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Micellar chromatographic partition coefficients and their application in predicting skin permeability

Yasser Shahzad

A thesis submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

The University of Huddersfield

2013

Abstract

The major goal for physicochemical screening of pharmaceuticals is to predict human drug absorption, distribution, elimination, excretion and toxicity. These are all dependent on the lipophilicity of the drug, which is expressed as a partition coefficient i.e. a measure of a drug's preference for the lipophilic or hydrophilic phases. The most common method of determining a partition coefficient is the shake flask method using octanol and water as partitioning media. However, this system has many limitations when modeling the interaction of ionised compounds with membranes, therefore, unreliable partitioning data for many solutes has been reported. In addition to these concerns, the procedure is tedious and time consuming and requires a high level of solute and solvent purity. Micellar liquid chromatography (MLC) has been proposed as an alternative technique for measuring partition coefficients utilising surfactant aggregates, known as micelles.

This thesis investigates the application of MLC in determining micelle-water partition coefficients ($\log P_{MW}$) of pharmaceutical compounds of varying physicochemical properties. The effect of mobile phase pH and column temperature on the partitioning of compounds was evaluated. Results revealed that partitioning of drugs solely into the micellar core was influenced by the interaction of charged and neutral species with the surface of the micelle. Furthermore, the pH of the mobile phase significantly influenced the partitioning behaviour and a good correlation of $\log P_{MW}$ was observed with calculated distribution coefficient ($\log D$) values. More interestingly, a significant change in partitioning was observed near the dissociation constant of each drug indicating an influence of ionised species on the association with the micelle and retention on the stationary phase. Elevated column temperatures confirmed partitioning of drugs considered in this study was enthalpically driven with a small change in the entropy of the system because of the change in the nature of hydrogen bonding. Finally, a quantitative structure property relationship was developed to evaluate biological relevance in terms of predicting skin permeability of the newly developed partition coefficient values. This study provides a better surrogate for predicting skin permeability based on an easy, fast and cheap experimental methodology, and the method holds the predictive capability for a wider population of drugs.

In summary, it can be concluded that MLC has the ability to generate partition coefficient values in a shorter time with higher accuracy, and has the potential to replace the octanol-water system for pharmaceutical compounds.

Dedication

This thesis is dedicated to my parents, without their love, support and encouragement, this would not have been possible.

Acknowledgments

I would like to express my sincere appreciation to my research supervisor, Dr. Laura J. Waters, whose expertise, understanding and patience added considerably to my graduate experience. I sincerely thank you for making my doctoral degree a wonderful learning experience with not only the technical content but also the philosophical aspects.

I must also extend my thanks to Dr. Peter Laity for his help in small angle X-ray scattering measurements.

I would also like to thank the University of Huddersfield for providing me a fee-waiver scholarship to pursue doctoral degree at the School of Applied Sciences. I would also like to thank the University of Huddersfield Graduate Centre for providing me with the much needed conference presentation funds throughout the duration of my PhD.

I would also like to thank all my colleagues who work in W1/28 and XG/04 for creating a friendly working environment, as well as to the technical staff of the School of Applied Sciences for their laboratory assistance.

To my parents, I thank you for everything you taught me growing up, and for instilling in me a desire to learn and the belief that hard work pays off no matter how long it takes.

Finally, I would like to thank my brother and sisters for their tremendous support and encouragement throughout this project.

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List of abbreviations

AN	Aggregation number
CCC	Counter-current chromatography
CMC	Critical micelle concentration
CN	Cyanopropyl
CTAB	Cetyl trimethylammonium bromide
DF	Degree of freedom
HPLC	High performance liquid chromatography
IAM	Immobilised artificial membrane
MEEKC	Microemulsion electrokinetic chromatography
MEKC	Micellar electrokinetic chromatography
MLC	Micellar liquid chromatography
MP	Melting point
MSE	Mean square error
MV	Molar volume
MW	Molecular weight
NSAIDs	Non-steroidal anti-inflammatory drugs
ODS	Octadecylsilyl
OECD	Organisation for Economic Co-operation and Development
PAMPA	Parallel artificial membrane permeability assay
PRESS	Prediction error sum of squares
PSA	Polar surface area
QSAR	Quantitative structure activity relationship
QSPR	Quantitative structure property relationship
SAXS	Small angle X-ray scattering
SDS	Sodium dodecyl sulfate
TLC	Thin layer chromatography
UV	Ultraviolet
VIF	Variance inflation factor

Chapter 1: An introduction to partition coefficients and micellar liquid chromatography

In the pharmaceutical industry, formulation of a new drug substance into a final dosage form requires substantial physical and chemical characterisation. For this purpose, preformulation studies are designed including the measurement of partition coefficients. Partition coefficient measurements also contribute to understanding the rate and extent of drug absorption. The partition coefficient is a measure of a drug's preference for the lipophilic or hydrophilic phases. When a drug substance is added in a mixture of two immiscible liquids, it distributes itself between the two phases and ultimately attains equilibrium at constant temperature. Transport of drug molecules across a biological membrane is governed by two processes; active and passive transport where active transport requires cellular energy while passive transport is related to the lipophilicity of the drug. As drugs have to pass through the biological membranes, which are composed of lipids and proteins, these membranes form a barrier for drugs (Dearden, 1985).

1.1. The importance of partition coefficients

Partition coefficients are often described as a measure of a compound's preference to reside in lipophilic and hydrophilic environments (Pliška et al., 2008). Being a physicochemical property, partition coefficients are of prime importance in drug discovery and development. The term 'lipophilicity' refers to the affinity of a molecule for a lipophilic environment (Everett, 1972), while 'hydrophilicity' refers to the affinity of a molecule for a hydrophilic environment. Lipophilicity or hydrophilicity is frequently measured through distribution of

molecules between biphasic systems; the most commonly used is an octanol-water system (Sangster, 1989). Lipophilicity is relevant in pharmacological studies, for example, absorption, distribution, metabolism and excretion (ADME) profiling (Van, 1987; Van de Waterbeemd et al., 2001; Oprea, 2002), the transport of molecules through cellular membranes (Avdeef, 2001) and in the assessment of environmental fate, toxicology and transport of organic chemicals (Duffus, 1993).

Historically, the importance of lipophilicity in connection with the biological activity of drugs was highlighted by Meyer (1899) and then Overton (1901) in their published work on narcosis using oil-water partition coefficients for the first time. This was followed by Guadette and Brodie (1959), who found a correlation between heptane-water partition coefficients of some drugs and penetration rate through the blood brain barrier. The first systematic research on partition coefficients was organised by Hansch and Fujita (1964) and then by Leo and co-workers (1971), introducing the octanol-water partition coefficient system as a reference to construct a quantitative structure-activity relationship (QSAR), known as Hansch analysis. This quantitative descriptor of lipophilicity, the partition coefficient (P) often expressed in its logarithmic form of logP, can be defined as the ratio of neutral species in organic (HA_{oct}) and aqueous (HA_{aq}) phases under equilibrium conditions, as expressed by Eq. (1.1):

$$P = \frac{[HA]_{oct}}{[HA]_{aq}} \quad (1.1)$$

Lipophilicity is known to be pH dependent, therefore it is crucial to determine partition coefficients at a range of pH values in order to get an appropriate predictor of biological activity. Hence, if the partition coefficients for neutral (P) and ionic (P_i) species are known, mathematically derived theoretical equations can be used to calculate the change in lipophilicity with respect to pH.

In the case of ionisable species, the term ‘distribution coefficient (D)’ is often used. For non-ionisable species, only the neutral form exists, therefore, P and D should be the same value. However, for ionisable species, there is a clear distinction between the partition and distribution coefficients in the pH range where these species exists in ionic forms (Kah and Brown, 2008). Mathematically, it can be expressed as:

$$P = \frac{[HA]_{oct}}{[HA]_{aq}} \approx D = \frac{[HA]_{oct} + [A_i^-]_{oct}}{[HA]_{aq} + [A_i^-]_{aq}} \quad (1.2)$$

where A_i^- is the concentration of the ionised species in octanol and the aqueous phase at a given pH. Assuming that only the neutral form of a species is present in the organic phase i.e. $[A_i^-]_{oct} = 0$, a value for D for an acidic compound can be derived from the pH and pK_a relationship:

$$D = \frac{P}{1 + 10^{(pH - pK_a)}} \quad (1.3)$$

Similarly, for basic compounds:

$$D = \frac{P}{1 + 10^{(pK_a - pH)}} \quad (1.4)$$

However, there is evidence that there is the presence of ionic species in the organic phase, thus a more complex approach is required to describe the distribution coefficient (Westall et al., 1985). In this case, the introduction of P_i (octanol-water partition coefficient for ionic species) is required, as highlighted in Eq. (1.5):

$$P_i = \frac{[A^-]_{oct}}{[A^-]_{aq}} \quad (1.5)$$

From equations (1.2) and (1.5), D can be more correctly defined as:

$$D = P \times [HA] + P_i \times [A^-]$$

Since:

$$[HA] = \frac{1}{1 + 10^{(pH - pK_a)}} \quad \text{and} \quad [HA] + [A^-] = 1$$

$$D = \frac{P}{1+10^{(pH-pK_a)}} + P_i \times \left(1 - \frac{1}{1+10^{(pH-pK_a)}}\right) \Rightarrow D = \frac{P+P_i \times 10^{(pH-pK_a)}}{1+10^{(pH-pK_a)}} \quad (1.6)$$

Similarly, for basic compounds:

$$D = \frac{P+P_i \times 10^{(pK_a-pH)}}{1+10^{(pK_a-pH)}} \quad (1.7)$$

Furthermore, the same principles can be extended to any amphoteric species that have both acidic and basic functional groups (Kah and Brown, 2008) to permit determination of relevant data for such compounds.

The octanol-water partition coefficient (logP) has been used as a physicochemical property as a descriptor of lipophilicity in a number of QSAR models. For example Hansch and co-workers (1987) elaborated the importance of a drug's lipophilicity in crossing the blood brain barrier successfully. It has also been found that highly lipophilic drugs are slowly eliminated from the body and may elicit central nervous system related problems. More recently, work published by Potts and Guy (1992) successfully elaborated the relationship between a drug's physicochemical properties, (for example lipophilicity and molecular weight) and permeability through the skin.

Lipophilicity and hydrophobicity are often used interchangeably in the literature, however, the term lipophilicity is scientifically dissimilar from hydrophobicity. As described earlier, lipophilicity is the affinity of a molecule for a lipophilic environment whilst hydrophobicity is the association of non-polar groups or molecules in an aqueous environment. The latter phenomenon arises from the tendency of water to exclude non-polar molecules (Duffus, 1993; Rutkowska et al., 2013) and can be expressed in steric terms including molar

volume, polarisability and molar refractivity (Giaginis and Tsantili-Kakoulidou, 2008). Lipophilicity encodes two major structural contributions, namely a bulk term (reflecting cavity formation, hydrophobic and dispersive forces) and a polar term (reflecting electrostatic interactions and hydrogen bonding). The former term refers to hydrophobicity whilst the latter refers to polarity (Gaillard et al., 1994b). The partition coefficient is an equilibrium property between organic and aqueous phases, therefore, it describes a balance between hydrophobicity and polarity (Rutkowska et al., 2013). On the basis of polar/nonpolar inter- and intramolecular interactions, lipophilicity can therefore be expressed by Eq. (1.8):

$$\text{Lipophilicity} = \text{Hydrophobicity} - \text{Polarity} \quad (1.8)$$

Changing the structural characteristics, for example the addition of substituents to aromatic or heterocyclic systems, conjugation within molecules or internal hydrogen bonds, affects intermolecular interactions, thus, influencing the lipophilicity (Bradshaw and Taylor, 1989; Gago et al., 1994; Vrakas et al., 2003). Additionally, the polar and nonpolar environments presented by the solvent may result in conformational changes within the molecules, thus, rendering them more lipophilic or more hydrophilic than expected in the given solvent (Gaillard et al., 1994a; Tsantili-Kakoulidou et al., 1999). Furthermore, ionisation of molecules has a significant effect on intermolecular interactions, therefore, limiting the validity of additive rules established for neutral compounds (Caron and Ermondi, 2007; Dellis et al., 2007). These issues are related to any biphasic system and are dependent on the solvent used. The difference among different biphasic systems, for example, octanol-water, gas-chloroform, chloroform-water, and water-alkane, can be described using solvatochromic analysis (Abraham et al., 1994; Abraham et al., 1999b). This is achieved by factorising the partition coefficient into steric and polar terms which describe various intermolecular interactions as expressed by Eq. (1.9) (Giaginis and Tsantili-Kakoulidou, 2008):

$$\log P = vW/100 + aA + bB + sS + eE + c \quad (1.9)$$

where V is the McGowan's characteristic molar volume, A and B are the solute's hydrogen-bonding acidity and basicity, respectively, S is a combined dipolarity/polarisability term reflecting the solute's capacity to elicit orientation and induction forces, E is the excess molar refractivity. v, a, b, s, e, c are constants derived by multiple linear regression analysis describing characteristics of the systems (Abraham and McGowan, 1987).

1.2. Biological relevance of lipophilicity

Lipophilicity is one of the most fundamental physicochemical properties influencing the biological activity of drugs. Lipophilicity dictates the absorption, distribution, metabolism and excretion of drugs and a key factor in the formulation of pharmaceuticals. Furthermore, the affinity of compounds towards lipophilic environments within biological systems helps in transporting them across membranes to the target sites where they illicit their biological effects (Rutkowska et al., 2013).

It is widely accepted that lipophilicity, solubility and ionisation are the key physicochemical parameters in the predictability of a drug's bioavailability, and significantly influences a drug's pharmacokinetic, pharmacodynamic and toxicological behavior (Wang and Skolnik, 2009; Van De Waterbeemd, 2009). This is exemplified by the fact that physicochemical properties are required for quantitative modeling of *in vitro* and *in vivo* biological data. Such physicochemical properties have proved to be useful in predicting drug permeation across biological membranes and barriers, for example skin, intestinal mucosa and the blood-brain-barrier (Kubinyi, 1995; Pagliara et al., 1999; Cross et al., 2003; Wang and Skolnik, 2009). For a variety of reasons, the oral route of drug delivery is the most desirable, therefore, much research has focused on optimising the absorption and bioavailability of drugs

following the oral route (Waterbeemd and Testa, 2009). Following on from absorption, lipophilic molecules can easily pass through the cell membrane (intercellular pathway) or cytoplasm (intracellular pathway) to reach the blood stream and from there the target site. Ionisation of drug molecules, along with many other factors, may limit absorption (Rutkowska et al., 2013) which can be an issue. The intestinal absorption of drugs has been studied through various *in vitro* techniques, for example, the Caco-2 cell line system (Hildago et al., 1989) and the physicochemical parallel artificial membrane permeability assay (PAMPA) (Kansy et al., 1998; Avdeef et al., 2007) which have remained the most popular approaches for estimating intestinal absorption. Although these models are mechanistic in nature their applicability has been questioned owing to the poor predictability found for some drugs (Verma et al., 2007).

Waterhouse (2003) demonstrated a relationship between a drug's lipophilicity and blood-brain-barrier permeation and explained situations in which lipophilicity could be a good predictor and an under-predictor, for example if the compound was highly lipophilic. These results were consistent with those published by Hansch and co-workers (1987) explaining reduced blood-brain-barrier uptake of highly lipophilic drugs as a consequence of non-specific plasma protein binding and a high uptake for moderately lipophilic drugs.

Correlating lipophilicity with skin permeability has remained an area of great interest in recent years and various mathematical models have been proposed for predicting skin permeability (Potts and Guy, 1992; Barratt, 1995; Potts and Guy, 1995; Abraham et al., 1995; Abraham et al., 1999a; Moss et al., 2002; Yamashita and Hashida, 2003). Despite its ability to correlate some biological properties, lipophilicity has been questioned several times, for example, isotropic organic-water partition coefficients have failed to encode some important recognition forces in biochemistry and molecular pharmacology, most notably ionic bonds,

which are important in modeling interactions of ionised species with biomembranes (Hartmann and Schmitt, 2004; Liu et al., 2011).

However, in general, lipophilicity is an established property that influences the distribution of drugs through drug-plasma protein interactions, consequently influencing pharmacokinetic and pharmacodynamic behaviour (Zoghbi et al., 2012). For example, the influence of lipophilicity on tissue distribution kinetics for some basic drugs has been reported by Yokogawa and co-workers (2002). Their study revealed that the uptake of basic drugs into lysosomes depended on both intra-lysosomal pH and drug lipophilicity, thus, a linear correlation was found between lipophilicity and lysosomal drug aggregation (Yokogawa et al., 2002). This relationship was further confirmed by Lazicek and Laznickova (1995) who described that the binding of some anionic drugs with plasma proteins and blood cells was dependent on drug lipophilicity.

A compound, after being absorbed and distributed in the body, is subjected to metabolism or biotransformation prior to elimination from the body. This usually occurs in two phases, namely phase I and phase II metabolism. The phase I metabolism pathway includes oxidation, reduction and hydrolysis. As a result of phase I metabolism, compounds are modified by the introduction of polar groups which reduce lipophilicity, thus facilitates elimination. In phase II, drugs are conjugated with charged species, namely glutathione, glycine or glucuronic acid resulting in formation of a less active complex. This complex often has a higher molecular weight with an associated impact on lipophilicity which restricts its diffusion across membranes and is therefore actively transported out of the body through renal and bile excretion (Rutkowska et al., 2013).

As long ago as the 19th century, it was established that lipophilicity may affect the toxicological properties of compounds, for example, it has been shown to be influential in cytotoxic studies performed on heart muscles (Halbach, 1990). Similarly, the toxicity of local anesthetics can be substantially increased at logP values above 3.0 because of a high plasma availability of these compounds and enhanced absorption into the blood-brain barrier (Nava-Ocampo and Bello-Ramírez, 2004). For all these reasons it is important to be aware of the lipophilicity of a compound and this is why it must always be determined for new chemical entities.

1.3. Octanol-water partition coefficients (logP_{ow})

The octanol-water system holds the ‘gold standard’ as a widely accepted reference system for describing the lipophilicity of compounds. Octanol offers some advantages over other organic solvents, namely the presence of hydrophobic chains with a polar head group, which correlates well with biological activity (Danielsson and Zhang, 1996; Giaginis and Tsantili-Kakoulidou, 2008). It also correlates well with soil sorption constants, water solubility and other parameters that are key in predicting biochemical and toxicological effects (Cairns et al., 1979). The hydroxyl group in the n-octanol molecule has a hydrogen bonding capability both as a donor and an acceptor, similar to a membrane, and a receptor’s hydrogen bond property. As biological membrane properties differ somewhat from the octanol-water system, some alternatives have been proposed to imitate different physiological membranes, for example, chloroform-water, dibutylether-water and propylene glycol diphosphate-water systems. In biological systems with no hydrogen bond donor or acceptor group available on the membrane, an alkane-water system or dichloroethane-water system would be preferable (Leahy et al., 1989; Avdeef et al., 1996; Hartmann and Schmitt, 2004). Although the amphiphilic properties of octanol partially resemble conditions for the biopartitioning of

compounds having moderate lipophilicity or hydrophilicity, the octanol-water system does not reflect biological partitioning (Rutkowska et al., 2013) for highly lipophilic or hydrophilic species which is why various alternative systems are under development.

1.4. Methods to measure partitioning

Numerous methods have been proposed to estimate partition coefficients for compounds including the previously discussed octanol-water system, reverse phase chromatography, micellar and microemulsion electrokinetics, liposome-water systems and computational systems using a fragmental approach. A brief summary of all these methods alongside some advantages and drawbacks is summarised in Table 1.1.

Table 1.1: Methods to determine or estimate the partition (Kah and Brown, 2008)

Method	Advantage	Drawback	Not applicable if	References
<i>Direct</i>				
Shake-flask	Widely used method, reliable	Time, quantity of product used, formation of octanol emulsions in water	$\log P > 6$; the tested substance dissociates (P_{ow} depends on concentration)	(OECD, 1995)
Slow stirring method	Avoids the formation of emulsions, reliable	Time, quantity of product used	The tested substance dissociates (P_{ow} depends on concentration)	(Brooke et al., 1986; De Bruijn et al., 1989)
<i>Chromatographic methods</i>				
Classical chromatographic approach RP-TLC and RP-HPLC	Rapid and cheap, does not require chemical quantification	Different retention mechanisms; poor reproducibility; pH range limited to 2-7.5	Very low P_{ow} values	(Sicbaldi and Finizio, 1993; Finizio et al., 1997; OECD, 2004)
Counter-current (CCC) and Immobilised artificial membrane (IAM) chromatography	Versatile and reliable method	CCC has low accuracy and resolution. IAM is costly and short life time of column	IAM is not applicable if volume phase ratio changes	(Berthod et al., 2009; Hartmann and Schmitt, 2004)
Micellar and microemulsion electrokinetic chromatography	Rapid and cheap	Requires the establishment of solvation parameter model	Ionic substance	(Poole and Poole, 2003)
<i>Others</i>				
Filter probe method	Rapid; well adapted to construct a $\log D/pH$ profile	Expensive equipment required, slow to set up	$\log P < 0.2$; no isosbestic point	(Takács-Novák et al., 1995; Leo, 2000)

Table 1.1: Continued....

Method	Advantage	Drawback	Not applicable if	References
pH metric method (or potentiometric method)	Rapid; well adapted to construct a logD/pH profile	Expensive equipment required	Insoluble compound	(Chamberlain et al., 1996; Avdeef, 2001)
<i>Calculation</i>				
Regressions with other properties	Quick and cheap	Result depends on the dataset		(Xing and Glen, 2002; Lü et al., 2007)
Computational method (fragmental approach)	Wide range of chemicals; reliable for neutral compounds; only reliable method for $P_{ow} > 5$	Not reliable for zwitterionic, tautomeric, charged compounds and strongly hydrogen-bonding compounds	Fragment not available in the software database	(Leo, 2000; Mannhold and Van De Waterbeemd, 2001; Machatha and Yalkowsky, 2005)

1.4.1. Direct measurement methods

The shake-flask method remains the most widely used experimental protocol utilising an octanol-water system and it has been designated as the standard method in the Organisation of Economic Cooperation and Development (OECD) guidelines for determining partition coefficients (OECD, 1995; Danielsson and Zhang, 1996). In the shake-flask method, a test substance is spiked in the octanol-water system in a separating funnel and shaken for 24 to 72 hours to attain an equilibrium as shown in Figure 1.1. The octanol and water phases are then separated and the concentration of substance in each layer is determined using standard analysis procedures involving either UV-spectrophotometry or high performance liquid chromatography (HPLC). From the ratio of a compound's concentration in each layer, the partition coefficient can be calculated using Eq. (1.1). The shake-flask method is a laborious and time consuming process that requires a very high purity of compound in relatively large amounts. Furthermore, it is only suitable over a narrow range of partition coefficient values i.e. -3 to +3 which limits its applicability. Glass and surface adsorption is another limitation that must be minimised to attain reliable results (Danielsson and Zhang, 1996). Furthermore, the aqueous phase often requires centrifugation after equilibration in order to remove the small droplets of octanol in the form of an emulsion created during shaking (Leo et al., 1971). Although, it may be possible to remove the emulsion that was formed during shaking, discrepancies occur in the values obtained (Hansch and Leo, 1979; Finizio et al., 1997). Furthermore, dissociation of molecules into the ionised form may also influence the partition coefficient, thus, this method is only applicable to compounds that are non-ionised (OECD, 1995).

Alongside the shake-flask method is the slow-stir method which follows similar principles but mixing is achieved at a very slow stirring speed in order to avoid the formation

of an emulsion which can hinder the measurement of true partition coefficients (Brooke et al., 1986; De Bruijn et al., 1989).

In summary, both methods are time consuming and expensive (in terms of high amount of compound requirement), and applicable to only neutral compounds or ionisable compounds in their neutral form, thus limiting their suitability and versatility.

1.4.2. Chromatographic methods

Various chromatographic approaches have been proposed to determine partition coefficients. One such approach is thin layer chromatography (TLC) which allows determination of lipophilicity in a relatively simple and fast way without utilising expensive equipment (Biagi et al., 1975; Biagi et al., 1992). Essentially, a compound is bound either covalently or by adsorption on a solid surface usually made of silica. This solid surface (or TLC plate) is then introduced to a mobile phase and movement of compound on the plate is recorded. The lipophilicity descriptor, namely retardation factor R_M is calculated from the retention factor, R_f as described in Eq. (1.10):

$$R_M = \log\left(\frac{1}{R_f - 1}\right) \quad (1.10)$$

where R_f is calculated on the basis of migration distances of a compound and the solvent front. The chromatographic parameter R_M^0 for pure water is obtained by extrapolating the linear relationship of R_M and various concentrations of organic modifier in the mobile phase as given in Eq. (1.11):

$$R_M = R_M^0 + S \times \Phi \quad (1.11)$$

where S is the slope of the linear plot and ϕ is the concentration of organic modifier.

Although TLC based logP determination overcomes some of the difficulties associated with the shake-flask method, results may be non-reproducible owing to the effects of the gradient of the mobile phase on the surface of the TLC plate (Giaginis and Tsantili-Kakoulidou, 2008).

The reverse phase-HPLC approach (OECD, 2004) utilises a stationary phase packed inside a column with a mobile phase that runs through the stationary phase, thus, retaining compounds onto the stationary phase which is then used to describe the lipophilicity. There is no chemical quantification required in this method, therefore, it is seen as a fast and cheap method (Kah and Brown, 2008). A number of studies have been published which describe the HPLC method for determination of octanol-water partition coefficients (Könemann et al., 1979; Garst and Wilson, 1984; Braumann, 1986; De Kock and Lord, 1987). The method encompasses the establishment of correlations between retention data (in terms of capacity factor of compounds of interest) and the known octanol-water partition coefficients of structurally similar compounds. It would not be wrong to say that this method is restricted to a homologous series of compounds and discrepancies often arise when suitable standards are unavailable. Another drawback is the narrow range of pH (usually from 3-7.5) for silica based columns to remain stable, therefore, this limits its suitability for ionic compounds (Danielsson and Zhang, 1996).

In addition to the above mentioned chromatographic methods, counter-current chromatography (CCC) and immobilised-artificial membrane (IAM) chromatographic approaches have been proposed as alternatives to the shake-flask method for measurement of partition coefficients. Counter-current chromatographic determination of logP_{ow} is attractive because it utilises a liquid stationary phase instead of a solid stationary phase which allows

partitioning of compounds between two immiscible liquids. For example, this technique has been successfully applied to a series of 23 ionisable compounds with a high correlation with experimental and computational logP values (Berthod et al., 1999), however, in a more recent study, a poor correlation was demonstrated (Carda-Broch and Berthod, 2003).

IAM chromatography utilises a stationary phase that is prepared from phospholipids covalently bonded to a propylaminosilica support material at monolayer densities, thus, mimics the lipid biomembrane on a solid surface (Taillardat-Bertschinger et al., 2003). IAM columns permit the use of an aqueous mobile phase without any addition of organic modifier which allows a direct measurement of logP values. Since the artificial membrane provides a closer mimic to the amphiphilic microenvironment of biomembranes ionic interactions are likely to be present which potentially makes it a superior technique (Taillardat-Bertschinger et al., 2003). Using this technique, lipophilicity is expressed as the retention capacity on an IAM column under isocratic conditions, assuming that the molar volume ratio of stationary and mobile phase does not vary from column to column (Hartmann and Schmitt, 2004; Valko, 2004). The lipophilicity scale of IAM chromatography has shown a good correlation with passive transport from the small intestine of rats, thus, endorsing its reliability as a predictor of lipophilicity (Genty et al., 2000). However, there are disadvantages, for example IAM columns tend to have shorter life-times due to the leaching of phospholipids (Taillardat-Bertschinger et al., 2003) and they are an expensive system to study, even for partitioning of compounds.

Micellar electrokinetic chromatographic (MEKC) and microemulsion electrokinetic chromatographic (MEEKC) approaches have been investigated for the estimation of partition coefficients (Herbert and Dorsey, 1995; Örnkov et al., 2005). Micellar electrokinetic

chromatography utilises a correlation between the partitioning of compounds in a micellar system (surfactant above its critical micellar concentration that forms an aggregate called a micelle) and the octanol-water partition coefficient through a linear solvation energy relationship (Hartmann and Schmitt, 2004). The retention factor in MEKC is derived from the migration time of solute of interest, an unretained solute and the micelle under electric current, thus, capacity factors are directly related to the solute's partitioning between the micelle and water.

It has been proposed to use small oil droplets in the micrometer size range instead of surfactant micelles to more closely mimic phospholipidic membranes (Örnkov et al., 2005). This technique has been named as microemulsion electrokinetic chromatography (MEEKC) which allows determination of partition coefficients in the log range of 0 to 5 for weakly acidic, basic or neutral compounds after ion suppression (Poole and Poole, 2003; Henchoz et al., 2010). However, results may not correlate well with logD values in the case of ionisable compounds (Poole and Poole, 2003).

1.4.3. Further methods to measure partitioning

In addition to the methods discussed above, several others are known including pH metric and filter probe methodologies (Chamberlain et al., 1996; Avdeef, 2001). These methods require expensive equipment and are not yet approved by the OECD.

Theoretical and computational methods have also been proposed to obtain logP values. There are at least 20 software systems available that have the capability to generate lipophilicity parameters from the structural fragments of a compound. Although results are fairly consistent with the direct measurement methods, discrepancies are observed in the case of compounds

that have interacting functional groups or complex functional groups that are not available in the software database (Finizio et al., 1997). A few examples of commonly used computational tools are discussed below;

a. AlogPs

This method, for the assessment of octanol-water partition coefficients, was developed on the basis of neutral network ensemble analysis of 12908 organic compounds. The atom and bond-type E-state indices and the number of hydrogen and non-hydrogen atoms were considered in calculations. A first selection of indices was performed by multiple linear regression analysis, and 75 input parameters were selected. Some of the parameters combined several atom-type or bond-type indices with similar physicochemical properties (Tetko et al., 2005; Tetko and Tanchuk, 2002).

b. IAlogP

This method is based on the set of 238 MolconnZ molecular indices generated from 13000 organic structures with accurately measured logP values. Interactive Analysis, using neutral networks technology to derive a set of 10 fold cross-validated networks, developed the IAlogP predictor (Tetko et al., 2005).

c. clogP

This method estimates interaction parameters for new fragments; clogP is based on an estimation of these interactions on the well-accepted principles defined by Hammett. Not only is clogP the most accurate calculation available but it produces results that are based on established chemical interactions, unlike other programs which are based solely on statistics (Ghose et al., 1998).

d. logP_{Kowwin}

This is an atom-fragment contribution method for octanol-water logP calculation. This method logP_{K_{ow}win} is based on data for 13058 organic compounds, for which accurate experimental partition coefficients logP are published (Meylan and Howard, 1995).

e. xlogP

This is an atom-additive method for octanol-water logP calculation. A large dataset of 1831 organic compounds was analysed by multivariate regression to derive the parameters. This method includes correction factors to account for intramolecular interactions. After including these correction factors, the final logP is described as:

$$\log P = \sum_i a_i A_i + \sum_j b_j B_j \quad (1.12)$$

where a_i and b_j are regression coefficients, A_i is the number of occurrences of the i th atom type, and B_j is the number of occurrences of the j th correction factor identified by this program (Wang et al., 1997).

f. miLogP

This method for logP prediction, developed at Molinspiration (miLogP 2.2), is based on group contributions obtained by fitting calculated logP values with experimental logP values for a training set of more than twelve thousand, mostly drug-like molecules (Rutkowska et al., 2013).

1.5. Micellar liquid chromatography

MLC is a form of high pressure liquid chromatography in which the mobile phase is composed of a micellar solution which is non-toxic to the environment i.e. preferential to conventional organic solvents. Aqueous solutions of surfactants above their critical micelle concentration (CMC) have the ability to arrange themselves into a well organised spherical aggregate known as a micelle. Generally, the outer surface of a micelle is hydrophilic while

the inner core of a micelle favours hydrophobic compounds. This kind of arrangement of surfactants in an aqueous solution allows them to be a highly effective mobile phase for micellar liquid chromatography (García Diez et al., 2004). Micelles provide a heterogeneous environment to solutes, therefore, solutes may experience different microenvironment polarities in a given micellar mobile phase (Khaledi and Breyer, 1989).

Like reverse phase high performance liquid chromatography (RP-HPLC), MLC utilises a non-polar stationary phase which is often a hydrocarbon chain and a polar liquid mobile phase to separate the compounds. MLC instrumentation is rather simple and identical to that of the RP-HPLC including an isocratic pump, an injector, a stationary phase column and a detector with the only difference being in the nature of the mobile phase. In RP-HPLC, mobile phases are usually composed of a mixture of water and an organic solvent, such as methanol or acetonitrile. Generally, when an analyte is injected into an HPLC, it partitions out of the mobile phase and interacts with the stationary phase (depending upon its polarity and hydrophobicity). This interaction leads to the retention of analyte on the stationary phase. If a compound is very polar, it will not significantly interact with the stationary phase, yet with a decrease in polarity, the residence time of analyte will increase in the column resulting in higher retention times (Meyer, 2010). MLC offers the introduction of micelles to the mobile phase without the addition of an organic solvent, thus, it permits the partitioning of an analyte between the micelle, an aqueous phase and the stationary phase to be determined. In addition to this, the presence of micelles maintains the solubility of nonpolar and hydrophobic solutes. Furthermore, without the addition of toxic organic solvents, MLC is a more environmental friendly and a low cost analytical technique compared with other systems.

Since the first report, MLC has been in constant development (Mutelet et al., 2003) and several research groups have explored different aspects of MLC, for example, separation of

mixture of solutes (Mao et al., 2004; Safa and Hadjmohammadi, 2005), separation of enantiomers (Gübitz and Schmid, 2006) and analysis of pharmaceutical compounds in formulations (Kulikov and Verushkin, 2008; Zhao et al., 2013).

1.5.1. Surfactants and micellisation

In MLC surfactant solutions above a specific concentration (where micelle formation occurs) are used as the mobile phase. Surfactants belong to a class of chemicals known as amphiphiles which means these compounds have a hydrophilic head group and a hydrophobic tail (Texter, 1999). The term ‘surfactant’ is a short form of ‘surface active agents’ which means any material, when used in low concentration, that is adsorbed onto the surface or at the interface of a system leading to an alteration in the interfacial free energies of the interface. The term ‘interface’ means a boundary between two immiscible systems or phases. The interfacial free energy refers to the minimum amount of work required to create that interface (Rosen and Kunjappu, 2012). When a surfactant molecule is dissolved in water, the hydrophobic part of the surfactant may alter the structure of water (by breaking the hydrogen bonding), thereby, rearranging the water molecules in the vicinity of the hydrophobic part and increasing the free surface energy of the solution. As a result, the system responds in a way to minimise the contact between the hydrophobic part and the water. Therefore, some of the surfactant molecules are expelled to the surface where the hydrophobic part is predominantly oriented towards the air (since air molecules are nonpolar, there is no dissimilarity between these two phases) so as to minimise the contact with the water molecules. On the other hand, the hydrophilic head group prevents the surfactants from being expelled from the water phase, thus, the hydrophilic group stays inside the water phase. In this way a concentrated monolayer of surfactant is developed at the interface which minimises the surface tension of water (Rosen, 2004a).

Surfactants are generally classified by the charge of the hydrophobic head group i.e. anionic, cationic, nonionic, and amphoteric or zwitterionic. The anionic surfactants are most commonly used because of their low cost and commercial availability in high purity. An anionic surfactant dissociates in aqueous solutions to give a negatively charged surface active portion and an inactive cation, commonly Na^+ or K^+ . The four main families of anionic surfactants are soaps, sulfonated compounds, alkylsulfates and alkylphosphates. Sodium dodecyl sulfate (SDS), which is an anionic surfactant from the alkylsulfate class, is the most widely used surfactant in MLC. Cationic surfactants are those that dissociate into a cationic amphiphile and an inactive anion, commonly Cl^- or Br^- . The most common cationic surfactants used in MLC contain a quaternary ammonium group, such as cetyl trimethylammonium bromide (CTAB). Nonionic surfactants have a polar group such as an ether, alcohol, carbonyl or amino group as their hydrophilic portion. Zwitterionic surfactants contain both positive and negative charges on the same molecule (Berthod and Garcia-Alvarez-Coque, 2000).

Surfactants and their physicochemical properties have been extensively studied over the years. It was firmly accepted from the very beginning that surfactants possess unusual bulk properties and indicated the presence of colloidal ions in surfactant solutions (Rosen, 2004b). The concept of ‘colloidal ions’ was given by James William McBain and co-workers to explain the conductive properties of sodium palmitic solutions (McBain et al., 1912). Later in 1936, the term ‘micelle’ was introduced and promoted for colloidal ions by G. S. Hartley in his book ‘Aqueous Solutions of Paraffin-Chain Salts, A Study in Micelle Formation’ (Hartley, 1936).

With the increase in surfactant concentration in solutions, the interface becomes more and more saturated with monomers, resulting in a substantial decrease in the interfacial tension and surface free energy. Further addition of surfactants abruptly changes the physicochemical

properties of the solution and the formation of micelle occurs. There is no definite concentration of surfactant at which spontaneous micellisation occurs, in fact, micelles exist above a narrow concentration range known as the critical micelle concentration (CMC). It should be noted that the formation of a micelle itself is a process of minimising the free energy of the solution. Therefore, micellisation is an alternative mechanism to surface adsorption of surfactants in order to reduce the contact with the water and a way of reducing the free energy of a system through micelle formation. It is noteworthy that the process of micellisation is predominantly observed in aqueous media because of the surfactant's ability to distort the water molecules. Therefore, micelles of size comparable to those formed in aqueous media are seldom observed in the non-aqueous solvents where surfactants have little chance of distorting the structure of the solvent or the surfactants have a very short hydrophobic tail if water is the solvent (Rosen, 2004b).

Micelles exist in dynamic equilibrium with monomers in solution. The structure of a micelle is dependent on the concentration of monomers in the solution. At a concentration equal to, or above, the CMC, the monomers tend to aggregate to acquire a spherical shape with the tail groups oriented inside the core and the head groups oriented outward facing i.e. towards the aqueous media. Generally, a certain number of monomers aggregate to form a micelle known as the aggregation number (AN). Different surfactants have different CMCs and AN s depending upon their physicochemical properties. Almost all surfactants will form spherical micelles at a concentration close to the CMC, however, ionic surfactants at higher concentrations will transform into elongated cylindrical, rod like, large lamellar and vesicular structures as shown in Figure 1.1. In the case of non-ionic surfactants, spherical micelles directly change into lamellar structures at higher concentrations (Moroi, 1992).

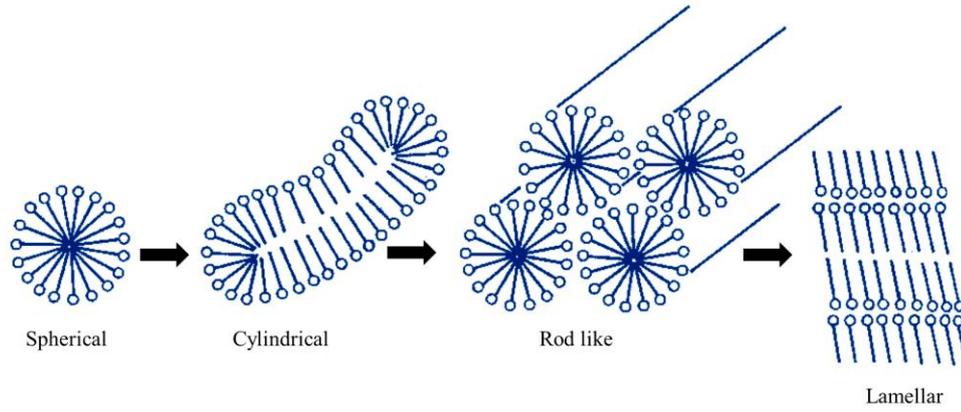


Figure 1.1: Transformation of micelles into different shapes on increasing surfactant concentration (Moroi, 1992)

Thermodynamically, micelle formation follows a pseudo-phase equilibria owing to their dynamic nature. The term ‘dynamic’ is used for micelles because micelles exist in a thermodynamic equilibrium i.e. there is a constant and very rapid interchange of surfactant monomers within a micelle, as well as the fact that micelles are continuously falling apart and new micelles are formed from a different set of monomers. The average lifetime of an individual micelle is approximately 10^{-3} to 1 second, while exchange of monomers in an existing micelle occurs within 10^{-8} to 10^{-4} seconds (Berthod and Garcia-Alvarez-Coque, 2000). The process of micellisation depends upon various interactions between: 1) the hydrophobic tail group and water, 2) hydrophobic interactions of the tail group with adjacent tail groups within the micellar core, 3) surfactant head groups with each other and 4) solvation of the head group in water (Berthod and Garcia-Alvarez-Coque, 2000; Eastoe, 2009). Based on these interactions, micellisation can be expressed mathematically as:

$$AN \cdot S \Leftrightarrow S_{AN} \quad (1.13)$$

and

$$AN \cdot S \Leftrightarrow mS + S_{AN} \quad (1.14)$$

where AN is the aggregation number, S is the concentration of given surfactant associating to

form a micelle, m is the number of free surfactant monomers, and S_{AN} is the micelle comprised of S monomers. When the surfactant monomers and micelles attain equilibrium, the equilibrium constant (K_{eq}) can be expressed as:

$$K_{eq} = \frac{[S_{AN}]}{[S]^{AN}} \quad (1.15)$$

The change in the standard Gibb's free energy (ΔG) of micelle formation can be expressed as:

$$\Delta G = -RT \ln K_{eq} \quad (1.16)$$

Where R is the gas constant and T is temperature in Kelvin. Equation 1.16 can be written as:

$$\frac{\Delta G}{AN} = -\frac{RT}{AN} \ln S_{AN} + RT \ln S \quad (1.17)$$

For micelles formed of sufficiently large aggregation number (AN), typically greater than 50, the first term on the right hand side of the equation can be neglected. Since S is the concentration of monomers to form a micelle, the above equation can be written in terms of CMC:

$$\Delta G \approx RT \ln CMC \quad (1.18)$$

The resultant ΔG of micellisation calculated from Eq. (1.18) will be negative implying that the micellisation is a spontaneous exothermic process. The change in entropy (ΔS) can then be expressed as:

$$\Delta S = \frac{d(\Delta G)}{dT} = R \ln CMC + RT \frac{d(\ln CMC)}{dT} \quad (1.19)$$

Knowledge of the CMC for a given surfactant is critical to ensure optimal experimental conditions. There are various factors that influence the CMC in aqueous solutions including hydrophilic and hydrophobic groups, counter ion effects (in the case of ionic surfactants),

electrolytic effects of added salts (ionic strength), effect of added organic solvents, effect of added solutes and temperature effects. Determination of the CMC has remained an area of great interest over the decades and various methods have been devised for the measurement of CMC including surface tension, conductivity, osmotic pressure, turbidity, dye absorbance and more recently isothermal titration calorimetry which is more accurate and sensitive than the former techniques (Berthod and Garcia-Alvarez-Coque, 2000; Waters et al., 2012a).

1.5.2. Stationary phase and surfactant adsorption in MLC

Liquid chromatographic columns are typically composed of silica based packing bonded with an R group to create a specific column chemistry. The silica particles are derivatised with chloro-silanes containing certain groups (alkyl, phenyl and cyanopropyl) that react with the surface hydroxyl groups (Si-OH) known as silanols. After binding of these groups to the silica surface, many silanols are left unreacted known as residual silanols. The residual silanols can have a pK_a range of 4-9.8 depending upon the silica used (Berthod, 1991).

