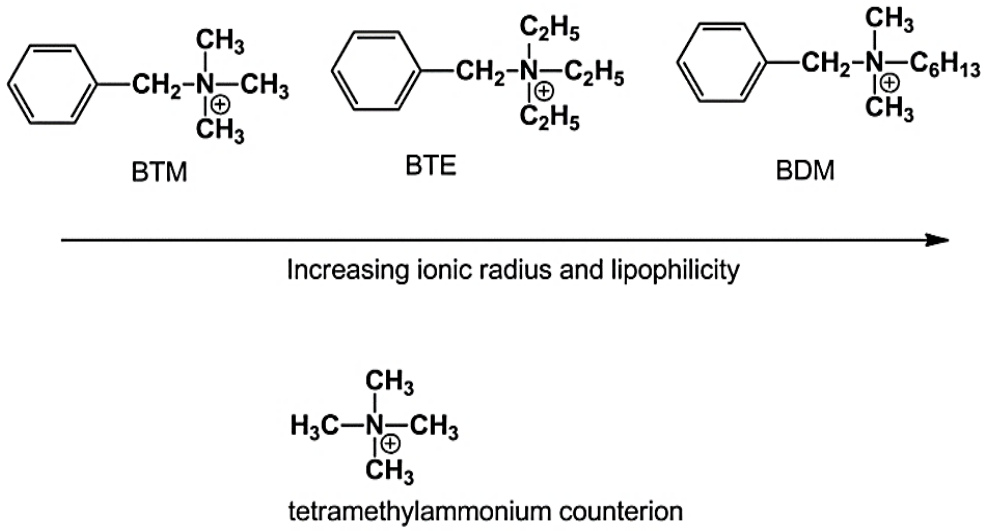


Figure 2-1: Quaternary ammonium test probes and tetramethylammonium (TMAA) competing counterion.

2.2 Method and Materials

2.2.1 Chemicals

HPLC grade acetic acid and acetonitrile were obtained from Fisher Scientific, Loughborough, UK and anhydrous acetonitrile (< 0.001% water) and molecular sieve were obtained from Sigma Aldrich, Dorset, UK. Tetramethylammonium hydroxide, benzyltrimethylammonium chloride (BTM), benzyltriethylammonium chloride (BTE) and benzyldimethylhexyl ammonium chloride (BDM), p-hydroxybenzoic acid, uridine, pentylbenzene and toluene were obtained from Sigma Aldrich, Dorset, UK.

2.2.2 Buffer preparation (stock solution)

Stock solutions of 100 mM and 200 mM of tetramethylammonium acetate buffer were made up for the preparation of mobile phases by dissolving respectively 3.00 g and 6.00 g of acetic acid in 300 ml of water and then were adjusted to pH 6 with tetramethylammonium-hydroxide. The stock solutions were then made up to volume of 500 ml with water.

2.2.3 Mobile phase preparation

The mobile phases for studying the effect of percentage of acetonitrile on retention of the test probes were prepared by mixing a fixed volume (50 mL) of 100 mM tetramethylammonium acetate buffer with varying volumes of water and acetonitrile to give the required percentage of modifiers in a 500 mL final volume.

Different mobile phase concentrations were used to study the effect of ionic strength at 80% and 90% acetonitrile. At 80% acetonitrile the mobile phases were prepared by diluting the 100 mM or 200 mM tetramethylammonium acetate buffer (pH 6) to 5, 10, 15 and 20 mM in a 500 mL volumetric flask and adding a fixed proportion of 400 mL or 450 mL of acetonitrile and varying amounts of buffer and water to give the required final ionic strength.

2.2.4 **Analytical HPLC columns**

Figure 2-2 shows the chemistry surface of Type B and C silica based columns. The study utilised Cogent hydride Silica C 100 Å (4 µm, 150 mm x 4.6 mm i.d.), Kromasil 60 Å and Kromasil 100 Å 5 µm silica gel (150 mm x 4.6 mm i.d.), ACE5 100 Å and 300 Å, 5 µm silica gel (150 mm x 4.6 mm i.d.) that were purchased from HiChrom Ltd., Reading, UK. In addition, the retention behaviour was studied on silica hydride (50 mm x 2.1 mm i.d) that was prepared especially for this study by removing silica gel from a commercially packed 100 Å ACE column, using a previously described method by Pesek [45] and was slurry packed into the 5 cm column. This column was provided by Dr. Melvin Euerby of Hichrom Ltd. The void volume (V_0) values for the columns were estimated by injecting toluene after the columns had been flushed with anhydrous acetonitrile, which was stored over molecular sieve, for 100 minutes at 1 $mLmin^{-1}$. The manufacturers' data for the columns is shown in **Table 2-1**.

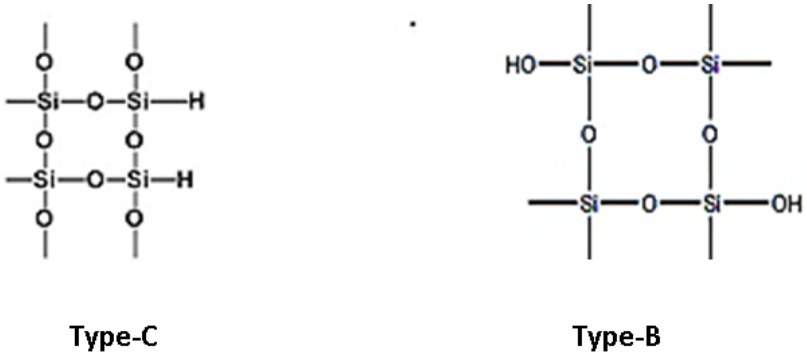


Figure 2-2: The chemical surface structure of Type-B silica and silica hydride (Type-C).

Table 2-1: Manufacturers data for the columns tested, (*) information not sated by manufacturers, (www.hichrom.co.uk).

Column (Å)	Particle Size (µm)	Pore Size (Å)	Surface Area (m^2/g)	V_0 (ml)	Pore Volume mg/mL	Packing density g/mL
ACE 100	5	100	300	2.1	*	*
ACE 300	5	300	100	2.1	*	*
Kromasil 60	5	60	540	2.3	1.2	0.45
Cogent Silica C	4	100	350	1.8	*	*
Kromasil 100	5	100	320	2.1	0.9	0.5

2.2.5 **HPLC Instrumentation**

HPLC analysis was carried out on a ThermoFinnigan HPLC system consisting of a P 4000 pump, P6000 diode array detector and an AS 4000 autosampler (ThermoFisher, Hemel Hempstead, UK) and Kontron 480 column oven **(**Kontron Instruments**,** Munich, Germany**)**. The mobile phase compositions were mixed off-line in the proportions required as described in **(section 2.2.3**) and the HPLC system was used in isocratic mode. The injection volume was 5 µL at 1 mL/min. flow rate and the column was kept in a column oven at 22˚C. The diode array detector was set to monitor specifically 263 nm and the full PDA range 200-350 nm.

2.2.6 Samples preparation

Samples (**Figure 2-1**) were prepared as stock solutions at 0.1% w/v in methanol and diluted to a concentration of 100 µg mL^{-1} with the appropriate mobile phase starting composition.

2.3 Results and Discussion

2.3.1 Analysis of Quaternary Ammonium Compounds

Previous studies found that high organic solvent and the associated lowering of dielectric constant affected the pK_a values of weak acids and bases, by increasing them and lowering them respectively [4, 136]. For this reason, quaternary ammonium test probes with a positive-charge were chosen to study the effect of organic solvent on retention in HILIC mode as these probes are pH-independent and will be ionised to the same extent no matter how high the levels of organic solvent. Also the permanently-charged competing counter-ion in the mobile phase, tetramethylammonium, was used to avoid any effect on its percentage ionisation due to the organic solvent content in the mobile phase. Therefore, the only charge which could be affected by the % organic solvent was that on the silanol groups within the silica gel stationary phases. The effect of % acetonitrile on the ionisation of silanol groups is uncertain exactly, there might be little effect if, as suggested by Melnikov *et al.* [135], there is a 0.5 nm water layer next to the surface of the silica gel

which is independent of the mobile phase composition. This layer is sufficiently thick for the silanol groups to have pK_a values in the normal range, and hence pH dependent ionisation, and be unaffected by the content of acetonitrile in the mobile phase. The order of charge density on the test probes is BTM> BTE> BDM and their lipophilicity is the reverse of this order (Log P values calculated by Chemdraw: -2.44, -1.05 and 1.07 respectively).

2.3.2 Plots of Log k against percentage of acetonitrile in mobile phase

Figure 2-3 shows the plots of log k for the test probes against % ACN for 60 Å and 100 Å Kromasil silica gel columns, a 300 Å ACE silica gel column and a Cogent Silica C column. In all cases the most retained test probe at 20% ACN is BTE. If the mode of retention at 20% ACN was predominantly ion exchange then it would have been expected that the more charge dense BTM probe would have the strongest retention.

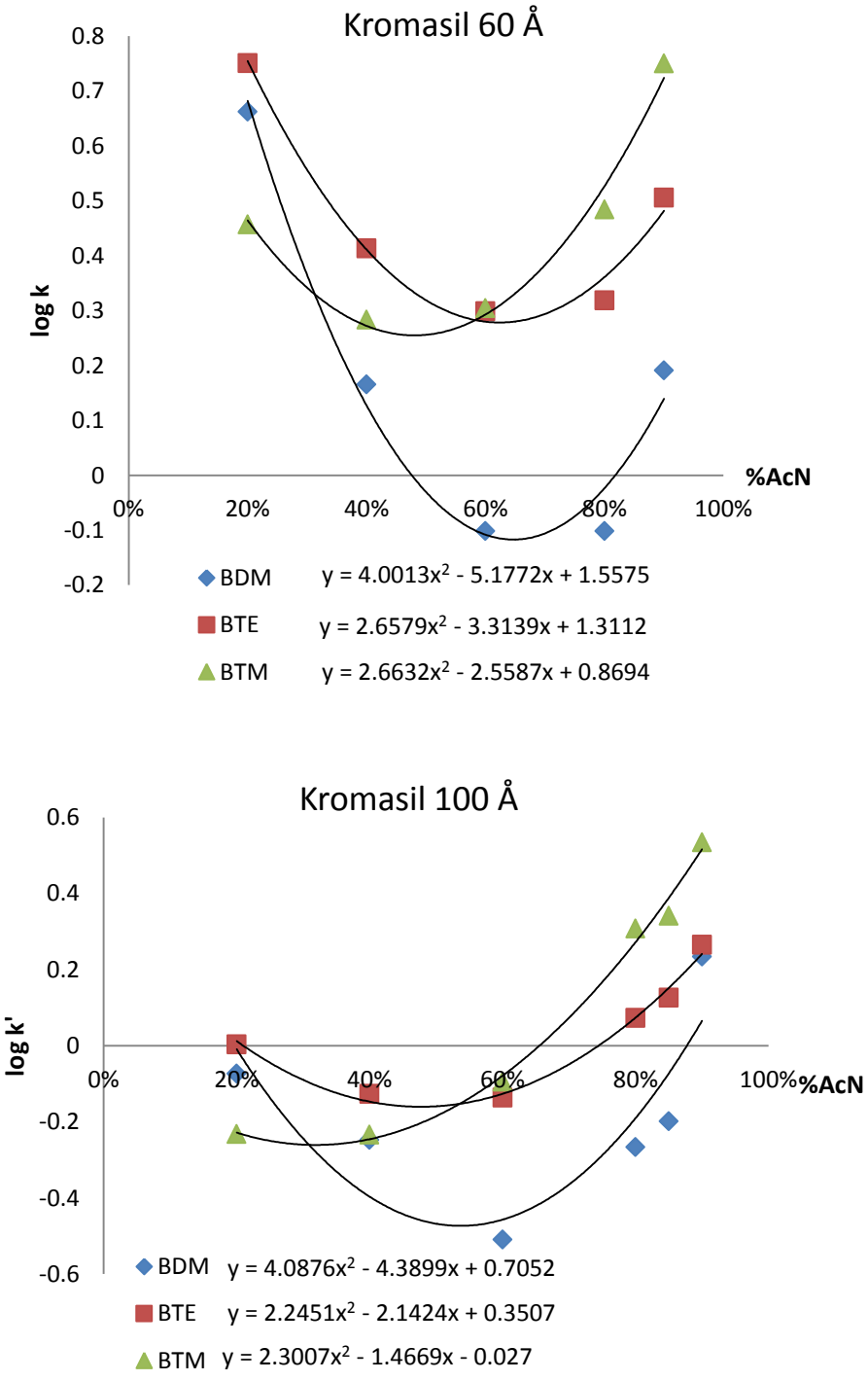


Figure 2-3: Plots of log k against % acetonitrile for the test probes (BTM▲, BTE ■, BDM ♦) on Kromasil 60, Kromasil 100 (all 4.6 mm x 150 mm) at constant buffer strength 10 mM TMAA .

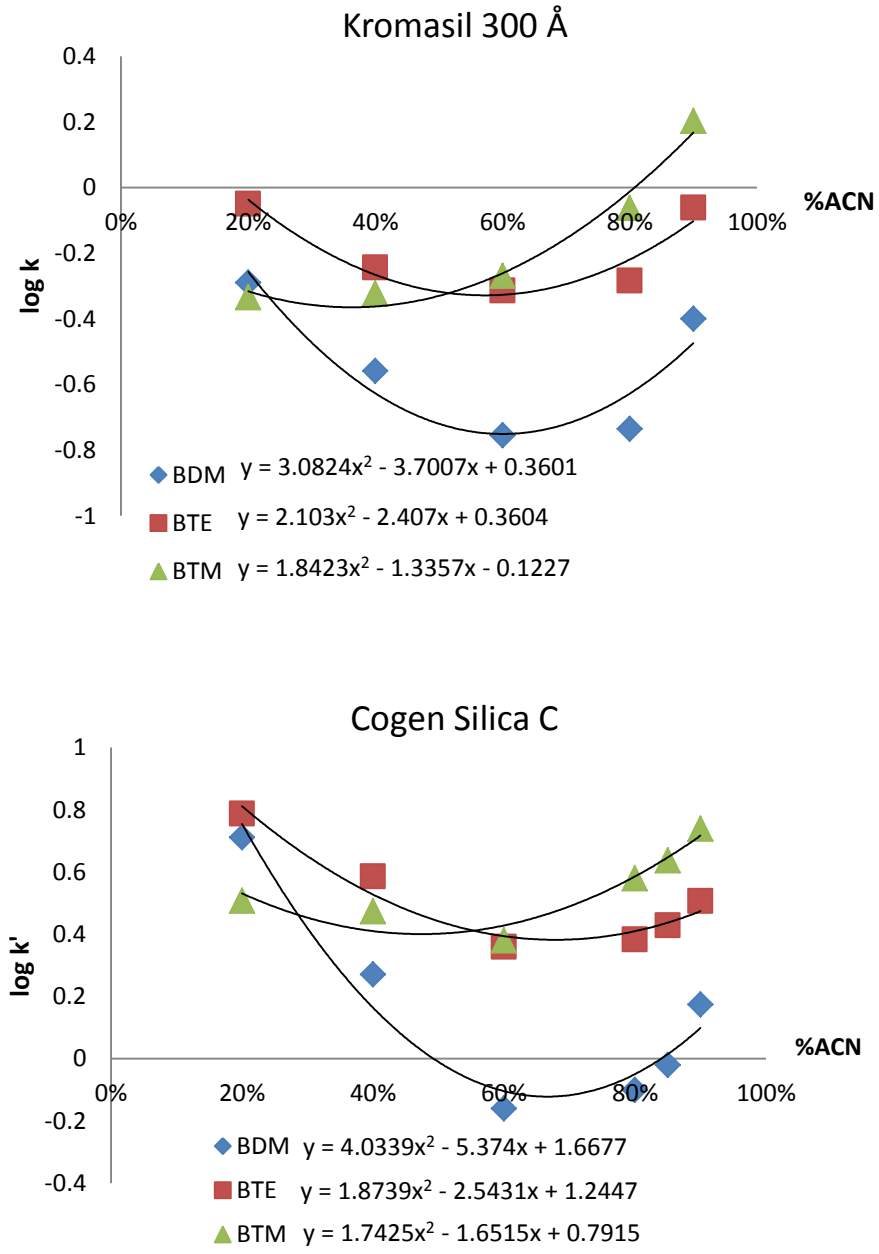


Figure 2-3 continued: Plots of log k against % acetonitrile for the test probes (BTM▲, BTE■, and BDM♦) on ACE 300 and Cogent Silica C (all 4.6 mm x 150 mm) at constant buffer strength 10 mM TMAA.

Due to the fact that the interaction between the lone pairs on the oxygen and the unoccupied d-orbitals of the flanking silicon atoms is very strong, it is unlikely for the siloxane bonds in the bare silica gel to hydrogen bond which qualifies silica gel to be lipophilic [52]. Thus it is possible to propose that a major component in the retention of the test probes at 20% ACN must be lipophilic interaction with the siloxane bonds on the silica gel surface. The retention of the least charge dense of the test probes BDM is almost as strong as BTE; this would suggest that the overall retention of these test probes at 20% ACN is due to a combination of lipophilicity and ion exchange. However, the BDM exhibits weaker ion exchange than the BTE due to its larger size, thus having less retention than BTE but strong enough lipophilic interaction to retain longer than the more charge dense BTM. Another indication of the lipophilic interaction at 20% ACN is the additional retention of BTE over BTM as if the ion-exchange interaction is the major at 20% ACN then the retention of BTM, which has the highest charge density, should be greater than those of BTE.

If the difference in the log k values for BTE and BTM at 20% ACN is taken as being approximately indicative of lipophilic interaction it can be seen from **Figure 2-3** that the lipophilic interaction is greatest for the high surface area Kromasil 60 Å silica gel (540 m^2/g) and for the Cogent Silica C column (350 m^2/g). As the % ACN increases in the mobile phase, the retention of the test probes initially falls as lipophilic interaction is reduced. Thus the minima in the plots of log k against % ACN at around 60% ACN indicate the

point where ion exchange becomes mainly responsible for the retention of the test probes. Above 60% ACN the hydrophilic interaction begins to exert an effect as illustrated in **Figure 2-3** the most hydrophilic and most densely charged probe, BTM, was the most sensitive to the establishment of HILIC layer which, as proposed by Melnikov *et al* [135], is composed of regions with varying degrees of water enrichment as well as fixed largely unvarying layer of water close to the surface of the silica gel. The retention of the other two probes also increases above 60% ACN although the retention of the BDM is generally lower at 90% ACN than at 20% ACN again indicating that it is exhibiting relatively weak ion exchange and, at 90% ACN, very little hydrophilic interaction. Taking the retention of BTM as being most indicative of the development of a HILIC layer, the 100 Å and the 300 Å ACE silica gel columns showed the most marked increase in log k up to 90% ACN although overall the 60 Å Kromasil column gives the strongest retention of BTM at 90% ACN.

2.3.3 The effect of ionic strength on retention in HILIC

The effect of buffer concentration was investigated in order to distinguish the relative contribution of HILIC and ion exchange to the retention on different columns and to evaluate the predominant retention mechanism. Therefore, the molarity of the TMAA buffer was varied from 5 to 20 mM within the mobile phases containing 80 % and 90% v/v ACN. **Table 2-2**

shows the retention times of the three probes at different ionic strengths on the five columns with 80 or 90% v/v acetonitrile in the mobile phase. It is clearly seen that as the ionic strength in the mobile phase increased, the retention time decreased in all tested columns with the Kromasil 60 Å column being the most retentive phase at 80 and 90% ACN.

Figure 2-4 displays the plots of log k against 1/mM for the tested columns at 80 and 90% ACN. It was expected to obtain linear plots if the retention mechanism is due to a single parameter as is accepted in models proposed for reversed-phase partitioning; however the plots are curved and it was possible to fit second order polynomial curves through the points indicating that a mixed mode retention mechanism was in operation. The r^2 values for the curves fitted according to second-order polynomials were > 0.99 apart from 5 out of the 36 curves, where r^2 was between 0.969 and 0.989.

Table 2-2: Retention times (minutes) for the test probes on five columns at 90 and 80 % acetonitrile with varying buffer strength.

	90% acetonitrile				80% acetonitrile			
Kromasil 60								
mM	5	10	15	20	5	10	15	20
BDM	8.9	5.5	4.6	3.9	6.0	4.0	3.4	3.0
BTE	15.7	9.2	7.5	6.2	11.5	7.0	5.8	5.0
BTM	24.6	14.6	11.9	9.7	14.6	9.1	7.4	6.4
Kromasil 100								
BDM	7.4	4.8	3.9	3.5	4.9	3.5	2.9	2.7
BTE	12.2	7.6	5.9	5.2	8.6	5.6	4.6	4.0
BTM	19.8	12.1	9.3	8.1	11.5	7.7	5.9	5.2
ACE 100								
BDM	5.5	3.9	3.4	2.9	4.3	3.2	2.8	2.6
BTE	8.5	5.8	4.8	4.0	7.0	4.6	3.9	3.5
BTM	12.4	8.7	7.1	5.8	9.4	6.1	4.9	4.3
ACE 300								
BDM	3.7	2.8	2.6	2.4	3.0	2.4	2.3	2.2
BTE	5.3	3.7	3.3	2.9	4.2	3.1	2.8	2.6
BTM	7.9	5.2	4.5	3.9	5.0	3.8	3.3	3.0
Cogent Silica C								
BDM	6.0	4.2	3.6	3.1	4.7	3.4	2.9	2.6
BTE	12.1	7.2	5.8	4.9	8.9	5.7	4.7	4.1
BTM	19.7	11.0	9.2	7.4	11.9	7.8	6.1	5.4

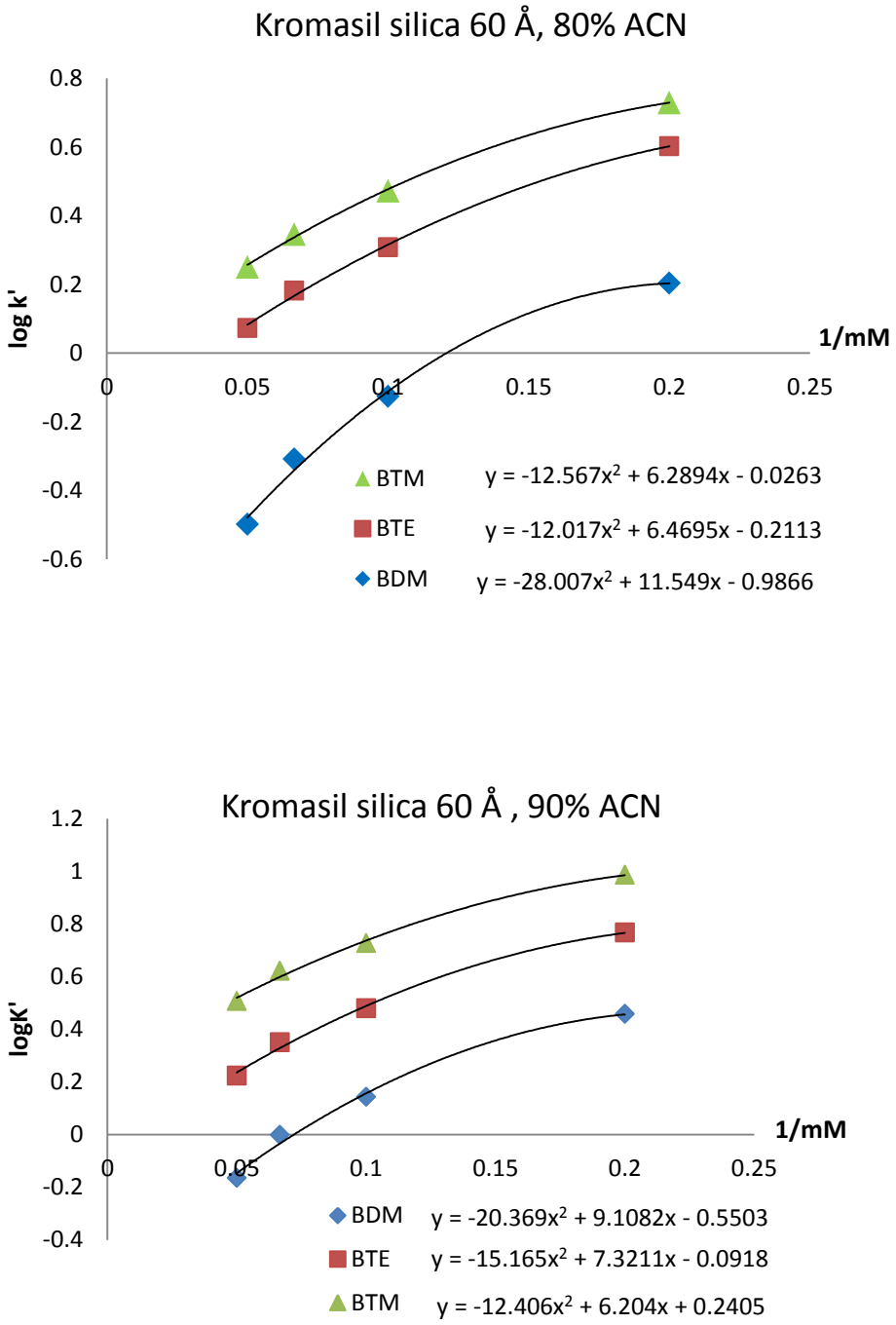


Figure 2-4: Plots of log k against the inverse of the buffer strength for the test probes (BTM▲, BTE ■, BDM ♦) on Kromasil 60 Å and Cogent Silica C columns with 80% ACN and 90% acetonitrile as the mobile phase modifier.

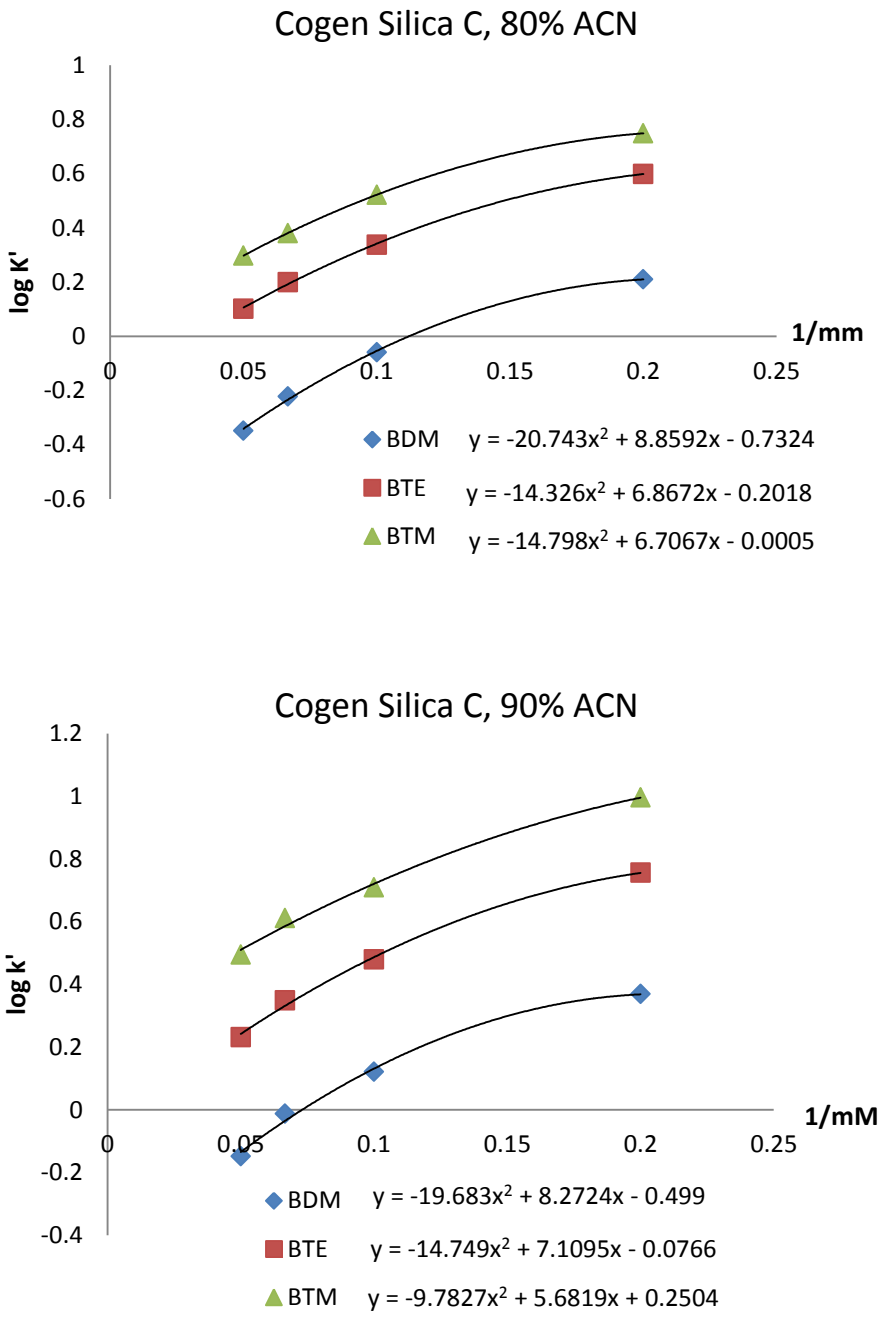


Figure 2-4 continued: Plots of log k against the inverse of the buffer strength for the test probes (BTM▲, BTE■, BDM♦) on Kromasil 60 Å and Cogent Silica C columns with 80% ACN and 90% acetonitrile as the mobile phase modifier.

According to the hypothesis proposed by McCalley [32] the intercepts for these retention plots with the Y-axis would occur with infinite ionic

strength in the mobile phase where there is no possibility of ion exchange interaction between the stationary phase and the test probes. Thus because of the absence of ion exchange interactions at infinite ionic strength the intercepts indicate the contribution of HILIC to the overall retention [32, 133].

An alternative approach has been proposed for estimating the relative contributions from ion -exchange and HILIC which involves plotting log of ionic strength against log k [137]. If a slope of -1 is obtained then the interaction can be regarded as being due entirely to ion exchange. The slopes for these plots are shown in **Table 2-3** and, since the slopes are largely > -1, they essentially reflect the same information obtained from the binomial plots which suggested that the interaction with the stationary phases is a mixture of ion exchange and HILIC.

Table 2-3: Intercepts for the polynomial curves fitted through the plots of log k against 1/ionic strength (mM) and the slopes obtained for plots of log k against log (mM).

Column	Analyte	Y intercepts for curves at 90% ACN	Intercepts for curves at 80% ACN	Slope of log k plotted against log mM at 90% ACN	Slope of log k plotted against log mM at 80% ACN
ACE 100 Å	BTM BTE BDM	-0.0974 -0.434 -0.876	-0.312 -0.511 -1.15	-0.724 -0.856 -0.984	-0.854 -0.920 -1.11
ACE 300 Å	BTM BTE BDM	-0.346 -0.756 -1.33	-0.693 -0.959 -2.14	-0.822 -0.944 -1.18	-0.826 -0.992 -1.68
Kromasil 60 Å	BTM BTE BDM	0.241 -0.0918 -0.550	-0.0263 -0.213 -0.987	-0.782 -0.889 -1.01	-0.793 -0.874 -1.14
Kromasil 100 Å	BTM BTE BDM	0.166 -0.173 -0.563	-0.1902 -0.371 -1.10	-0.788 -0.871 -0.968	-0.812 -0.885 -1.15
Cogent Silica C	BTM BTE BDM	0.250 -0.0766 -0.499	-0.00050 -0.202 -0.732	-0.817 -0.862 -0.843	-0.756 -0.827 -0.923
ACE Silicon Hydride based on 100 Å	BTM BTE BDM	-0.166 -0.504 -0.916	-0.636 -0.975 -4.01	-0.692 -0.7685 -1.02	-0.933 -1.15 -1.17

The overall percentage contribution of HILIC to the retention factors for the probes was estimated from the intercepts which obtained from the polynomial plots for each buffer-strength as shown in **Table 2-4**. From this data it can be clearly seen that increasing the ionic strength increases the percentage of HILIC contribution to the overall retention and this varies

between about 15% at 5 mM buffer strength to *ca* 50% or over at 20 mM buffer strength. In addition, the data in **Table 2-3** show no significant variation in the contribution of HILIC to retention between either the three probes or between the different columns at different buffer strengths, in most cases, indicating some link between hydrophilic partitioning and ion-exchange interaction. This is clearest at 20 mM, where the RSD overall percentage contributions from HILIC for all the probes run on all the columns is ± 16 %. Furthermore this suggests that hydrophilic partitioning increases or decreases in proportion to the ion-exchange capacity of a stationary phase. The degree of correlation between k values of the probes at 90 % ACN and the surface area per gram for the different stationary phases is shown in **Figure 2-5** and indicates that retention increases with stationary phase surface area for all the probes.

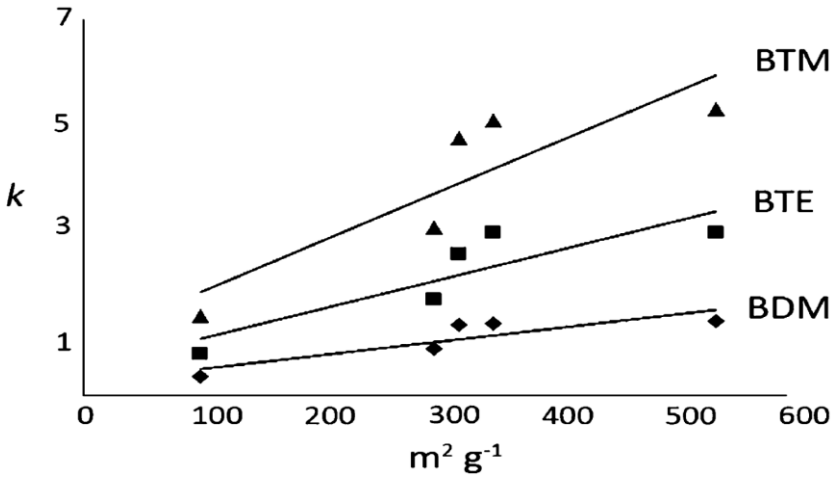


Figure 2-5: Plot of retention times for the test probes (BTM ▲, BTE■, BDM ♦) on five columns, with acetonitrile/10 mM TMAA buffer (90:10) as the mobile phase, against silica gel surface area.

Table 2-4: The variation in the percentage contribution of HILIC to the overall capacity factor for the different columns estimated from the intercepts of the polynomial plots of log k against 1/ionic strength.

	90% acetonitrile				80% acetonitrile			
Kromasil 60 Å								
mM	5	10	15	20	5	10	15	20
BDM	9.8	20.2	28.1	40.9	6.3	13.4	20.4	31.4
BTE	13.9	26.9	36.2	48.4	15.5	30.5	40.7	52.4
BTM	18.0	32.6	41.7	54.2	17.5	31.7	42.3	52.7
Kromasil 100 Å								
BDM	10.9	21.1	32.4	41.0	5.9	12.1	19.6	29.4
BTE	13.9	25.5	37.2	45.9	13.6	25.8	36.4	46.6
BTM	17.4	30.7	43.2	51.3	14.4	24.1	35.4	43.8
ACE 100 Å								
BDM	8.1	14.9	21.5	33.3	6.7	13.6	21.9	31.5
BTE	12.1	20.9	29.0	40.9	13.3	26.3	36.8	47.6
BTM	16.3	25.6	33.4	45.1	14.2	25.6	36.8	46.0
ACE 300 Å								
BDM	6.1	13.7	20.5	32.9	1.6	5.1	7.9	19.1
BTE	11.9	23.6	32.9	45.2	10.8	22.4	31.6	43.6
BTM	17.8	33.1	43.3	56.5	14.3	25.1	35.6	45.2
Cogent Silica C								
BDM	13.6	23.9	32.6	44.6	11.4	21.2	30.8	41.3
BTE	14.7	27.8	37.5	49.1	15.8	28.8	39.7	49.7
BTM	17.9	34.8	43.6	56.9	17.8	30.0	41.6	50.3

Figure 2-6 shows BTM chromatograms on the five tested columns, the negative peak after the analyte peak is probably indicative of the elution of the chloride counter ion which is present in the test compounds. Taking the behaviour of BTM as being most indicative of hydrophilic partitioning/ion exchange, the chromatograms show the greatest retention for BTM on Kromasil 60 Å column which contains silica gel with the highest surface area/g. The Kromasil 100 Å column is the next most retentive column for BTM and it shows considerably stronger HILIC interaction than the ACE 100 Å silica column which has a slightly smaller surface area per gram of silica gel. The next greatest HILIC contribution to the retention of BTM was on the Silica C column, which is based on a 100 Å silica gel with a high surface area (**Table 2-1**) and which behaves just like a silica gel column where the retention of the test probes depends on ionic strength despite that fact that it is claimed that there are very few free silanol groups in this stationary phase. The low surface area 300 Å column exhibits very little HILIC interaction. The silicon hydride column which was prepared in house exhibited slightly stronger HILIC interaction than the 100 Å ACE silica from which it was prepared but much less HILIC interaction than the Cogent silica C column. Thus while it is not possible from the manufacturer's data to state that hydrophilic partitioning/ion exchange interactions are directly proportional to surface area per gram of the silica gel, there is a strong trend in this direction.

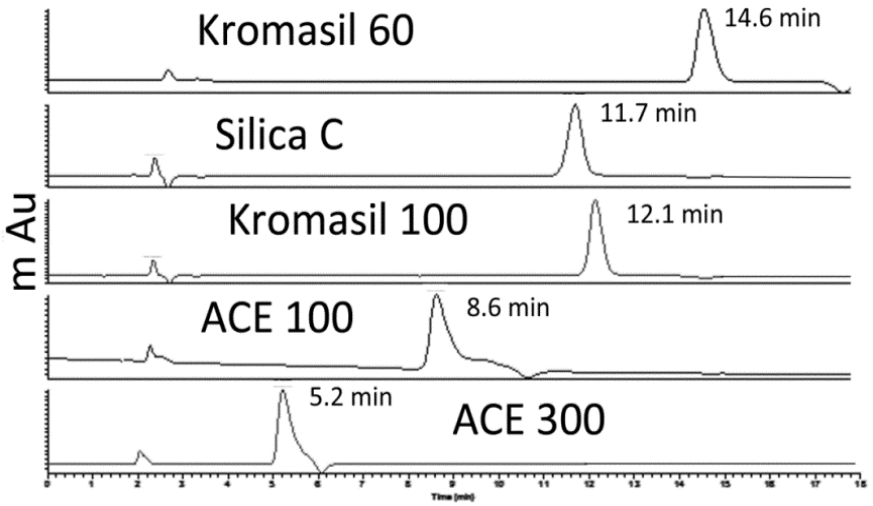


Figure 2-6: HPLC chromatograms for the test probe BTM run on five columns with 90% ACN as mobile phase modifier and 10 mM TMAA pH 6.0 as buffer. UV detection set at 263 nm.

In order to further differentiate ion exchange and HILIC contributions to the retention mechanism, a neutral and an acidic test probe were run on the Kromasil 60 Å, the Kromasil 100 Å and the Cogent Silica C columns. **Figure 2-7 A** shows the plots of the log k values for the two test probes against percentage ACN on the Kromasil 60 Å column. The plots obtained reflect the low lipophilicity of the probes, which are not strongly retained with 20 % v/v ACN in the mobile phase. Above 60 % v/v ACN, there is a marked increase in retention as the HILIC effect increases and there is no possibility for additional ion-exchange contribution to retention of the acidic and neutral probes. **Figure 2-7 B** displays the retention of the two test probes at 80% ACN against varying buffer concentration in the mobile phase from 5 to 20mM, the plot showed no marked effect evident from

plotting 1/ionic strength against the retention factors of the uridine (neutral probe).

In contrast, the retention factor of hydroxybenzoic acid (HBA) increased with increasing ionic strength suggesting that ionic repulsion effects were being overcome as the concentration of the TMAC counter-ion increased. Increase the percentage of organic to 90% ACN with varying the ionic strength of buffer showed no significant effect on the retention of uridine or HBA (**Figure 2-7 C)**. The behaviour of HBA with a higher organic content mobile phase might be explained by the fact that the concentration of the TMAC counter-ion is likely to be higher in the HILIC layer with 90 % ACN in the mobile phase, since its partition coefficient will be altered in favour of the aqueous phase.

