# Evaluation and Application of Stationary Phase Selectivity for Drug Analysis

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## Abstract

Despite the wide range of HPLC stationary phases available for reversed-phase high-performance liquid chromatography (RP-HPLC) and the in-depth studies using probes to highlight differences between them, there is very little in the way of stationary phases which offer selectivity that is substantially different from that offered by the very commonly used alkyl-silicas. Therefore, the primary aim of the research programme was to explore and try to exploit LC stationary phases which offered genuinely different selectivity to alkyl-silicas for typical drug applications. Chiral stationary phases (CSP) potentially had different selectivity and in this context a secondary aim was to explore aspects of the enantioselectivity of CSP as well as their chemical selectivity.

Claims of orthogonal selectivity had been made for pentafluorophenyl (PFP) phases and phases exhibiting the hydrophilic interaction liquid chromatography (HILIC) mode. However, the Ultra PFP phase was found to be very similar in selectivity to ACE 5 C18 for both amitriptyline and acemetacin related compounds. The ZIC-HILIC phase was shown to behave as a reversed-phase material at high aqueous content in the mobile phase. There was some indication of selectivity orthogonal to that of ACE 5 C18 with low aqueous content in the mobile phase but this occurred at low retention and with mobile phases unsuitable for use with C18 phases in coupled (column or phase) systems. Nonetheless the work carried out shed more light on the mechanisms taking place in the HILIC mode which is currently attracting so much interest. Also it was possible to put ZIC-HILIC to good use for polar plant metabolites and other applications.

Chiral stationary phases (CSP) also offered the prospect of selectivity orthogonal to that of C18 phases. Given the proliferation of such phases though and the fact that it would be useful to use CSP that gave chiral separations for a broad spectrum of compound classes as well as giving orthogonal separations between different compounds, it was decided to carry out comparative studies of CSP classes in order to identify any redundancies and to seek out CSP that were complementary to one another. The Regis Whelk-O1 CSP was shown to be much superior to other higher-generation Pirkle-concept CSP such as DACH-DNB and ULMO. Also it was shown to be complementary to the Chiralcel OD derivatised

polysaccharide CSP and that both had something to offer alongside the widely used Chiralpak AD derivatised polysaccharide CSP. It was also found that a series of Chiralcel OD clones were virtually identical to Chiralcel OD and similarly for Chiralpak AD clones. Chiralpak IA, an immobilised version of Chiralpak AD, was not markedly less enantioselective than Chiralpak AD. Chiralcel OJ was less enantioselective than Chiralpak AD but the gap in performance was not as wide as between Whelk-O1 and the other Pirkle-concept CSP. The information gathered during these studies should prove to be of enormous value for further work in chiral LC method development screening.

Before embarking on applications work utilising the stationary phase selectivity that had been found, a study was carried out on the effectiveness of the high efficiencies obtainable with short run times through ultra-performance liquid chromatography (UPLC). It was found that, for a range of pharmaceutical applications, that it was still necessary in each case to adjust selectivity before increasing speed through working at higher temperatures with faster flow rates. In the course of this work some exceptionally high speed separations for example for paroxetine and related substances, benzodiazepines and flurbiprofen and related substances, were developed.

With respect to the evaluation of CSP as orthogonal phases to alkyl silicas under reversed-phase conditions, the Whelk-O1 CSP showed promise. However on closer inspection it was found that the Whelk-O1 CSP had very similar selectivity to the alkyl silica phase, ACE 5 C18, and deviation from this only occurred in instances when there was interaction with the chiral recognition site to give a separation of enantiomers. This prompted the notion that, rather than using Whelk-O1 in a coupled column system with ACE 5 C18, it could be used on its own for the separation of both trace enantiomer and all other related substances. This was shown to be possible using (*S*)-naproxen, laevokalim and (*S*)-flurbiprofen as illustrative examples.

The evaluation of the enantioselectivity of CSP led to an optimised resolution (suitable for scaling up for preparative work) of the enantiomers of the former ‘legal-high’ drug, mephedrone, on Whelk-O1 under normal phase conditions. It was also shown that the infrequently used Chiralcel OJ derivatised polysaccharide

CSP was ideal for developing an assay to determine trace amounts of (*R*)-nicotine in (*S*)-nicotine.

Overall, the information obtained on stationary phase selectivity and retentivity through evaluation and application will be of great value in HPLC and UHPLC column selection and also selection of orthogonal phases for coupled column systems but, ultimately, moving forward, most value may be in aiding the design of two-dimensional LC systems for complex mixture analysis. This would particularly apply to the use of CSP with reversed-phase eluents in achiral-chiral systems.

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## List of Abbreviations

| | |
|---|---|
| AGP | $\alpha_1$-acid glycoprotein |
| COMET | comprehensive orthogonal method evaluation technology |
| CSP | chiral stationary phases |
| DAD | diode array detector |
| 2D-LC | two dimensional liquid chromatography |
| ELSD | evaporative light scattering detector |
| GC | gas chromatography |
| HILIC | hydrophilic interaction liquid chromatography |
| HPLC | high performance liquid chromatography |
| I.D. | internal diameter |
| LC | liquid chromatography |
| MS | mass spectrometry |
| NP-HPLC | normal-phase HPLC |
| NMR | nuclear magnetic resonance spectroscopy |
| ODS | octadecylsilyl silica |
| PAT | process analytical technology |
| POPLC | phase optimized liquid chromatography |
| QC | quality control |
| R&D | research and development |
| RP-HPLC | reversed-phase HPLC |
| SAX | strong anion exchanger |
| SCX | strong cation exchanger |
| SFC | supercritical fluid chromatography |
| THF | tetrahydrofuran |
| TEA | triethylamine |
| TFA | trifluoroacetic acid |
| TLC | thin layer chromatography |
| UPLC | ultra performance liquid chromatography |
| UV-VIS | ultra violet-visible |

# 1. General Introduction

## 1.1 Overview

The research programme described in this thesis involves studies in the general area of the selectivity and, to a lesser extent, retentivity, of stationary phases in high-performance liquid chromatography (HPLC; or nowadays used almost interchangeably with the abbreviation LC, with the generally accepted meaning of 'modern liquid chromatography') relating to pharmaceutical applications. Therefore it is appropriate to first consider HPLC in general, its importance in pharmaceutical R&D, its development and the role of stationary phase selectivity and retentivity in developing separations.

## 1.2 High-Performance Liquid Chromatography (HPLC)

Analytical methods play a vital role in underpinning all the activities that take place in the Discovery, Development and Manufacture of drugs (Fig.1). At each stage of the cycle there is a close relationship between analysts and other pharmaceutical scientists which not only affects the speed with which progress to the market can be made (e.g. rapid analysis of toxicology formulations, rapid metabolism studies in Discovery, cleaning validation in process development) but also can have a profound influence on the actual direction of R&D (e.g. the advent of commercially-

available chiral stationary phases (CSP) which transformed the way in which chiral drugs were developed; active metabolites being isolated by semi-preparative HPLC, identified by nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) and ultimately replacing the pre-existing development compound).

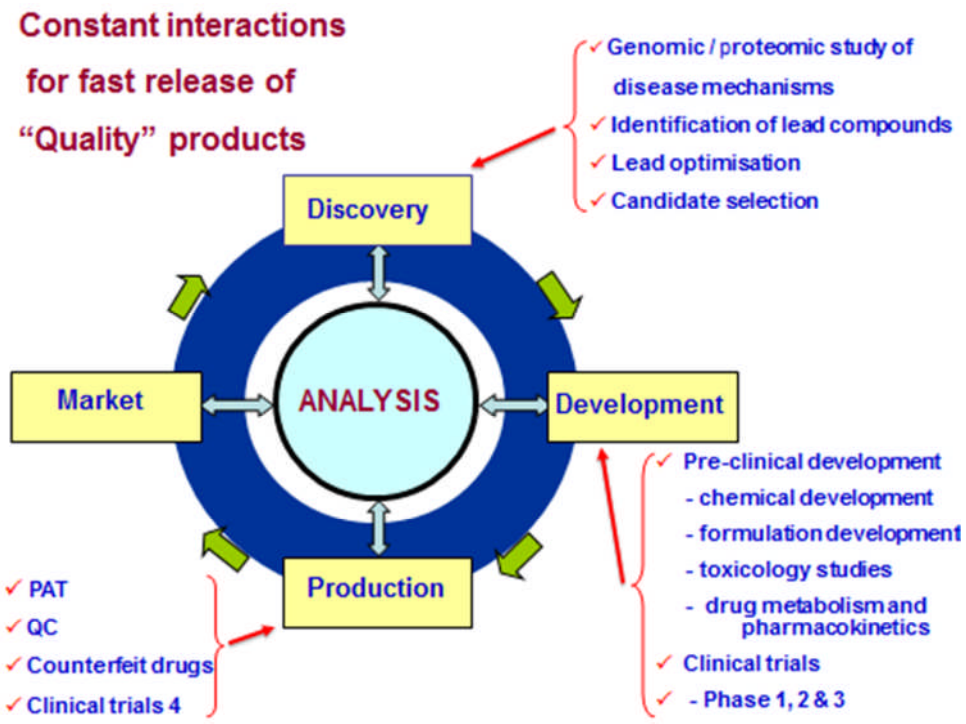



Figure 1.1 Role of analysis in pharmaceutical R & D showing its interactions at each stage from Discovery to the final product released to the market. (Concept used based on theme developed by W.J. Lough)

However, despite the many vital interactions with research and development activities that influence the direction of projects and help speed up progress to the market, the importance of analysis is perhaps best recognised for its role in providing assurance of Drug Quality without which the safety and efficacy of medicinal products cannot be guaranteed. Therefore, drug analysis is aimed to ensure that the expected levels of the drug substances are present in the end medicinal products and that the levels of the related substances which may be present in it are at or below toxic levels. The analysis of a drug substance involves qualitative analysis to separate and identify the drug substance and all of the drug related impurities present and quantitative analysis to determine their quantities. However, as is apparent from some of the applications given as examples in Fig. 1.1, analysis also plays an important role with respect to drug products, drugs in biological fluids and miscellaneous other sample types.

Methods involving chromatography are the most widely used in pharmaceutical R&D. Chromatographic techniques such as HPLC are employed very frequently in methods for drug analysis because they are *specific* i.e. the value measured arises from the analyte (that what it is intended to measure) and only the analyte. This specificity is dependent on achieving chromatographic resolution by separating all the peaks obtained from the peak arising from the analyte (and ideally from one another). In this way when peaks are measured the analyte and only the analyte is determined.

Chromatography is a separation process which involves the differential migration of all the components of a sample which are distributed between a stationary and mobile phase. The partition takes place while the components in the mobile phase pass through the column which contains the stationary phase. The speed of migration of each component through the column is governed by the affinity of each of them either to one or both phases. Modern high-performance liquid chromatography (HPLC/LC) is a considerably advanced form of the column liquid chromatography which was first introduced in 1903(Lough and Wainer, 1995; Ettre, 1980).The first systematic study using a technique known as “column chromatography” on chlorophyll extracts in petroleum was reported by Russian botanist Michael Tswett on March 21$^{st}$, 1903 (Ettre, 1980). From 1013 onwards, L.S. Palmer an American scientist used chromatography to separate the pigments in plants and dairy products. Later in 1930 Edgar Lederer a German scientist made an attempt to enhance the work of Tswett and Palmer in using chromatography in his investigational study on the pigments in egg yolk. The work carried out by Martin and Synge in 1941 (Ettre, 1980) on partition chromatography involved silica wetted with water and the use of an indicator. In 1943 they discovered paper chromatography using cellulose. Martin and Synge too produced the first mathematical treatment of chromatographic theory and won the Nobel Prize in 1952.Thin-layer chromatography (TLC) was introduced by Stahl in 1956 using a stationary phase adsorbent on a glass plate. The adsorbent was mixed to a binder to stick it to the glass plate. In 1952, Martin and his co-worker James were able to introduce gas chromatography (GC) using a gaseous mobile phase and a stationary phase consisting of an involatile viscous liquid coated on a solid

support to analyse volatile compounds. During the late 1960s more research work was still being carried out on GC. However, by using supercritical fluids as a mobile phase instead of using a gas, it was possible to develop to supercritical fluid chromatography (SFC) which was more applicable to involatile compounds. In 1957 Golay introduced the open tubular GC column in which the stationary phase was coated on the inner wall of a capillary column instead of packing solid particles in a wider column. Such columns became popular commercially in the early 1980's when glass columns were replaced by counterpart fused silica columns. These became the basis for modern capillary GC.

LC has moved on from the chromatographic theory of Martin and Synge in the 1940s, to the first instruments using 'normal-phase HPLC' in the late 1960swith systems from Waters Associates (Buie, 2010) and DuPont (Snyder *et al*., 2010) and beyond the late 1970s (Lough, 2000; Snyder *et al*., 2010)when 'reversed-phase HPLC' (Fig. 1.2) became the most prevalent mode of the technique.