Surfactant adsorption on the stationary phase has a dramatic effect on the retention behavior of solutes resulting in changes in the surface properties of the stationary phase. Surfactant monomers coat the stationary phase pores, thus minimising the silanophilic interactions. The amount of adsorbed surfactant increased in the order: bare silica < cyanopropyl < methyl < octyl < oca decyl, which agrees with the decreasing stationary phase (Ruiz-Ángel et al., 2009). Surfactant interactions with the stationary phase have been extensively studied and determined as surfactant monomers tend to adsorb onto the stationary phase. Lavine and coworkers examined the surfactant interactions with different silica modified stationary phases, namely cyanopropyl, C18 and C8. They found that the hydrophobic alkyl tail of SDS was associated with the bonded layer of the C8 and C18 stationary phases,

while the sulfate polar group of SDS protrudes out of the bonded layer (Lavine et al., 1994). Conversely, the polar head group of CTAB is incorporated within the bonded phase, with the tail group oriented outward. For the cyano-bonded phase (Figure 1.2), both the CTAB and SDS surfactants became tightly bound and entwined within the bonded phase through electrostatic interactions (Lavine et al., 1996).

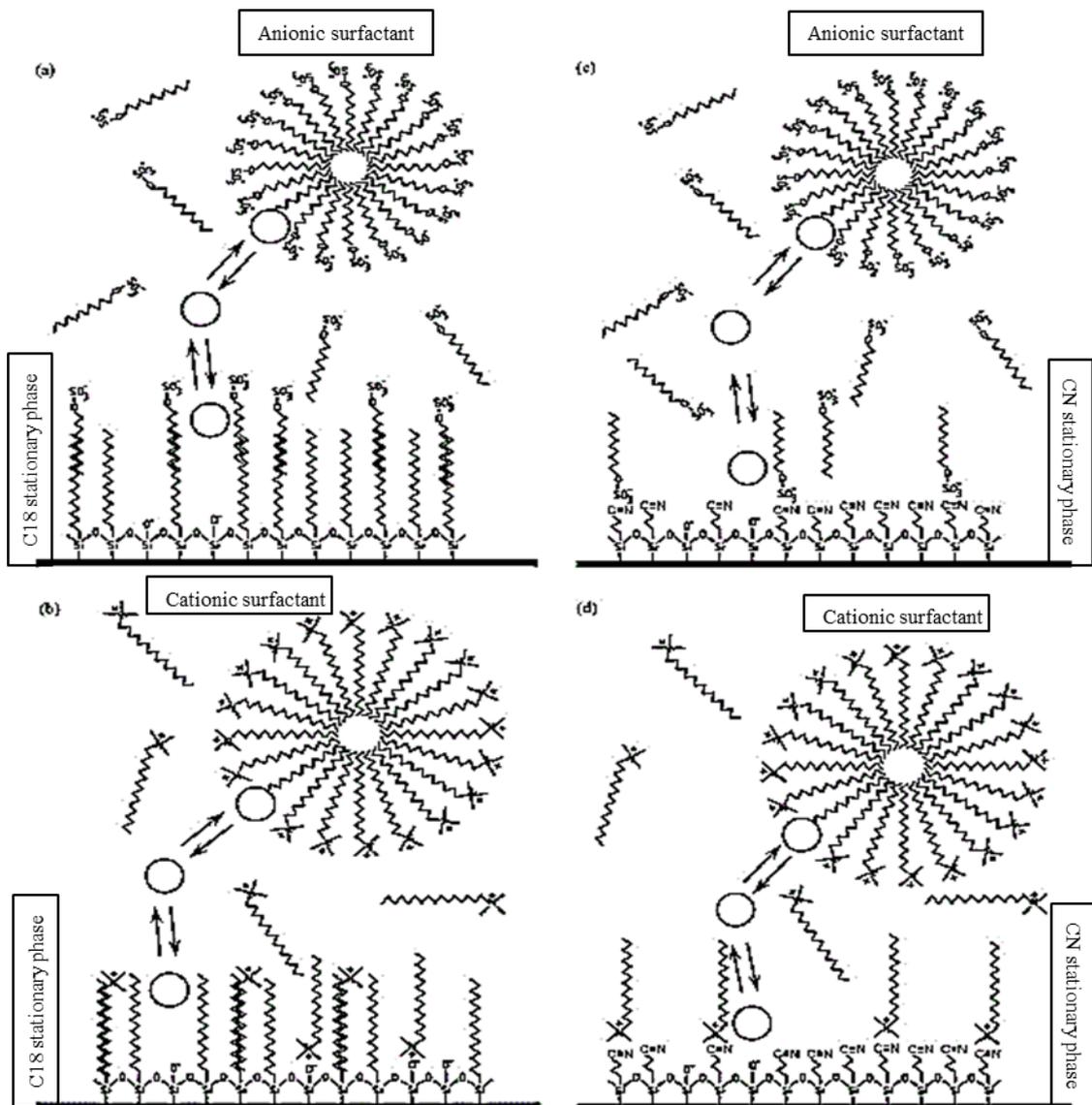


Figure 1.2: Solute environment in a chromatographic system using octadecyl-bonded (a & b) and cyanopropyl-bonded phases (c & d) with anionic SDS (a & c) and cationic CTAB (b & d) (Ruiz-Ángel et al., 2009)

1.5.3. Interaction of solute with micelle and stationary phase

The retention behaviour of compounds can be explained by the interactions between the solutes, the surfactant- modified stationary phase and the micelle. It has been reported that neutral solutes eluted with non-ionic and ionic surfactants, and charged solutes eluted with non-ionic surfactants will only be affected by non-polar, dipole-dipole and proton donor-acceptor interactions. However, charged solutes will interact electrostatically with ionic surfactants. Also, the steric effect can be an important parameter in such solute/surfactant interactions (Marina et al., 1996; Ruiz-Ángel et al., 2009).

In the case of electrostatic attractions, charged solutes can be strongly retained in MLC giving rise to a combination of attraction and hydrophobic interactions. Conversely, charged solutes cannot be retained by the stationary phase owing to electrostatic repulsion and elute at the dead volume, unless significant hydrophobic interactions with the modified stationary phase exist.

For most solutes, retention times decrease with increasing the surfactant concentration in MLC. However, those which interact sufficiently with the micelle, elution strength increases with increasing surfactant concentration. According to their elution behaviour with a micellar mobile phase, solutes have been classified into three categories: solutes binding to the micelle, non-binding and anti-binding solutes (Armstrong and Stine, 1983). Solutes that bind to micelles show decreased retention with increasing surfactant concentration in the mobile phase, whereas, for solutes having no interaction or binding with micelle (non-binding), the retention remains unaltered with respect to surfactant concentration. A less common interaction, namely anti-binding causes an increase in retention with increasing surfactant content in the micellar mobile phase. Electrostatic repulsion is an important issue in anti-binding behaviour, since anti-

binding solutes with anionic or cationic surfactants are negatively or positively charged, respectively. However, positively charged compounds eluted with a cationic surfactant and a negatively charged compound with an anionic surfactant may exhibit binding behaviour owing to hydrophobic interactions (Ruiz-Ángel et al., 2009).

Since ionic surfactants tend to adsorb on the C8 or C18 bonded phases (Figure 1.2a & b), the negatively charged layer for SDS and positively charged for CTAB, will elute at the dead volume owing to electrostatic repulsions. On the contrary, when using C1 or cyano-bonded phases (Figure 1.2c & d), which do not adsorb a large amount of surfactants, the anti-binding behaviour is observed. This could be a consequence of a compound being strongly excluded or repelled from the micelle, which forces the solute onto the stationary phase, where it is retained due to hydrophobic interactions. Armstrong and Stine studied the effect of ionic strength and behaviour of solutes from anti-binding to binding behaviour with a small change in the micellar environment which proved the electrostatic nature of the implied interactions (Armstrong and Stine, 1983). However, it seems that for the transition from anti-binding to binding, the hydrophobicity of the charged solute must be sufficiently high to associate with the non-polar portion of the micelle, once electrostatic repulsion has been minimised by salt addition (Ruiz-Ángel et al., 2009).

1.5.4. Armstrong's partitioning model

The partitioning process of compounds in MLC is much more complicated than that for HPLC because of the presence of three types of equilibria (Armstrong and Nome, 1981) i.e. between the stationary phase and water (P_{SW}), between the micelles and water (P_{MW}), and between micelles and the stationary phase (P_{SM}) as illustrated in Figure 1.3.

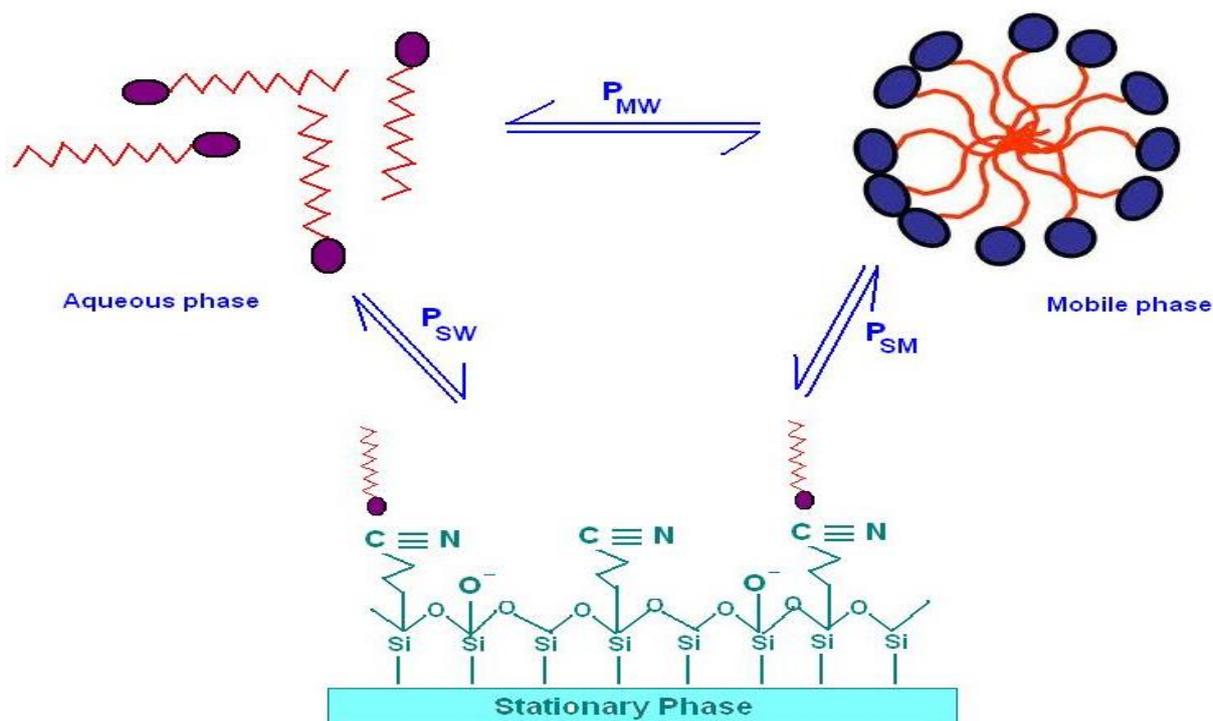


Figure 1.3: Three phase equilibria in micellar liquid chromatography; adapted from Armstrong and Nome (1981)

Armstrong and Nome (1981) derived the partition coefficients from the relationship of the capacity factor (k') with the concentration of micelles in the mobile phase. The capacity factor is derived from the retention times of solute (t_r) and the dead time (t_0) as expressed in Eq. (1.20):

$$k' = \frac{t_r - t_0}{t_0} \quad (1.20)$$

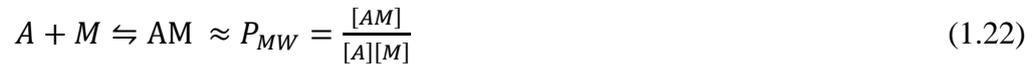
On the basis of three phase equilibria, Armstrong and Nome (Armstrong and Nome, 1981) proposed an equation which can possibly calculate micelle-water partition coefficients for solutes as expressed in Eq. (1.21):

$$\frac{1}{k'} = \left[\frac{v(P_{MW}-1)}{\phi \times P_{SW}} \right] \times C_M + \frac{1}{\phi \times P_{SW}} \quad (1.21)$$

where, k' is the capacity factor of solute, v is the molar volume of the surfactant, P_{MW} is the partition coefficient of solute between micelle and water, φ is the phase volume ratio (stationary phase volume/mobile phase volume), C_M is the micellar concentration of the surfactant (total surfactant concentration minus critical micellar concentration), and P_{SW} is the partition coefficient between water and stationary phase.

Unlike Armstrong and Nome, Arunyanart and Cline Love (Arunyanart and Cline Love, 1985) explained the three phase equilibria in terms of following equations:

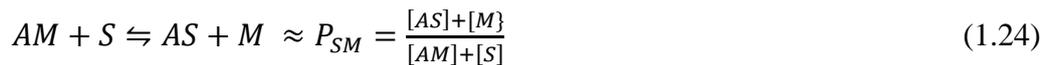
(1) Association of the solute (A) in the bulk aqueous phase with the micelle:



(2) Association of the solute in the bulk aqueous phase with the stationary phase:



(3) Direct transfer of solute in the micelle to stationary phase:



The magnitude of solute retention expressed as the capacity factor (k') is defined as (Arunyanart and Cline Love, 1985):

$$k' = \varphi \frac{[AS]}{[A]+[AM]} \quad (1.25)$$

Substituting equilibrium constants (P_{AM} and P_{SW}) in the Equation (1.25) gives:

$$k' = \frac{\varphi \times P_{SW}}{1 + P_{MW}[C_M]} \quad (1.26)$$

On taking inverse of the Eq. (1.26) and then rearranging it:

$$\frac{1}{k'} = \frac{P_{MW}}{\varphi \times P_{SW}} [C_M] + \frac{1}{\varphi \times P_{SW}} \quad (1.27)$$

Since molar volume of surfactant (v) remains constant with respect to surfactant concentration and can be neglected from Equation (1.21), which gives a similar expression as given by Equation (1.27). Experimental values for k' obtained at various surfactant concentrations can be used to plot a graph of $1/k'$ and C_M . From this linear relationship the ratio of the slope and intercept represents P_{MW} , while P_{SW} is calculated from the intercept. Furthermore, if P_{MW} and P_{SW} are known, the partition coefficient between stationary phase and micelle can be calculated using the relationship given in Eq. (1.28):

$$P_{SM} = \frac{P_{SW}}{P_{MW}} \quad (1.28)$$

The combined effects of these three equilibria would lead to the different elution patterns for different solutes. By the use of different micellar mobile phases, the capacity factor of a compound on a single stationary phase would result in different values of P_{SM} and P_{MW} but identical P_{SW} values. On the other hand, if the micellar mobile phase was constant, P_{MW} values will be the same for two different stationary phases but P_{SW} and P_{SM} would differ (Armstrong and Nome, 1981; Gago et al., 1987).

Armstrong's partitioning model has been validated by several research groups and some of them have proposed variations (Foley and May, 1987; Foley, 1990; Jandera and Fischer, 1996). Waters and co-workers (2007) have used the normalised value of the k' to evaluate micelle water partition coefficients for alkyl phthalate esters. More recently, Armstrong's model has been used to determine micelle-water partition coefficients (Waters et al., 2012b) as an alternative to the traditional octanol-water for a series of structurally and pharmacologically

similar pharmaceutical compounds from the class of non-steroidal anti-inflammatory drugs (NSAIDs). This will be discussed in detail in Chapter 4.

1.6. Applications of MLC

Micellar liquid chromatography has proved to be a versatile analytical technique with various applications. Since there is no need for a high purity of sample to be injected for analysis, direct injection of physiological fluid (for example, serum, plasma, urine) in MLC has made it a superior technique over other chromatographic approaches (Khaledi, 1997). This ensures rapid screening of samples in quick succession of time since sample preparation steps are far less than conventional chromatography where a high purity of sample is required. One such example is the direct injection of a urine sample into MLC which permits analysis of beta-blocker drugs with a shorter run time (Martínez et al., 1999).

MLC has been successfully applied as a separation technique for the analysis of pharmaceutical compounds and it has proved to be a better alternative of commonly used ion-pair chromatography (Nishi, 1997). Hydrophilic drugs, usually unretained in RP-HPLC, are often retained in MLC owing to ionic interactions with the charged surfactant molecules. Capella-Peiró and co-workers developed a rapid screening method for analysing corticosteroids in various pharmaceutical formulations using direct injection in MLC (Capella-Peiró et al., 2002). Similarly, other classes of drugs such as phenethylamines (Gil-Agustí et al., 2000), tetracycline (Caballero et al., 2002) and tricyclic antidepressants (Ruiz-Angel et al., 2003) have been successfully separated using MLC.

To this end, MLC will be explored in depth as a potential alternative to the traditional octanol-water system for acquiring partition coefficients for compounds of pharmaceutical interest. In particular, the effect of the mobile phase pH and experimental temperature on the partitioning process will be the focus of this study as these have never been investigated prior

to this study. This is an important consideration for MLC as it may ultimately expand, or potentially limit, its potential as an alternative technique to determine partition coefficients.

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Chapter 2: Summary of scientific literature, aim and objectives

By far, the most common method of determining a partition coefficient is the shake flask method using octanol and water as partitioning media. Hydrophobic compounds can cause practical difficulties in measuring the octanol-water partition coefficient and may not truly represent the biological system because of structural differences (Seeman, 1972). Lipophilicity as a molecular parameter encodes both polar and hydrophobic intermolecular forces. However, when expressed by partition coefficients measured in the traditional octanol-water system, lipophilicity fails to encode some important recognition forces, most notably ionic bonds, which are of particular importance when modeling the interaction of ionised compounds with membranes, therefore, un-reliable partitioning data for many solutes has been reported. In addition to these concerns, the traditional octanol-water partition coefficient determination procedure is tedious and time consuming and requires a high level of solute and solvent purity. Furthermore, the use of toxic organic solvent is another drawback of this technique (Hartmann and Schmitt, 2004; Khaledi and Breyer, 1989).

The traditional view of passive transport is that hydrophilic compounds (particularly ions) do not permeate into and across biological membranes. However, recent experimental evidence invalidates this assumption and documents the distribution of ion pairs and the passive transport of ionic species. Indeed, it is increasingly clear that the behavior of ionisable drugs in the body is controlled by the interaction of both their neutral and ionised forms with biological membranes. The importance of partitioning of ionic and zwitterionic species in biphasic media

has been emphasised in connection with their pharmacokinetic and pharmacodynamic behaviour, and hydrophobic as well as electrostatic interactions are expected between biological membranes and ionised compounds (Taillardat-Bertschinger et al., 2003).

The major goal for physicochemical screening is to predict human drug absorption, distribution, elimination, excretion and toxicity and these are all dependent on the lipophilicity. There is a clear necessity to replace the octanol-water partitioning process with a relatively more suitable process which can possibly mimic the biological partitioning process of pharmaceuticals and can generate more reliable data in a shorter period of time. More recently micellar liquid chromatography (MLC) based partition coefficients for pharmaceutical compounds have been reported (Waters and Kasprzyk-Hordern, 2010) as a reliable alternative to octanol-water. This technique utilises an aqueous mobile phase containing surfactants over a certain concentration where these surfactants exist in the form of aggregates known as micelles. Micelles partially resemble biological membranes owing to the presence of the hydrophilic head and a hydrophobic tail, therefore, MLC is a potential alternative to the traditional octanol-water system.

2.1. Aims

The aim of this research is to investigate and refine a simple method to acquire partitioning and thermodynamic data associated with the partitioning process for pharmaceutical compounds. Furthermore, the partitioning data acquired in this research will be exploited in order to evaluate its biological relevance for transdermal drug delivery systems.

2.2. Objectives

The specific objectives to support the aims of this thesis are as follows:

1. To develop a fast and reliable method of partition coefficient determination for pharmaceuticals which can mimic biological partitioning
2. To study the influence of compounds used in this study on the size and shape of micelles
3. To study the effect of pH on partition coefficient determination by micellar liquid chromatography
4. To study the effect of temperature on partition coefficient determination by micellar liquid chromatography
5. To compare and evaluate the partitioning data obtained as an alternative method to predict skin permeability

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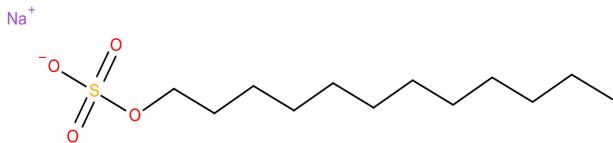
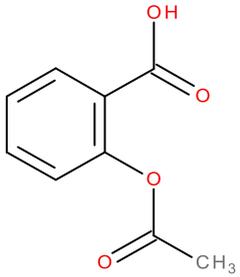
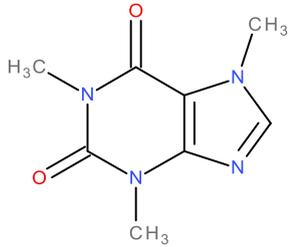
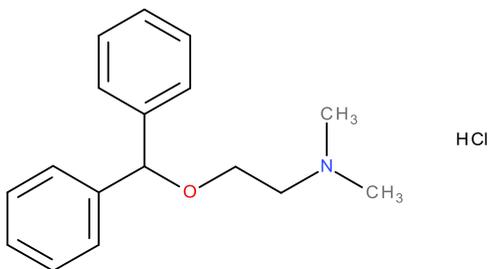
Chapter 3: Materials and Methods

3.1. Chemicals

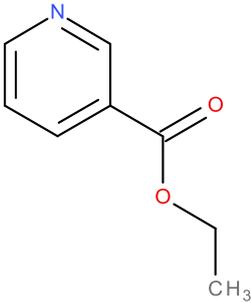
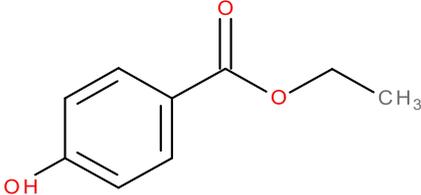
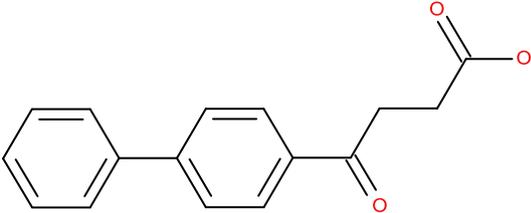
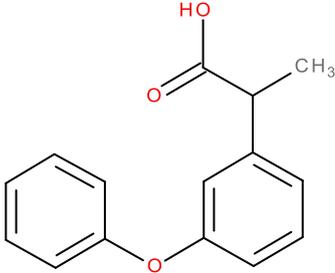
Sodium dodecyl sulfate (SDS), electrophoresis grade, was purchased from Fisher Scientific (Loughborough, UK) with a minimum purity of 99 %. Test solutes were: acetylsalicylic acid 99 % (Acros Organics, Geel, Belgium), caffeine 97 % (Sigma-Aldrich, Dorset, UK), diphenhydramine hydrochloride 99 % (Acros Organics, Geel, Belgium), ethyl nicotinate 98 % (Sigma-Aldrich, Dorset, UK), ethyl paraben 98 % (Sigma-Aldrich, Dorset, UK), fenbufen 99 % (TCI, Oxford, UK), fenoprofen 99 % (TCI, Oxford, UK), flurbiprofen 99 % (TCI, Oxford, UK), haloperidol 99 % (Sigma-Aldrich, Dorset, UK), hexyl nicotinate (TCI, Oxford, UK), ibuprofen 99 % (BASF, Cheshire, UK), indomethacin 99 % (Sigma-Aldrich, Dorset, UK), ketoprofen 99 % (TCI, Oxford, UK) lidocaine 98 % (Sigma-Aldrich, Dorset, UK), methyl nicotinate (Fluka, Dorset, UK), methyl paraben 98 % (Sigma-Aldrich, Dorset, UK), naproxen 99 % (Sigma-Aldrich, Dorset, UK), nicotinic acid (Sigma-Aldrich, Dorset, UK), paracetamol 99 % (Sigma-Aldrich, Dorset, UK), procaine hydrochloride 97 % (Sigma-Aldrich, Dorset, UK), salicylic acid 98 % (Fisher, Loughborough, UK), thiourea 97 % (Vickers Lab. Ltd., Pudsey, UK) and urea 97 % (Vickers Lab. Ltd., Pudsey, UK) were used as received. De-ionised water was used throughout the experiments.

The structure of chemicals along with their physicochemical properties is presented in Table 3.1.

Table 3.1: Physicochemical properties of chemicals

Sodium Dodecyl Sulfate		Structure
Molecular weight ¹	288.4 g/mol	
Charge	Anionic	
CMC	8 mM	
AN	62	
Micellar shape	Spherical	
CAS no.	151-21-3	
Acetylsalicylic acid		
Molecular weight ¹	180.2 g/mol	
pK _a ¹	3.5	
LogP _{ow} ²	1.19	
Charge	Anionic	
λ _{max} ³	278 nm	
CAS no.	50-78-2	
Caffeine		
Molecular weight ¹	194.2 g/mol	
pK _a ¹	0.52	
LogP _{ow} ²	-0.07	
Charge	Neutral	
λ _{max} ³	254 nm	
CAS no.	58-08-2	
Diphenhydramine hydrochloride		
Molecular weight ¹	206.3 g/mol	
pK _a ¹	4.41	
LogP _{ow} ²	3.6	
Charge	Cationic	
λ _{max} ³	221 nm	
CAS no.	15687-27-1	

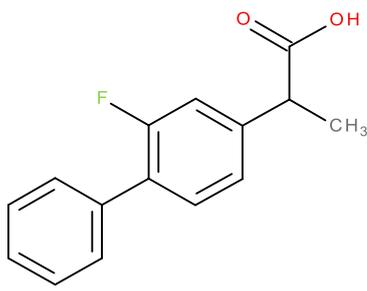
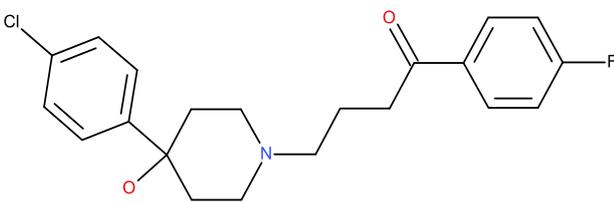
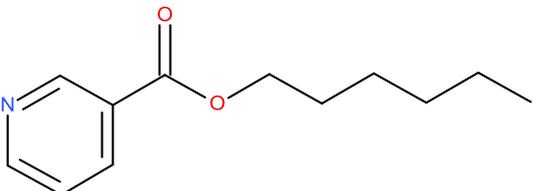
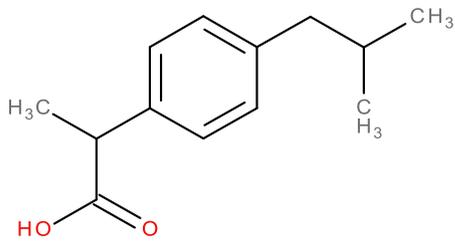
¹ Molecular weight and pK_a (dissociation constant) were generated from ACD/Labs, RSC, UK² LogP_{ow} from literatures (Waters et al., 2012, Waters et al., 2013)³ Taken from Clark's drug analysis

Ethyl nicotinate		Structure
Molecular weight ¹	151.2 g/mol	
pK _a ¹	3.3	
LogP _{ow} ²	1.3	
Charge	Cationic	
λ _{max} ³	261 nm	
CAS no.	614-18-6	
Ethyl paraben		
Molecular weight ¹	166.2 g/mol	
pK _a ¹	8.3	
LogP _{ow} ²	2.47	
Charge	Anionic	
λ _{max} ³	254 nm	
CAS no.	120-47-8	
Fenbufen		
Molecular weight ¹	254.3 g/mol	
pK _a ¹	4.55	
LogP _{ow} ²	3.2	
Charge	Anionic	
λ _{max} ³	285 nm	
CAS no.	36330-85-5	
Fenoprofen		
Molecular weight ¹	242.3 g/mol	
pK _a ¹	4.2	
LogP _{ow} ²	3.45	
Charge	Anionic	
λ _{max} ³	272 nm	
CAS no.	53746-45-5	

¹ Molecular weight and pK_a (dissociation constant) were generated from ACD/Labs, RSC, UK

² LogP_{ow} from literatures (Waters et al., 2012, Waters et al., 2013)

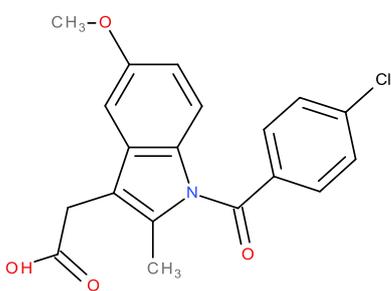
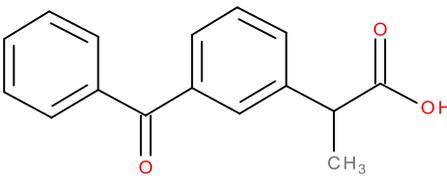
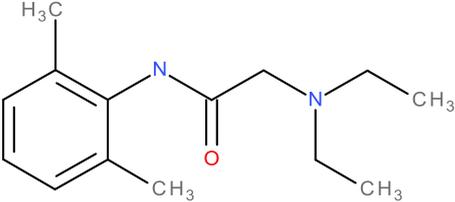
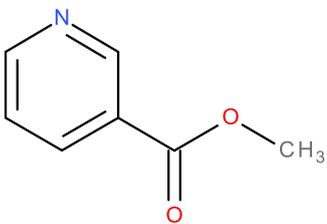
³ Taken from Clark's drug analysis

Flurbiprofen		Structure
Molecular weight ¹	244.3 g/mol	
pK _a ¹	4.14	
LogP _{ow} ²	3.7	
Charge	Anionic	
λ _{max} ³	247 nm	
CAS no.	5104-49-4	
Haloperidol		
Molecular weight ¹	357.9 g/mol	
pK _a ¹	8.3	
LogP _{ow} ²	3.49	
Charge	Neutral	
λ _{max} ³	245 nm	
CAS no.	52-86-8	
Hexyl nicotinate		
Molecular weight ¹	207.3 g/mol	
pK _a ¹	3.21	
LogP _{ow} ²	3.5	
Charge	Cationic	
λ _{max} ³	260 nm	
CAS no.	23597-82-2	
Ibuprofen		
Molecular weight ¹	206.3 g/mol	
pK _a ¹	4.41	
LogP _{ow} ²	3.6	
Charge	Anionic	
λ _{max} ³	221 nm	
CAS no.	15687-27-1	

¹ Molecular weight and pK_a (dissociation constant) were generated from ACD/Labs, RSC, UK

² LogP_{ow} from literatures (Waters et al., 2012, Waters et al., 2013)

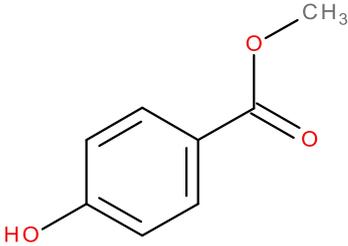
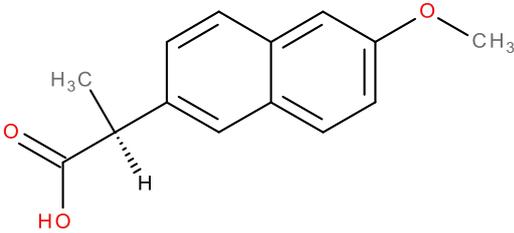
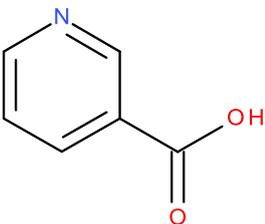
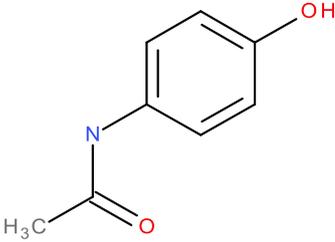
³ Taken from Clark's drug analysis

Indomethacin		Structure
Molecular weight ¹	357.8 g/mol	
pK _a ¹	4.18	
LogP _{ow} ²	3.51	
Charge	Anionic	
λ _{max} ³	230 nm	
CAS no.	53-86-1	
Ketoprofen		
Molecular weight ¹	254.3 g/mol	
pK _a ¹	4.23	
LogP _{ow} ²	2.7	
Charge	Anionic	
λ _{max} ³	262 nm	
CAS no.	22071-15-4	
Lidocaine		
Molecular weight ¹	234.3 g/mol	
pK _a ¹	7.9	
LogP _{ow} ²	2.3	
Charge	Cationic	
λ _{max} ³	220 nm	
CAS no.	137-58-6	
Methyl nicotinate		
Molecular weight ¹	137.1 g/mol	
pK _a ¹	3.19	
LogP _{ow} ²	0.8	
Charge	Cationic	
λ _{max} ³	264 nm	
CAS no.	93-60-7	

¹ Molecular weight and pK_a (dissociation constant) were generated from ACD/Labs, RSC, UK

² LogP_{ow} from literatures (Waters et al., 2012, Waters et al., 2013)

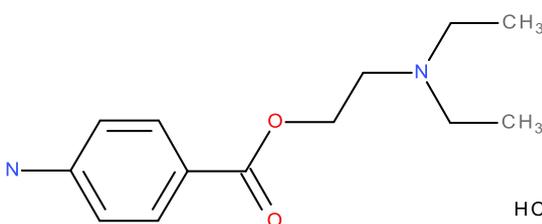
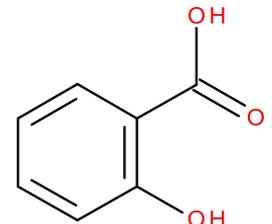
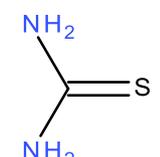
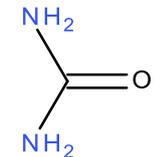
³ Taken from Clark's drug analysis

Methyl paraben		Structure
Molecular weight ¹	152.1 g/mol	
pK _a ¹	8.4	
LogP _{ow} ²	1.96	
Charge	Anionic	
λ _{max} ³	257 nm	
CAS no.	99-76-3	
Naproxen		
Molecular weight ¹	230.3 g/mol	
pK _a ¹	4.8	
LogP _{ow} ²	3.2	
Charge	Anionic	
λ _{max} ³	262 nm	
CAS no.	22204-53-1	
Nicotinic acid		
Molecular weight ¹	123.1 g/mol	
pK _a ¹	4.8	
LogP _{ow} ²	0.4	
Charge	Anionic	
λ _{max} ³	263 nm	
CAS no.	59-67-6	
Paracetamol		
Molecular weight ¹	151.2 g/mol	
pK _a ¹	9.8	
LogP _{ow} ²	0.5	
Charge	Neutral	
λ _{max} ³	243 nm	
CAS no.	103-90-2	

¹ Molecular weight and pK_a (dissociation constant) were generated from ACD/Labs, RSC, UK

² LogP_{ow} from literatures (Waters et al., 2012, Waters et al., 2013)

³ Taken from Clark's drug analysis

Procaine hydrochloride		Structure
Molecular weight ¹	236.3 g/mol	
pK _a ¹	9.0	
LogP _{ow} ²	2.2	
Charge	Cationic	
λ _{max} ³	294 nm	
CAS no.	51-05-8	
Salicylic acid		
Molecular weight ¹	138.1 g/mol	
pK _a ¹	13.06	
LogP _{ow} ²	2.26	
Charge	Anionic	
λ _{max} ³	236/298 nm	
CAS no.	69-72-7	
Thiourea		
Molecular weight ¹	76.1 g/mol	
pK _a ¹	13.0	
LogP _{ow} ²	-0.95	
Charge	Neutral	
λ _{max} ³	238 nm	
CAS no.	62-56-6	
Urea		
Molecular weight ¹	60.1 g/mol	
pK _a ¹	14.3	
LogP _{ow} ²	-2.11	
Charge	Neutral	
λ _{max} ³	280 nm	
CAS no.	57-13-6	

¹ Molecular weight and pK_a (dissociation constant) were generated from ACD/Labs, RSC, UK

² LogP_{ow} from literatures (Waters et al., 2012, Waters et al., 2013)

³ Taken from Clark's drug analysis

3.2. Instrumentation

The HPLC system comprised of a Severn Analytical solvent delivery system (SA6410B). A Rheodyne injector with 20 μL loop volume was attached for sample injection. Separation of solutes was performed using a reverse phase cyanopropyl bonded stationary phase column (spherisorb 5 μL , 15 cm \times 4.6 mm i.d). The temperature of the column was maintained using a Jones Chromatography column heater and chiller (Model 7950). A UV detector (Waters UV/Vis, 2487) was attached with the HPLC system for the detection of eluents.

3.3. Mobile phase

The mobile phase was prepared by dissolving an accurately weighed amount of SDS in de-ionised water over a concentration range of 0.01 – 0.03 M. The mobile phase was sonicated to ensure removal of air bubbles prior to the start of experiments. Mobile phase was freshly prepared on a daily basis and the pH of the mobile phase was measured with a pre-calibrated pH meter (Jenway 3305, UK).

3.4. Measurement of dead time (t_o)

An important point in MLC is the determination of dead time (t_o), which is the time taken by the solvent front to reach the detector. In literature, various procedures have been documented that describe the measurement of dead time including injection of water (Pramauro et al., 1988), salt solutions such as NaNO_3 (Borgerding et al., 1989), sodium or potassium iodide (Pramauro and Pelizzetti, 1983), or organic solvents such as methanol or acetonitrile (Khaledi et al., 1987; Khaledi, 1988). The criteria described in the literature to locate the dead time uses the position of the maximum or minimum perturbation in the chromatogram, i.e. the time from the injection to the appearance of the first deviation in the base line. The use of small

compounds like sodium or potassium iodide is based on the assumption that they will not interact with the stationary phase and their appearance at the detector reflects the dead time. However, these methods remain questionable for assessing the real dead times, although the measured times were proven suitable for this purpose (Torres-Lapasió et al., 1996).

In this work, dead time was measured by either injecting water or acetonitrile (20 μ L) through the column and the time to the first major deviation recorded as exemplified in Figure 3.1. This procedure was repeated for all surfactant concentrations (0.01 – 0.03 M) with at least ten replicates to get a reliable value for dead time. The average t_o value was 50 seconds which was used in all experiments for the determination of capacity factor k' using Equation 3.

It should be noted that a new UV detector was installed on the HPLC system during this study which resulted in an alteration in the length of the HPLC tubes. Therefore, it was necessary to determine the dead time value for that system. The resultant dead time was recorded to be 20 seconds which was used for the calculation of capacity factors.

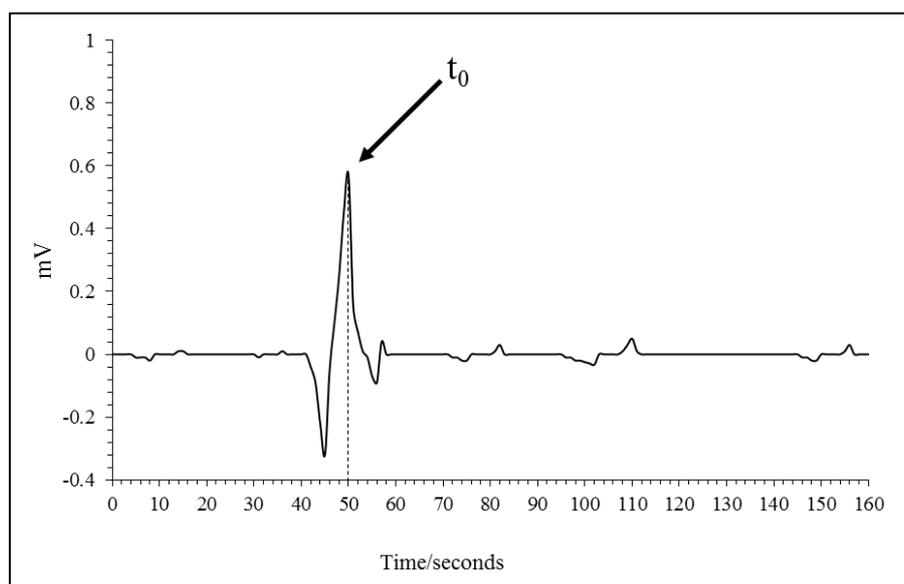


Figure 3.1: Dead time of the column at 0.03 M surfactant concentration

3.5. Small angle X-ray scattering (SAXS) measurements

Micelle-water partition coefficients measured through MLC depend on the geometry of micelles, therefore, it was necessary to investigate the effect of added drug on the size of micelles in the studied range of surfactant concentration.

Two-dimensional small-angle X-ray scattering (2D-SAXS) patterns were measured using a Nanostar camera (Bruker). This apparatus was fitted with an air-cooled microbeam X-ray generator, operated at 50 kV and 600 μ A, producing Cu K α radiation (wavelength, $\lambda_x = 0.15418$ nm). The scattered light was collected using a Vantec 2000 detector, which resolved the intensity patterns into 2048×2048 pixels. A lead beamstop was suspended in front of the detector, to block the direct (i.e. unscattered) light. The sample-to-detector distance was 0.678 m, as determined by calibration against a silver behenate reference, corresponding to a range of the scattering angle (2θ) of roughly 0 to 5°.

Samples consisting of 0.05 M sodium dodecyl sulfate (SDS) in water were held in quartz capillaries (ca. 1.5 mm internal diameter). This was compared with samples containing 0.2 mM ibuprofen (anionic), paracetamol (neutral) and lidocaine (cationic). The maximum working concentration of SDS in MLC was 0.03 M which did not give any significant scattering using Nanostar, therefore, for the proof of concept a concentration of 0.05 M was selected. Measurements were performed at an ambient temperature (ca. 25 °C inside the sample chamber). To prevent leakage, the sample chamber was maintained at atmospheric pressure, with mylar windows to separate it from the rest of the optical path, which was held under vacuum. Acquisition times of 12 hours were used and samples were measured in duplicate, to further improve the signal-to-noise ratio.

‘Background’ scattering due to the sample capillaries, air in the sample chamber, the X-ray windows and any other elements of the apparatus was determined using a blank solution

consisting of 0.005 M SDS solution (i.e. below the critical micelle concentration). The resulting intensity patterns were subtracted from the SAXS data, after suitable scaling, to allow for sample absorbance.

Since the scattering patterns were circularly symmetrical, the intensity maps were resolved into radial plots of intensity against scattering angle, by performing azimuthal integrations, using the SAXS for Windows software provided. The resulting intensity curves were plotted against the modulus of the scattering vector, which is defined as:

$$q = \frac{4\pi}{\lambda_x} \sin \theta \quad (3.1)$$

where q is the scattering vector, λ is the radiation wavelength and θ is the scattering angle.

The micelle structures were analysed by fitting an appropriate model to the scattering data for each SDS concentration, using the ‘Solver’ tool in Excel (Microsoft). It was assumed that the electron densities in the micelle core (assumed to be pure hydrocarbon) and the continuous liquid medium (assumed to be pure water) remained constant, while the average size of the micelle, the thickness and composition of its surface layer, the thickness of a further bound layer and an overall intensity factor were allowed to change, in order to fit the model to the data.