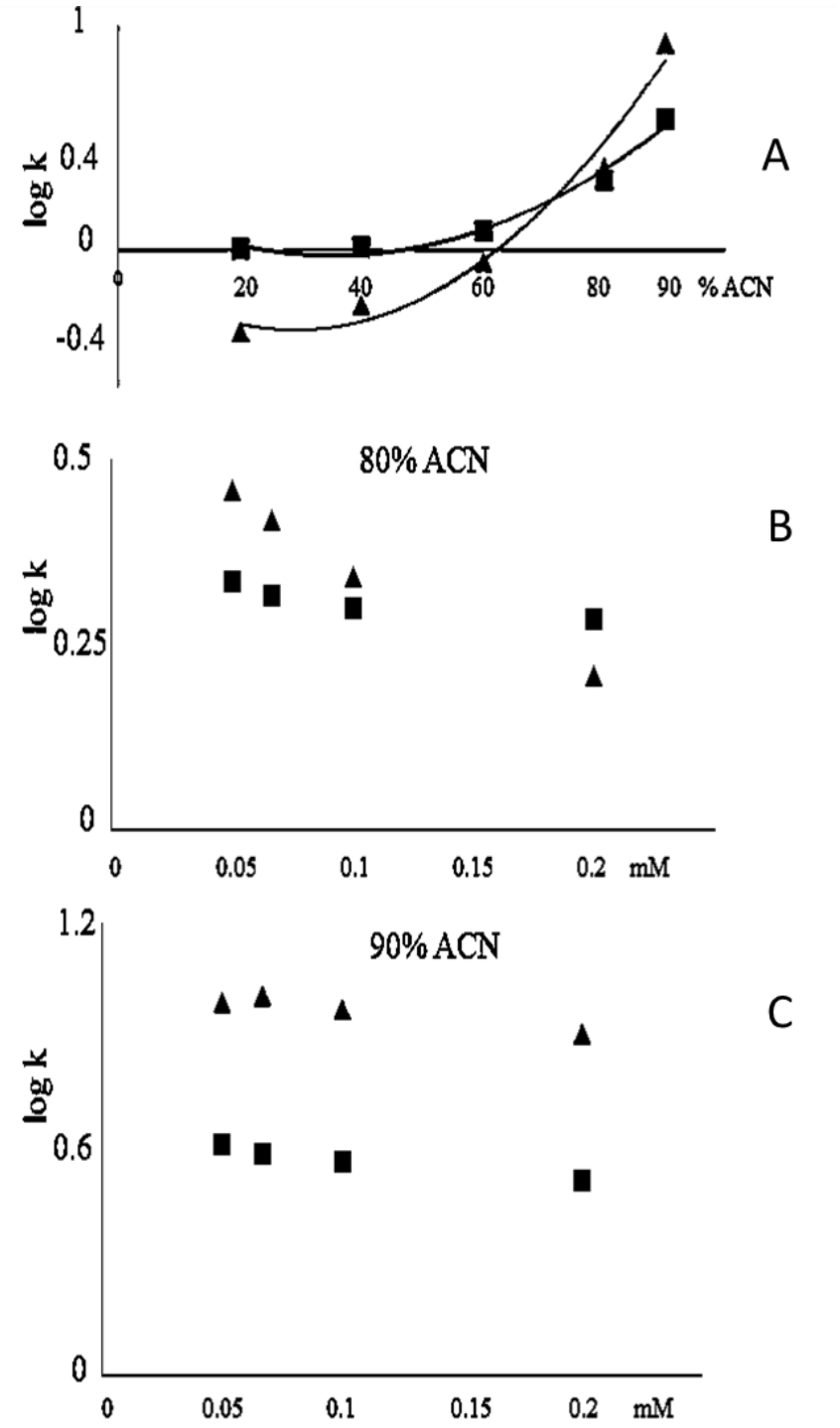


Figure 2-7: [A] plot of log k against % acetonitrile, [**B**] and [**C**] plots of the effect of 1/ionic strength (mM) on retention of a neutral probe (uridine ■) and an acidic probe (*p*-hydroxybenzoic acid ▲) at 80 and 90% ACN in the mobile phase respectively on Kromasil 60 Å column**.**

Toluene and pentylbenzene were used to get some estimation of the thickness of the absorbed water layer on the silica gel columns based on the idea of McCalley and Neue [25]. Since it was proposed that some of the HILIC activity resides in water rich regions rather than pure water [135] the estimation was determined in 100% anhydrous acetonitrile with no modifier being added. The lipophilic pentylbenzene should be more sensitive to the thickness of the HILIC layer because it would be less likely to partition into regions of the HILIC layer which are enriched with water and thus should exhibit less retention.

The values of the void volume, V_0 for the probes at different percentage of anhydrous acetonitrile is shown in **Table 2-5**. The experiments were carried out at a flow rate of 1 mL min^{-1} thus retention volumes and retention times are equivalent. As might be expected the 60 Å Kromasil column had the largest V_0 with an apparent volume of 2.3 mL.

Table 2-5: Void volume data for the pentylbenzene (Pb) and Toluene test probes on the different columns with 100%, 80% and 90% anhydrous ACN with 10 mM TMAC buffer in the mobile phase.

Column	**V_0 Toluene 100% ACN**	**V_0 Toluene 90% ACN**	**V_0 Toluene 80% ACN**	**V_0 Pb 100% ACN**	**V_0 Pb 90% ACN**	**V_0 Pb 80% ACN**
Kromasil 60 Å	2.3	1.9	1.9	2.2	1.8	1.6
Kromasil 100 Å	2.1	1.8	1.7	2.1	1.8	1.6
Cogent Silica C	1.8	1.7	1.6	1.8	1.7	1.5

From the data of pore volume and packed density of silica gel which are presented in **Table 2-1** it would be expected for the 150 mm x 4.6 mm Kromasil 60 Å column with (2.5 cm^3 total volume) to have an internal-pore volume of 2.5 **x** 0.45 **x** 1.2 = 1.35 ml (total volume **x** packing density **x** pore volume). This result suggests that the internal pore volume produces most of the additional contribution to the total V_0 value.

In the mobile phase containing acetonitrile/buffer (90:10) there was a marked decrease in the residence time of toluene from 2.3 to 1.9 min. indicating a decrease in available internal-pore volume of 0.4 mL. This equates to about 29.6% of the internal pore volume of the column being occupied by water. This is higher than the volume estimated by McCalley and Neue which was around 6.9 % occupation of the internal pore volume at 90% acetonitrile, however, their estimates were made without buffer in the mobile phase where there would be much less ionisation of the silanol groups of the silica gel. McCalley and Neue observed a large change in the thickness of the water layer on the silica gel moving between 10% and 20% water in the mobile phase with the % of the internal pore volume occupied by water being around 11.1% at 20% water.

In the current study there was a negligible change in the residence time of toluene upon moving from 10% to 20% buffer indicating a stable water layer close to the surface of the silica gel. This observation agrees with the remarkable increase in the retention times of the quaternary ammonium

test probes between 20% and 10% buffer driven by decrease the affinity for the high organic solvent content in the mobile phase. In other words, increasing the organic content in the mobile phase is increasing its lipophilicity and thus the hydrophilic test probes will retain longer in the polar water-rich layer in the stationary surface at 90% ACN.

The residence time of the pentylbenzene probe with 10% buffer in the mobile phase was similar to that of toluene but there was a marked decrease in V_0 of 0.2 mL to 1.7 mL with 20% buffer in the mobile phase suggesting that there might be an increase in a water enriched layer into which the very lipophilic probe does not partition.

The 100 Å Kromasil column, as might be expected, had a lower V_0 than the 60 Å column of 2.1 mL (from the data in **Table 2-1**, its internal pore volume would be expected to be 1.125 mL) and moving to 10% buffer reduced the residence volume of toluene by 0.3 mL suggesting that about 26% of the original internal pore volume is occupied by water which is in line with the lower HILIC contribution to retention on the 100 Å Kromasil column.

The Cogent Silica C column behaved differently from the silica gel columns apparently having a smaller total pore volume of 1.8 mL. The data to enable calculation of the internal pore volume of the Silica C column is not available. Although moving from 10% to 20% buffer there was only a 0.1 mL decrease in the pore volume occupied by water similar to silica gel, the behaviour of Silica C is not identical. A recent study used micro-

calorimetry to measure water absorption on a silica hydride column in comparison with the bare silica gel from which it was prepared [138] and showed that the silica hydride surface adsorbed methanol more strongly than the bare silica gel and it was proposed that this was due to bonding with Si-H groups. It was also observed that the water excess adsorption on the silica hydride column was lower than on silica gel which is consistent with our current observations of a lower effect on the internal pore volume for this phase.

2.4 Conclusion

Although it might seem that much work has been carried out in trying to understand the HILIC mechanism, there is still a lack of knowledge of the processes which occur on the simplest HILIC phase, silica gel. The majority of HILIC phases use silica gel as a base and even with very efficient surface modification many silanol groups remain within these phases and will have a role in the overall HILIC mechanism. While different silica gels have been explored previously with regard to their HILIC properties there has been no systematic study of the influence of silica gel surface area of HILIC properties. Most studies have used basic test probes and counter-ions with a charge which is dependent on the % of acetonitrile in the mobile phase and this factor has been eliminated from the current study. It was observed that lipophilicity was the major determinant of retention of the quaternary

ammonium probes at 20% acetonitrile and the most highly charged probe BTM was the least retained on all of silica gel columns and on the silicon hydride column. Overall the retention of the probes at 20% acetonitrile was due to a balance between ion-exchange and lipophilicity. The plots of log k against the percentage of acetonitrile at constant ionic strength gave "U" shaped plots with the lipophilic and HILIC/ion exchange interactions being at a minimum at around 50-60% acetonitrile. The log k values obtained in HILIC/ion exchange mode at 80 -90% acetonitrile depended on the surface area of the silica gel with the Kromasil 60 Å column giving the strongest retention. The Cogent Silica C column, where the retention mechanism is not exactly known, behaved in very similar manner to a silica gel column with a pore size somewhere between 100 Å and 60 Å and despite not being identical to the purely silica gel based columns; it behaves in a very similar to silica gel in HILIC mode. McCalley proposed a model [32] which was later used by Bicker *et al.* to separate the relative contributions of ion-exchange and HILIC for different HILIC columns [109]. Thus plots of log k against 1/ionic strength were fitted with second order polynomial curves and that the intercepts of these curves with the Y-axis of the plots indicated the relative contribution of HILIC to the overall retention mechanism also this model shows how HILIC and ion-exchange are necessarily linked as the percentage contributions of HILIC to overall retention at a given ionic strength were similar for all the tested columns.

In the current study it was clearly observed that an increase in silica gel surface area led to an increase in hydrophilic interaction.

For bases the contribution of HILIC to overall retention increases as the ionic strength of the competing counter ion is increased, therefore to promote the contribution of HILIC to retention then higher ionic strength is required. However, the binomial curve fitting used to assess the contribution of HILIC to overall retention neglects the effect the ionic strength in the HILIC layer. The ionic strength of the mobile phase modifier is likely to be much higher in the HILIC layer than in the bulk mobile phase since ionic modifiers will partition strongly into the aqueous layer. This hypothesis should be investigated in an extension of the current study since it provides another parameter that can be used to control the HILIC process. The level of such modifier partitioning should also depend on the particular counter ions used, both anions and cations.

Chapter 3

Retention and Selectivity of Cogent Hydride™ based stationary phases in Hydrophilic Interaction Chromatography

3 Retention and Selectivity of Cogent Hydride™ based stationary phases in Hydrophilic Interaction Chromatography

3.1 Introduction

Although the HILIC mechanism on the simplest HILIC system, silica gel, is still not fully understood, the observations of our previous study (**Chapter 2**) clearly showed that increasing the surface area will increase the hydrophilic interaction in silica gel stationary phase. It was also observed that higher ionic strength is required to promote the contribution of HILIC to the retention of basic probes. The synthesis of HILIC phases are based on silica gel which is contributes to the overall retention in HILIC due to the remaining silanol groups even with efficient surface modifications. An attempt to overcome the remaining silanols which contribute to the retention in HILIC is the development of silica hydride stationary phases. In this material modified silica should contain very few silanols on the surface. One approach to prepare a diverse range of functionalised stationary phases is to create a silica hydride surface and then to modify it further with different organic groups through hydrosilation reactions [44, 45, 49]. Thus, a hydride surface is initially generated through the silanization process and then a second step (hydrosilation) is used to attach the desired organic functionalities such as (cholesterol or phenyl) which are included in this study.

Already, many interesting aspects of the separation performance of silica-hydride stationary phases have been identified [33, 46, 47]. One of the most promising features of the stationary phases based on silica hydride is their ability to retain both polar and non-polar compounds. This unique property is due to a combination of the bonded R-group as well as the hydride surface. An addition advantage of silica hydride stationary phases is that they can be used either with the high-aqueous content mobile phases or the high-organic content mobile phases for the retention of various compounds. To achieve such separation capabilities normally requires the use of more than one type of stationary phase (often referred to as multi-dimensional chromatography) or a sample-preparation strategy that removes a particular class (*e.g.,* hydrophobic, hydrophilic, or ionic) of compounds prior to a specific separation or analysis being undertaken. The latter approach necessitates a second analytical protocol to determine the compounds removed if complete identification of the sample is desired.

The predominant retention mechanism in HILIC separation is not always easily predictable. It can depend not only on the characteristics of the analytes but also on the selection of mobile and stationary phase compositions. The objective of this study is to evaluate the retention mechanism and chromatographic behaviour of acidic and basic analytes that differ in the degree of hydrophobicity under hydrophilic interaction chromatography (HILIC) conditions. The study was conducted on different hydride based stationary phases to explore the potential application of

these columns toward improving HILIC selectivity. The tested hydride columns include cogent silica hydride, cogent phenyl hydride and cogent cholesterol UDC.

The comparison of separation characteristics depends on the different functionalities of these columns. For example, the surface of silica hydride column is populated with Si-H (silica hydride) groups so it is weakly hydrophobic and should have less strongly adsorptive properties. The second phase, phenyl hydride column, can potentially provide additional selectivity between compounds through π–π interactions between the phenyl group on the stationary surface and functional group from the analyte. In UDC cholesterol column the shape differences between analytes could lead to secondary selectivity. The data from the three stationary phases was used to elucidate the roles of the retention mechanisms.

3.2 Method and materials

3.2.1 Chemicals

Ammonium acetate, ammonium formate, HPLC grade methanol and HPLC grade acetonitrile were obtained from Fisher Scientific (Loughborough, UK). HPLC water was prepared in house using a Milli Q purification system. The basic and acidic compounds used in the tests were either from Sigma

Aldrich (Dorset, UK), or European Pharmacopoeia standards, or were part of an in house stock donated by companies over the years.

3.2.2 Chromatographic HPLC columns

The cogent columns were manufactured by MICRO SOLV and were obtained from the distributor HiChrom Limited, Reading, UK.

- Cogent phenyl hydride based stationary phase, **Figure 3-1**, 15 cm x 4.6 mm i.d., 4 µm, has a hydrophobic surface and selectivity for aromatic compounds.

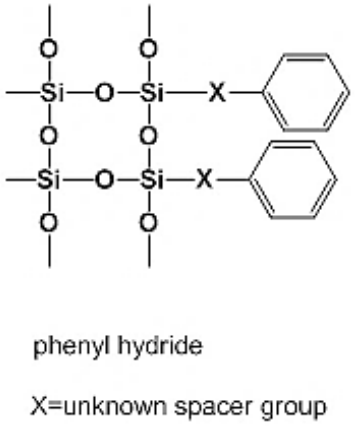
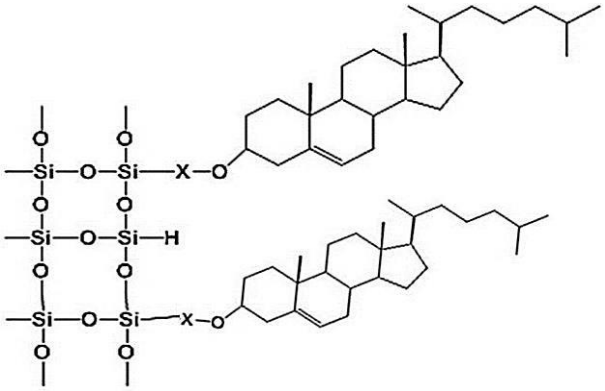


Figure 3-1: Chemical structure of the phenyl hydride stationary phase surface.

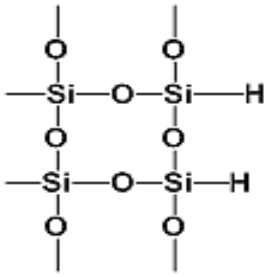
- Cogent UDC cholesterol, **Figure 3-2**, 15 cm x 4.6 mm i.d., 4 µM, is highly hydrophobic; the cholesterol is bonded directly to the silica hydride surface via a direct silicon-carbon bond.



Cholesterol hydride
X= $C_9H_{18}C=O$

Figure 3-2: Chemical structure of the cholesterol hydride stationary phase surface. http://mtc-usa.com/hplc/cogent_chem_udc.asp

- Silica hydride column, **Figure 3-3**, 15 cm x 4.6 mm i.d. 4 µm, is populated with (Si-H) functional groups instead of silanols which makes the surface slightly hydrophobic and it should adsorb and desorb solvents much more easily than ordinary silica.



Type C silica

(Silica C)

Figure 3-3: Chemical structure of the silica hydride stationary phase surface. Source http://mtc-usa.com/hplc/cogent_typec_silica.asp

3.2.3 **Instrumentation**

HPLC analysis was carried out on a Thermo-Finnigan HPLC system consisting of a P 4000 pump, UV 6000 PDA detector and an AS 3000 auto-sampler. The mobile phase compositions were mixed off-line in the proportions required and the HPLC system was used in isocratic mode; 10 µl of sample was injected. The flow rate was 1 ml/min and the column was run at 22°C in a column oven. The PDA-UV detector was used. The void volume of the columns was determined according to the minor disturbance peak in the baseline produced by injecting 10 µl of pure methanol. The values for Log P of the probes were calculated by ChemDraw Ultra software, Cambridgesoft, Massachusetts, USA.

3.2.4 **Mobile phase preparation**

A 400 mM of ammonium acetate (AmAct) buffer was prepared by weighing accurately 15.42 g of the salt and dilutes it in a 500 mL of purified water. The pH 6.9 of the salt in water was measured by pH meter and the required molarity of 20 mM was achieved by mixing with acetonitrile, ACN, in the proportion of 5% or 10% of the buffer. If a higher proportion of aqueous phase was required, water was added to the mobile phase mixture to make up the volume, (*e.g.* 10:90, v/v) of AmAct:ACN would require 5:5:90 of AmAct:H_2O:ACN. All mobile phases were degassed prior the experiments.

3.2.5 **Sample preparation and information**

The tested analytes include acidic, **Figure 3-4**, and basic, **Figure 3-5**, probes were prepared as stock solutions of 1 mg/mL in methanol then it was diluted prior to analysis to 0.1 mg/mL in mobile phase.

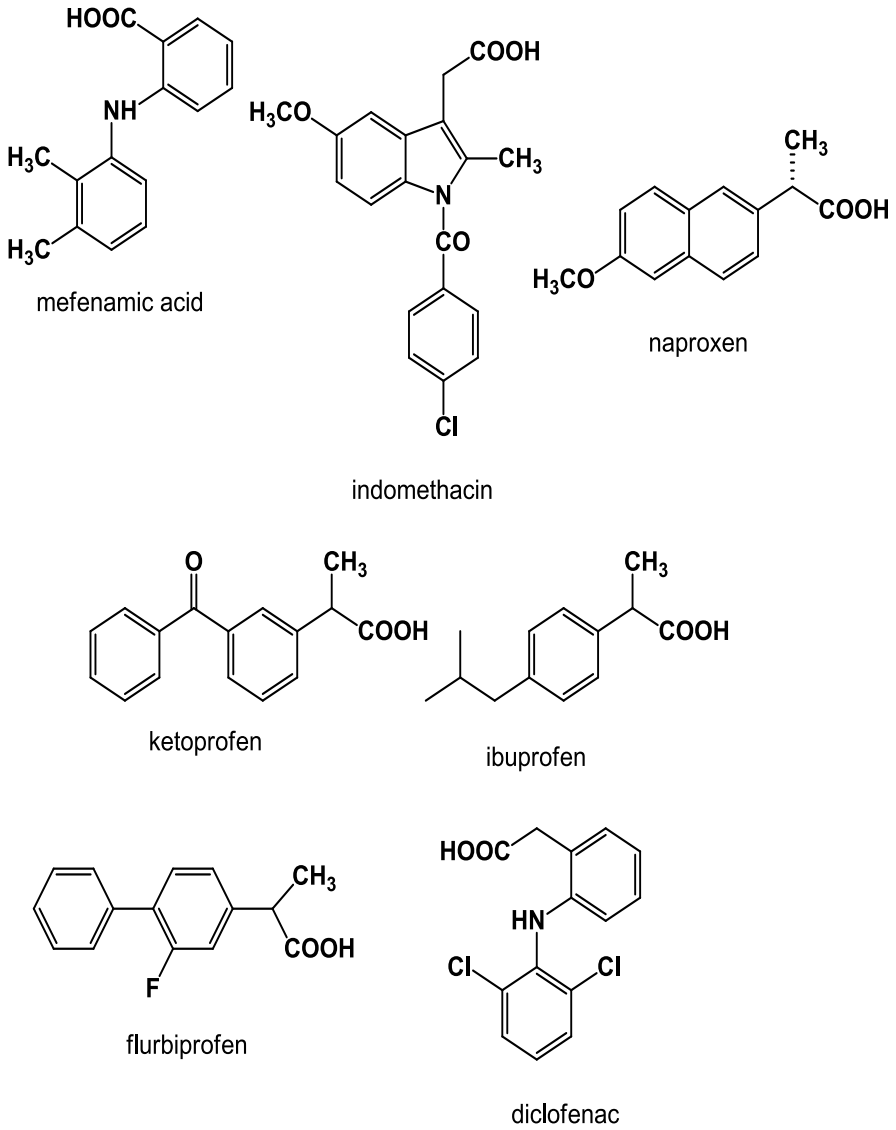


Figure 3-4: Chemical structure of acidic test probes used in the study.

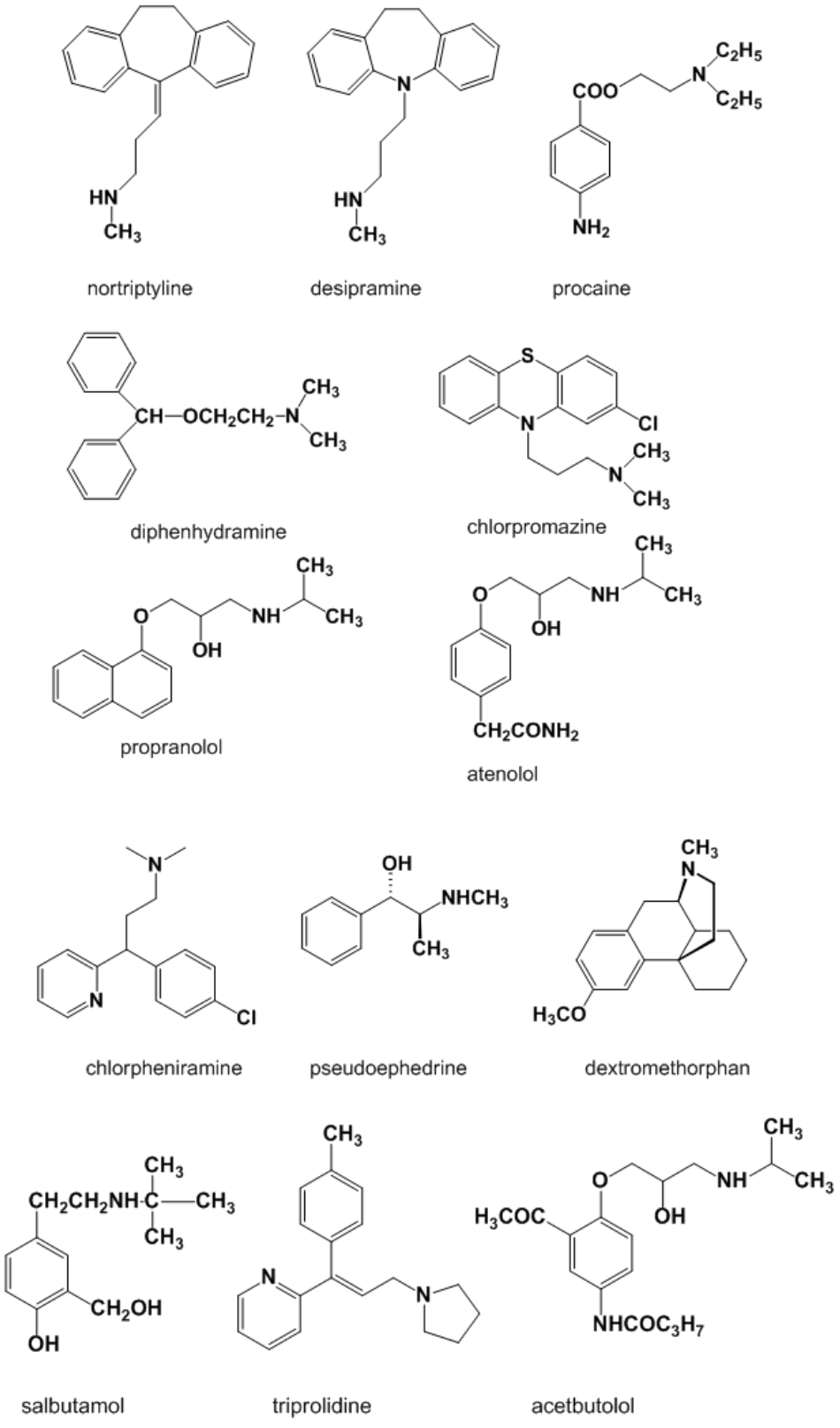


Figure 3-5: Chemical structure of basic test probes used in the study.

3.3 Results & Discussions

The retention behaviour of a range of basic and acidic analytes that differ in lipophilicity was investigated on cogent phenyl, cogent cholesterol and cogent silica hydride columns under HILIC conditions. Lipophilicity is a very important molecular descriptor in HILIC separation since it correlates with water solubility. Lipophilicity can therefore be measured by log P, which reflects the equilibrium partitioning for a molecule between a polar (aqueous) and non-polar phases. The calculated log P was obtained from *Clarke's analysis of drugs and poisons,* Moffat *et al.* (2011) the expected values (Exp) were calculated by ChemDraw Ultra software, Cambridgesoft, Massachusetts, USA. The study utilised two mobile phases contained 20mM ammonium acetate buffer at pH 6.9 in the proportion of 5:95 or 10:90 buffer: ACN, the flow rate set at 1 mL/min and UV detection utilised a PDA. The analysis condition was the same for all columns in this study and the columns were kept in column oven during experiments to maintain the same conditions.

I. Acidic test probes information

Table 3-1: Properties of acidic test probes used in the study.

DRUG	Calculated Log P	pK_a	Molecular weight	% Ionisation at pH 6.9
Mefenamic acid	5.1	4.2	265.32	98.44
Indomethacin	4.3	4.5	373.83	99.6
Naproxen	2.8	4.2	245.29	99.8
Ketoprofen	2.8	4.5	254.28	99.6
Ibuprofen	3.7	4.4	206.28	99
Flurbiprofen	3.8	4.2	244.26	99.7
Diclofenac	4.7	4.2	297.14	99.8

3.3.1 Retention properties of acidic models

The retention behaviour of the analytes was investigated under isocratic conditions as a function of different lipophilicity at different composition of hydro-organic mobile phases. In the present study, ACN was investigated as the organic modifier of the mobile phase, as it is the solvent of choice for HILIC. The effect of water concentration on the retention data of seven acids was tested at constant content of 20 mM ammonium-acetate buffer at 90% and 95% ACN (v/v) for all the Cogent™ columns also the impact of changing buffer type on retention was investigated by using ammonium

formate buffer. Although the acids were lipophilic their partition coefficient will be reduced by > 200 at pH 6.9 of the ammonium acetate buffer.

The three stationary phases shared similar selectivity in terms of retention time increases as the acetonitrile content in mobile phase increased from 90% to 95%; however a different elution order was noticed. Theoretically, the more hydrophilic the analytes, the more the partitioning equilibrium is shifted towards the adsorbed water layer on the stationary phase, and, thus the more the analytes are retained. Consequently, for the tested non-polar acidic probes which are used in this study, it would be expected that the most hydrophobic probe would elute earlier than the least-hydrophobic ones in the hydrophilic system.

The retention time was investigated for group of lipophilic analytes as a function of acetonitrile content in the mobile phase on three hydride-based columns; according to **Table 3-1**, the most lipophilic probe with the highest log P value is diclofenac followed by mefenamic acid whereas naproxen and ketoprofen have the lowest log P value among the lipophilic probes. The expected order for elution based on the lipophilic data of the compounds from the highest to the lowest lipophilicity is as follow:

Diclofenac>mefenamic acid>flurbiprofen>Ibuprofen>Indomethacin>ketoprofen>naproxen

Although the behaviour of some test probes was un-expected, the three hydride-based stationary phases retained the most lipophilic probe, mefenamic acid, least which agrees with the theory of HILIC. The retention time of each probe on the three cogent columns is presented in **Table 3-2**. Looking more closely at the data it is evident that the HILIC effect is weaker on all tested columns when 90% ACN in the mobile phase is applied, however increasing the content of acetonitrile to 95% produces a larger HILIC retention and a noticeable increase in retention for the all probes was observed.

Table 3-2: The average retention of n=3 (in minutes) for the acidic test probes on the three tested columns at 90 and 95% ACN in mobile phase, containing 20 mM ammonium acetate buffer, pH 6.9.

		Silica-hydride		**Phenyl-hydride**		**Cholesterol-UDC**	
Acid Identity	Exp. Log P	90%ACN	95%ACN	90%ACN	95%ACN	90%ACN	95%ACN
Mefenamic Acid	5.4	2.4	4.5	2.0	3.4	6.2	7.0
Diclofenac	4.1	2.8	5.5	2.3	4.5	8.5	9.2
Ibuprofen	3.8	3.4	6.8	2.7	4.9	9.8	10.9
Flurbiprofen	4.0	3.5	8.2	2.8	6.0	11.4	12.8
Indomethacin	3.6	3.6	8.9	3.5	8.1	12.7	18.0
Naproxen	3.0	3.9	8.7	2.9	6.5	11.3	12.8
Ketoprofen	3.3	4.0	9.9	2.9	7.2	12.7	14.4

Although it is well established that a hydrophilic surface holds water when exposed to a mixture of organic solvent and water, HILIC may include weak electrostatic interactions under high organic solvent conditions. In HILIC mode, the elution order for the acidic probes is not as expected on all columns as shown in **Table 3-2.** Probes with high log P values are retained longer than others with lower log P values. In HILIC mode, all columns seem to share similar selectivity and mefenamic acid elutes before diclofenac which have very close log P values of 4.03 and 4.1 respectively.

Looking specifically at the retention data of each phase, the silica hydride column shows a strong HILIC effect although it is claimed that the water accumulation of silica hydride phases is minimal. It is possible that there might be an ion exchange mechanism playing a role. The accumulation of ammonium ions in a static aqueous layer or associated with the surface of the stationary could be high and the concentration would be greater than the 20 mM added to the mobile phase. All the tested acids are stronger than acetic acid which provides acetate the competing counter ion in the current case. This might encourage the generation of an *in situ* ion exchange layer in a manner similar to that generated by a lipophilic anion or cation in reversed phase chromatography. The elution order with both mobile phases, contained 90% and 95% ACN, was the same on the hydride phase.

In RP chromatography, the selectivity of phenyl phases is often explained by the π-π interactions available through the phenyl ring. However, in HILIC mode partitioning mechanism is believed to be the mechanism of retention as the application of high organic content mobile phase is well established to form water rich layer on the surface of the stationary phase. According to the retention data in **Table 3-2** for the phenyl phase, a significant increase in retention time of all tested probes is clearly seen when moving from 90 to 95% ACN content in the mobile phase. At 90% ACN naproxen and ketoprofen have the same retention time however the selectivity was different at 95% ACN when naproxen eluted a minute before ketoprofen.

Although the degree of ionisation of indomethacin and naproxen should be the same because they have the same ionisable carboxylic group, it was observed that the former which has relatively higher log P value, 4.00, is more strongly retained on the phenyl phase than the latter, log P 3.00. This behaviour might be explained by the presence of an indole group in the indomethacin molecule which could impart additional polarity in the molecule and could undergo hydrogen bonding on top of that due to carboxylic acid and ether groups. It is also possible that there could be charge transfer interactions between the phenyl rings on the stationary phase and the extended pi-excessive indole ring within indomethacin. Thus it would seem that under HILIC conditions its partition coefficient cannot be predicted from previous experimental data or prediction.

The ionic strength of the aqueous HILIC layer will be quite strong since the ammonium acetate modifier will accumulate in that layer because it is more soluble in water than in acetonitrile. Thus perhaps it is not surprising that the observed partition coefficient differs from that obtained from partitioning between water and an organic solvent. Comparing the retention behaviour of phenyl hydride column to the silica hydride column; the increase in retention times when increasing the organic content from 90 to 95% acetonitrile is somewhat less for the phenyl hydride column suggesting that the hydrophilic layer built up on this column might be less than that formed on the silica hydride stationary phase. This would be expected since the surface is partly occupied by the phenyl containing stationary phase ligand.

Generally, the retention times of all the acids were longer on the UDC cholesterol column than on the silica hydride and phenyl hydride columns. This suggests a greater contribution from lipophilic interaction on the UDC cholesterol column which populated with large lipophilic ligands attached to the surface. However, although lipophilicity would appear to have a role the most lipophilic compounds, mefenamic acid and indomethacin, have the lowest retention times on this phase. Interestingly, at 90% ACN, the most hydrophilic, ketoprofen, and the most lipophilic, indomethacin, probes have the same retention time 12.7 min. suggesting an equal contribution of hydrophilic and lipophilic interactions, however; when increasing the organic content in the mobile phase to 95% ACN an even

stronger lipophilic interaction occurs with an increase in the retention of indomethacin to 18.0 minutes. Despite differences in the intrinsic hydrophobicity of these compounds was found to significantly affect their retention behaviour, it is difficult to explain the mechanism of retention but it is possible that the separation is governed by a dual mechanism where the retention increases as a result of hydrophilic interaction which re-enforces hydrophobic interaction with the hydrophobic ligand on this column, **Figure 3-6**.

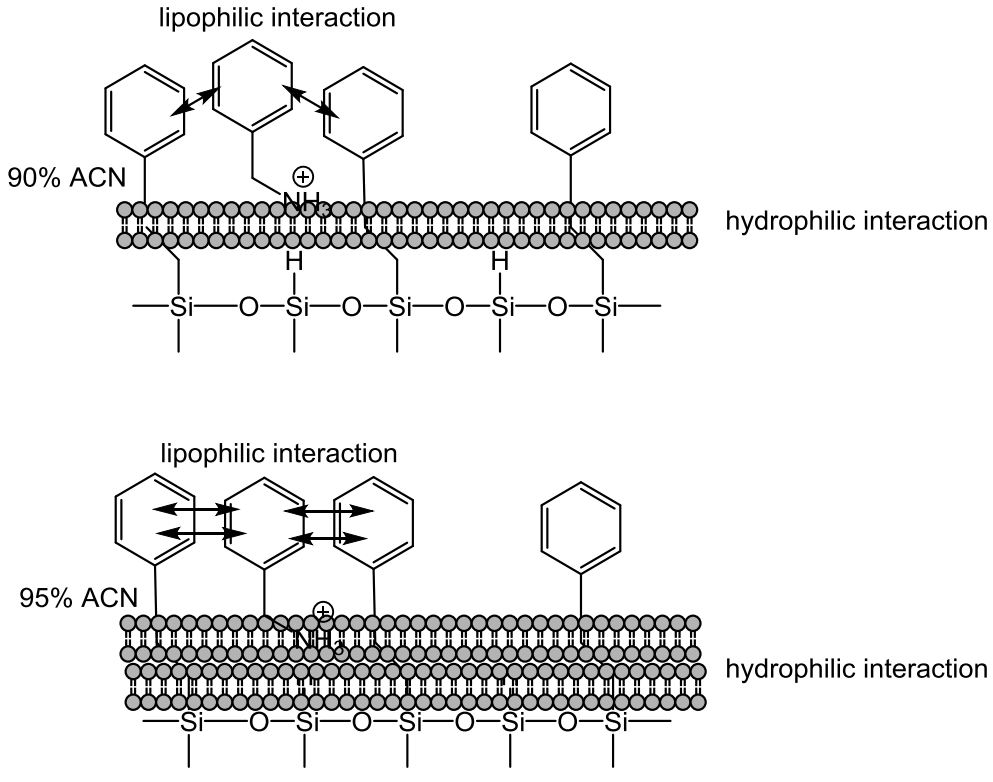


Figure 3-6: The process of promoting lipophilic interaction on the stationary phase as the hydrophilic interaction increased.

The variability of the retention of the acids under the same operating conditions at 90% ACN and 95% ACN over a short interval of time on each column was expressed in terms of %RSD (Relative standard deviation) as shown in **Table 3-3**. All test probes showed relatively acceptable reproducibility of the retention with RSD within average range 0.00-0.78% and 0.00-1.66% for 90% and 95% respectively for all tested columns with the UDC cholesterol column producing the lowest RSD values.

Table 3-3: The variation in retention times for acidic probes on the three tested columns with mobile phases containing 90% and 95% ACN and 20 mM ammonium acetate buffer pH 6.9.

		%RSD. n=3 Silica-hydride phase		%RSD. n=3 Phenyl-hydride phase		%RSD. n=3 Cholesterol-UDC phase	
Acid Identity	Pk_a	90%ACN	95%ACN	90%ACN	95%ACN	90%ACN	95%ACN
Mefenamic Acid	4.2	0.77	1.66	0.00	0.41	0.08	0.71
Diclofenac	4.2	0.34	0.96	0.61	0.31	0.18	0.10
Ibuprofen	4.4	0.61	0.79	0.7	1.16	0.41	0.36
Flurbiprofen	4.2	0.14	0.90	0.78	1.18	0.35	0.27
Indomethacin	4.5	0.26	1.52	0.00	1.33	0.08	0.00
Naproxen	4.2	0.00	0.94	0.00	0.95	0.31	1.13
Ketoprofen	4.5	0.00	1.04	0.32	0.47	0.39	0.20

The retention pattern of the acids is illustrated in **Figure 3-7** on all tested Cogent columns. It is clear from the plot that all acidic compounds exhibited a significant increase in the retention time as the percentage of acetonitrile in the mobile phase increased from 90% to 95% on all tested columns which supports the theory of HILIC. However, all of these acids have significant distribution coefficients into organic solvent since even when they are 99.9% ionised their partition coefficients will be in the range 5-10 and thus they should move quickly through the columns unless there are extra contribution mechanisms. The increase in retention time when acetonitrile content increases could be explained by the differences in the thickness of the water-rich layer which is established on the surface of the phase as suggested by McCalley and Neue [25]. Unexpectedly, the most lipophilic Cogent cholesterol UDC column showed the greatest ability to retain acidic compounds under HILIC conditions. This high retention in the cholesterol column is probably due to combination of lipophilic and HILIC-type interactions, since this column has a very bulky hydrophobic ligand. It is surprising with such a large hydrophobic ligand attached to the surface that there is still HILIC interaction and it would seem more likely that the retention is due to the formation of an in situ ion-exchange phase.

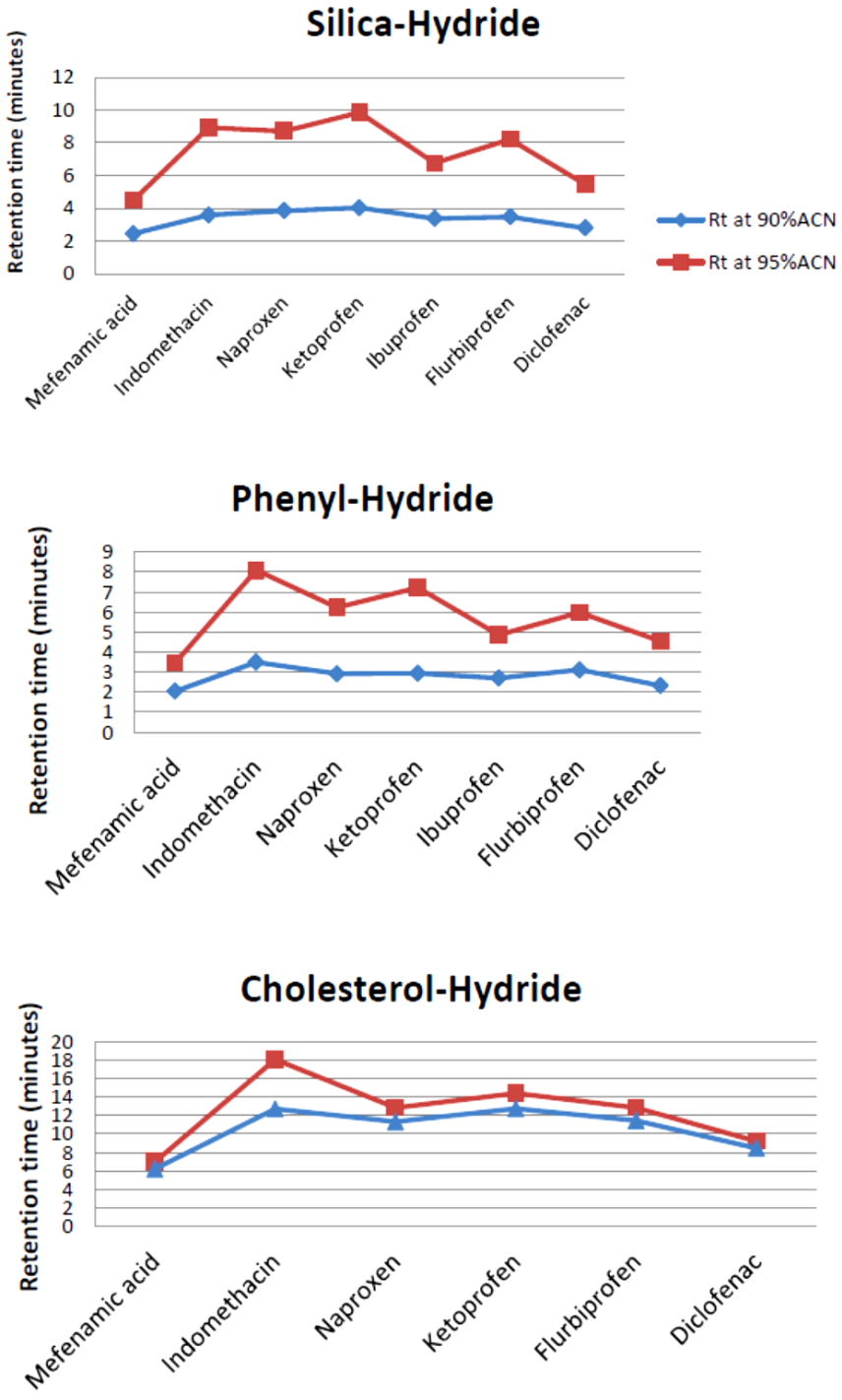


Figure 3-7: The retention behaviour of acidic analytes on the three Cogent columns; silica-hydride, phenyl- hydride and cholesterol-UDC columns (15 cm x 4.6 mm i.d., 4 µm), with mobile phases contained 90 and 95% of acetonitrile and 20 mM ammonium acetate buffer, pH 6.9.

The chromatographic retention and peak shape for some of the tested acidic drugs with mobile phases containing 90 and 95% ACN and 20 mM ammonium acetate buffer is shown in **Figure 3-8** (**A, B** and **C**) on the three tested columns. It was observed that some peak shapes are not good at the higher percentage of acetonitrile, where the acids will be more ionised, possibly because of charge repulsion of the ionised acid by remaining accessible negatively charged silanol groups or through the development of a mixed retention mechanism. While sharp peaks were obtained for the silica-hydride phase, the phenyl-hydride and cholesterol UDC produced broad peaks and in some cases split-peaks obtained. In general, the results were unexpected and the retention mechanism still not clearly confirmed on these phases.