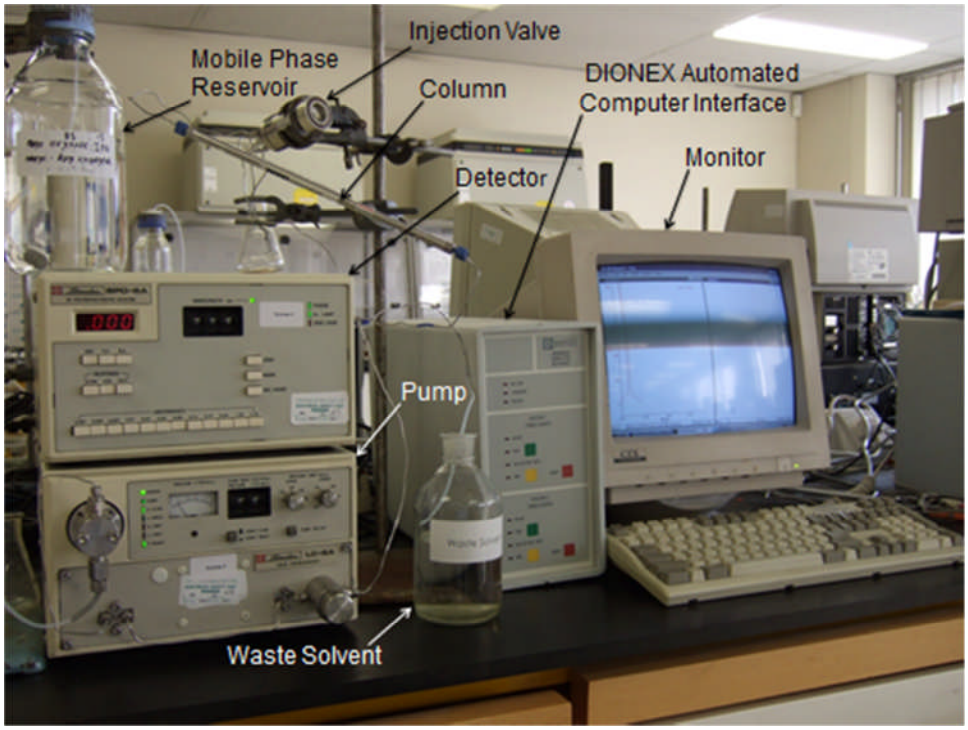



Figure 1.2 A typical isocratic HPLC System, consisting of a pump, detector, column, advanced computer interface, computer, monitor, mobile phase reservoir, injection valve and waste solvent bottle.

Reversed-phase HPLC separations (Lough, 2000; Majors, 1980) are performed using non-polar stationary phases with more polar mobile phases, usually consisting of a mixture of a polar organic solvent, such as methanol, acetonitrile etc, and water or an aqueous buffer. The separation of a mixture of components present in a drug sample is determined by the hydrophobicities of the analytes, since the levels of hydrophobicity governs the extent of the interaction of each analyte with the non-polar stationary phase. The retention mechanism of RP-HPLC is such that the non-polar regions of analyte molecules are repelled by the polar water molecules in mobile phase, which are present away from the hydrophobic stationary phase, driving the analyte molecules towards a less polar

organic layer which surrounds the non-polar stationary phase surface, allowing it to interact with the analyte molecules (Riley, 1995; Lough, 2000).Therefore, polar or more polar compounds elute before non-polar or less polar compounds, as they are less repelled by water molecules and are less likely to be driven towards the stationary phase surface(Fig. 1.3).

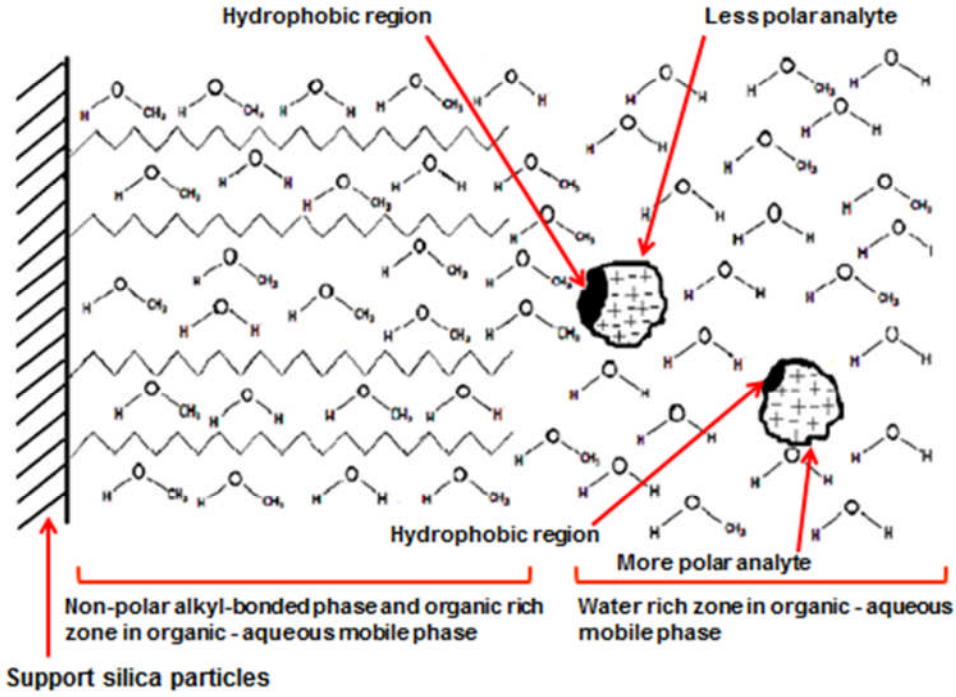



Fig. 1.3 RP-HPLC (partition) retention mechanism on non-polar alkyl-bonded silica stationary phase illustrating a repulsion of hydrophobic regions of an analyte molecule in the water-rich zone in the organic – aqueous mobile phase and attraction/interaction of this in a relatively non-polar zone close to the stationary phase.

In HPLC, the retention mechanism is governed by hydrophobicities/polarities of stationary phase, mobile phase and analytes and therefore, retention could be altered by changing any one or more of the parameters such as the type of stationary phase (bonded phase with longer alkyl chain or to a less non-polar or polar bonded phase), the content of the organic component in mobile phase, the column temperature, the pH and the ionic strength of the mobile phase (Majors, 1980; Riley, 1995; Lough, 2000).In normal-phase HPLC, polar stationary phases like silica, are used withn on-polar mobile phases and the components present in the sample, interact with the polar stationary phase differently, depending upon their polarities, allowing non-polar or less polar compounds to elute early while retaining polar or less non-polar compounds for a longer time. As drugs tend to be polar ionisable compounds they are generally well retained in normal phase HPLC and there may be difficulties involved in dissolving the drug in the mobile phase. Also basic drugs invariably give tailed peaks because of interactions with strongly acidic sites on the weakly acidic silica. Hence RP-HPLC is usually preferred for pharmaceutical analysis. In RP-HPLC, the mobile phase pH plays a vital role in the retention mechanism, as acidic molecules become less ionised and less polar with much lower pH of the mobile phase than their p$K_a$ values, while basic molecules become less ionised and less polar with higher mobile phase pH than their p$K_a$ values, giving a long retention in both cases. The mobile phase pH could be changed as desired by using a suitable phosphate buffer salt. The use of concentration of the salt, also have an effect on retention as the increased ionic strength of the mobile phase increases the retention times (Lough, 2000). In RP-HPLC, increasing the proportion of organic component in mobile phase reduces

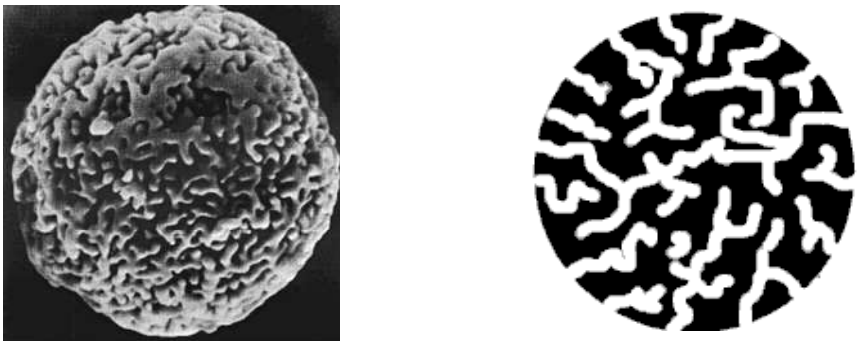
repulsion of analyte molecules towards stationary phase, by water molecules in mobile phase and reduces the retention time, due to reduction of analyte interactions with stationary phase. Replacing methanol with acetonitrile or THF in mobile phase reduces the retention due to the differences of polarity of such solvents, the lower polarity resulting in the hydrophobic component of the analyte molecules being more compatible with the mobile phase thereby reducing the holding times of analyte molecules in the organic layer. By a similar rationale, the retention increases with the use of alkyl-bonded silicas with longer alkyl chains (C4 to C8 or C18) and decreases with the use of phenyl or CN bonded silicas (Majors, 1980; ACE HPLC columns guide, 2010).Increased temperatures reduce mobile phase viscosity and enhance the speed of mobile phase mass transfer, resulting in reduced retention times, while improving efficiency.

### 1.3 Drivers for progress in HPLC

In the late 50s and in the 60s there was rapid growth in the pharmaceutical industry which gave rise to an unmet need for analytical methodology for the determination of involatile compounds. This was one of the main drivers for the development of modern liquid chromatography (LC).

The main factor leading to the step improvement which led from column liquid chromatography giving birth to HPLC was a reduction in the size of the particles used for the stationary phase. Column liquid chromatography improved greatly with the development of technology and the use of very small particles for the solid

adsorbent stationary phase, led HPLC to become a much improved instrumental technique, which has good resolving power, speed and detection in qualitative and quantitative analysis. The pioneers of HPLC such as Huber, Kirkland, Knox, Snyder and Scott played a major role in these developments. During the1970s, HPLC columns were improved by moving from 40 μm pellicular particles which consist of a non-porous core covered with a thin layer to 10 μm totally porous, irregularly shaped particles and later, in the mid- to late 1970s, to 5 μm totally porous, spherical particles with a narrow particle size distribution.



(a) (b)

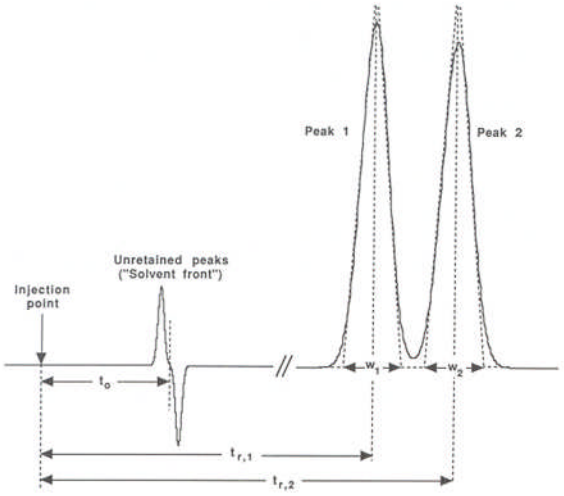
[(a) http://hplc.chem.shu.edu/NEW/HPLC_Book/Adsorbents/particl1.gif

(b) http://www.lcresources.com/resources/getstart/pores.gif]

Figure 1.4 5 μm totally porous spherical particle; even in the late 1970s, it was possible to pack columns containing these into stable beds and low 'dead volume' equipment which could handle the back pressure generated was available.

## 1.4 Emphasis on Resolution

As indicated earlier, chromatography is widely used because it has good specificity, resolving all the components of a mixture from one another before making a measurement. To do this, column efficiency, retention and selectivity (Fig. 1.4, 1.5) may be manipulated.




**capacity factor** $k = \frac{t_1 - t_0}{t_0}$

**selectivity** $\alpha = \frac{k_2}{k_1}$

**efficiency** $N = 16\left(\frac{t}{w}\right)^2$

**resolution** $R_s = \frac{(t_2 - t_1)}{1/2(w_1 + w_2)}$

Figure 1.5 A baseline separation, showing a separation of two components present in a mixture (Adapted from University of Sunderland lecture notes, W. J. Lough).

## 1.5 Resolution Equation

The qualitative relationship between resolution and efficiency, capacity factor, selectivity and retentivity (Fig. 1.4) may also be expressed quantitatively by substituting the expressions for efficiency, selectivity and capacity factor into the expression for resolution. After some rearranging, this gives the Purnell Equation (Riley, 1995; Smith and Braithwaite, 1996).

$$R_s = \frac{1}{4}\sqrt{N}.\frac{k}{(1+k)}.\frac{(\alpha - 1)}{\alpha}$$

*Eqtn. 1.1* Purnell Equation

As given in the Purnell Equation above, resolution ($R_s$) between two peaks depends on three factors, an efficiency factor (N), a selectivity factor (α) and a retention factor (*k*). Therefore, increased resolution could be achieved by maximising the contribution of any of these three factors. If two peaks are baseline resolved their $R_s$ value is approximately 1.5 (Riley, 1995) (Fig. 1.5).