3.6. Chromatographic procedure

Mobile phase was pumped through the chromatographic system using the solvent delivery system (SA 6410B) with a flow rate of 1.35 mL per minute. Each 0.2 mM drug sample was prepared in an aqueous solution of SDS equivalent to the mobile phase concentration. The concentration of drug in each sample was kept to a minimum to avoid saturation of the column with the drug and to refute the chances of any change in the micelle size and geometry. Each

20 μL sample was injected via the Rheodyne injector into the system. UV detection was set at a specified wavelength (λ_{max}) for each drug as given in Table 3.1.

Each run was repeated six times to achieve statistically significant retention time (t_r) data. After every sixth run, the mobile phase was diluted to the next specified concentration using de-ionised water. The system was left for 25-30 minutes for equilibrium to be attained and repeated for all specified mobile phase concentrations. From the data obtained, capacity factors were calculated using Eq. (1.2). The partition coefficient was measured by plotting the inverse of the capacity factor ($1/k'$) against the micelle concentration (C_M) where C_M is the micelle concentration obtained by subtracting the CMC from the total surfactant concentration.

3.6.1. pH controlled experiments

Experiments were conducted at a controlled pH of the mobile phase over a range from 3.0 to 7.0 (i.e. the operational pH range for cyanopropyl column) values. The pH of the mobile phase was adjusted using a 0.1 M phosphate buffer system. All the samples were prepared in the mobile phase of respective pH. When necessary, an acidic pH was achieved by adding 10 % H_3PO_4 .

For skin permeability predictions (Chapter 7), chromatographic data was collected at a mobile phase pH of 5.5 at $35 \pm 1^\circ\text{C}$ in order to mimic the skin pH conditions.

3.6.2. Temperature controlled experiments

Temperature of the mobile phase and sample was controlled by immersion in a thermostatic water bath maintained at the same temperature as that of the stationary phase. A Jones column heater was used to systematically maintain the column temperature. Retention data was obtained over a range of temperatures (295 K – 317 K) to allow determination of the thermodynamics of the partitioning process. The experimental P_{MW} was used to calculate the

change in the Gibb's free energy (ΔG) as described by Eq. (1.18). Thermodynamic data was obtained by applying the van't Hoff isochore which may permit the calculation of a change in enthalpy (ΔH) and entropy (ΔS) of the partitioning process from the slopes and intercepts of the plots.

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Chapter 4: Determination of chromatographic based partition coefficients for pharmaceutical compounds

This chapter highlights the measurement of partition coefficients and the mechanisms behind the retention of compounds in micellar liquid chromatography (MLC). The impact of physicochemical properties of the compounds on their retention on the stationary phase and interaction with the micelles will be explored in depth. Micelle-water partition coefficients will be determined using Armstrong's partitioning model and values will be compared with those from literature based on other techniques.

4.1. Introduction

This research uses MLC to determine micelle-water partition coefficients for compounds of pharmaceutical interest as an alternative to traditional methods. The unique selectivity of such systems is a result of the micellar core, which offers a hydrophobic site of interaction for non-polar drugs in an aqueous phase. This 'protection' is based on the association of the solute with the micelle through a combination of factors including adsorption, electrostatic, and steric interactions (Armstrong and Nome, 1981; Berthod et al., 1986a). Micelles in aqueous solutions offer various microenvironments: the hydrophobic core, the head group region in contact with water and an aqueous region outside the micelle, thus, closely mimicking biomembranes (Ingram et al., 2013). This creates a unique situation in which solutes can occupy various locations inside the micelle or on the surface of micelle and face various polarities in the given micellar mobile phase (Khaledi and Breyer, 1989). Therefore, retention behaviour in micellar liquid chromatography is dissimilar to that with more conventional hydro-organic mobile phases, such as those currently used in the pharmaceutical industry.

Utilisation of micelles in MLC for partition coefficient determination has recently been considered as a superior and potential alternative technique to those used for determining traditional octanol-water partition coefficients (Waters and Kasprzyk-Hordern, 2010; Waters et al., 2007). MLC accounts for three types of partition coefficients, as described in Chapter One, which makes MLC a technique that has the ability to quickly, precisely and unequivocally evaluate the partition coefficients of solutes between micelle and water (P_{MW}) for a chosen surfactant system.

Most of the previous reports for determining solutes in MLC make use of a micellar mobile phase containing an organic modifier, usually a short chain alcohol, to improve the chromatographic efficiency (García-Alvarez-Coque et al., 1997). However, the presence of an organic modifier in the micellar mobile phase may alter the CMC, AN and polarity of the aqueous phase which results in an additional interaction with the solute. This study has involved the development of a method that does not require an organic modifier, thus overcoming this issue.

It is widely accepted that surfactant adsorption onto porous silica based columns drastically affects the chromatographic retention, owing to the surface properties of the stationary phase. The decreasing order of surfactant adsorption on various stationary phases of different polarities as reported by Berthod and co-workers (1986a; 1986b) is octadecyl (C18), octyl (C8), methyl (C1), cyanopropyl (CN) and bare silica. Thus, the more hydrophobic the stationary phase, the more surfactant will be adsorbed, i.e. adsorption of surfactants on CN based columns is lower than C8 and C18 columns. Therefore, it is possible to state that the distribution of solute between the aqueous phase and micelles (P_{MW}) would largely represent the partitioning behaviour of solutes if a CN based column is used. Additionally, the nature of interactions that are established between solutes, surfactant-modified stationary phases and micelles also influences the partitioning behaviour. On the basis of these interactions, solutes

either bind to the micelle (binding solutes), do not bind to the micelle (non-bonding) or are repelled by the micelle (anti-binding) (Armstrong and Stine, 1983).

It should be noted that there are certain assumptions applied to the Armstrong model that describe the retention of the solutes in MLC (Ruiz-Ángel et al., 2009):

- 1- The aggregation number and geometry of micelles is not altered with increasing concentration of surfactants.
- 2- The CMC is not altered with the addition of small amounts of solutes in the chromatographic system
- 3- Binding of surfactant to the stationary phase reaches saturation level at, or before, the CMC of the surfactant is reached.
- 4- The stoichiometry of the solute-micelle complex is 1:1.

In this set of experiments, a CN based reverse phase column was employed in an attempt to take advantage of a reduced interaction between the stationary phase and the twelve carbon containing surfactant, namely SDS for the determination of partition coefficients of selected compounds.

4.2. Results and discussion

4.2.1. Small angle X-ray scattering (SAXS) measurements

Small-angle X-ray scattering is a powerful tool for determining the structure of colloidal particles, macromolecules and macroions in solution. Particularly in the case of micelles, this method can in principal measure parameters such as the micelle geometry (size and shape) and extent of water penetration. Geometry of micelle, especially those formed from surfactant monomers, depends upon various factors including added drug molecules or salt to

the surfactant solution. In this study, the effect of added drug (cationic, neutral and anionic) on the size of micelles was studied using SAXS.

SAXS patterns for 0.05 M SDS solutions with and without drugs are presented in Figure 4.1. All the SAXS patterns shown a peak at about $q = 1.7 \text{ nm}^{-1}$, characteristic of the intramicellar form factor (Itri and Amaral, 1993). The presence of a second inner peak around $q = 0.4 \text{ nm}^{-1}$ is a fingerprint of an interference function due to the interaction between charged micelles (Itri and Amaral, 1993). However, there was no difference observed in these peaks in the presence of drugs (Figure 4.1), confirming a stable geometry of the micelle with the addition of anionic, neutral and cationic drugs. Since the scattering patterns were circularly symmetrical (not shown here), the shape of the micelles was deemed spherical at the studied surfactant concentration range with and without the addition of drugs.

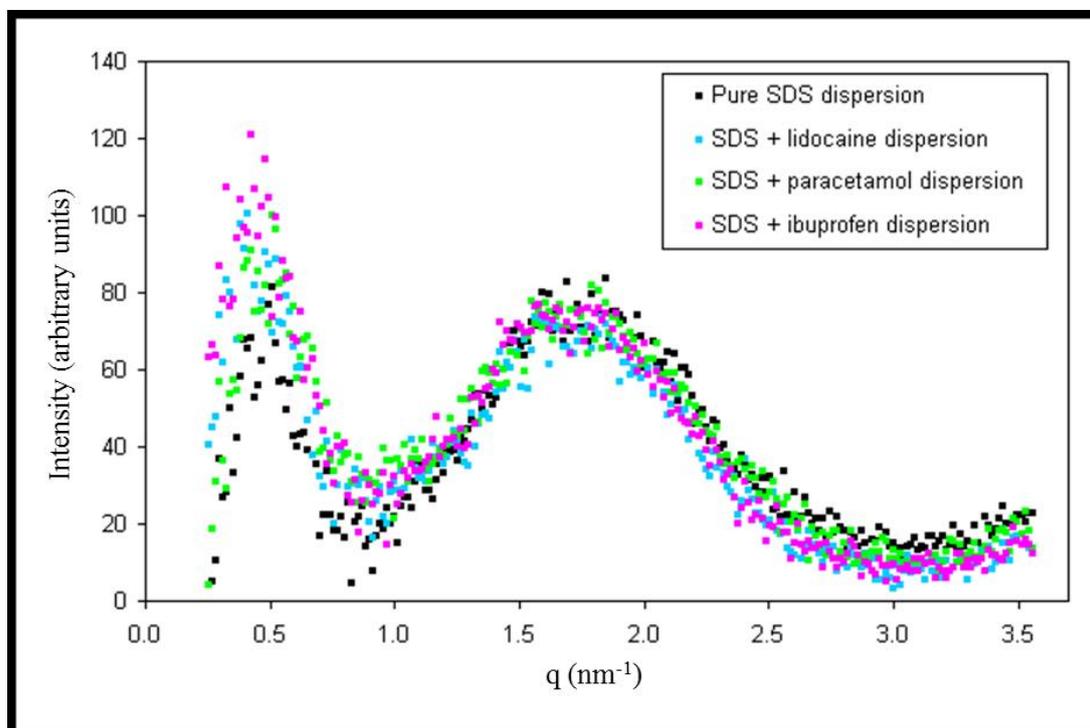


Figure 4.1: Plots of intensity vs. modulus of the scattering vector, 0.05 M SDS solutions

4.2.2. Micelle-water partition coefficients

Micelle-water partition coefficients were determined for seven drugs using SDS and MLC. An accurate determination of P_{MW} requires a precise modelling of retention times especially when the efficiencies achieved are rather low. The retention model in MLC relates a solutes capacity factor with the mobile phase composition, thus avoiding the dependency of the model on the flow rate of mobile phase. However, the capacity factor is prone to errors if the dead time is unreliable. Torres-Lapasió and co-workers (Torres-Lapasió et al., 1996) have described the influence of dead time on the prediction of capacity factors for five different compounds. According to their findings, the end results were severely affected by the value taken for the dead time and indicated large errors in the determination of physicochemical properties, for instance the association constants between micelles and the solutes. Therefore, a precise dead time measurement was necessary before proceeding with the MLC based partition coefficients measurement. The mean value for the dead time at all surfactant concentrations was 50 seconds or 20 seconds as explained earlier (see section 3.4).

To cover a broad range of solutes, anionic drugs (ibuprofen and ketoprofen), cationic drugs (diphenhydramine, lidocaine and procaine) and neutral drugs (paracetamol and caffeine) were chosen for an evaluation of MLC to determine partition coefficients. The chromatograms of the seven drugs considered in this study (at various surfactant concentrations) are presented in Figures 4.2 – 4.8.

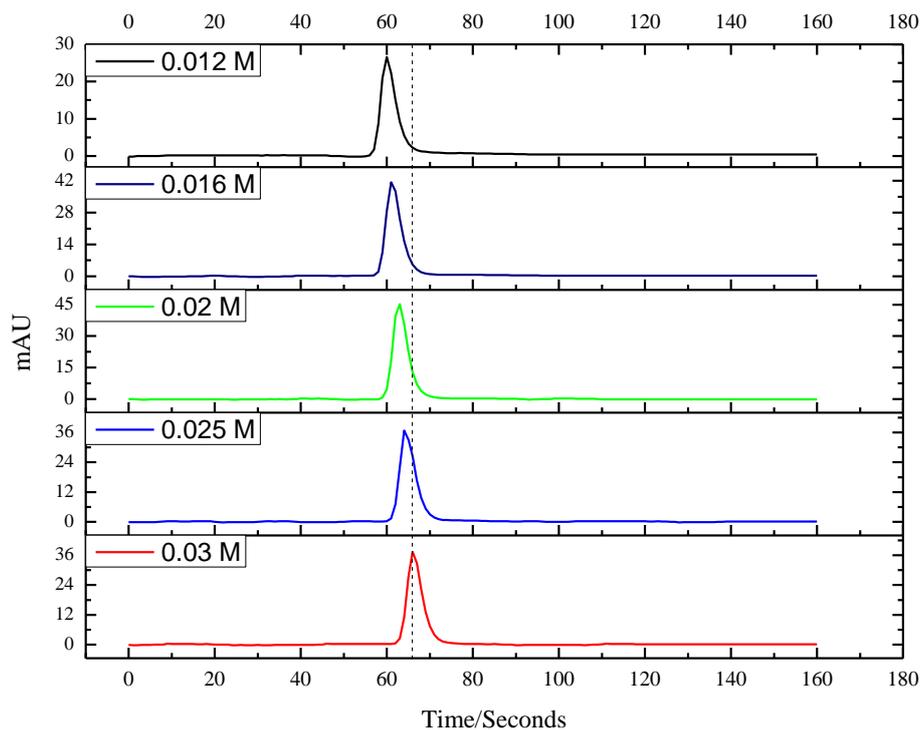


Figure 4.2: Chromatograms of ibuprofen at room temperature obtained using MLC

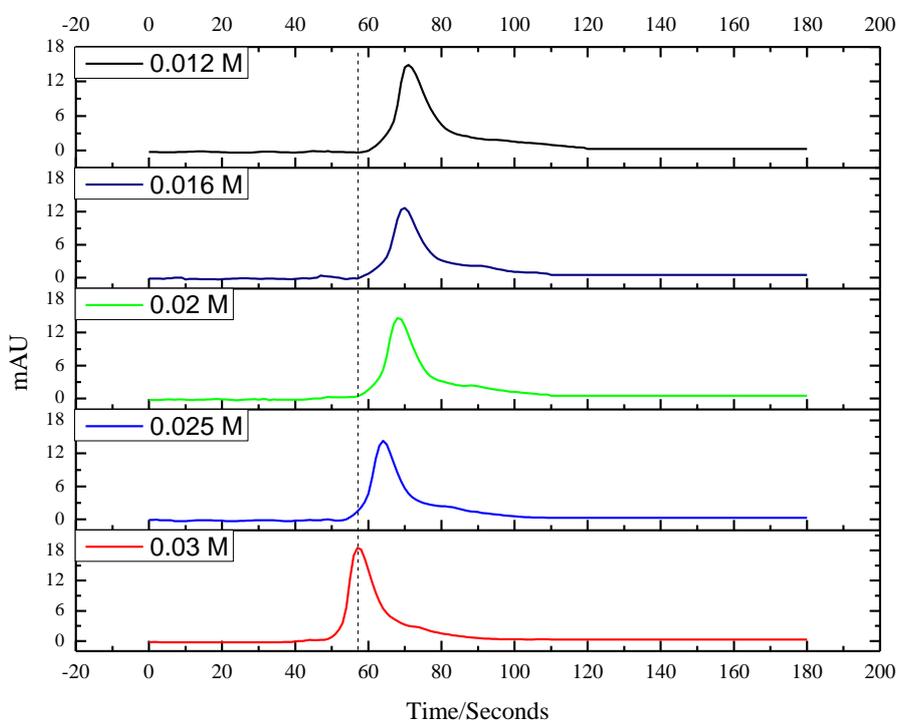


Figure 4.3: Chromatograms of ketoprofen at room temperature obtained using MLC

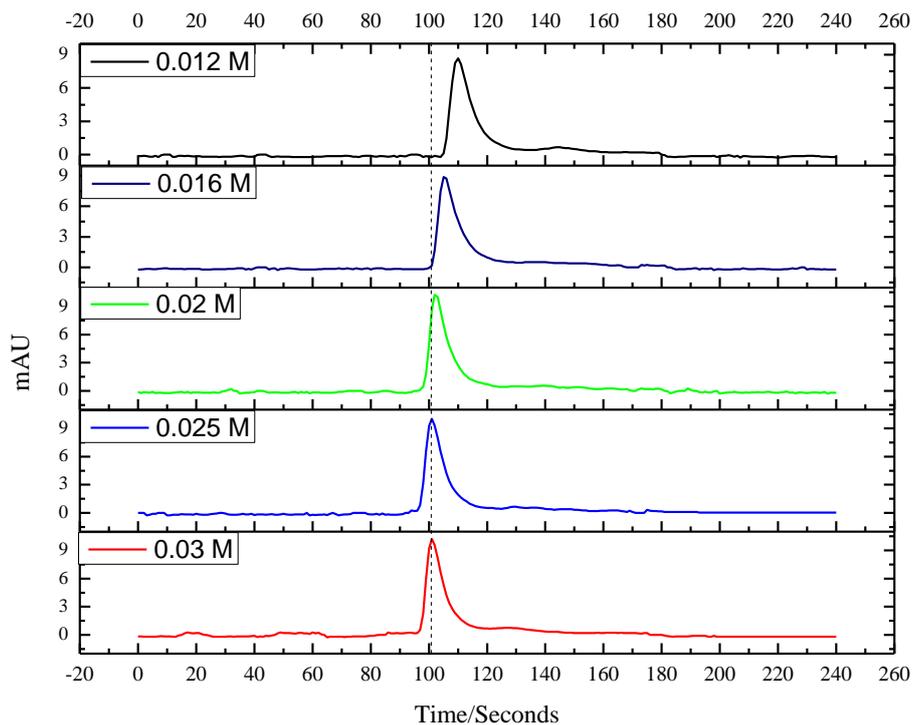


Figure 4.4: Chromatograms of caffeine at room temperature obtained using MLC

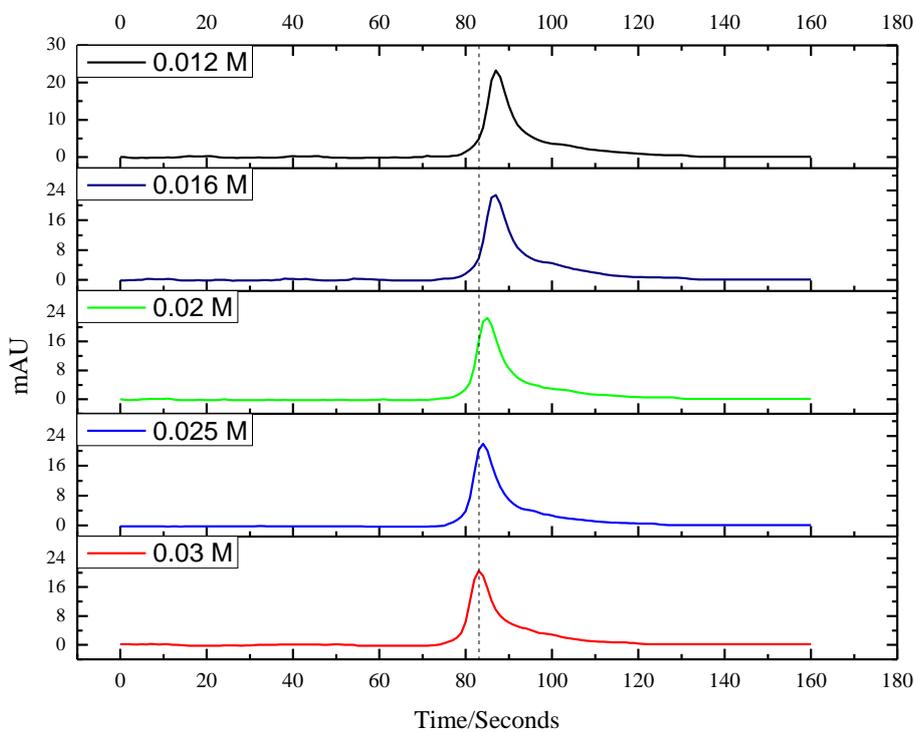


Figure 4.5: Chromatograms of paracetamol at room temperature obtained using MLC

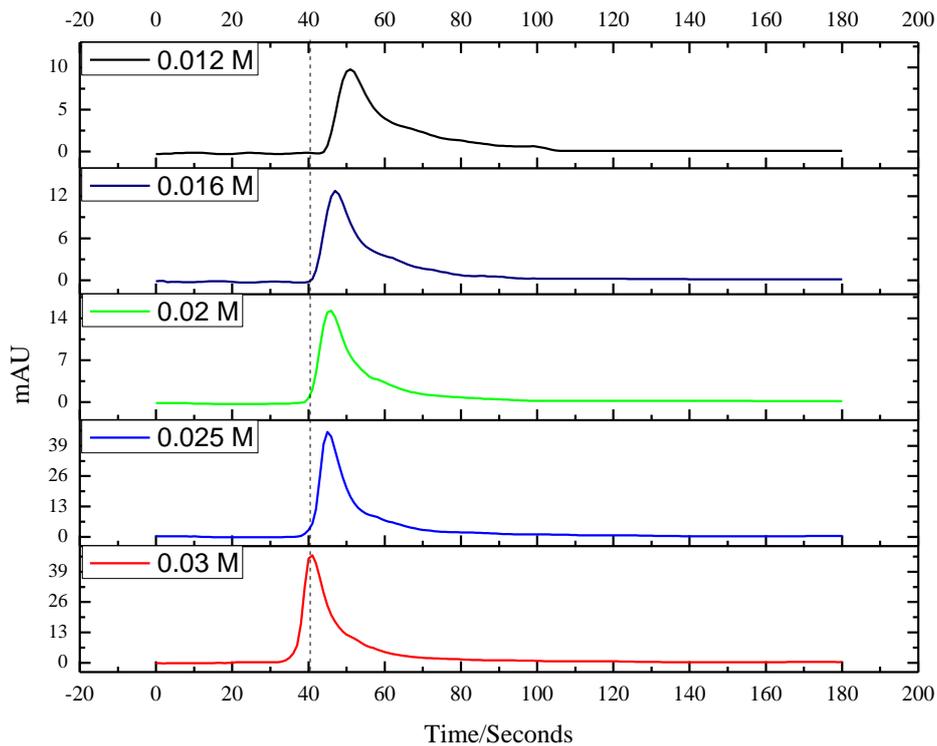


Figure 4.6: Chromatograms of diphenhydramine at room temperature obtained using MLC

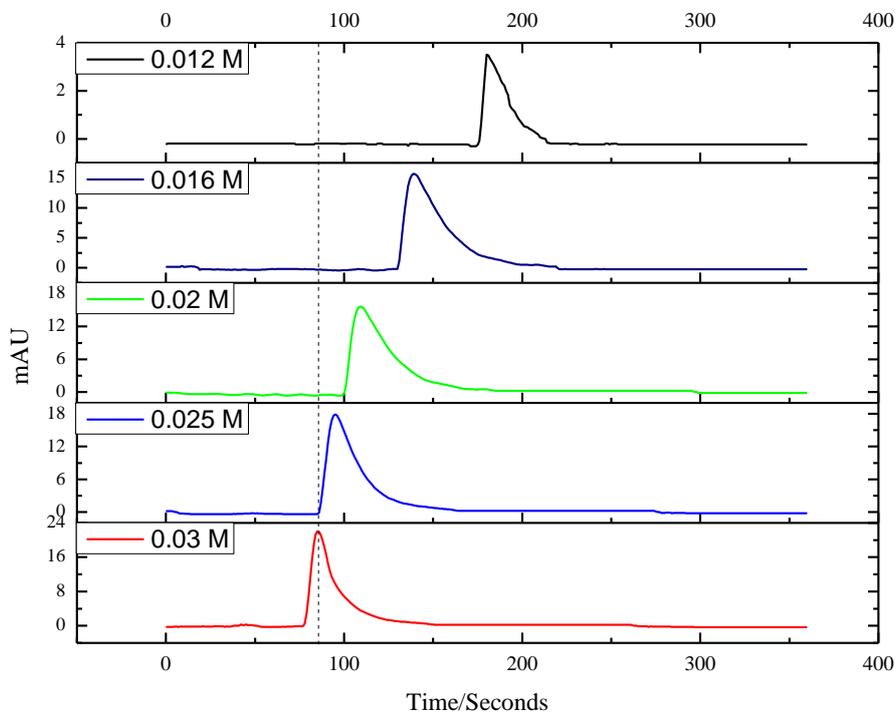


Figure 4.7: Chromatograms of lidocaine at room temperature obtained using MLC

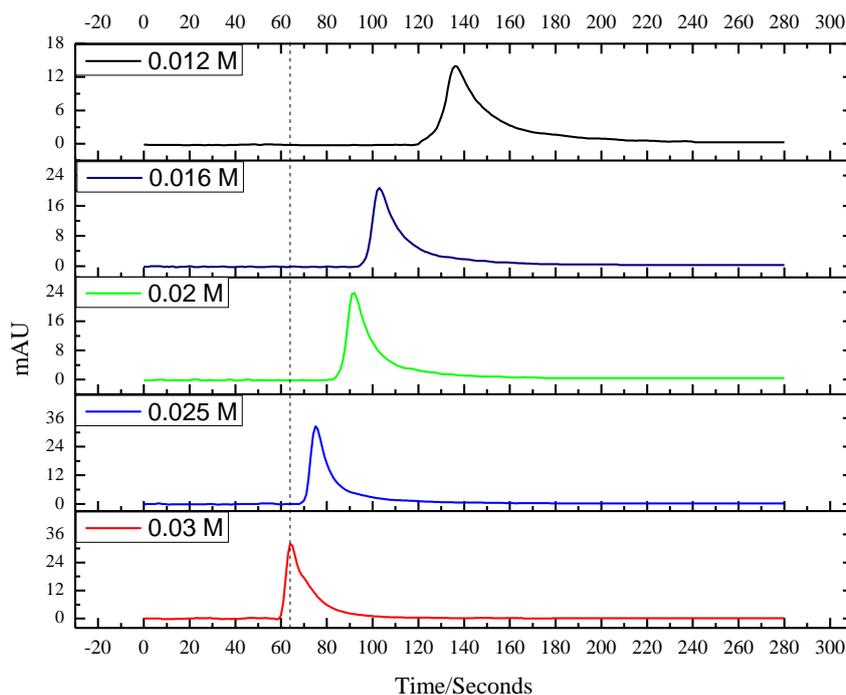


Figure 4.8: Chromatograms of procaine at room temperature obtained using MLC

It should be noted that the pH of the mobile phase, consisting of the highest (0.03 M) and the lowest (0.012 M) SDS concentration, was recorded before injecting samples into the MLC system in order to probe the state of the drugs within the column which could foresee the possible interactions between solutes, stationary phase and the micelles. The pH of the mobile phase was consistently in the range of 5.6 – 6.2 which suggests all the solutes were in their ionised form. The retention behaviour depends on how a drug interacts with the surfactant modified stationary phase and the micelle. Obviously, the charge on the ionised species will be the most important factor in describing these interactions. Drugs with both positive and negative charges, i.e. relatively neutral drugs overall, would have non-polar, dipole-dipole and/or proton donor-accepter interactions. The charged species of the drugs will interact electrostatically with the ionic surfactant, i.e. anionic drugs are expected to be repelled by the net negative charge on the head groups of the micelles, while cationic drugs will have an attraction for the negatively charged SDS micelles. Depending on the degree of ionisation, this

will result in an ‘apparent’ rather than ‘absolute’ value for the P_{MW} . Ionised drugs will have an increased aqueous solubility, compared with un-ionised, and therefore the ‘apparent’ P_{MW} will appear lower in value than an absolute value (Waters and Kasprzyk-Hordern, 2010).

Capacity factors (k') were calculated from the retention times of solutes at five surfactant concentrations, collected at an ambient temperature (295 K), with the relevant chromatographic data for the seven drugs presented in Tables 4.1 – 4.7.

Table 4.1: A summary of chromatographic data for ibuprofen

SDS conc. (M)	Mean retention time (t_r) in seconds	k'	$1/k'$	C_M (M)
0.030	66.2	0.33	3.05	0.022
0.025	64.3	0.29	3.44	0.017
0.020	63.3	0.27	3.69	0.012
0.016	61.8	0.24	4.15	0.008
0.012	60.8	0.22	4.53	0.004

Table 4.2: A summary of chromatographic data for ketoprofen

SDS conc. (M)	Mean retention time (t_r) in seconds	k'	$1/k'$	C_M (M)
0.030	59.0	0.29	3.43	0.022
0.025	64.0	0.40	2.49	0.017
0.020	67.0	0.47	2.14	0.012
0.016	69.0	0.51	1.96	0.008
0.012	72.0	0.58	1.73	0.004

Table 4.3: A summary of chromatographic data of caffeine

SDS conc. (M)	Mean retention time (t_r) in seconds	k'	$1/k'$	C_M (M)
0.030	100.0	1.00	1.00	0.022
0.025	101.0	1.02	0.98	0.017
0.020	103.0	1.06	0.94	0.012
0.016	106.0	1.12	0.89	0.008
0.012	111.0	1.22	0.82	0.004

Table 4.4: A summary of chromatographic data of paracetamol

SDS conc. (M)	Mean retention time (t_r) in seconds	k'	$1/k'$	C_M (M)
0.030	82.5	0.66	1.52	0.022
0.025	84.0	0.69	1.45	0.017
0.020	85.5	0.72	1.39	0.012
0.016	87.0	0.75	1.33	0.008
0.012	88.0	0.77	1.30	0.004

Table 4.5: A summary of chromatographic data of diphenhydramine

SDS conc. (M)	Mean retention time (t_r) in seconds	k'	$1/k'$	C_M (M)
0.030	41.0	1.02	0.98	0.022
0.025	45.0	1.21	0.82	0.017
0.020	45.8	1.25	0.80	0.012
0.016	47.0	1.31	0.76	0.008
0.012	51.0	1.51	0.66	0.004

Table 4.6: A summary of chromatographic data of lidocaine

SDS conc. (M)	Mean retention time (t_r) in seconds	k'	$1/k'$	C_M (M)
0.030	84.8	0.70	1.42	0.022
0.025	93.3	0.87	1.15	0.017
0.020	109	1.19	0.84	0.012
0.016	141	1.83	0.55	0.008
0.012	191.2	2.84	0.35	0.004

Table 4.7: A summary of chromatographic data of procaine

SDS conc. (M)	Mean retention time (t_r) in seconds	k'	$1/k'$	C_M (M)
0.030	65	0.44	2.27	0.022
0.025	77	0.54	1.85	0.017
0.020	92	0.78	1.28	0.012
0.016	102	1.04	0.96	0.008
0.012	136	1.60	0.63	0.004

Once the relevant chromatographic data was obtained, the inverse of the capacity factors ($1/k'$) were plotted against the concentration of micelles (C_M) in the mobile phase. This firstly requires subtraction of the CMC of SDS from the total amount of surfactant used. The CMC of SDS considered in this study was 0.008 M (Waters et al., 2007). As a result, linear plots were obtained with an appreciable linearity (R^2 ranged from 0.919 - 0.997) as shown in Figures 4.9 – 4.15.

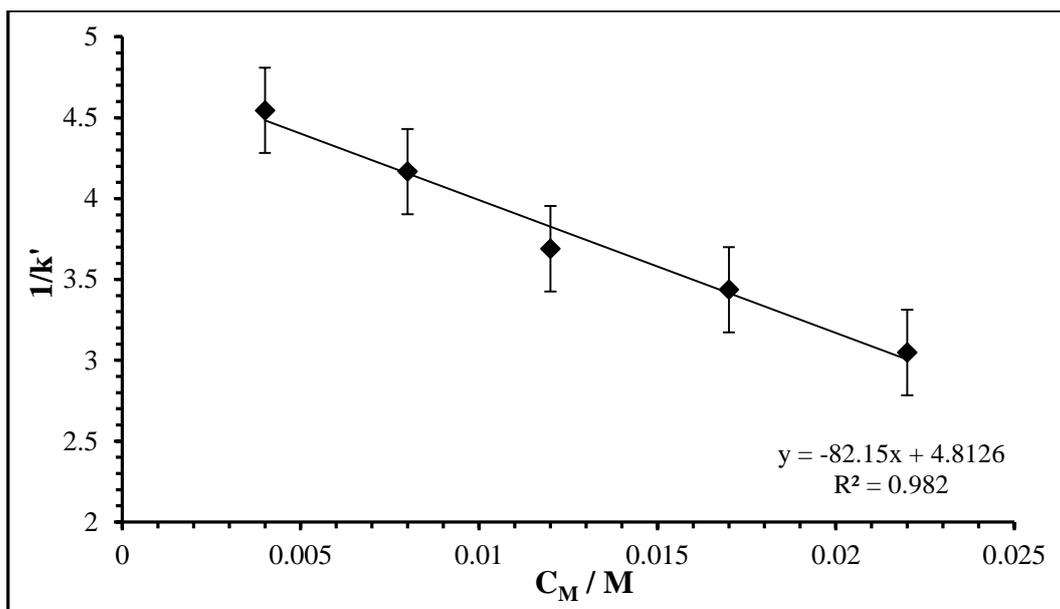


Figure 4.9: Plot of $1/k'$ of ibuprofen as a function of C_M in an un-buffered SDS mobile phase at room temperature

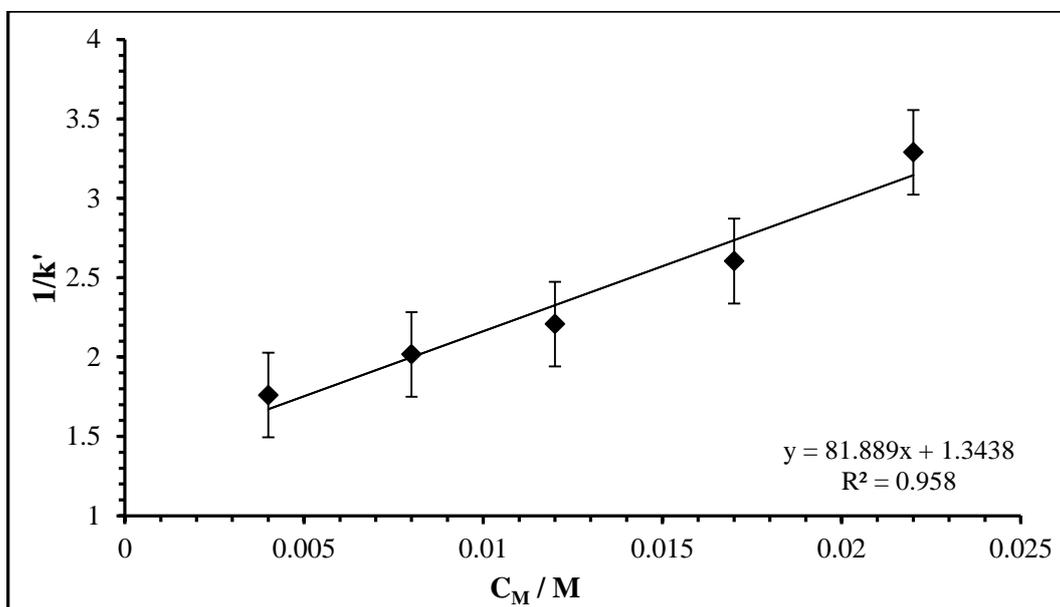


Figure 4.10: Plot of $1/k'$ of ketoprofen as a function of C_M in an un-buffered SDS mobile phase at room temperature

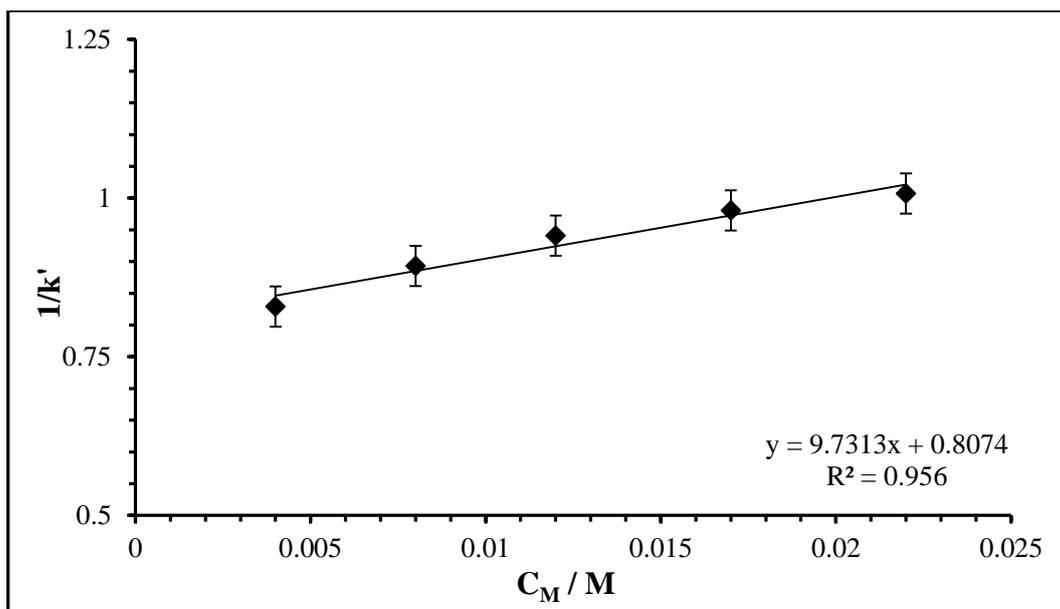


Figure 4.11: Plot of $1/k'$ of caffeine as a function of C_M in an un-buffered SDS mobile phase at room temperature

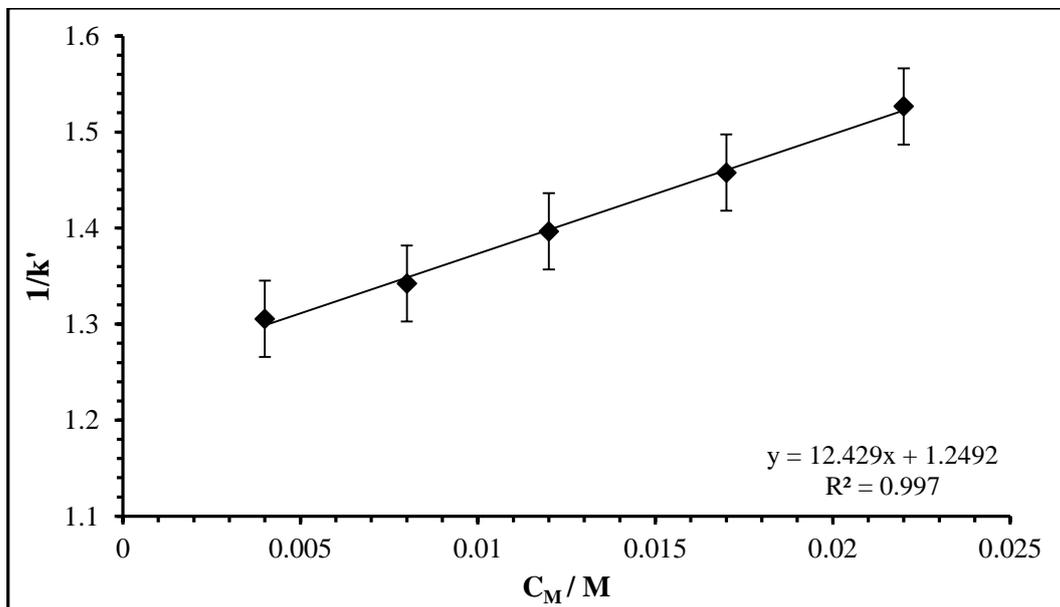


Figure 4.12: Plot of $1/k'$ of paracetamol as a function of C_M in an un-buffered SDS mobile phase at room temperature

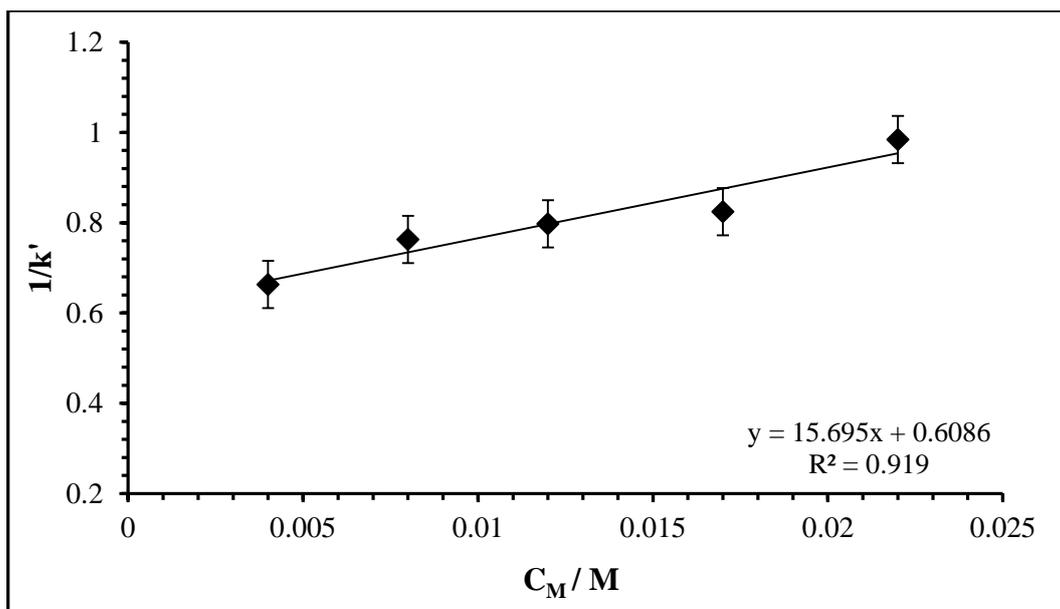


Figure 4.13: Plot of $1/k'$ of diphenhydramine as a function of C_M in an un-buffered SDS mobile phase at room temperature

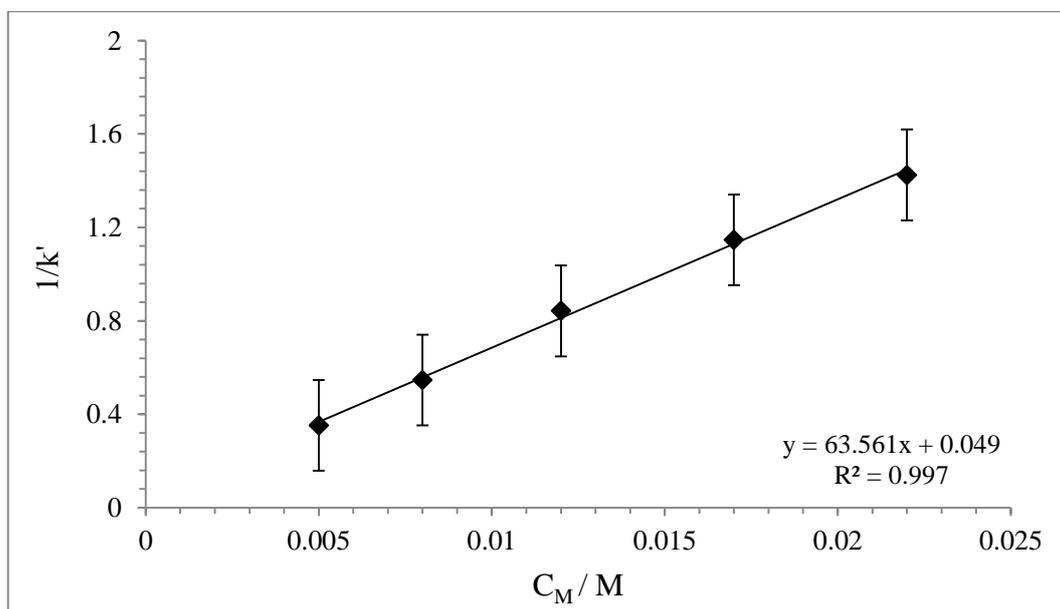


Figure 4.14: Plot of $1/k'$ of lidocaine as a function of C_M in an un-buffered SDS mobile phase at room temperature

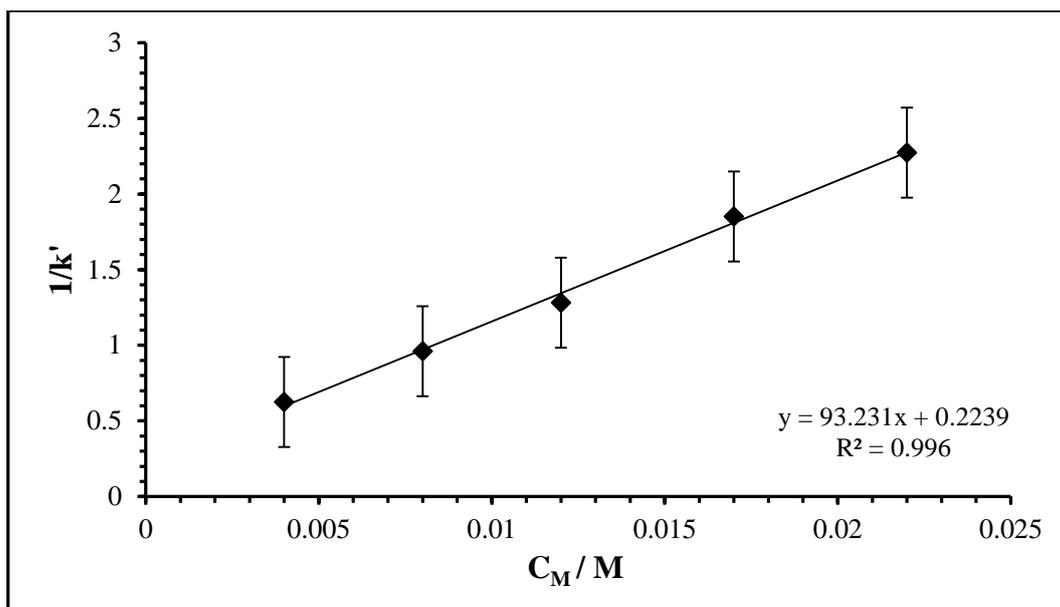


Figure 4.15: Plot of $1/k'$ of procaine as a function of C_M in an un-buffered SDS mobile phase at room temperature

The micelle-water partition coefficient (P_{MW}) was determined from the ratio of the slope and the intercept obtained from the relationship of the inverse of the capacity factors ($1/k'$) and the concentration of micelles (C_M), as described by Armstrong and Nome (1981) and Arunyanart and Cline Love (1985). The experimental P_{MW} values obtained from MLC and their logarithmic form ($\log P_{MW}$) for seven model drugs are listed in Table 4.8 along with the literature octanol-water partition coefficient ($\log P_{OW}$) values.