The correlations between the physicochemical properties of the test probes and retention behaviour are not simple and pK_a may also have a bearing on retention. The retention of the acids is unexpectedly strong given that they are all hydrophobic compounds and even when highly ionised will still have a high organic partition coefficient. It is possible that at high levels of acetonitrile that the concentration of ammonium ions in the HILIC layer is sufficiently high to generate an *in situ* anion exchange phase with ammonium ions being loosely bound to the silica gel surface. Yet, a clear picture is difficult to be drawn but a working hypothesis is that the phases accumulate water on the surface and the ammonium acetate in the mobile phase also accumulates in this layer at concentrations much

higher than the 20 mM starting concentration to form a very concentrated charged phase. The positive charge in this layer increases with the percentage of acetonitrile in the mobile phase results in increasing the retention of the negatively charged acids. This explains some of the observations. An additional point is that on the cholesterol column the lipophilic interactions are also increased by the probes being drawn into the charged layer.

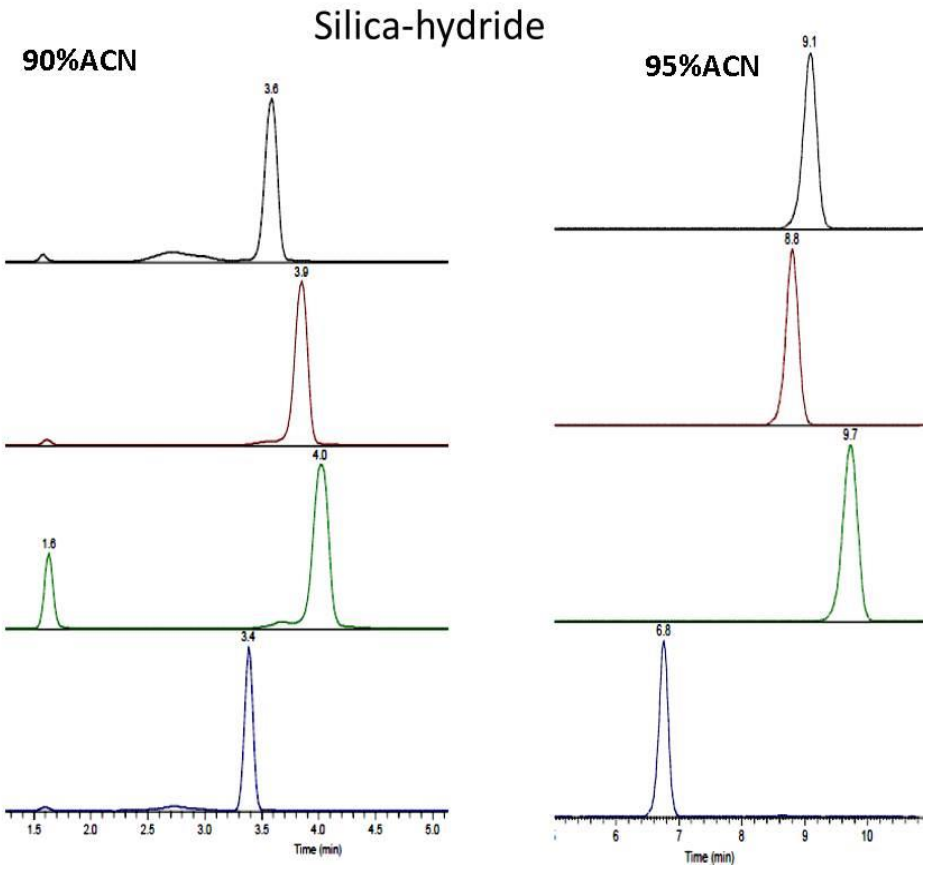


Figure 3-8: A) Chromatograms for indomethacin, naproxen, ketoprofen and ibuprofen from the top to the bottom, on the silica-hydride column with mobile phases contained 20 mM ammonium acetate / acetonitrile (10:90) or (5:95).

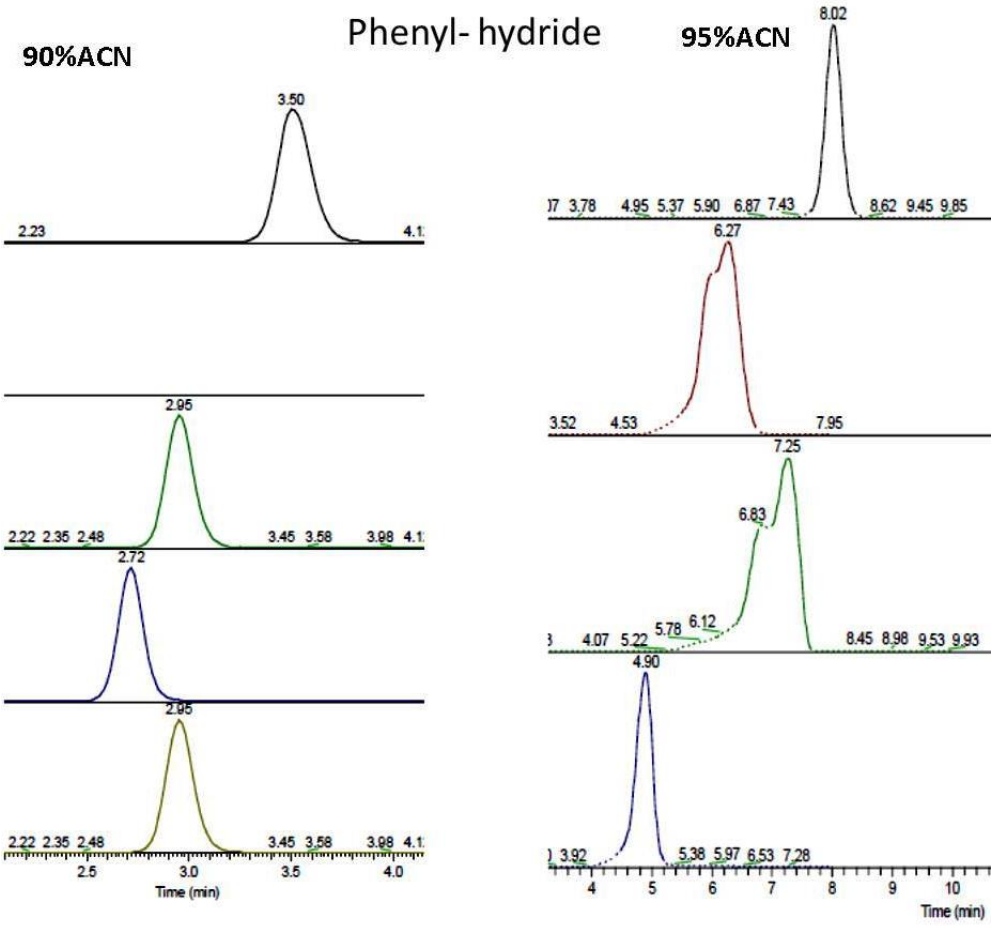


Figure 3-8 continued: B) Chromatograms for indomethacin, naproxen, ketoprofen and ibuprofen from the top to the bottom, on the phenyl-hydride column with mobile phases contained 20 mM ammonium acetate / ACN (10:90) or (5:95).

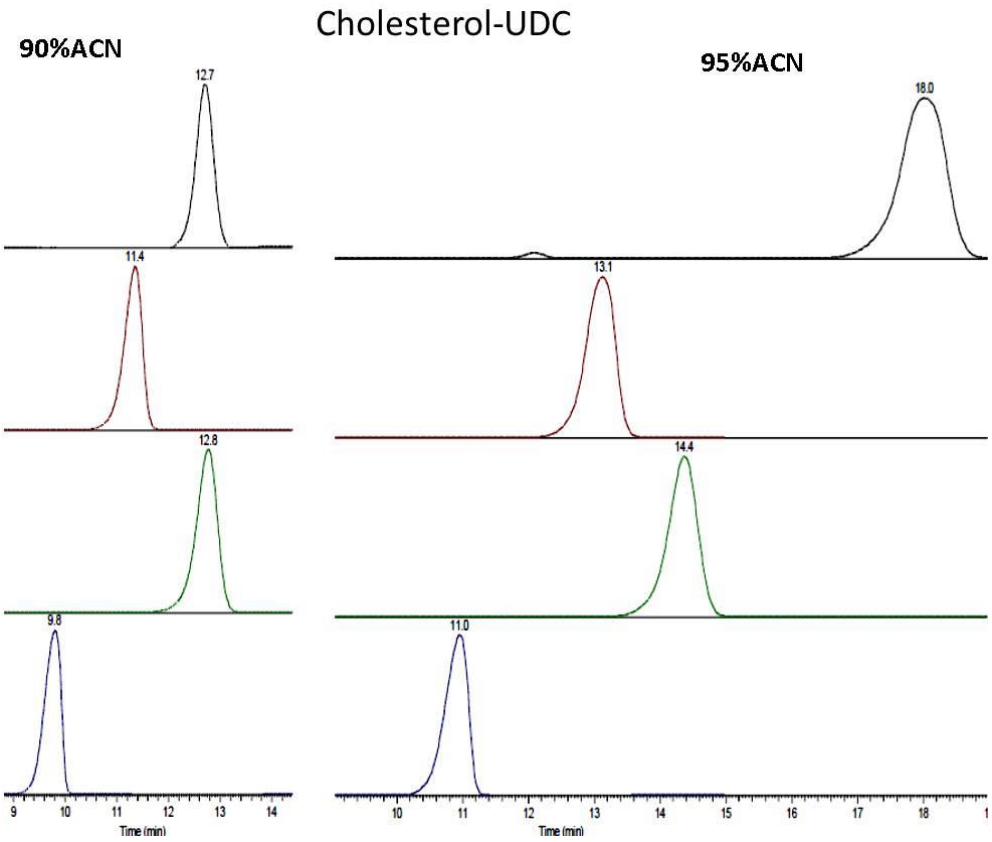


Figure 3-8 continued: C) Chromatograms for indomethacin, naproxen, ketoprofen and ibuprofen from the top to the bottom, on the cholesterol UDC column with mobile phases contained 20 mM ammonium acetate / ACN (10:90) or (5:95).

Changing the buffer modifier to ammonium formate reduced the retention times significantly for indomethacin, naproxen and ketoprofen when compared with the retention times obtained with ammonium acetate at 95% acetonitrile, **Figure 3-9**. Thus it seems very likely that the retention is due to an ion-exchange mechanism. If the mechanism was HILIC then the indomethacin would be less retained than the more polar naproxen and ketoprofen. Although the exact pK_a values of the acids under the conditions of the analysis are not known and they might differ from the

reported experimental values (**Table 3-3**), electrostatic and/or lipophilic interactions seem to be strongly involved in the retention mechanism under HILIC conditions for the hydride based stationary phases.

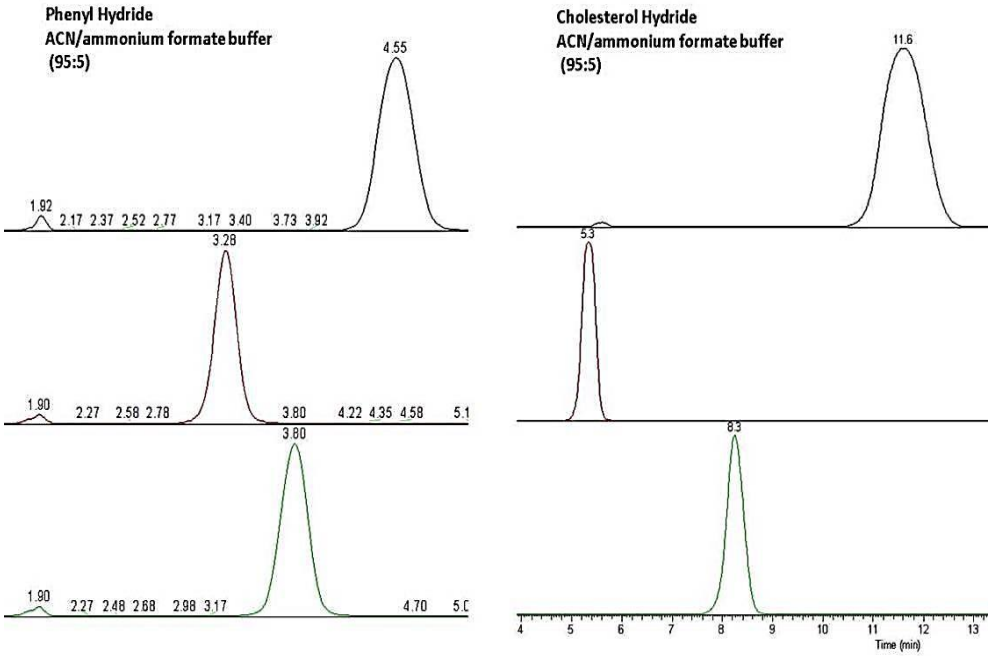


Figure 3-9: Chromatograms for indomethacin, naproxen, ketoprofen and ibuprofen from the top to the bottom, on phenyl hydride and cholesterol UDC cogent columns with mobile phase contain 20 mM ammonium formate in water / acetonitrile (5:95, v/v).

The HILIC partitioning theory is based on only circumstantial evidence and designing and experiment would not necessarily be easy as changing the basic counter-ion might have an affect but unless the counter-ion had the same pK_a as ammonia in adjusting to the same pH the concentration of

acetate would change. Thus the requisite for experimental design of changing one variable at a time seems difficult to fulfil.

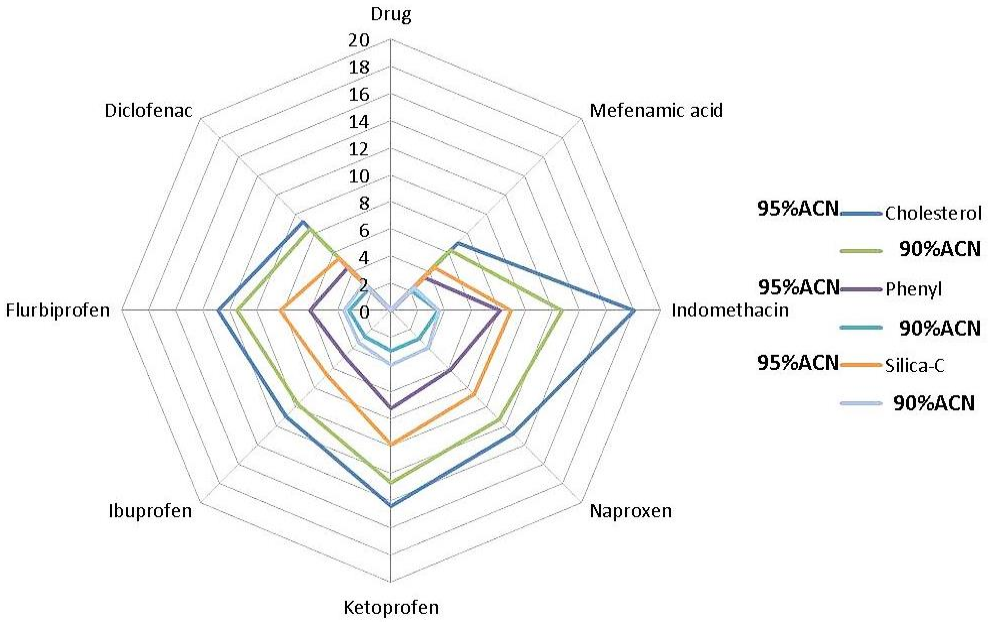


Figure 3-10: Spider chart presents the retentivity of the tested columns for the acidic probes in HILIC conditions, 90 and 95% ACN.

The cholesterol UDC phase with higher acetonitrile content in the mobile phase was the most retentive condition for the lipophilic acids, **Figure 3-10**. Data suggested that HILIC is more than just a simple partitioning and it may include weak electrostatic interactions under high organic solvent conditions used for retention. Work under HILIC condition provides a balance between mechanisms which in most cases is required for separation selectivity.

3.3.2 The retention properties of basic drugs on cogent hydride- based stationary phases

The retention behaviour was investigated in HILIC conditions on the three Cogent columns for basic probes that are different in their lipophilicity and range from very polar, log P (0.23), to highly non-polar, log P (5.2), analytes. The columns included Cogent phenyl, Cogent cholesterol and Cogent silica hydride stationary phases. The mobile phases contained 20 mM ammonium acetate buffer (pH 6.9) in the proportion of 5:95 or 10:90 buffer: acetonitrile, the flow rate set at 1mL/min and UV detection utilised was by PDA. The analysis condition was identical for all columns in this study and the columns were kept in a column oven during the experiments to maintain the same condition.

II. Basic test probes information

The chemical structure of the test probes is shown in **Figure 3-5** (Section **3.2.5**) and the chemical information of each basic analyte used in the study is presented in **Table 3-4** below.

Table 3-4: Chemical information for the basic test probes used in the study.

DRUG	Calculated Log P	Molecular Weight (g/mol)	% Ionisation at pH 6.9
Nortriptyline	4.3	263.38	99.9
Desipramine HCl	4.5	302.84	100
Procaine HCl	2.5	272.77	99.2
Diphenhydramine HCl	3.2	291.82	99.2
Chlorpromazine HCl	5.3	355.33	99.6
Propranolol HCl	2.8	259.34	99.7
(±)Chlorpheniramine malate	3.2	390.86	99.5
Pseudoephedrine HCl	0.9	201.69	99.9
Dextromethorphan	3.9	271.40	96.2
Atenolol	0.57	266.34	99.8
Triprolidine HCl	3.7	278.39	28.5
Acebutolol	1.7	336.43	99.7
Salbutamol	0.6	239.31	99.6 100

3.3.2.1 Retention behaviour of the basic drugs

Table 3-5 lists the basic probes which were used in the study from the highest to lowest log P value and the average retention time for each probe on the three tested columns is also included for the mobile phases which contained 90% and 95% of acetonitrile at constant buffer strength of 20 mM ammonium acetate, pH 6.9.

The retention data is not available for all test probes as some of them did not give any peak during the analysis run time on the silica hydride phase but from the available data it can be seen that the most hydrophilic base, atenolol, exhibited a strong retention at 90% and 95% ACN whereas the most lipophilic base, chlorpromazine, was the first to elute from the hydrophilic system being not detected at 95% ACN as it is probably eluted at the void volume, indicating a strong HILIC- like mechanism in the retention of these bases. In many cases the lipophilic bases were not strongly retained however increasing the percentage of acetonitrile produced a significant increase in the retention of all the bases apart from chlorpheniramine which showed a slight decrease in retention from 8.00 to 6.8 minutes with the most marked increases being for the hydrophilic atenolol and salbutamol. There is no surface ligand on the column attached to the hydride surface and the data suggested that silica hydride phase has HILIC-like properties for bases when utilising it with a high organic mobile phase and these properties could increase with increasing composition of organic modifier.

Table 3-5: The average experimental retention data of basic probes on the three cogent silica hydride-based columns with 20 mM ammonium acetate (pH 6.9) in mobile phase containing 90% or 95% acetonitrile.

		Silica-hydride phase		Phenyl-hydride phase		Cholesterol-hydride phase	
DRUG identity	Exp. Log P	90% ACN	95% ACN	90% ACN	95% ACN	90% ACN	95% ACN
Chlorpromazine	5.2	3.4	--	16.1	16.5	22.02	20.5
Nortriptyline	4.5	4.4	6.4	13.3	24.9	32.6	27.1
(±)Chlorpherinamine	4.00	8.00	6.8	40.3	44.1	13.1	17.3
Desipramine	3.9	4.5	--	13.4	25.4	33.9	29.4
Triprolidine	3.8	6.3	--	43.3	52.4	42.0	37.6
Propranolol	3.4	4.5	--	8.9	13.4	13.1	17.5
Dextromethorphan	3.4	6.2	6.6	29.6	39.6	45.5	39.3
Diphenhydramine	2.8	3.6	--	10.1	11.9	9.8	8.9
Salbutamol	1.8 1.00	15.4	35.7	6.6	16.7	33.2	32.00
Procaine	1.3	4.2	--	7.8	8.00	6.8	6.4
Acebutolol	0.94	8.4	11.5	9.7	19.3	27.2	23.9
Pseudoephedrine	0.90	8.9	--	--	--	--	--
Atenolol	0.23	18.4	29.2	12.00	26.5	46.4	40.8

Different behaviour was observed on phenyl hydride phase which strongly retained the relatively lipophilic bases, chlorpherinamine and triprolidine with the latter being the most retained probe to 43.3 and 52.4 minutes at 90% and 95% ACN in the mobile phase respectively while the hydrophilic bases exhibited a low retention compared to the lipophilic ones. Generally, increasing the acetonitrile content in the mobile phase led to an increase in the retention of all tested probes. It is interesting to note that the two bases, dextromethorphan and propranolol, which have the same log P values, exhibited very different retention time with the former having around 20% extra retention on the phenyl hydride phase. This observation is unexpected but possibly the presence of the methoxy group on dextromethorphan encourage the pi-pi interaction with the phenyl stationary surface in addition to the hydrophilic interaction and this assumption could be proved by the tailing effect which the dextromethorphan experienced as shown in **Figure 3-11** and in many cases this tailing is explained by more than one retention mechanism and one mechanism is overloaded. The behaviour although does not seem to be fully explained sometimes by the small or no differences in the polarity it is suggesting the possible presence of other interactions.

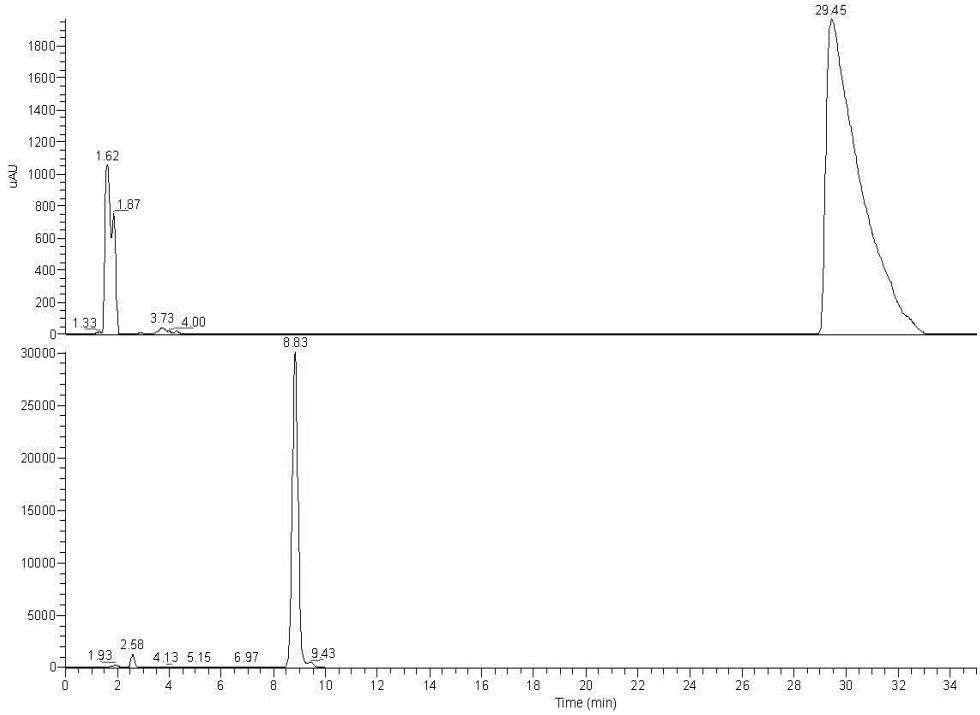


Figure 3-11: Chromatograms for dextromethorphan (top) and propranolol (bottom) on Cogent phenyl hydride column at 90% ACN in a mobile phase contained 20 mM ammonium acetate buffer,pH6.9.

Despite the significant polarity difference of atenolol, 0.23, and dextromethorphan, 3.4, they are more or less exhibited very similar strong retention above 40.0 minutes on the UDC cholesterol hydride stationary phase when 90% ACN was utilised in the mobile phase. However, increasing the content of acetonitrile in the mobile phase to 95% led to decrease in the retention of the hydrophilic atenolol and acebutolol while the retention of the most lipophilic bases chlorpherinamine and propranolol increased. The correlation of log P value and the retention affinity on this phase is not clear and RP-like mechanisms seem to be operated even at high organic content in the mobile phase. The

hydrophobicity of the cholesterol phase seems to have strong control over the separation and, even when using a high organic content, the hydrophobic properties of the phase will still affect the retention of the probes. **Figure 3-12** illustrates the pattern of retention on phenyl hydride and cholesterol hydride columns but the data for silica hydride column is not presented because the lack of retention information for some probes which didn't produce a peak during the analysis.

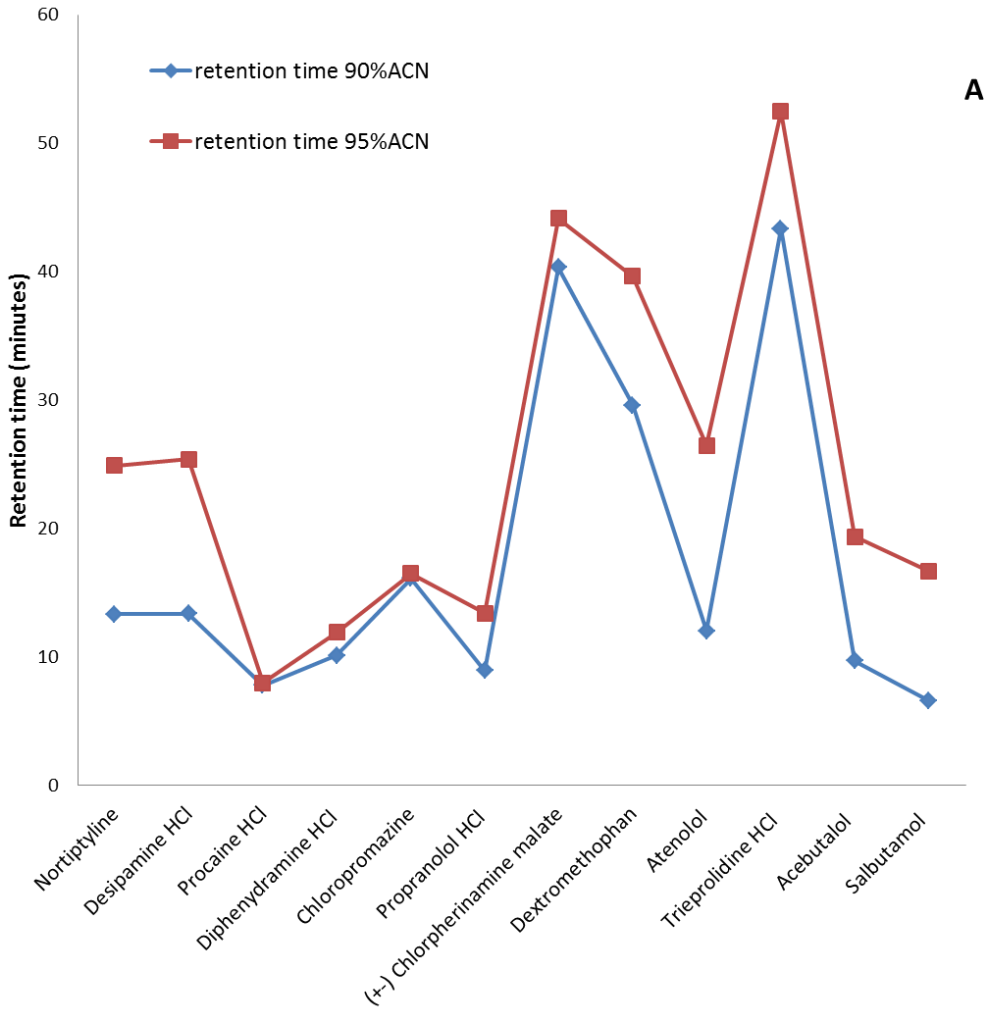


Figure 3-12: The retention trend of the basic probes on [**A**] cogent phenyl-hydride and [**B**] cogent cholesterol hydride stationary phases with mobile phase containing 90% or 95% ACN and 20 mM ammonium acetate.

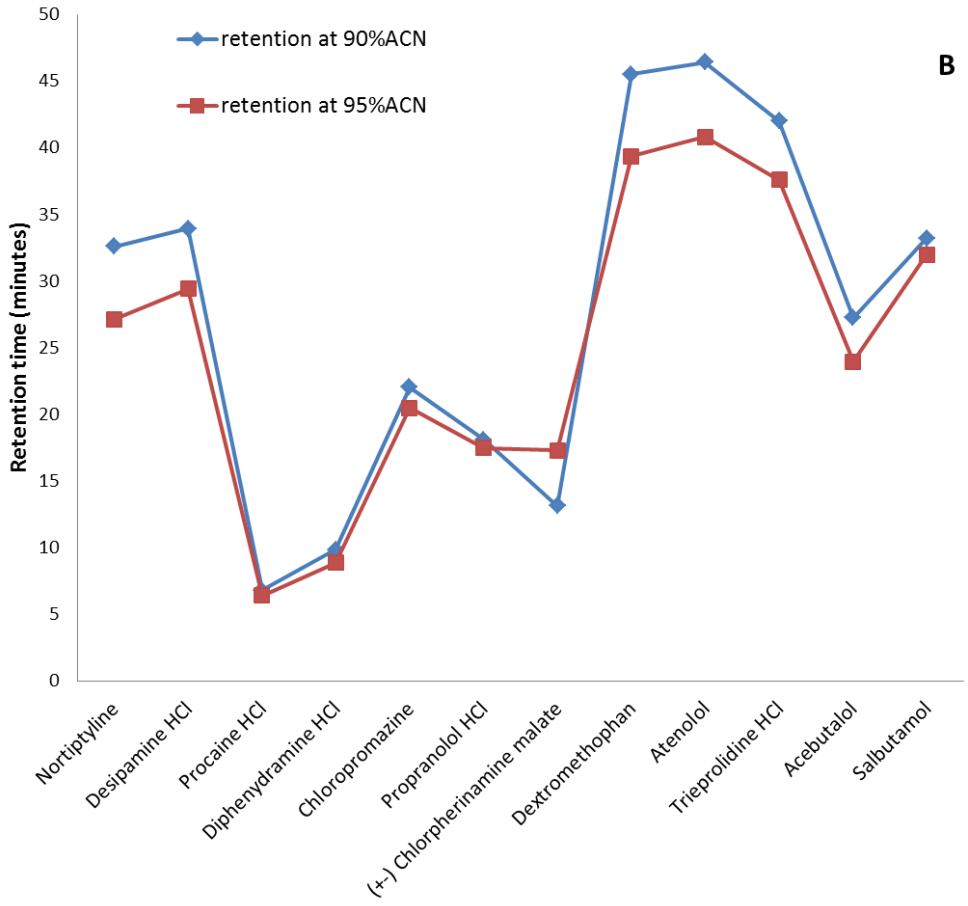


Figure 3-12 Continued: The retention trend of the basic probes on [**A**] cogent phenyl-hydride and [**B**] cogent cholesterol hydride stationary phases with mobile phase containing 90% or 95% ACN and 20 mM ammonium acetate.

The reproducibility of retention for the bases seem to be an issue on these phases as presented in **Table 3-6**, the %RSD of some probes exceeded ≤ 1.0 FDA guidelines [139] and the peak shapes in most of the chromatograms are broad or splitting. The inconsistency in retention might be due to mixed retention mechanisms faced by the analyte and the greatest variations were found in the cholesterol-hydride column which has a bulky ligand on its surface and might produce mixed retention mechanism. It is also of note that the peaks which seem to split into two clear peaks on the cholesterol phase are chiral compounds in a mixture containing two enantiomers. Cholesterol possesses many chiral centres so one could speculate that chiral selectivity is being observed. Although silica hydride stationary phase did not give retention data for all tested bases, the available data demonstrated % RSD slightly above limit for some bases while others had good values $<$ 1.

Table 3-6: The variation in retention times for basic probes on the three tested columns with mobile phase containing 90% or 95% ACN and 20 mM ammonium acetate buffer pH 6.9.

		%RSD. n=3 Silica-hydride phase		%RSD. n=3 Phenyl-hydride phase		%RSD. n=3 Cholesterol-hydride phase	
Acid Identity	**Pk_a**	**90% ACN**	**95% ACN**	**90% ACN**	**95% ACN**	**90% ACN**	**95% ACN**
Chlorpromazine HCl	9.3	0.14	--	0.15	1.9	2.2	0.78
Nortriptyline	10.1	1.63	0.32	1.1	1.15	0.00	1.97
(±)Chlorpherinamine malate	9.2	0.65	0.54	1.15	4.45	9.2	5.79
Desipramine HCl	10.4	0.91	--	1.06	1.00	0.75	8.40
Triprolidine HCl	6.5	0.71	--	0.48	0.57	3.48	2.82
Propranolol HCl	9.5	1.10	--	0.58	1.94	2.8	0.57
Dextromethorphan	8.3	1.32	0.36	0.73	1.33	6.1	0.70
Diphenhydramine	8.98	0.99	--	0.14	1.27	5.0	1.98
Salbutamol **pK_{a1}** (phenolic hydroxyl group) **pK_{a2}** (secondary amine group)	9.3 10.3	0.06	0.66	1.22	1.39	4.5	2.19
Procaine HCl	9.0	0.22	--	0.12	1.57	1.99	1.65
Acebutolol	9.4	1.61	1.53	0.97	3.2	6.00	3.55
Pseudoephedrine	9.8	0.55	--	--	--	--	--
Atenolol	9.6	0.44	0.66	1.41	0.83	5.8	1.18

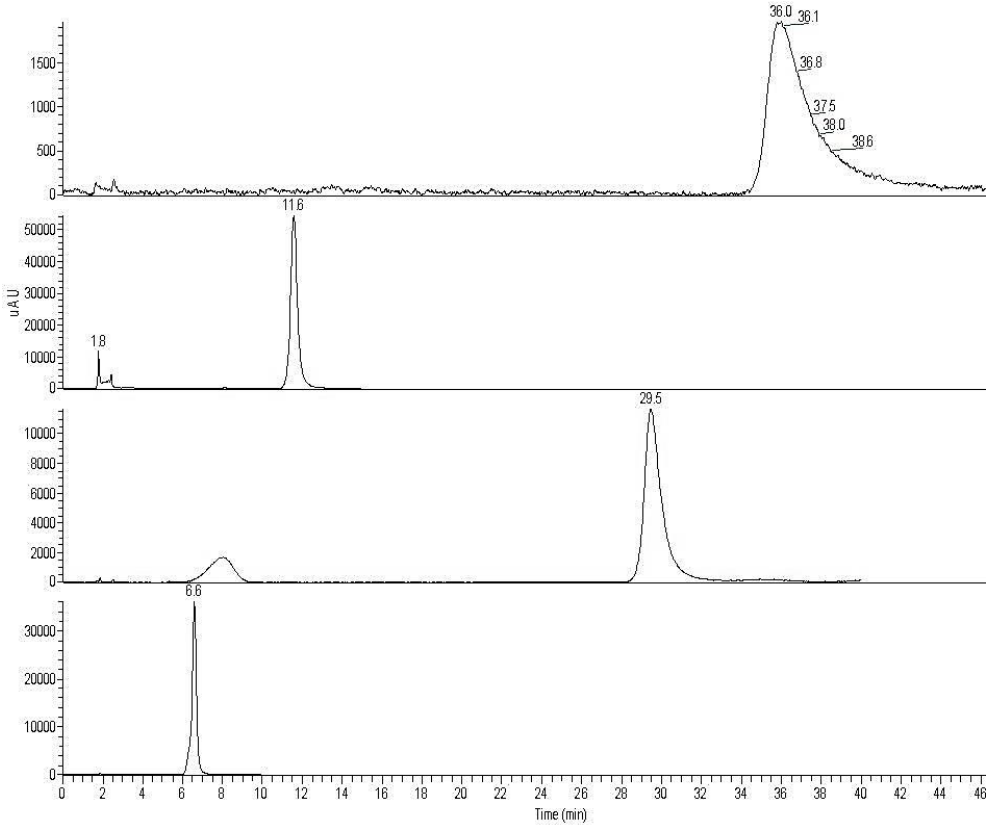


Figure 3-13: Chromatograms for salbutamol, acebutolol, atenolol and dextromethorphan, from top, on a cogent hydride column, the mobile phase contain 20 mM ammonium acetate in water/acetonitrile (5:95, v/v).

The tailing and splitting behaviour of the bases on the phenyl hydride phase, **Figure 3-14**, indicates the possibility of two or more interactions governing the retention such as HILIC interaction re-enforcing lipophilic or π-π interactions as described in **Figure 3-15**, even at high organic content in the mobile phase. The UDC-cholesterol hydride phase also produced poor peak shapes, **Figure 3-14**, and it is probably due to the lipophilic interaction which is still having an effect even under high organic conditions. The selectivity offered by the phenyl hydride and the cholesterol hydride

columns is very unusual and more experiments will be required in order to fully understand the behaviour.

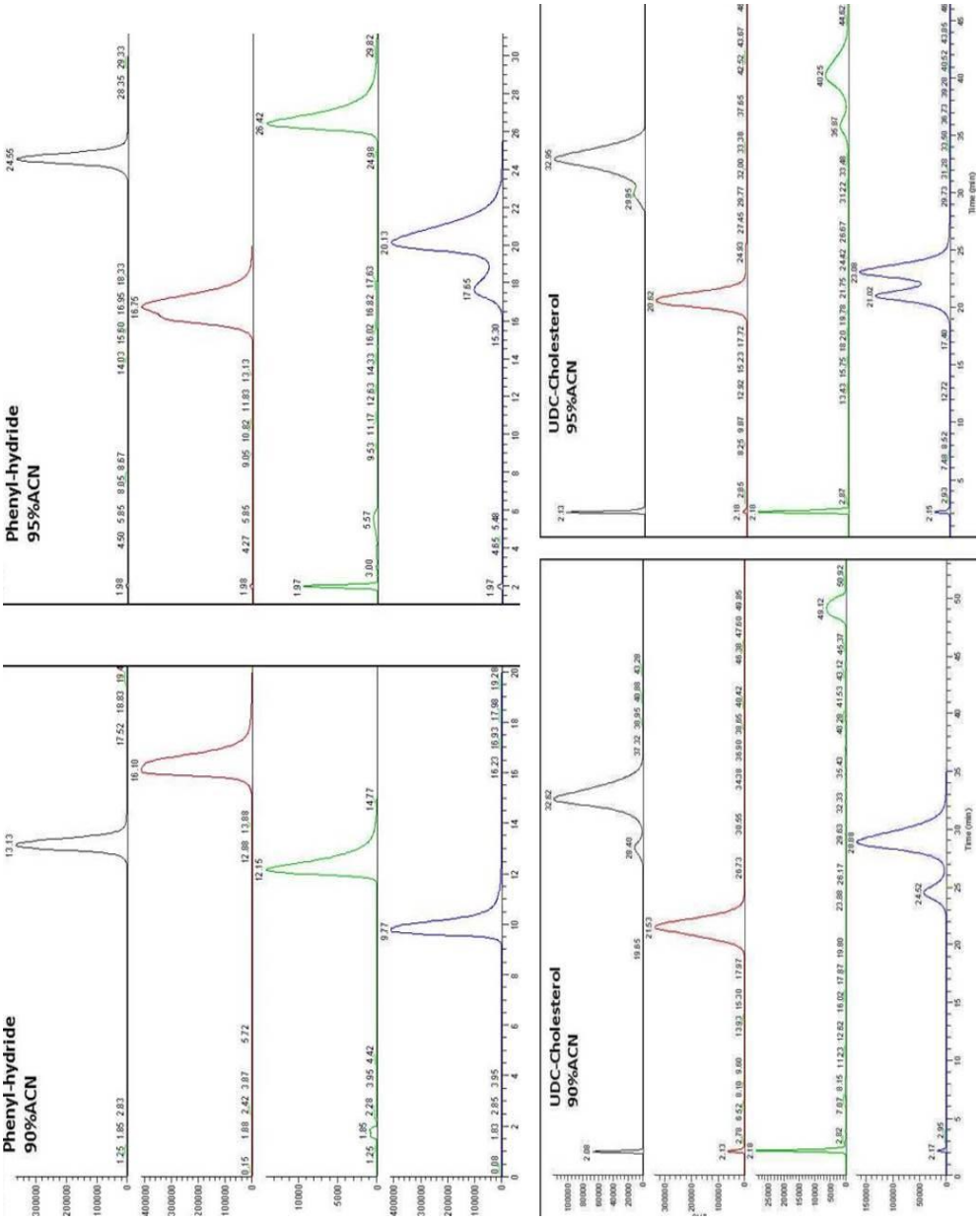


Figure 3-14: Chromatograms for nortiptyline, chlorpromazine, atenolol and acebutolol on a Cogent phenyl and cholesterol hydride columns, the mobile phase contained 20 mM ammonium-acetate in water/acetonitrile (5:95 and 10:90 v/v).

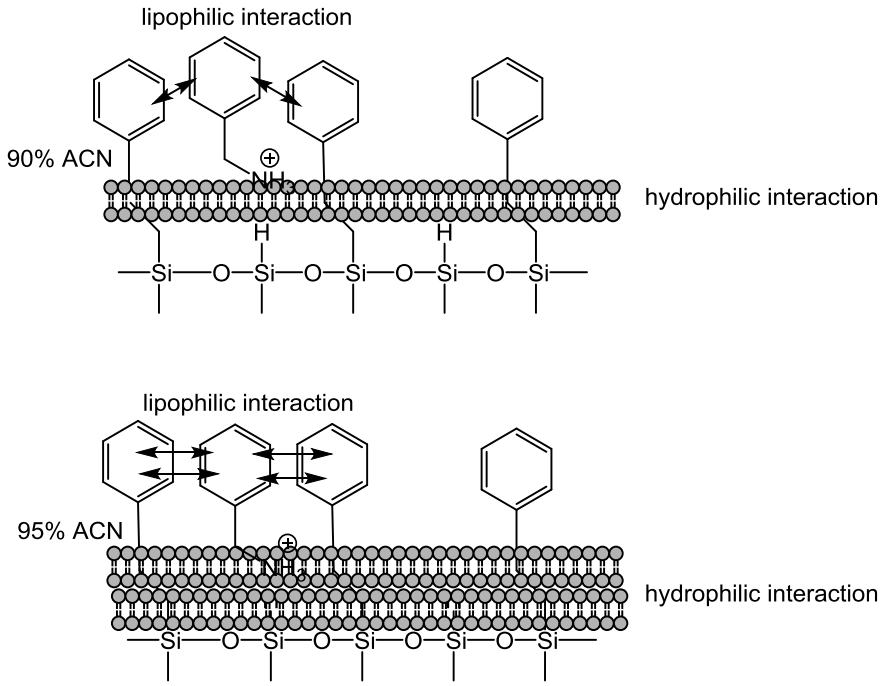


Figure 3-15: The process of promoting lipophilic interaction on the stationary phase as the hydrophilic interaction increased.