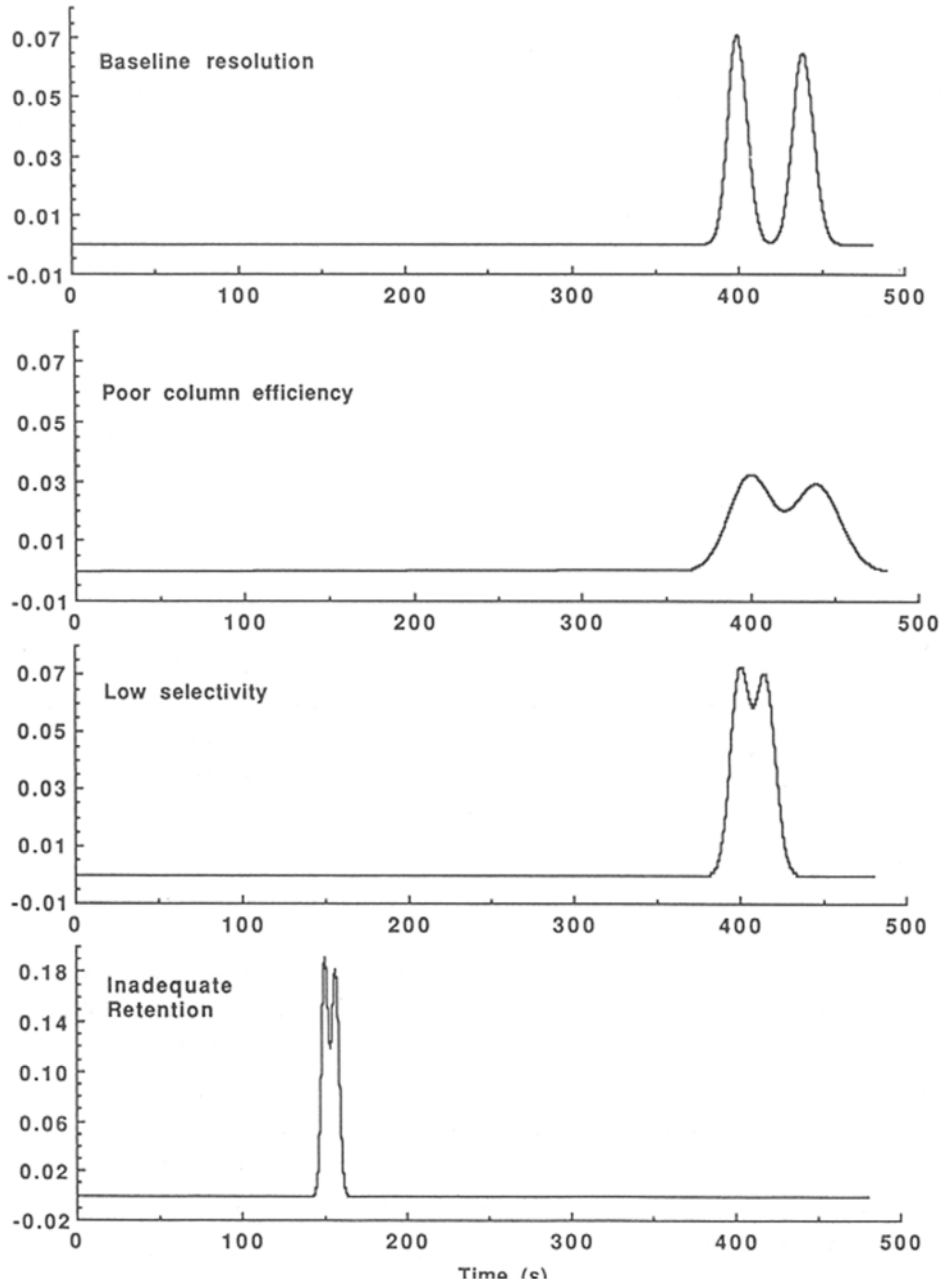



Figure 1.6 Effect of selectivity (α), column efficiency (N) and retention (*k*) on resolution (adapted from Snyder and Kirkland (1986)).

In reversed phase LC, greater affinity for the bonded phase will give longer retention timesand hence high *k* values. As indicated by the Purnell Equation, better resolution may be obtained by increasing *k*(e.g. by using a less strong mobile phase). This will not be the best option as changing mobile phase in occasional cases may also have an effect on selectivity but much more importantly will give rise to longer analysis times. (Fig. 1.6)

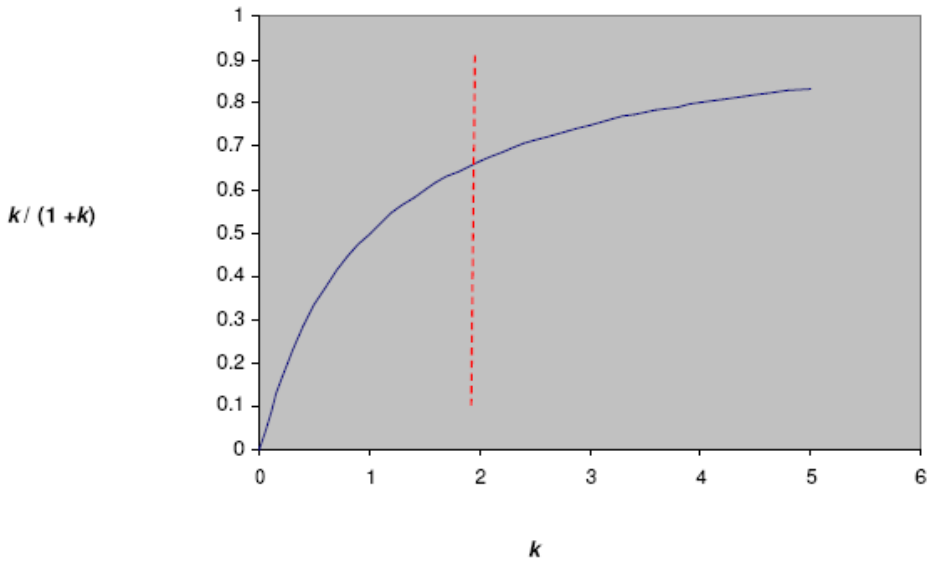



Figure 1.7 Relationship of retention factor in Purnell Equation (*k*/1+*k*) against retention (*k*). Increasing *k* up to ~2 has a significant effect on resolution but beyond this there are diminishing benefits

However, the sharpness of the peaks could be improved by increasing the column efficiency (N) which is also the number of theoretical plates. Therefore, as an alternative option to achieve high resolution, N could be increased by decreasing particle size. The use of smaller particles, 3 µm, was an option that was explored as far back as in the early 90s (Henry, 2009; de Biasi *et al*., 1987). It enabled the use of increased flow rates without sacrificing efficiency in order to achieve fast analysis times. However, this approach may not give very high resolution, due to the fact that $R_S$ is not directly proportional to N, but to the square root of N. Therefore doubling the number of theoretical plates by reducing the particle size will increase resolution only by 40% (Riley, 1995) or in other words, it is required to increase N by a factor of 4, in order to double $R_S$ (Lindsay, 1992). Despite this and despite practical obstacles, much of the recent developments in achieving fast HPLC have been in the use of sub-2 µm particles. The sub-2 µm particle stationary phases are fairly expensive and due to the high back pressures generated, this approach requires highly expensive specialised instrumentation. For example the pioneering Waters AcQuity UPLC system (Fig. 1.7) was introduced in to the UK in 2004 (Waters, 2004). The impact of UPLC is addressed in more detail in Chapter 4. In later developments by competing instrument manufacturer's and from Waters themselves instruments have been introduced which possess both HPLC and U-HPLC capability, U-HPLC being the more generic terminology favoured by manufacturers other than Waters.


Figure 1.8 A Waters ACQUITY UPLC system at the University of Sunderland showing the modern stacked modules configuration favoured by manufacturers to give a reduced ‘footprint’

From the resolution equation, it is apparent from the above discussion that at the outset of the research programme out of the three factors governing resolution, that, especially given that there were no further step improvements in efficiency changes envisaged, selectivity could be most easily manipulated to optimise HPLC separation. Selectivity can be increased by changing the "chemistry" of the separation either by changing one or more of silica support, bonded phase and

mobile phase. The improvement of selectivity may be useful even for the use of shorter columns, smaller particles and with higher flow rate in fast analysis.

## 1.6 Role for Selectivity

5µm ODS silica spherical particles were in general use by the early 1980s and well into the 2000s were still being used more commonly than 3 µm or sub 2 µm particles. Accordingly, for difficult separation problems, manipulation of selectivity seemed to be an attractive option rather than attempting to achieve resolution simply by increasing efficiency (Fig. 1.8). As for the equivalent plot for the retentivity factor (Fig. 1.6), there is a reduced rate of increase in resolution towards higher values on the x-axis. However, in this case, this is of little significance since at selectivity values greater than 2 it is difficult not to achieve separation.

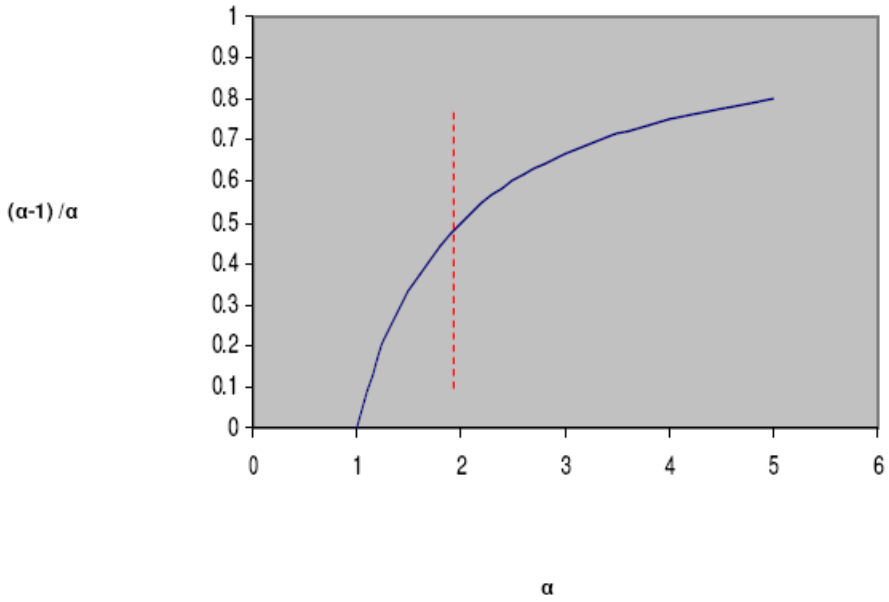



Fig. 1.9 Variation of selectivity factor (proportional to resolution) with selectivity. For the relatively difficult separations that are being dealt with in HPLC (I.e. $\alpha$<2), there will be a sharp rise in resolution with a rise in selectivity, **α**.

Further light may be shed on the importance of selectivity by further consideration of the Purnell Equation (Chapter 4).

In terms of ease of manipulating selectivity, 'expert' chromatographers would be able to consider analyte properties and make adjustments in the mobile phase (ratio of organic to aqueous components, pH, ionic strength, nature of organic component and/or buffer salt) as necessary. However by the end of the 1980s automated computer-driven mobile phase optimisation software/products were being developed (Dolan *et al*., 1989).

In chromatographic method development, it is vital to determine suitable chromatographic conditions which could give a reasonable retention for the main peak with an acceptable peak shape. This is usually considered to be a pre-requisite before moving on to improving resolution to ensure the specificity of the method being developed (Lough and Wainer, 2005; Lindsay, 1992). Decisions on the type of column, the type of solvent, the solvent strength, the additives, the pH and the temperature are dependent on the nature of the analyte and steps involving changes in these conditions are made to arrive at the desired retention and peak shape. Once this has been achieved, mobile phase optimisation steps are carried out in order to obtain a baseline separation of all the components present in the sample. To achieve this, the mobile phase could be further manipulated / fine-tuned, again by altering the proportions of organic component, changing pH and ionic strength and the level of additives such as ion-pairing agents and masking agents in the mobile phase to change the pH and the ionic strength.

## 1.7 Stationary Phase Developments

During the same time period (early 1990s to mid 2000), there was only a limited amount of work carried out on stationary phase selectivity. Most of the developments on stationary phases were aimed at reducing peak tailing and increasing reproducibility.

Only ~50% (Sunseri *et al*, 2003) of the surface silanols are expected to be derivatised as the size of molecules of derivatising reagents are much larger than silanols and also the pore size of silica particles are relatively smaller and therefore the accessibility to the silanol groups is restricted (Sunseri *et al*, 2003; HPLC Columns Technical Guide 2004, Thermo Electron Corporation). The different types of silanol groups such as ‘isolated’, ‘vicinal’ and ‘geminal’ silanols, which may still be remaining in the silica face even after derivatisation, can cause unwanted silanol interactions with the analytes, due to the acid properties of the remaining groups. Moreover, the silanol groups which are already H-bonded to adjoining silanol groups tend to be activated due to the presence of metallic impurities and could cause problems (Lough, 2000).The peak tailing and reduced efficiency of the peaks are caused by such properties. A base deactivation process (Riley, 1995; Thermo, 2003) will transform a silica surface to a much more homogeneous one with all the same type of silanol groups before derivatisaton and any remaining silanol groups after the derivatisation will be less interactive and become ‘friendly’. These interactions are further reduced by end capping. The basic molecules may require further shielding from even a few residual silanol groups that may be present even after end-capping. The addition of a masking agent such as N,N-dimethyloctylamine at concentrations in the order of 0.0005M to the mobile phase was used to achieve this (Riley, 1995). Fortunately the use of such reagents is nowadays only rarely necessary. Improvements have been made by, for example, using monochlorosilane rather than trichlorosilane alkyl-derivatising agents. The monochlorosilanes could be reacted with the hydroxylated silica surface.to attach one alkylsilane group to one silanol group to form a

‘monomolecular organic layer, which may minimise the free silanol groups on the surface. Further improvements were made by using ultra-pure silica and a range of different ‘shielding’ groups such as $^{t}$butyl- or more polar embedded groups such as carbamates.