Table 4.8: MLC based partition coefficients for seven compounds

	Compound	P_{MW}	$\log P_{MW}$	$\log P_{OW}$	$\log D^*$ at mobile phase pH
Anionic	Ibuprofen	16.83 ± 0.34	1.23 ± 0.01	3.6 (Waters et al., 2012)	1.9
	Ketoprofen	60.93 ± 4.25	1.78 ± 0.04	2.7 (Waters et al., 2012)	1.1
Neutral	Caffeine	12.94 ± 1.05	1.12 ± 0.04	-0.07 (Waters et al., 2012)	-0.6
	Paracetamol	10.29 ± 0.40	1.01 ± 0.02	0.5 (Machatha and Yalkowsky, 2005)	0.5

Cationic	Diphenhydramine	25.69 ± 0.17	1.41 ± 0.00	3.3 (Liu et al., 2003)	0.4
	Lidocaine	1297.14 ± 13.74	3.11 ± 0.45	2.4 (Thomas et al., 2007)	0.4
	Procaine	416.25 ± 9.34	2.62 ± 0.33	2.1 (Waters et al., 2012)	-0.6

(Results are presented as mean ± SD; $n = 5$); *Calculated from ACD software (ACD/Labs, RSC, UK).

In MLC, it is expected that an increase in the concentration of surfactant in the mobile phase results in a decreased retention of solutes. However, this is not true if the interaction between solute and micelle is weak or the solute is electrostatically repelled (Ruiz-Ángel et al., 2009). It has been previously reported that only completely nonpolar solutes can reside in the micellar core (Attwood and Florence, 1983). Although most hydrophobic compounds are predominantly non-polar and only a small fraction of the molecule contains polar groups, the latter would affect the location of the solute in the micelle.

Ibuprofen and ketoprofen are weakly acidic drugs having pK_a 's of 4.41 (Waters et al., 2012) and 4.23 (Waters et al., 2012), respectively. It has been previously reported that the experimental octanol-water partition coefficient of ibuprofen and ketoprofen are 3.5 and 2.67 at 298 K, respectively (Waters et al., 2012). At the mobile phase pH, carboxylic groups on both drugs are fully exposed to the aqueous environment of the mobile phase which brings a net negative charge on the ionisable group, thus, making these drugs anionic. In the case of ibuprofen, the retention was increased with increasing surfactant concentration (Figure 4.9) which depicts an anti-binding of ibuprofen with anionic surfactant. The electrostatic force of repulsion repelled the drug molecule from the micelle thus forcing it to be retained on the stationary phase through hydrophobic interactions. Since cyano (CN) based columns tend to adsorb less surfactant, it is expected that these stationary phases offer hydrophobic interactions with solutes in the presence of surfactants and anti-binding phenomena can be observed. On

the contrary, conventional C8 and C18 columns tend to adsorb larger amounts of surfactant, therefore, anti-binding phenomena are hardly observed in those stationary phases (Ruiz-Ángel et al., 2009).

The experimental $\log P_{MW}$ for ibuprofen was found to be 1.23 ± 0.01 which suggests it preferred the aqueous phase slightly more than expected. This means the ionised form of ibuprofen has been electrostatically repelled from entering the hydrophobic micellar core. Further to this argument, the decrease in retention at lower surfactant concentration may be attributed to overcoming the electrostatic repulsion between negatively charged micelles and ibuprofen as less micelles are present in the mobile phase. Therefore, the presence of more ibuprofen molecules than SDS micelles, saturates the available micelles, thus, elution of ibuprofen was closer to the column's dead time because of the low adsorption of micelles onto the surfactant saturated stationary phase.

Ketoprofen is a slightly less hydrophobic drug than ibuprofen ($\log P_{OW} = 2.7$) (Waters et al., 2012), and has shown an increased retention with a decrease in surfactant concentration (Figure 4.10). Although, ketoprofen is ionised at the mobile phase pH, it appears to favour the hydrophobic micellar core more than expected. This could be a result of the polarity of ketoprofen which is less than ibuprofen. Ketoprofen is structurally more apolar than ibuprofen, therefore, a higher $\log P_{MW}$ (1.78 ± 0.04) value was achieved. Water tends to expel apolar solutes which counter-balance the electrostatic repulsion between the negatively charged ketoprofen and SDS micelle, therefore, there is more chance of ketoprofen residing inside the hydrophobic core of the micelle. The resultant increase in retention time is a result of the direct transfer of solute from micelle to the stationary phase where it is adsorbed slightly longer than expected owing to the hydrophobic interactions.

Micelle-water partition coefficients for cationic drugs, namely diphenhydramine, lidocaine and procaine were also determined to compare with the partitioning of anionic drugs. It was expected that cationic drugs would bind to the micelles through electrostatic interactions and have more chance of residing in the micellar core as a result of hydrophobic interactions. The octanol – water partition coefficients for diphenhydramine, lidocaine and procaine are 3.3, 2.4 and 2.1, respectively (Waters et al., 2013; Liu et al., 2003). The dissociation constants (pK_a) for diphenhydramine, lidocaine and procaine are 8.9, 7.9 and 9.0 respectively (Waters et al., 2013; Liu et al., 2003). It should be noted that all three cationic drugs are in their ionised form at the mobile phase pH, therefore, binding to the micelle would be significant for these drugs. On closely analysing the retention behaviour of these drugs (Figures 4.6 – 4.8), all three drugs have strong binding to the micelle and the retention time increased as the concentration of surfactant decreased in the mobile phase. Although significant peak tailing was observed at lower surfactant concentrations (as a result of the basicity of these compounds), this was not an issue in the calculation of micelle – water partition coefficients.

The experimental $\log P_{MW}$ values for diphenhydramine, lidocaine and procaine were found to be 1.41 ± 0.00 , 3.11 ± 0.45 and 2.62 ± 0.33 , respectively as shown in Table 4.8. These compounds are in their ionised form, therefore, $\log P_{MW}$ values reported here correspond to their ‘apparent’ partition coefficients. The $\log P_{MW}$ for diphenhydramine was the lowest among the three studied cationic drugs suggesting its preference for the aqueous phase despite its high $\log P_{ow}$ (3.14) (Liu et al., 2003). This result is further supported by the $\log D$ value of 0.4 at the mobile phase pH which clearly shows its increased preference for the aqueous phase. More interestingly, diphenhydramine was not retained on the CN column for longer times at various surfactant concentrations (Figure 4.6), which might be attributed to the self-micellisation of this drug. Diphenhydramine has colloidal properties and an amphiphilic structure. It has been reported that light-scattering measurements on these compounds have clearly produced plots

with distinct inflection points identical to the CMC inflection points for conventional surfactant molecules (Attwood and Florence, 1983). This may have affected the results obtained.

Lidocaine and procaine, which both belong to the same class of local anaesthetics, have shown a strong association with the micelle which is evident from the $\log P_{MW}$ values of both compounds. Since both compounds are in their ionised form, it is expected that a strong electrostatic attraction would exist between each cationic drug and the negatively charged surfactant head group. Furthermore, it would seem that the drug is distributed in the micellar core as well as on the surface of the micelle which is evident from the higher $\log P_{MW}$ values. At lower surfactant concentrations, the number of available micelles is significantly reduced and saturation of micelles would occur. This results in a relatively high amount of free drug available to interact with the stationary phase, thus, extending the retention times (Figures 4.7 and 4.8).

LogD values of 0.4 and -0.6 for lidocaine and procaine, respectively, at the pH of the mobile phase suggest that these drugs should prefer the aqueous phase in their ionised form. Conversely, this was not true in the presence of the micellar mobile phase and both drugs have shown a high preference for the micelles as a result of hydrophobic and electrostatic interactions. Although, $\log P_{MW}$ and $\log P_{OW}$ are relatively similar for lidocaine and procaine in this study, it can still be said MLC provides a better mimic of biological partitioning since it encodes electrostatic, as well as hydrophobic, interactions.

Two neutral drugs (compounds that acquire both negative and positive charges on ionisation) were used to compare chromatographic data with the anionic and cationic drugs. This was achieved by determining MLC based partition coefficients for two model drugs, namely caffeine and paracetamol. Caffeine is a freely water soluble drug with a $\log P_{OW}$ value of -0.07 (Waters and Kasprzyk-Hordern, 2010), while paracetamol is a slightly hydrophobic

drug with a $\log P_{OW}$ value of 0.5 (Machatha and Yalkowsky, 2005). The retention of caffeine and paracetamol increased with decreasing concentration of surfactant in the mobile phase (Figures 4.11 and 4.12). This represents a normal binding phenomenon in MLC which shows a preference by the compounds for the micelles. The experimental $\log P_{MW}$ values were found to be 1.12 ± 0.04 and 1.01 ± 0.02 for caffeine and paracetamol, respectively, which is higher than the traditionally determined $\log P_{OW}$ values. This increase in partition coefficient value was attributed to the preference of caffeine and paracetamol to either reside in the hydrophobic core of the micelle or stay at the surface (palisade layer) of the micelle. On closely analysing the retention behaviour of these two drugs, the retention time for caffeine was from 100 to 111 seconds and that for paracetamol was 82 to 88 seconds at all surfactants concentrations (Table 4.3 and 4.4). Although, the retention time was higher than that for most of the anionic and cationic drugs, there was no significant difference in the retention times between the highest (0.03 M) and lowest (0.012 M) surfactant concentrations. Therefore, it is possible to hypothesise that both of these drugs were mainly located at the surface (or palisade region) of the micelle, i.e. some part of the drug molecule would be facing the aqueous phase resulting in the retention of drug through interactions with the stationary phase.

In short, the ionisable drugs studied have demonstrated a superior micelle-water partitioning process, compared with the traditional octanol-water system which considers only the un-ionised form of drugs, and therefore fails to describe partitioning of ionised compounds such as those considered in this study.

4.3. Conclusion

MLC based partition coefficients for anionic, neutral and cationic drugs were evaluated. Owing to the structural resemblance of micelles to biomembranes, MLC is a potential

alternative to the traditional octanol-water system for the accurate measurement of partition coefficients. Data acquisition is quick and the results are reproducible. Drugs with various polarities have shown a broad range of lipophilicity in terms of $\log P_{MW}$ values. In all cases, partitioning of drugs solely into the micellar core was not the only factor, in fact, interaction of charged and neutral species with the surface of the micelle was an important factor in explaining partition coefficients determined through MLC.

However, drugs usually have ionisable groups, which may interact differently with the micelle and the stationary phase, therefore, systematic control of ionisation through controlling the pH of the mobile phase would be extremely beneficial.

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Chapter 5: Influence of mobile phase pH on partitioning

¹This chapter highlights pH effects on the measurement of partition coefficients using micellar liquid chromatography (MLC). The impact of ionisation of the compounds on their retention on the stationary phase and interaction with micelles will be explored in depth. From this, correlations will be developed with pH, ionisation and computational logD values.

5.1. Introduction

Retention behaviour of acidic solutes is greatly influenced by the pH of the mobile phase in conventional reverse phase HPLC. Small changes in pH can significantly alter chromatographic retention, particularly when it is close to the dissociation constant (pK_a) of the solute (Palmisano et al., 1989; Haginaka et al., 1989; Love and Fett, 1991). However, literature reporting pH effects on micellar chromatographic retention of compounds is scarce, especially with reference to the cyano-bonded stationary phase. Most reports suggest that the mobile phase pH has a fundamental impact on the gradient of the inverse of capacity factor with micelle concentration (Arunyanart and Cline Love, 1985; Rodgers and Khaledi, 1994). This is because retention of organic solutes on alkyl-bonded stationary phases showing acid-base behaviour is highly dependent on the pH of the mobile phase (García-Alvarez-Coque et al., 1997). Retention of the acidic and basic species of an organic solute will differ in the micellar pseudophase as a consequence of shifts in the dissociation constant in the micellar mobile phase. Previous reports also suggest that the retention of acidic and basic species

¹ Sections of this chapter have been published, see Appendix A

follows sigmoidal behaviour at varying pH values (Arunyanart and Cline Love, 1985; Rodgers and Khaledi, 1994; García-Alvarez-Coque et al., 1997).

As discussed in Chapter 4, cyanopropyl (CN) columns interact in a different way with ionic surfactants compared with conventional alkyl-bonded columns. Firstly, adsorption of ionic surfactants is minimal on the neutral stationary phase. Secondly, the surfactant hydrophobic tail is oriented towards the mobile phase, thus offering hydrophobic interactions (Ruiz-Ángel et al., 2009), therefore, cyano-bonded stationary phases generally have the capability to retain various ionic species.

In this chapter, micelle – water partition coefficient values were determined over a range of mobile phase pHs for five non steroidal anti-inflammatory drugs (NSAIDs). For comparison, ACD labs software (ACD labs, RSC, UK) based computational logD values were used instead of the more traditional logP_{OW} which are only valid for the neutral species (Klein et al., 1988).

5.2. Theoretical considerations

Retention in reverse phase chromatography for solutes that exhibit acid-base behavior depends on the pH of the mobile phase. The relationship between pH and p*K*_a for ionisable solutes is well established through Henderson-Hasselbalch equations (Horváth, 1977). The degree of ionisation of a given solute is based on the pH of the solution. Arunyanart and Cline-Love (Arunyanart and Cline Love, 1985) and Rodgers and Khaledi (Rodgers and Khaledi, 1994) studied the effect of pH on the MLC retention of weak acids and bases. These authors derived an equation that describes the observed behaviour in terms of pH and micellar concentration. For a monoprotic acid (solute with one solvated proton H⁺), the dissociation in the mobile phase is governed by the following equilibrium:



where A⁻ is the dissociated acid and H⁺ is the solvated proton.

For simplicity, solvated proton concentration will be denoted by h in the proceeding equations. The equilibrium constant can then be described as:

$$K_H = \frac{[HA]}{[A]h} \quad (5.2)$$

where h is the proton concentration and K_H is the protonation constant, the capacity factor of a chromatographic system can then be represented as:

$$k' = \varphi \frac{[AS]+[HAS]}{[A]+[HA]+[AM]+[HAM]} \quad (5.3)$$

where $[AS]$ and $[A]$ are the concentrations of the non-protonated species present in the stationary phase and the bulk aqueous phase, respectively, and $[HAS]$ and $[HA]$ represent the concentrations of the respective acidic species. Lastly, $[AM]$ and $[HAM]$ represent the concentrations of the basic and acidic species associated with the micelle, respectively.

The solute retention in the chromatographic system studied here is assumed to be case of several partition equilibria (or reversible association) between dissociated or un-dissociated solute, micelles and the stationary phase (García-Alvarez-Coque et al., 1997). This equilibrium can be described as:

(1) Association of the acidic species with the micelle:



$$K_{HAM} = \frac{[HAM]}{[HA][M]} \quad (5.5)$$

(2) Partitioning of the acidic species with the stationary phase:



$$P_{HAS} = \frac{[HAS]}{[HA]} \quad (5.7)$$

(3) Association of the basic species with the micelle:



$$K_{AM} = \frac{[AM]}{[A][M]} \quad (5.9)$$

(4) Partitioning of the basic species with the stationary phase:



$$P_{AS} = \frac{[AS]}{[A]} \quad (5.11)$$

By substituting Eq. (5.2, 5.4 – 5.11) with Eq. 5.3, and changing $K_{AS} = \phi P_{AS}$ and $K_{HAS} = \phi P_{HAS}$, the following Eq. will be obtained:

$$k' = \frac{K_{AS} + K_{HAS} K_H h}{1 + K_H h + K_{AM} [M] + K_{HAM} K_H h [M]} \quad (5.12)$$

On rearranging Eq. (5.12):

$$k' = \frac{\frac{K_{AS} + K_{HAS} K_H h}{1 + K_H h}}{1 + \frac{K_{AM} + K_{HAM} K_H h}{1 + K_H h} [M]} = \frac{K_{AS}^H}{1 + K_{AM}^H [M]} \quad (5.13)$$

where K_{AS}^H and K_{AM}^H are conditional constants with respect to the proton concentration that show a sigmoidal dependence with pH.

Eq. (5.13) can be re-written as follows:

$$k' = \frac{\frac{K_{AS}}{1 + K_{AM} [M]} + \frac{K_{HAS}}{1 + K_{HAM} [M]} + \frac{1 + K_{HAM} [M]}{1 + K_{AM} [M]} K_H h}{1 + \frac{1 + K_{HAM} [M]}{1 + K_{AM} [M]} K_H h} \quad (5.14)$$

Eq. (5.14) has been reported previously (Arunyanart and Cline Love, 1985, Rodgers et al., 1993) indicating a sigmodal behaviour dependence on pH between the retention of acidic and basic species, thus validating the theoretical models.

5.3. Results and discussion

Generally, all previous studies have considered octadecylsilyl (ODS) bonded columns to establish retention-surfactant-pH relationships for the organic acids and bases (García-Alvarez-Coque et al., 1997; Torres-Lapasió et al., 2000; Ruiz-Angel et al., 2004; Pous-Torres et al., 2009). However, Arunyanart and Cline Love (1985) have shown a comparison between ODS and CN bonded columns in relation to the retention of some organic acids and bases with respect to surfactant concentration and pH of the mobile phase. Retention of solutes on ODS and CN based columns differ in terms of surfactant adsorption capacity. ODS columns do adsorb SDS surfactant monomers with the negative head group in contact with the mobile phase, such that the surface is charged and will repulse anionic species. On the other hand, CN based columns tend to adsorb a negligible amount of surfactant monomers, thus, presenting a higher capacity of retaining anionic species (Arunyanart and Cline Love, 1985).

This study investigates the retention and partitioning behaviour of five structurally related hydrophobic drugs (NSAIDs) with reference to the mobile phase pH. Some of the relevant physicochemical properties including the dissociation constant (pK_a) and octanol-water partition coefficients ($\log P_{ow}$) are presented in Table 3.1. Since cyano bonded columns only function over a narrow pH range (2-8), it was vital to select a pH range that does not produce any drastic effects on the column. It was decided that a pH range of 3-7 would be sufficient to elicit various ionisation levels of drugs and also corresponds to physiological conditions. The aqueous pK_a for all studied drugs is from 4.1 to 4.5 (Waters et al., 2012),

therefore, it was expected that various percentages of ionic species would exist at the studied range of mobile phase pH.

5.3.1. Retention and pH relationship

Theoretically, application of the models described in Section 5.2 should produce a sigmoidal behaviour in the studied range of pH (i.e. 3 - 7). However, deviations from the theoretical outcomes were achieved and no sigmoidal behaviour was obtained when capacity factors were plotted against pH over a range of surfactant concentrations as presented in Figures 5.1 – 5.5. Arunyanart and Cline Love (1985) reported that the pH of the mobile phase has significant importance on the retention of monoprotic acids. They found that at low pH values the neutral acid k' values decrease, and at high pH the anionic conjugate bases k' values increase with increasing SDS concentration on an ODS stationary phase. However, the opposite of what has previously been reported (Arunyanart and Cline Love, 1985) was seen in this study, i.e. anionic conjugate bases k' values were decreased with increasing SDS concentration (Figure 5.1 – 5.5). The fact is ODS columns tend to adsorb more anionic surfactant than cyano-bonded stationary phases, therefore, the elution behaviour versus pH for weak acids on cyano columns would be the opposite to that observed in ODS columns.

The separation process in micellar liquid chromatography for neutral species is mainly controlled by the hydrophobic interactions of the compound with the micellar system and the stationary phase (Arunyanart and Cline Love, 1985). However, electrostatic attraction, or repulsion, affects the retention behaviour for ionised species. This behaviour was evident in this study where neutral species show a high affinity for the micellar hydrophobic core through hydrophobic interactions, thus eluted quickly. Once the pH of the mobile phase was raised above the pK_a of each drug, negatively charged species were electrostatically repelled from the

micelle thus interaction with the stationary phase was increased. This resulted in delayed elution of the drugs from the column which is confirmed from high capacity factor values (Figures 5.1 – 5.5). Overall, a similar trend with a comparable retention on the stationary phase was seen for all the drugs studied here. This is because of the similar ionisation constants and the hydrophobicity of these compounds.

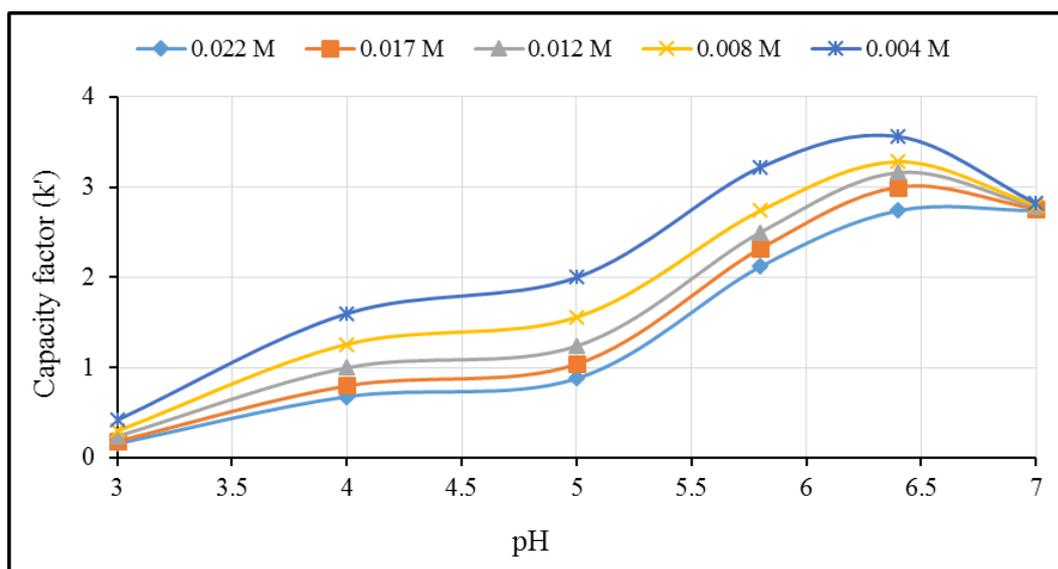


Figure 5.1: Plot of capacity factors of ibuprofen at various mobile phase pHs

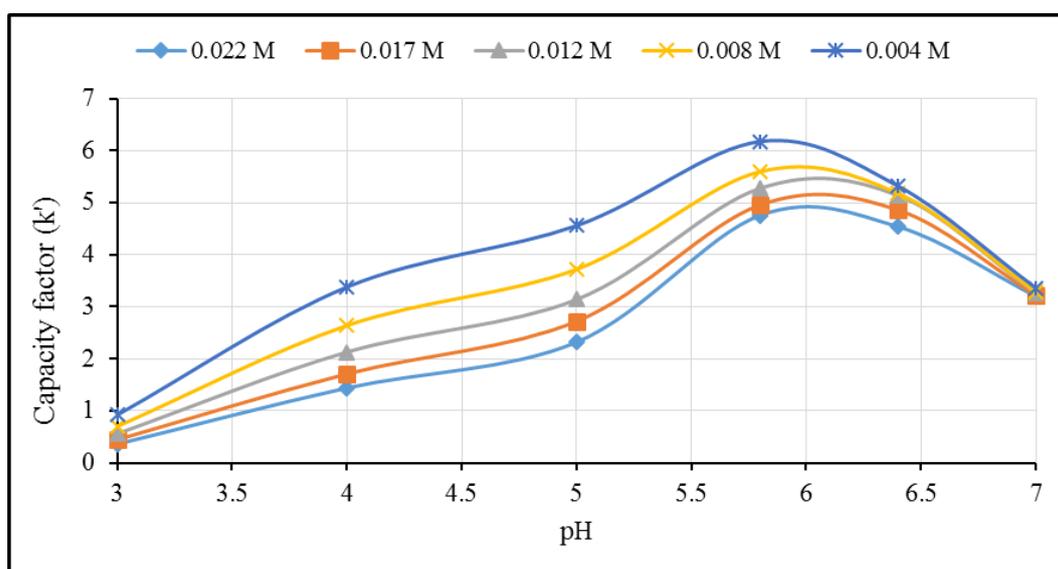


Figure 5.2: Plot of capacity factors of ketoprofen at various mobile phase pHs

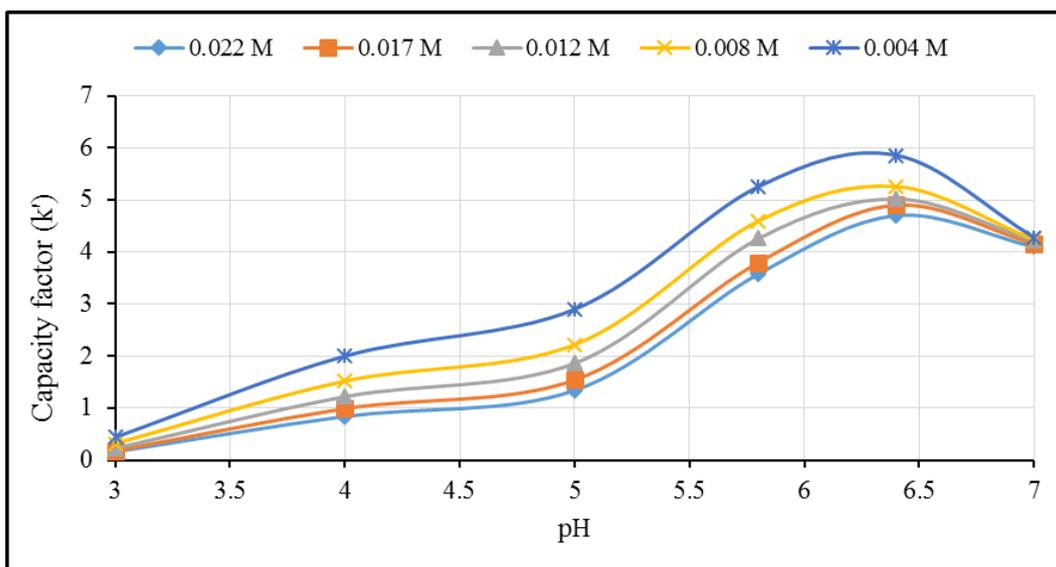


Figure 5.3: Plot of capacity factors of flurbiprofen at various mobile phase pHs

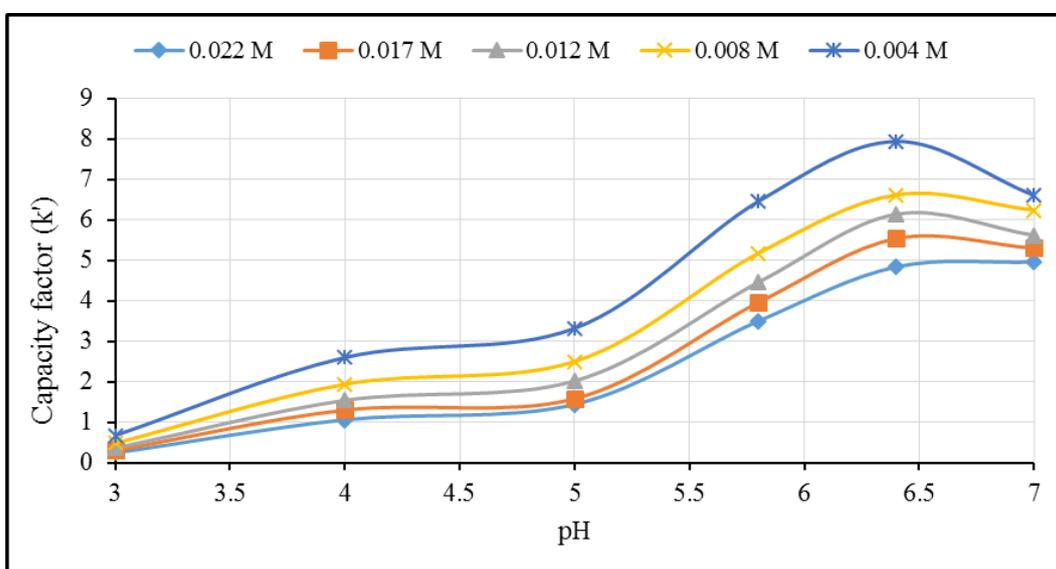


Figure 5.4: Plot of capacity factors of fenbufen at various mobile phase pHs

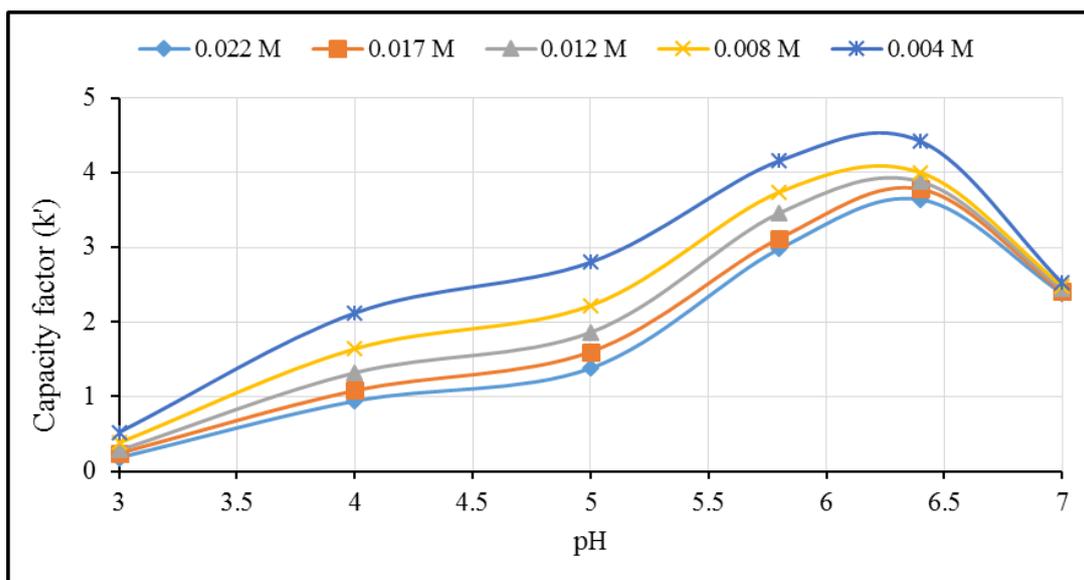


Figure 5.5: Plot of capacity factors of fenopufen at various mobile phase pHs

5.3.2. Partition and ionisation relationship

The extent of ionisation for each compound at a pH range of 3-7 was calculated using the Henderson-Hasselbalch relationship for monoprotic weak acids as given by Eq. (5.15):

$$\% \text{ ionisation} = \frac{10^{(pH-pK_a)}}{1 + 10^{(pH-pK_a)}} \times 100 \quad (5.15)$$

Table 5.1 shows the percent ionisation of the five drugs at six different pH values of the mobile phase. The lowest ionisation was observed at pH 3 which is lower than the pK_a of all drugs and the drugs are predominantly in their non-ionised form. At pH values above the pK_a , an anionic form of the drugs dominates.

Table 5.1: Percent ionisation of five drugs at six different pH values

Drug	pK _a	% ionisation at various pH					
		3	4	5	5.8	6.4	7
Ibuprofen	4.41	3.74	28.01	79.55	96.09	98.99	99.74
Ketoprofen	4.23	5.56	37.06	85.48	97.38	99.33	99.83
Flurbiprofen	4.14	6.76	42.01	87.87	97.86	99.45	99.86
Fenbufen	4.55	2.74	21.99	73.81	94.68	98.61	99.65
Fenoprofen	4.20	5.94	38.69	86.32	97.55	99.37	99.84

In theory, the anionic form of a drug should experience electrostatic repulsion from the anionic micelles, therefore, will be retained on the stationary phase longer than in its non-ionised form. Attempts were made to correlate the retention behaviour of compounds in the chromatographic system, in terms of the micelle-water partition coefficients, with corresponding percent ionisation and computational logD values over a range of pHs of the mobile phase as presented in Figures 5.6 – 5.10. As can be seen from these figures a significant decrease in the logP_{MW} was achieved at a pH above the pK_a of the compounds. This is because the compounds studied were mainly in their neutral form at a pH below their dissociation constants and have relatively more affinity for the hydrophobic core of the micelle than the bulk aqueous phase. As the pH was increased above the dissociation constant, the compounds were ionised to a greater extent and therefore more soluble in the bulk aqueous phase rather than the hydrophobic core of the micelle. Another reason for this was the electrostatic repulsive forces driving the anionic species of the drugs away from the micelles. As a result, relatively higher retention times were achieved for the ionised form of the drugs because of an increased affinity for the bulk aqueous phase and, consequently, for the cyano-bonded stationary phase.

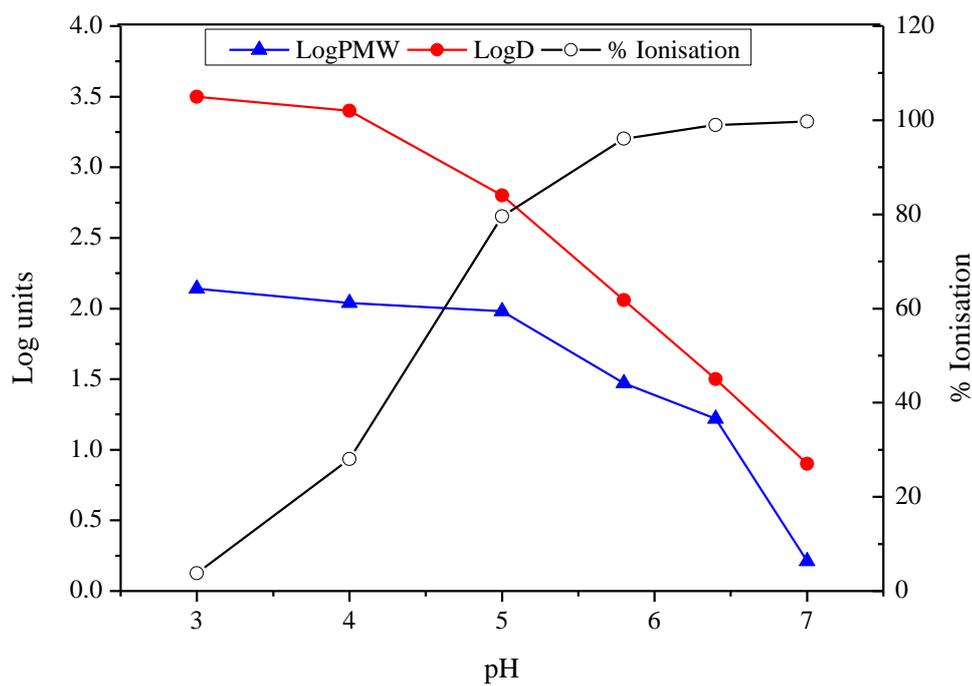


Figure 5.6: Relationship of $\log P_{MW}$, $\log D$ and % ionisation of ibuprofen

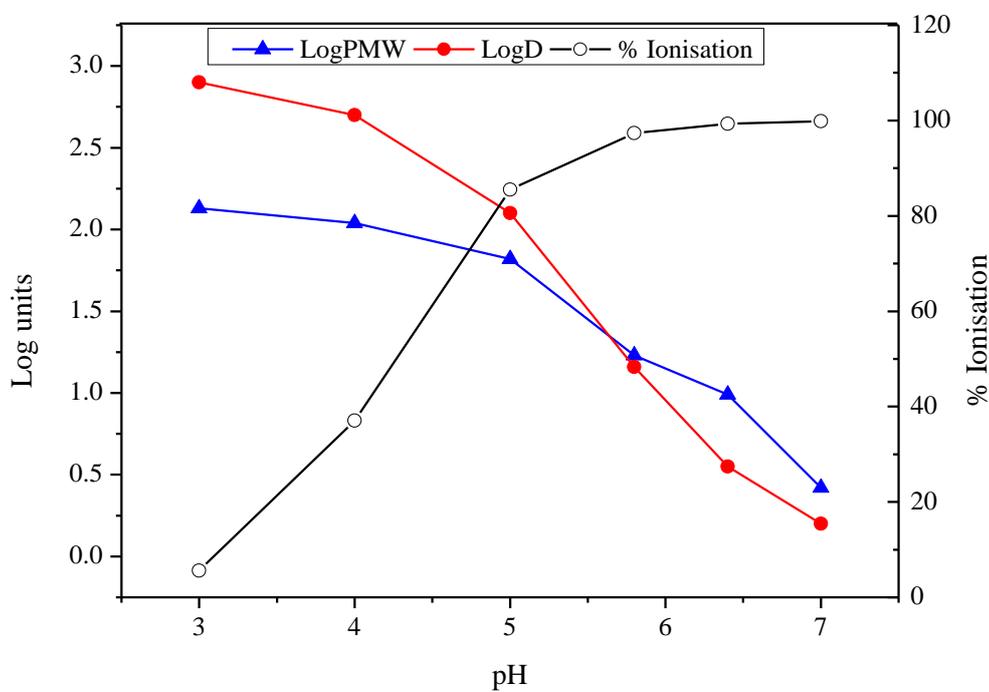


Figure 5.7: Relationship of $\log P_{MW}$, $\log D$ and % ionisation of ketoprofen

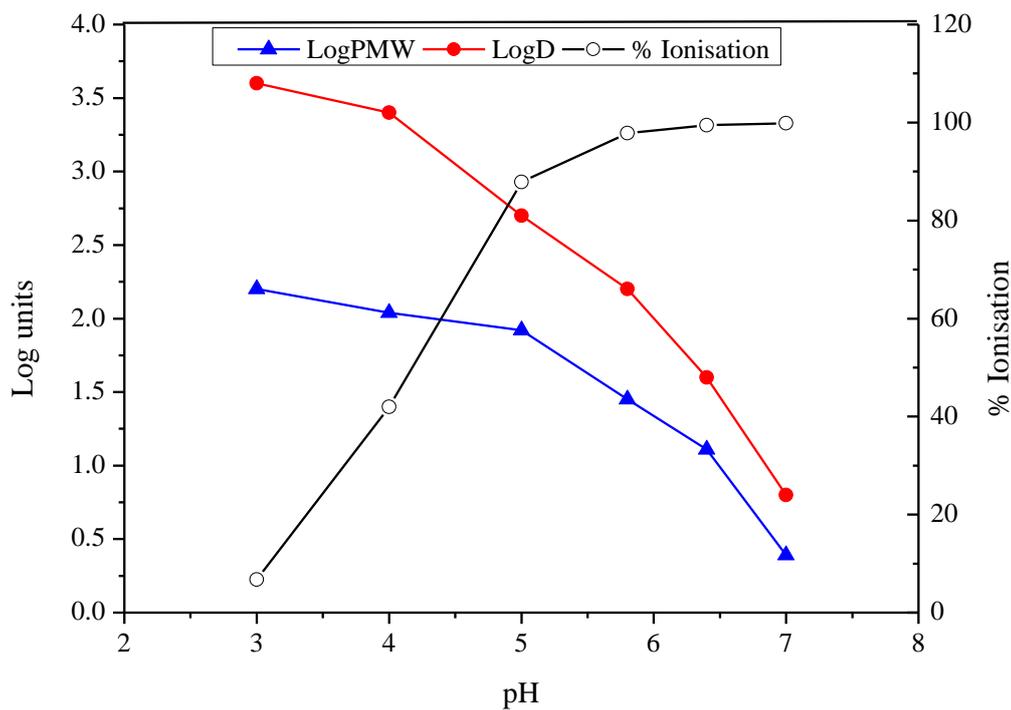


Figure 5.8: Relationship of $\log P_{MW}$, $\log D$ and % ionisation of flurbiprofen

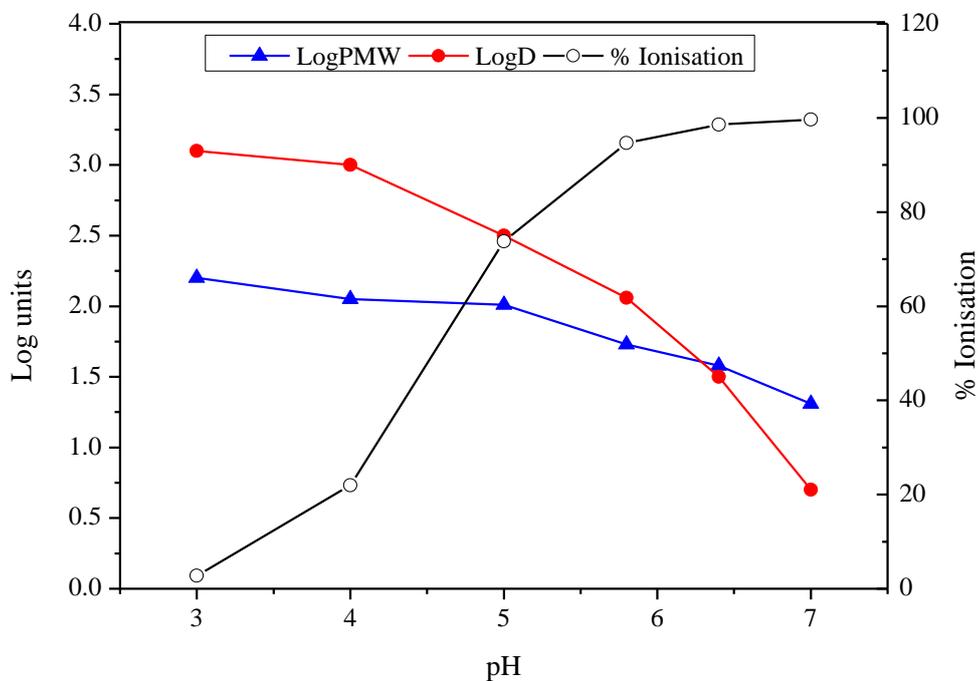


Figure 5.9: Relationship of $\log P_{MW}$, $\log D$ and % ionisation of fenbufen

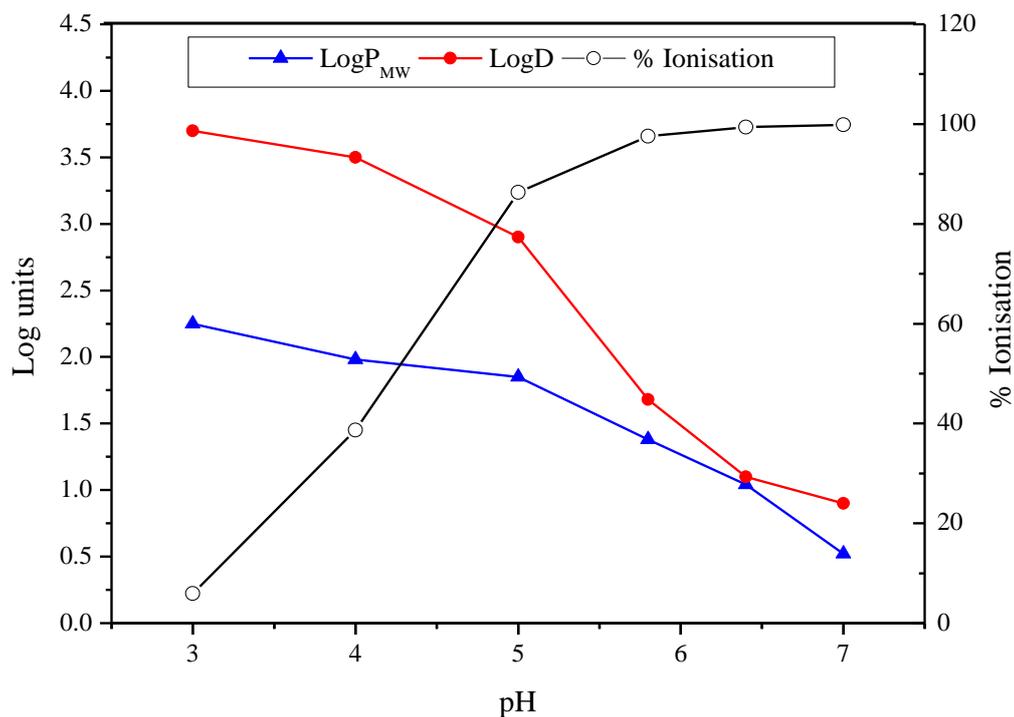


Figure 5.10: Relationship of $\log P_{MW}$, $\log D$ and % ionisation of fenopfen

5.3.3. $\log P_{MW}$ and $\log D$ relationship

The traditional approach of describing partitioning, namely the octanol-water partition coefficient does not account for the ionised species (Klein et al., 1988), therefore, computational $\log D$ values were obtained from the ACD i-Lab software (RSC, UK). This was achieved by carefully analysing and mapping the $\log D$ vs. pH graphs for each drug at specified pH values used in this study. Table 5.2 shows $\log P_{MW}$ and $\log D$ values while Figures 5.11 and 5.12 illustrate the $\log P_{MW}$ and $\log D$ values at six different pH values of the mobile phase.