The two bases chlorpheniramine and chlorpromazine have very similar pK_a values but the log P value of latter is much higher than that of the former, yet the retention time of chlorpheniramine is much longer than that of chlorpromazine thus retention cannot be due to partition coefficient. A recent study found that the selectivity in the phenyl hydride phase in RP-chromatography was believed to arise from π–π interactions, while differences in shape selectivity were provided by UDC Cholesterol column [140]. For example, the molecular size of the basic probes procaine and dextromethorphan is almost the same *ca.* 272.8 g/mol and 271.4 g/mol respectively, however the retention time of the former is 40 minutes less than the latter on the UDC-cholesterol hydride phase. On the other hand,

the difference in the molecular size between salbutamol and nortriptyline is over 100 g/mole but this difference did not affect the retention behaviour of these probes which have similar retention values of 33.2 and 32.6 minutes respectively. The retention behaviour of bases in cholesterol hydride column under HILIC condition seems complicated and unpredictable, thus there is a need for further investigations. Overall, the contribution of the stationary phase to the retention mechanism in HILIC in respect to the data on phenyl and cholesterol hydride based stationary phases illustrates the importance of understanding the secondary interactions from these column types to eliminate the attributes of inappropriate contribution and improve peak shapes for better separation.

3.3.3 Effects of buffer type on the retention of basic drugs in HILIC

The acetate counter ion in the buffer was replaced by formate to study the effect of the buffer type and pH in the retention of the basic drugs in HILIC. The ammonium formate buffer was prepared in the same way as the acetate in **section 3.2.4**, however to achieve the 400 mM of the formate buffer a 12.62 g of the salt was weighed out accurately and dissolved in a 300 mL purified water, the pH was adjusted by adding a dropwise of ammonia solution to obtain pH 6.9 then water was added to made up the final volume of 500 mL. The required amount of the buffer, 5%, was mixed

with acetonitrile to obtain 20 mM of ammonium formate in 95% of acetonitrile in the mobile phase. Acetonitrile (ACN) is preferentially used as organic modifier for HILIC, although it is known to be much poorer solvent for buffer and salts than methanol (MeOH), and tetrahydrofuran (THF) is even worse [104]. However, there was no solubility issues observed during the preparation of HILIC buffer.

Generally, a marked decrease in the retention of the basic drugs was observed when ammonium formate buffer was applied in the HILIC system. Presumably because the ammonium concentration is slightly higher in this case than in the ammonium acetate mobile phase since there is very little buffer capacity in the formate salt. Thus the main difference is the replacement of acetate with formate. Also the solvation energy of acetate is higher than that of formate (acetate -389 kJ mol^{-1} and formate -368 kJ mol^{-1}) [141]. This higher solvation energy for acetate could mean that it has a greater affinity for water and thus increases the thickness of the HILIC water layer. This would result in a greater HILIC contribution to the retention of the bases when acetate is used and thus greater retention times. On the phenyl hydride phase, the retention time was reduced for all the test probes except for the hydrophilic salbutamol and the lipophilic chlorpheniramine which show slight increases in their retention times. In the absence of silanol groups it is difficult to explain the behaviour of the test compounds on the phenyl hydride phase; however this observation could be explained by a contribution of hydrogen-bond and/or π- π

interactions in addition to portioning. Although an improvement in the peak shapes was observed for the basic drugs which exhibited less retention in the Cogent cholesterol stationary phase when ammonium formate was used as the mobile phase modifier, the repeatability of the retention time for most of the probes was very poor as shown in **Figure 3-16**, the retention time of desipramine varies over time suggesting that it is taking time for the mobile phase and stationary phase to equilibrate. Current data highly suggests that the strong retentivity of the cholesterol phase for the lipophilic probes under HILIC condition is due to accessibility to the lipophilic surface for a lipophilic interaction.

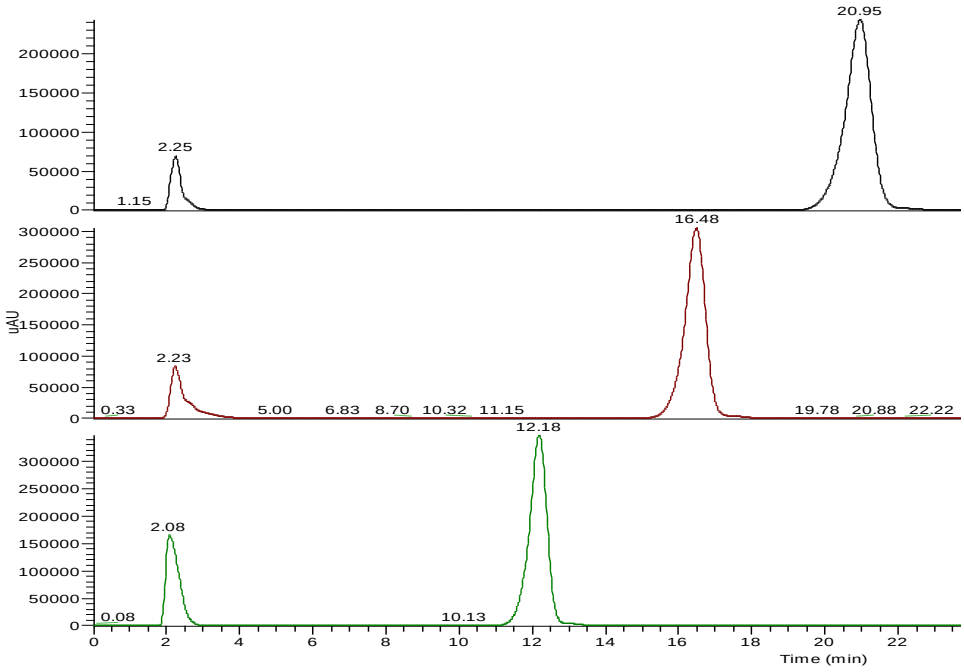


Figure 3-16: The variation in the retention time of desipramine , 0.1 mg/mL, in the Cogent cholesterol-UDC stationary phase, the mobile phase contain 20 mM ammonium-formate (pH 6.9)/ACN (5:95, v/v).

With the formate buffer it is expected for the water-rich layer to be thicker than with the acetate since more formate ions will accumulate on the surface and partitioning should be stronger but the low retention on these phases suggest although partitioning occurs at high organic content in the mobile phase there is still accessibility for further electrostatic and/or lipophilic interaction and the buffer type plays a role to promote the partitioning in HILIC.

3.4 Conclusions Based upon Log P as a Factor

It is interesting that the two bases triprolidine and chlorpheniramine both of which contain a pyridine ring are the most strongly retained on the phenyl hydride phase. The manufacturer claims that the phenyl ring in the phenyl hydride column is particularly available to interact with analytes because the solvation of the silica hydride surface is low. Aromatic rings can interact via charge transfer (π-π interactions) and for this to occur; a π-acid (electron acceptor) and a π-base (electron donor) is required. Thus interactions will occur between electron rich rings and electron deficient rings. The phenyl ring in the silica hydride column can be considered as an electron rich since it does not have electron withdrawing substituents attached. Pyridine rings are known to be π-deficient systems; this is thought to result from the electronegative nitrogen atom withdrawing electrons from the double bond system within the ring. The nitrogen takes the place of a carbon atom within the aromatic system but since it is more electronegative the carbon in contrast to benzene, the carbons in the ring are electron deficient as electrons are withdrawn towards the nitrogen atom. Thus it is possible that the π-deficient pyridine rings in chlorpheniramine and triprolidine are interacting with the phenyl rings in the phenyl-hydride stationary phase via charge transfer which would explain the unusually strong retention of these two analytes.

The other contributing factor to retention is HILIC but if the increase in retention from 90% to 95 % ACN is compared for the Cogent hydride column as shown in **Table 3-7**, where the only possible interaction is HILIC-like, and the phenyl hydride column then in most cases there are greater increases in retention time on the phenyl hydride column. The only exception is for salbutamol. For example acebutolol has quite a low log P value and would be expected to have a strong HILIC interaction and the HILIC interaction might be expected to be greatest on the unmodified silica hydride surface where the whole surface is available for interaction rather than being partly occupied by phenyl groups. However, the increase in retention for acebutolol is greatest on the phenyl hydride column when moving from 90% to 95% acetonitrile and a similar observation can be made for dextromethorphan. Thus in addition to HILIC and lipophilic partitioning there has to be a third unknown interaction.

Although the purpose behind the synthesis of hydride phases is to overcome the problem of silanol group ionisation and contribution to the retention mechanism, the data in this study concluded that even under extreme HILIC condition (acetonitrile ≥90%) there is still electrostatic contribution from the residual silanols on the hydride surface, therefore the problem has not been eliminated. Based on that, the phenyl and cholesterol hydride-based stationary phases will also experience a degree of HILIC-like and/or lipophilic interaction. Consequently, the attribute of the stationary surface seems to be only minimised on these types of phases

which produced a unique model of interactions and selectivity despite the physical and/or chemical properties of the analyte; it will experience mixed-mode of mechanisms. Hence the observations made in this investigation should provide useful guidance in design and selection of stationary phases for HILIC interaction.

Table 3-7: Retention time differences (in minutes) between 90% and 95% ACN on cogent phenyl and hydride columns.

Drug	**Increase in retention time between 90 and 95% acetonitrile (min.)**	
	Cogent Hydride	**Cogent phenyl hydride**
Nortriptyline	2	11.6
Desipramine	-	12
Procaine	-	0.2
Diphenhydramine	-	1.8
Chlorpromazine	-	0.4
Propranolol	-	4.5
(+-) Chlorpheniramine	1.2	3.8
Pseudoephedrine	-	-
Dextromethorphan	0.4	10
Atenolol	10.8	14.5
Triprolidine	-	9.1
Acebutolol	3.1	9.6
Salbutamol	20	10.1

This conclusion is based on the retention behaviour of the tested probes in the light of log P values. All retention behaviour on the silica hydride, phenyl hydride, and cholesterol hydride which was discussed in this section

using log P was re-visited, in the next section, using log D values to see if taking into consideration ionisation made any differences to the inferences that could be drawn.

3.5 Introduction to Distribution Coefficient

The intrinsic lipophilicity (P) of a compound refers only to the equilibrium of the unionised drug between the aqueous phase and the organic phase. The overall ratio of an ionised drug between the phases can be described as the distribution coefficient (D) also known as the apparent log P, log P_{app}, to distinguish it from the intrinsic lipophilicity (P) which is a constant [142]. The term has become widely used in recent years to describe, in a single term, the effective lipophilicity of a compound at a given pH taking into account both its intrinsic lipophilicity and its degree of ionisation [142]. While log P is thermodynamically constant and is not affected by pH, log D is pH dependent and thus the distribution of ionised/unionised forms of a molecule can be varied in octanol/water phases at different pH. The distribution coefficient (D) for a monoprotic organic acid (AH) is the ratio of the experimentally measured concentrations of the solute's forms , ionised and unionised, in octanol divided by the sum of such concentrations of its forms in the aqueous phase, which can be defined as [142]:

For acidic compounds:

$$\text{Log D} = \log P_{app} = \frac{[AH] + [A^-]_{octanol}}{[AH] + [A^-]_{water}} \qquad \textbf{Eq. 1.3}$$

For basic compounds:

$$\text{Log D} = \log P_{app} = \frac{[B] + [BH^+]_{octanol}}{[B] + [BH^+]_{water}} \qquad \textbf{Eq. 2.3}$$

Partitioning of a compound between aqueous and lipophilic (organic) phases is an equilibrium process. Both ionised and unionised forms of the molecule will partition into octanol as well as in water. However, the amount of ionised/unionised will vary with pH. This traditional view is shown schematically for acidic and basic compounds in **Figure 3-17 (A** and **B)** for acids and bases respectively [142].

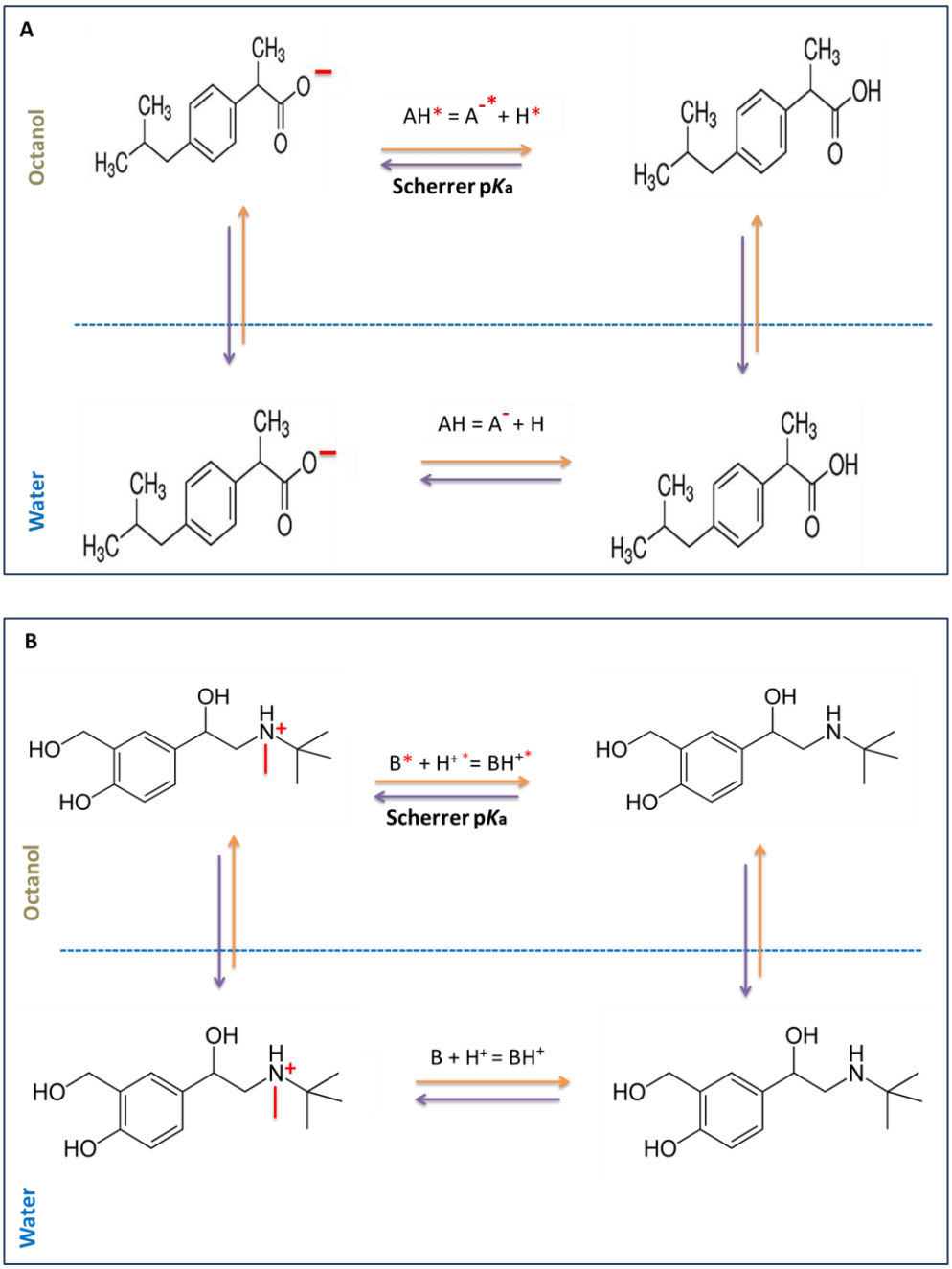


Figure 3-17: Equilibrium presses of acid/base portion into organic-aqueous phase.

The relationship between log D and log P for monoprotic organic acids can be expressed as:

$$\mathbf{Log\ D\ =\ log\ P\ -\ log\ [1+\ 10^{(pK_a - pH)}]} \qquad \textbf{Eq. 3.3}$$

While for monoprotic organic bases the corresponding relationship is given by:

$$\mathbf{Log\ D\ =\ log\ P\ -\ log\ [1+\ 10^{(pH - pK_a)}]} \qquad \textbf{Eq. 4.3}$$

From these equations, it is possible to predict the effective lipophilicity (log D) of an acidic or basic compound at any pH value. The data required in order to use the relationship in this way are the intrinsic lipophilicity (log P), the dissociation constant (pK_a), and the pH of the aqueous phase. The percentage ionisation was calculated using the following equation [3]:

$$\textbf{\% Ionisation} = \frac{100}{1+10^{x(pH-pKa)}} \textbf{ x 100} \qquad \textbf{Eq. 5.3}$$

Where x = -1 for acidic drug and 1 for basic drug.

The correlation between pH and log D suggests that increasing the pH (for acids) will decrease the proportion of unionised drug available to distribute into the organic medium. However, for bases the proportion of unionised drug available to distribute into the organic medium will increase.

Therefore, it is expected to have long retention for probes with low log D values under typical HILIC conditions.

Although it is well established that a hydrophilic surface holds water when exposed to mixtures of organic solvent and water, HILIC is more than just a simple partitioning, and may include polar interactions as well as hydrophobic interactions for molecules with low affinity for water. Moreover, ionisable molecules may experience some electrostatic mechanisms. Alpert [1] considered dipole–dipole interactions, and hydrogen bonds may contribute to partitioning into the stationary phase layer. Hydrogen bonding, especially when using low-water mobile phase, probably contribute also hydrogen-donor interactions, between neutral polar species, make important contributions to the mechanism of separation. Electrostatic interactions can play an important role with ionisable molecules due to the ionisation of residual silanol groups on the silica surface, which cannot be removed or blocked because of steric effects of bonded ligands HILIC [143]. This may cause strong polar (hydrogen-bonding and dipole– dipole) interactions between the basic groups and the ionised residual silanols of the support. However, presence of buffering salts in the mobile phase can decrease electrostatic interactions through disruption [143, 144].

3.5.1 Distribution Coefficient as Measure of Lipophilicity for Acidic Probes

At the experimental pH of 6.9, all the acidic probes will be almost completely ionised, see **Table 3.8**. Therefore, the distribution coefficient for the probes at the experimental pH has been adopted as the standard measure of lipophilicity in order to explain the retention behaviour in the HILIC system. The more lipophilic a molecule is, the more soluble it is in a lipophilic organic phase but when a molecule is ionised, it can form more hydrophilic electrostatic interactions and subsequently has less affinity for the organic phase. Therefore, ionisation of a molecule leads to the accumulation of the hydrophilic form in the aqueous phase; consequently they have more affinity to a hydrophilic layer in HILIC. In contrast, its lipophilic form will have more affinity for the organic phase producing less retention in HILIC. For the determination of log D values, aqueous pK_a values have been used. However the mobile phases used contain at least 90% acetonitrile. In non-aqueous solution, lower relative static permittivity is accompanied by decreased ion stabilisation, and this is manifested in increased pK_a values for acids which will decrease their ionisation [4, 143]. However, measuring non-aqueous pK_a values is difficult and outside the scope of this work. Also in HILIC mode, the adsorbed layer of water on the stationary phase will have a much lower concentration of acetonitrile and so the use of aqueous pK_a values would seem practically more applicable.

Table 3-9 presents the retention time for all acidic test probes on the silica hydride, phenyl hydride and cholesterol hydride columns with hydro-organic mobile phases containing 90 and 95% ACN. All acidic probes experienced an increase in their retention as the concentration of acetonitrile increased from 90 to 95 in the mobile phase, which appears to confirm the HILIC behaviour of these acids on the selected phases. Log D value for acids were calculated from the predicted log P and pk_a, values obtained from ChemAxon's online software (https://chemicalize.com/welcome).

Table 3-8: Physiochemical properties of the acidic probes involved in the HILIC study. Log D for acids was calculated from **Equation 1.3** (**section 3.5**) and the percentage ionisation was calculated from **Equation 5.3**, (**section 3.5**). The predicted log P and pK_a values obtained from ChemAxon's online software (https://chemicalize.com/welcome).

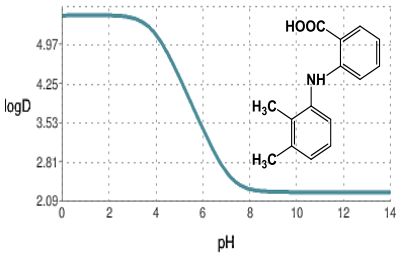
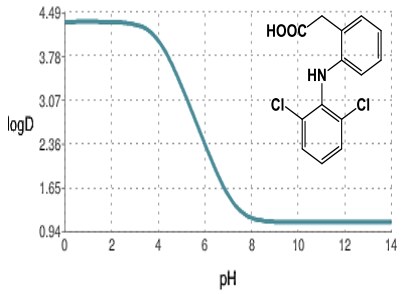
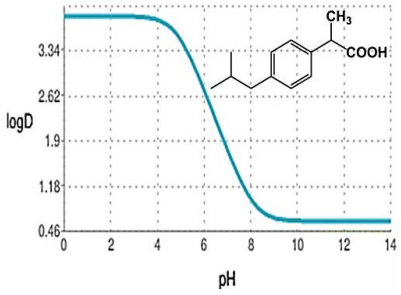
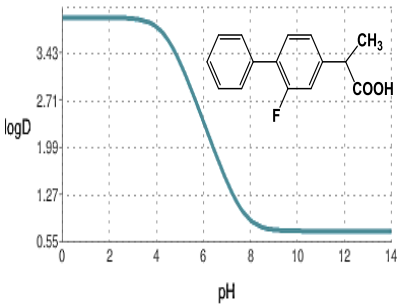
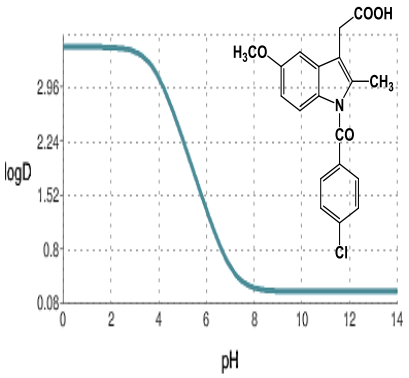
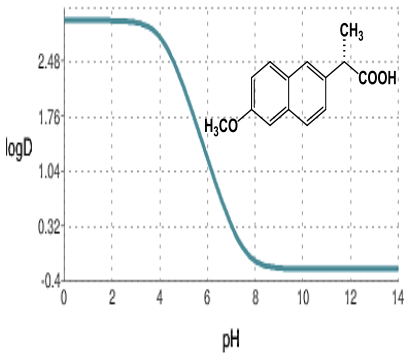
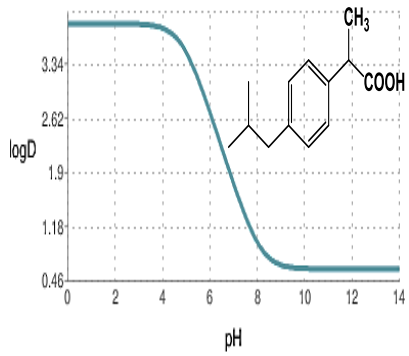
Acid identity	**pk_a**	**% Ionisation at pH 6.9**	**Log D at pH 6.9**	**Log P**	**Log D *vs.* pH**
Mefenamic acid	5.1	99.90	2.39	5.4	
Diclofenac	4.0	99.87	1.40	4.1	
Ibuprofen	4.9	99.12	1.8	3.8	
Flurbiprofen	4.4	99.67	1.42	4.00	

Table 3-8 continued

Acid identity	pk_a	% Ionisation at pH 6.9	Log D at pH 6.9	Log P	Log D *vs.* pH
Indomethacin	3.8	99.92	0.5	3.6	
Naproxen	4.2	99.80	0.3	3.00	
Ketoprofen	3.9	99.90	0.6	3.3	

From **Table 3-9**, it was observed that mefenamic acid which has the highest log D value (2.4), has the shortest retention on all tested columns compared to other probes as expected in the HILIC system confirming, unexpectedly, the formation of the hydrophilic layer on the surface of the "lipophilic" stationary phases. However, the correlation of retention with log D values of the probes was poor; it can be seen that although diclofenac has a lower log D, 1.4, than ibuprofen, 1.8, ibuprofen retained longer on all columns indicating that the retention might not be caused solely by the distribution effect. Similar observation was recorded for flurbiprofen and diclofenac which have almost the same log D value of (1.42) and (1.40) respectively. However flurbiprofen retained longer on all phases; possibly the presence of fluorine increases the polarity [145] resulting in equilibrium-shift toward the hydrophilic layer. On the silica-hydride phase, it was expected that the acidic probes would have a pure partitioning mechanism as the columns have no ligands attached to their surface. According to the silica-hydride column manufacturer's claim, "these columns have virtually no silanols remaining (< 2%), and so they do not have a strong association with water and other solvents" [146]. Despite ketoprofen having a relatively higher log D value (0.6) than naproxen (0.3) and indomethacin (0.43), it was the most retained probe on the silica-hydride phase compared to the other compounds. Interestingly, phenyl-hydride and cholesterol hydride phases offered a different selectivity for the acidic probes under the same conditions. It was observed that

indomethacin retained longer than ketoprofen and interestingly on the cholesterol hydride phase, ketoprofen and indomethacin had the same retention, 12.7 min, with 95% ACN in the mobile phase.

Table 3-9: Retention data for the acidic probes on the three tested columns under HILIC condition. Ombile phase conataning either 90:10 or 95:5 acetonitirile: ammonium acetate aqueous buffer (20 Mm), pH6.9. All the retention is the mean of (n=3) and (*) refers to split peak.

	Silica hydride retention (min.)			**Phenyl hydride retention (min.)**			**Cholesterol-hydride retention (min.)**		
Acid Identity	**90% ACN**	**95% ACN**	**t_R diff.**	**90% ACN**	**95% ACN**	**t_R diff.**	**90% ACN**	**95% ACN**	**t_R diff.**
Mefenamic Acid **Log D 2.39**	2.4*	4.5	2.1	2.0	3.4	1.4	6.2	7.0	0.8
Diclofenac **Log D 1.4**	2.8	5.5	2.7	2.3	4.5	2.2	8.5	9.2	0.7
Ibuprofen **Log D 1.8**	3.4	6.8	3.4	2.7	4.9	2.2	9.8	10.9	1.1
Flurbiprofen **Log D 1.42**	3.5	8.2	4.7	2.8	6.0	3.2	11.4	12.8	1.4
Indomethacin **Log D 0.43**	3.6	8.9	5.3	3.5	8.1	4.6	12.7	18.0	5.3
Naproxen **Log D 0.3**	3.9	8.7	4.8	2.9	6.5	3.6	11.3	12.8	1.5
Ketoprofen **Log D 0.6**	4.0	9.9	5.9	2.9	7.2	4.3	12.7	14.4	1.7

Table 3-9 also shows that with 95% ACN, indomethacin (log D 0.43) has the longest retention of 8.1 min on the phenyl-hydride column, and up to 18.0 min on the cholesterol-hydride. Despite the log D of indomethacin being very close to naproxen, log D 0.3, the latter had a significant shorter retention than indomethacin on the phenyl-hydride and cholesterol-hydride phases under the same conditions. The electron transfer interaction between the phenol and the indole ring of indomethacin was possibly the cause of the long retention on the phenyl-hydride column, while the strong retention on the cholesterol hydride column suggests the contribution of an extra interaction in addition to HILIC partitioning. On the cholesterol-hydride phase, despite the marked difference in log D of flurbiprofen, 1.42, and naproxen, 0.3, both probes seem to have very similar retention with 90% and 95% ACN, **Table 3-9**. This observation indicates that in a HILIC system the underlying molecular process of retention and its correlation with solute lipophilicity are not sufficiently understood yet. There is certainly partitioning but it also appears that additional interactions are taking place in the retention of acids on the cholesterol-hydride phase. It is concluded that this phase may possess a unique function of well-retaining compounds with different physiochemical properties. It is also appears that under HILIC conditions the separation mechanism is complex and there are other factors such as the chemistry of stationary phase surface in addition to the nature of tested molecules that could affect the retention in the hydrophilic layer. It was interesting to note

that on the cholesterol-hydride column, retention increased with increasing acetonitrile concentration in the mobile phase which somehow confirming that HILIC-type interaction still occurs with such a large hydrophobic ligand attached to the surface.

The increase in the retention of all acidic probes when the acetonitrile content increased in the mobile phase indicates that a water layer forms on the surface of all the tested stationary phases. However, the greatest difference in retention when the acetonitrile increased from 90 to 95% was observed in the silica hydride column whilst the cholesterol hydride phase showed a little increase in retention, see **Table 3-9**. On the silica hydride and the phenyl hydride phases, the increase in retention of acids when the acetonitrile percentage increased from 90 to 95% in the mobile phase was observed. However, acidic probes experienced a slight increase in retention on the cholesterol hydride column, apart from indomethacin which clearly responds to the formation of the hydrophilic layer when the acetonitrile percentage increased to 95% in the mobile phase. Surprisingly, the amount of increase in the retention of indomethacin was the same, 5.3 min, on the silica hydride and the cholesterol hydride phases see **Table 3-9**, regardless the differences in the surface properties of the two columns which could have an effect on the in situ hydrophilic layer which is formed under HILIC condition. On conventional silica columns, the driving force for the formation of this water layer is the interaction of silanol groups with water. Surprisingly, the retention here strongly indicates the

formation of a hydrophilic water layer but the factors responsible for its formation are not clear, since according to manufacturer the amount of residual silanols in silica hydride columns is low (< 2%) [146]. Are there more residual silanol groups than expected?

3.5.2 Distribution Coefficient as Measure of Lipophilicity for the Basic Probes on the tested Columns

All of the basic probes which are used in the HILIC study contain ionisable groups and are likely to be charged at the experimental pH 6.9, **Table 3-10**. Therefore, it was expected for bases with low log D to have more partition equilibrium towards the immobilised water layer on the stationary phase, and thus more retention. The log D for the basic probes was calculated at pH 6.9 using the predicted log P and pK_a for the singly ionised species obtained from ChemAxon's online service. **Table 3-11** re-shows the retention data of all basic probes on the silica hydride, phenyl hydride and cholesterol-hydride columns for the two HILIC mobile phases used.

Table 3-10: physiochemical properties of the acidic probes involved in the HILIC study. Log D for bases was calculated from **Equation 2.3** (**section 3.5**) and the percentage ionisation was calculated from **Equation 5.3** (**section 3.5**). The predicted log P and pK_a values obtained from ChemAxon's online software (https://chemicalize.com/welcome).

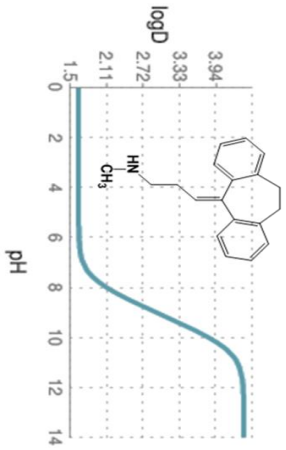
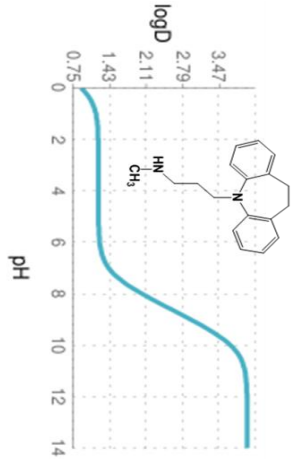
Base	% Ionisation	pk_a	Log D at pH 6.9	Pred. Log p	Log D vs. pH plot
Nortriptyline	99.9	10.47	0.86	4.43	
Desipramine HCl	100.0	10.02	0.78	3.9	

Table 3-10 continued

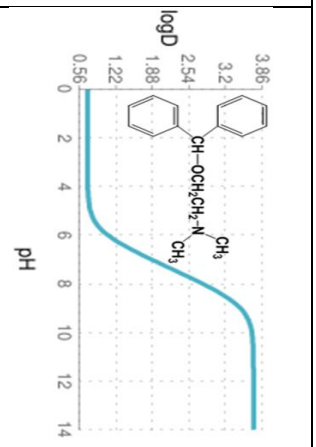
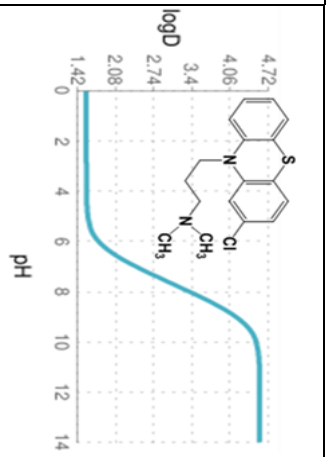
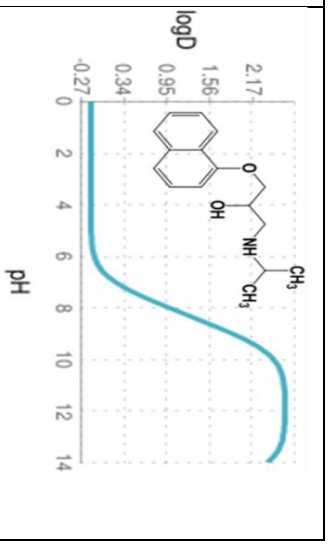
Base	% Ionisation	pk_a	Log D at pH 6.9	Pred. Log p	Log D vs. pH plot
Diphenhydramine	99.2	8.87	0.58	3.65	
Chlorpromazine	99.6	9.2	2.24	4.54	
Propranolol	99.7	9.67	- 0.19	2.58	

Table 3-10 continued

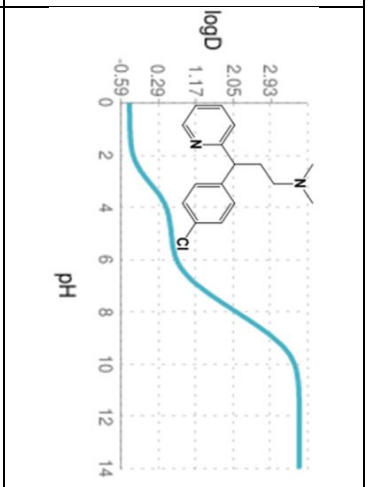
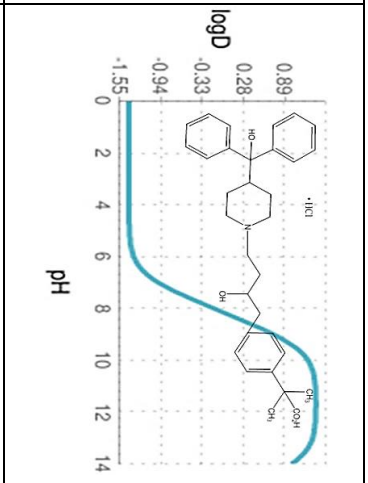
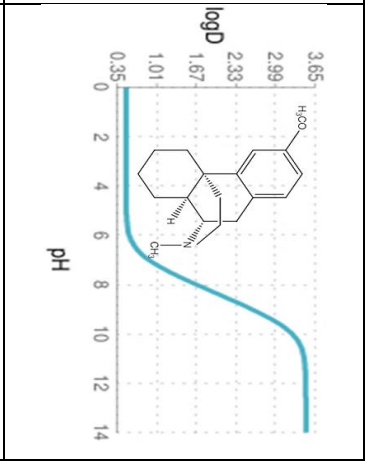
Base	% Ionisation	pk_a	Log D at pH 6.9	Pred. Log p	Log D vs. pH plot
Chlorpheniramine malate	99.5	9.47	1.01	3.58	
Pseudoephedrine	99.9	9.52	- 1.30	1.32	
Dextromethorphan	96.2	9.85	0.54	3.49	

Table 3-10 continued

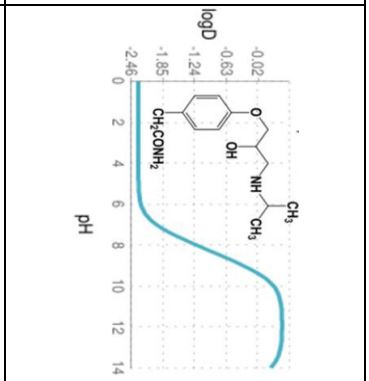
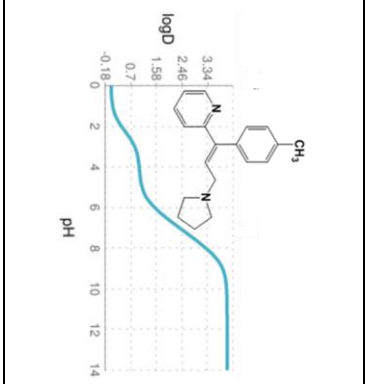
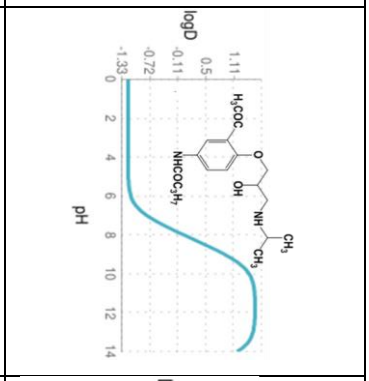
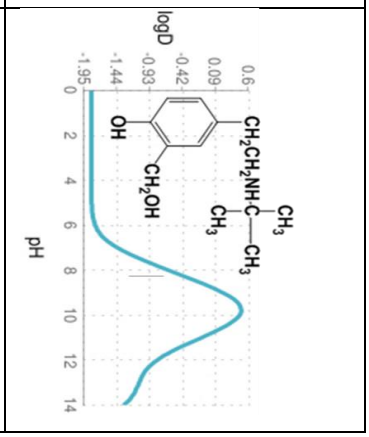
Base	% Ionisation	pk_a	Log D at pH 6.9	Pred. Log p	Log D vs. pH plot
Atenolol	99.8	9.67	- 2.34	0.43	
Triprolidine	28.5	8.64	2.30	4.05	
Acebutolol	99.7	9.57	- 1.14	1.53	
Salbutamol	99.6 100	9.40	- 2.16	0.34	

Table 3-11: Retention data for the basic probes on the three tested columns under HILIC condition. The mobile phase conataning either 90:10 or 95:5 acetonitirile: 20 Mm ammonium acetate aqueous buffer, pH6.9.

	Retention on silica-hydride (min)			**Retention on phenyl-hydride (min)**			**Retention on cholesterol-hydride (min)**		
Base	**90% ACN**	**95% ACN**	**Diff. in t_R**	**90% ACN**	**95% ACN**	**Diff. in t_R**	**90% ACN**	**95% ACN**	**Diff. in t_R**
Nortriptyline	4.4	6.4	2	13.3	24.9	11.6	32.6	27.1	5.5
Desipramine HCl	4.5	*	*	13.4	25.4	12	33.9	29.4	4.5
Procaine HCl	4.2	4.2	0	7.8	8.00	0.2	6.8	6.4	0.4
Diphenhydramine	3.6	*	*	10.1	11.9	1.8	9.8	8.9	0.9
Chlorpromazine	3.4	*	*	16.1	16.5	0.4	22.02	20.5	1.52
Propranolol	4.5	*	*	8.9	13.4	4.5	13.1	17.5	4.4 ↑
(±)Chlorphenir-amine malate	8.0	6.8 ↓	1.2	40.3	44.1	3.8	13.1	17.3	4.2↑

Table 3-11 continued

	Retention on silica-hydride (min)			Retention on phenyl-hydride (min)			Retention on cholesterol-hydride (min)		
Base	**90% ACN**	**95% ACN**	**Diff. in t_R**	**90% ACN**	**95% ACN**	**Diff. in t_R**	**90% ACN**	**95% ACN**	**Diff. in t_R**
Pseudoephedrine	8.9	*	*	*	*	*	*	*	*
Dextromethorphan	6.2	6.6	0.4	29.6	39.6	10	45.5	39.3	6.2
Atenolol	18.4	29.2	10.8	12.0	26.5	14.5	46.4	40.8	5.6
Triprolidine	6.3	*		43.3	52.4	9.1	42.0	37.6	4.4
Acebutolol	8.4	11.5	3.1	9.7	19.3	9.6	27.2	23.9	3.3
Salbutamol	15.4	35.7	20.3	6.6	16.7	10.1	33.2	32.0	1.2

***** = fully retained

On the silica hydride column, some of the basic compounds were not detected because they are fully retained. However, most of the detected probes showed an increase in retention as the content of acetonitrile increased, apart from (±) chlorpheniramine which experienced a decrease in the retention when the % ACN from 8.0 to 6.8 min, see **Table 3-10/3-11**. It was observed that compounds with similar pK_a did not show the same trend, suggesting this is not due to a change in ionisation or increasing the percentage of acetonitrile. The reason for this decrease is not known yet.

On the phenyl hydride column, the increase in the % ACN led to a significant increase for some of the bases while others showed a slight or negligible increase in the retention. As presented in **Table 3-10/3-11**, although some bases have almost the same log D, such as *chlorpromazine* (2.24) and *triprolidine* (2.30), their retention is very different. *Triprolidine* experienced very strong retention when the % ACN increased to 95%, but this was not observed with chlorpromazine. It would appear that lipophilicity (as measured by log D) is having little effect on the retention of the compounds, and other mechanisms must be taking place. These observations may suggest the contribution of more mechanisms where other types of interactions than a partitioning mechanism come into effect.

Procaine appeared to show some interesting behaviour on the three phases tested. On the silica-hydride column its retention did not change with increasing acetonitrile content in the mobile phase. Only a very small

increase was observed on the phenyl-hydride phase, whilst on the cholesterol phase, a slight decrease was observed. The reason for the slightly different behaviour does not appear to be related to its pK_a or lipophilicity as other compounds have similar properties. This slightly anomalous behaviour may warrant further investigations.