Other developments on stationary phases included the development of stationary phases that could be used at extremes of pH. One such material was the styrene – divinylbenzene co-polymer phase, PRP-1, which could be used at high pH to estimate log P values of bases (Lough, 2000) because of its almost ‘pure’ hydrophobic partition (or interaction) retention mechanism. On the other hand, ‘Hypercarb’ (Fig. 1.9) is significantly different from traditional silica-based stationary phases, both in retentivity and selectivity. Hypercarb contains a non-derivatised fully porous graphite carbon surface which shows a porosity of 75% or more. The carbon atoms in the phase are hexagonally arranged and the phase is free from functional groups. The unique retention of the Hypercarb phase can separate a large number of highly polar and ionised compounds and geometric isomers (Pereira, 2010; Hypercarb HPLC Columns Technical Guide 2004, Thermo Electron Corporation). Another advantage of Hypercarb is that it is stable at pH 0-14 with any solvent at very high temperatures like 200 $^{0}$C because it is free from functional groups and silica. The retention behaviour of the Hypercarb phase is said to be governed by two main factors, firstly the ability of the analyte molecules to disperse onto the fully porous stationary phase and into the mobile phases and secondly, the ability of the charged functional groups of the analytes to generate charge-induced interactions on the polarisable graphite surface depending on the

ability of such functional groups to fit onto the flat surface, exhibiting a unique retention mechanism. For this reason, according to the manufacturers, it is usually expected that retention on Hypercarb occurs, according to the polarity order as well as the order of the molecular area of analytes, giving longest retention to the most polar analytes with less steric effects. (Hypercarb HPLC Columns Technical Guide 2004; Yulia & Row, 2005)

In contrast to C18 silica phases, the electrostatic interactions of polar compounds with the Hypercarb phase have a major impact on the retention mechanism of such compounds rather than their hydrophobicity order (Forgacs, 2002).

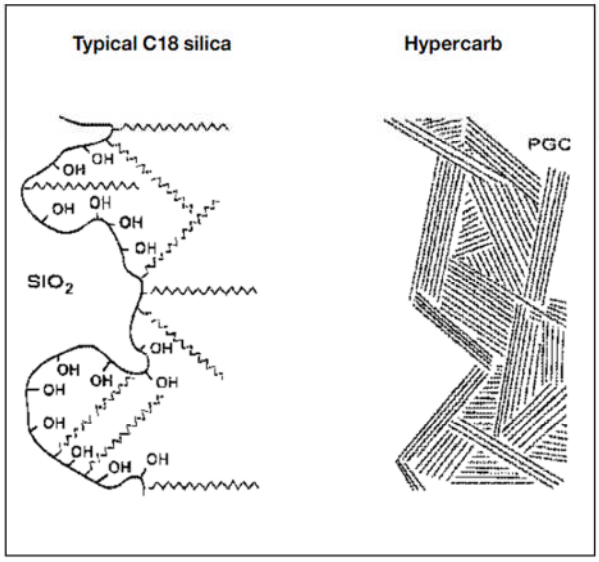



Figure 1.10 Surface comparison between ODS bonded silica and Hypercarb porous graphite carbon. Technical guide (2002), Hypercarb columns, Thermo Hypersil-Keystone.

(http://www.bioszeparacio.hu/_user/downloads/Thermo%20Electron/TG01-06.pdf)

Good pH stability was also later obtained via the preparation of ‘hybrid’ phases (Hambleton, 1995; Silva and Collins, 2008). Particles containing organosiloxane groups are formed in the surface structure of the phase and surface-bonding is performed to attach any reversed-phased groups such as C8 and C18 to the phase. The methylsiloxane groups which are present in hybrid particles reduce the concentration of residual silanol groups in the phase and therefore reduce the peak tailing effect even for highly basic analytes, while improving the efficiency of the peaks. As already indicated, these materials have the added advantage of improved pH stability being insoluble out with the pH 2.5 – 7.5 working range of traditional silica particles.

## 1.8 Importance of Stationary Phase Selectivity

As previously discussed, the manipulation of selectivity is very important in attempting to improve chromatographic resolution. Much of the time this can be done conveniently by mobile phase optimisation, for example using an experimental variable such as mobile phase pH that would increase in retention of one or more analytes and decrease in retention of another one or more (Lough, 2000). However there are specific instances when resorting to stationary phase selectivity is more appropriate. (difficult separations, chiral separations being an obvious subset of this; medium complexity applications using serial coupling of columns containing phases of orthogonal selectivity (Lough, Perera unpublished work on degradants in fenbendazole formulations) (subsequently commercialised as POPLC, Bischoff Chromatography, 2006); very complex mixtures (using 2D-LC

with columns of orthogonal selectivity)). However, the interest in stationary phase selectivity giving rise to this research programme was initially triggered by the then Big Pharma approach to LC method development throughout the Drug Development phase.

In method development strategies of UK based pharmaceutical companies such as Pfizer Global Research with their “Comprehensive Orthogonal Methodology” (COMET) (Soo, 2003) approach (Fig. 1.11), 3-4 reversed-phase HPLC columns with different selectivity are run in parallel, each column trying to uncover drug related substances that are not observed by using the other columns. However, approaches such as this will be not as effective as they could be if stationary phases that are only marginally different from one another are being used in the parallel column screens in rapid method development. In the context of this fast moving area of development, one of the key problems which needed to be addressed was, to identify chromatographic stationary phases, which are genuinely orthogonal to one another in their selectivity with reversed phase mobile phases.

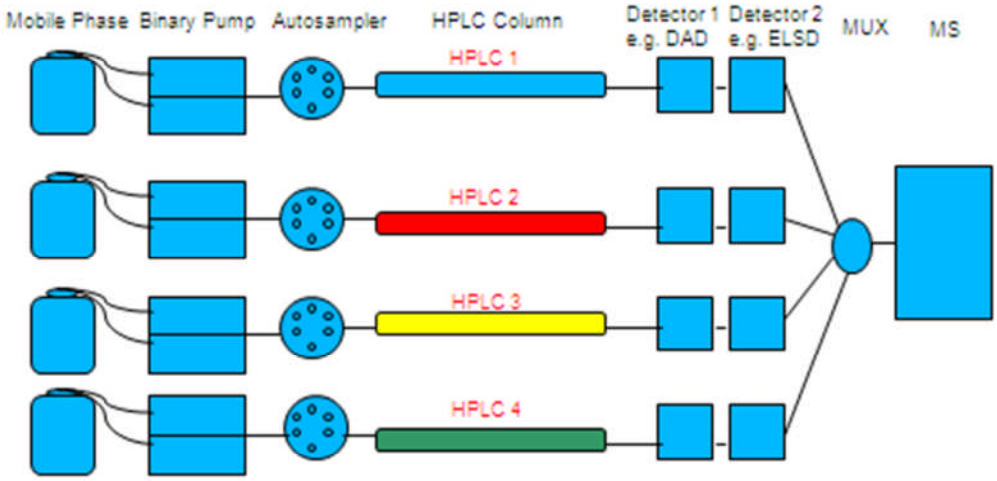



Figure 1.11 Screening approach to HPLC method development. The concept depends on the columns being used in parallel containing phases of 'orthogonal' selectivity (Craig Donnelly, oral presentation to final year students of BSc. (Hons) Chemical and Pharmaceutical Science, University of Sunderland, 2005.)

## 1.9 Historical Developments in using Selectivity

Previous research work which was carried out in the University of Sunderland, helped identify sets of genuinely orthogonal stationary phases and also to exploit them in mixed stationary phase (coupled column) systems to reduce run times and avoid gradient mobile phases in a drug related substance assay. In work by Soo (Soo,2003), it was demonstrated that only Hypercarb, cation exchange phases and anion exchange phases were genuinely orthogonal to C-18 reversed phase silica materials. Given this and the fact that it is not always easy to use the same

mobile phases for these phases as for the reversed-phase materials, there was a clear need for further studies to identify more stationary phases with orthogonal selectivity to the very commonly used C-18 reversed phase silicas. Indeed, although perhaps surprisingly considering the long history of the development of stationary phases for HPLC, there was a need to develop new stationary phases that will exhibit unusual selectivity when operated with polar organic – aqueous mobile phases.

In further work by Soo (Soo, 2003), Henderson (R. Henderson, Final Year Project, BSc Chemical and Pharmaceutical Science, 2004) and then Perera (R. W. H. Perera, Final Year Project, BSc Chemical and Pharmaceutical Science, 2005) it was possible to exploit tailored coupling of columns containing phases with orthogonal selectivity (mixtures of stationary phases with orthogonal stationary phases were also used) to arrive at very robust methods suitable for routine analysis of complex mixtures. Independently, a similar approach had subsequently been promoted commercially by Bischoff Chromatography
(Bischoff Chromatography, 2006), albeit using phases which are far from orthogonal. With phases with genuinely orthogonal selectivity, this coupled column(s) or mixed phases approach seemed to have considerable promise.

Perhaps the most successful exploitation of the designer phase/column mixture approach prior to the commencement of this research programme was the stability indicating assay developed for fenbendazole veterinary products(R. Henderson, Final Year project, BSc Chemical and Pharmaceutical Science, 2004). In this case

it was possible to prepare the optimum mix of C-18 and –SCX phases that would resolve the fenbendazole degradants well away from the neutral excipients present without adding significantly to the run time for the fenbendazole. There is no reason at all to suspect that this approach might not be generic for all products containing basic drugs and neutral excipients or, with different phases, for other types of drugs and excipients. It would be important therefore to quickly generate a number of illustrative examples e.g. of pharmacopoeial assays of basic drug products and one illustrative example for an acidic drug product.

The first type of application to which the designer phase/column mixture was applied was the determination of drug related substances in order to avoid the use of complex gradient systems. This worked well, using C-18 and –SCX phases, for a few proprietary drugs. However it was found that in using –SAX and C-18 phases for an assay of flurbiprofen the mixed phase approach offered no advantage. While the –SAX had orthogonal selectivity to C-18, the main reason for this was because it showed no selectivity for the carboxylate analytes and its presence therefore only served to attenuate the retention on C-18. Because of this, the application of the mixed phase approach to drug related substances assays using phases that are orthogonal to C-18 other than ion-exchangers needed to be explored.

## 1.10 Aims and Objectives

Given the state-of-the-art in LC with respect to achieving resolution at the outset of the programme the main aims were (a) to investigate the retention and selectivity properties of HPLC stationary phases (current and new arrivals) which were expected to give unique or orthogonal selectivity, and (b) to extend the exploitation of such phases from model illustrations to important applications to give more convenient assays with isocratic mobile phases and much reduced run times that would be suitable for use in routine analysis.

Of particular interest within these broad aims were the objectives of exploring the enantioselectivity of chiral stationary phases (CSP) in the context of redundancies in chiral LC method development and the retentivity and ‘chemical’ selectivity of CSP under reversed-phase LC conditions.

# 2. Evaluation of Achiral Stationary Phases for Orthogonal Selectivity

## 2.1 Introduction

As set out in the General Introduction (Chapter 1), stationary phase selectivity is of interest not just for difficult separations of pairs of compounds that cannot be resolved using mobile phase optimisation or high efficiency but also for method development, parallel screening, serial coupling of columns (now named commercially as POPLC (POPLC Bischoff Chromatography, 2006) and 2D-LC (Horvath *et al*., 2009). In previous work at Sunderland (Soo, 2003) it had been found for a wide range of ODS silicas that they only differed in selectivity by virtue of the degree of residual silanol groups remaining on the silica surface. Newer, ODS phases with ultra-inert, high purity silica showed some selectivity differences from older ODS silicas. However, these differences were not marked enough to be considered to give orthogonality or to give rise to retention order switches for drug separations. Also, since these separation differences were a consequence of differences in numbers of residual silanol groups, clearly using residual silanols to manipulate selectivity is not a good idea for basic drugs because of the accompanying peak tailing. Only Hypercarb and ion-exchange phases showed genuine orthogonal selectivity. Therefore, it was aimed to explore further options that had become available at the outset of the research programme and also to investigate their use in some applications that might seem suitable to be exploited by that mode.

## 2.2 Experimental

### 2.2.1 Instrumentation

#### 2.2.1.1 HILIC retentivity and selectivity evaluation

The HPLC system used consisted of a Shimadzu (Milton Keynes, UK) LC-10AD pump and SPD-10A UV-VIS detector. A manual 7125 loop injection valve, fitted with a 20 µl loop (Rheodyne, Kotati, Ca, USA), was used for loading samples. Data was collected using a Dionex PC-based data system with Automated Computer Interface and *AI* 450 Chromatographic Automation Software Release 3.33 (Leeds, UK). The water purifier ELGA Option 3 used was from (ELGA, High Wycombe, Bucks., UK).The sonicator used to degas mobile phases was from GS Group-ULTRAWAVE Ltd, Cardiff, CF2 1YY.

#### 2.2.1.2 ULTRA-Pentafluorophenyl (ULTRA-PFP) retentivity and selectivity evaluation.

Instrumentation used was as for 2.2.1.1.