Table 5.2: Effect of mobile phase pH on experimentally determined logP_{MW} values compared with calculated logD values for five drugs

Drug	pH 3.0		pH 4.0		pH 5.0		pH 5.8		pH 6.4		pH 7.0	
	logP _{MW}	logD										
Fenbufen	2.20	3.1	2.05	3.0	2.01	2.5	1.73	1.8	1.58	1.3	1.31	0.7
Fenoprofen	2.25	3.7	1.98	3.5	1.85	2.9	1.38	2.1	1.04	1.6	0.52	1.0
Flurbiprofen	2.20	3.6	2.04	3.4	1.92	2.7	1.45	2.3	1.11	1.3	0.39	0.8
Ibuprofen	2.14	3.5	2.04	3.4	1.98	2.8	1.47	2.2	1.22	1.4	0.21	0.9
Ketoprofen	2.13	2.9	2.04	2.7	1.82	2.1	1.23	1.3	0.99	0.9	0.42	0.2

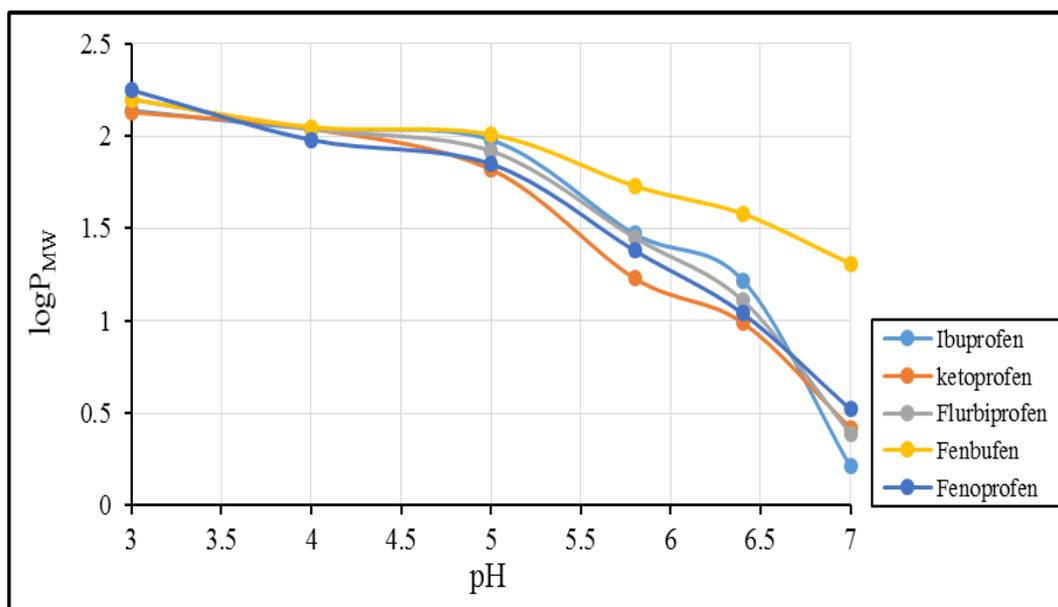


Figure 5.11: $\log P_{MW}$ of five drugs at various mobile phase pH values

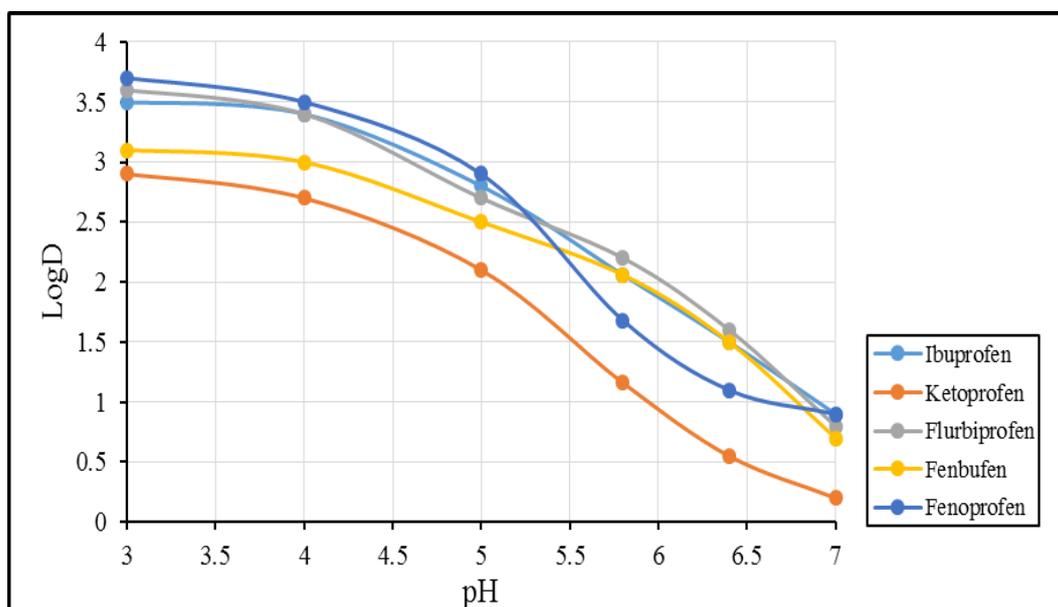


Figure 5.12: ACD software calculated $\log D$ values of five drugs at various pH values

The data suggest that the $\log D$ is a reasonable predictor of the MLC derived partitioning values across the range of drugs studied. While absolute values of $\log D$ and

$\log P_{MW}$ significantly differ (Table 5.2), the change in both values with respect to pH is consistent from pH 5 to 7. Interestingly while the relationship between the measured $\log P_{MW}$ and pH shows deviation from linearity around the pK_a 's of the drug substance, the calculated $\log D$ values are better correlated with pH across the entire range of pH studied. This may suggest either an oversimplification of the calculational approach to ionisation or enhanced sensitivity of the MLC technique in reporting partitioning data near the pK_a of the analyte in the experimental solvent system.

This relationship is further explained on the basis of correlation between experimental and computational values. The micelle-water partition coefficient ($\log P_{MW}$) values at different mobile phase pH were linearly regressed against computational $\log D$ values at similar pH conditions (Figures 5.13 – 5.17) using Origin software version 8.5 (Northampton, USA). Two parameters, namely R^2 and Pearson's correlation coefficient were assessed in order to explain the correlation between $\log P_{MW}$ and $\log D$ values. The Pearson's correlation coefficient is a numerical measure of the strength of the relationship between two random variables (Pearson, 1901). The value of the correlation coefficient varies from -1 to 1. A positive value means that the two variables under consideration have a positive linear relationship (i.e., an increase in one corresponds to an increase in the other) and are said to be positively correlated. A negative value indicates that the variables considered have a negative linear relationship (i.e., an increase in one corresponds to a decrease in the other) and are said to be negatively correlated. The closer the value is to +1 or -1, the stronger the degree of linear dependence. The R^2 values for ibuprofen, ketoprofen, flurbiprofen, fenbufen and fenoprofen were found to be 0.943, 0.963, 0.962, 0.974 and 0.954, respectively. Pearson's correlation coefficients were found to be in the range of 0.945-0.987. These values show a good correlation between the chromatographic partition coefficients and the computational values despite the fact there are mechanistic differences between the two techniques.

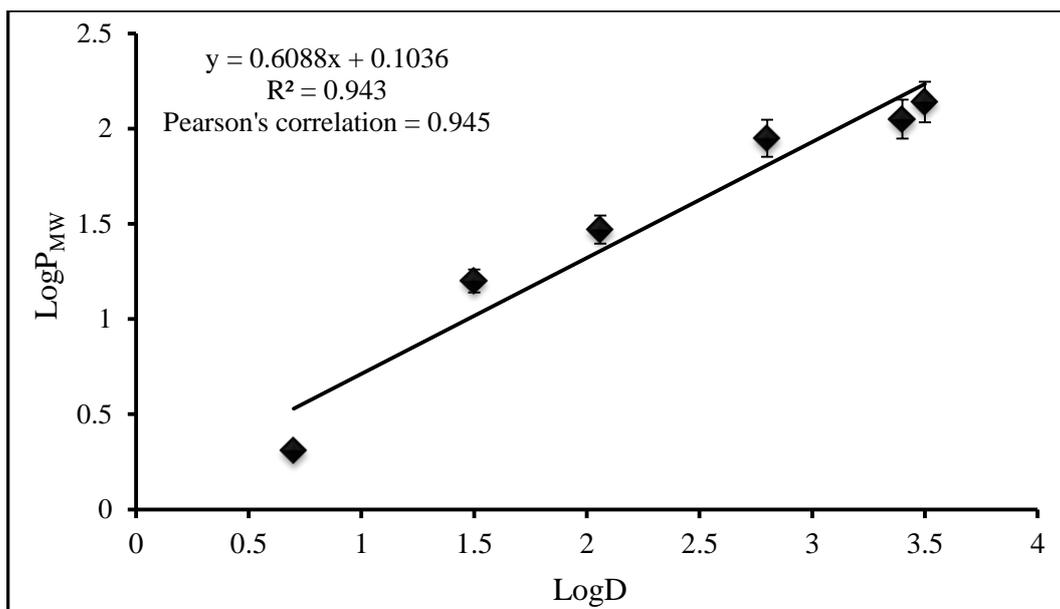


Figure 5.13: LogP_{MW} and logD relationship for ibuprofen

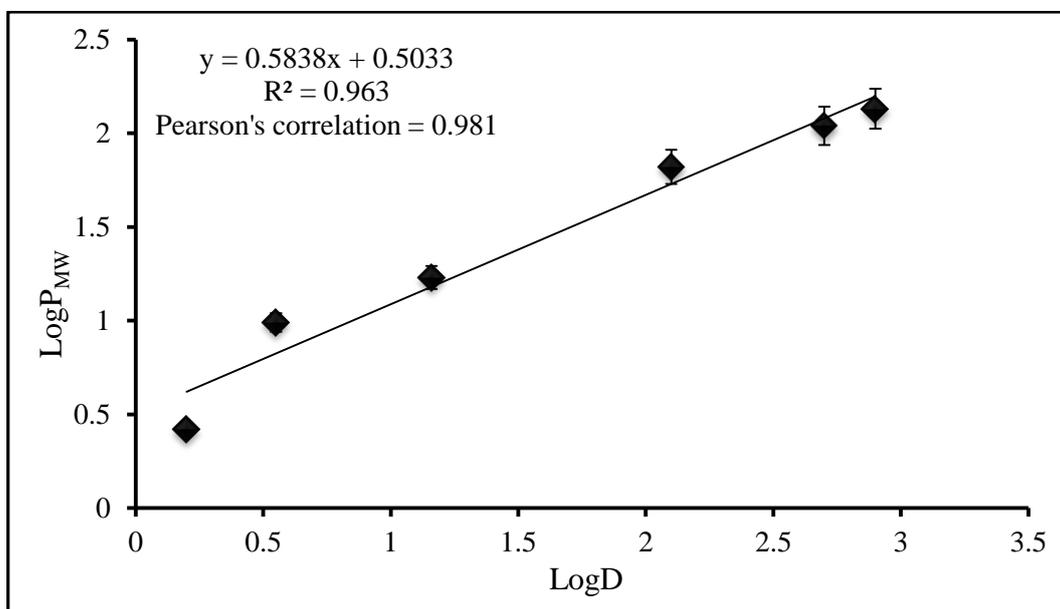


Figure 5.14: LogP_{MW} and logD relationship for ketoprofen

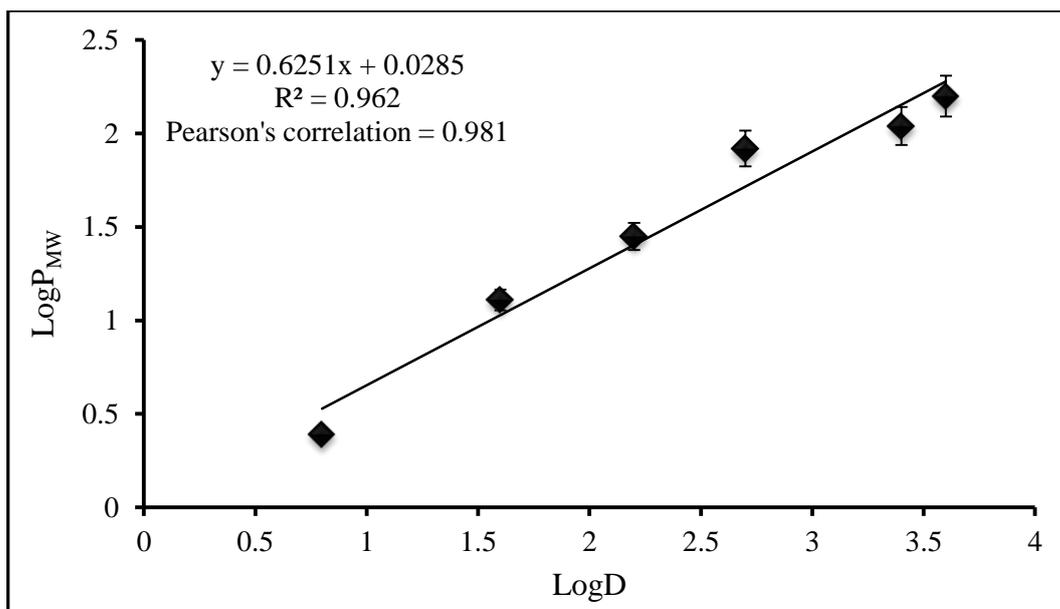


Figure 5.15: LogP_{MW} and logD relationship for flurbiprofen

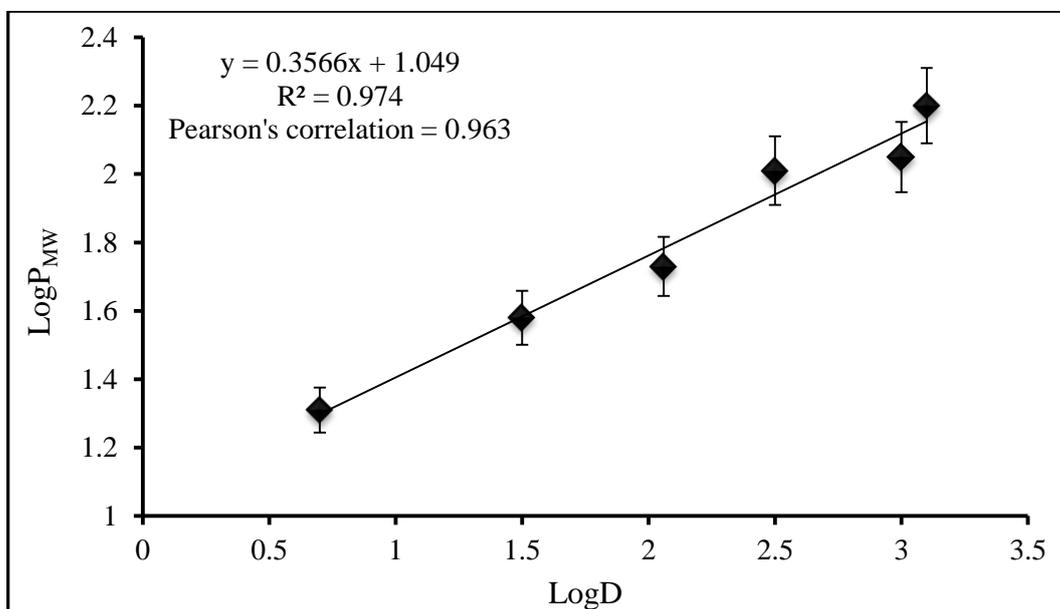


Figure 5.16: LogP_{MW} and logD relationship for fenbufen

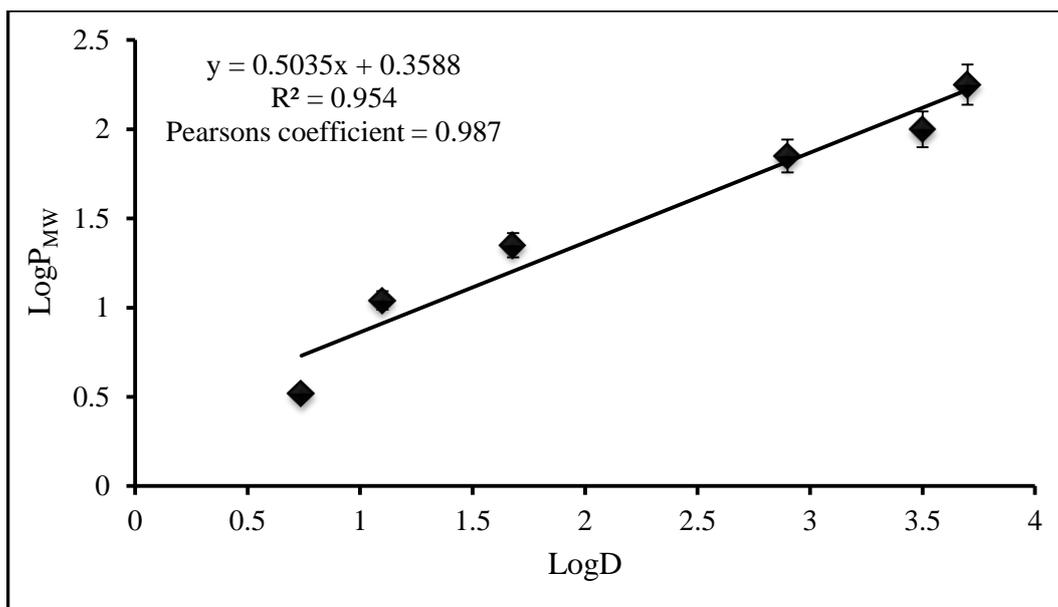


Figure 5.17: LogP_{MW} and logD relationship for fenopfen

5.4. Conclusions

It can be seen that for the five drugs under investigation in this study the pH of the mobile phase significantly influenced the partitioning behaviour observed using MLC. In particular, a strong correlation of logP_{MW} was observed with calculated logD values. More interestingly, a significant change in partitioning was observed near the pK_a of each drug indicating an influence of ionised species on the association with the micelle and retention on the stationary phase.

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Chapter 6: Effect of elevated column temperatures on the partitioning of compounds

¹*This chapter deals with the effects of elevated column temperatures on the partitioning of seven compounds of different ionic nature using micellar liquid chromatography (MLC). A series of column temperatures (295 K – 317 K) were selected with the aim to evaluate the possible thermodynamic parameters involved in the partitioning of these drugs.*

6.1. Introduction

Chromatographic retention in conventional HPLC has been described in terms of thermodynamic properties (Melander et al., 1968; Colin et al., 1978). The thermodynamic theory has satisfactorily accounted for the interactions of the solute with the mobile phase and the stationary phase. Melander and coworkers applied the van't Hoff equation to reverse phase chromatography as described in Eq. (6.1) (Melander et al., 1968):

$$\ln k' = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \ln \varphi \quad (6.1)$$

where k' is the capacity factor, R is the gas constant, T is the temperature in Kelvin and φ is the volume phase ratio.

The resultant van't Hoff plots give enthalpies and entropies of transfer of the solute from the mobile phase to the stationary phase. Since micellar liquid chromatography is a form of reverse phase HPLC, the effect of temperature on retention may provide information concerning the thermodynamics of the partitioning of compounds in micellar chromatographic processes. However, the use of thermodynamic properties in describing micellar liquid chromatography has been limited. Dorsey and coworkers directly applied Eq. (6.1) to describe

¹ Sections of this chapter have been published, see Appendix A

the thermodynamics of the partitioning in micellar liquid chromatography (Dorsey et al., 1983). More recently, Waters and coworkers (2007) have explained the thermodynamic function of the transfer of solutes from one phase to another phase which can lead to the determination of a change in the Gibbs free energy (ΔG).

$$\Delta G = -RT \ln P_{MW} \quad (6.2)$$

where R is the gas constant ($8.314 \text{ J.K}^{-1}.\text{mol}^{-1}$) and T is the temperature in Kelvin at which the partitioning process occurred.

Partitioning is a thermodynamic process and determination of whether a micelle-solute interaction at equilibrium is enthalpy- and/or entropy-driven can be achieved by thermodynamic analysis. An enthalpy-driven process is usually associated with the formation of new bonds such as hydrogen bonds and van der Waals interactions, whereas an entropy-driven process is usually characterised by the displacement of ordered water molecules coupled with the formation of new hydrophobic interactions (Leffler, 1963; Kim, 2001). Previous studies have shown that application of the van't Hoff isochore on partitioning data over a range of temperatures can facilitate calculation of the change in enthalpy (ΔH) and entropy (ΔS) of the system (Congliang et al., 2007; Waters et al., 2007).

This work presents partitioning of a range of pharmaceutical compounds at a series of elevated column temperatures in an attempt to investigate the thermodynamics of the partitioning process, especially with reference to physicochemical characteristics of compounds. This will be achieved by selecting pharmaceutical compounds of different ionic states in solution i.e. anionic (fenbufen, fenoprofen, flurbiprofen, ibuprofen and ketoprofen), cationic (procaine) and neutral (paracetamol).

6.2. Results and discussion

Temperature has a major impact on retention, selectivity, and resolution in chromatographic separations (Oberleitner et al., 2002). In chromatographic systems, the distribution coefficient of a solute between the mobile phase and stationary phase is a function of the difference in free energy of the solute in the two phases (Martin, 1949). Thus, a better understanding of the partition mechanism of a solute between micellar pseudophase and stationary phase is important to control selectivity.

Chromatographic retention of various solutes was determined at a range of elevated column temperatures (i.e. from 295 K to 317 K) using micellar mobile phase. From the linear relationship of the inverse of capacity factors ($1/k'$) and the micellar concentration (C_M), micelle water partition coefficients were deduced at each studied temperature. The influence of temperature on the retention of seven solutes considered in this work is presented in Figures 6.1 to 6.7.

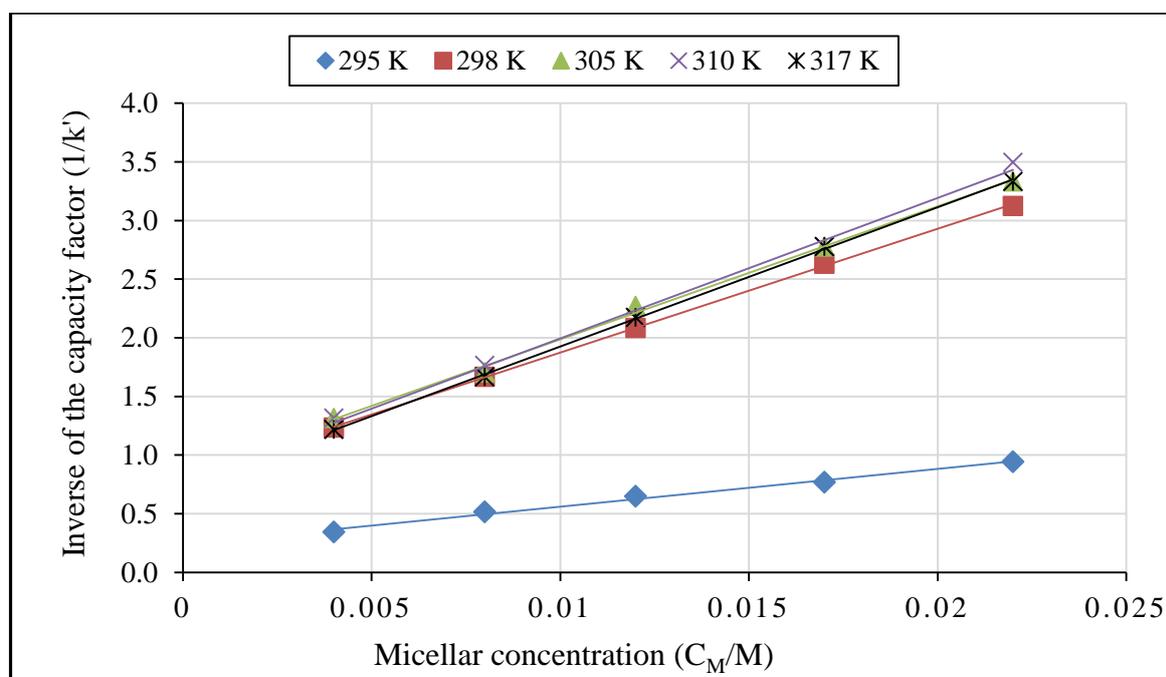


Figure 6.1: Plots of inverse of the capacity factors vs. micellar concentration for fenbufen at various temperatures

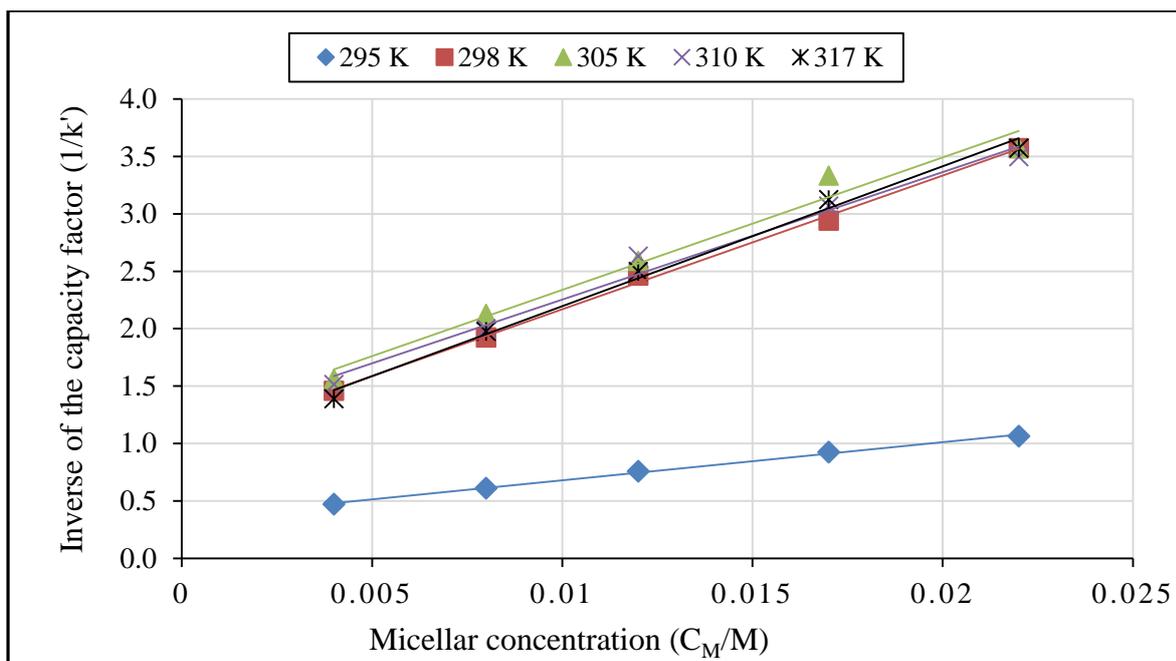


Figure 6.2: Plots of inverse of the capacity factors vs. micellar concentration for fenopropfen at various temperatures

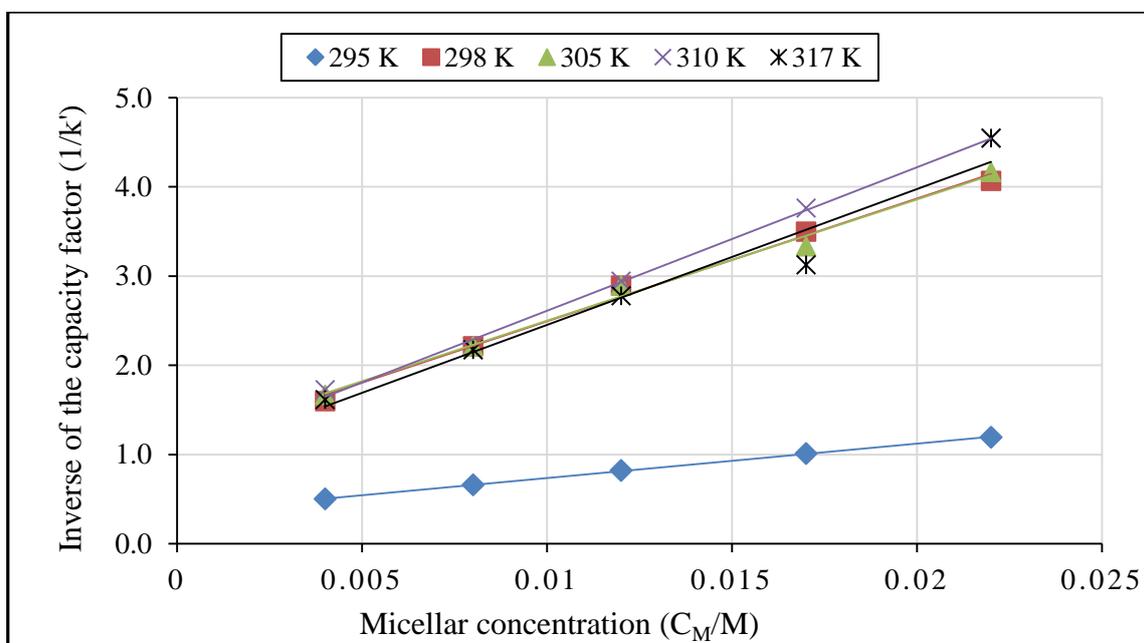


Figure 6.3: Plots of inverse of the capacity factors vs. micellar concentration for flurbiprofen at various temperatures

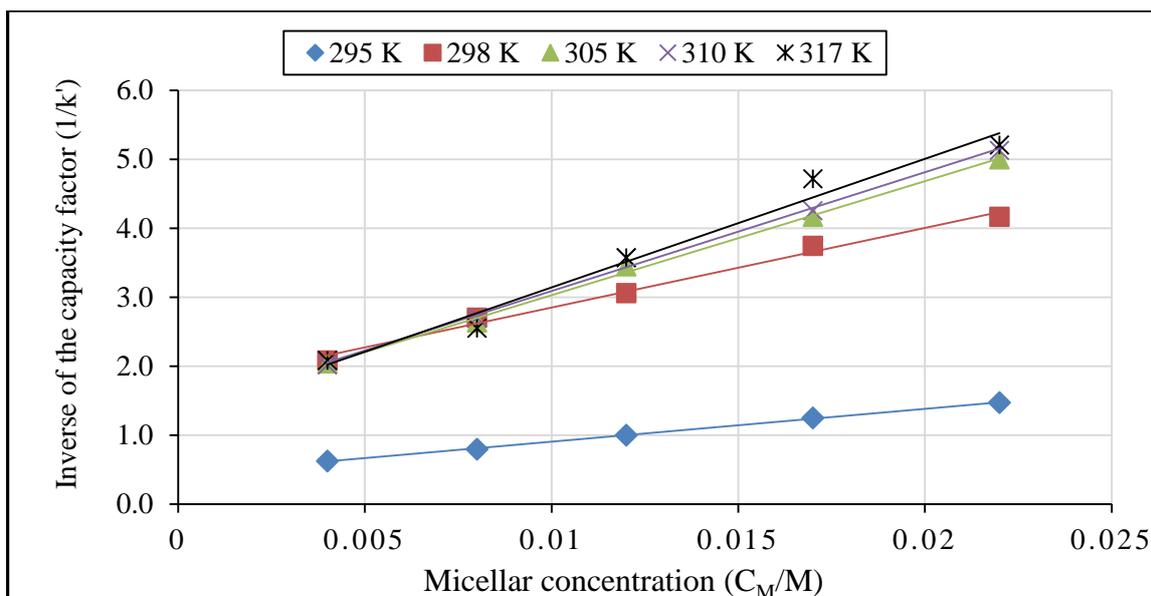


Figure 6.4: Plots of inverse of the capacity factors vs. micellar concentration for ibuprofen at various temperatures

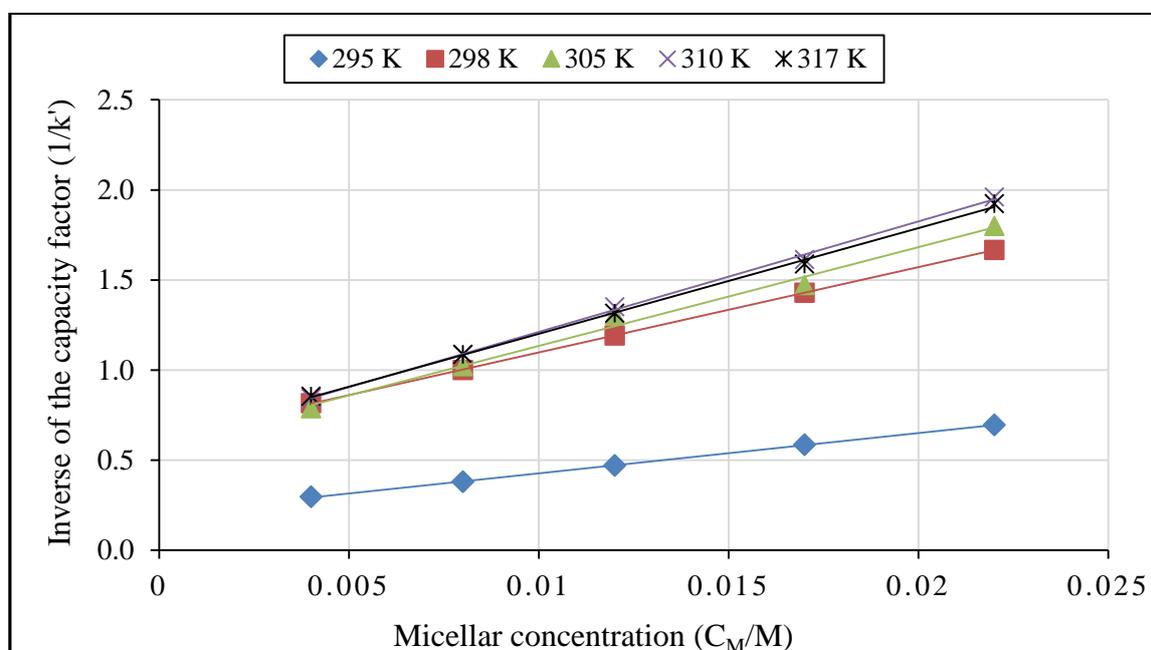


Figure 6.5: Plots of inverse of the capacity factors vs. micellar concentration for ketoprofen at various temperatures

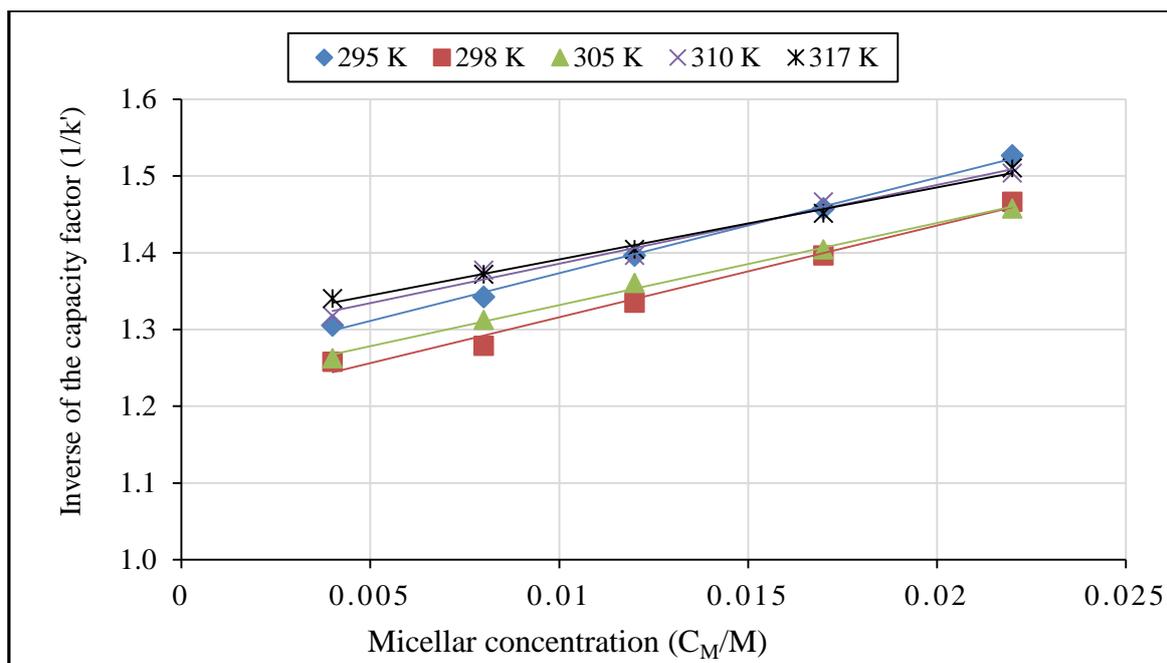


Figure 6.6: Plots of inverse of the capacity factors vs. micellar concentration for paracetamol at various temperatures

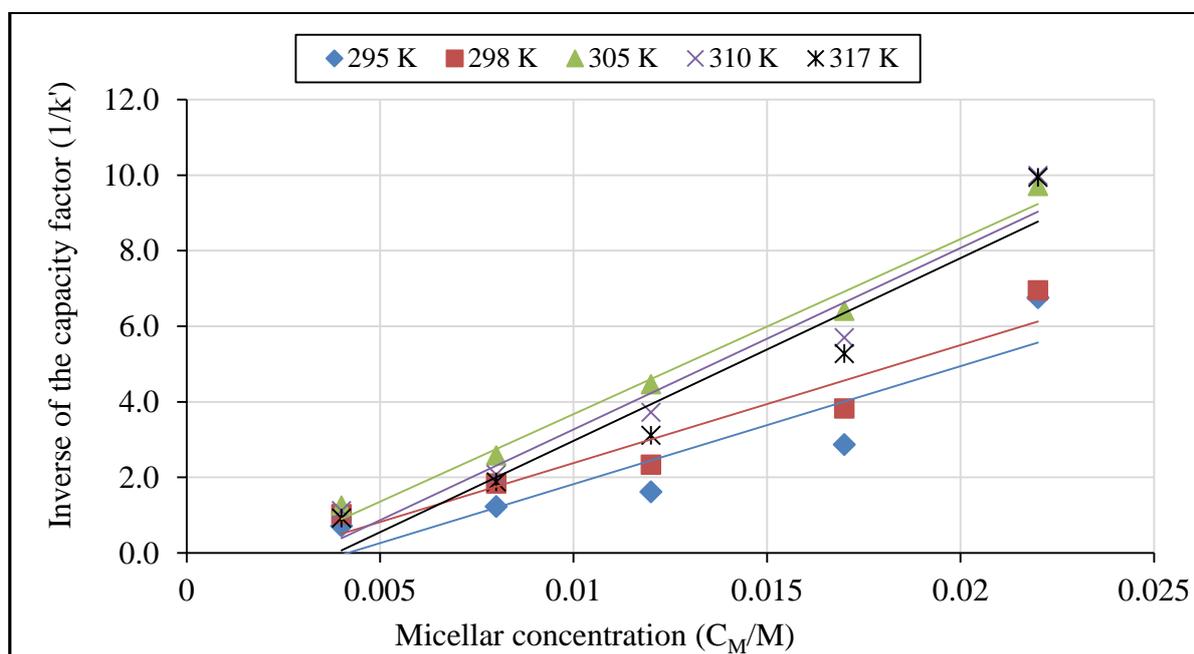


Figure 6.7: Plots of inverse of the capacity factors vs. micellar concentration for procaine at various temperatures

Table 6.1 represents the micelle-water partition coefficients at each studied temperature along with the slopes and intercepts of the linear relationships of $1/k'$ vs. C_M .