The correlation between the elution order and log D for the basic probes, **Figure 3-18**, on the phenyl hydride phase shows no direct correlation between the retention of the probes and their log D. However data below value of log D = 0 appears to suggest a HILIC-partitioning mechanism; as the log D decreases, retention appears to increase (but, the trend is not simple and the number of compounds is only five). As the log D is increased to above zero, it would appear that more lipophilic retention behaviour starts to take place, but the trend is not clear. Probes with significant difference in their log D but having almost the same retention may indicate there might still many silanol groups which remain active and effectively contribute to the separation mechanism. This is different than the manufacturer's claim; it would be interesting to measure the silanol activity in future work. It is also obvious that lipophilic interactions on this phase can be very important.

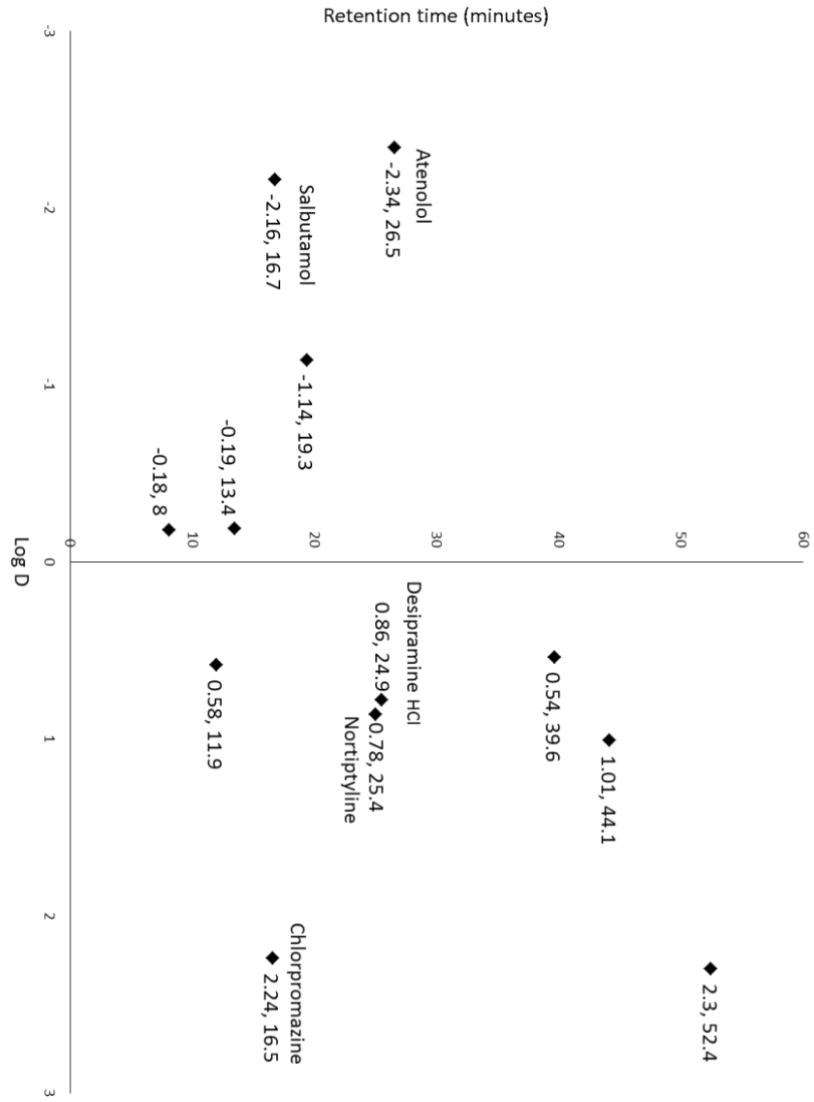


Figure 3-18: The correlation between the tested bases and log D on the phenyl hydride column. The mobile phase contained (5%) 20 mM ammonium acetate: 95% ACN.

Different retention behaviour was observed on the cholesterol hydride column where the retention of most of the bases *decreased* as the acetonitrile content increased from 90 to 95% which would suggest an RP-like retention mechanism rather than HILIC. Interestingly the retention of two probes, propranolol and chlorpheniramine, *increased* as the content of

acetonitrile increased, which would suggest a HILIC- type mechanism for these compounds. This difference does not appear to be pK_a or log D related as other compounds with similar values show a different effect, see **Table 3-11**.

Looking more closely into the cholesterol-hydride column, **Table 3-12,** despite that triprolidine has the highest log D (2.3) and atenolol has the lowest (- 2.3), both have the longest retention. Such behaviour despite log D differences is interesting, and suggesting that cholesterol-hydride phase has unique properties which allow the retention of compounds with different physiochemical characteristics. It is worth to mention that cholesterol hydride phase is the most lipophilic phase among other tested columns, and so the strong retention of probes with the highest log D values could indicate additional hydrophobic interactions under HILIC mode. It is also clear that lipophilic interactions on this phase are very important to the overall retention mechanism.

Table 3-12: Retention data for the basic drugs on the cholesterol-hydride column against Log D. The mobile phase contained 95% acetonitrile: 5% ammonium acetate buffer 20 mM, pH 6.9.

Base	**Log D**	**Retention on cholesterol hydride with 95% ACN**
Triprolidine	2.302	37.6
Chlorpromazine	2.238	20.5
(±)Chlorpheniramine malate	1.009	17.3
Nortriptyline	0.860	27.1
Desipramine HCl	0.780	29.4
Diphenhydramine	0.580	8.9
Dextromethorphan	0.540	39.3
Procaine HCl	-0.183	6.4
Propranolol	-0.191	17.5
Acebutolol	-1.141	23.9
Pseudoephedrine	-1.301	--
Salbutamol	-2.161	32.00
Atenolol	-2.341	40.8

The correlation between the elution order and log D values on the cholesterol-hydride phase is shown in **Figure 3-19**. Data suggested a possibility of hydrophilic and lipophilic interaction operating simultaneously as very similar retention can be observed for very different log D values. It would appear that, due to the presence of HILIC type behaviour on the cholesterol column that, surprisingly, an immobilised water-rich layer still can be formed on the surface of the column,

presumably via hydrogen bonding interactions from residual silanol groups. However, these immobilised water molecules cannot prevent the lipophilic interactions between the analyte and the stationary phase. Therefore, hydrophobic interaction as well as hydrophilic ones is believed to operate on the cholesterol hydride phase under HILIC condition.

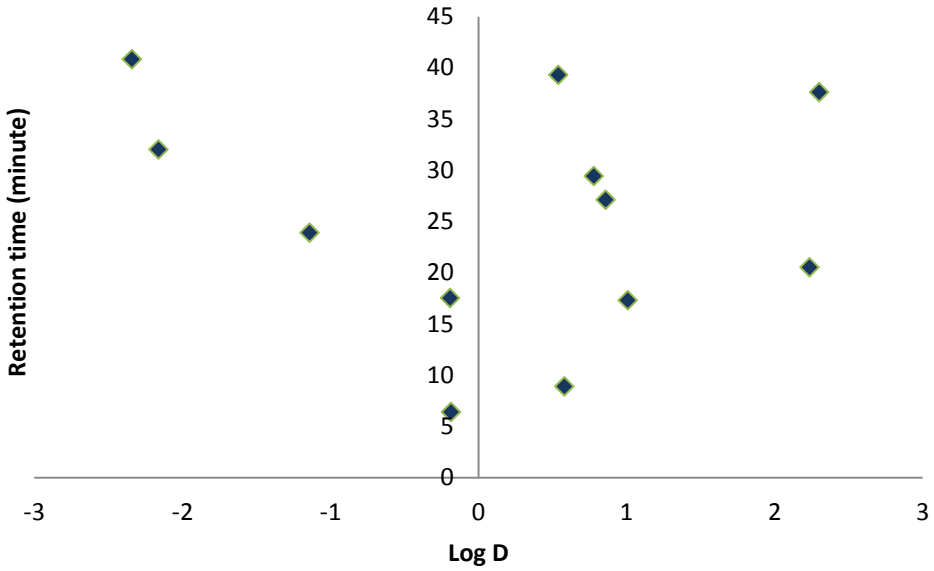


Figure 3-19: Retention data vs. Log D values the basic probes on the cholesterol hydride column. Mobile phase containing 95:5% acetonitrile: 20 mM ammonium acetate buffer, pH 6.9.

3.6 Conclusions Based upon Log D as a Factor

It is commonly believed that in HILIC mode, the mobile phase forms a water-rich layer on the surface of the polar stationary phase vs. the water-deficient mobile phase, creating a liquid-liquid extraction system. Accordingly, the separation mechanism assumed to base on the differential distribution of the analyte between the acetonitrile-rich mobile phase and a water-enriched layer adsorbed onto the hydrophilic stationary phase. Therefore, it was assumed that the distribution coefficient (D) would, hopefully, be a better descriptor of the lipophilicity of the probes and could possibly explain the retention behaviour in HILIC layer.

Data on the silica-C columns suggests that the retention mechanism is complex, consisting of: partitioning between a layer of water held on the surface and the bulk mobile phase; polar interactions with polar functional groups (i.e. silanol groups); and reversed-phase type interactions with hydrophobic portions of non-polar ligands (which could include π-π interactions). This complex mechanism leads to different retention patterns on different stationary phases. Unlike the chemically-bonded ligands on the surface of the stationary phase, the water layer which is formed on the stationary phase surface is only dynamically immobilised under HILIC mode, which may explain the attribution of the surface functional group. The behaviour of the acidic and basic probes suggests that even lipophilic analytes may penetrate through the water-layer to

directly interact with the surface functional moiety although this hypothesis has yet to be confirmed. No matter whether or not “non-polar” analytes could penetrate through the water-layer, it is true that different functional groups have displayed their specific retention behaviour on different hydride-based stationary phases. The outcome of this study is interesting and unexpected, thus more investigations are required to explore how the water-rich layer is formed on the base of the lipophilic-hydride columns. The predominant retention mechanism in a HILIC separation is not persistent and it can be varied especially when different analytes, mobile and stationary phases are applied. The current study to determine the correlation log D of the analytes and their retention factor when the ionic contribution held constant was inconclusive, as the mechanism of HILIC separations is quite complex with retention being affected by partitioning as well as lipophilic interaction, hydrogen bonding or other unknown mechanisms [12]. Also it appears that different surface offer different selectivity to the overall retention in HILIC. Further work needs to be performed to extrapolate these claims.

Chapter 4

Study the contribution of Silica surface to the Overall Retention Mechanism in HILIC using Kromasil® Silica gel Stationary Phase and the applications of HILIC in the impurity profiling in drugs and metabolite profiling in biological samples

4 Study the contribution of Silica surface to the Overall Retention Mechanism in HILIC using Kromasil® Silica gel Stationary Phase and the applications of HILIC in the impurity profiling in drugs and metabolites profiling in biological sample

4.1 Introduction

HILIC chromatography is a complex system involving partition, polar, and ion-exchange interactions. The key of using any chromatographic mode is to have some idea of where the greatest differences lie with respect to the different analyte physiochemical attributes and invoke the most selective retention mechanisms by choosing the most appropriate phase or phases to screen. The previous investigation suggested a strong HILIC interaction on the hydride-based stationary phases but as the surface is attached to some kind of ligands, the contribution of the surface is involved and affects the retention mechanism.

A good starting point for method development is to identify the stationary phase with the appropriate mechanisms that best retain and differentiate the analytes along with offering a desirable peak shape. Thus, understanding the interactions that different stationary phases provide and applying that knowledge to the separation can greatly facilitate the analytical methods in HILIC chromatography. At a minimum, determining the attributes of the stationary phase can be used to eliminate those phases that are inappropriate.

Our recent published study [147] was found that under HILIC conditions the retention mechanism is combination of lipophilic interaction with the siloxane groups of silica gel when working with 20% ACN in the mobile phase because the least charge dense probe benzyldimethylhexyl ammonium chloride (BDM) was almost as strong as the benzyltriethyl ammonium chloride (BTE) which has higher charge dense than BDM but less than benzyltrimethyl ammonium chloride (BTM) which exhibited the lowest retention at 20% ACN. BTE was the most retentive probe at 20% ACN which suggest the contribution of ion-exchange to the overall retention. However, increasing the acetonitrile content above 60% showed that the hydrophilic interaction begins to exert an effect on the most hydrophilic and densely charged probe, BTM, which was the most sensitive to the establishment of the HILIC layer which, as proposed by Melnikov *et al,* is composed of regions with varying degrees of water enrichment as well as fixed largely unvarying layer of water close to the surface of the silica gel [135].

This study will focus on the chromatographic behaviour of acidic and basic drugs on Kromasil silica gel 60 Å which has a great surface area (540 m^2/g) which allows the formation of large volume of water pseudo-stationary phase under HILIC conditions. The set of acidic and basic probes (used in the previous chapter) that are different in the degree of polarity were used to monitor retention behaviour and observe the contribution of the electrostatic and/or lipophilic interaction to the overall retention starting

from reverse phase condition at low acetonitrile content of 20% and increasing the percentage to 90% and 95% in the mobile phase as described in **Figure 4-1**.

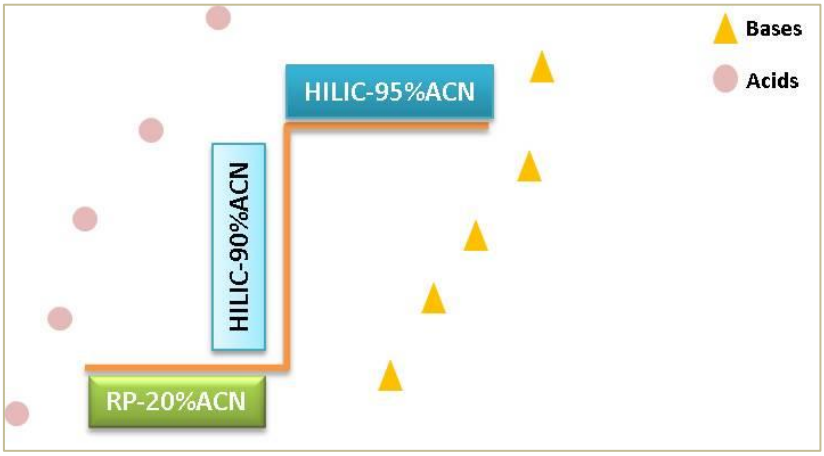


Figure 4-1: Overall study plan.

4.2 Method and materials

4.2.1 Chemicals

Ammonium acetate, HPLC grade methanol and HPLC grade acetonitrile were obtained from Fisher Scientific (Loughborough, UK). HPLC water was prepared in house using a Milli Q purification system. The basic and acidic compounds used in the tests were either from Sigma Aldrich (Dorset, UK), or European Pharmacopoeial standards, or were part of an in house stock donated by companies over the years.

4.2.2 Mobile Phase preparation

The mobile phase consists of ammonium acetate buffer was prepared by dissolving the required amount of base in water to obtain a concentration of 400 mM. Then the final molarity required of 20 mM was achieved by mixing with acetonitrile in the proportion of 5% or 10% of the mobile phase final volume for HILIC study. If a higher proportion of aqueous phase was required, water was added to complete the final volume, *e.g.* (10:90 v/v) buffer:ACN would require (5:5:90 v/v/v) of buffer:H_2O:ACN. For the reversed phase study the mobile phase consisted of 20 mM ammonium acetate buffer was achieved by mixing an appropriate amount of the 400 mM buffer stock with acetonitrile in the proportion of 80:20 buffer: ACN (v/v) and if a higher proportion of aqueous phase was required water was added to the make up the volume (*e.g.* 80:20 v/v) buffer:ACN would require 5:75:20 of buffer: H_2O: ACN. The pH of the buffers, 6.9, was measured and recorded before mixing with acetonitrile in the mobile phase. All mobile phases were degassed prior to use.

4.2.3 Samples Preparation

A set of acidic, **Figure 4-2**, and basic, **Figure 4-3**, drugs were used as models for retention behaviour. A stock solution of each drug was prepared in methanol as 1 mg/ mL and was diluted to, 100 µg mL^{-1}, with the

appropriate mobile phase which contains 20 mM of ammonium acetate buffer, pH 6.9, and acetonitrile in proportion of 5:95 or 10:90 (v/v).

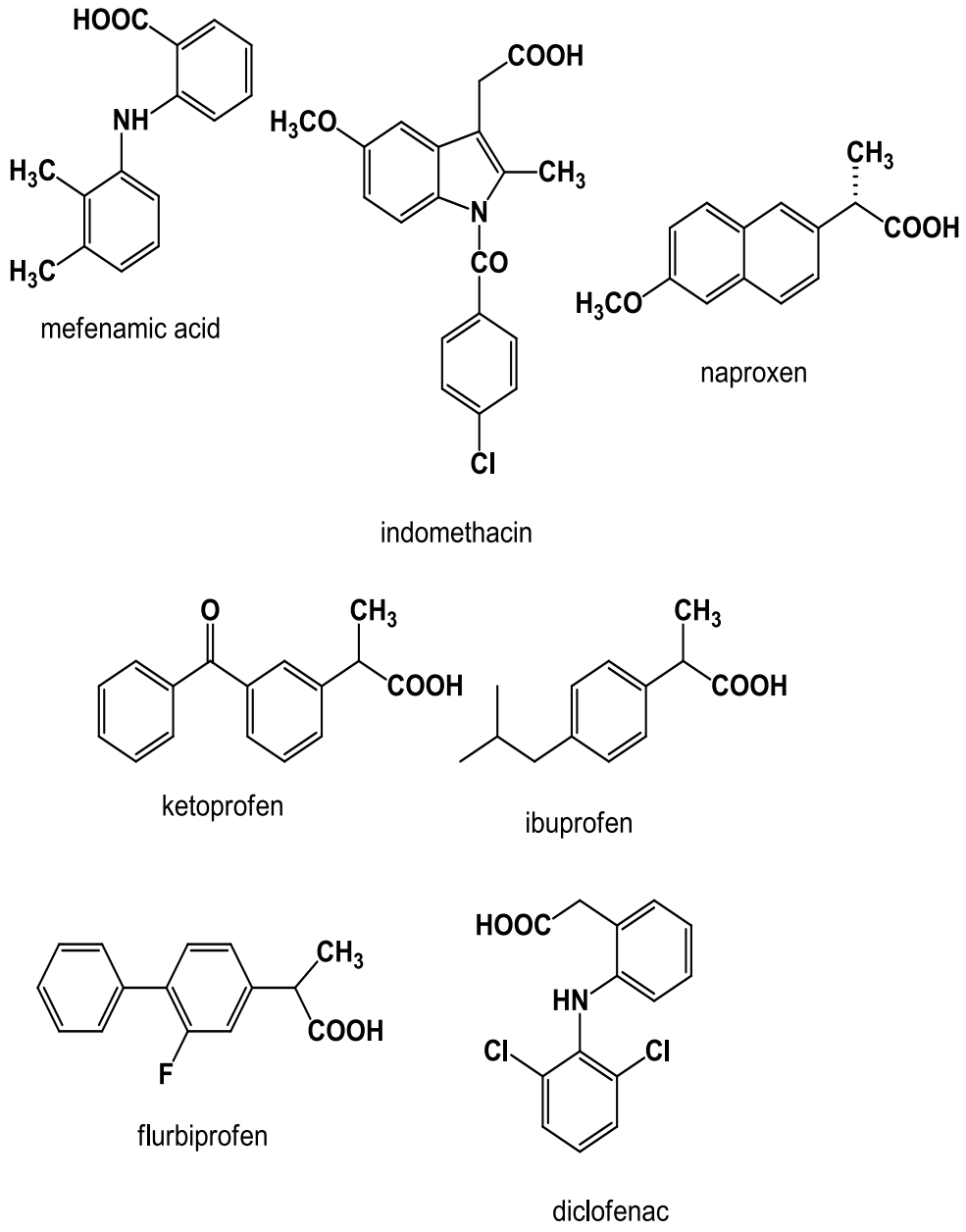


Figure 4-2: Chemical structure of the acids models used in the study.

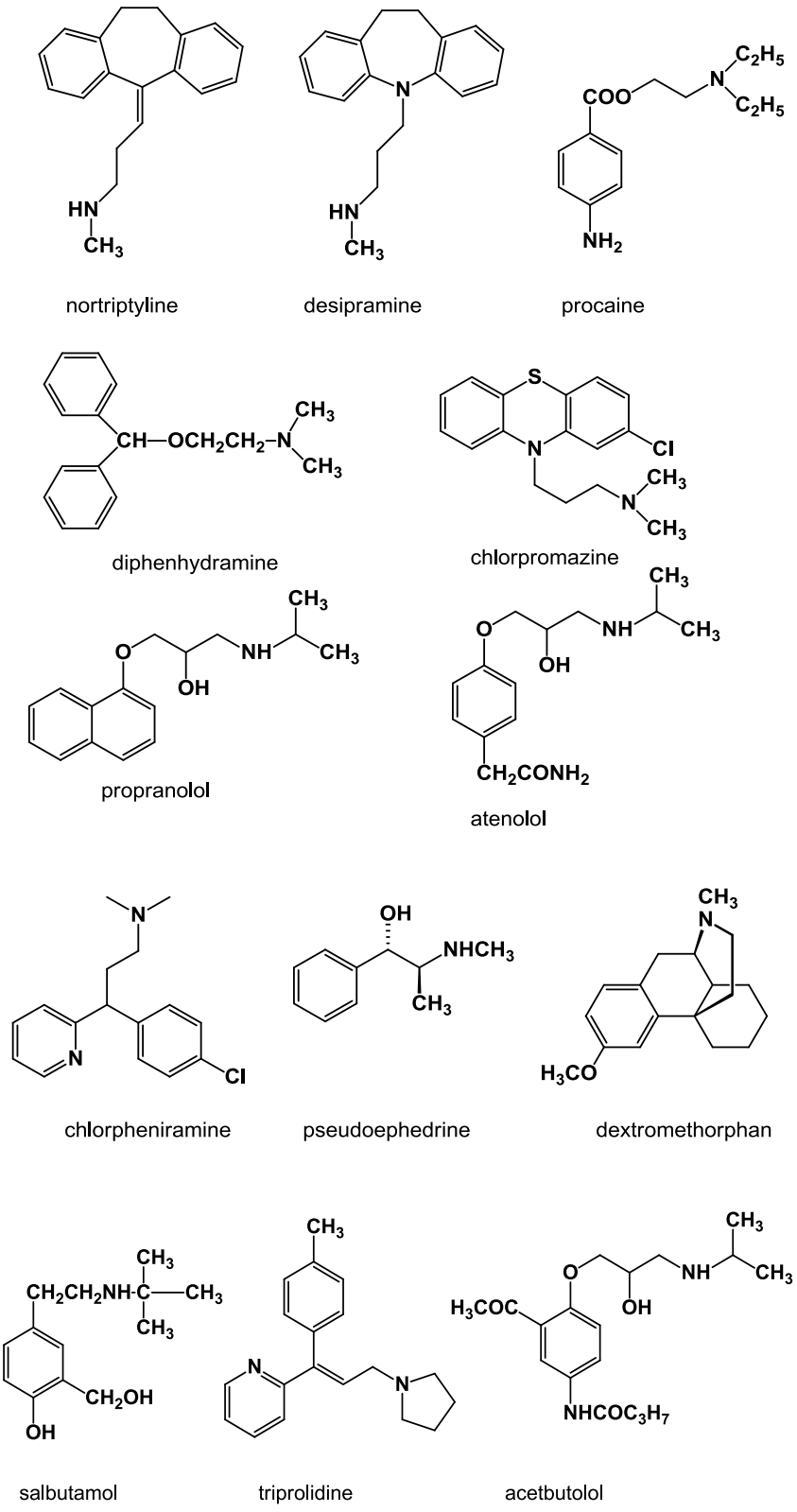


Figure 4-3: chemical structures of basic models used in the study.

4.2.4 Instrumentation

HPLC analysis was carried out on a ThermoFinnigan HPLC system consisting of a P 4000 pump, UV 6000 PDA detector and an AS 3000 autosampler. The mobile phase compositions were mixed off-line in the proportions required and the HPLC system was used in isocratic mode; 10 µl of sample was injected. The flow rate was 1 mL/min and the column was run at room temperature. The PDA-UV detector was used. The void volume of the column was determined according to the minor disturbance peak in the baseline produced by injecting 10 µl of pure methanol. The analytical column, Kromasil 60 Å 5 µm silica gel (150 mm x 4.6 mm i.d.), was purchased from HiChrom Ltd., Reading, UK. The column was kept in a column oven during the analysis to minimise the contribution of temperature in to the retention.

4.3 Results and Discussion

An isocratic HILIC analysis was carried out to study the retention behaviour of the acidic and basic analytes on the Kromasil silica gel stationary phase. Only ACN was investigated as the organic modifier of the mobile phase because it is the solvent of choice for HILIC. The mobile phase consists of constant strength of 20 mM ammonium acetate buffer in water and acetonitrile content varied between 20%, 90% and 95%. Distinctively different retention behaviour was observed for the acidic probes on the

bare silica gel column, and although the acids are relatively hydrophobic they experienced a strong retention in the hydrophilic system while the electrostatic repulsion effect was clearly seen in RP conditions.

4.3.1 Chromatographic Retention of Lipophilic acids on Silica gel column in HILIC

The chemical properties and the retention data for the acidic probes are shown in **Table 4-1**. Generally, all acidic probes exhibited a marked increase in the retention time in the silica gel column as the acetonitrile content in the mobile phase increased.

Table 4-1: The retention data of the acidic probes under different mobile phase conditions and their physiochemical information. (*) indicates a splitting peak and (**) strong splitting. Log D and the percentage ionisation were calculated from **Equation 1.3** and **Equation 3.3** respectively in **(section 3.5, Chapter 3)**. The predicted log P and pK_a values obtained from ChemAxon's online software (https://chemicalize.com/welcome). t_R = retention time in minute.

Acid identity	**pK_a**	**Log P**	**Log D**	**% Ionisation**	**t_R at 20% ACN**	**t_R at 90% ACN**	**t_R at 95% ACN**
Mefenamic acid	4.2	4.03	2.39	99.8	1.62	2.5 **	4.8 *
Diclofenac	4.2	4.1	1.40	99.8	1.67	3.2	6.5
Flurbiprofen	4.2	4.00	1.42	99.7	1.64	3.8 *	10.1
Ibuprofen	4.4	3.8	1.8	99.0	1.68	3.7	8.2
Indomethacin	4.5	3.6	0.5	99.6	1.67	4.0	9.8
Ketoprofen	4.5	3.3	0.6	99.6	1.66	4.4	10.7
Naproxen	4.2	3.0	0.3	99.8	1.66	4.2	9.4

At 20% ACN, the electrostatic repulsion of all acidic probes is clearly seen as the silanol surface and the acidic probes will be negatively charged at pH 6.9. Increasing the concentration of acetonitrile in the mobile phase to 90% led to a significant increase in the retention of the acidic probes as illustrated in **Figure 4-4**. This increase in the retention is caused by the formation of the HILIC layer on the silica surface where the analytes will partition. The retention of the acids is unexpectedly strong since they are all hydrophobic and even when highly ionised will still have a high partition coefficient, as previously shown in **Table 4-1**. It is possible that at high levels of acetonitrile the concentration of ammonium ions in the HILIC layer is sufficiently high to generate an in situ anion exchange phase with ammonium ions being loosely bound to the silica gel surface or a mechanism could be based on hydrophilic ion pair formation in a manner analogous to lipophilic ion pair formation in reversed phase chromatography.

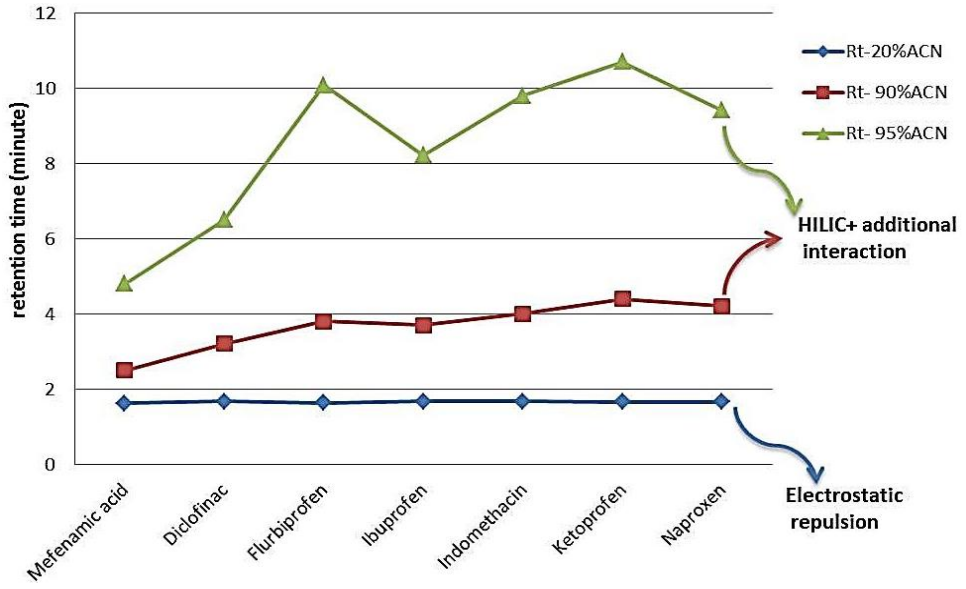


Figure 4-4: Plot of average retention time of acidic analytes at 20%, 90% and 95% ACN and with 20 mM ammonium acetate buffer at pH 6.9, on kromasil silica gel column.

The acidic probes exhibited the greatest retention on the silica gel surface at 95% acetonitrile in the mobile phase. **Figure 4-4** shows a significant increase in the retention of all probes moving from 90% to 95% ACN. This observation agrees with the hypothesis of HILIC where it is believed that when the concentration of acetonitrile increases, water interacts more strongly with the surface of the polar stationary phase, in this case the bare silica. In this case, acetonitrile cannot interact with residual silanols on the stationary phases, so they are uncovered and water molecules can be adsorbed onto them [148 -150]. Presumably high concentrations of organic solvent in the mobile phase cause the polar salt in to partition preferentially into the water-rich pseudo-stationary phase. The presence of

more solvated ions in this phase would increase its volume, potentially leading to stronger retention of solutes. It was also observed that with 95% ACN in the retention time of the most hydrophilic probe ketoprofen, log D = 0.6, retained as strong as the relatively lipophilic flurbiprofen, log D = 1.42. However, interestingly, Diclofenac (log D 1.40) which has almost the same log D as flurbiprofen experienced a significant lower retention but ibuprofen which has higher log D of 1.8 retained longer than diclofenac. The fluorine is known to have the greatest electronegativity of all elements which could increase the polarity of the compound and might explain the long retention in the hydrophilic layer. Because of the similar behaviour, it is difficult to clearly distinguish the performance of the Kromasil column from that of the Cogent hydride phase.

Generally all acidic probes produced excellent sharp peak shapes on the silica gel column apart from mefenamic aid and flurbiprofen which exhibited splitting effects as shown in **Figure 4-5**. The long retention of flurbiprofen over the mefenamic acid at 95% ACN in the mobile phase, despite both acids have the same log P and pK_a values ,**Table 4-1**, could be explained by the presence of fluorine substituent in the flurbiprofen which adds more polarity to the molecule.

At 20% ACN, the peak shapes suggested that both acids exhibited more or less a single retention mechanism while the splitting effect at 90% ACN indicates the exert of secondary interaction in addition to HILIC. The

splitting effect was overcome at 95% ACN for flurbiprofen while mefenamic acid still experienced the splitting but the effect is smaller which could indicate the strong HILIC effect over the secondary interaction at 95% ACN.

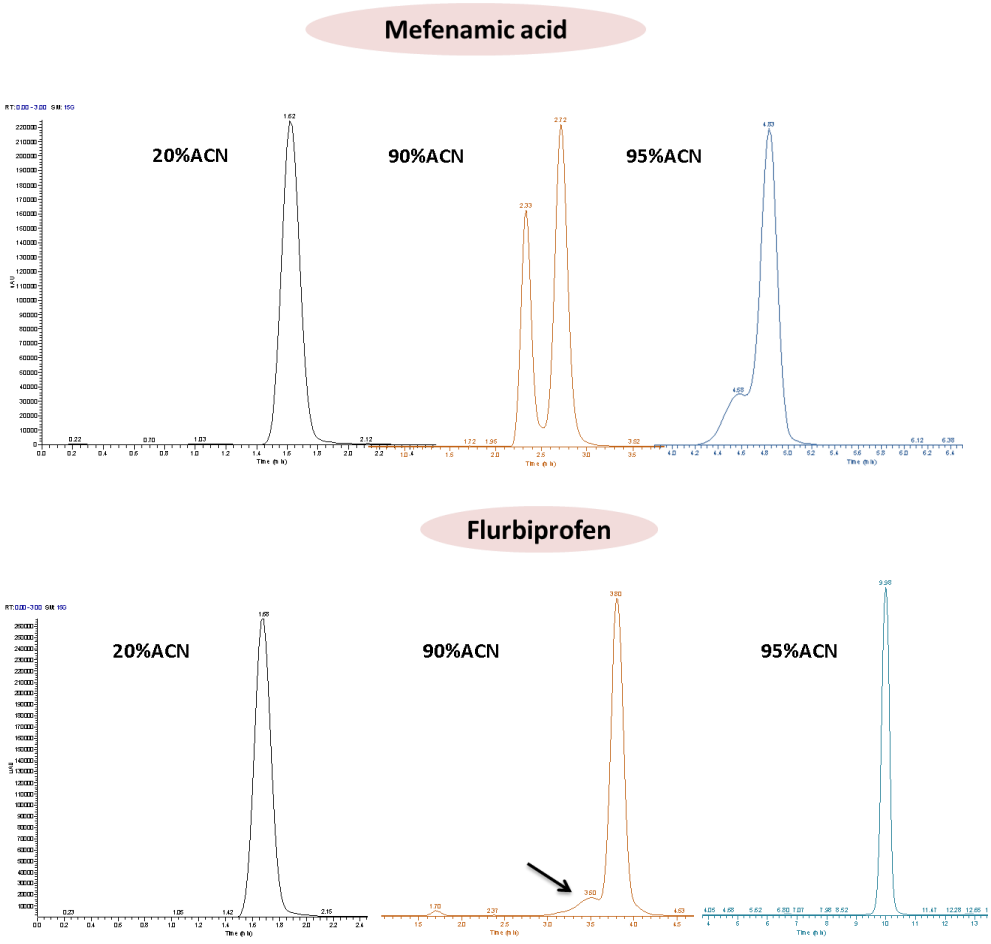


Figure 4-5: The effect of acetonitrile content in the mobile phase on the retention and peak shape of mefenamic acid and flurbiprofen.

Figure 4-6 shows plots of the retention time for the acids verses their log D values at different percentages of acetonitrile in the mobile phase.

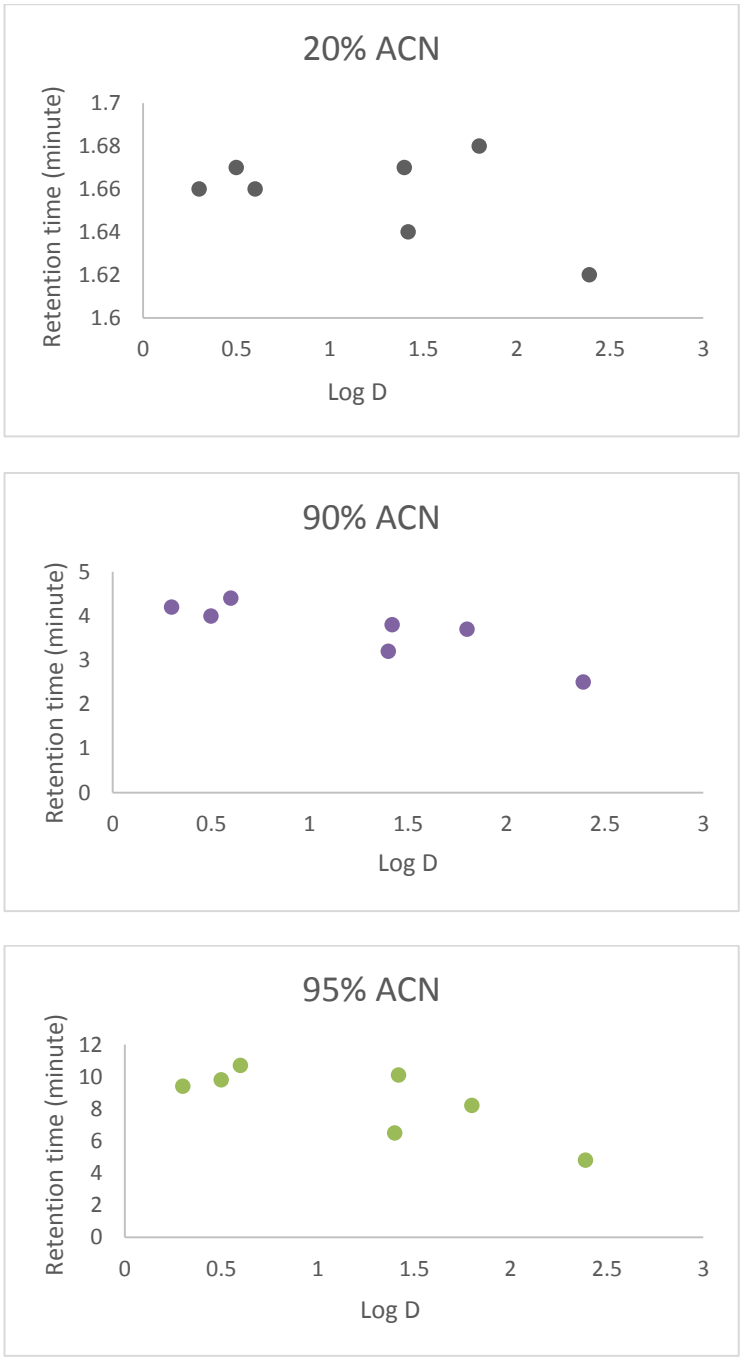


Figure 4-6 : Plots show the trend of retention time of acids against their log D, at different concentration of ACN in the mobile phase; 20%, 90% and 95% ACN.

At 20% ACN, the plot of retention verses log D shows no clear trend. There is possibly a general decrease in retention with log D but this is not clear. A lipophilic retention mechanism would show increased retention with log D. This is not happening here, as would be expected for a polar stationary phase such as silica.

At 90% ACN, the formation of the hydrophilic layer is very clear as the retention of the acids increased and an inverse correlation can be seen with Log D suggesting hydrophilic interactions. A further increase in the retention of acids was observed with 95% ACN and again the plot of retention verses log D shows an inverse correlation between Log D and the retention. This would appear to confirm the exert of hydrophilic interactions when the organic content is increased to high levels (>80%).

Plots of the retention of acids verses their log P, are shown in **Figure 4-7**. Trends are somewhat similar to the log D plots. However, it is interesting to note that retention of compounds with log P around 4 show very different retentions; giving the plots a vertical drop in data at high log P values. This would appear to confirm the importance of using log D instead of log P to take into consideration the ionised state of the molecules.

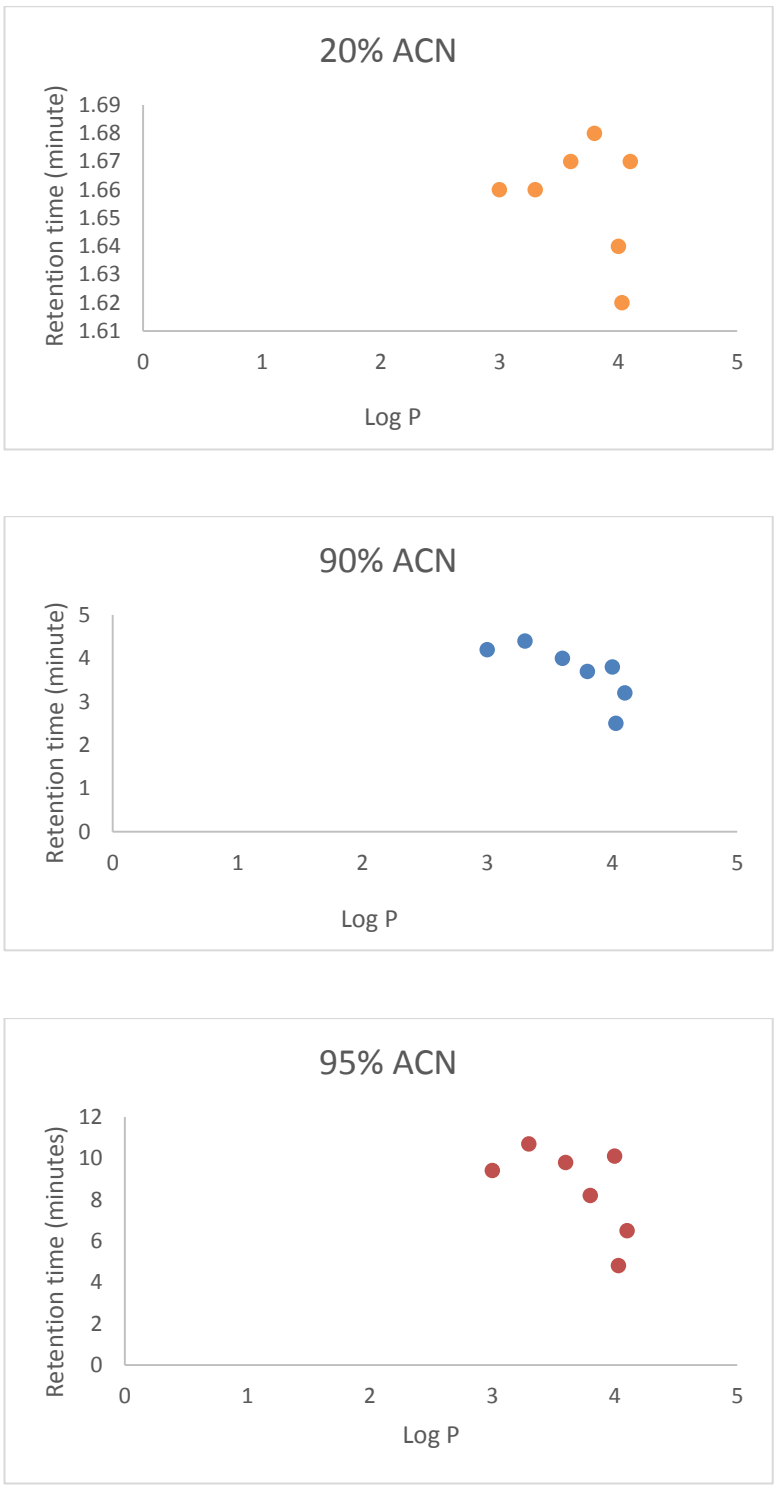


Figure 4-7: Plots show the trend of retention time of acids against their log P at different concentration of ACN in the mobile phase; 20 to 90 and 95% ACN.