#### 2.2.1.3 HILIC application for plant secondary metabolites

Instrumentation used was as for 2.2.1.1.

#### 2.2.1.4 HILIC application for acemetacin and related substances

Instrumentation used was as for 2.2.1.1.

#### 2.2.1.5 HILIC Application for urine constituents and amitriptyline metabolites

The HPLC system used consisted of a Dionex GP40 gradient pump (Severn Analytical, Wellington House, Cheshire, UK), Linear UV/VIS 200 detector with Dionex U120 Universal Interface (Dionex Corporation, Sunnyvale, USA) and *AI* 450 Chromatographic Automation Software Release 3.33 (Leeds, UK). Injections were made using a Thermo Separation Products autosampler fitted with a 20 µl loop. The water purifier ELGA Option 3 used was from (ELGA, High Wycombe, Bucks., UK).The sonicator used to degas mobile phases was from GS Group-ULTRAWAVE Ltd, Cardiff, CF2 1YY.

#### 2.2.1.6 LC assays for anti-bacterial products

Instrumentation used was as for 2.2.1.1.

#### 2.2.1.7 Application of a coupled column approach to develop a method for baclofen and its impurity A

The automated HPLC system, Agilent 1290 Infinity(Agilent Technologies, (76337) Waldbronn, Germany) used for loading and running samples, consisted of a

G4220A 1290 binary pump, G4226A 1290 sampler, G1316C Infinity TCC (Thermostatted Column Compartment) and a G4212A Infinity DAD detector. Data was collected using Agilent ChemStation software. Water purifier ELGA Option 3 used was from (ELGA, High Wycombe, Bucks., UK).

### 2.2.2 Materials and Methods

#### 2.2.2.1 HILIC retentivity and selectivity evaluation

ZIC – HILIC (250 mm x 4.6 mm I.D.) (SeQuant AB, 907 19 UMEA , Sweden), ACE 5 C18 (250 mm x 4.6 mm I.D.) and ACE 5 CN (250 mm x 4.6 mm I.D.), (Advanced Chromatography Technologies, Aberdeen, Scotland, UK), were used during the course of the study. Mobile phases were prepared using HPLC – grade methanol (HPLC far-UV grade, formic acid (Sigma-Aldrich, Poole, Dorset, UK), ammonium acetate, ammonium formate (BDH Laboratory Supplies, Poole, Dorset, UK). Amitriptyline and its metabolites were from Dr. I. W. Wainer, National Institute of Aging, Baltimore, USA. The urine metabolites such as creatinine, pseudouridine, hippuric acid, L-phenyl alanine, tyrosine, 4-hydroxy-benzoic acid, xanthurenic acid,phenyl acetic acid, ferulic acid, urea, indolyl-3-acryloyl glycine (IAG) and kynurenic acid, used in this study were from the collection of preparation of various samples available in Sunderland Pharmacy School.

For both the studies of 12 selected urine metabolites and amitriptyline and its metabolites, sample solutions of each compound in the two sets at 0.2 mg $ml^{-1}$ in

methanol – water (50:50, v/v) were prepared. 20 µl of each solution was injected initially onto an ACE 5 C18 (250 mm x 4.6 mm I.D.) column and later onto ACE 5 CN (250 mm x 4.6 mm I.D.) and ZIC–HILIC columns. Mobile phases were prepared by adding ammonium acetate to methanol – water mixtures (in the range 10 - 90% methanol, v/v) to achieve a concentration of 0.01 M and by adding ammonium formate to achieve a concentration of 0.02 M and then adding 2 ml of formic acid for every 1 L of mobile phase (or *pro rata* for different volumes). A flow rate of 1.0 ml $min^{-1}$was used throughout the experiments and UV detection at 254 nm (acemetacin and related substances) or 239 nm (amitriptyline and metabolites) was used. Water was distilled and doubly de-ionised using an ELGA Option 3 Water purifier(ELGA, High Wycombe, Bucks., UK). All work was carried out at ambient temperature (22-25 degrees)

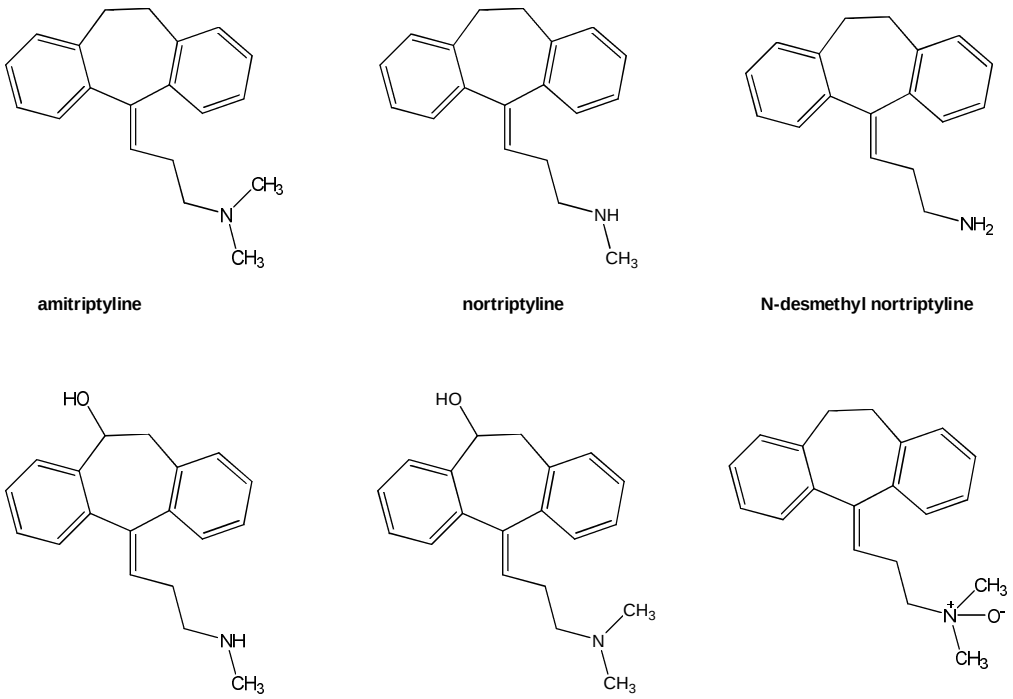



Figure 2.1 Amitriptyline and metabolites

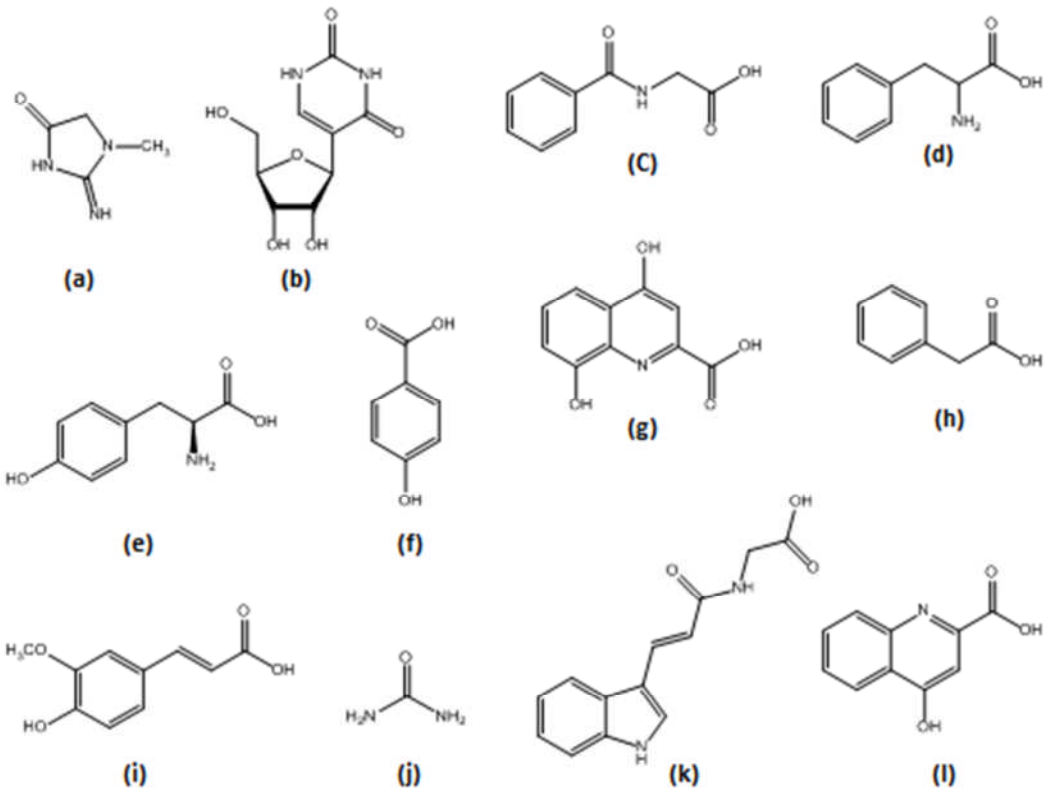



Figure 2.2 Urinary metabolites [(a) creatinine, (b) pseudouridine, (c) hippuric acid, (d) L-phenyl alanine, (e) tyrosine, (f) 4-hydroxy-benzoic acid, (g) xanthurenic acid(h) phenyl acetic acid, (i) ferulic acid, (j) urea (k) indolyl-3-acryloyl glycine (IAG) and (l) kynurenic acid].

### 2.2.2.2 Ultra PFP evaluation

Ultra PFP (250 mm x 4.6 mm I.D) (Thames Restek, Saunderton, Buckinghamshire, United Kingdom) and ACE 5 C18 (150 mm x 4.6 mm I.D.) (Advanced Chromatography Technologies, Aberdeen, Scotland, UK) columns were used during the course of the study. Mobile phases were prepared using

HPLC – grade methanol (HPLC far-UV grade, ammonium acetate, (BDH Laboratory Supplies, Poole, Dorset, UK). All acemetacin and its metabolites and/or potential related substances were from Sigma–Aldrich (Poole, Dorset, UK). Amitriptyline and its metabolites were from Dr. I. W. Wainer, National Institute of Aging, Baltimore, USA.

For both studies using acemetacin and its metabolites and/or related substances and amitriptyline and its metabolites, solutions of each compound in the two sets at 0.1 mg $ml^{-1}$ in methanol – water (50:50, v/v) were prepared. 20 µl of each solution was injected onto Ultra PFP (250 mm x 4.6 mm I.D.) and ACE 5 C18 (150 mm x 4.6 mm I.D.) columns. Mobile phases were prepared by adding ammonium acetate to methanol - water mixtures (in the range 10 - 50% methanol, v/v) to achieve a concentration of 0.01 M.A flow rate of 1.0 ml $min^{-1}$was used throughout the experiment and UV detection at 239 nm or 254 nm was used to monitor amitriptyline and its metabolites or acemetacin and its related substances respectively. Water was distilled and doubly de-ionised using an ELGA Option 3 Water purifier (ELGA, High Wycombe, Bucks., UK).All work was carried out at ambient temperature (22-25 degrees)

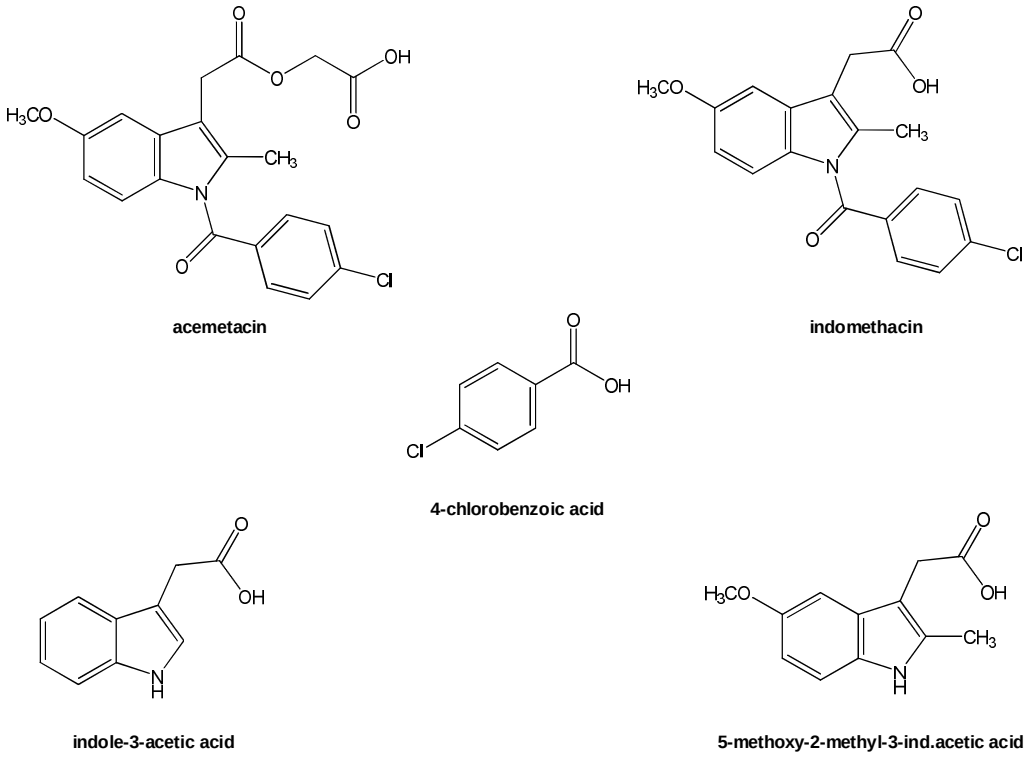



Figure 2.3 Acemetacin and related substances

2.2.2.3 HILIC application for plant secondary metabolites

A ZIC–HILIC (250 mm x 4.6 mm I.D.), (SeQuant AB, 907 19 UMEA, Sweden) column was used during the course of the study. Mobile phases were prepared using HPLC – grade methanol (HPLC far-UV grade), formic acid (Sigma-Aldrich, Poole, Dorset, UK) and ammonium formate (BDH Laboratory Supplies, Poole, Dorset, UK). The plant constituents, gossypol, gallic acid, codeine, physostigmine and quercetin were obtained from Sigma-Aldrich, Poole, Dorset, UK and theobromine was obtained from Sigma Chemical Co., Poole, Dorset, UK.