Table 6.1: Partition coefficient measurement at various column temperatures

Drugs	Temp./K	Slope	Intercept	R²	P_{MW}	LogP_{MW}
Fenbufen	295	32.1	0.24	0.992	133.8	2.13
	298	105.4	0.82	0.999	128.5	2.11
	305	113.3	0.85	0.998	133.3	2.12
	310	119.8	0.80	0.996	149.8	2.18
	317	118.6	0.74	0.999	160.3	2.20
Fenoprofen	295	33.3	0.35	0.997	95.1	1.98
	298	116.4	1.00	0.998	116.4	2.07
	305	115.5	1.18	0.976	97.9	1.99
	310	111.0	1.14	0.984	97.4	1.99
	317	122.0	0.98	0.992	124.5	2.10
Flurbiprofen	295	38.5	0.35	0.999	110.0	2.04
	298	137.5	1.12	0.992	122.8	2.09
	305	135.8	1.14	0.992	119.1	2.08
	310	158.2	1.05	0.996	150.7	2.18
	317	152.3	0.93	0.953	163.8	2.21
Ibuprofen	295	47.7	0.43	0.999	110.9	2.04
	298	115.5	1.69	0.991	68.3	1.83
	305	165.4	1.37	0.998	120.7	2.08
	310	172.3	1.36	0.978	126.7	2.10
	317	186.6	1.28	0.996	145.8	2.16

Table 6.1: continued....

Drugs	Temp./K	Slope	Intercept	R²	P_{MW}	LogP_{MW}
Ketoprofen	295	22.3	0.20	0.999	111.5	2.05
	298	47.4	0.62	1.000	76.5	1.88
	305	54.8	0.59	0.989	92.9	1.97
	310	61.3	0.60	0.998	102.2	2.01
	317	58.7	0.61	0.999	96.2	1.98
Paracetamol	295	12.4	1.24	0.997	10.0	1.00
	298	11.9	1.19	0.985	10.0	1.00
	305	10.7	1.22	0.996	8.8	0.94
	310	10.2	1.28	0.982	7.9	0.90
	317	9.73	1.30	0.992	7.5	0.87
Procaine	295	284.5	0.84	0.832	338.7	2.53
	298	345.7	1.65	0.911	209.5	2.32
	305	518.5	2.38	0.985	217.9	2.34
	310	523.7	2.73	0.947	191.8	2.28
	317	536.0	3.28	0.923	163.4	2.21

Figure 6.8 represents slopes obtained from the plots of $1/k'$ vs. C_M for five drugs, namely fenbufen, fenoprofen, flurbiprofen, ibuprofen and ketoprofen at five different column temperatures. An increase in column temperature does increase the slope, however, a clear correlation was not observed in the case of fenoprofen and flurbiprofen. In fact, these drugs have shown a more complex behaviour at elevated column temperatures. On the contrary, slopes for fenbufen, ibuprofen and ketoprofen either remained almost unchanged above 298 K, or increased as seen in the case of ibuprofen. A more general perception could be taken from these relationships that, more or less, a slight increase in slope was seen in all cases above 298 K.

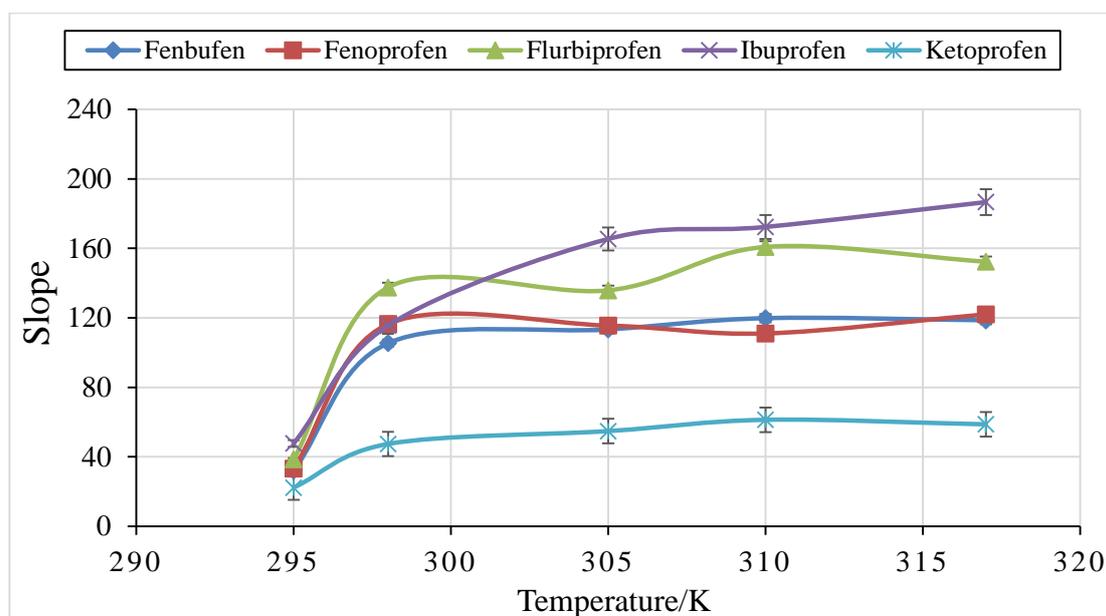


Figure 6.8: Comparison of slopes obtained from $1/k'$ vs. C_M plots for five anionic drugs over a range of temperatures

Previously it was reported that an increase in the slope or intercept with respect to column temperature generally represents preference of a solute for the bulk aqueous phase over micellar pseudophase (Tomasella and Love, 1990). Taking into consideration this fact, an increase in the slope values obtained here would mean that anionic drugs considered in this

study favoured the bulk aqueous phase at the studied range of column temperatures. If this was true, longer retention times would be achieved at elevated temperatures because of the fact that more the drug in the bulk aqueous phase, the more is available to interact with the cyanopropyl based stationary phase and less contribute to the micelle-water partition coefficient. On the contrary, a decrease in retention time of five anionic drugs was recorded which shows preference of these drugs for the micellar pseudophase. In line with the findings described in Chapter 3, adsorption of SDS on cyanopropyl columns is far less than the conventional ODS columns (C1, C8 and C18) (Berthod et al., 1986), therefore, justifying the outcomes of this study. The retention mechanism at ambient temperature (295 K) was quite different from that seen at elevated column temperatures. Longer retention times were seen at an ambient temperature, which showed preference for the bulk aqueous phase. These results are graphically illustrated in Figures 6.1 – 6.5.

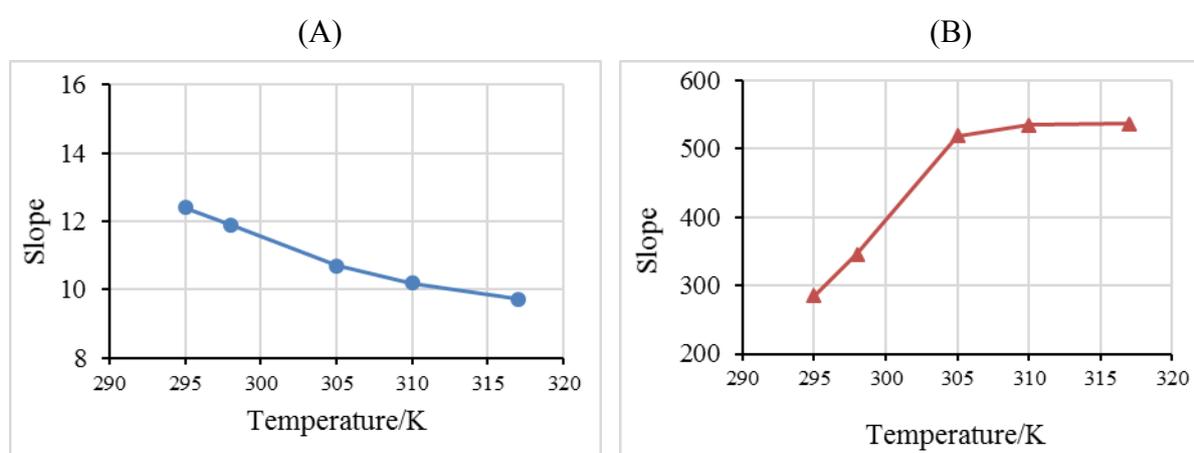


Figure 6.9: Slopes obtained from $1/k'$ vs. CM plots for paracetamol (A) and procaine (B) over a range of temperatures

Paracetamol, a neutral drug, interacted differently with the micelles and the cyano bonded stationary phase. An increase in column temperature had almost no effect on the retention of this drug with a slight decrease in the slope values. A slight decrease in partition coefficient (see Table 6.1) at an elevated column temperature shows a preference of paracetamol for the bulk aqueous phase. In the case of the cationic drug, procaine, results obtained in this study

were in line with those previously reported where an increase in slope (Figure 6.9 B) showed a preference for the bulk aqueous phase (Tomasella and Love, 1990). At an elevated column temperature, procaine showed slightly higher affinity for the bulk aqueous phase which resulted in a marginal decrease in the partition coefficient (Table 6.1).

One of the objectives of this study was to evaluate thermodynamic parameters including the change in free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) values as the solutes transfer from the bulk aqueous phase to the micellar pseudophase as a function of temperature. Under linear chromatographic conditions, the temperature dependence of the partition coefficient of a given solute can be expressed by replacing k' with P in the van't Hoff equation (6.1) (Coym and Dorsey, 2004):

$$\ln P = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \ln \varphi \quad (6.3)$$

where P is the partition coefficient of a solute between the two phases, ΔH and ΔS are the enthalpy and entropy change associated with the transfer of solute from one phase to another, R is the gas constant, T is the temperature, and φ is the volume phase ratio, which is the ratio of volume of stationary phase to the volume of mobile phase. Berthod and coworkers observed that the adsorbed surfactant on the bonded phase remains constant with micellar mobile phases (Berthod et al., 1986). Therefore, the volume phase ratio (φ) would remain constant and can be neglected from Eq. (6.3). Thus, the slope of the plot between the natural logarithm of P_{MW} and $1/T$ would give the enthalpy of solute transfer as given by the following equations (Tomasella and Love, 1990):

$$\text{Slope} = -\frac{\Delta H}{R} \quad (6.4)$$

and

$$\text{Intercept} = \frac{\Delta S}{R} \quad (6.5)$$

A distinct nonlinearity in van't Hoff plots was observed when the inverse of the temperature ($1/T$) was plotted against the natural logarithm of micelle-water partition coefficient ($\ln P_{MW}$) as shown in Figure 6.10. Nonlinear van't Hoff plots indicate a temperature dependence of the partitioning of drugs from bulk aqueous phase to the micellar pseudophase.

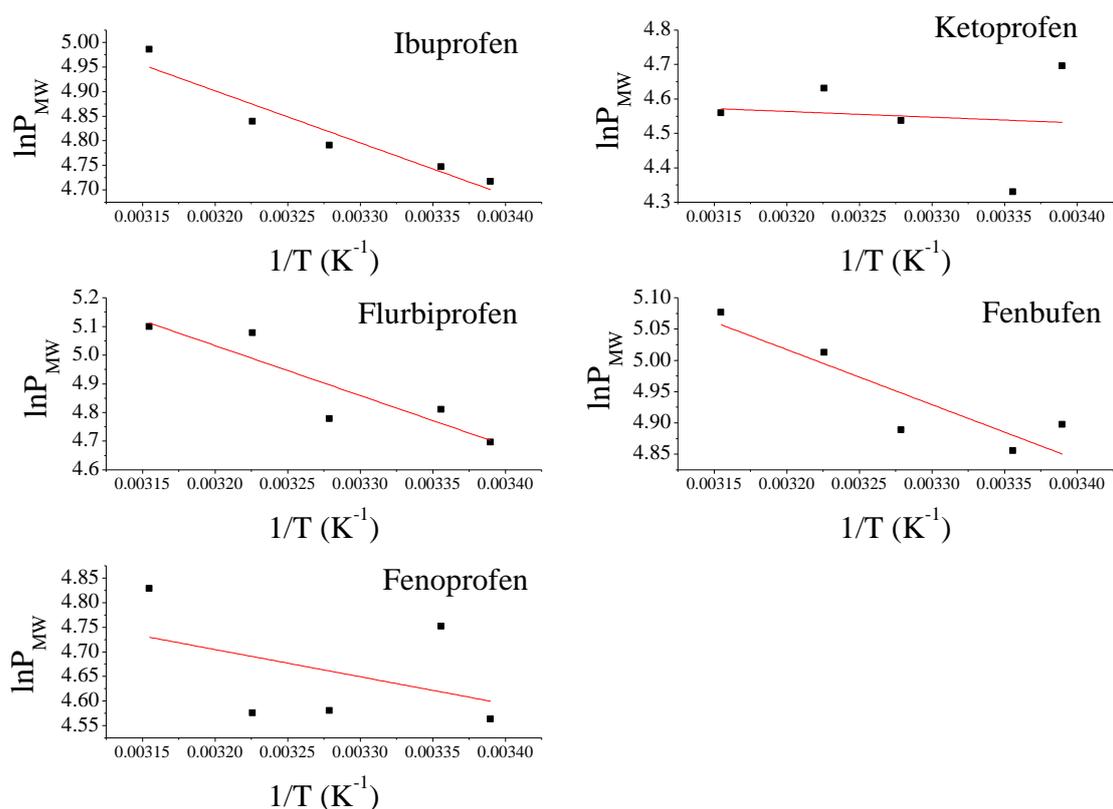


Figure 6.10: van't Hoff plots for five drugs at 295 K, 298 K, 305 K, 310 K and 317 K

However, an improved linearity was achieved when two data points (298K and 305 K) were excluded and $\ln P_{MW}$ was plotted at three temperatures (295 K, 310 K and 317 K) which led to the calculation of thermodynamic parameters of interest. Figures 6.11 – 6.16 are graphical representations of the van't Hoff plots at various temperatures.

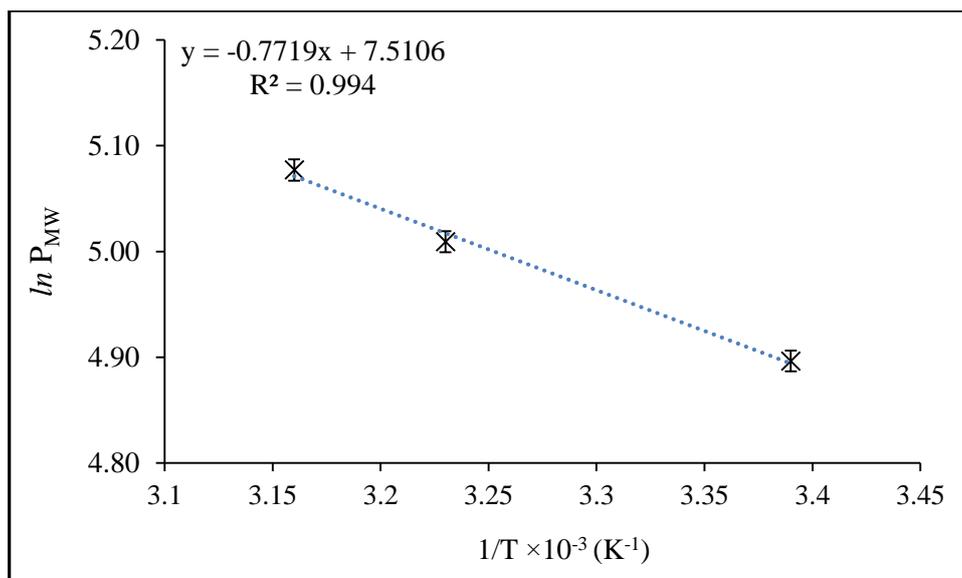


Figure 6.11: van't Hoff plot for fenbufen at 295 K, 310 K and 317 K

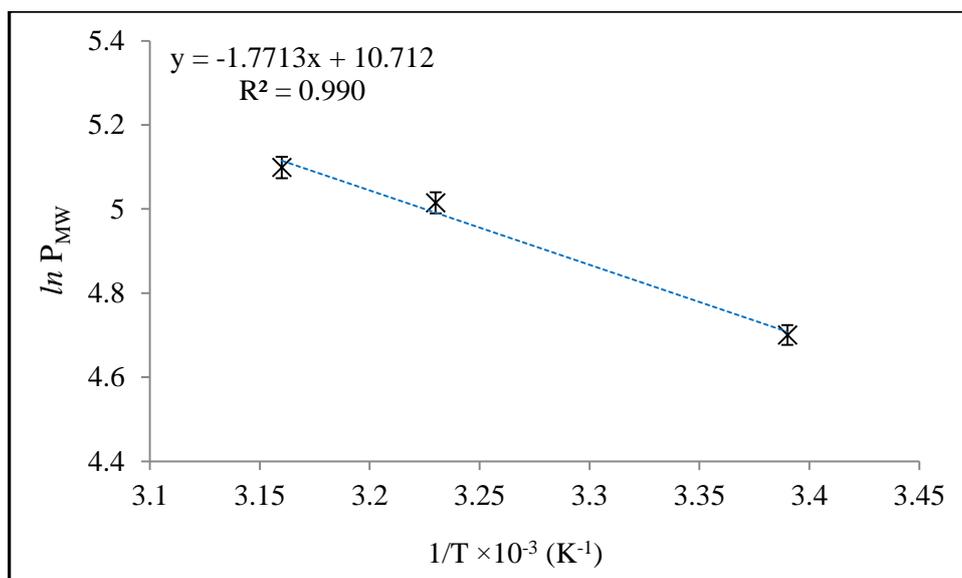


Figure 6.12: van't Hoff plot for flurbiprofen at 295 K, 310 K and 317 K

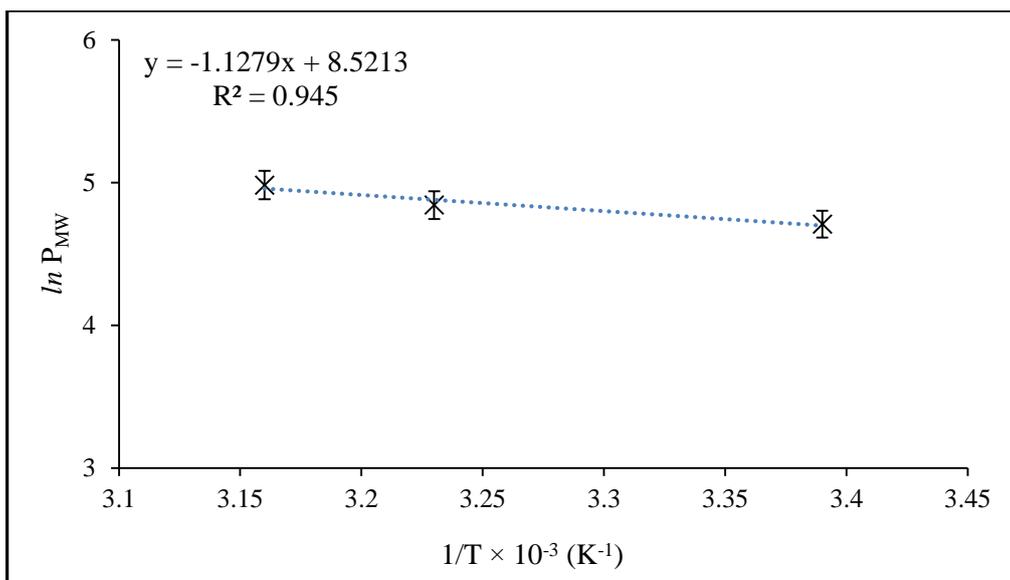


Figure 6.13: van't Hoff plot for ibuprofen at 295 K, 310 K and 317 K

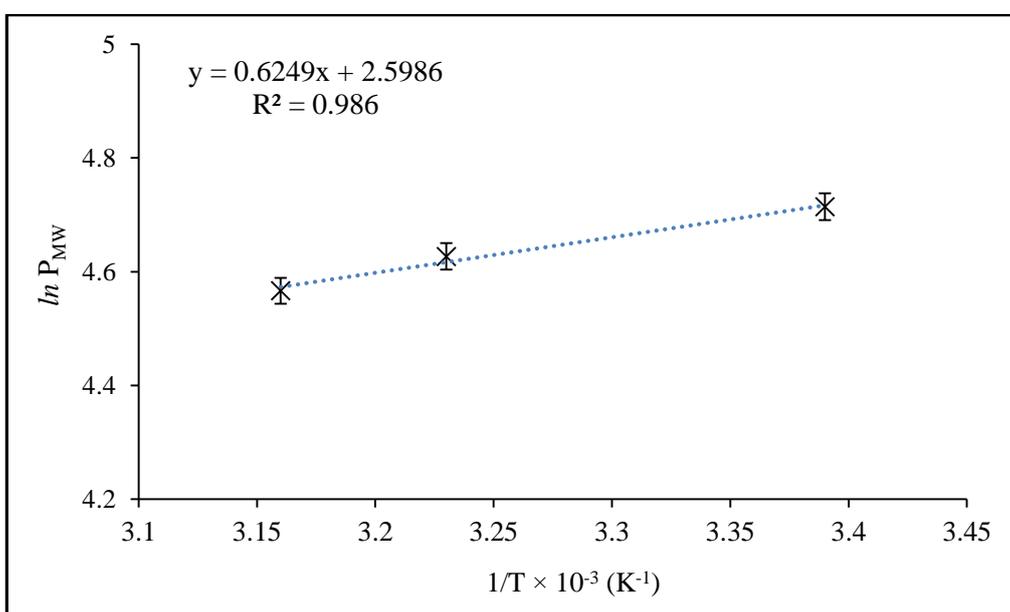


Figure 6.14: van't Hoff plot for ketoprofen at 295 K, 310 K and 317 K

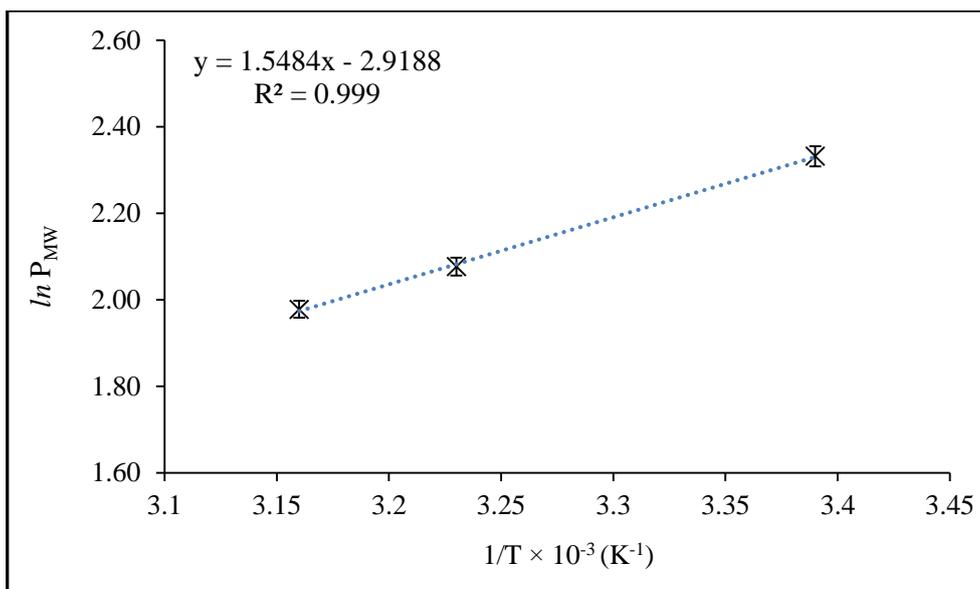


Figure 6.15: van't Hoff plot for paracetamol at 295 K, 310 K and 317 K

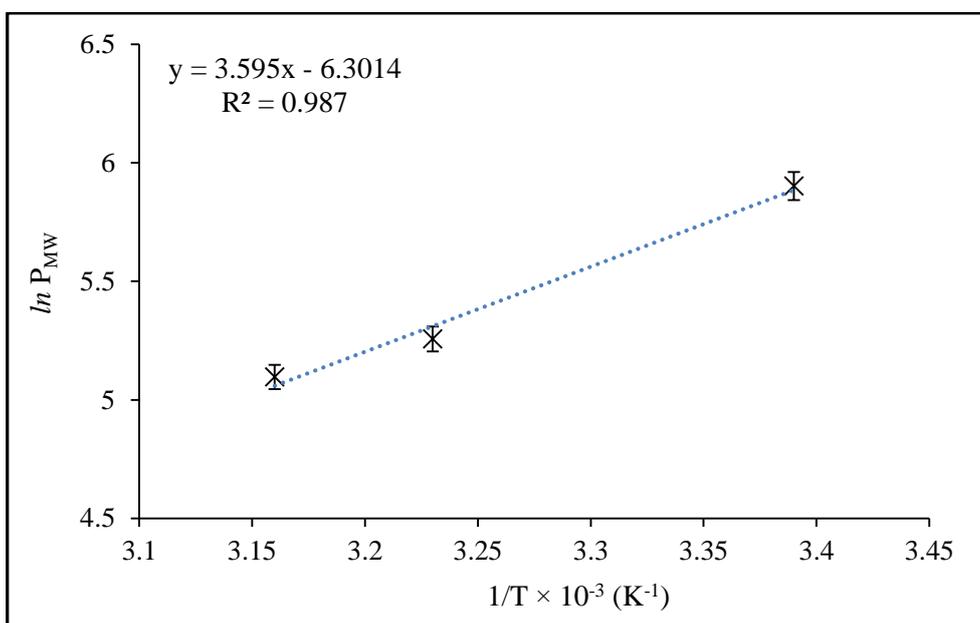


Figure 6.16: van't Hoff plot for procaine at 295 K, 310 K and 317 K

The thermodynamic parameters obtained from van't Hoff plots are summarised in Table 6.2. The ΔG values were negative for all compounds indicating that the partitioning of

these compounds from the bulk aqueous phase to the micellar pseudophase was thermodynamically favoured. Since the van't Hoff plots were presumably independent of the phase ratio (ϕ), the linearity would suggest a constant enthalpy of transfer from the bulk aqueous phase to the micellar pseudophase at three temperatures (295 K, 310 K and 317 K). Higher values of the change in enthalpy (ΔH) as compared to entropy (ΔS) suggest that the partitioning process was enthalpically driven. Fenbufen, flurbiprofen and ibuprofen showed positive enthalpies which were attributed to a less hydrophobic interaction with the stationary phase as these solutes were largely in the core of the micelles (Lee and Hoffman, 1994). However, negative enthalpy values of ketoprofen, paracetamol and procaine demonstrated hydrophobic interactions with the stationary phase because of an increasing preference for the bulk aqueous phase for these compounds.

Table 6.2: Change in Gibbs free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) of various compounds

Drugs	ΔG (kJ. mol ⁻¹)	ΔH (kJ. mol ⁻¹)	ΔS (kJ. mol ⁻¹ . K ⁻¹)
Fenbufen	-12.77 ± 0.69	6.42 ± 0.79	0.062 ± 0.001
Flurbiprofen	-12.42 ± 0.81	14.73 ± 2.30	0.089 ± 0.009
Ibuprofen	-12.80 ± 0.70	9.38 ± 1.19	0.071 ± 0.007
Ketoprofen	-11.55 ± 0.45	-5.20 ± 0.61	0.022 ± 0.002
Paracetamol	-5.43 ± 0.19	-12.87 ± 1.39	-0.024 ± 0.009
Procaine	-14.64 ± 0.63	-29.88 ± 2.79	-0.052 ± 0.009

Previous studies have demonstrated that a significant amount of water is present in palisade and stern layers of conventional micelles (El Seoud, 1997; Mukerjee and Ko, 1992).

The chemistry on, and around, the surface of the micelle is influenced through the water molecules residing in the palisade and stern layers of the micelle. It has been suggested that these water molecules can influence the polarity and the hydrogen bonding character of the micelle (Mukerjee and Ko, 1992; Poole and Poole, 1997). Additionally, all the compounds have hydrogen bond donating and/or hydrogen bond accepting functional groups on the molecules, hydrogen bonding is believed to play a more significant role than the hydrophobic interaction (Akbay et al., 2007). Therefore, the chemistry on, and around, the micellar surface can be altered by temperature. An increase in temperature may increase the ionisation of the water molecules as well as of the compounds, thus the interactions between the micelles, stationary phases and the solutes can be significantly influenced by temperature.

The entropy values were negative for paracetamol and procaine which shows their preference for the aqueous phase. In contrast, positive entropy values were obtained for anionic drugs, namely fenbufen, flurbiprofen, ibuprofen and ketoprofen. This can be attributed to the contribution from hydrophobic interactions between the solutes and the micellar pseudophase. It is reasoned that the presence of a hydrophobic solute forces the water molecules surrounding the solute to form a dense, ordered network of hydrogen bonds with each other to minimise their contact with the hydrophobic solute within the palisade or stern layer of the micelle, thereby, lowering the total entropy of the aqueous system. This ordered network of water molecules will disappear upon transfer of hydrophobic solutes from the polar aqueous solution into the relatively hydrophobic stationary phase. Consequently, the total entropy of the system increases for anionic drugs compared with neutral (paracetamol) and cationic (procaine) drugs.

6.3. Conclusions

This study evaluates the impact of elevated column temperature on the partitioning of compounds of varying physicochemical properties. This allowed determination of thermodynamic parameters involved in the partitioning process. Negative ΔG values indicated partitioning as a spontaneous exothermic process. The partitioning of seven drugs considered in this study were enthalpically driven with a small change in the entropy (ΔS) of the system which was possibly because of the change in the nature of hydrogen bonding of water at elevated temperatures and the hydrophobic interactions presented by the drugs and the micelle.

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Chapter 7: Modeling skin permeability with micellar liquid chromatography

**Previous chapters described MLC based micelle-water partition coefficients as a potential alternative to the traditional octanol – water method. This chapter highlights the practical applicability of this newly developed system in a more real world scenario. The data acquired using MLC will be used in developing a quantitative relationship with human skin permeability and other physicochemical properties of commonly used pharmaceutical compounds. The derived relationship will then be compared with the established quantitative relationships which are built upon the traditional octanol-water partition coefficients.*

7.1. Introduction

The skin, together with the mucosal linings of the digestive and respiratory tract, is the largest organ of the human body and protects the internal body structure from external hostility such as micro-organism entry, radiation as well as limiting water loss from the body (Meidan, 1996; Silva et al., 2012). Transdermal delivery of pharmaceutical compounds has remained a challenging domain for pharmaceutical scientists over the years. Generally, it was assumed that skin is impermeable to pharmaceutical compounds especially ionic species (Sun et al., 2008). However, with an advancement in understanding the structural properties of skin, penetration of various ionic compounds has been documented in the 1950's and 1960's (Blank et al., 1957; Malkinson, 1958; Stoughton et al., 1960; Stoughton, 1962).

The skin consists of three major layers: the epidermis, the dermis and subcutaneous fat or hypodermis (Figure 7.1). The epidermis is the outermost layer of the skin which is

* Sections of this chapter have been published, see Appendix A

characterised as the thinnest part of the skin and its thickness varies depending upon the location of the skin. It is further divided into 5 distinct layers: the stratum corneum or horny layer; the stratum lucidum; the stratum granulosum; the stratum spinosum; and the stratum basale. The stratum corneum is composed of compact, dehydrated and keratinised multi-layered cells that are embedded in a matrix of lipid lamellas that descend from the secreted content of the lamellar bodies, which gives the stratum corneum a brick and mortar organisation (Moss et al., 2002). Although it is the thinnest and smallest compartment of the skin it represents the main barrier function of the skin (Barry, 1983).

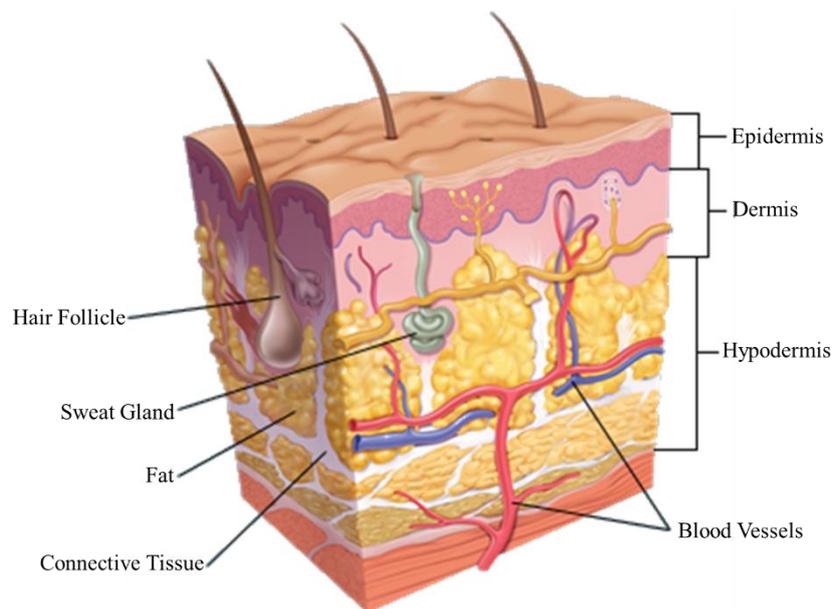


Figure 7.1: Structure of the skin (Barry, 1983)

The dermis is connected by the basement membrane to the stratum basale. This junction is of folded appearance, supporting the exchange of nutrients and other physiological substances with the unvascularised epidermis. The main components of the dermis are collagen and elastin fibers that form a vast network of filamentous and amorphous connective tissue that provides strength and flexibility to the skin (Schaefer and Redelmaier, 1996). Furthermore, the dermis accommodates cellular residents such as fibroblasts, endothelial cells, mast cells and,

under conditions of inflammation, macrophages, lymphocytes and leucocytes. The tissue of the dermis has an extensive vascular network that is involved in nutrition, thermal regulation and immune responses. A variety of appendages, such as hair follicles or sweat and sebaceous glands are also derived from this tissue. Underneath the dermis is the hypodermis that contains subcutaneous fats which represent a source of energy for the body. It provides support and mechanical cushioning for the overlying skin. It also acts as a thermal insulator and a shock absorber (Moss et al., 2002).

In recent years, skin has been extensively studied as an alternative route for drug delivery. Transdermal drug delivery facilitates the passage of a drug substance through the skin into the general circulation where it can have systemic effects, thus bypassing the hepatic first-pass effect. This route offers several other advantages over the oral route; for example, avoidance of untoward gastro-intestinal effects, controlled drug release over a long period of time and patient compliance (Shahzad et al., 2012; Silva et al., 2012).

7.2. Modelling skin permeation

An ideal situation for a pharmaceutical research and development setup is to predict the percutaneous absorption of a potential drug candidate from its physicochemical and structural properties. This is important because thousands of new compounds (which could be potential drug candidates) are being synthesised and extensively tested through various *in vitro* and *in vivo* models. However, these initial measurements can take a long time and face a series of ethical, economical and legislative difficulties; for example, ethical approval is required when animals or humans are involved in the experiments and there is increasing legislation in the risk assessment of industrial chemicals (Martínez-Pla et al., 2003; Moss et al., 2009; Chen et al., 2010). To overcome these difficulties, several mathematical models have been proposed to predict skin permeability of compounds from their structural parameters (Potts and Guy, 1992;

Potts and Guy, 1995; Lien and Gao, 1995; Barratt, 1995b; Abraham et al., 1995a; Abraham et al., 1997). These mathematical models are based on quantitative structure-permeability relationships (QSPR) correlating experimental skin permeability to physicochemical properties such as an octanol – water partition coefficient (logP), molecular weight (MW), melting point (MP), molecular volume (MV), hydrogen bond donor and acceptor, polarity and other molecular structure descriptors.

The quantitative structure-permeability relationships not only help in understanding the science behind skin permeation but also provide an invaluable help in analysing the data that results from the mechanistic studies of skin permeation. Developing a QSPR usually occurs in three distinct steps:

1. Gathering biologically relevant data

The foremost and the most important part in any QSPR development is to acquire a set of biological relevant data that has been obtained during real time *in vitro* or clinical investigations. The quality, applicability and predictability of QSPR rely upon the source data.

2. Gathering physicochemical properties and molecular descriptors

With respect to skin permeation, QSPR model development is often dependent on the physicochemical properties and/or structural and molecular descriptors of a permeant. Some of the important and routinely used physicochemical and molecular properties are:

A. Lipophilicity

Lipophilicity is the single most informative physicochemical property in medicinal chemistry and also a meaningful parameter in quantitative structure-activity relationships (QSARs) (Geinoz et al., 2004), for example prediction of skin permeability (Tayar et al., 1991).

B. Molecular size

Molecular size, either in terms of molecular weight (MW) or molecular volume (MV), plays a distinct role in membrane permeation (Xiang and Anderson, 1994, Bunge and Cleek, 1995). Generally speaking, in simple liquid systems, drug diffusivity (D_i) is inversely dependent on the size of the permeant whilst an exponential relationship exists in organised media such as polymers and the stratum corneum (Kasting, 1987). This relationship can be expressed mathematically by Eq. (7.1):

$$D_i = D_o \cdot e^{-B \cdot (MV)} \quad (7.1)$$

Where D_o is the diffusivity of a hypothetical molecule having zero molecular volume, and B is a constant. This implies that drug diffusivity across the stratum corneum decreases with an increase in MV or MW, as both of these are interdependent.

C. Solvatochromic parameters

A linear solvation free-energy relationship (LSER) is the most widely used QSPR approach applied to develop reliable predictive models (Kamlet and Taft, 1976; Taft and Kamlet, 1976; Taft et al., 1985). These predictive models are based on molecular descriptors that are fundamental in predicting molecular properties in the areas of practical and academic interest. These descriptors may be experimentally determined properties or theoretically derived quantities (Panayiotou, 2012). LSER correlates a given molecular property (S_p), for example partitioning, retention, or permeation (Pagliara et al., 1997; Pagliara et al., 1995; Abraham et al., 1994) with the solvatochromic parameters as follows:

$$\log S_p = v \cdot V_w + p \cdot \pi^* + a \cdot \alpha + b \cdot \beta + c \quad (7.2)$$

Where v , p , a , and b are the regression coefficients while c is a constant that includes the information on the analysed system. V_w is van der Waals volume, π^* is dipolarity/polarisability, α and β are the hydrogen-bond donor acidity and hydrogen-bond acceptor basicity, respectively.

It should be noted that Eq. 7.2 deals with the process that includes only neutral solutes. More recently, Abraham and Acree have explained the importance of ionic species in LSER analysis (Abraham and Acree Jr, 2010a) and they have incorporated additional terms that refer to ion-solvent interactions for cations and anions i.e. j^+j^+ for cations and j^-j^- for anions, as shown in Eq. (7.3):

$$\log S_p = v. V_w + p. \pi^* + a. \alpha + b. \beta + c + j^+ j^+ + j^- j^- \quad (7.3)$$

The improved LSER model (with the addition of ionic species) has proven to be a better predictive model for the partition of solutes in water/organic solvents (Abraham and Zhao, 2005; Abraham and Acree Jr, 2010b) and in water/liposome systems (Zhang et al., 2011) as well as permeation through the blood-brain-barrier (Abraham, 2011).

D. Other molecular properties

In addition to the above mentioned properties, there are few other parameters that have been used in developing QSPR. For example the solubility parameter (SP), polar surface area (PSA) (which is directly related to the hydrogen bonding of the solute with the solvent) and melting points (MP) of the solutes have been documented (Sloan et al., 1986; Riviere and Brooks, 2005). The solubility parameter is related to the solubility of a permeant in the stratum corneum of the skin. A molecule that is highly soluble in the stratum corneum might not be soluble in other layers of the skin; hence it might have limited

skin permeation. Therefore, the use of SP will provide an indication of lipophilic compounds likely to exhibit this behaviour (Sun et al., 2008).

3. Modeling techniques

The final step in developing a QSPR model is the technique or method used to create a link between experimental or clinical data of a series of compounds and their physicochemical properties and/or molecular and structural descriptors. This is usually achieved by employing a range of statistical and mathematical techniques including multiple linear regression analysis, multiple non-linear regression analysis, least squares analysis, neural network algorithms and Gaussian algorithms (Livingstone, 1995).

7.3. Methodology

7.3.1. Data mining and generation of descriptors

The physicochemical properties and molecular descriptors, along with the published permeability data, for twenty two compounds were acquired through data mining of the literature available along with the use of ACD/labs database services at Daresbury, UK. Results from the MLC technique permitted the development of a dataset that contained $\log P_{MW}$ values for 22 different compounds along with their physicochemical parameters, such as molecular weight (MW) and published permeability values (K_p).

Physicochemical properties of interest for all the compounds considered in this work are presented in Table 7.1 while a general scheme of modeling methodology is presented in Figure 7.2.

Table 7.1: Physicochemical parameters of selected compounds

Drug	Experimental log <i>K_p</i>	Literature log <i>P_{ow}</i>	Log <i>P_{MW}</i>	MW	<i>pK_a</i>	MP	HA	HD	<i>V_M</i>	PSA
Urea	-3.8 (Martínez-Pla et al., 2003)	-2.11 (Martínez-Pla et al., 2003)	-0.5	60.1	14.3	134.0	3	4	49.5	69.1
Thiourea	-4.02 (Martínez-Pla et al., 2003)	-0.95 (Martínez-Pla et al., 2003)	-0.2	76.1	13.0	182.0	2	4	57.3	84.3
Nicotinic acid	-4.6 (Dal Pozzo et al., 1991)	0.40 (Dal Pozzo et al., 1991)	-0.3	123.1	4.8	236.6	3	1	95.1	50.2
Methyl nicotinate	-2.05 (Le and Lippold, 1995)	0.80 (Le and Lippold, 1995)	1.13	137.1	3.2	39.0	3	0	120.5	39.2
Salicylic acid	-2.2 (Assessment, 1992)	2.26 (Assessment, 1992)	0.43	138.1	13.1	159.0	3	2	100.3	57.5
Ethyl nicotinate	-2.21 (Dal Pozzo et al., 1991)	1.30 (Dal Pozzo et al., 1991)	1.47	151.2	3.3	8.0	3	0	137.1	39.2
Acetaminophen	-2.75 (Assessment, 1992)	0.50 (Assessment, 1992)	0.69	151.2	9.8	170.0	2	2	105.4	49.3
Methyl Paraben	-1.21 (Nicoli et al., 2008)	1.96 (Nicoli et al., 2008)	1.15	152.1	8.4	127.0	3	1	125.7	46.5
Sodium salicylate	-4.26 (Martínez-Pla et al., 2003)	2.06 (Martínez-Pla et al., 2003)	-0.01	160.1	3.0	160.0	3	1	100.3	57.5
Ethyl paraben	-1.39 (Nicoli et al., 2008)	2.47 (Nicoli et al., 2008)	1.43	166.2	8.3	117.0	3	1	142.2	46.3
Butyl nicotinate	-1.48 (Le and Lippold, 1995)	2.27 (Le and Lippold, 1995)	1.84	179.2	3.2	5.0	3	0	170.1	53.0
Aspirin	-2.14 (Degim et al., 1998)	1.19 (Degim et al., 1998)	0.91	180.2	3.5	135.0	4	1	139.5	63.6
Caffeine	-3.50 (Lian et al., 2008)	-0.07 (Lian et al., 2008)	0.92	194.2	0.5	238.0	2	0	134.6	58.4
Ibuprofen	-1.4 (Degim et al., 1998)	3.57 (Waters et al., 2012)	1.74	206.3	4.4	76.0	2	1	200.3	37.3
Hexyl nicotinate	-1.09 (Le and Lippold, 1995)	3.50 (Le and Lippold, 1995)	1.91	207.3	3.2	5.0	3	0	203.1	39.2
Naproxen	-2.54 (Degim et al., 1998)	3.20 (Degim et al., 1998)	1.57	230.3	4.8	155.0	3	1	178.3	46.5
Lidocaine	-1.7 (Thomas et al., 2007)	2.40 (Thomas et al., 2007)	1.77	234.4	7.9	68.5	2	1	228.3	32.3
Flurbiprofen	-2.56 (Charoo et al., 2008)	3.70 (Waters et al., 2012)	1.68	244.3	4.1	112.0	3	1	203.6	37.3
Ketoprofen	-2.57 (Hadgraft et al., 2000)	2.70 (Waters et al., 2012)	1.46	254.3	4.2	96.0	3	1	212.2	54.4
Diclofenac	-3.1 (Hadgraft et al., 2000)	4.50 (Hadgraft et al., 2000)	1.64	296.2	3.9	157.0	3	2	182.9	49.3
Indomethacin	-3.15 (Hadgraft et al., 2000)	3.51 (Hadgraft et al., 2000)	1.88	357.8	4.2	155.0	4	1	269.5	68.5
Haloperidol	-4.04 (Azarbayjani et al., 2010)	3.49 (Azarbayjani et al., 2010)	1.57	375.9	8.3	151.5	4	1	303.2	40.5

*MW (g/mol) is the molecular weight; *pK_a* is the dissociation constant; MP (°C) is the melting point; HA and HD, number of hydrogen bond acceptor and donator respectively; PSA (Å²) is polar surface area and *V_M* (cm³.mol⁻¹) is the molar volume. All these descriptors were generated from ACD/Labs RSC, UK.