4.3.2 Chromatographic Retention of the Lipophilic bases on Silica gel column in HILIC

In HILIC, it is thought that the more hydrophilic the analytes, the more the partitioning equilibrium is shifted towards the adsorbed water layer on the polar stationary phase, and, thus the more the analytes are retained. The data in **Table 4-2** show the retention behaviour for the basic probes at different organic percentage in the mobile phase. In general, the retention of the most hydrophilic probes increases with increasing % acetonitrile while most lipophilic probes decreased. However, unexpected behaviour was observed for some lipophilic probes which retained longer than the least lipophilic probes in HILIC conditions.

Figure 4-8 displays the retention trend of all basic probes at 20%, 90% and 95% ACN. The retention of salbutamol significantly increased at 95% ACN and it was the most retained probe whereas at 20% ACN it was the first to elute from the system and it experienced a negligible increase in the retention when acetonitrile content increased from 20% to 90%. This strongly supports the establishment of the hydrophilic layer at high organic content, 95% ACN.

Table 4-2: The retention data of the basic models and their chemical properties at the experimental condition. (*) splitting effect (t) tailing effect and (f) fronting. Log P and pK_a values obtained from ChemAxon's online software (https://chemicalize.com/welcome). t_R = the retention time in minutes.

Base identity	**pK_a**	**Exp. Log P**	**t_R at 20% ACN**	**t_R at 90% ACN**	**t_R at 95% ACN**	**% Ionisation at pH 6.9**
Chlorpromazine	9.3	5.2	9.1^t	3.8*	5.0	99.6
Nortriptyline	10.1	4.5	6.2^t	5.1	7.6	99.9
Chlorpheniramine	9.2	4.0	14.9^t	8.3	9.9	99.5
Desipramine	10.4	3.9	5.9^t	5.4*	8.4^f	100
Triprolidine	6.5	3.8	17.2^t	6.3	7.8	28.5
Propranolol	9.5	3.4	5.3	4.9	8.7	99.7
Dextromethorphan	8.3	3.4	12.5^t	7.0	7.4	96.2
Diphenhydramine	8.98	2.8	7.2	4.2	5.8	99.2
Salbutamol	9.3-10.3	1.8 - 1	4.5	11.9	28.6	99.6-100
Procaine	9.0	1.3	7.5	4.5	5.4	99.2
Acebutolol	9.4	0.9	6.2	8.4	11.9	99.7
Pseudoephedrine	9.8	0.9	5.2	9.5	18.5	99.9
Atenolol	9.6	0.2	5.3	15.7	26.7	99.8

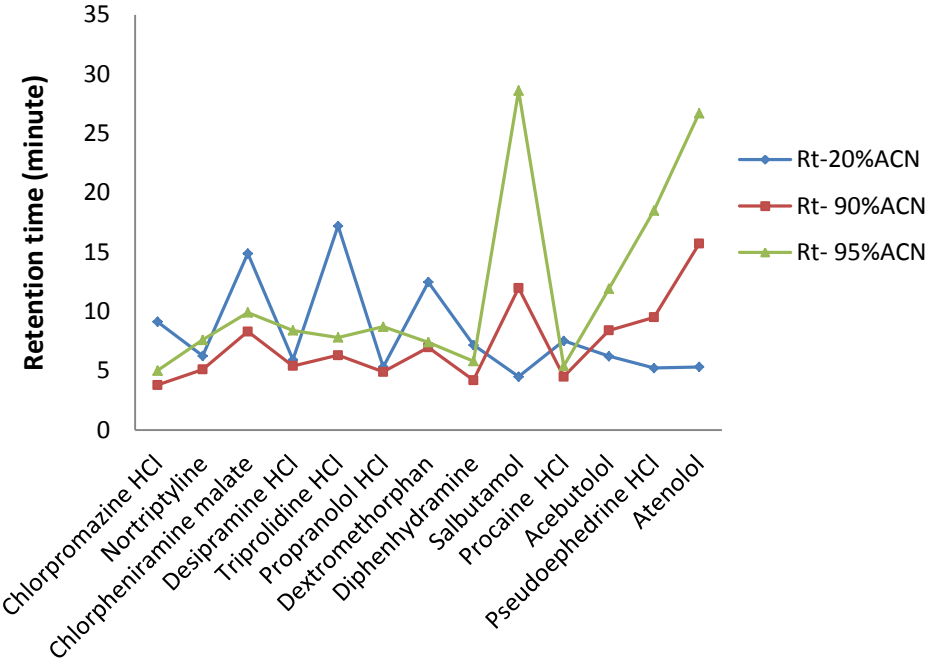


Figure 4-8: Plot of average retention time of basic analytes at 20%, 90% and 95% ACN with 20 mM ammonium acetate buffer at pH 6.9, on Kromasil silica gel column.

In reversed phase condition, the strong retention of triprolidine at 20% ACN was expected as it has the lowest pK_a value and therefore it will be the least ionised and the most sensitive probe to the lipophilic interaction. However, when the acetonitrile content increased in the mobile phase, the most hydrophilic probes (as measured by log $D_{6.9}$), atenolol and salbutamol, experienced the strongest retention. This observation is expected because under RP conditions, the retention is governed by lipophilic interactions. As shown in Table 4-3, triprolidine has the highest log D of 2.3, which gives it strong retention. This is also the case with chlorpromazine. However, some compounds with lower log D values

(e.g. chlorpheniramine) have stronger retention. This would suggest that other mechanisms are occurring. The retention at 90 and 95% ACN will based on hydrophilic interactions, thus it would be expected that low log D probes such as atenolol (-2.34) and salbutamol (-2.16) will retain longer. This observation strongly suggests a strong lipophilic interaction at 20% ACN. However, hydrophilic interaction is predominant at 90 and 95% ACN.

Plots of retention time verses log $D_{6.9}$ are shown in **Figure 4-9**. Generally it would appear that at 20% ACN retention time increased with log D. However, the trend is not completely clear and some compounds with similar log D values have very different retention times. There is less scatter in the data below log D = 0 and relatively little change in retention. This would suggest that log D has little effect when the compounds are hydrophilic. The results would suggest there is some lipophilic interactions with compounds of higher log D (> 0).

Table 4-3: Log D and the percentage ionisation for the bases were calculated from **Equation 2.3** and **Equation 3.3** respectively in (**section 3.5, Chapter 3)**. The predicted log P and pK_a values obtained from ChemAxon's online software (https://chemicalize.com/welcome).

Base identity	**pK_a**	**Predicted Log P**	**Log D at pH 6.9**
Chlorpromazine	9.3	5.2	2.24
Nortriptyline	10.1	4.5	0.86
± Chlorpheniramine malate	9.2	4.0	1.01
Desipramine HCl	10.4	3.9	0.78
Triprolidine	6.5	3.8	2.30
Propranolol	9.5	3.4	-0.19
Dextromethorphan	8.3	3.4	0.54
Diphenhydramine	8.98	2.8	0.58
Salbutamol	9.3-10.3	1.8 - 1	-2.16
Procaine	9.0	1.3	-0.18
Acebutolol	9.4	0.9	-1.14
Pseudoephedrine	9.8	0.9	-1.30
Atenolol	9.6	0.2	-2.34

Figure 4-9 A shows that at 90% ACN the retention pattern appeared to reverse. Decreasing the log D value from just under zero to below (-2) showed a clear increase in retention. Interestingly, increasing the log D further appeared to have little significant effect on retention, and the scatter of the data possibly increased. Increasing the acetonitrile content to 95% led to a very similar effect. The results suggest that retention is closely correlated to log D when the compounds are hydrophilic (log D < 0) when in HILIC mode. It is interesting to note that for more hydrophobic compounds (log D > 0) there appears to be little correlation of retention with log D.

It is not clear from these results if this lack of correlation when log D > 0 is due to a secondary mechanism or some other cause. One possibility is that a minimum hydrophilicity is required to get the compounds into the HILIC water-layer. Once the compounds become to hydrophobic they are partially excluded from the water layer reducing retention. However, more work needs to be performed to confirm this mechanism. Plots of Log P verses retention for the compounds are shown in **Figure 4-9 B**. As can be seen from the diagrams, retention trends for log P plots showed very similar behaviour to those of log D verses retention, although there does appear to be slightly more scatter in the data, possibly highlighting the effect of ionisation.

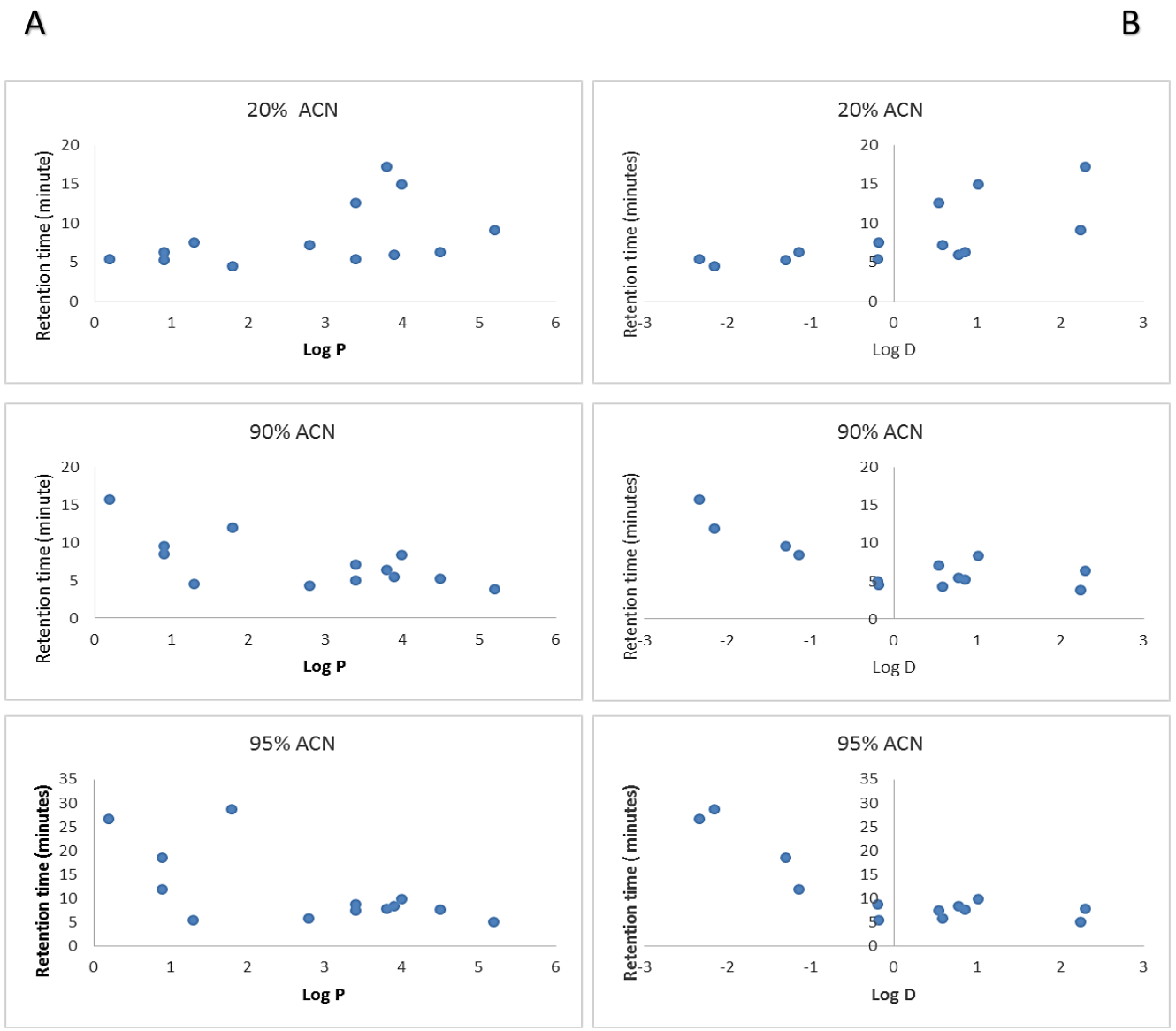


Figure 4-9: Plots show the trend of retention time of bases against their log P and log D with different ACN concentration in the mobile phase; 20 to 90 95% ACN.

There is some correlation between low retention in HILIC mode and high retention in RP conditions. Dextromethorphan for instance is moderately retained in HILIC mode and strongly retained in reversed phase mode. In this phase ion exchange interactions might also play a part and it might be expected to be strongest for the secondary amines pseudoephedrine and desipramine where the methyl substituted amine has a small ionic radius. However, only pseudoephedrine is strongly retained in HILIC mode. Dextromethorphan is a tertiary amine with a lower charge density and a relatively low pK_a value and thus its strong retention at 20% ACN suggests that the most important determinant of retention is lipophilic interaction and the same argument can be used for chlorpheniramine.

Although propranolol and procaine has very similar log D of (-0.19) and (-0.18) respectively, they exhibited very different retention at 20% ACN. However, propranolol and atenolol exhibited exactly the same retention time, 5.3 min, at 20% ACN whereas the retention of atenolol increased significantly when ACN content increased in the mobile phase. This behaviour can be explained by the combination of lipophilic and electrostatic interactions at 20% ACN, however, increasing the % ACN reduced the lipophilic effect and the hydrophilic interaction started to take place on the surface. At 95% ACN, the establishment of the hydrophilic layer is expected which lead to early elution of the most lipophilic probes. However, it was interesting to see the retention of triprolidine, the most

lipophilic probe, log D 2.3, is longer than the retention of nortriptyline, log D 0.86.

The presence of the electrostatic interaction in addition to the lipophilic affects at 20% ACN can be observed in the retention of nortriptyline, 6.3 min, and acebutolol, 6.2 min, which have a significant difference in log D values; however they exhibited a similar retention time.

Interesting retention behaviour was observed for chlorpromazine and procaine at high organic content despite the significant difference in their lipophilicity, they almost exhibited the same retention time at 95% ACN but the latter retains longer at 90% ACN. This observation in some cases complex but it suggests the operation of mixed-mode mechanism and it indicates the strong contribution of the stationary phase to the overall retention even at high organic content.

The nature of the salt used as mobile phase modifier was also critical, since replacing ammonium acetate by ammonium formate caused a marked deterioration of peak shapes and changes in the elution order (data not shown). It would have been interesting to study the lipophilic retention of bases at a higher pH, also study the acids at a lower pH since in ammonium acetate they were highly ionised as was the silica gel, producing repulsion and thus no retention

The peak shapes for some of the basic probes is shown in **Figure 4-10**. The tailing effect is strongly shown at 20% ACN for the most lipophilic probes

whereas the most hydrophilic probes, salbutamol and atenolol, produced sharp and excellent peak shapes despite having lower retention. The tailing effect of the lipophilic probes could be due to combination of electrostatic and lipophilic interactions at 20% ACN. Increasing the % of acetonitrile improved the peak shape of the basic probes noticeably apart from the most lipophilic probe, chlorpromazine, which experienced a splitting effect in the base-line at 90% ACN but relatively sharp peak at 95% ACN.

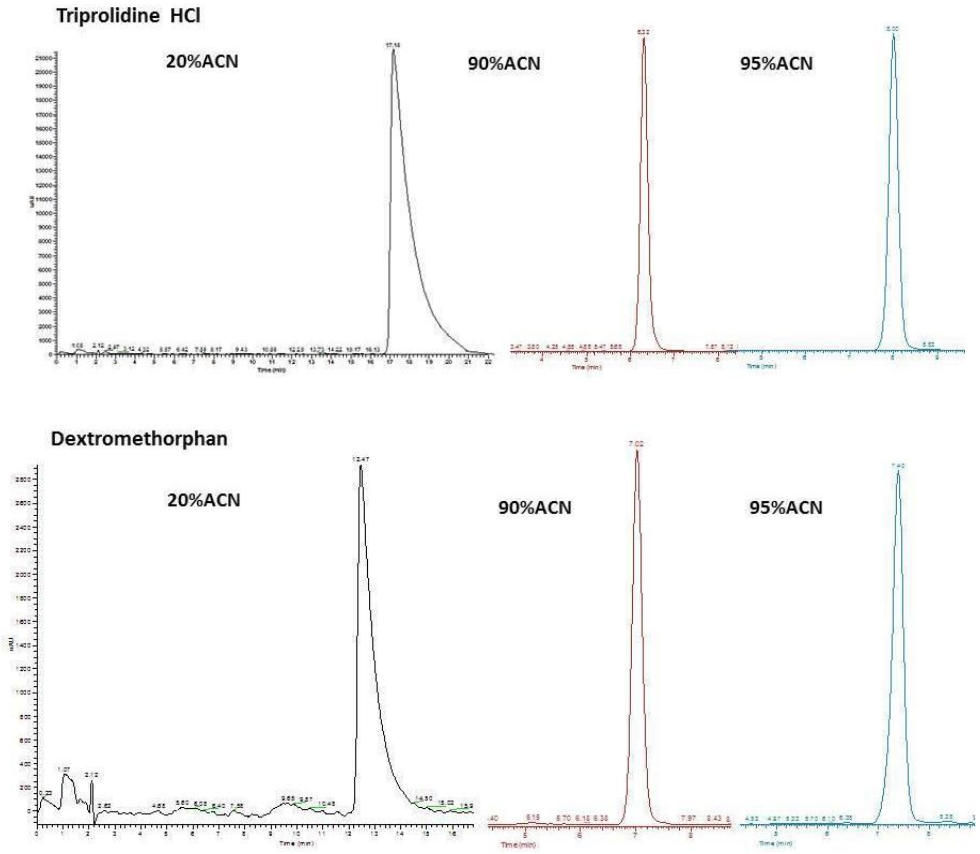


Figure 4-10: The effect of acetonitrile content in the mobile phase on the retention and peak shape of basic probes at 20%, 90% and 95% ACN on the Kromasil silica gel column.

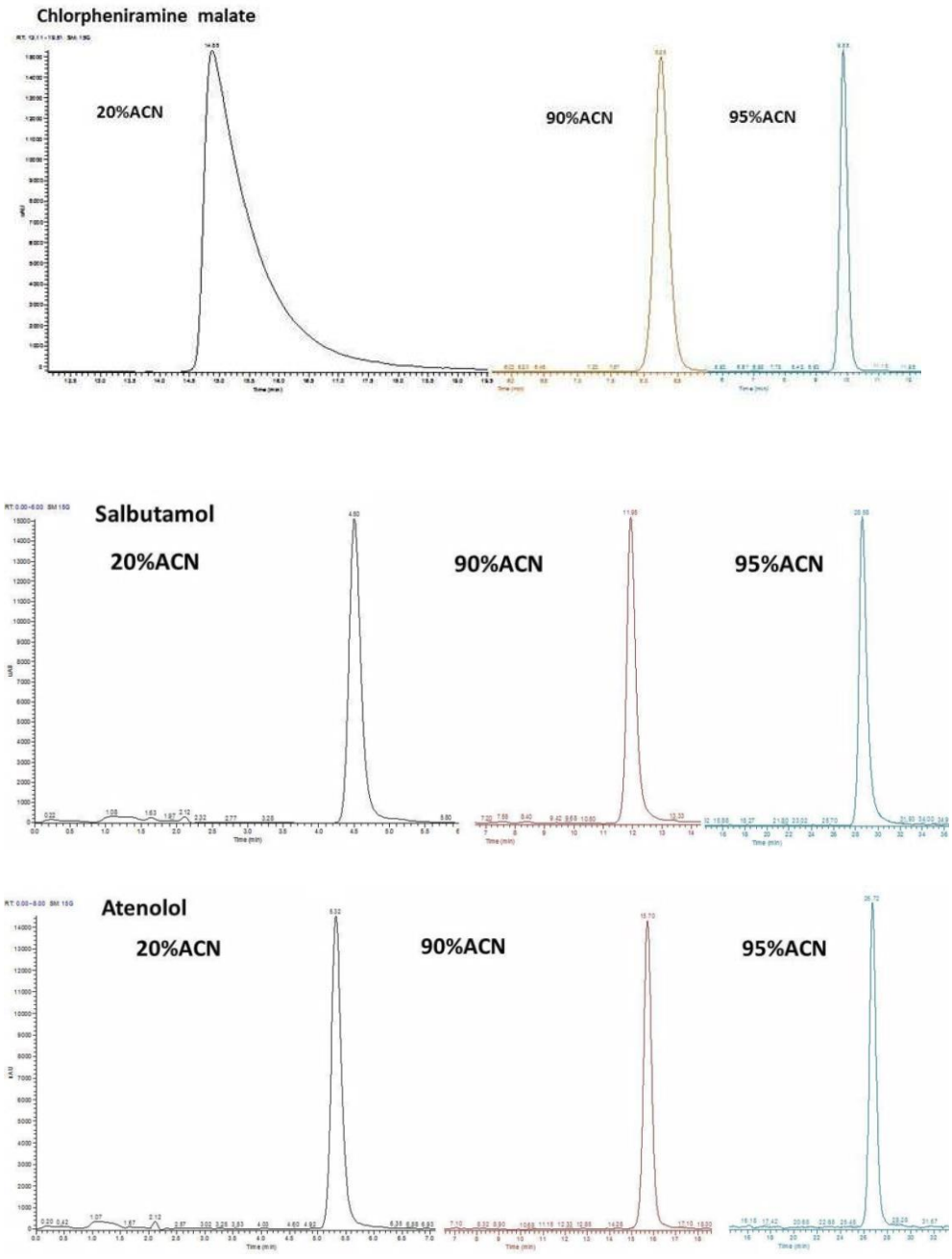


Figure 4-10 continued: The effect of acetonitrile content in the mobile phase on the retention and peak shape of basic probes at 20%, 90% and 95% ACN on the Kromasil silica gel column.

4.4 Application of Kromasil silica column in the analysis of metabolites in plasma and urine

Hydrophilic interaction liquid chromatography (HILIC), although not a new technique, has joined a recent renaissance with the introduction of robust and reproducible stationary phases. It is consequently finding application in metabolomics studies, which have traditionally relied on reversed phases (RPs), since the bio-fluids are predominantly aqueous and thus contain many polar analytes. The compatibility of the high organic solvent mobile phases with mass spectrometry has increased the adoption of hydrophilic chromatography in studies of complex aqueous metabolomics. The ability of a bare silica gel column to retain range of different analytes under HILIC conditions ranged from very hydrophilic to highly lipophilic raise the interest to apply this column in the profiling of metabolites in urine and plasma by using an alternating HILIC with reversed phase interaction by programming down and up a solvent gradient. Particularly in plasma there are lipid molecules which do not retain strongly in HILIC mode and if such strategy were successful it would be possible to offer two types of selectivity on the same column.

4.4.1 Sample treatment

For HILIC analysis (0.2 mL) of urine was diluted with (0.8 mL) of acetonitrile, the sample was centrifuged and the supernatant was used for analysis. For

reversed phase analysis of urine a sample (0.2 mL) free of particulates was taken and diluted with (0.8 mL) of HPLC grade water. For HILIC analysis of plasma (0.2 mL) of plasma was taken and (0.8 mL) of acetonitrile was added, the sample was centrifuged and the supernatant was used for analysis. For reversed phase analysis of plasma, (0.5 mL) of plasma was mixed with (0.5 mL) of acetonitrile. The sample was centrifuged and the supernatant (0.5 mL) was mixed with (0.5 mL) of HPLC grade water.

4.4.2 **LC-MS Analysis**

The Kromasil silica column was coupled to an Exactive Orbitrap mass spectrometer operated in positive negative switching mode. The spray needle voltage was set to 4.5 kV on positive ion mode and -4.0 kV in negative ion mode. The sheath and auxiliary gases were set at 50 and 17 arbitrary units respectively. The resolution of the instrument set at 50,000 and it scanned between 75 and 1500 amu with the capillary heated to 250°C. The mobile phase consists of two reservoir bottles; **A** contained 20 mM ammonium acetate, pH 6.9, in water and **B** contained 100% ACN. The gradient run started serially from 100% B (0 min) to 100% A (30 min) and hold for (10 min). This was followed by 100% A (0 min) to 100% B (30 min) and hold for (10 min) in reversed phase mode. The flow rate was 0.3 ml/min. and the data was processed by using Xcalibur 2.1.

4.4.3 **Results**

Figure 4-11 shows the extracted ion chromatograms for leucine and isoleucine, choline and lysine in plasma in HILIC mode. The peak shapes are good although the resolution of leucine and isoleucine is only partial. However, the column works well for the analysis of typical biomolecules including the polar diamine lysine.

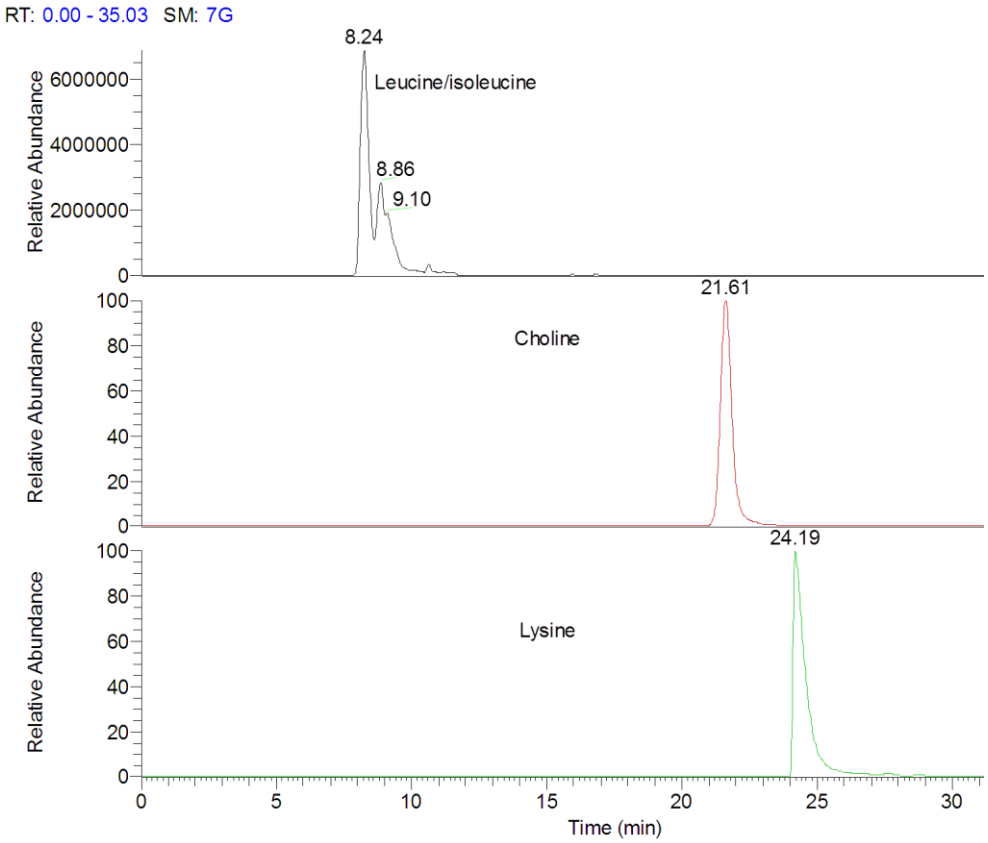


Figure 4-11: Extracted ion traces for leucine, isoleucine, choline and lysine in human plasma on a Kromsil column in HILIC mode (conditions as described in **section 4.4.2**).

Due to the high polarity of these compounds, lysine would be expected to elute at the void volume of the column in the reversed phase mode with high aqueous content in the moble phase. However, there was a significant retention for choline and lysine ,**Figure 4-12**, which may be attributed to ion exchange interactions with the ionised silica gel surface. This was observed for the quaternary ammonium test probes studied in **chapter 2**.

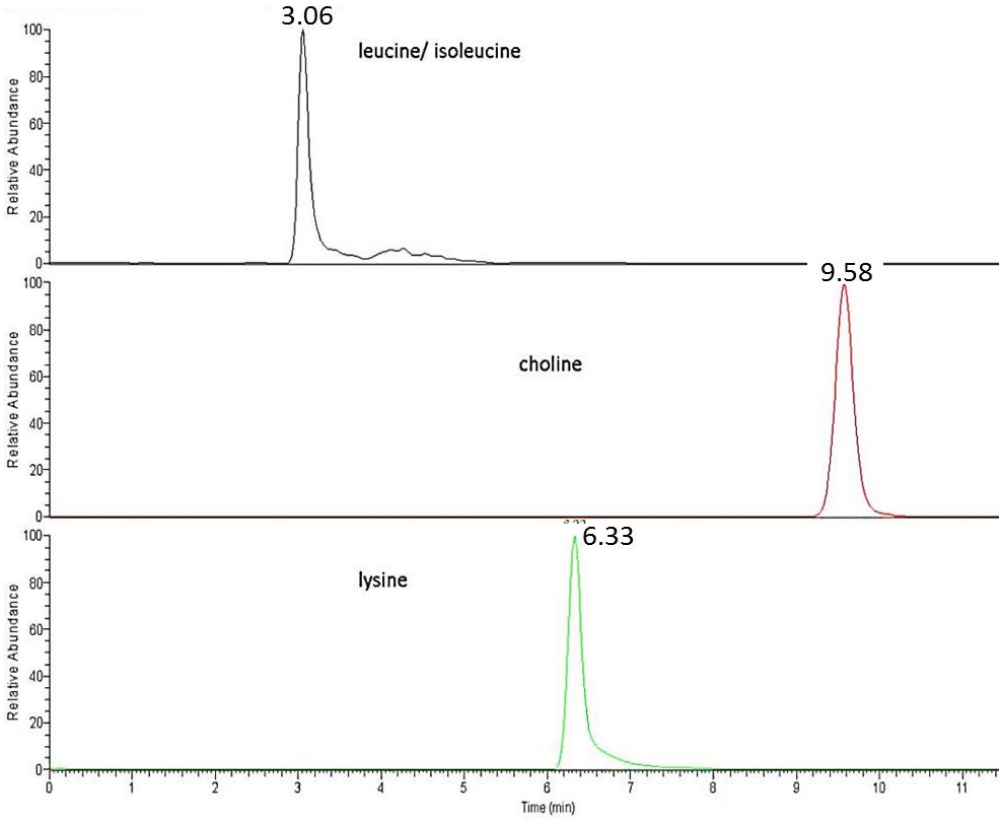


Figure 4-12: Extracted ion traces for leucine, isoleucine, choline and lysine in human plasma on a Kromasil silica column in reversed phase mode **(**conditions as described in **section 4.4.2).**

Figure 4-13 shows chromatograms for the basic compounds arginine, carnitine and acetyl choline in human plasma in reversed phase mode. The retention data also indicated that these compounds are retained via ion exchange interactions with the ionised silica gel surface.

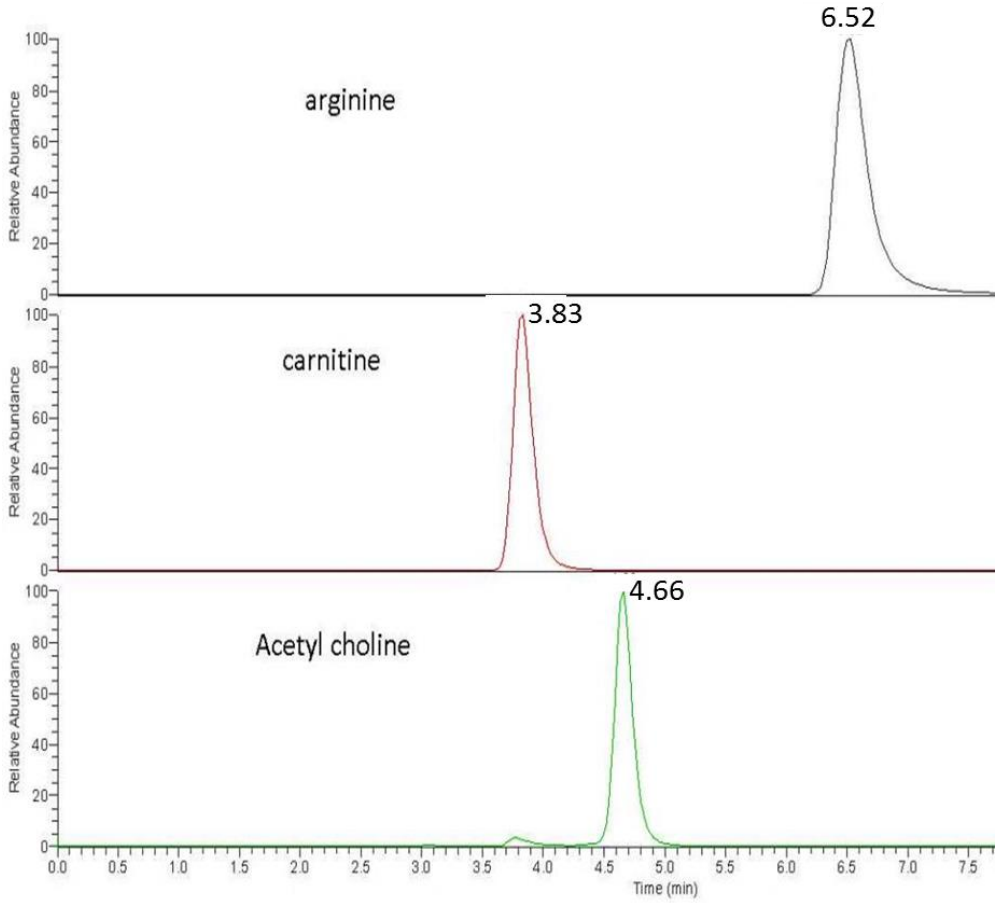


Figure 4-13: Extracted ion traces for arginine, carnitine and lysine in human plasma on a Kromasil silica column in reversed phase mode **(**conditions as described in **section 4.4.2).**

Figure 4-14 shows the traces for two acidic compounds, ketoglutaric acid and succinic acid, and a neutral compound glucose on the Kromasil column in HILIC mode.

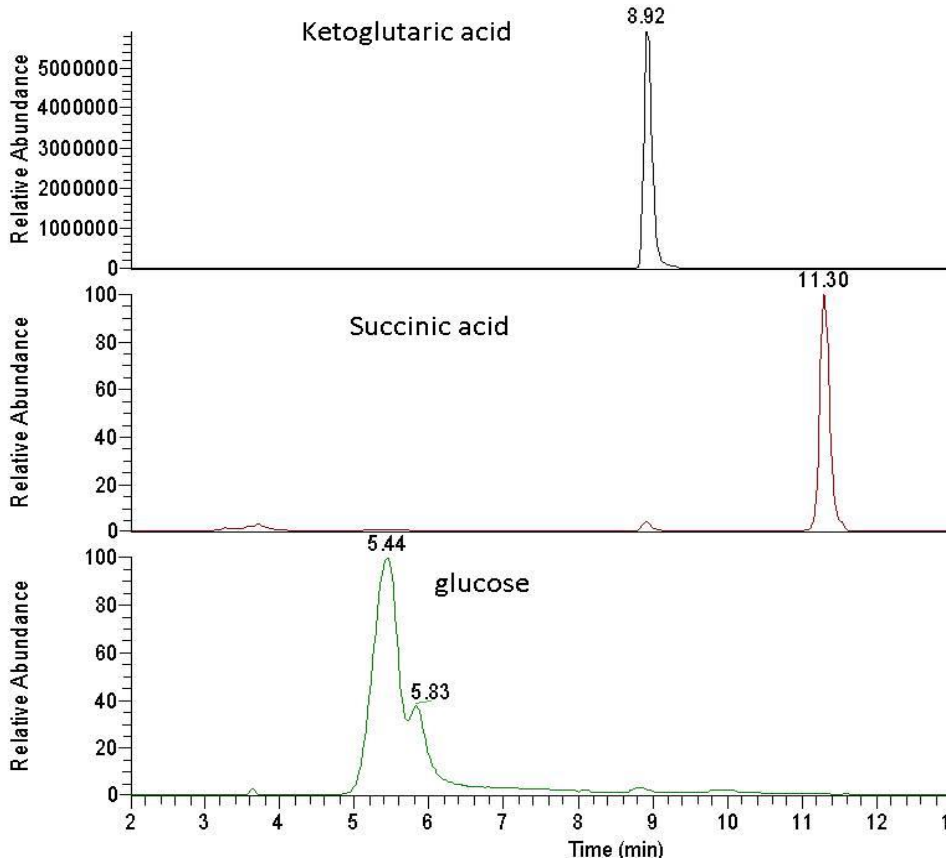


Figure 4-14: LC-Ms for ketoglutaric acid, succinic acid and glucose in human plasma in HILIC mode on a Kromasil column, (conditions as described in **section 4.4.2**).

While an appreciable retention was observed for these compounds in HILIC mode, in reversed phase mode there was no retention, **Figure 4-15**, and in fact ketoglutaric and succinic acid eluted before the void volume due to the electrostatic repulsion by the ionised silanol groups.

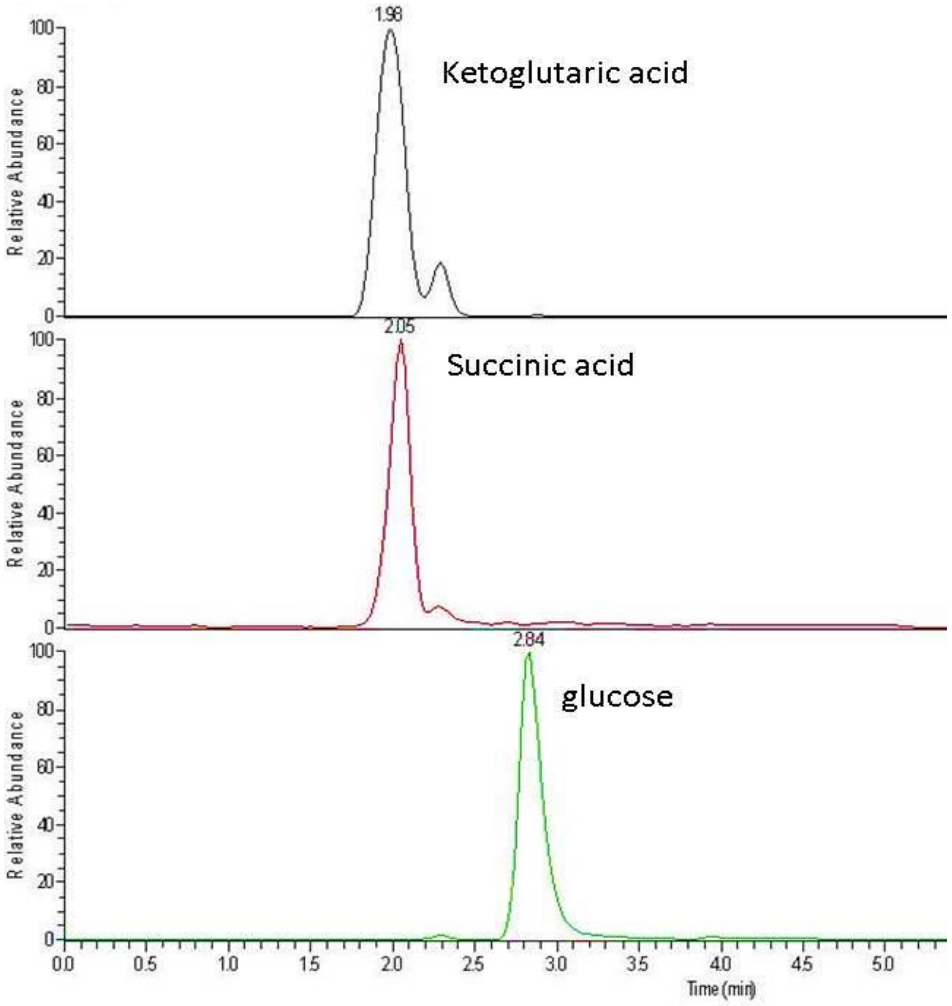


Figure 4-15: LC-MS for ketoglutaric acid, succinic acid and glucose in human plasma in reversed phase mode on a Kromasil column, **(**conditions as described in **section 4.4.2).**

Examining the more lipophilic compounds in plasma revealed that although there was a lipophilic interaction the peak shape was not good with very wide peaks being produced. **Figure 4-16** displays the extracted ion chromatograms for stearic acid, decanoyl, palmitoyl, and stearoyl carnitine in plasma on the Kromasil silica column operated in reversed phase mode. The peaks for the carnitines and stearic acid have shown somewhat better

shape. The order of elution of the carnitines supports the fact that lipophilic interactions are being observed with their order of elution being in order of lipophilicity.

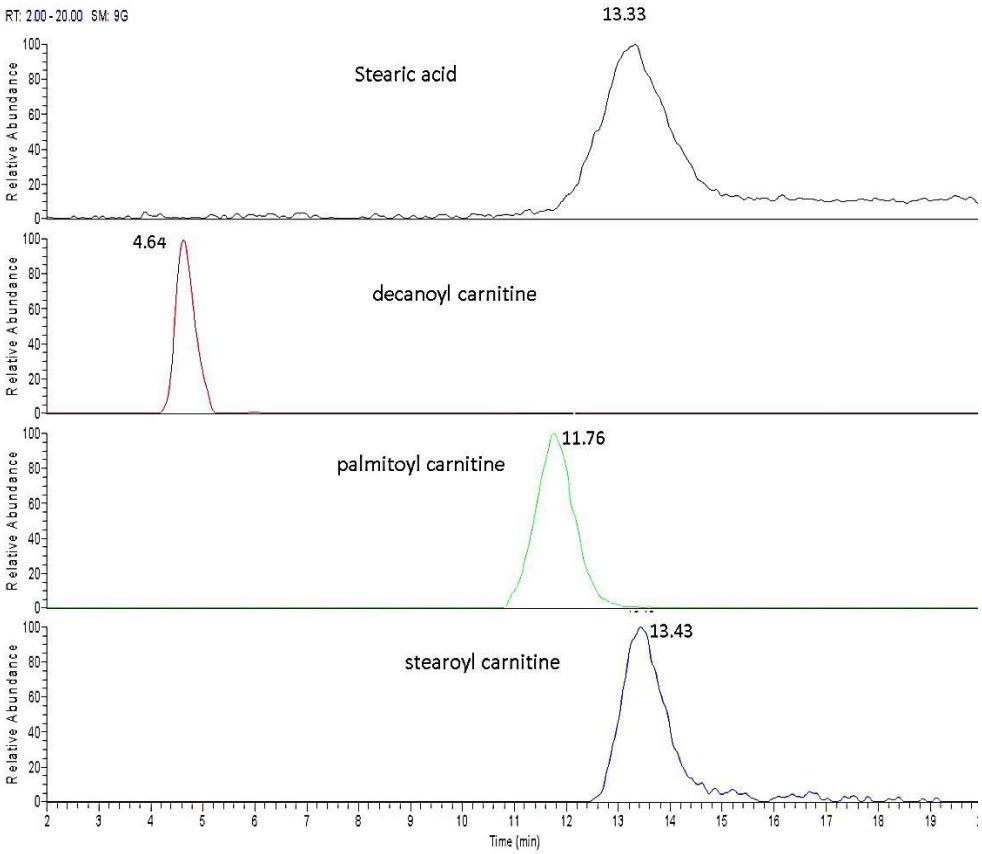


Figure 4-16: LC-Ms for stearic acid, decanoyl, palmitoyl, and stearoyl carnitine in human plasma in reversed phase mode on a Kromasil column.