Plant metabolite solutions (containing theobromine, gossypol, gallic acid, codeine, quercetin or eserine) at 0.1 mg $ml^{-1}$ in methanol – water (95:05, v/v) were prepared and 20 µl of each solution was injected on to the ZIC – HILIC column (250 mm x 4.6 mm I.D.). A mixture was prepared adding all plant constituents at 1.0:1.5:2.0:10.0:1.0:1.5 (v/v/v/v/v/v) respectively and the mixture was further diluted to 1:1 with mobile phase. Mobile phase was prepared by adding 0.02 M ammonium formate to methanol – water mixture (95:05, v/v). For every 1 L of mobile phase, 2 ml of formic acid was added (or *pro rata*) for different volumes. UV detection at 254 nm was used to monitor the plant constituents. A flow rate of 1.0 ml $min^{-1}$ was used throughout the experiment. Water was distilled and doubly de-ionised using an ELGA Option 3 Water purifier (ELGA, High Wycombe, Bucks., UK). All work was carried out at ambient temperature (22-25 degrees).

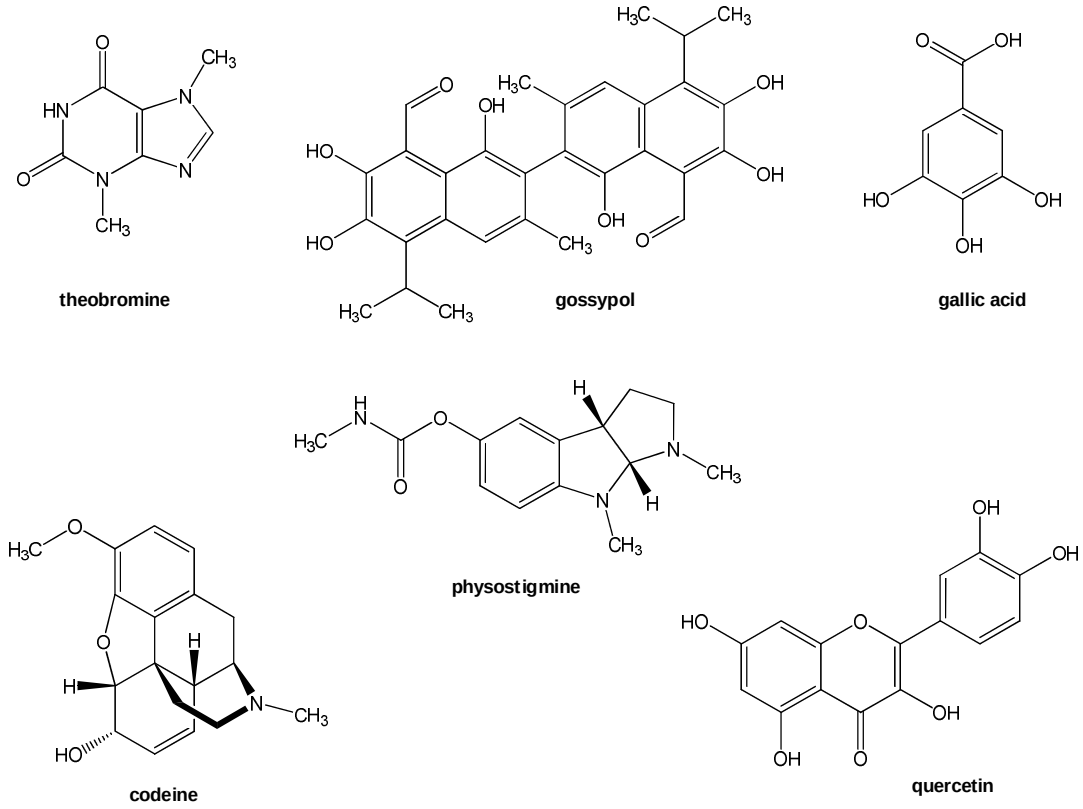



Figure 2.4 Plant secondary metabolites

### 2.2.2.4. HILIC application for acemetacin and its metabolites

ZIC–HILIC (250 mm x 4.6 mm I.D.) (SeQuant AB, 907 19 UMEA, Sweden) and ACE 5 C18 (150 mm x 4.6 mm I.D.) (Advanced Chromatography Technologies, Aberdeen, Scotland, UK) columns were used during the course of study. Mobile phases were prepared using HPLC – grade methanol (HPLC far-UV grade), formic acid (Sigma-Aldrich, Poole, Dorset, UK), ammonium acetate, ammonium formate

(BDH Laboratory Supplies, Poole, Dorset, UK). All acemetacin and related substances were from Sigma–Aldrich (Poole, Dorset, UK).

The sample solutions of acemetacin and its metabolites and/or related substances were, prepared at concentrations of 0.2 mg $ml^{-1}$ using the mobile phase.20 µl of each solution was injected onto ZIC-HILIC (250 mm x 4.6 mm I.D.) and ACE 5 C18 (150 mm x 4.6 mm I.D.) columns. Finally, a mixture containing all compounds was run on a combined ACE 5 C18 and ZIC-HILIC system in which both phases were coupled in series. Mobile phases were prepared by adding 0.01 M ammonium acetate to methanol in water mixtures (in the range 10 - 50% methanol). A flow rate of 1.0 ml $min^{-1}$was used when individual compounds were run and a low flow rate, 0.2 ml $min^{-1}$ was used when the mixture of all compounds were run on coupled column system. UV detection at 254 nm was used to monitor acemetacin and its related substances. Water was distilled and doubly de-ionised using an ELGA Option 3 Water purifier (ELGA, High Wycombe, Bucks., UK). All work was carried out at ambient temperature (22-25 degrees).

2.2.2.5 HILIC application for urine constituents, amitriptyline and its metabolites

ZIC–HILIC column (250 mm x 4.6 mm I.D.) (SeQuant AB, 907 19 UMEA, Sweden), ACE 5 C18 (250 mm x 4.6 mm I.D.) and ACE 5 CN (250 mm x 4.6 mm I.D.) (Advanced Chromatography Technologies, Aberdeen, Scotland, UK) columns were used during the course of study. Mobile phases were prepared using HPLC–grade methanol (HPLC far-UV grade), formic acid (Sigma-Aldrich, Poole, Dorset,

UK), ammonium acetate, ammonium formate (BDH Laboratory Supplies, Poole, Dorset, UK). Amitriptyline and its metabolites were from Dr. I. W. Wainer, National Institute of Aging, Baltimore, USA. All the urine metabolites were from the collection of preparation of various samples available in Sunderland Pharmacy School.

The sample solutions of amitriptyline and its metabolites and urine constituents were prepared at concentrations of 0.2 mg $ml^{-1}$ using the mobile phase. 20 µl of each solution was injected on to ZIC-HILIC (250 mm x 4.6 mm I.D.) and ACE 5 CN (250 mm x 4.6 mm I.D.) columns. Mobile phases were prepared adding ammonium acetate to methanol - water mixtures (in the range 10 - 50% methanol) and adding 0.02 M ammonium formate to methanol – water mixtures with 2 ml of formic acid for every 1 L of mobile phase(or *pro rata* for different volumes). A flow rate of 1.0 ml $min^{-1}$was used throughout the experiment and UV detection at 254 nm was used to monitor acemetacin and its related substances. Water was distilled and doubly de-ionised using an ELGA Option 3 Water purifier (ELGA, High Wycombe, Bucks., UK). All work was carried out at ambient temperature (22-25 degrees)

2.2.2.6 Development of LC assays for antibacterial products

ZIC–HILIC column (250 mm x 4.6 mm I.D.) (SeQuant AB, 907 19 UMEA, Sweden) and ACE 5 C18 (150 mm x 4.6 mm I.D.) (Advanced Chromatography Technologies, Aberdeen, Scotland, UK) and PLRP-S 4000A (5 µm) (50 mm x 4.6

mm I.D.) (Polymer Laboratories, Church Stretton, Shropshire, UK) columns were used during the course of study. Mobile phases were prepared using HPLC–grade methanol (HPLC far-UV grade), formic acid (Sigma-Aldrich, Poole, Dorset, UK), ammonium acetate, ammonium formate (BDH Laboratory Supplies, Poole, Dorset, UK). The samples of each component of two anti-bacterial products, polyaminopropyl biguanide, bronopol, chlorhexidine digluconate, IPA, tegotain AFB and cetrimide were from AGMA Ltd (Haltwhistle, Northumberland, UK).

The UV absorbance $\lambda_{max}$ was obtained using each component in two products with water as the solvent. All the samples solutions except IPA, were prepared at concentrations of 0.2 mg $ml^{-1}$ using the mobile phase and 20 µl of each solution was injected onto ACE 5 C18 (150 mm x 4.6 mm I.D.), Spherisorb 5 SCX (100 mm x 4.6 mm I.D.), ACE 5 AQ (150 mm x 4.6 mm I.D.), ZIC-HILIC, PLRP-S 4000A (50 mm x 4.6 mm I.D.) and H 5 ODS columns, using mobile phases containing mixtures of methanol – water (in the range 0% - 50% methanol, v/v) and acetonitrile – water (70% - 90%, v/v) initially with 0.01M perchloric acid and subsequently with 0.01M phosphate buffer, 0.02 M ammonium acetate and 0.02 M ammonium formate and 0.03% formic acid. Water was distilled and doubly de-ionised using an ELGA Option 3 Water purifier(ELGA, High Wycombe, Bucks., UK). All the work was carried out at ambient temperature (22-25 degrees).

2.2.2.7 Application of coupled orthogonal phases approach to develop a method for baclofen and its impurity A

The sample solutions of baclofen and its impurity A were prepared at concentrations of 0.2 mg $ml^{-1}$ using the mobile phase. 10 µl of each solution was injected on to ZIC-HILIC (250 mm x 4.6 mm I.D.) and ACE 5 CN (250 mm x 4.6 mm I.D.) columns. Mobile phases A and B were prepared adding ammonium acetate to methanol - water mixtures (in the range 10- 50% methanol) and adding 0.02 M ammonium formate to methanol – water mixtures with 2 ml of formic acid for every 1 L of mobile phase(or *pro rata* for different volumes). A flow rate of 1.0 ml $min^{-1}$was used at initial stage and 2.0 ml $min^{-1}$was used at final stage of the experiment and UV detection at 254 nm was used to monitor acemetacin and its related substances. Water was distilled and doubly de-ionised using an ELGA Option 3 Water purifier (ELGA, High Wycombe, Bucks., UK). All work was carried out at ambient temperature (22-25 degrees).

## 2.3 Results and Discussion

### 2.3.1 HILIC retentivity and selectivity evaluation

The HILIC mode of LC was causing some interest at the time of the outset of this research programme. (Pellett *et al*., 2006)The ZIC-HILIC phase, for example, had been reported to be suitable for the separation of polar and hydrophilic compounds as it was being talked of in terms of being orthogonal to reversed phase liquid

chromatography (RPLC). Analytes such as amino acids, peptides, carbohydrates, plant extracts and various other polar compounds that usually have little or no retention in reversed phase HPLC are expected to have strong retention on the HILIC column with very high levels of organic component (above 80-85%), especially acetonitrile, in the mobile phase. For ZIC-HILIC the zwitterionic stationary phase, sulfobetaine groups (SeQuant Innovators in Chemical Analysis, 2006; 'Laboratorytalk', 2007) is attached to porous silica. The separation is said to be achieved by a hydrophilic partitioning mechanism which is caused by weak electrostatic interactions. An important feature of the HILIC mode is that the low content of both water and buffer salt in the mobile phase enables higher detection sensitivity with the most compatibility in many LC-MS (Hemtrom and Irgum, 2006; Boersema, 2008)applications.

As alluded to above, LC using the HILIC mode has occasionally been stated as being orthogonal to the reversed-phase (RP) mode (Pellett *et al*., 2006; Boersema, 2008). However, consideration of the retention mechanism of the HILIC (Fig. 2.5) and RP modes suggests that HILIC will simply give reversed retention order to RP-HPLC rather than orthogonal selectivity. Accordingly it was sought to examine the comparative selectivity of HILIC and reversed-phase LC by using sets of compounds in each of which the compounds were structurally related.