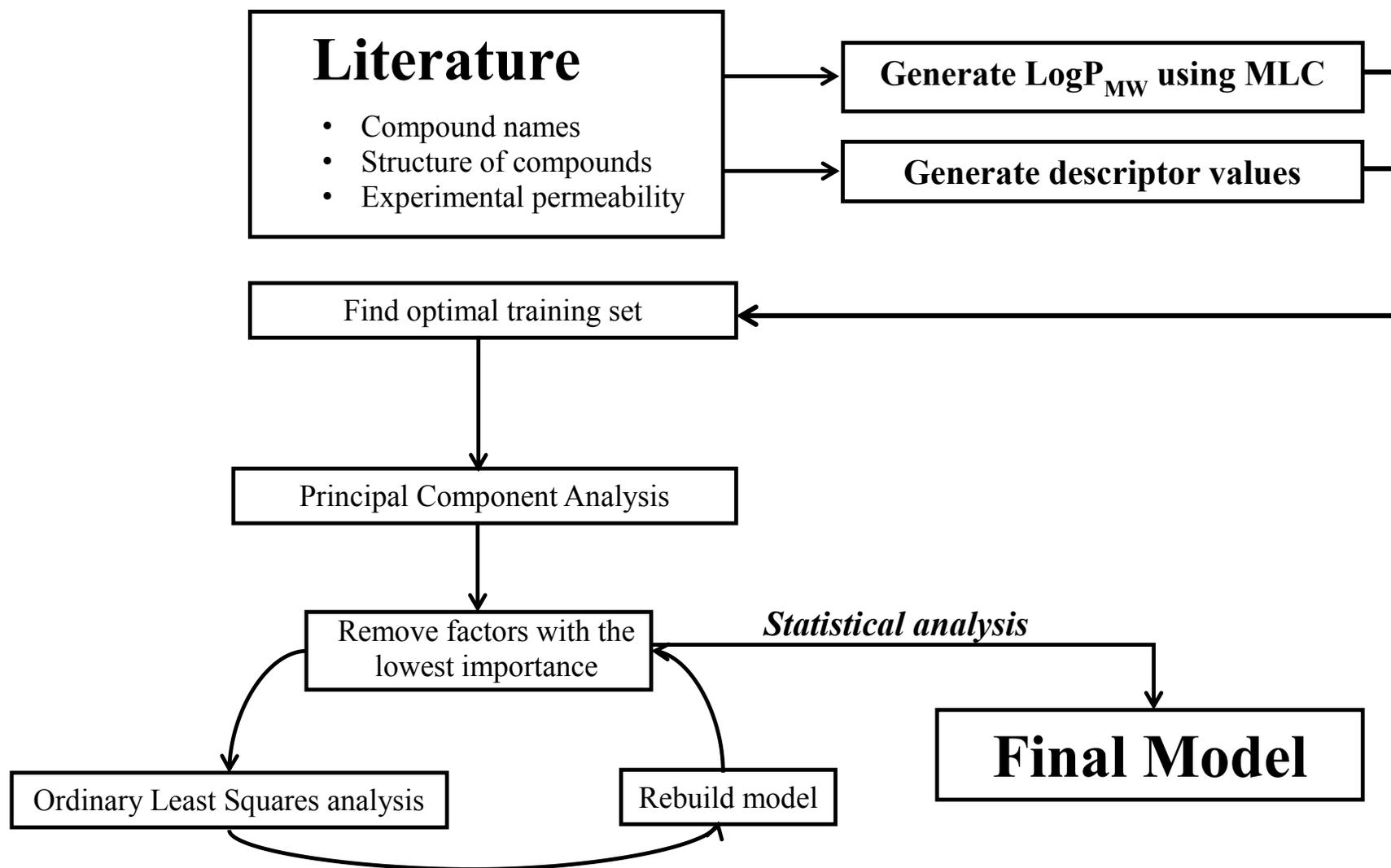


Figure 7.2: Schematic representation of modeling methodology

7.3.2. Inclusion of $\log P_{MW}$ in the Potts and Guy prediction model

Before developing a full-fledged prediction model, the present dataset of 22 compounds was fitted to the Potts and Guy prediction model (Potts and Guy, 1992) which holds the gold standard in modeling skin permeability based on the structure property relationship between physicochemical properties and permeability of compounds.

The Potts and Guy permeability model is expressed by the following equation:

$$\log K_P = -2.72 + 0.71 \times \log P_{OW} - 0.0061 \times MW \quad (7.4)$$

where K_P is the permeability coefficient, P_{OW} is the octanol – water partition coefficient and MW is the molecular weight.

This was achieved in two steps:

- (i) The data of 22 compounds were fitted to the Potts and Guy model equation using traditional octanol-water partition coefficients ($\log P_{OW}$).
- (ii) The data of 22 compounds were fitted to Potts and Guy model equation using recently developed micelle-water partition coefficients ($\log P_{MW}$).

7.3.3. Model development based on $\log P_{MW}$

Initially, two parallel regression analyses were conducted, with experimental K_P values regressed against $\log P_{MW}$ or $\log P_{OW}$ values. In both cases molecular weight was included as an additional covariate for consistency with previous analyses (Potts and Guy, 1992). The fit of both models to the data, and the predictive capability of both models (using the prediction error sum of squares (PRESS) statistic), were compared to determine the most appropriate partition coefficient for inclusion in a regression model.

Further multiple regression analyses were undertaken, considering additional recorded parameters in conjunction with the partition coefficient parameter found to provide the best summary of the data in the above models, to determine an optimum parsimonious model for the full complement of drugs in the data set. Checks for multicollinearity were performed on all variables before consideration for inclusion in a multiple model. Residual analysis was also undertaken to check the underlying regression assumptions. The variables remaining in the optimal model were assessed for significance and relative importance using standardised coefficients and associated p -values.

As a result of the relatively small sample size, and variations in the drugs selected for investigation between this and previous studies, cross-validation techniques were also employed to assess the predictive capability of the preferred regression model. These techniques included determination of the Wherry adjusted R^2 statistic and an approximate R^2 for prediction derived from the PRESS statistic, R^2_{PRED} , which can be used to compare a number of candidate model structures for the same data set. The Wherry statistic indicates how much variability in experimental K_p values would be accounted for from a prediction equation derived in the population from which the sample was drawn, but does not indicate how well the derived equation will predict on other samples of drugs from the same population. The PRESS-based R^2_{PRED} statistic measures the external predictive power of the model (i.e. how well the model is likely to predict responses in a new experiment).

Data splitting was not utilised as a cross-validation method, in order to avoid obtaining prediction equations based on the very small numbers of drugs; by contrast, the PRESS-based approach comprises 22 validations, each of sample size 21.

Drugs with potentially high influence on the regression model were identified by calculation of Studentised residuals, leverage statistics and Cook's distances for all drugs in the data set,

in order to provide insights of the suitability of the chosen drugs in the data set to derive the prediction model. A set of potentially highly influential drugs was derived, comprising drugs which met one or more of the following criteria:

- (i) Drugs whose Studentised residual lay in excess of 3 standard deviations from the mean (for a data set of the current size, it might be expected that about 1 observation would be in excess of 2 standard deviations from the mean);
- (ii) Drugs whose leverage value indicated it to be very different to other drugs in the sample (according to the commonly-applied criteria that a leverage value above $2(p+1)/n$, where p is the number of predictors in the model and n is the number of items in the sample). In the current data set, this would amount to any drug with a leverage of 0.272 or more, based on a set of leverage values which would be expected to have a mean value of 0.091;
- (iii) Drugs considered being potentially influential according to Cook's distance, D_i (assumed to be represented in drug i by an associated D_i value of greater than 1).

A sensitivity analysis using a reduced data set with potentially highly influential drugs was conducted, to determine whether these drugs had a detrimental impact on model predictive capability.

7.4. Results and discussion

Partition coefficient ($\log P_{MW}$) values for twenty two compounds considered in this study using MLC, as described in the previous chapters, along with the literature octanol-water partition coefficient ($\log P_{OW}$) values, published skin permeability data in terms of permeability coefficient ($\log K_p$) and other associated physicochemical parameters are listed in Table 7.1.

7.4.1. Relationship between experimental and predicted permeability using the Potts and Guy model

Initially, traditional octanol-water partition coefficients for 22 compounds were fitted to the Potts and Guy (P&G) model in order to investigate if this model holds predictive ability beyond the dataset originally used to develop the model. Fitting data to the Potts and Guy equation resulted in predicted permeability coefficient values which were then compared with the experimental permeability coefficient values in order to probe a relationship between experimental and predicted values. This was achieved by applying simple linear regression on experimental and predicted permeability coefficient values. A summary of the statistics is given in Table 7.2.

Table 7.2: Summary of the statistics

Goodness of Fit Statistics	P&G with logP_{ow}	P & G with logP_{MW}
<i>Observations</i>	22	22
<i>Sum of weights</i>	22	22
<i>DF</i>	20	20
<i>R²</i>	0.247	0.771
<i>Adjusted R²</i>	0.209	0.760
<i>MSE</i>	0.910	0.276
<i>Press RMSE</i>	1.027	0.579

It can be seen from the results of linear regression that a poor R^2 and adjusted R^2 statistics were achieved when the logP_{ow} of 22 compounds in the dataset was used in the P&G model for prediction of permeability coefficient. This implies that the P&G model is not an accurate predictor of skin permeability beyond the dataset originally used to construct this model. The Potts and Guy model was predominantly based on the dataset compiled by Flynn and co-workers (Flynn et al., 1990). This discrepancy has been highlighted by Lun Tak Lam

and co-workers in previous studies (Lam et al., 2010). They highlighted that the composition of the dataset has a significant effect on the outcome and nature of the derived QSPR model. Further to this argument, the dataset considered by Flynn and co-workers (Flynn et al., 1990) predominantly consisted of compounds with a $\log P_{ow}$ value of less than 2.0. This has been argued by Moss and co-workers (2009) that the constrained lipophilicity parameter in the dataset may provide a limited picture of skin permeability, thus, limits the applicability of the model.

Figure 7.3 depicts the relationship between experimental and predicted permeability coefficients while Table 7.3 summarises the predicted permeability coefficients as a result of inclusion of $\log P_{ow}$ in the Potts and Guy model. It can be seen from the Figure 7.3 that a poor correlation was achieved when permeability coefficients were predicted using octanol-water partition coefficients as a lipophilicity descriptor in the Potts and Guy model thus endorsing a limited predictive capability of this model.

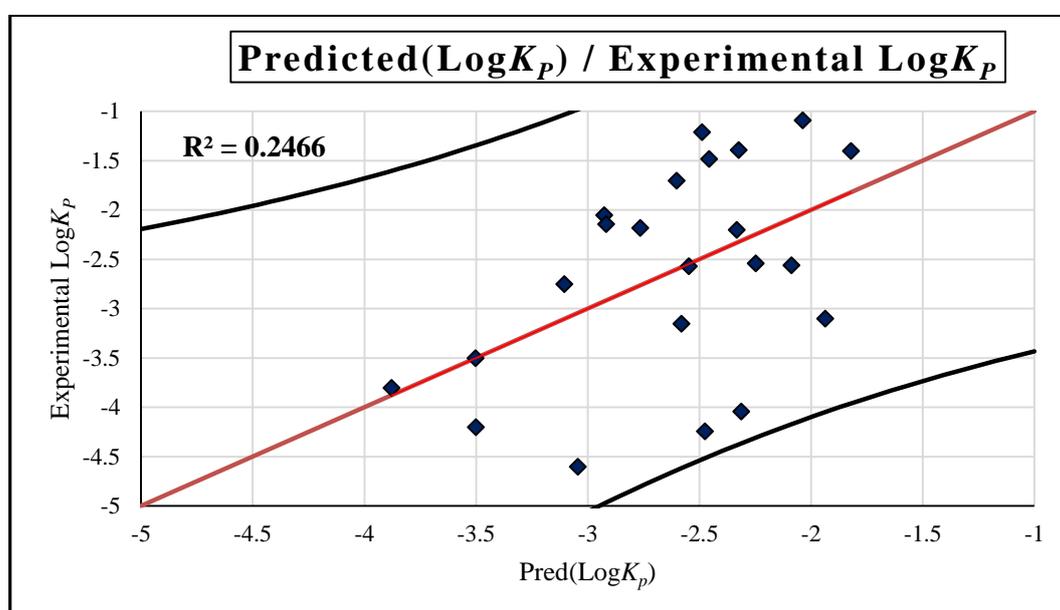


Figure 7. 3: Simple linear regression between experimental and predicted $\log K_P$ using Potts and Guy model after incorporation of $\log P_{ow}$

Table 7.3: Prediction using logP_{ow} in the Potts and Guy Model

Drug	Experimental logK_P	Predicted logK_P
Urea	-3.80	-4.58
Thiourea	-4.02	-3.95
Nicotinic acid	-4.60	-3.19
Methyl nicotinate	-2.05	-2.99
Salicylic acid	-2.20	-1.99
Ethyl nicotinate	-2.21	-2.72
Acetaminophen	-2.75	-3.29
Methyl Paraben	-1.21	-2.26
Sodium salicylate	-4.26	-2.23
Ethyl paraben	-1.39	-1.98
Butyl nicotinate	-1.48	-2.20
Aspirin	-2.14	-2.97
Caffeine	-3.50	-3.95
Ibuprofen	-1.40	-1.14
Hexyl nicotinate	-1.09	-1.49
Naproxen	-2.54	-1.85
Lidocaine	-1.70	-2.44
Flurbiprofen	-2.56	-1.58
Ketoprofen	-2.57	-2.35
Diclofenac	-3.10	-1.33
Indomethacin	-3.15	-2.41
Haloperidol	-4.04	-1.96

In the second step of analysis, logP_{ow} was replaced with the recently developed lipophilicity parameter, namely micelle-water partition coefficient (logP_{MW}). Subsequently, the predicted permeability coefficient values were linearly regressed against the experimentally determined permeability coefficient values. Statistical parameters, for example R² statistic, adjusted R² statistic and PRESS statistic, were considered to compare the prediction values. A summary of the statistics is given in Table 7.2.

Simple linear regression analysis revealed an improved correlation between experimental and predicted permeability coefficients as shown in Figure 7.4. The predicted permeability coefficient values are presented in Table 7.4. This confirmed that the $\log P_{MW}$ is a better predictor of skin permeability compared with the traditional $\log P_{OW}$ owing to a close mimic of biological partitioning. Therefore, these initial results were the basis of developing a full predictive model based on multiple linear regression analysis including $\log P_{MW}$ instead of $\log P_{OW}$ along with several other physicochemical and molecular descriptors.

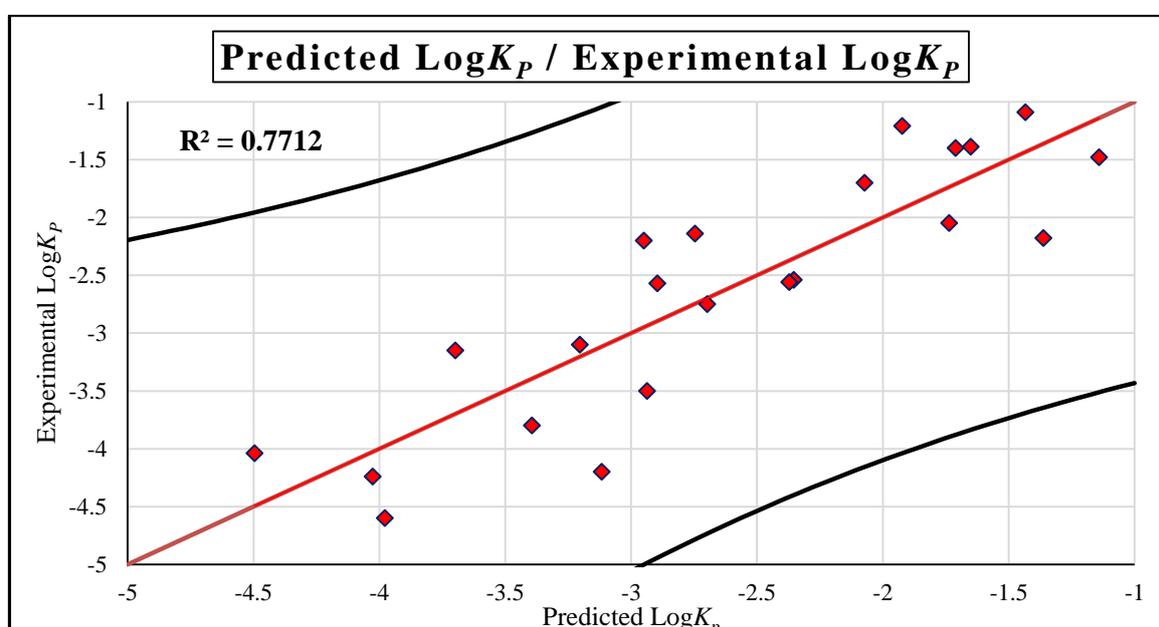


Figure 7. 4: Simple linear regression between experimental and predicted $\log K_p$ using Potts and Guy model after incorporation of $\log P_{MW}$

Table 7.4: Prediction using logP_{MW} in the Potts and Guy Model

Drug	Experimental logK _P	Predicted logK _P
Urea	-3.80	-3.44
Thiourea	-4.02	-3.32
Nicotinic acid	-4.60	-3.68
Methyl nicotinate	-2.05	-2.75
Salicylic acid	-2.20	-3.26
Ethyl nicotinate	-2.18	-2.59
Acetaminophen	-2.75	-3.15
Methyl Paraben	-1.21	-2.83
Sodium salicylate	-4.26	-3.70
Ethyl paraben	-1.39	-2.71
Butyl nicotinate	-1.48	-2.50
Aspirin	-2.14	-3.17
Caffeine	-3.50	-3.25
Ibuprofen	-1.40	-2.74
Hexyl nicotinate	-1.09	-2.62
Naproxen	-2.54	-3.01
Lidocaine	-1.70	-2.89
Flurbiprofen	-2.56	-3.02
Ketoprofen	-2.57	-3.23
Diclofenac	-3.10	-3.36
Indomethacin	-3.15	-3.56
Haloperidol	-4.04	-3.89

7.4.2. Development of new model using Multiple Linear Regression Analysis

Various physicochemical properties of drugs (Table 7.1) were considered for inclusion in a multiple regression model including logP_{MW} and molecular weight, and using a backward elimination modeling strategy. Molar volume was found to be very strongly correlated with molecular weight ($r = 0.940$, $p < 0.001$), and inclusion of both these parameters in a multiple

regression model led to unacceptably high levels of the variance inflation factor (VIF) statistic. Deletion of the molar volume parameter resulted in levels of the VIF within acceptable limits; hence this parameter was not considered further in any subsequent analysis. Simple regression models with either $\log P_{MW}$ as the sole covariate ($R^2 = 0.427$), $\log P_{OW}$ as the sole covariate ($R^2 = 0.135$), or molecular weight as the sole covariate ($R^2 = 0.001$) did not provide a particularly good summary of the data.

A regression analysis including only $\log P_{MW}$ and molecular weight as covariates was found to have an R^2 statistic of 0.829 (95 % confidence interval 0.716 – 0.914) and a PRESS statistic of 5.16. A regression analysis including only molecular weight and $\log P_{OW}$ as covariates was found to have an R^2 statistic of 0.350 (95 % confidence interval 0.072 – 0.628) and a PRESS statistic of 20.1. Hence $\log P_{MW}$ in the presence of molecular weight provided a substantially better summary of the data than $\log P_{OW}$ in the presence of molecular weight for the full complement of drugs in the study; and the PRESS statistics indicated the model including molecular weight and $\log P_{MW}$ to show superior predictive capability to a model including molecular weight and $\log P_{OW}$.

The unadjusted R^2 statistic of 0.829 derived from the current data indicates that the fit of the sampled drugs to the model is good, with about 83 % of variance in the outcome measure being accounted for by $\log P_{MW}$ and MW values. This value is comparable with unadjusted R^2 values obtained by several other researchers investigating the relationship between skin permeability and partition coefficients using a variety of different sets of drugs, reported by Moss *et al.* (2002).

The high consistency of the adjusted R^2 and R^2_{PRED} statistics does not suggest any evidence of model or data inadequacies in the current model.

The optimal parsimonious model was found to exclude all the remaining additional variables, with only the originally included variables of $\log P_{MW}$ and molecular weight (MW) remaining in the final model. Hence this model was considered to provide the best summary of the data. This model also avoided inclusion of large numbers of predictor variables in an analysis based on a limited number of data points.

The optimal model so obtained was as follows:

$$\log K_P = -2.24 + 1.83 \times \log P_{MW} - 0.0123 \times MW \quad (7.5)$$

Hence a 1-unit increase in values of $\log P_{MW}$ is associated with an increase of 1.83 units in $\log K_P$ values, controlling for molecular weight. A 95% confidence interval for the $\log P_{MW}$ parameter is given by (1.43, 2.27); a 95% confidence interval for the MW parameter is given by (-0.0162, -0.00847).

The model illustrated in Figure 7.5 is the regression plot of experimental and predicted values while Figures 7.6 and 7.7 depict partial regression plots.

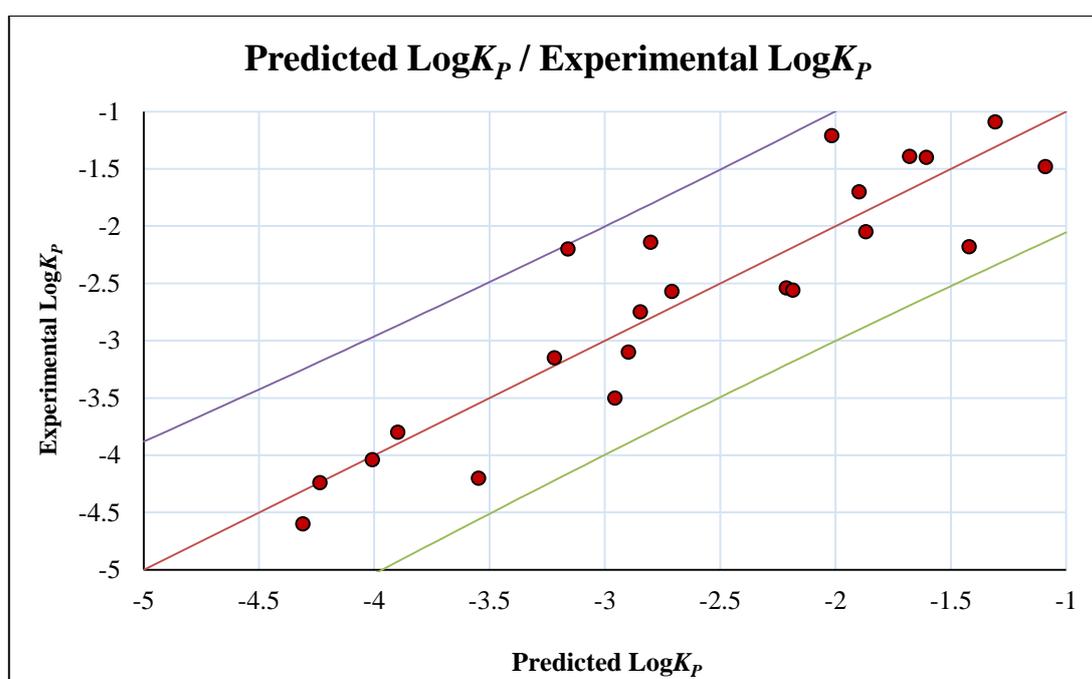


Figure 7. 5: Regression plot of experimental $\log K_P$ values against predicted $\log K_P$

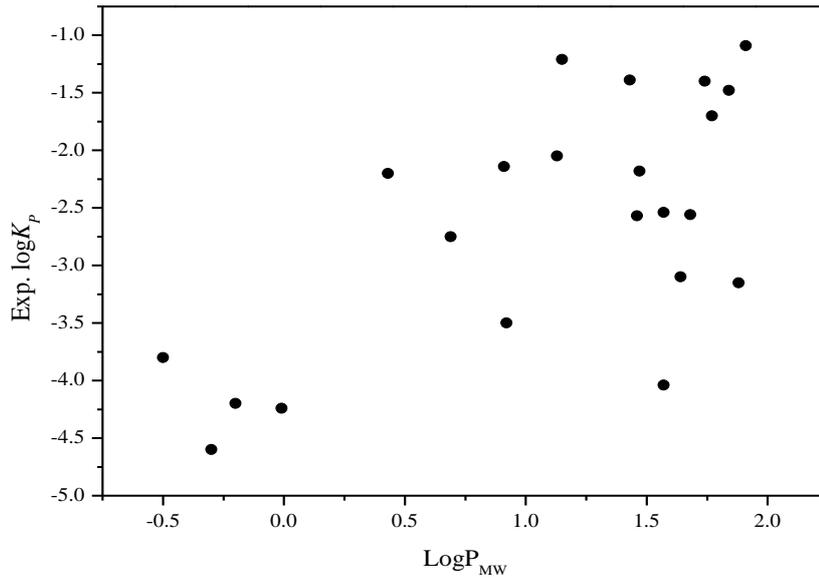


Figure 7.6: Partial regression plots of experimental $\log K_P$ values against $\log P_{MW}$

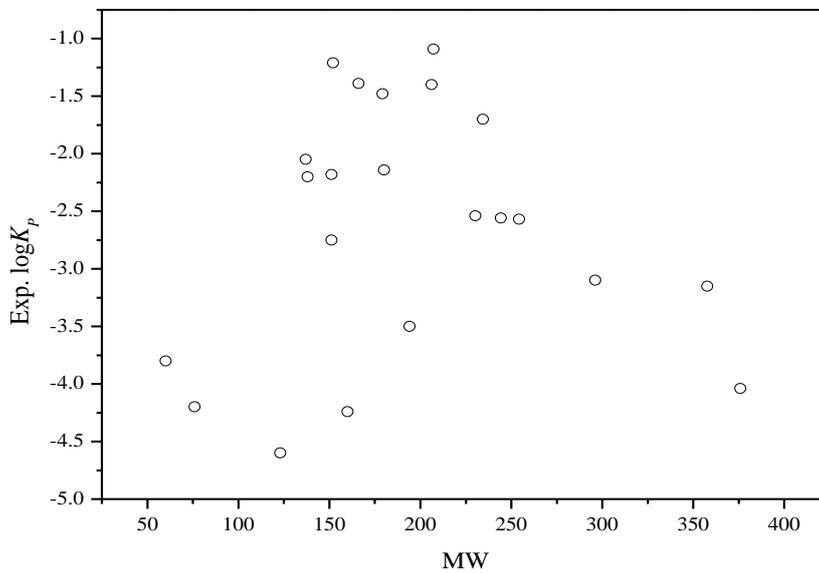


Figure 7.7: Partial regression plots of experimental $\log K_P$ values against MW

The R^2_{PRED} value of 0.786 implies that it could be expected that a model including just $\log P_{\text{MW}}$ values and molecular weight would explain about 79% of the variability in observations made in new drugs not currently considered. This may be compared with variability of about 83% in the existing data set being explained by the model. Hence the overall predictive capability of the model appears to be very good, in terms both of the high values of the R^2_{PRED} statistic, and the consistency between the R^2 statistics.

The deletion of a high-leverage drug from the data set results in a negligible change to the adjusted R^2 and the R^2 for prediction values, implying the model is not dependent on “good” drugs for its predictive capability, and hence provides further evidence that the model should be reasonably robust to variations in the subset of drugs utilised in its generation.

A residual analysis did not detect any marked relationship between residuals and predicted values (Figure 7.8), indicating no evidence for violation of regression assumptions.

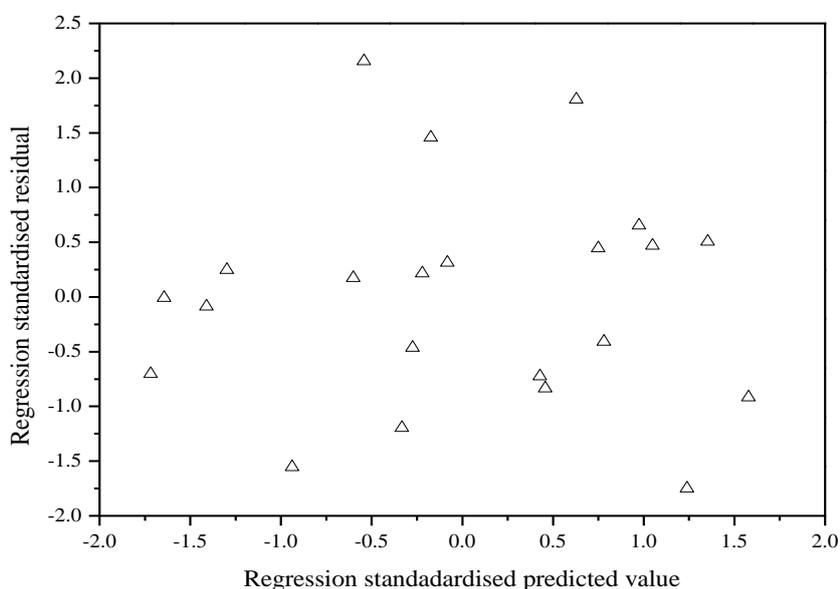


Figure 7.8: Residual plot for optimal regression model

The magnitude of the standardised coefficients (1.301 for $\log P_{MW}$ and 0.907 for molecular weight) indicated that $\log P_{MW}$ was of greater importance in the model. Both covariates were statistically significant ($t_1 = 9.61$, $p < 0.001$ for $\log P_{MW}$; $t_1 = 6.70$, $p < 0.001$ for molecular weight), with the model F-ratio also being statistically significant ($F_{2,19} = 46.2$, $p < 0.001$).

The corresponding adjusted R^2 statistic of the optimum model derived above was 0.811, which might be described as a large effect. The approximate R^2 value for prediction based on the PRESS statistic was found to be 0.786: hence this statistic showed very good agreement with the corresponding adjusted R^2 statistic.

None of the drugs in the data set were found to have high residuals or be influential according to the calculated Studentised residuals or Cook's distance values. The highest absolute Studentised residual (2.15), was associated with salicylic acid; while the highest value of Cook's distance of 0.190 was associated with thiourea. Haloperidol was found to have a relatively high leverage value of 0.355 (i.e. above the calculated cut-off value for the current data set of 0.272). However, the predicted experimental $\log K_p$ value associated with this drug of -4.01 was close to the actual experimental value obtained of -4.04; hence the overall influence of this drug on the data set would not be expected to be excessive.

Deleting haloperidol from the data set resulted in a negligible change in the R^2 statistic from 0.811 to 0.813 (95 % confidence interval 0.689 – 0.937); with a corresponding change in the R^2_{PRED} statistic from 0.786 to 0.767.

Although hard to compare, as datasets vary, in comparison with published data, it can be seen that this model is as good as, or better than, several existing models, including that of Potts and Guy (Potts and Guy, 1992) with an R^2 value of 0.67, and Dearden et al. (Dearden, 1990) with an R^2 value of 0.83, despite the latter being based on a more complicated six

parameter system. It is noteworthy that using the current drug set of the current study, the replacement of the $\log P_{OW}$ parameter in the equation derived by Potts and Guy with the $\log P_{MW}$ parameter results in a substantially better model fit. The R^2 value of 0.35 using the $\log P_{OW}$ parameter indicates that only 35% of the variability in observations may be accounted for by $\log P_{OW}$ and molecular weight in conjunction; less than half that accounted for by $\log P_{MW}$ and molecular weight in conjunction. Furthermore, the PRESS statistic associated with the Potts and Guy model formulation applied to the current data set was found to be around 4 times greater than the corresponding statistic derived from the model formulation including the $\log P_{MW}$ parameter. Hence the predictive capability of our model is substantially better than the Potts and Guy model.

Several published models provide greater values for R^2 using a range of alternative parameters (Lien and Gaot, 1995; Barratt, 1995a; Potts and Guy, 1995; Abraham et al., 1995b; Abraham et al., 1997). However, despite these latter models appearing to provide enhanced predictive qualities there are many reasons for the incorporation of this particular model in the prediction of skin permeability. The first of these is in the practical benefits of chromatographically measuring a partition coefficient compared with the more traditional shake-flask technique. For example, measurements are obtained with comparative rapidity, accuracy, precision and with reduced organic solvent waste and thus deemed both economically and environmentally favourable.

7.5. Conclusion

A systematic study of predicting human skin permeability of compounds using chromatographic measurements has been presented in this work. Incorporation of chromatographically derived micelle-water partition coefficients ($\log P_{MW}$) substantially improved the predictability of the existing model which was based on octanol-water partition

coefficients ($\log P_{ow}$). The correlation was even more improved when $\log P_{MW}$ values for a dataset of 22 compounds were modeled in combination with their respective physicochemical properties. This study provides a better surrogate for predicting skin permeability based on an easy, fast and cheap experimental methodology, and the method holds the predictive capability for a wider population of drugs from the same dataset and also on different samples of drugs.

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Chapter 8: Conclusions and future work

Hydrophobicity is an important property of drugs, being closely related to the process of transportation to the target site. The most popular hydrophobic parameter is logP which is extensively used in pharmaceutical and biomedical sciences, for example, knowledge of logP values is important to study pharmacokinetics and the behaviour of xenobiotic substances in organisms. The logarithm of a partition coefficient has traditionally been measured in an octanol-water system. However, this system of measuring partition coefficients has not been widely accepted in research and the subsequent development of new drug entities for several reasons. This includes a tedious and time consuming methodology that utilises large amounts of materials, along with the fact octanol is an isotropic medium that cannot represent the anisotropic nature of biomembranes. Several chemical models have been proposed, such as micelles or liposomes, utilising much more complex techniques including gas chromatography, calorimetry, NMR and so on. All these methodologies are associated with problems in practice, and have therefore also not been widely accepted.

More recently, the use of aqueous micellar solutions in reverse phase liquid chromatography gave birth to micellar liquid chromatography (MLC). Amphiphilic surfactants have polar head groups which favour aqueous environments and non-polar (or hydrophobic) tails. These surfactant monomers above a certain concentration, known as the critical micelle concentration (CMC), tend to aggregate to acquire a form known as micelles. Micelles offer different microenvironments to solutes, compounds which are hydrophilic remain outside the micelle while those which are hydrophobic tend to reside in the inner core of the micelle. Micelle liquid chromatography has traditionally been used for separation and partitioning of

organic compounds. The partitioning of compounds is based on the Armstrong model which describes three types of equilibrium i.e. between the micelle and aqueous phase, between the aqueous phase and stationary phase, and the micelle and stationary phase. However, prior to this work, only the retention factor, k' , in MLC has been used to indicate hydrophobicity, and P_{MW} has not been given any attention although it is derived from the relation of the inverse of the capacity factor and micellar concentrations. The difference between k' and P_{MW} is clear as the former not only includes partitioning between the micelle and water, but also between the stationary phase and water, and between the stationary phase and micelle. Therefore, this thesis proposes a potential alternative to the traditional octanol-water system, to acquire partition coefficients for compounds of pharmaceutical relevance.

MLC showed promise as a fast and reliable tool to acquire partition coefficient values. Most importantly, it highlighted many factors that contributed to the hydrophobicity determinations including hydrophobic and electrostatic interactions of the solutes bearing ionisable groups with the micelle. It became obvious that drugs with ionisable groups, may interact differently with the micelle, without changing the geometry of micelles as revealed by SAXS measurements, and the stationary phase, therefore, systematic control of ionisation through controlling the pH of the mobile phase would be extremely beneficial.

Controlling the pH of the mobile phase provides various microenvironments within MLC. The scarcity of partition coefficients over the studied range of pH values using the traditional octanol-water system limited its comparison with MLC based partition coefficients. Therefore, software based calculated logD (clogP) values were used to compare with the experimental values. The pH appeared to significantly influence the partition coefficient values obtained from MLC, and these values showed a strong correlation with the computational logD

values. Furthermore, a specific influence of the nature of the stationary phase on the partition coefficients was seen. This is explained as cyanopropyl bonded stationary phases tend to adsorb less surfactant compared with ODS bonded phases, therefore the retention of compounds is largely based on the solute present in the bulk aqueous phase. Hydrophobic compounds would therefore prefer the micellar core and their retention time would be less than for those that prefer the aqueous phase.

Experiments were conducted over a range of temperatures (295 K – 317 K) to acquire the thermodynamic parameters involved in the partitioning process. The partitioning of drugs in this study was enthalpically driven with negative ΔG values indicating the process was spontaneous.

Finally, the newly developed partition coefficient values were evaluated for their biological relevance in terms of their predictability of transport of compounds through skin (Chapter 7). A large dataset comprising of $\log P_{MW}$ values for 22 compounds was acquired through MLC and fitted with the other physicochemical parameters that are important for skin permeability. An improved correlation and predictability of the model compared with the Potts and Guy' model was achieved, thus endorsing the suitability of MLC based micelle-water partition coefficients as a potential alternative to the traditional octanol-water system.

It can be concluded that MLC has the ability to generate partition coefficient values in a shorter time with higher accuracy, and has the potential to replace the octanol-water system because of the partial structural resemblance of micelles to biomembranes.

Future work

There are many areas of potential opportunity to expand this work, including:

(a) Use of different surfactants

This work reports on determining partition coefficients using an anionic surfactant, sodium dodecyl sulfate. There is a possibility of incorporation of cationic surfactants, such as cetyltrimethylammonium bromide or zwitterionic surfactants, such as Brij to acquire partition coefficients.

In addition to that, mixed micellar systems comprising of anionic and cationic surfactants would provide additional opportunity to study the partitioning of compounds.

(b) Use of different stationary phases

Different stationary phases i.e. C₈ or C₁₈ with different pore sizes could be used to compare partition coefficients with values acquired using the cyano bonded stationary phase. This may provide values of biological relevance that could possibly mimic other bodily systems.

(c) Expansion of logP_{MW} dataset

This thesis has described a potential application of logP_{MW} in predicating skin permeability. This can be further refined by the addition of more compounds in the dataset. This will not only increase the predictability but also reliability of the model proposed.

Appendix A

Peer reviewed publications

1. pH effects in micellar liquid chromatographic analysis for determining partition coefficients for a series of pharmaceutically related compounds
Current Pharmaceutical Analysis, Vol. 8 (3), 272-277, 2012
2. Modelling skin permeability with micellar liquid chromatography
European Journal of Pharmaceutical Sciences, Vol. 50 (3-4), 335-340, 2013

Oral presentation

Evaluating micelle-water partition coefficients to assist prediction of skin permeability
UKPharmSci, Nottingham, 2012

Poster presentations

1. Micelle-Water Partition Coefficients: A potential alternative to octanol-water partition coefficients for pharmaceutical compounds
Swiss Pharma Science Day, Bern, Switzerland, 2011
Conference proceedings published in *Swiss Pharma*, Vol. 33 (10), p. 35, 2011
2. Evaluating Micelle-Water Partition Coefficients to assist Prediction of Skin Permeability
UKPharmSci, Nottingham, 2012

pH Effects in Micellar Liquid Chromatographic Analysis for Determining Partition Coefficients for a Series of Pharmaceutically Related Compounds

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Abstract: Five drugs were studied using micellar liquid chromatography (MLC) to determine micelle-water partition coefficients (P_{mw}) over a range of mobile phase pH values and column temperatures. In all cases the sodium dodecyl sulphate mobile phase utilised a CN reversed-phase column with UV detection, optimised for the λ_{max} of each drug. The pH of the mobile phase was systematically varied over the range 3 to 7 pH units, incorporating values above and below the pK_a 's of the drugs studied. From this it was possible to determine MLC based values of P_{mw} and establish their relationships with pK_a values, software predicted partition coefficients (logP), dissociation constants (logD) and published partitioning data (log P_{ow}). This study also considered the relationship between column temperature, from 294K to 317K, and P_{mw} . For all five drugs it was found that P_{mw} decreased with increasing pH implying a systematic increased preference for the drug to remain in the aqueous phase rather than partition into the micellar phase. In addition, the partition coefficient displayed a linear relationship with log D over the pH range for each drug with a 'break-point' observed at the pK_a for each drug. With respect to increasing temperature, the results were non-linear indicating that there is no general relationship for these drugs with temperature. Overall, it was found that MLC is suited to the measurement of partition coefficients for pharmaceutical compounds yet it should be noted that both pH and temperature play a significant role in the values obtained.

Keywords: HPLC, logP, Micellar Liquid Chromatography, MLC, partition coefficients, pH.

INTRODUCTION

Micellar liquid chromatography (MLC) is a well established form of HPLC that incorporates a chosen surfactant in the mobile phase at a concentration significantly above the critical micellar concentration (CMC) [1]. This creates the formation of micelles with both hydrophobic and hydrophilic regions within the aqueous mobile phase. If an additional compound is then introduced into this system it may reside in the micellar core, remain outside of the micelle or equilibrate between both, i.e. establish a micelle-water partition coefficient (P_{mw}) [2]. For drugs this value can be of significance as it has recently been proposed as an alternative to the traditional octanol-water partition coefficient (P_{ow}) that is fundamental in the development process of all new drugs [3]. This is important because it is known that the efficacy of a drug relies upon its ability to partition through biological systems repeatedly from organic to aqueous environments and the partitioning value obtained is a reflection of this physicochemical property. Therefore, if MLC is able to provide a more accurate and precise prediction of the *in vivo* drug partitioning behaviour it should be considered as a superior alternative to P_{ow} .