Certainly it is clear that the surface is quite lipophilic since the stearic acid is strongly retained by the column despite being quite highly ionised at pH 6.9, it might be that some of the loss of peak shape is due to charge repulsion and a lower pH might be used to improve the chromatography.

Thus it might be possible to choose better mobile phase and explore this further. Yet it would be attractive in metabolic profiling experiments to have different selectivity in one column and although silica gel has been used as a stationary phase for many years it can still offer some surprises.

4.5 Application of Kromasil Silica Gel to Impurity Profiling by LCMS in HILIC Mode

The LC-MS conditions were as described in **section 4.4.2**. Atenolol was prepared as 1mg/mL solution in methanol and 10 µl was injected into the LC-MS.

4.5.1 **Results**

The ESI spectrum for atenolol is shown in **Figure 4-17** and the main impurities in atenolol are shown in **Figure 4-18**. The figure suggested several impurities were observed and eluted earlier than atenolol indicating that they might be either neutral or larger cations. The mass spectrum of the impurities is shown in **Figure 4-19**, and based on the elemental compositions for the impurities which are shown in **Table 4-4** it is possibly belongs to a propionyl ester form of the molecule. The proposed structures for the impurities are displayed in **Figure 4-20**, yet, only one of these impurities is listed in the EP and to confirm the structure MS^2 is required.

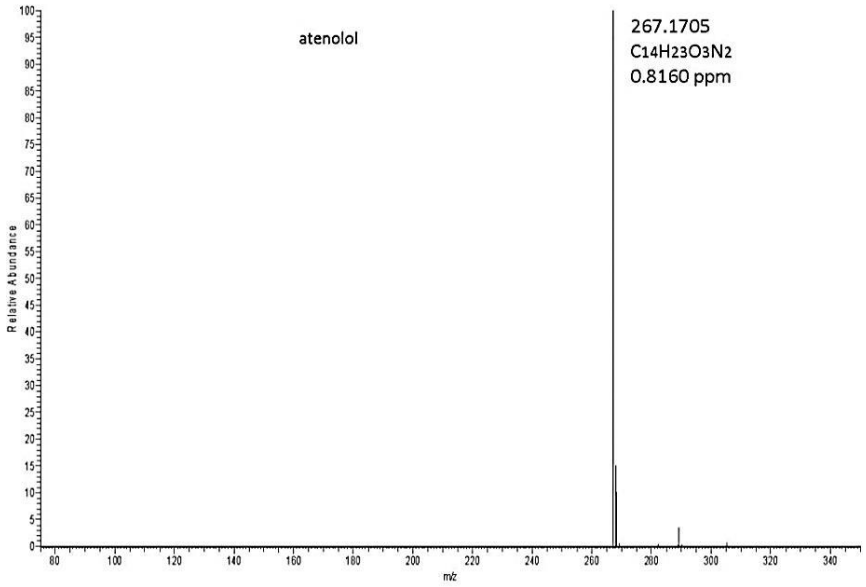


Figure 4-17: ESI spectrum of atenolol.

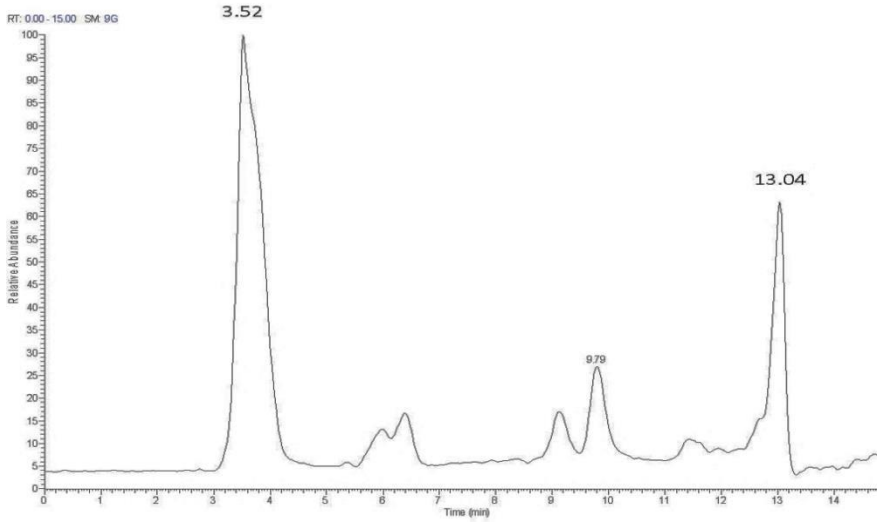


Figure 4-18: Main impurities in the atenolol sample, total ion current.

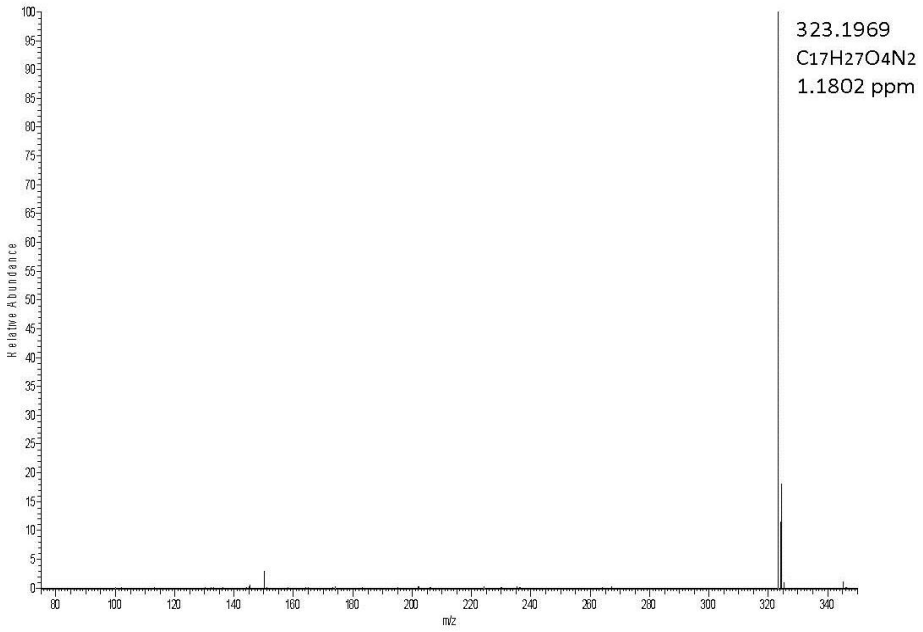
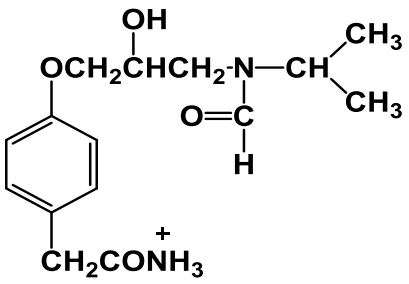


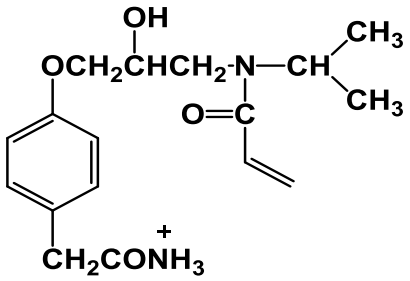
Figure 4-19: Mass spectrum of impurity in atenolol, possibly a propionyl ester.

Table 4-4: Elemental composition for the main impurities in atenolol, all masses were within 2 ppm of the proposed elemental composition.

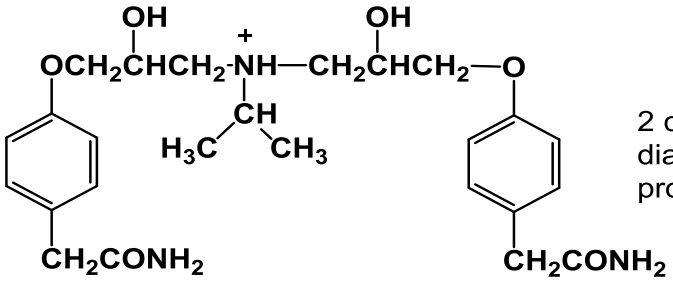
t_R (min)	m/z	Elemental composition
3.5	295.1693	$C_{15}H_{23}N_2O_4$
3.6	321.1810	$C_{17}H_{25}N_2O_4$
5.9	474.2602	$C_{25}H_{36}N_3O_6$
6.4	474.2602	$C_{25}H_{36}N_3O_6$
9.1	323.1968	$C_{17}H_{27}N_2O_4$
9.8	323.1968	$C_{17}H_{27}N_2O_4$
13.0	282.1700	$C_{15}H_{24}NO_4$



Exact Mass: 295.1652



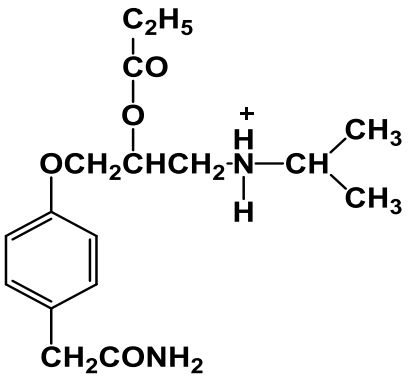
Exact Mass: 321.1809



2 chiral centres therefore diastereoisomers produce two peaks.

Exact Mass: 474.2599

EP listed impurity



Exact Mass: 323.1965

Figure 4-20: Proposed structures for the impurities in atenolol listed in **Table 4-4.**

4.6 System Suitability Testing (SST) of the Kromasil Column under HILIC Mode

4.6.1 Definition

SST is used to verify the accuracy of a specific method for a particular analysis including resolution, column efficiency, and repeatability of a chromatographic system to ensure it is adequate. The chromatographic systems used for most pharmaceutical analyses such as assays of the active ingredients, impurity determinations, and dissolution testing (measuring the dissolution rate for a particular form of dosage) must pass a set of predefined acceptance criteria (SST limits) before sample analysis can operate.

4.6.2 SST Parameters

The system suitability tests represent an integral part of the method and it is used to ensure the consistency of the performance of the chromatographic system. It is one of the most critical factors in developing pharmaceutical drug substances and drug products of reliable data for the HPLC analytical methods. The acceptable limit for SST parameters is summarised in **Table 4-5** according to Food and Drug Administration, FDA, guidelines.

Table 4-5: Food and Drug Administration (FDA) Guideline for system suitability test [139].

Test	**Specification**
Capacity Factor (K')	K'>2
Precision	RSD≤1%
Repeatability (RSD)	n ≥ 5
Resolution	Rs >2
Tailing Factor (T)	T ≤ 2
Number of theoretical plate (N)	N > 2000

1. Capacity Factor (k')

The capacity factor displays the relation between the time spent by a compound in a stationary phase and the time it spends in the mobile phase; it refers to as k' and it is a unitless quantity given by the equation:

$$k' = \frac{t_r - t_0}{t_0} \qquad \textbf{Eq. 1.4}$$

The higher the value of k', the greater is the retention of the compound on the column and ideally k' should be greater than 2.0 [3].

2. Precision (%RSD)

Precision is the measure of the replicates of the data values to each other for a number of measurements under the same analytical conditions [3] and it can be expressed by:

$$\%\text{RSD} = \frac{SD}{mean} * 100 \qquad \text{Eq. 2.4}$$

3. Tailing Factor

This term is used to define the quality of separation and to describe the chromatographic peaks. The ideal peak shape will have a symmetrical shape or a Gaussian peak shape on a flat baseline. A peak can be shifted from its ideal shape to become asymmetrical, flatten and become broader, or the baseline can rise [3]. One of the common shifts away from a Gaussian peak is when the back half of the peak falls away. This effect is most clearly seen close to the baseline and is known as peak tailing. There are two main methods for defining peak tailing; the tailing factor and the asymmetry factor, as described below, these two values will typically be similar for the same peak, although cannot be directly converted [2]. Both factors were used in the current study and defined as:

- Tailing factor **(T_f)** is widely used in the pharmaceutical industry. **Figure 4-21** presents the calculation of tailing factor where **a** and **b** are the peak half-widths at 5% of the peak height.

- Asymmetry Factor **(A_s)** also used to measure the peak tailing but in this case the peak width is defined at 10% of peak height, **Figure 4-22**, where **a** and **b** are the peak half-widths, at 10% of the peak height.

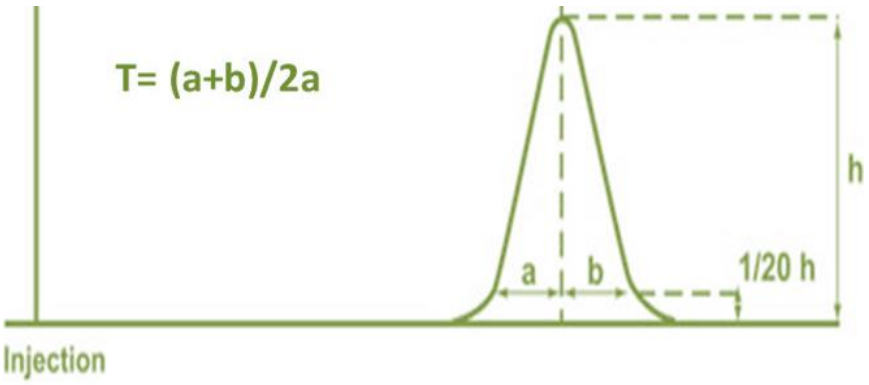


Figure 4-21: Tailing factor of a chromatographic peak at 5% peak hieght.

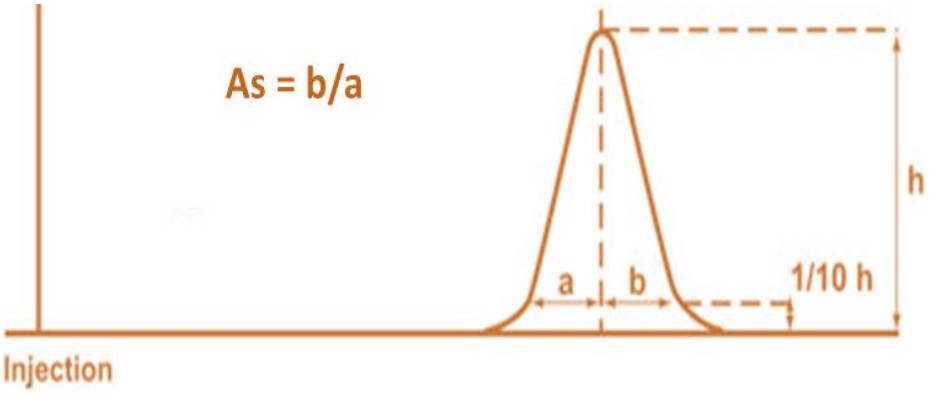


Figure 4-22: Asymmetry factor of a chromatographic peak at 10% peak height**.**

4. Number of Theoretical plate (N)

Also known as column efficiency, the number of theoretical plates is a mathematical concept which can be calculated using the equation below. Theoretical plate numbers are an indirect measure of a peak width at a specific retention time [2], and it can be calculated from the equation:

$$N = 5.54 \left(\frac{t_r}{w_h}\right)^2 \qquad \textbf{Eq. 3.4}$$

Where;

t_r is the retention time (minute)

w_h is the peak width at half height (in unit of time)

5. Linearity

Linearity measures the ability of the method to produce test results that are directly proportional to analyte's concentration within a given range [3]. The range of linearity is checked by injections of 3 to 6 concentrations of the reference standards (in triplicates). The analytical curve was obtained with three concentrations of the standard solution (0.1, 0.01 and 0.001 mg/mL).

6. Robustness

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters [3]. Examples of typical variations in the case of Liquid chromatography are:

• Influence of variations of pH in a mobile phase

• Influence of variations in mobile phase composition

• Different columns (different lots and/or suppliers)

• Temperature

• Flow rate

In the current study the influence of mobile phase composition was examined by deliberately alerting the molarity of buffer from 20 to 18 mM.

4.6.3 SST of the Kromasil column under HILIC conditions

System suitability testing is essential for the assurance of the quality performance of the chromatographic system. In practice, each method submitted for validation should include an appropriate number of system suitability tests defining the necessary characteristics of that system.

There is an unfounded opinion that the robustness and stability of HILIC methods is poor. Thus, in order to test this opinion SST was carried out for the Kromasil silica column (15 cm x 4.6 mm i.d, 5 μm) also the robustness of this system was examined under HILIC mode. The acidic and basic test probes detailed in **section 4.2.3**, **Figures 4-2 and Figure 4-3**, respectively for the acids and the bases were used with a mobile phase consisting of 95% ACN where most of HILIC effect was observed and ammonium acetate

buffer at constant strength, 20 mM, was applied at pH 6.9. The peak width at half height was calculated manually then the number of theoretical plates, tailing factor, asymmetry, and the precision were calculated from the equations (detailed in **section 4.6.2**). The linearity of the data was acquired from plotting three different concentrations of the analyte against its respond, as a function of peak area. For accurate validation, six points are required for linearity but as the purpose for this part is to check the performance under HILIC condition, the minimum number of three concentrations was used.

4.6.3.1 Results

The data for system suitability parameters of acidic probes is shown in **Table 4-5**. The capacity factor of all probes is within or above the specification limit which is required. The tailing factor for the tested acids under HILIC mode was good ≤ 1. However, the asymmetry factor is not as good possibly due to the fronting of some peaks which could be solved by adjusting the injection volume. For %RSD of triple injections, some probes showed within the range values and some exceeded the limit of ≤1.

Table 4-6: System suitability parameters of the acidic probes on a kromasil silica column (15 cm x 4.6 mm, 5 µm i.d.), mobile phase contain 95% ACN: 20 mM ammonium acetate 5%, pH 6.9; where (t_0) =1.63 minutes and (L) =0.15 m**.**

Analyte	Theoretical Plates (N) Per 15 cm	Tailing (T_f)	Asymmetry (As)	%RSD of Rt n=3	Linearity (r^2)	Capacity Factor (K')
Mefenamic acid **A1**	5460	0.9	0.72	±0.10	--	2.00
Indomethacin **A2**	6783	0.9	0.73	±0.48	0.9988	5.0
Naproxen **A3**	9600	0.9	0.78	±0.13	0.9995	4.8
Ketoprofen **A4**	6065	1.0	0.90	0	0.9306	5.5
Ibuprofen **A5**	4563	0.8	0.67	±2.49	--	4.0
Flurbiprofen **A6**	4920	0.9	0.70	±1.63	0.9947	5.3
Diclofenac **A7**	6973	0.8	0.57	±1.31	0.9992	3.0

The linearity for mefenamic acid and ibuprofen could not be predicted because the peaks were not detected at lower concentration; however the remaining probes, **Figure 4-23,** showed acceptable linearity of three concentrations and all responses were accurate. The efficiency of the Kromasil stationary phase under HILIC condition as a function of theoretical plate's number is demonstrated above in **Table 4-6**. The obtained N value for all tested acidic probes is above the required limits indicating good

efficiency in HILIC mode for this column. The behaviour of the lipophilic acids in HILIC mode although quite unexpected certainly offers an alternative selectivity.

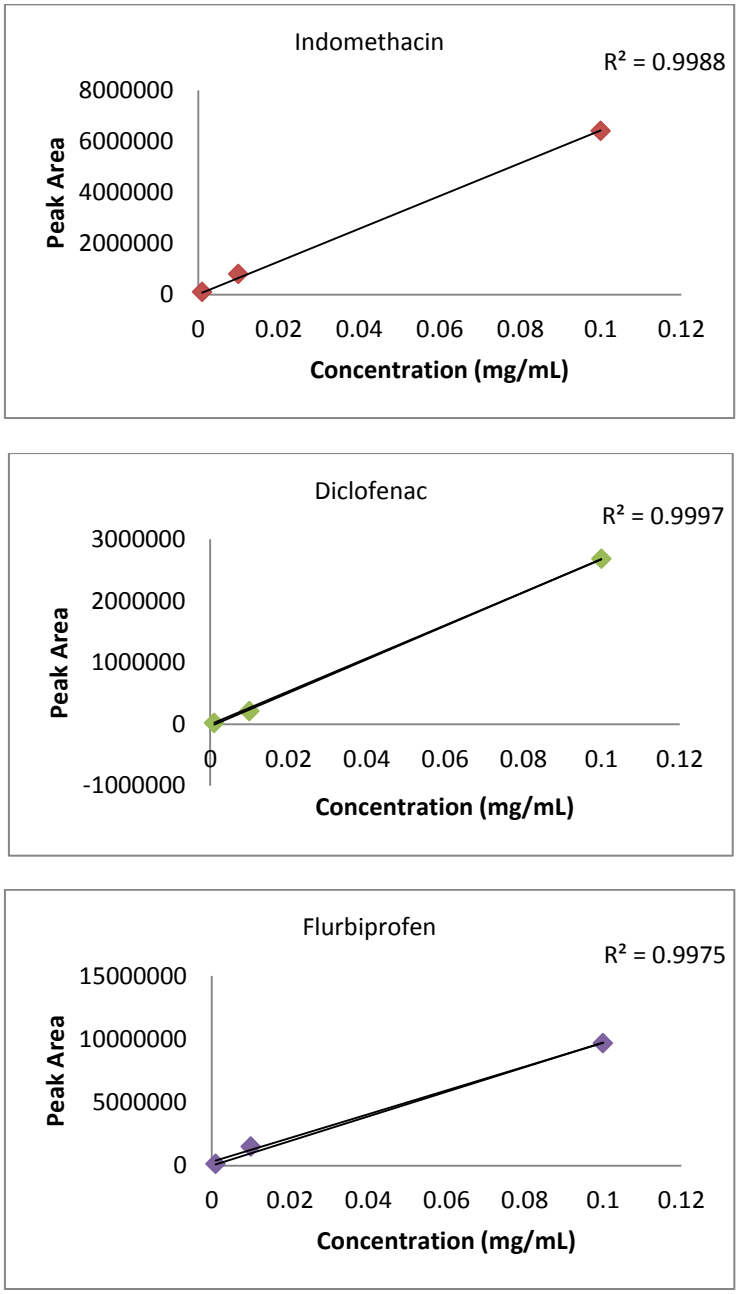


Figure 4-23: Linearity plots of some tested acidic probes.

The system suitability parameters were assessed also for the basic probes, **Table 4-7**, on the Kromasil silica column. The capacity factor was > 2 for all the bases. The tailing factor was found to have good values of less than 2 apart from pseudoephedrine which experienced some tailing. The r^2 values of calibration curves for the bases, **Figure 4-24,** are generally good and close to 1. Apart from atenolol, chlorpromazine and propranolol the % RSD of the retention for basic drugs are relatively good under HILIC conditions. Again the Kromasil stationary phase shows largely great performance under HILIC conditions according to the number of theoretical plates which is much greater than 2000 FDA specification.

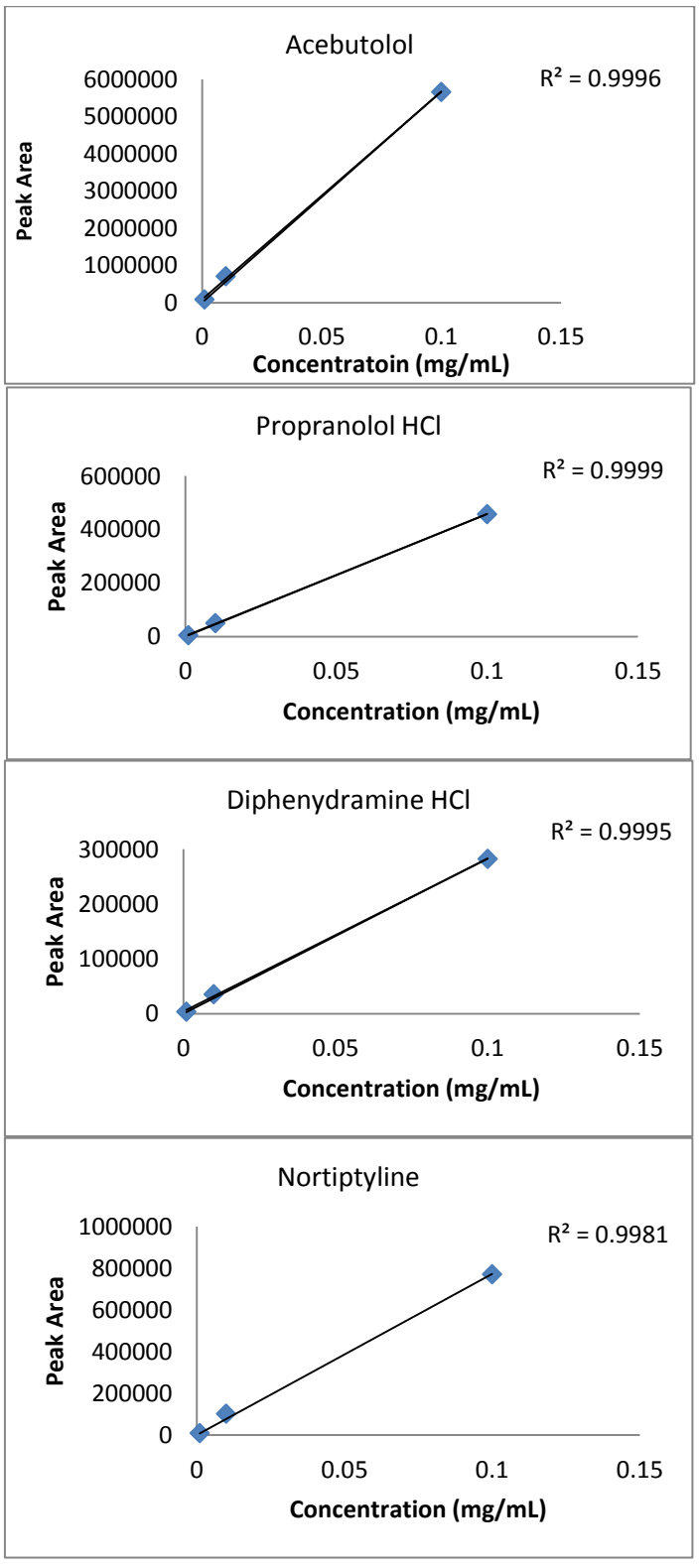


Figure 4-24: Linearity plot of some tested basic probes.

Table 4-7: System suitability data of the basic probes on a Kromasil silica gel column (15 cm x 4.6 mm, 5µm), mobile phase 95% ACN: 20 mM ammonium acetate 5%, pH 6.9; where (t_0) =1.63 minutes and (L) =0.15 m.

Analyte	Theoretical plates per column	T_f	A_s	%RSD of retention n=3	r^2	K'
Nortiptyline **B1**	1153	1.0	1.00	±0.12	0.9981	3.7
Desipramine **B2**	9451	0.8	0.66	±0.20	0.9971	4.1
Procaine **B3**	3867	1.1	1.21	±0.18	0.9969	2.3
Diphenhydramine **B4**	1040	1.2	1.32	±0.08	0.9989	2.6
Chlorpromazine **B5**	8125	0.9	0.79	±4.33	0.9672	2.2
Propranolol **B6**	9794	1.0	1.09	±2.86	0.9998	4.4
Chlorpheniramine **B7**	9754	1.1	1.25	±0.29	0.9936	11.0
Pseudoephedrine **B8**	8613	2.3	3.50	±0.51	--	10.4
Dextromethorphan **B9**	7833	0.8	0.60	±0.17	--	3.5
Atenolol **B10**	1077	1.1	1.24	±2.71	--	15.4
Triprolidine **B11**	7860	1.0	1.10	±0.92	0.9974	3.9
Acebutolol **B12**	8102	1.1	1.12	±0.248	0.9988	6.3
Salbutamol **B13**	8545	1.5	2.10	±0.029	--	16.5

Figure 4-25 shows some examples of chromatograms for acidic and basic test probes in the assessment of robustness test based on slightly modify the concentration of ammonium acetate buffer in the mobile phase. Moving from 20 to 18 mM of salt concentration in the mobile phase produced a significant change in the retention data; also the quality of peak shapes was affected. Apparently, the robustness of HILIC method is sensitive to small changes and may lead to a drift in chromatographic information of acids and bases in addition to the poor peak shape. However, the chromatography seems more stable with higher amounts of ammonium acetate in the mobile phase and this remains to be explored.

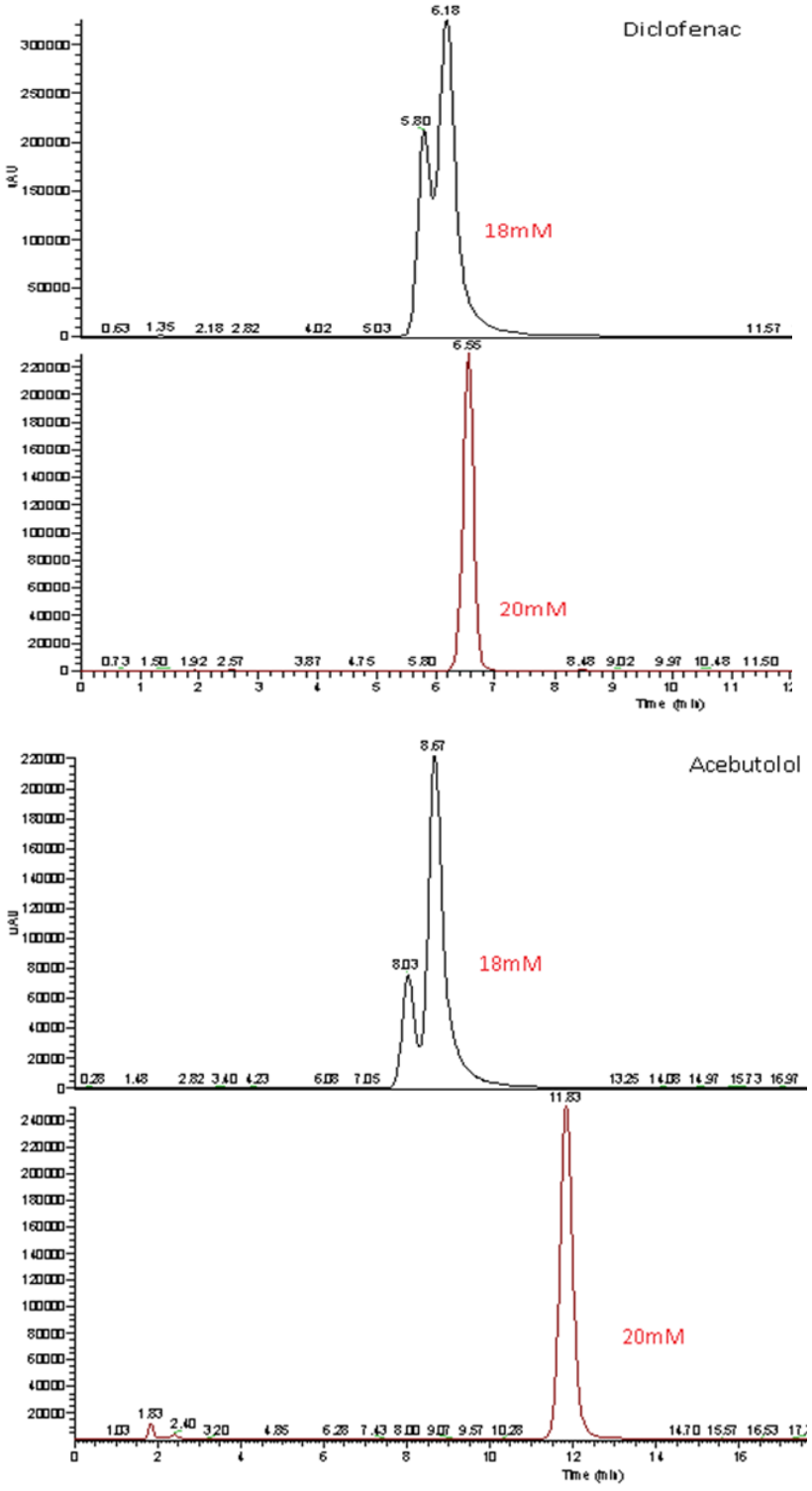


Figure 4-25: Chromatographic retention of some tested acidic and basic probes in the robustness study on the Kromasil stationary phase. The mobile phases contain 95% ACN: 18 mM ammonium acetate compared to chromatograms obtained with 20 mM ammonium acetate, both pH 6.9 and flow rate 1 mL min^{-1} .

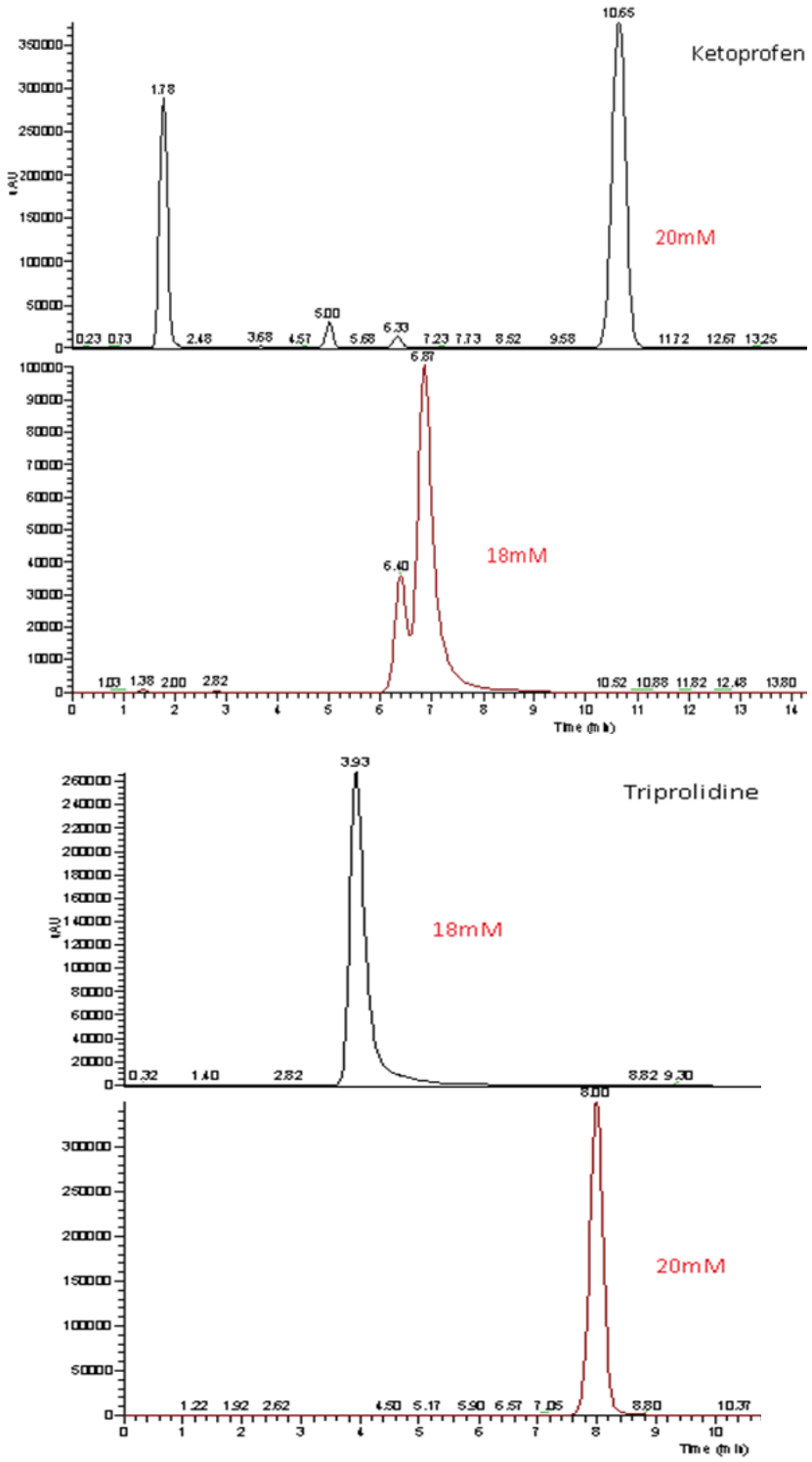


Figure 4-25 continued: Chromatographic retention of some tested acidic and basic probes in the robustness study on the Kromasil stationary phase. The mobile phases contain 95% ACN: 18 mM ammonium acetate compared to chromatograms obtained with 20 mM ammonium acetate, both pH 6.9 and flow rate 1 mL min^{-1}.

4.7 Conclusion

The acids exhibited a strong retention with high-organic mobile phases and it remains to be explained how a lipophilic acid can be strongly retained by a HILIC system. In practical terms such a system might not be particularly useful in routine QC applications but it might offer alternative selectivity in systems such as impurity profiling and would also be useful in bio-analytical analysis where samples containing high levels can be injected directly following a solvent crash. The basic test probes also showed a strong retention with high-organic mobile phase and tended to give better peak shapes than the acids. Trends of the retention of acids verse their log P and log D is somewhat similar, but ionisation of compounds caused significant differences in the retention behaviour of some compounds. The correlation of retention to log $D_{6.9}$ appeared to improve when in HILIC mode, but more work is needed before this can be confirmed. The results with acids would appear to confirm the importance of using log D instead of log P to predict retention behaviour, to take into consideration the ionised state.

Generally, the correlation between retention behaviour of bases verses their log D appeared to be stronger when compounds were analysed in HILIC mode. As with acids the correlation of retention with log D appeared stronger than with log P, but the differences were not so clear. Similar trends were observed with both log P and log D. It would appear that ionisation of basic drug molecules, given the similar trends observed, has a

more consistent affect with basic drugs than acids. More work is needed to confirm this.

The general scattering plot of log D verses retention indicate that lipophilic interaction is governing the retention at low organic content while HILIC-type interactions take place when the organic content increased in the mobile phase. In general, peak shape is more of a problem for basic than acidic compounds in reversed phase mode and HILIC can offer an alternative form of chromatography providing better peak shapes. As in the case of acids, there are advantages in impurity profiling and bio-analysis in applying a HILIC method. The observations of this study strongly suggest the contribution of the stationary phase surface to the overall mechanism even at high organic content in the mobile phase despite the formation of large solvated hydrophilic layer. Although it is still unexplained how the surface could attribute significantly under such conditions, the data in accordance with the findings in **Chapter 3** for the retention behaviour of the lipophilic analytes on the hydride based stationary phases under HILIC mode. The contribution of strong electrostatic interaction in silica-C based stationary phases is surprising; however is observed in previous studies when the surface of silica hydride was compared with an ordinary silica gel column [147, 43].

Chapter 5

Application of Hydrophilic Interaction Chromatography (HILIC) in the Separation and Analysis of Designer Drugs

5 Application of Hydrophilic Interaction Liquid Chromatography (HILIC) in the Separation and Analysis of Designer Drugs

5.1 Introduction

The "designer drugs" are analogs or derivatives of controlled substances that have been designed to mimic the pharmacological effects of the original drug, while avoiding classification as illegal and/or detection in standard drug tests. New designer drugs are constantly emerging onto the illicit drug market and it is difficult to validate and maintain comprehensive analytical methods for accurate detection of these compounds. Chemical modifications of these substances can be very subtle, leading to virtually unlimited structural variation

In the last few years there has been a striking increase in the sale of *"legal highs"*. These chemicals may be bought through the internet at low cost and are sometimes pure compounds which display highly similar chemical structures to existing and illegal drugs of abuse [151]. The (±) 4'-methylmethcathinone or mephedrone ,**Figure 5-1**, is a synthetic beta-ketoamphetamine that is structurally similar to methcathinone, related to cathinone (a psychoactive compound found in Khat) which has found prominence in the recreational drug scene as a *"legal high"* replacement for controlled stimulants including amphetamines such as methamphetamine and MDMA (*i.e.* Ecstasy). These drugs with a stimulant effect on the central nervous system (CNS) can cause physical and psychological addiction which induces temporary improvements in mental

and/or physical function when used in high quantities. In April 2010, mephedrone was classified as a Class B, Schedule 1 substance under the Misuse of Drugs Act (1971). Our previous study reported a full synthesis, analytical profiling and development of robust and validated chromatographic methods (GC and HPLC) for the detection and quantification of mephedrone both in its pure form and in the presence of a number of common adulterants [152]. Consequently, new chemically-designed forms of mephedrone, **Figure 5-1**, have been emerged onto the illicit drug market. These derivatives have similar chemical properties to mephedrone and may present significant analytical challenges to forensic scientists engaged in the routine screening of seized drug samples. Therefore, drug manufacturers have an opportunity to evade detection and prosecution.

Recently Power *et al.* [153] reported the separation of mephedrone regioisomers, namely: 2-methylmethcathinone (2-MMC); 3-methylmethcathinone (3-MMC) and 4-methylmethcathinone (4-MMC), **Figure 5-1,** using GC-MS with non-polar stationary phase. Under high thermal stress mephedrone regioisomers might experience stability issue; therefore there was a critical need to develop a liquid chromatographic method for the detection of these substances.