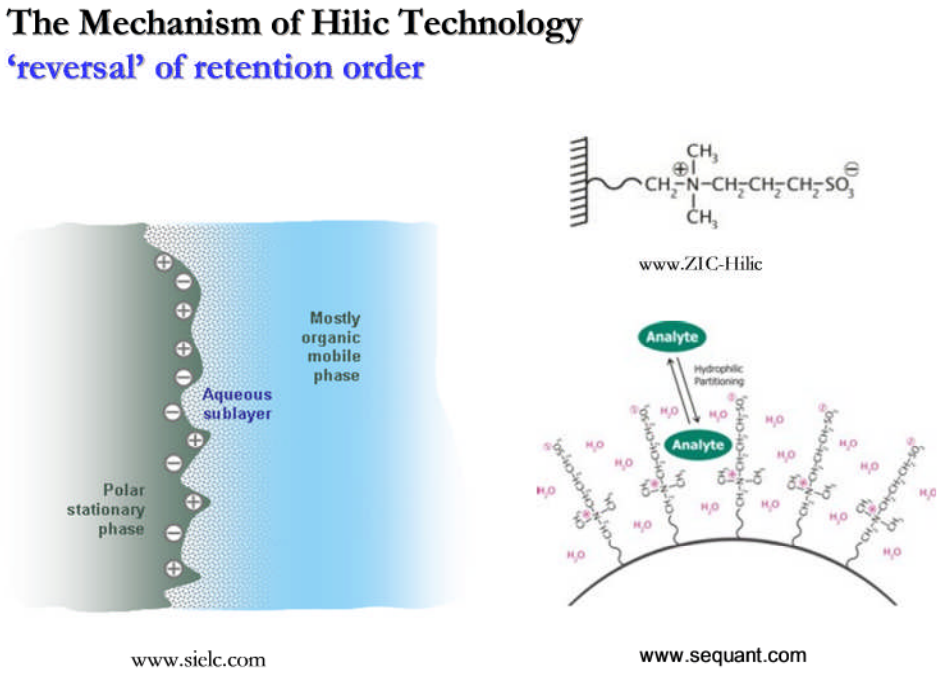



Figure 2.5 Structure and basic principles of operation of the ZIC-HILIC stationary phase (http://kvcv.be/downloads/hplc/14h15%20SeQuant_ZIC-HILIC_Seminar_Grobbendonk_061012.pdf).

The comparison of ZIC-HILIC and ACE 5 C18 selectivity was carried out using a drug, amitriptyline and a set of its metabolites. The rationale for this was related to the fact that drug metabolites are generally much more polar than the drug itself and consequently a gradient RP-HPLC method is frequently needed for the determination of a drug and its metabolite. Logically, if as suggested ZIC-HILIC gives a reversal of retention order, then it should be possible to couple C18 and ZIC-HILIC columns in order to arrive at a simple isocratic method.

#### 2.3.1.1 Selectivity Comparison

Selectivities were compared in a similar fashion to the previous Sunderland studies by Soo (Soo, 2003) by plotting *k* or log *k* on a ZIC-HILIC column *v* that on a RP-HPLC column and by counting the percentage of compound pairs for which the retention order was switched (reversed selectivity would be expected to give rise to a 100% switch of compound pairs whereas ~50% would be expected for orthogonal selectivity). However, some problems were encountered in arriving at suitable mobile phases that would allow an appropriate comparison between HILIC and RP-HPLC i.e. significant but not excessive retention needed on both phases. Even with as much as 90% methanol in the mobile phase there was very little retention on the ZIC HILIC column. What was more to the point, there was no retention on ACE 5 C18 with this mobile phase. Therefore the percentage aqueous component had to be increased to 30% to obtain significant retention on both columns. Also, possible outliers were a problem for amitriptyline metabolites $r^2$ = 0.0521 but = 0.8750 (Fig. 2.6) when the N oxide is considered as an outlier. Further, while $r^2$ values were generally less than 0.75 it was difficult to assess whether, say, 0.3 is any more orthogonal than 0.5. Therefore, it was decided to resort to the much more practical retention reversal indicator. It was apparent, using this, that the HILIC selectivity was more, but not exactly, the same as that of RP-HPLC rather than being orthogonal or reverse. These results suggest that it is erroneous to refer to a HILIC column because HILIC column can clearly operate in a non-HILIC mode. Related to this is that currently there are a number of different

types of phase which are said to be HILIC and can operate in the HILIC mode but are different in other respects (McCalley, 2010).

With respect to the potential application of coupling HILIC mode and RP-HPLC mode in order to facilitate an isocratic method when otherwise a gradient method might have been necessary, this was achieved, subsequent to this study, by Hanna-Brown (Louw *et al*., 2008). However, on the evidence here, by the compound set would have needed to be very unusual and specially selected for this to have been achieved.

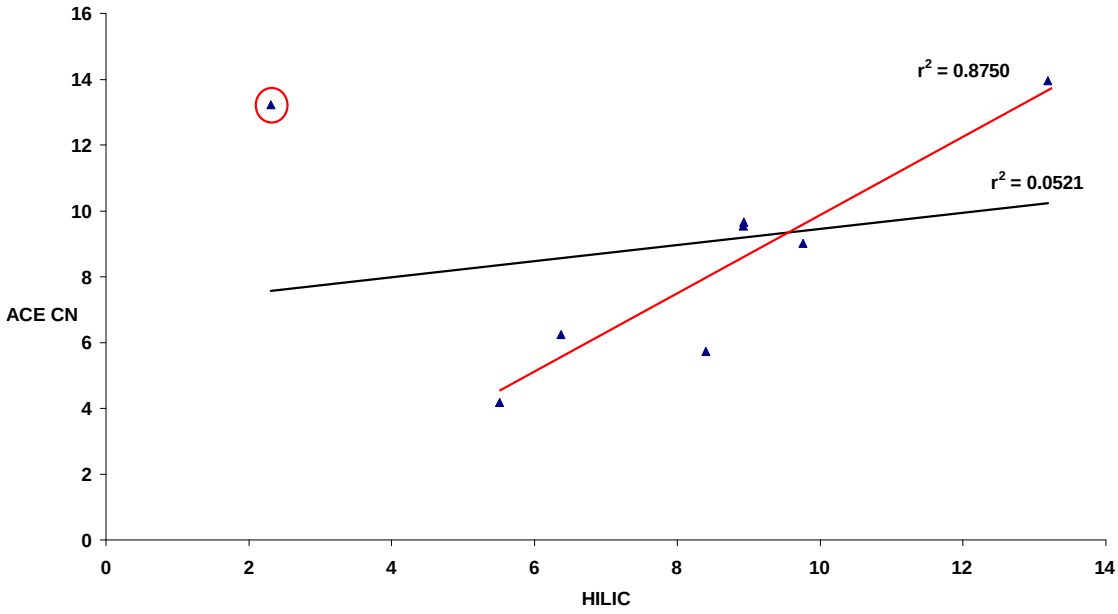



Figure 2.6 *k* of ACE CN v *k* of HILIC for amitriptyline metabolites

### 2.3.2 Evaluation of Ultra PFP (Pentafluorophenyl) Phase

Ultra PFP was selected because of promise of phases with potential for $\pi$–$\pi$ interactions in previous work (Soo, 2003). However until recently there had been few such phases commercially available. Therefore, a study of PFP phase was taken into consideration with the aim of exploring any different selectivity that PFP could offer over the modern alkyl phases or even over the phases containing cyano, amide, urea and carbamate linkages which had also been shown to be similar in selectivity to alkyl RP-HPLC phases by Soo (Soo, 2003). However,PFP which is a fluorine-substituted stationary phase was said to have shown unique retention and selectivity for certain groups of analytes (Restek Catalogue, page 318 - www.restek.com), as it was believed that the electronegativity of fluorine in PFP phases has a major impact on the polar attraction of the phase towards such analytes giving longer retention times. Furthermore, due to the strong interaction forces of the PFP phase towards some analytes, it had been said to have altered the phase behaviour from reversed phase to normal phase, while using reversed phase mobile phase containing wat6er (Supelco e-article, http://www.sigmaaldrich.com/img/assets/3560/T404055H1.pdf).

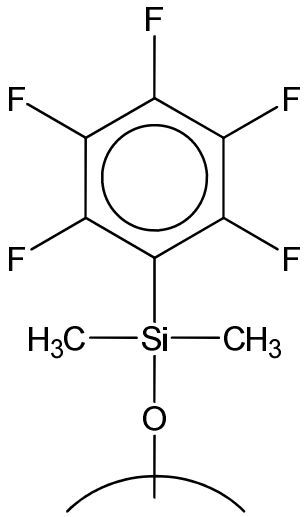



Figure 2.7 Ultra Pentafluorophenyl Phase

Amitriptyline and its metabolites had been earmarked as a set for compounds which might be well suited for an application of the ZIC-HILIC phase but they were obviously also useful as a set that could be used to compare the selectivity of Ultra PFP and ACE 5 C18 (Table 2.1, Fig. 2.8).

Table 2.1 *k* for RPLC of amitriptyline and related substances on Ultra PFP (100 mm X 4.6 mm I.D.) and ACE 5 C18 (150 mm X 4.6 mm I.D.); mobile phase – [methanol - water (50:50, v/v)], 0.01M ammonium acetate; UV detection at 239 nm.

| **Compound** | **ACE 5 C18** | **Ultra PFP** |
|---|---|---|
| amitriptyline HCl | 4.68 | 0.9 |
| amitriptyline -N-oxide | 1.9 | 0.5 |
| E-10-OH amitriptyline | 3.64 | 0.7 |
| Z-10-OH amitriptyline | 14.41 | 1.71 |
| nortriptyline HCl | 2.77 | 0.76 |
| N-desmethyl nortriptyline HCl | 23.82 | 2.71 |
| E-10-OH nortriptyline | 15.07 | 1.63 |
| Z-10-OH nortriptyline | 21.86 | 2.74 |

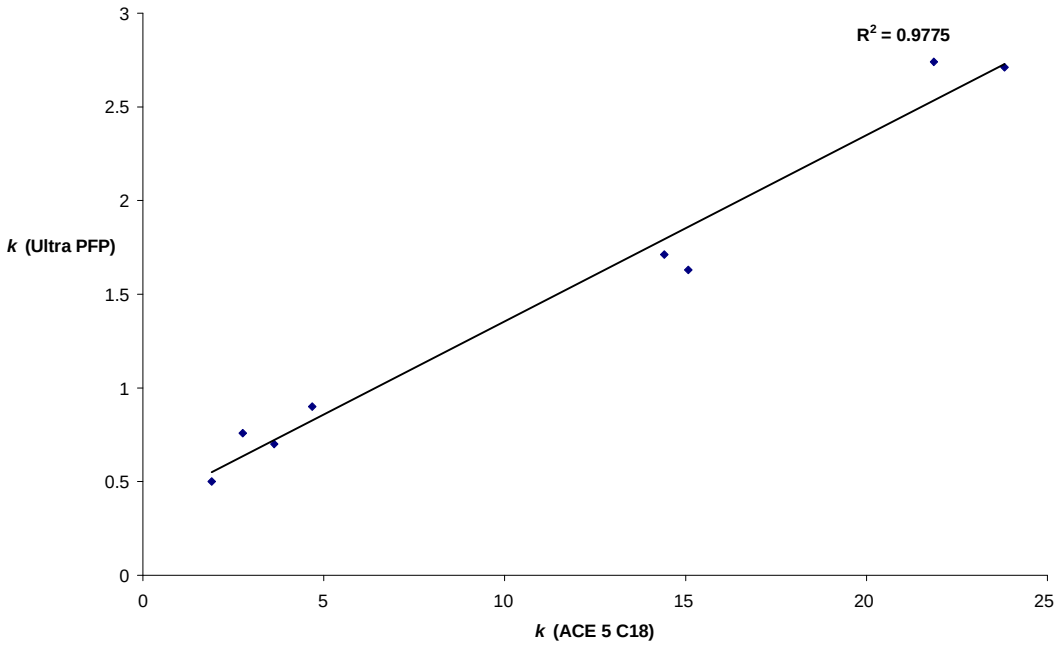



Figure 2.8 *k* of Ultra PFP (100 mm X 4.6 mm I.D.) v *k* of ACE 5 C18 (150 mm X 4.6 mm I.D.) for RP-HPLC of amitriptyline and related substances; mobile phase – [methanol - water (50:50, v/v)]; 0.01M ammonium acetate; UV detection at 239 nm.

Surprisingly, or perhaps not surprisingly in light of the previous work by Soo, the results with this compound set suggested that Ultra PFP was very similar in selectivity to ACE 5 C18.

Just in case this was a unique feature of the compound set being used, a comparison was also made using acemetacin and structurally-related substances, some of which might also potentially be metabolites (Table 2.2, Fig. 2.9).

As for the amitriptyline and metabolites set, there was a high degree of correlation between Ultra PFP and ACE 5 C18. This might have been due at least in part to the data points being clustered into two separate groups. However, that said, there no encouragement for the notion that the two phases were orthogonal to each other. It was thought that the orthogonal / complementary / unique / etc selectivity often claimed for phases such as this might be a consequence of them having been tested primarily with especially designed ‘probe’ compounds (Euerby, 2003).