MLC has already shown promise as an analytical technique in related areas [4], for example, the determination of

quinolones in milk and egg samples [5], where the lack of the need for prior protein elimination is a key advantage. Vitamin analysis has also exploited the application of MLC where the mobile phase has been optimised to simultaneously detect seven water soluble vitamins using UV detection and sodium dodecyl sulphate has been used in the mobile phase [6]. MLC has frequently been employed in the analysis of substituted aromatic hydrocarbons [7-9], especially with respect to determining correlations with P_{ow} values over a series of compounds [10]. For example, hydrophobicity measurements with experimental P_{mw} values are known to be comparable with those reported for P_{ow} in certain cases [11].

Since the first reports of MLC systems in the early 1980s [12, 13], modifications such as variations in the choice of surfactant [14-16] and addition of organic solvents to the mobile phase have been reported in the literature [17-20]. More recently, the use of MLC to provide additional information regarding the partitioning process of environmental compounds has been reported at elevated temperature studies, facilitating the determination of associated thermodynamic data [21].

A second variable of interest in this work is the effect of pH of the mobile phase on the partitioning data obtained for the pharmaceutical compounds. Other investigators in this area suggest that there is an effect of acidity on retention of ionisable solutes with ionic surfactants [22-25]. In particular, with a cyano-bonded column modified with SDS the retention of a weak acid decreases with pH whereas for weak

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bases the strongest retention occurs in the more basic solutions.

Previous research has considered the application of MLC for pharmaceutical compounds [26-28] yet not, as in this paper, with particular emphasis on buffer pH with respect to the pKa of the drug and the temperature of the study. This work considers five non-steroidal anti-inflammatory drugs (NSAIDs), namely ibuprofen, ketoprofen, fenbufen, flurbiprofen and fenoprofen that have structural similarities yet exhibit differing literature partition coefficients [29]. There are many traditional approaches to predicting and measuring partition coefficients including the frequently used 'slow-stir' method [30] alongside computational methods such as theoretical calculation using the clogP program [31], counter-current chromatography [32] amongst others [33]. With all methods of prediction there are associated errors in extensions of laboratory data to *in vivo* partitioning systems which is why it is so vital to develop and validate a more suitable alternative such as MLC. In summary, this paper investigates the application of MLC using a cyanopropyl column with SDS as the mobile phase for five related compounds of pharmaceutical interest. In particular, this work explores the influence of the pH of the mobile phase and experimental temperature to probe the physicochemical parameters involved in the partitioning process. This information is essential to optimise the analytical technique to fully exploit the potential application of MLC as an alternative to traditional P_{ow} measurements or those more recently developed [34].

EXPERIMENTAL

2.1. Materials

For all MLC experiments, the mobile phase consisted of sodium dodecyl sulphate (SDS), used as purchased from Fisher Scientific, Loughborough, UK (99%) and was chosen as a suitable surfactant based on previous work [21]. This was diluted with distilled water as necessary to achieve each desired concentration (10-30 mM). The five drugs considered in this work were: fenbufen (Fluka, Dorset, UK), fenoprofen (Sigma Aldrich, Dorset, UK), flurbiprofen (TCI, Oxford, UK), ibuprofen (BASF, Rhineland-Palatinate, Germany) and ketoprofen (TCI, Oxford, UK), used as purchased with a minimum purity of $\geq 97\%$, with each at a concentration of 0.2 mM. For experiments at specified pH values the following compounds were required to act as buffering agents: 0.1M di-sodium hydrogen phosphate and 0.1M sodium di-hydrogen phosphate with 10% phosphoric acid (Sigma Aldrich, Dorset, UK) for pH adjustment to provide a series of mobile phases of pH 3.0, 4.0, 5.0, 5.8, 6.4 and 7.0. No organic modifier was used in this work.

2.2. Methods

Degassed mobile phase solvent was pumped through the system using a solvent delivery system (Severn Analytical, Model 6410B, Gloucester, UK) with a flow rate of 1.35mL per minute to optimise subsequent data resolution. The reverse phase cyanopropyl column (Spherisorb 5 μ m, 15cm x 4.6 mm i.d.; Waters, Hertfordshire, UK) was placed in a column oven (Jones Chromatography, Model 7950, Lakewood, US) at 294K for pH optimisation experiments and from

294K to 317K for temperature optimisation experiments with a pH 4 mobile phase. This column was chosen based on previous work [21]. 20 μ L samples of 0.2 mM drug (solute) were injected via a Rheodyne injector (Perkin Elmer, Model 7725, Cambridge, UK) with the solute immersed in a water bath (Grant, Wiltshire, UK) maintained at the same temperature as the column. A Waters 2487 UV detector (Waters, Hertfordshire, UK), set at a wavelength appropriate for each drug (221 – 285nm), produced a peak indicating the retention of the solute within the column as a function of time. Data were recorded and then analysed to obtain capacity factors and each run was repeated six times to ensure reasonable accuracy and precision were achieved.

From these results it was possible to calculate the micelle/water partition coefficient at each temperature through plotting the SDS concentration (after subtraction of the CMC) with the inverse of the capacity factor. A study of partitioning as a function of temperature involves knowledge of the CMC of the surfactant at each experimental temperature. This means that P_{mw} calculated at each temperature involved subtraction of a different CMC. Previous research [35] has established a CMC for each temperature from 294-323 K therefore these values were used in this analysis. Calculation of clogP data was achieved using a software prediction program (ACD/Labs, Berks, UK) using a fragmental method based on the chemical structure of the compound [36]. It should be noted that the addition of salts to micellar solutions causes a phenomenon known as 'salting out' which decreases the solvent activity by ion-solvent binding. As a consequence, values obtained for the partition coefficients will be incorrectly increased beyond their real values. Secondly, salts will decrease the interfacial potential at the micelle surface which will allow more binding of solute to micelle to occur which again is undesirable. It is for this reason that excess salt was not added to the mobile phase during the course of these experiments, i.e. ionic strength was not maintained as a constant.

RESULTS AND DISCUSSION

In all MLC experiments firstly the pH of the mobile phase and secondly the temperature of the column was controlled and systematically varied over a specified range to determine the potential existence of relationships between these variables and related physicochemical properties.

Previous research has established that there is no significant shift in pK_a values of similar compounds to these drugs with SDS present in solution [37]. Calculated lipophilicities based on each drug's chemical structure were compared with the experimentally determined P_{mw} results at pH 7 in addition to pK_a values and established octanol-water partition coefficients (P_{ow}) (Table I).

It can be seen in Table I that there is little correlation between the values obtained using MLC at pH 7.0 and those obtained either through calculation or from literature implying significant differences in the partitioning process between MLC and the simple octanol/water shake flask method. In addition, there is little correlation between the values obtained using MLC and the small differences in pK_a between the five compounds. As a consequence of these results it was decided that a full investigation should be under-

Table 1. Comparison of Experimentally Determined $\log P_{mw}$ Values at pH 7.0 with Software Based (clogP) Partitioning Data and Established Octanol-Water Partitioning Data ($\log P_{ow}$)

Drug	pK_a	pH 7.0 $\log P_{mw}$	clogP	$\log P_{ow}$
Fenbuten	4.55	1.31	3.1	3.20 [36]
Fenoprofen	4.20	0.52	3.7	3.45 [36]
Flurbiprofen	4.14	0.39	3.7	4.2 [29]
Ibuprofen	4.41	0.21	3.5	3.6 [29]
Ketoprofen	4.23	0.42	2.9	2.7 [34]

Table 2. Effect of Mobile Phase pH on Experimentally Determined $\log P_{mw}$ Values Compared with Calculated Dissociation Constant Values ($\log D$) for Five Drugs

Drug	pH 3.0		pH 4.0		pH 5.0		pH 5.8		pH 6.4		pH 7.0	
	$\log P_{mw}$	$\log D$										
Fenbuten	2.20	3.1	2.05	3.0	2.01	2.5	1.73	1.8	1.58	1.3	1.31	0.7
Fenoprofen	2.25	3.7	1.98	3.5	1.85	2.9	1.38	2.1	1.04	1.6	0.52	1.0
Flurbiprofen	2.20	3.6	2.04	3.4	1.92	2.7	1.45	2.3	1.11	1.3	0.39	0.8
Ibuprofen	2.14	3.5	2.04	3.4	1.98	2.8	1.47	2.2	1.22	1.4	0.21	0.9
Ketoprofen	2.13	2.9	2.04	2.7	1.82	2.1	1.23	1.3	0.99	0.9	0.42	0.2

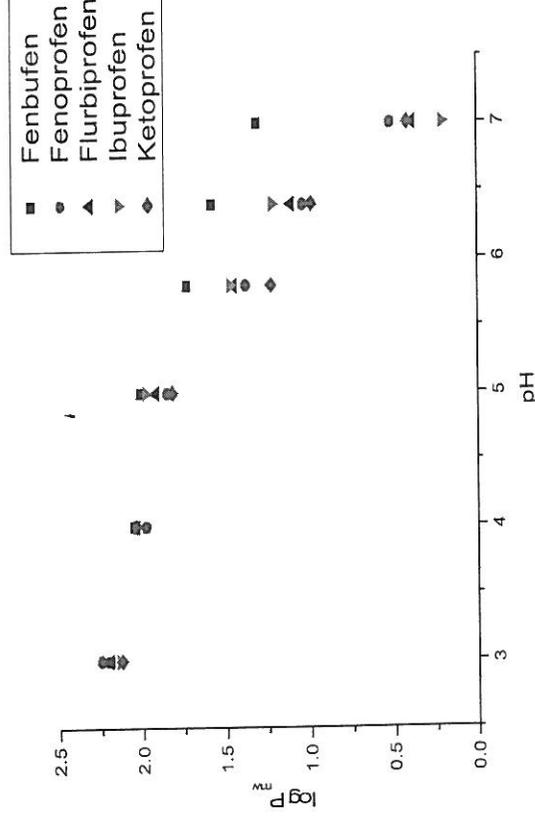


Fig. (1). Relationship observed between the experimentally determined $\log P_{mw}$ values and mobile phase pH for the five drugs using MLC.

taken to consider the five compounds over a range of pH values both above and below the pK_a of each drug.

P_{mw} values were determined for all five drugs in six separate mobile phases ranging in pH from 3 to 7 at 294K and compared with calculated dissociation constant values ($\log D$) based on the chemical structures of the drugs (Table 2). All drugs have a pK_a between 4.1 and 4.6 therefore as the pH is increased above this region more drug is ionised and partitioning into the lipophilic phase becomes less favourable.

The effect of the pH on the surfactant is assumed to be negligible as the pK_a of SDS is below the lowest pH considered in this work.

It was observed that the relationship between $\log P_{mw}$ with pH (Table 2) was reasonably linear from pH 5 to 7 for all compounds with a general trend of a change in slope at a value around the pK_a for each drug (Fig. 1). Correlation (R^2) values between $\log P_{mw}$ and pH from pH 5 to 7 were; 0.985 for fenbuten, 0.984 for fenoprofen, 0.944 for flurbiprofen, 0.867 for ibuprofen and 0.973 for ketoprofen.

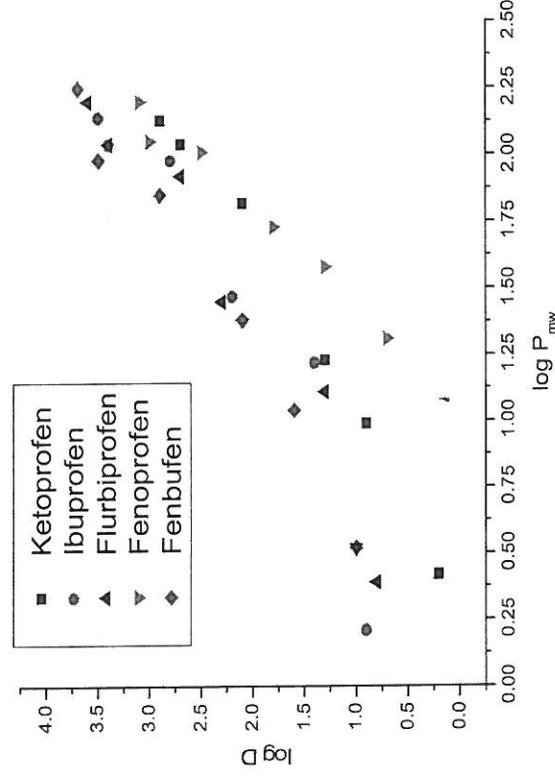


Fig. (2). Relationship between the calculated $\log D$ and experimental $\log P_{mw}$ values for the five drugs.

Table 3. Comparison Of Experimentally Determined $\log P_{mw}$ Values for five NSAIDs at Five Specified Temperatures with Software Based (clogP) Partitioning Data and Established Octanol-Water Partitioning Data ($\log P_{ow}$)

	294K $\log P_{mw}$	298K $\log P_{mw}$	305K $\log P_{mw}$	310K $\log P_{mw}$	317K $\log P_{mw}$	Calculated clogP	$\log P_{ow}$
Fenbufen	2.05	2.11	2.12	2.18	2.21	3.10	3.20 [36]
Fenopropfen	1.98	2.06	1.99	1.98	2.09	3.70	3.45 [36]
Flurbiprofen	2.04	2.09	2.08	2.21	2.22	3.70	4.20 [29]
Ibuprofen	2.04	1.83	2.08	2.10	2.17	3.50	3.60 [29]
Ketoprofen	2.04	1.88	1.97	2.01	1.98	2.91	2.70 [34]

The relationship between the $\log D$ and $\log P_{mw}$ values for each drug is revealed in Fig. (2). The data suggest that the $\log D$ is a reasonable predictor of the MLC derived partitioning values across the range of drugs studied.

While absolute values of $\log D$ and $\log P_{mw}$ significantly differ (Table 2), the change in both values with respect to pH is consistent from pH 5 to 7. Interestingly while the relationship between the measured $\log P_{mw}$ and pH shows deviation from linearity around the pKa's of the drug substance, the calculated $\log D$ values are better correlated with pH across the entire range of pH studied. This may suggest either an oversimplification of the calculational approach to ionisation or enhanced sensitivity of the MLC technique in reporting partitioning data near the pKa of the analyte in the experimental solvent system.

P_{mw} values were determined for the same drugs at five controlled column temperatures from 294K to 317K and compared with calculated partitioning data based on the chemical structures of the drugs and previously reported octanol-water partitioning data (Table 3).

From the data shown in Table 3 it can be seen there is no clear relationship between temperature and partitioning using MLC as was expected. Previous work [3] has indicated that an increase in temperature would encourage the compounds

to more favourably partition into the micellar structure thus resulting in increased P_{mw} values. For these compounds this trend was not observed and a potential 'minimum' value was seen for some drugs that mirrors the effects observed when considering the relationship between temperature and CMC of sodium dodecyl sulphate (SDS).

CONCLUSIONS

In summary, it can be seen that for the five compounds under investigation in this work the pH of the mobile phase significantly effects the partitioning data observed using MLC. In particular, the relationship between $\log P_{mw}$ and $\log D$ is clear with a defined change occurring around the pKa of each drug. It is not possible to state at this time if this relationship could be applied to a wider range of ionisable compounds although it is envisaged this may be possible. In addition, it can be said that the temperature of the column does not correlate directly with the partitioning data obtained, contrary to what was to be expected. At present, the reason for this lack in trend is unknown but it is thought it may be associated with a modification to the CMC in the presence of drug and current work is being undertaken to investigate this further.

Overall, it can be seen that MLC is a suitable technique to measure the partitioning of these drugs between micelles

and water although careful attention must be paid to the pH of the mobile phase and control of the experimental temperature as both of these factors influence the values obtained.

ACKNOWLEDGEMENTS

The authors acknowledge the support of the University of Huddersfield, UK.

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Received: October 13, 2011

Revised: January 10, 2012

Accepted: January 11, 2012



Modelling skin permeability with micellar liquid chromatography



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ARTICLE INFO

Article history:

Received 24 May 2013

Received in revised form 16 July 2013

Accepted 2 August 2013

Available online 13 August 2013

Keywords:

Permeability
Chromatography
Modelling
Micellar
Partition coefficient

ABSTRACT

This study evaluates the potential application of micellar liquid chromatography (MLC) to predict skin permeation with a series of model compounds. MLC has previously been found to be useful in the prediction of partition coefficient values ($\log P$) for pharmaceutical compounds, yet has not been incorporated in skin permeability models prior to this work. This article provides statistically supported data that this technique enhances the ability to predict the permeability of similar drugs through the skin (K_p). The replacement of a traditional physicochemical parameter, namely the octanol–water partition coefficient ($\log P_{ow}$) with a chromatographically determined value ($\log P_{mw}$), results in a quantitative partition–permeability relationship that is robust to variation. MLC offers many benefits compared with the traditional techniques employed to obtain $\log P$ values.

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1. Introduction

Drug permeation through human skin is an increasingly important delivery route, often described by the associated permeability coefficient (K_p). It is widely known that discrepancies often occur between the *in vivo* reality (where the stratum corneum controls the rate of intercellular passive diffusion) and *in vitro* based models (Walters, 2002). Moreover, even models based on human *in vivo* data can experience orders of magnitude of discrepancy from observed data (Farahmand and Maibach, 2009) highlighting the complexity of such phenomena. A significant body of research exists assessing a variety of model based systems as their potential benefits are recognised widely. These models are often described as quantitative structure–permeability relationships (QSPRs) (Moss et al., 2002) with their main shortfall arising from the inconsistency in the values used to derive them. For example, the heterogeneity of the data, in terms of skin origin and experimental conditions, has been shown to contribute to the residual variance in existing models (Geinoz et al., 2004). In addition, drug permeation through human skin is significantly influenced by the degree and nature of charge on the drug molecule which is hard to accommodate within the majority of proposed models (Zhang et al., 2012) and often studies are restricted to one specific series of compounds (Hadgraft et al., 2000). This often prevents proposed models from being more widely applicable as they are only valid for a designated group of drugs, rather than all drugs, which would be far more useful for analytical purposes. One particular challenge

is attempting to retain the simplicity of a model so that it is widely used within the scientific community yet maintain the validity to explain complex real-world data (Mitragotri et al., 2011); for example, retaining the predictive ability of the model in the presence of transdermal enhancers (Moss et al., 2012) or different vehicles (Ghafourian et al., 2010). In some cases it has even been proposed that it may be best to apply both QSPRs and other models such as Gaussian process-based machine learning methods (Brown et al., 2012), with some claims that the latter may have significantly improved predictivity compared with the former (Lam et al., 2010). Several permeability correlation models have become very well established in the scientific community for the prediction of K_p and are regarded as an ideal initial step in the development and characterisation process for pharmaceutical compounds. Often models are based on parameters such as the lipophilicity and molecular weight (or size) of a drug (Magnusson et al., 2004; Ng et al., 2012; Potts and Guy, 1992; Singh and Roberts, 1996), although other, related physicochemical parameters have been investigated in an attempt to improve the predictivity of the models. Another approach employed to predict the ability of a drug to permeate the skin is to employ *in vitro* based model systems, for example those that have been successfully used to predict other biological permeations; such as those based on the blood–brain barrier (Sinkó et al., 2012), using electrokinetic chromatography (Wang et al., 2009), simple silicone membrane skin models (Khan et al., 2005; Waters et al., 2013) and most recently, chromatographic techniques (Hidalgo-Rodríguez et al., 2013). Simple models, such as a silicone membrane, are particularly suited to studies that require comparative values, for example, with variations in the presence of additional excipients or temperature

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changes (Waters et al., 2013) but may struggle to achieve absolute data.

Recently, micellar liquid chromatography (MLC) has been proposed as an alternative, and potentially superior, method for determining the lipophilicity of a compound by applying a modified form of the chromatographic technique to determine micelle-water partition coefficients (Waters and Kasprzyk-Hordern, 2010; Waters et al., 2007). MLC is an established form of High Performance Liquid Chromatography (HPLC) that incorporates a chosen surfactant in the mobile phase at a series of concentrations significantly above the critical micellar concentration (CMC). This creates the formation of micelles with both hydrophobic and hydrophilic regions within the aqueous mobile phase: i.e. a similar environment to that present in skin. A sample of compound under investigation is then injected into this surfactant-based mobile phase under controlled parameters, such as buffer pH and temperature. Monitoring the movement of a drug within MLC leads to a more accurate and precise prediction of drug partitioning compared with octanol-water based systems, i.e. P_{ow} . MLC has been shown to be a very simple, cheap and fast method for the determination of micelle-water partition coefficient values ($\log P_{mw}$) (Waters et al., 2012). Furthermore, it is suitable for compounds over a range of lipophilicities, ensuring it can be applied to the study of a range of drugs with varying acidity or basicity with analysis simply requiring measurement of the retention time for the sample at each surfactant concentration. This flexibility is essential if the technique is to be applied to predict the behaviour of drugs as they regularly exist in a variety of ionisable forms.

Previous research has considered the application of MLC for pharmaceutical compounds, and in some cases even to evaluate skin permeability (Martínez-Pla et al., 2003, 2004), yet not, as in this paper, with the incorporation of experimentally determined $\log P_{mw}$ values in a model to predict drug permeability, K_p . The model developed in this work is based on a sample system comprised of 22 compounds with the application of statistical analysis to develop an optimum model for predictive purposes that can be applied to a far wider range of drugs than those identified in this study. The replacement of $\log P_{ow}$ with $\log P_{mw}$, and the subsequent model development, is intended to provide a superior estimation of drug permeability through human skin *in vivo* compared with existing models. In addition to this main benefit, MLC requires no organic solvent, unlike octanol based measurements, and is faster to determine experimentally to an equivalent degree of reproducibility.

2. Materials and methods

2.1. Materials

Sodium dodecyl sulphate (SDS) was the mobile phase surfactant for all experiments, used as purchased from Fisher Scientific, Loughborough, UK (99%) and diluted with distilled water as necessary to achieve each concentration from 10 to 30 mM. The 22 compounds considered in this work were: methyl nicotinate (Fluka, Dorset, UK), acetaminophen, ethyl nicotinate, ethyl paraben, haloperidol, indomethacin, lidocaine, methyl paraben, naproxen, nicotinic acid, sodium salicylate (Sigma Aldrich, Dorset, UK), butyl nicotinate, diclofenac, flurbiprofen, hexyl nicotinate, ketoprofen (TCl, Oxford, UK), ibuprofen (BASF, Cheshire, UK), acetyl salicylic acid (Acros Organics, Geel, Belgium), thiourea, urea (Vickers Lab. Ltd., Pudsey, UK), caffeine and salicylic acid (Fisher, Loughborough, UK), used as purchased with a minimum purity of $\geq 98\%$, with each at a concentration of 0.2 mM. All experiments were conducted at a specified pH value of 5.5, chosen to mimic the pH of human skin, with 0.1 M di-sodium hydrogen phosphate and 0.1 M sodium di-

hydrogen phosphate with 10% phosphoric acid (Sigma Aldrich, Dorset, UK) as buffering agents. No organic modifier was used in this work.

2.2. Micellar liquid chromatography method

A similar system to that previously reported was the basis of the methodology employed in this study (Waters et al., 2012). Degassed surfactant-based mobile phase was pumped through the chromatographic system (Severn Analytical SA 6410B) with a reverse phase cyanopropyl column (Spherisorb 5 μ m, 15 cm \times 4.6 mm i.d.) and a flow rate of 1.35 mL per minute. 20 μ L samples of 0.2 mM drug (solute) were injected via a Rheodyne injector. A UV detector (Waters 2487), set at a wavelength appropriate for each drug (221–285 nm), produced a peak indicating the retention of the solute within the column as a function of time. Data were recorded and then analysed to obtain capacity factors and each run was repeated five times to ensure reasonable accuracy and precision were achieved. With a fixed pH for the mobile phase it should be noted that the degree of ionisation between compounds may vary yet the pH of the solution was measured before and after the addition of each compound and confirmed to remain at a value of 5.5. This is also of importance when evaluating values for $\log K_p$ derived from literature sources as the pH of the solvent may have an effect on the degree of ionisation and hence the resultant value obtained (Zhang et al., 2012).

In agreement with previous work (Waters et al., 2012), it was possible to calculate a value for $\log P_{mw}$ by plotting the surfactant concentration (after subtraction of the CMC, i.e. 8.0 mM) with the inverse of the capacity factor. It should be noted that ionic strength was not maintained as a constant during this work, in line with previous studies.

2.3. Data analysis

Results from the MLC technique permitted the development of a dataset that contained $\log P_{mw}$ values for 22 different compounds along with their physicochemical parameters, such as molecular weight (MW) and published permeability values (K_p). Analysis focussed on the development of a quantitative partition-permeability relationship. Data analysis was conducted using IBM SPSS statistical software, Version 20.0.

Initially two parallel regression analyses were conducted, with experimental K_p values regressed against $\log P_{mw}$ or $\log P_{ow}$ values. In both cases molecular weight was included as an additional covariate for consistency with previous analyses (Potts and Guy, 1992). The fit of both models to the data, and the predictive capability of both models (using the prediction error sum of squares (PRESS) statistic), were compared to determine the most appropriate partition coefficient for inclusion in a regression model. Further multiple regression analyses were undertaken, considering additional recorded parameters in conjunction with the partition coefficient parameter found to provide the best summary of the data in the above models, to determine an optimum parsimonious model for the full complement of drugs in the data set. Checks for multicollinearity were performed on all variables before consideration for inclusion in a multiple model. Residual analysis was also undertaken to check the underlying regression assumptions.

The variables remaining in the optimal model were assessed for significance and relative importance using standardised coefficients and associated p -values.

As a result of the relatively small sample size, and variations in the drugs selected for investigation between this and previous studies, the predictive capability of the preferred regression model was also assessed using the Wherry adjusted- R^2 statistic and an approximate R^2 for prediction, R^2_{PRED} , derived from the

PRESS statistic, which can be used to compare a number of candidate model structures for the same data set. The Wherry statistic indicates how much variability in experimental K_p values would be accounted for from a prediction equation derived in the population from which the sample was drawn, but does not indicate how well the derived equation will predict on other samples of drugs from the same population. The R^2_{PRED} statistic gives an indication of the external predictive power of the model (i.e. how well the model is likely to predict responses in a new experiment), and hence more accurately reflects the far wider applicability of the model.

Drugs with potentially high influence on the regression model were identified by calculation of Studentised residuals, leverage statistics and Cook's distances for all drugs in the data set, in order to provide insights of the suitability of the chosen drugs in the data set to derive the prediction model. A set of potentially highly influential drugs was derived, comprising drugs which met one or more of the following criteria:

- (i) Drugs whose Studentised residual lay in excess of 3 standard deviations from the mean (for a data set of the current size, it might be expected that about 1 observation would be in excess than 2 standard deviations from the mean).
- (ii) Drugs whose leverage value indicated it to be very different to other drugs in the sample (according to the commonly-applied criteria that a leverage value above $2(p+1)/n$, where p is the number of predictors in the model and n is the number of items in the sample). In the current data set, this would amount to any drug with a leverage of 0.272 or more, based on a set of leverage values which would be expected to have a mean value of 0.091.
- (iii) Drugs considered to be potentially influential according to Cook's distance, D_i (assumed to be represented in drug i by an associated D_i value of greater than 1).

A sensitivity analysis using a reduced data set with potentially highly influential drugs was conducted, to determine whether these drugs had a detrimental impact on model predictive capability.

3. Results

Partitioning values for 22 compounds were measured in this study using the micellar chromatographic technique previously described, with the resultant calculated $\log P_{\text{mw}}$ values shown in Table 1. Table 1 also provides published octanol–water partition coefficient ($\log P_{\text{ow}}$) values, plus all associated physicochemical parameters of the drugs analysed in the study.

A regression analyses including only molecular weight and $\log P_{\text{mw}}$ as covariates was found to have an R^2 statistic of 0.829 (95% confidence interval 0.716–0.914) and a PRESS statistic of 5.16. A regression analyses including only molecular weight and $\log P_{\text{ow}}$ as covariates was found to have an R^2 statistic of 0.350 (95% confidence interval 0.072–0.628) and a PRESS statistic of 20.1. Hence $\log P_{\text{mw}}$ in the presence of molecular weight provided a substantially better summary of the data than $\log P_{\text{ow}}$ in the presence of molecular weight for the full complement of drugs in the study; and the PRESS statistics indicated the model including molecular weight and $\log P_{\text{mw}}$ to show superior predictive capability to a model including molecular weight and $\log P_{\text{ow}}$.

Simple regression models with either $\log P_{\text{mw}}$ as the sole covariate ($R^2 = 0.427$), $\log P_{\text{ow}}$ as the sole covariate ($R^2 = 0.135$), or molecular weight as the sole covariate ($R^2 = 0.001$) did not provide a particularly good summary of the data.

All other measured variables on the drugs investigated in the current study were considered for inclusion in a multiple regression model including $\log P_{\text{mw}}$ and molecular weight, and using a backward elimination modelling strategy. Molar volume was found to be very strongly correlated with molecular weight ($r = 0.940$, $p < 0.001$), and inclusion of both these parameters in a multiple regression model led to unacceptably high levels of the variance inflation factor (VIF) statistic. Deletion of the molar volume parameter resulted in levels of the VIF within acceptable limits; hence this parameter was not considered further in any subsequent analysis.

The optimal parsimonious model was found to exclude all the remaining additional variables, with only the originally included variables of $\log P_{\text{mw}}$ and molecular weight (MW) remaining in the final model. Hence this model was considered to provide the best

Table 1
Physicochemical parameters for 22 selected drugs.

Drug	Exp. $\log K_p$	$\log P_{\text{mw}}$	MW	$\log P_{\text{ow}}$	pK_a^a	MP	HA ^a	HD ^a	V_M^a (cm ³ mol ⁻¹)	PSA ^a
Urea	−3.8 (Martínez-Pla et al., 2003)	−0.5	60.1	−2.11 (Martínez-Pla et al., 2003)	14.3	134	3	4	49.5	69.1
Thiourea	−4.02 (Martínez-Pla et al., 2003)	−0.2	76.1	−0.95 (Martínez-Pla et al., 2003)	13	182	2	4	57.3	84.3
Nicotinic acid	−4.6 (Dal Pozzo et al., 1991)	−0.3	123.1	0.4 (Dal Pozzo et al., 1991)	4.8	236.6	3	1	95.1	50.2
Methyl nicotinate	−2.05 (Le and Lippold, 1995)	1.13	137.1	0.8 (Le and Lippold, 1995)	3.19	39	3	0	120.5	39.2
Salicylic acid	−2.2 (Assessment, 1992)	0.43	138.1	2.26 (Assessment, 1992)	13.06	159	3	2	100.3	57.5
Ethyl nicotinate	−2.21 (Dal Pozzo et al., 1991)	1.47	151.2	1.3 (Dal Pozzo et al., 1991)	3.3	8	3	0	137.1	39.2
Acetaminophen	−2.75 (Assessment, 1992)	0.69	151.2	0.5 (Assessment, 1992)	9.86	170	2	2	105.4	49.3
Methyl Paraben	−1.21 (Nicoli et al., 2008)	1.15	152.1	1.96 (Nicoli et al., 2008)	8.4	127	3	1	125.7	46.5
Sodium salicylate	−4.26 (Martínez-Pla et al., 2003)	−0.01	160.1	2.06 (Martínez-Pla et al., 2003)	3	160	3	1	100.3	57.5
Ethyl paraben	−1.39 (Nicoli et al., 2008)	1.43	166.2	2.47 (Nicoli et al., 2008)	8.3	117	3	1	142.2	46.3
Butyl nicotinate	−1.48 (Le and Lippold, 1995)	1.84	179.2	2.27 (Abraham et al., 1995)	3.2	5	3	0	170.1	53.02
Aspirin	−2.14 (Degim et al., 1998)	0.91	180.15	1.19 (Degim et al., 1998)	3.5	135	4	1	139.5	63.6
Caffeine	−3.50 (Lian et al., 2008)	0.92	194.2	−0.07 (Lian et al., 2008)	0.52	238	2	0	134.6	58.4
Ibuprofen	−1.4 (Degim et al., 1998)	1.74	206.3	−3.5 (Waters et al., 2012)	4.41	76	2	1	200.3	37.3
Hexyl nicotinate	−1.09 (Le and Lippold, 1995)	1.91	207.3	3.5 (Abraham et al., 1995)	3.21	5	3	0	203.1	39.2
Naproxen	−2.54 (Degim et al., 1998)	1.57	230.3	3.2 (Degim et al., 1998)	4.84	155	3	1	178.3	46.5
Lidocaine	−1.7 (Thomas et al., 2007)	1.77	234.4	2.4 (Thomas et al., 2007)	7.96	68.5	2	1	228.3	32.3
Flurbiprofen	−2.56 (Charoo et al., 2008)	1.68	244.3	3.7 (Waters et al., 2012)	4.14	112	3	1	203.6	37.3
Ketoprofen	−2.57 (Hadgraft et al., 2000)	1.46	254.3	2.7 (Waters et al., 2012)	4.23	96	3	1	212.2	54.4
Diclofenac	−3.1 (Hadgraft et al., 2000)	1.64	296.2	4.5 (Hadgraft et al., 2000)	3.96	157	3	2	182.9	49.3
Indomethacin	−3.15 (Hadgraft et al., 2000)	1.88	357.8	3.51 (Hadgraft et al., 2000)	4.18	155	4	1	269.5	68.5
Haloperidol	−4.04 (Azarbayjani et al., 2010)	1.57	375.9	3.49 (Azarbayjani et al., 2010)	8.3	151.5	4	1	303.2	40.5

^a pK_a is the dissociation constant; MP the melting point; HA and HD the number of hydrogen bond acceptor and donor respectively; V_M the molar volume; and PSA is the polar surface area. All these descriptors were generated from ACD/Labs, Daresbury, UK.

summary of the data. This model also avoided inclusion of large numbers of predictor variables in an analysis based on a limited number of data points.

The optimal model so obtained was as follows:

$$\log K_p = -2.24 + 1.83 \log P_{mw} - 0.0123 MW$$

Hence a 1-unit increase in values of $\log P_{mw}$ is associated with an increase of 1.83 units in $\log K_p$ values, controlling for molecular weight. A 95% confidence interval for the $\log P_{mw}$ parameter is given by (1.43, 2.27); a 95% confidence interval for the MW parameter is given by (-0.0162, -0.00847).

The model is illustrated in Fig. 1 (three-dimensional regression plot) and Fig. 2 (partial regression plots) below.

A residual analysis did not detect any marked relationship between residuals and predicted values (Fig. 3), indicating no evidence for violation of regression assumptions.

The magnitude of the standardised coefficients (1.301 for $\log P_{mw}$ and 0.907 for molecular weight) indicated that $\log P_{mw}$ was of greater importance in the model. Both covariates were statistically significant ($t_1 = 9.61$, $p < 0.001$ for $\log P_{mw}$; $t_1 = 6.70$, $p < 0.001$ for molecular weight), with the model F -ratio also being statistically significant ($F_{2,19} = 46.2$, $p < 0.001$).

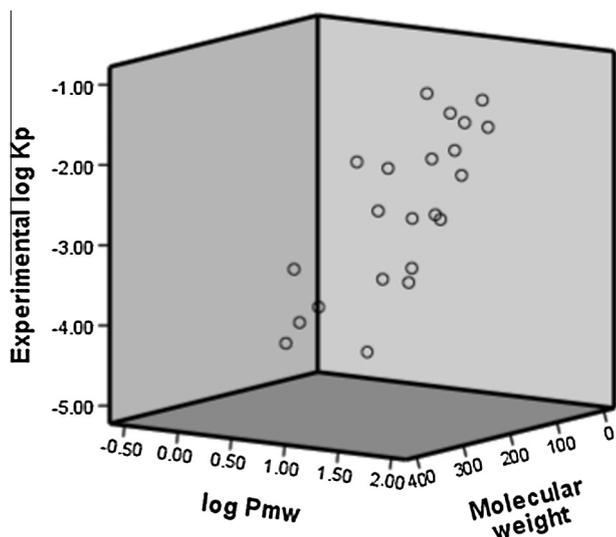


Fig. 1. 3-Dimensional plot of regression of experimental $\log K_p$ values against $\log P_{mw}$ and MW.

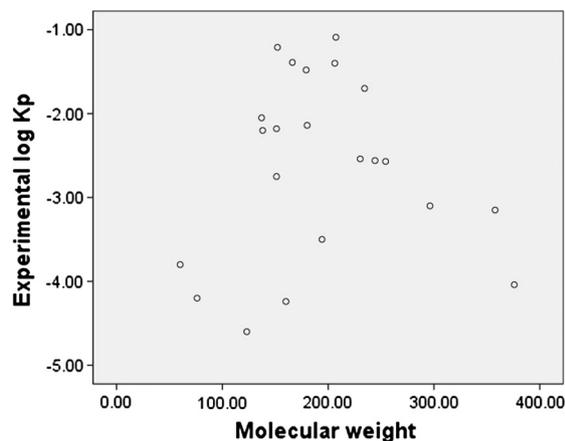
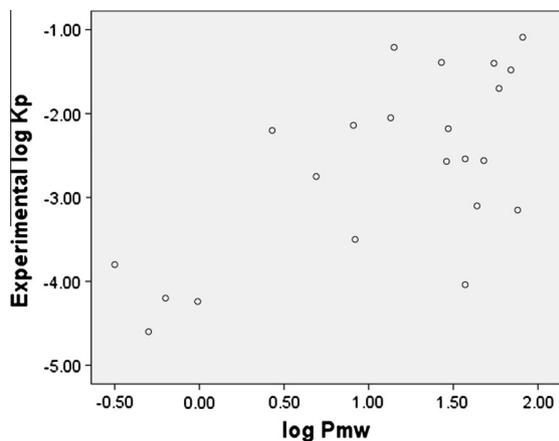


Fig. 2. Partial regression plots of experimental $\log K_p$ values against $\log P_{mw}$ and MW.

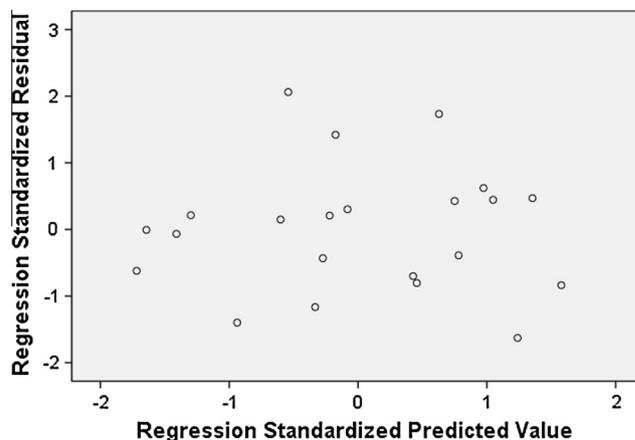


Fig. 3. Residual plot for optimal regression model.

The corresponding adjusted- R^2 statistic of the optimum model derived above was 0.811, which might be described as a large effect. The approximate R^2_{PRED} value was found to be 0.786. The close agreement with the corresponding adjusted- R^2 statistic indicates that the model does not over-fit the data.

None of the drugs in the data set were found to have high residuals or be influential according to the calculated Studentised residuals or Cook's distance values. The highest absolute Studentised residual (2.15), was associated with salicylic acid; while the highest value of Cook's distance of 0.190 was associated with thiourea. Haloperidol was found to have a relatively high leverage value of 0.355 (i.e. above the calculated cut-off value for the current data set of 0.272). However, the predicted experimental $\log K_p$ value associated with this drug of -4.01 was close to the actual experimental value obtained of -4.04; hence the overall influence of this drug on the data set would not be expected to be excessive.

Deleting haloperidol from the data set resulted in a negligible change in the R^2 statistic from 0.811 to 0.813 (95% confidence interval 0.689–0.937); with a corresponding change in the R^2_{PRED} statistic from 0.786 to 0.767.

4. Discussion

The unadjusted R^2 statistic of 0.829 derived from the current data indicates that the fit of the sampled drugs to the model is good, with about 83% of variance in the outcome measure being accounted for by $\log P_{mw}$ and MW values. This value is comparable

with unadjusted R^2 values obtained by several other researchers investigating the relationship between skin permeability and partition coefficients using a variety of different sets of drugs, reported by Moss et al. (2002).

The high consistency of the adjusted- R^2 and R^2_{PRED} statistics does not suggest any evidence of model or data inadequacies in the current model.

The R^2_{PRED} value of 0.786 implies that it could be expected that a model including just $\log P_{\text{mw}}$ values and molecular weight would explain about 79% of the variability in observations made in new drugs not currently considered. This may be compared with variability of about 83% in the existing data set being explained by the model. Hence the overall predictive capability of the model appears to be very good, in terms both of the high values of the R^2_{PRED} statistic, and the consistency between the R^2 statistics.

The deletion of a high-leverage drug from the data set results in a negligible change to the adjusted- R^2 and the R^2_{PRED} values, implying the model is not dependent on a full complement of “good” drugs for its predictive capability, and suggests that the model may be robust to minor variations in the subset of drugs utilised in its generation.

Although hard to compare, as datasets vary in size, in comparison with published data, it can be seen that this model is as good as or better than several existing models, including that of Potts and Guy (1992) with an R^2 value of 0.67, and Dearden (1990) with an R^2 value of 0.83, despite the latter being based on a more complicated six parameter system. It is noteworthy that using the drug set of the current study, the replacement of the $\log P_{\text{ow}}$ parameter in the equation derived by Potts and Guy with the $\log P_{\text{mw}}$ parameter results in a substantially better model fit. The R^2 value of 0.35 using the $\log P_{\text{ow}}$ parameter indicates that only 35% of the variability in observations may be accounted for by $\log P_{\text{ow}}$ and molecular weight in conjunction; less than half that accounted for by $\log P_{\text{mw}}$ and molecular weight in conjunction. Furthermore, the PRESS statistic associated with the Potts and Guy model formulation applied to the current data set was found to be around 4 times greater than the corresponding statistic derived from the model formulation including the $\log P_{\text{mw}}$ parameter. Hence the predictive capability of our model is substantially better than the Potts and Guy model.

Several published models provide greater values for R^2 using a range of alternative parameters (Abraham et al., 1995, 1997; Barratt, 1995; Lien and Gaot, 1995; Potts and Guy, 1995). However, despite these latter models appearing to provide enhanced predictive qualities there are many reasons for the incorporation of this particular model in the prediction of skin permeability. The first of these is in the practical benefits of chromatographically measuring a partition coefficient compared with the more traditional shake-flask technique. For example, measurements are obtained with comparative rapidity, accurately, precisely and with reduced organic solvent waste and thus deemed both economically and environmentally favourable. Secondly, this model is a good predictor of the outcome not only on the set of drugs studied in this work, but, as suggested by the R^2_{PRED} statistic, may also be applicable to other groups of compounds, as appears to be the case with on-going studies. This is often not considered in similar studies.

5. Conclusions

The chromatographically determined value of the octanol-water partition coefficient (P_{mw}) represents a substantial improvement on the traditional physicochemical parameter (P_{ow}) in the prediction of skin permeability coefficients in the study data set. In addition to the octanol-water partition coefficient, molecular weight is an important predictor of skin permeability coefficients. Predictive analysis suggests that a model based on $\log P_{\text{mw}}$ values

and derived from a set of 22 drugs would show good predictive capability in a wider population of drugs, and also on a different sample of drugs. This level of predictive capability has not been proven in other studies utilising models based on physicochemical parameters.

In the future it is anticipated that the ability of such systems to predict physicochemical data *in vitro* will become more and more important as the scientific community are encouraged to remove *in vivo* methods of analysis. For example, the recent change in the EU regulations on cosmetic testing have already sparked a far wider discussion on this topic which will continue to grow over the next few years.

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