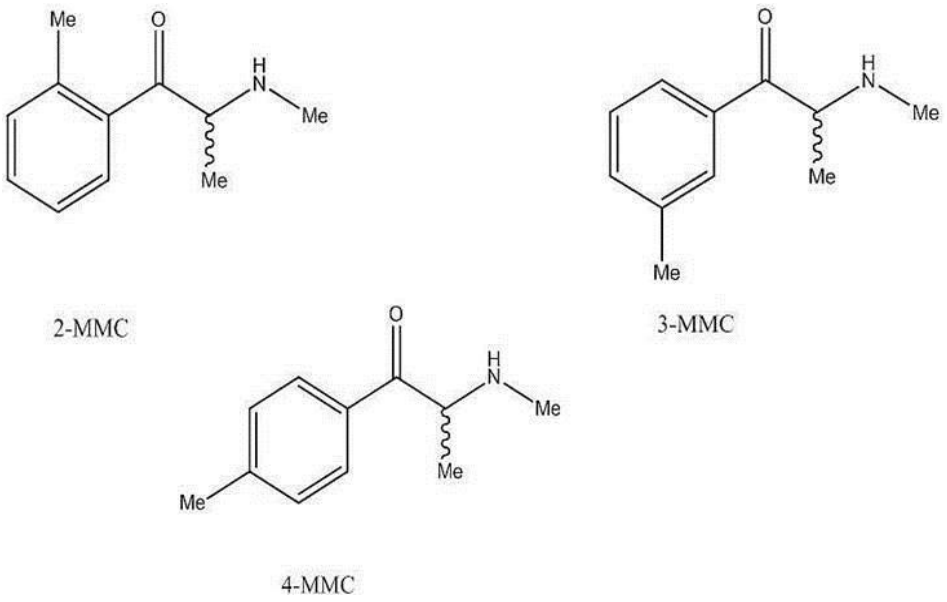


Figure 5-1: The Chemical structure of (±) 2-methylmethcathinone; (±) 3- methylmethcathinone and (±) 4- methylmethcathinone, pK_a 8.2.

The separation of regioisomers is challenging due to the similarity not only in structure, but also in chromatographic properties. Reversed phase and normal phase chromatography have been applied to regioisomers separation with some success [4]. However, HILIC has not been well established as a viable technique for the separation of regioisomers. Therefore, this chapter will examine the application of HILIC in the separation of cathinone regioisomers in comparison to reverse phase chromatography in HPLC.

5.2 Method and Materials

5.2.1 Chemicals

All laboratory reagents and solvents were of commercial quality (> 99% purity) and obtained from either Sigma Chemical- Aldrich Germany or AnalaR® BDH laboratory supplies, and used without further purification. The three isomers 2-MMC, 3-MMC and 4-MMC were synthesised at Strathclyde Institute of Pharmacy and Biomedical Science, HPLC grade water was obtained using a DIRECT-Q® 3 System at 25°C (processed to a resistance of 18 MΩ/cm). Samples were weighed by difference using analytical balance. The samples were dissolved in the appropriate solvent. All analytical solutions were prepared in class A volumetric glassware and stored in refrigerator in the absence of light to avoid sample degradation.

5.2.2 Synthesis of mephedrone

2-MMC and 3-MMC were synthesised, by Dr. Sutcliffe, as the method reported by Power *et al.* [153] while 4-MMC was synthesised as the method reported by Santali *et al*. [152].

5.2.3 Stationary Phases

The analytical columns, **Figure 5-2**, were purchased from HiChrom Ltd., Reading, UK. For RP separation three stationary phases were applied including ACE 3 C_{18} (150 mm x 4.6 mm, i.d., 3 µm), ACE 3 PFP (150 mm x 4.6

mm, i.d., 3 µm) and ACE 3 AR (150 mm x 4.6 mm, i.d., 3 µm) columns. For HILIC study Kromasil silica gel column (150 mm x 4.6 mm, i.d., 5 µm) was utilised.

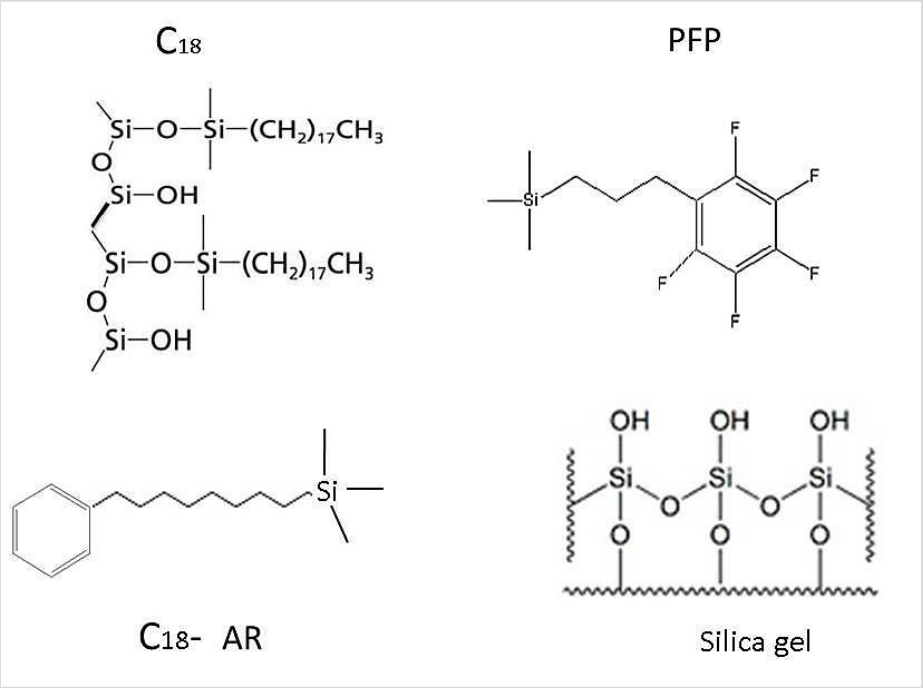


Figure 5-2: Chemical structure of the stationary phases used in the study.

5.2.4 Preparation of buffer for Reverse phase and HILIC Analysis

For the reverse phase study, 100 mM of ammonium formate (6.3 g, 99 % pure) was mixed with in ultra-pure HPLC grade water (800 mL) and the pH was adjusted by drop wise addition of formic acid (98 – 100 % pure) to pH 3.5. The stock solution was transferred into to a 1000 mL volumetric flask and made up to the volume with ultra-pure HPLC grade water. The exact

weight used in preparation was 6.300 g. For the HILIC study 1% w/v of ammonium acetate (5.00 g, 99 % pure) was mixed with ultra-pure HPLC grade water (500 mL) and the recorded pH was 6.9. The exact weight used in preparation was 5.200 g containing 0.8 mM. The pH of solutions was measured by using a pHenomenal™ pH meter 1000L- VWR.

5.2.5 Mobile Phase preparation

For the RP study, three mobile phases A, B and C, which differ in the organic-aqueous compositions, were prepared by mixing an appropriate proportion of 100 mM ammonium-formate buffer (pH 3.5) with methanol (MeOH) or acetonitrile (ACN) as described in **Table 5-1**. The final buffer concentration of 10 mM was constant for the all used mobile phases. Each mobile phase was run under three different temperatures 20, 40 and 60˚C on three different reversed phase columns.

Table 5-1: Mobile phase composition for RP study**.**

Mobile phase	Composition at pH 3.5	Ratio(% v/v)
A	100 mM ammonium-formate: MeOH or ACN	90:10
B	100 mM ammonium-formate: MeOH or ACN	80:20
C	100 mM ammonium-formate: MeOH or ACN	70:30

The mobile phase for the HILIC study consists of 0.8 mM of ammonium acetate buffer (pH 6.9) and only acetonitrile was used as organic modifier in the mobile phase in the proportion of (25:25:950) buffer:H_2O:ACN v/v in total volume of 1000 mL. All mobile phases were degassed prior to use.

5.2.6 Sample Preparation

A stock solution of each isomer was prepared by accurately weigh out 10.00 mg of 2-mmc, 3-mmc and 4-mmc separately into 10 mL volumetric flasks then dissolved with methanol to give three solutions each contains 1000.0 µg /mL of each isomer. The mixture solution was prepared by weigh out 10.00 mg/mL of each isomer into one 10 mL volumetric flask then the mixture was diluted with methanol to give solution containing 1000 µg /mL of each isomer. The samples were prepared for injection by pipetting the appropriate amount of the stock solution and diluting it with either RP or HILIC mobile phases to give final concentration of 20 µg /mL.

5.3 Instrumentation

5.3.1 High-performance liquid chromatography

HPLC performed with a ThermoFinnigan HPLC system consisting of a P4000 pump, UV 6000 PDA detector and an AS3000 autosampler. The mobile phase compositions were mixed off-line in the proportions required and the HPLC system was used in isocratic mode, 10 µl of sample was injected.

The flow rate was 1.00 mL/min and the analysis was conducted at room temperature. A PDA detector was used. The void volume of the columns was determined according to the minor disturbance peak produced by injecting 10 µl of toluene for HILIC and uracil for RP. Data analysis was carried out using Xcalibur for LC. DryLab was applied for the experimental data by the HiChrom Ltd., Reading, UK.

5.3.2 **LC-MS**

The electrospray ionisation, ESI, interface was operated in a positive/negative polarity switching mode. The spray voltage was 4.5 kV for positive mode and 4.0 kV for negative mode. The temperature of the ion transfer capillary was 275°C and sheath and auxiliary gas was 50 and 17 arbitrary units respectively. The full scan range was 75 to 1200 m/z for both positive and negative modes with settings of Automatic Gain Control (AGC) target and resolution as Balanced and High (1E6 and 50,000) respectively. The data were recorded using Xcalibur 2.1.0 software package (Thermo Fisher Scientific).

5.3.3 **NMR Method**

Approximately 50 mg of solid 2-MMC was dissolved in 50 mL of HILIC mobile phase containing 0.8 mM ammonium acetate -ACN in proportion of 5: 95% **(**pH 6.9). The solution was left for 24 h then 10 mL of the solution

,containing 10 mg of 2-MMC, was transferred into a funnel and was frozen under 80°C for 24 h then dried for 27 h. The dried 2-MMC sample was then dissolved in 0.6 mL of DMSO-d_6, deuterated solvent, and was transferred into a standard 5 mm NMR tube. The NMR spectroscopic data were carried out on a JEOL-LA400 -NMR spectrometer system (^{1}H NMR at 400 MHz) (JEOL Ltd, UK) using TMS as internal standard, Chemical shifts are given in ppm and coupling constants are in Hz.

5.4 Results and Discussion

The ACE 3 C_{18} stationary phase is an octadecyl-silica phase, it is the most widely used in reversed-phase chromatography and recommended for basic compounds. The ACE 3 PFP offers an alternate selectivity; the fluorine atoms are incorporated in an aromatic ring in this phase and make it act as a Lewis acid, thus it will interact with analytes able to donate electrons (Lewis base). The PFP phase also offers dipole-dipole or hydrogen bonding interaction with analytes in addition to the shape selectivity [154]. The last phase used for RP separation was the ACE 3 AR which combines a ligand consisting of a C_{18} chain with integral phenyl functionality, thus combining the benefits of both C_{18} and phenyl characteristics into a single phase. Thus, it is recommended for compounds containing aromatic substitutes and regioisomers separations [155]. For the HILIC study, a Kromasil silica gel column was used to provide maximum surface area for interaction.

Reverse phase chromatography was attempted in the separation of mephedrone isomers by using different reverse phase columns [C_{18} (ODS), C_{18}- AR and C_{18}- PFP] and also different analytical conditions. Each isomer was injected separately into the HPLC system to identify peaks then a mixture of the three isomers was analysed. The RP analysis conducted with three mobile phase compositions (70, 80 and 90% ammonium-formate , pH 3.5) at three different column temperatures (20, 40 and 60˚C) and two different organic modifiers (methanol or acetonitrile). **Figure 5-3** shows the chromatographic separation of the three isomers on the tested RP-columns at 20˚C with 20% methanol or acetonitrile, chromatographic data for all applied conditions is not shown. However, there was no separation obtained for the three isomers therefore DryLab was applied to search the optimum point of separation between isomers. The resolution map for isomers, **Figure 5-4,** in methanol showed the best resolution value was obtained with the AR column; however no base line separation achieved suggesting no promising separation under such conditions for the tested columns in RP. It was observed that the elution order of methanol and acetonitrile was the same and the co-elution was shown in both organic solvents with the critical pair 3-MMC and 4-MMC. However, the selectivity for methanol was higher than for acetonitrile since it showed some promise for separation on the C_{18} column. The PFP phase offered the most retention for the analytes.

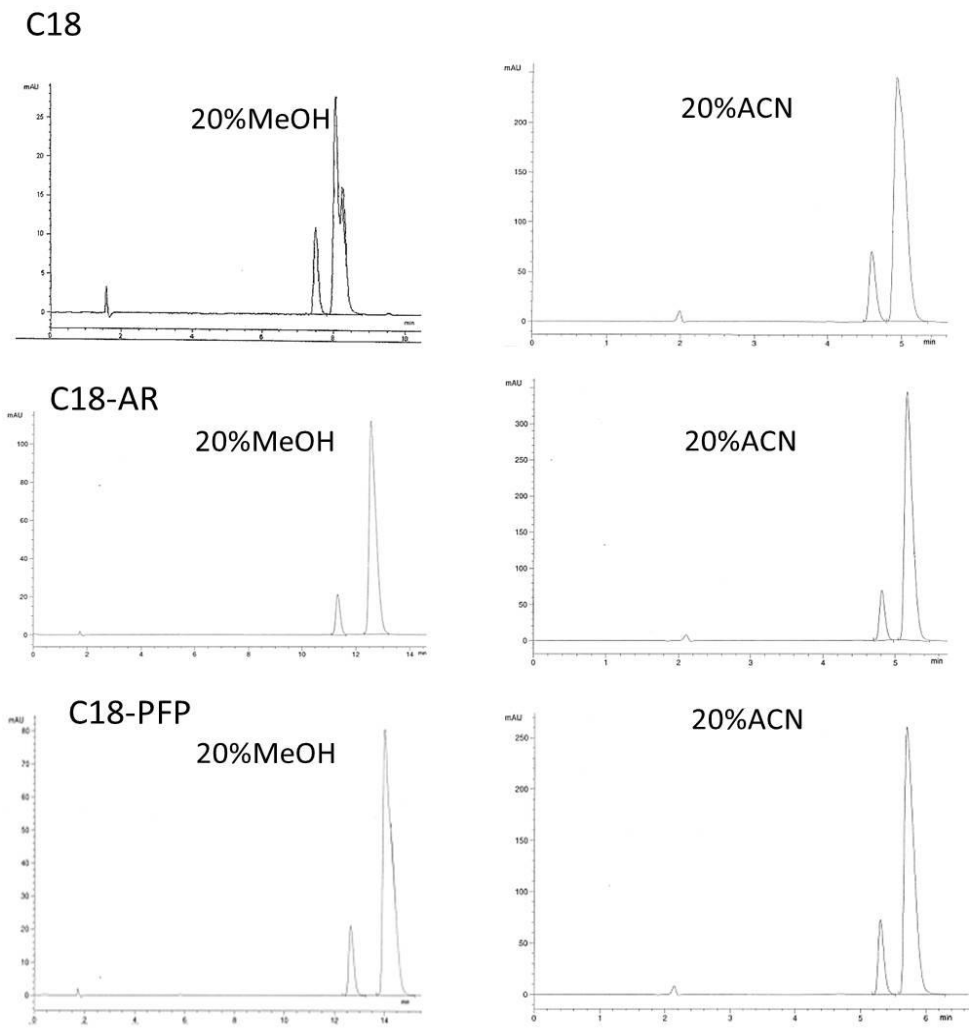


Figure 5-3: Chromatographic result for a mixture of three isomers on RP columns (15 cm x 4.6 mm i.d, 3 µm), mobile phase (20% MeOH or ACN/80% ammonium formate buffer, pH 3.5) UV 258 nm, 20˚C column temperature, flow rate 1 mL/min.

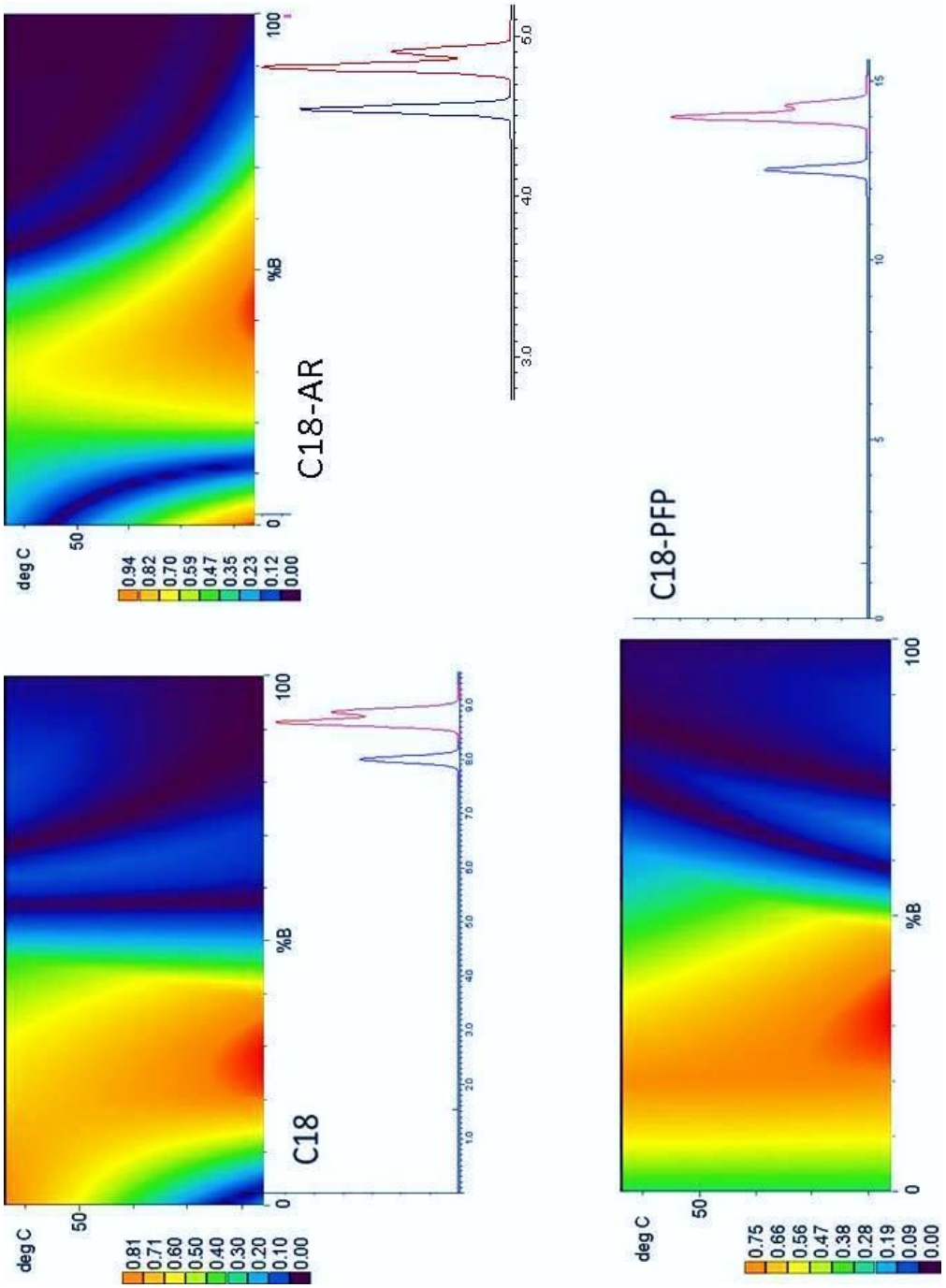


Figure 5-4: Resolution map for all critical pairs. Colour scale on the left indicates the minimum resolution that is predicted for a particular colour in the resolution map. The *x* axis is the gradient time and the *y* axis is the temperature.

In comparison to RP separation, a HILIC system using 95% ACN on a polar silica column offered different selectivity and it successfully separated the peaks of mephedrone's isomers, although the resolution is low compared to the limit ≥ 1.5, in this case it was acceptable baseline separation as no co-elution was observed and each isomer eluted separately as shown in **Figure 5-5**. However, a stability issue was observed for the 2-MMC which over time exhibited either rearrangement or degradation.

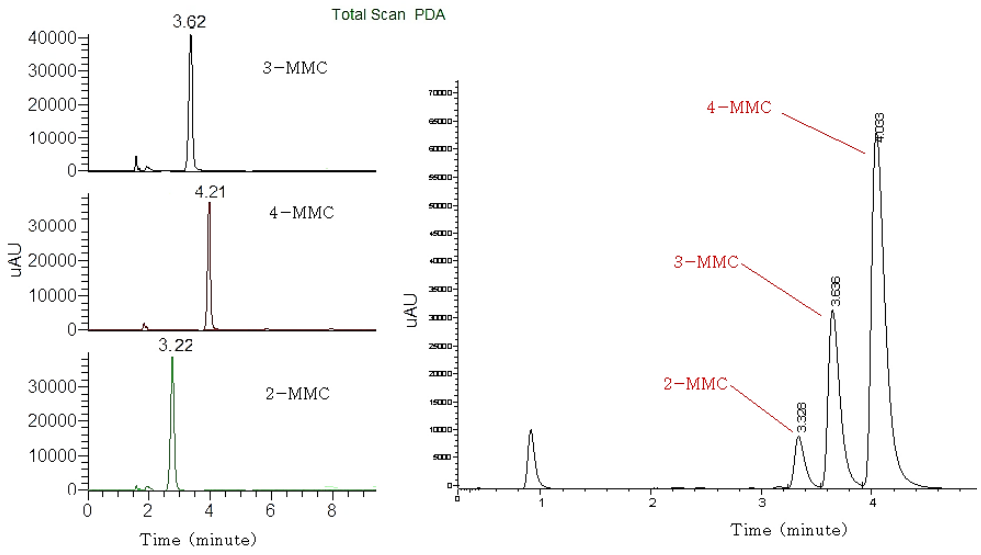


Figure 5-5: chromatographic separation of the three cathinone regioisomers on a Kromasil 5 µm silica gel SP (150 mm x 4.6 mm), mobile phase contain 95% ACN-5% [0.8 mM] ammonium acetate, pH 6.9, flow rate 1 mL/min.

Despite the fact that the 2-MMC isomer was freshly prepared and was kept in dark vial away from light and heat, the stability issue was still present. The degradation of the 2-MMC was associated with the appearance of an early peak at 0.8 min. and the intensity of the corresponding peak increased over time as the 2-MMC intensity decreased in a parallel **Figure 5-6**. The investigation involved 60 injections overnight with 10 minutes between each injection and the analysis operated in HILIC conditions 95:5% (v/v) acetonitrile: ammonium acetate.

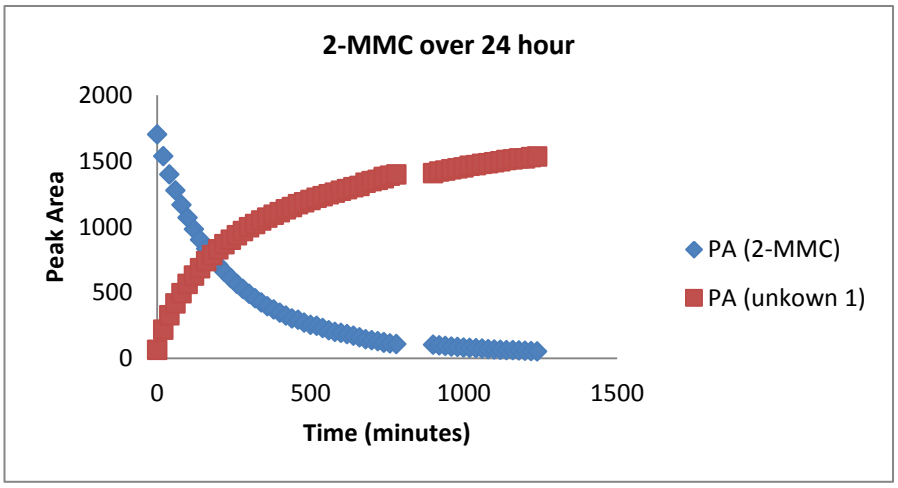


Figure 5-6: The degradation kinetics of 2-methymethcathinone.

An early peak appeared as the 2-MMC decreased in intensity and finally disappeared. It is assumed that this early peak was not interacting with the stationary phase because of steric hindrance due to isomer-rearrangement. Further investigations include NMR and LC-MS studies were conducted in order to identify this unknown peak.

5.5 LC-MS Study of 2-MMC degradation

LC-MS with HILIC analytical conditions were applied with 95% ACN: 5% ammonium acetate buffer pH 6.9; the flow rate was reduced to 0.4 mL/min to be compatible with LC-MS conditions. **Table 5-2** shows the obtained formula of 2-MMC and the unknown peak. Data suggested the formula of the unknown is $C_{11}H_{14}ON$ with two hydrogens having been lost from the original compound. The result assumed a rearrangement of the compound after losing two protons, thus NMR was required for further identification. The NMR spectrum of the degradant was exactly the same as that of pure 2-MMC, **Figure 5-7**, and this phenomenon remains unexplained. It is probable that what is occurring is a reversible equilibrium between two forms of the molecule.

Table 5-2: The LC-MS of 2-MMC under HILIC condition (95% ACN-5% ammonium acetate buffer), Kromasil silica column (150 mm x 4.6 mm).

Formula	Retention time (minutes)	Mass	compound/fragment
$C_{14}H_{16}ON$	6.78	178.12	2-MMC
$C_{11}H_{14}ON$	1.73	176.11	Unknown compound

5.6 NMR Identification for 2-MMC degradant

The 1H NMR spectra recorded a de-shielded three-hydrogen singlet at 249 ppm (NHCH3); a slightly de-shielded methyl singlet attributable to the methyl attached the aromatic ring (ArCH3, 2.6 ppm) and finally a methyl doublet (CHCH3, 1.34 ppm). A broad signal at 9.5 ppm was consistently observed and corresponded to the ammonium salt protons. 1H NMR spectrum, **Figure 5-7**, indicated that the degradant is not present and the spectrum corresponds to 2-methylmethcathinone as demonstrated in **Table 5-3**.

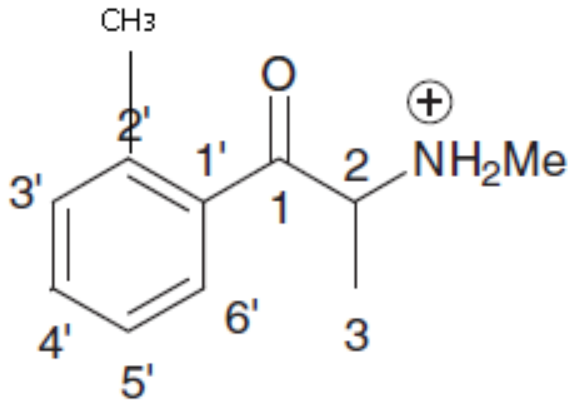


Table 5-3: H^1 NMR spectrum data (400 MHz), d6-DMSO) of 2-MMC HCl (10 mg/0.6 mL).

Position	**δ_H (ppm) [n, multiplicity)**
1	-
2	5.07,q(1H)
3	1.34,d (3H)
NH	9.5,br S(1H)
ARCH3	2.6,S(3H)
1’	-
2’-CH3 ($NHCH_3$)	2.49,s (3H)
3’	7.5 (1H)
4’,5’	7.4 (2H)
6’	7.9, d (1H)

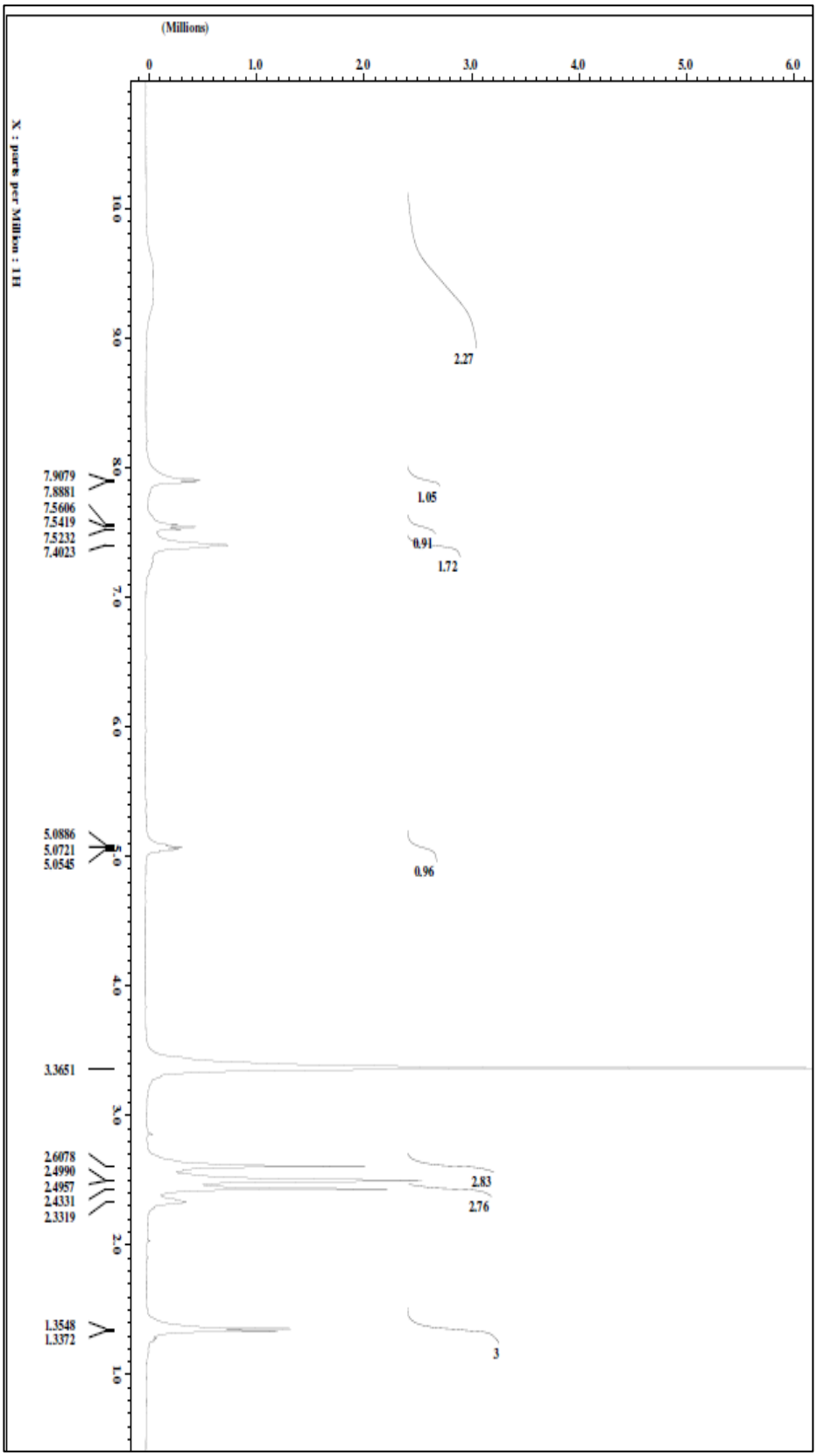


Figure 5-7: H^1 NMR spectrum (400 MHz), d6-DMSO) of 2-MMC HCl (10 mg/ 0.6 mL).

5.7 Conclusion

A simple, suitable and economical HPLC method was described for the separation of new designer drugs, 2-MMC, 3- MMC and 4-MMC. A baseline separation (**Figure 5-5**) was achieved on the Kromasil 5 µm silica gel column (150 mm x 4.6 mm). However the stability of the 2-MMC was an issue that is affected the quality of separation. The results of LC-MS analysis suggested a loss of two protons from the main compound, 2-MMC, and therefore a rearrangement of the compound might explain the weak interaction with the stationary phase. However, if in HILIC the main retention occurs on the hydrophilic layer the rearrangement of the compound should not affect the partitioning especially the silica column has no ligands attached, thus the retention would be due to partitioning/lipophilic or electrostatic interaction. The unknown compound might repelled from the HILIC system and experienced a steric hindrance effect as it eluted in the void volume. The NMR data suggested the signals are belong to a pure 2-MMC compound with no change in structure indicating that the rearrangement might be reversible. A further investigation to identify the unknown compound is required or modifications the analytical condition which could affect the stability. The HILIC technique on a bare silica stationary phase showed a great power in the separation of regioisomers compounds whereas it was not possible to achieve resolution on a reversed phase columns and/ or conditions. The method is currently being developed further by collaborators.

Chapter 6

Conclusion and Final Remarks

6.1 General Conclusion

The HILIC technique is a powerful method for the separation of different lipophilic compounds as well, surprisingly as very lipophilic ones. An extensive study to explore the mechanism of retention for HILIC like properties was attempted for silica gel based stationary phases with different pore sizes including ACE 100 Å, ACE 300 Å and Kromasil 60 Å in addition to a Cogent silica C 100 Å column. Three quaternary ammonium basic test probes BTM, BTE and BDE that were different in the charge density were used to characterise the retention properties of these columns. The retention behaviour of these bases was investigated with 20, 40, 60, 80 and 90% of acetonitrile at constant buffer strength of 10 mM tetramethylammonium acetate (pH 6.0) in the mobile phase. The U shape plot of log k against the percentage of acetonitrile suggests that at 20 % acetonitrile the retention is due to a balance between ion-exchange and lipophilicity with the lipophilic and HILIC/ion exchange interactions being at a minimum at around 50-60% acetonitrile in the mobile phase. The Kromasil 60 Å column produced the strongest retention of the test probes in comparison with the larger pore size columns at 80 and 90% acetonitrile, indicating that the surface area of the silica gel plays a significant role in the retention in HILIC/ion exchange mode. Although Cogent silica C column and purely silica gel based columns are different in properties; the

behaviour of silica C column is very similar to silica gel column with a pore size somewhere between 100 Å and 60 Å.

The second part of the study investigated the contribution of the ionic strength of buffer on the retention of bases in HILIC mode. The buffer concentration range from 5- 20 mM was studied. It was observed that the contribution of HILIC to overall retention of bases increases as the ionic strength of the competing counter ion is increased. The binomial curve fitting used to assess the contribution of HILIC to the overall retention neglects the effect of the ionic strength in the HILIC layer. The ionic strength of the mobile phase modifier is likely to be much higher in the HILIC layer than in the bulk since ionic modifiers will have affinity to partition strongly into the polar aqueous layer.

The type C silica phase was successfully used in HILIC mode. Therefore, the application of HILIC using some commercially available bonded hydride-based stationary phases was addressed using 13 bases and 7 acids which varied in their lipophilicity. The investigation was performed on cogent phenyl, cogent silica hydride and cogent cholesterol columns under HILIC conditions. The study utilised two mobile phases at high acetonitrile percentage of 90 and 95% in the mobile phase which contained a constant buffer strength of 20 mM ammonium acetate. The effect of buffer type in the retention of test probes was also addressed using ammonium formate buffer. The acidic probes exhibited a significant increase in the retention

time as the percentage of acetonitrile increased from 90 to 95%. It was observed that the silica hydride stationary phase is more retentive for the acidic probes than the phenyl hydride suggesting that the hydrophilic layer which formed on phenyl column might be less than that on the silica hydride. This would be expected since the surface is partly occupied by the phenyl containing stationary phase ligand.

Despite the high lipophilic properties of all tested acids, even when they are 99.9% in their ionised form, the retention was strong and their retention in the basis of the hydrophilic interaction remains unexplained. The greatest retention in HILIC conditions was shown in the Cogent cholesterol UDC column despite the fact that this column has a very bulky hydrophobic ligand in comparison to the other tested silica hydride-based columns. The mechanism of retention of the acidic test probes was not primarily lipophilic and possibly due to a combination of lipophilic and HILIC-type interactions since this column have a very bulky hydrophobic ligand. It is surprising with such a large hydrophobic ligand attached to the surface that there is still HILIC interaction and the possibilities of this behaviour might be the formation of an in situ ion-exchange phase and/ or lipophilic interactions with accessible stationary surface.

The results for the basic test probes were quite unexpected and the retention mechanism not clearly explained. The retention of the basic probes was greatest on the phenyl hydride column when the acetonitrile

content increased from 90 to 95%, although HILIC interaction might be expected to be greatest on the unmodified silica hydride surface where the whole surface is available for interaction rather than being partly occupied by phenyl groups. Thus in addition to HILIC and lipophilic partitioning there has to be a third unknown interaction and the contribution of the stationary phase surface to the retention should be examined. For this purpose, the acidic and basic test probes were examined on a simple system silica gel column. The analysis was conducted under HILIC and reversed phase conditions on a Kromasil silica column. For the HILIC study the mobile phase contained 90 and 95% acetonitrile and for the RP study 80% of ammonium acetate buffer at constant strength of 20 mM and 20% ACN was used. The study attempted to explain the retention behaviour under HILIC mode in a simple silica gel column. The observations of the study confirm the formation of the hydrophilic layer when high organic content is applied to the mobile phase. The use of log D and log P values to predict retention for acids and bases generally indicates that log D is more relevant and predictive of retention behaviour. This certainly appears to be the case with acidic compounds, but the results for bases is no so clear, but the reasons for this are not known. More work need to be done to investigate the retention behaviour of compounds and its correlation to lipophilicity to produce definitive results. The results do however appear confirm that the charged state of the compounds is critically important in HILIC mode when considering the extent of retention.

In the reversed phase analysis the retention was a combination between lipophilic and ion exchange interactions. Surprisingly, in HILIC condition the lipophilic effects seem to still have an effect in the retention even at high organic content in the mobile phase and the formation of the solvated layer on the surface. There was some correlation between the low retention in HILIC mode and high retention in RP. Although the significant differences in lipophilic properties of the tested probes, they showed strong retention in HILIC as the hydrophilic ones. The least hydrophobic probes were the most sensitive to the establishment of HILIC layer indicating the predominance of HILIC interaction. The contribution of stationary phase to the overall retention was clearly demonstrated and further studies are needed to eliminate or control the effect for optimal separation conditions. Comparing the retention of acids on the Kromasil silica gel stationary phase to a silica hydride column it was observed that although there is strong electrostatic repulsion on the ordinary silica gel, it retained acids more than the silica hydride column under HILIC conditions with flurbiprofen exhibited exactly the same retention on both phases at 95% ACN. Again the basic probes were strongly retained on the silica gel surface in HILIC conditions with two bases, triprolidine and acebutolol, exhibited exactly the same retention on both phases. The retention of moderate and less lipophilic bases was found relatively stronger on the hydride surface. Generally, the un-modified silica hydride column was also found to have lipophilic retention properties. The application of mobile

phases with different acetonitrile content concluded that with low organic content the retention on an unbounded silica phase is governed by lipophilic interactions. However, as the acetonitrile content is increased to $\geq$ 90%, a hydrophilic layer is produced which allows HILIC type interactions to take place. This gives significant retention of polar compounds.

The validation for HILIC method on a Kromasil silica gel column was attempted by testing various system suitability parameters and it presented good efficiency; however the precision of some test probes was low, possibly due to the mixed-retention mechanism faced by each analyte, but in general it shows the potential and versatility of this stationary phase. In addition, the hydrophilic chromatography is useful in the analysis of wide range of compounds with differing polarities and has been shown to be very useful in the analysis of metabolites.

The HILIC method was successfully applied to the separation of cathinone regioisomers on the Kromasil silica gel stationary phase. The separation was achieved under HILIC conditions, which was not observed under reversed phase stationary and conditions. However, a stability issue was raised for one of the isomers, 2-MMC, which exhibited degradation that affected the separation while the 3-MMC and 4-MMC could be separated. An investigation to identify the unknown peak was carried out but did not yield a clear answer. Thus a further study could be conducted to evaluate the separation and avoid the degradation of the critical isomer. In

conclusion, HILIC is a powerful technique for the analysis of various compounds which have been challenging in reverse phase chromatography.

6.2 Final Remarks

HILIC proved a powerful chromatographic method for the analysis of acidic, basic and neutral compounds with different range of polar properties. HILIC could be useful for drug impurities as it offers different kinds of selectivity and it can be used to provide rapid methods for separation. The high organic solvent content of the mobile phase would be readily adaptable to UHPLC methods since the high organic content results in low back pressures.

This work has not found the “ultimate” stationary phases for HILIC; i.e. a phase that gives a “pure hydration” layer around a stationary phase. In this work the nature of both the stationary and mobile phases would appear to have a large effect on retention behaviour. It is also obvious from this work that the retention mechanism in HILIC is multi-faceted and more work is required to fully elucidate the mechanisms. One the organic content in the mobile phase is high enough for HILIC-type separations, then both the nature of the stationary phase (polarity, bonded groups, residual silanols) and the nature of the mobile phase (pH, buffers) have a large effect on retention. However, as mentioned above more work is needed to fully

understand the interactions taking place; it is obvious that HILIC is more than just partitioning.

The application of HILIC would appear to be very useful for metabolism studies and it can be used for challenging analysis problems.

Overall there are many questions regarding the mechanism of HILIC separation remaining at the end of study, which provides plenty of scope for future investigations.

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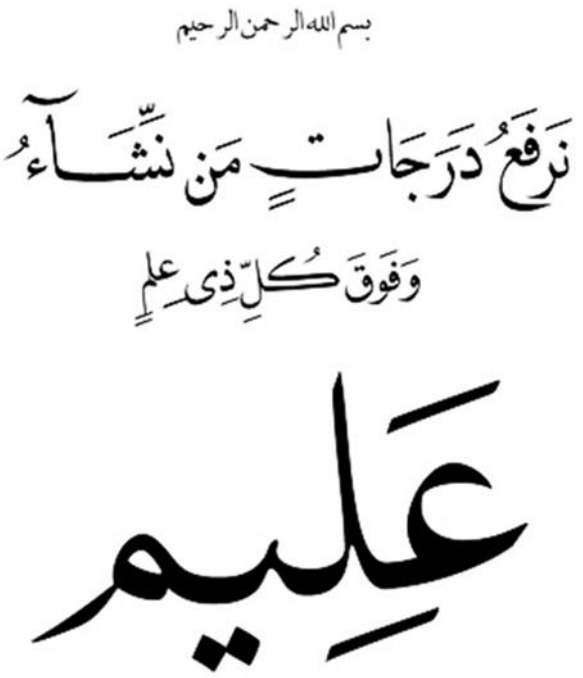

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