Table 2.2 *k* for RPLC of acemetacin and related substances on Ultra PFP (100 mm X 4.6 mm I.D.) and ACE 5 C18 (150 mm X 4.6 mm I.D.); mobile phase – [methanol - water (50:50, v/v)], 0.01M ammonium acetate; UV detection at 254 nm.

| **Compound** | **ACE 5 C18** | **Ultra PFP** |
|---|---|---|
| indomethacin | 13.35 | 1.09 |
| 4-chloro-benzoic acid | 0.55 | 0.15 |
| acemetacin | 17.62 | 1.26 |
| indole-3-acetic acid | 0.09 | 0.07 |
| 5-methoxy-2-methyl-3-indole acetic acid | 0.16 | 0.09 |

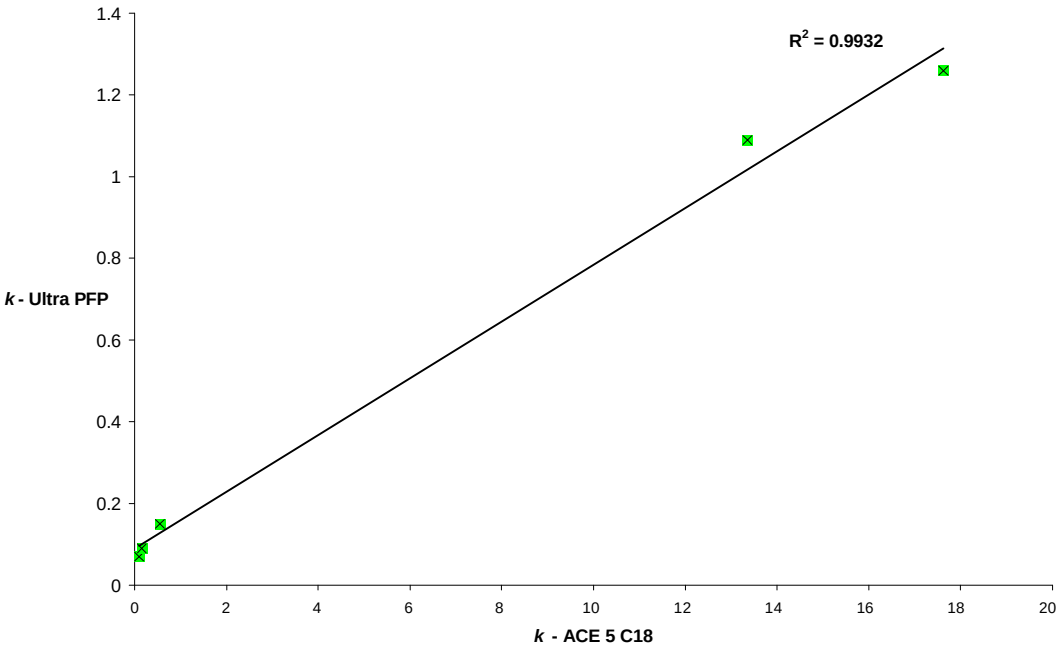



Figure 2.9 *k* of Ultra PFP (100 mm X 4.6 mm I.D.) v *k* of ACE 5 C18 (150 mm X 4.6 mm I.D.) for HPLC of acemetacin and related substances; mobile phase – [methanol - water (50:50, v/v)], 0.01M ammonium acetate; UV detection at 254 nm.

### 2.3.3 HILIC Applications: plant secondary metabolites

From the studies on evaluation of ZIC-HILIC phase and, more so, from subsequent studies reported in the literature (McCalley and Neue, 2008; Clinchrom.com, A mini-tutorial in ZIC-HILIC), it was apparent that the phase might offer benefits in retaining polar compounds with mobile phases containing very high percentage of organic component and a salt, due to its claimed nature of giving reversal of selectivity with such conditions. It was also obvious that the

mobile phases with high methanol content would be much more compatible with LC-MS as it would be, more easy to evaporate off solvent which may be having much less proportion of aqueous, most likely 5-10% or even less. Therefore, it was decided to look at very polar plant constituents which may require LC with MS detection as the first application (Hemtrom and Irgum, 2006; Boersema, 2008) of the ZIC-HILC phase.

For the set of plant secondary metabolites, the use of the ZIC-HILIC phase allowed the compounds to be eluted in a mobile phase with a very high methanol content and scope for more retention and resolution with mobile phase optimisation (including further increase in % methanol). Clearly, in the context of preparative isolation, this was a major advantage over the water-rich mobile phases that would be needed for RP-HPLC. Due to the high content of methanol in mobile phase, the solvent from the fractions collected could be easily evaporated for further analysis of fractions by HPLC and other techniques or on-line LC-MS for identification of their structures, could be easily used.

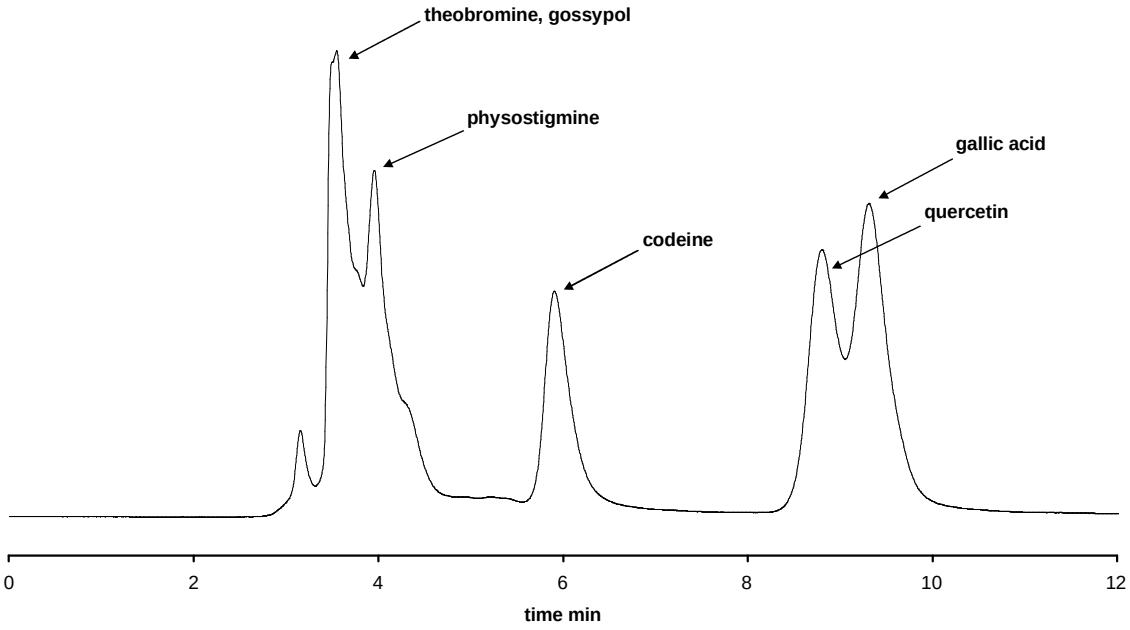



Figure 2.10 LC of a set of secondary plant metabolites on ZIC-HILC (250 mm x 4.6 mm I.D.); mobile phase – [methanol - water (95:5, v/v)], 0.02 M ammonium formate, formic acid 2ml/L; flow rate - 1.0 ml $min^{-1}$; UV detection at 254 nm; ambient temp. 22-28$^{o}$ C.

The illustrative secondary plant metabolites were not all resolved from one another but the main point had been to demonstrate that these compounds could be eluted in mobile phases containing low percentages of water. No comparison with ACE 5 C18 was carried out for these compounds as it was already known (S. Myers, Final Year Project, BSc. Chemical and Pharmaceutical Science, 2007, P. Kharabe, Current research student for M.Phill. Programme) that a high proportion of aqueous component was needed for their retention in RP-HPLC.

2.3.4 HILIC applications: Acemetacin and related substances

For the ZIC-HILIC column, it was generally not possible to obtain a common mobile phase that gave retention on both ZIC-HILIC and ACE 5 C18. However, this proved possible for acemetacin related substances albeit only because exclusion chromatography was observed on the ZIC-HILIC column at mid-range % methanol. While retention on the ZIC-HILIC phase was minimal with this mobile phase it, nonetheless, was sufficient to allow some useful crossover of retention to give a better separation on a coupled column than on either of the columns individually (Fig 2.11).

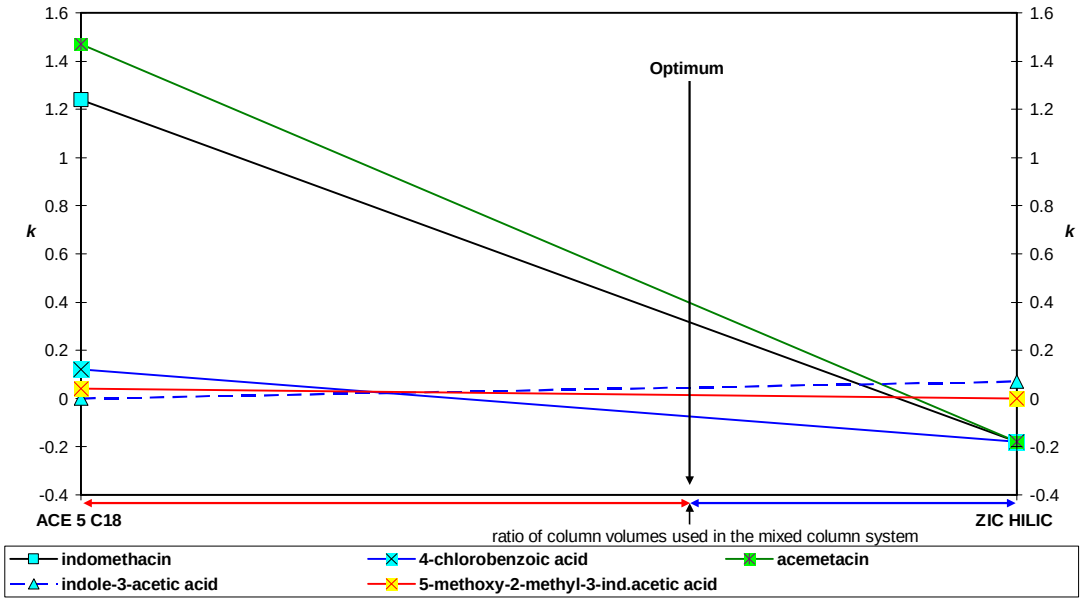



Figure 2.11 *k* values of LC for acemetacin and its metabolites on ACE 5 C18 (250 mm x 4.6 mm I.D.) and ZIC-HILIC (250 mm x 4.6 mm I.D.) indicating optimum proportions for a mixed phase or coupled column system; mobile phase – [methanol – water (70:30, v/v)], 0.01 M ammonium acetate; UV detection at 254 nm.

In this part of the study, it was demonstrated that better separation of all substances present in a mixture could be achieved using the most appropriate column-volume ratio as indicated in Fig. 2.11 above. Therefore, it proved possible to couple ACE-5-C18 and HILIC columns in series so that acemetacin and its related substances could be resolved from one another without a very long run time or the need for gradient elution.

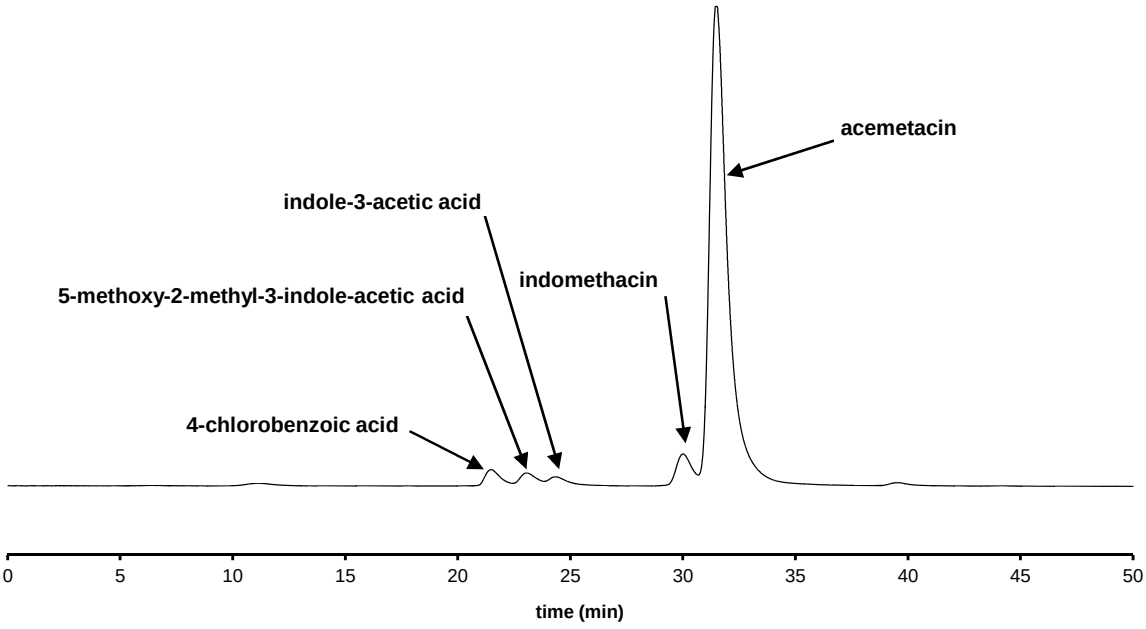



Figure 2.12 LC of acemetacin and related substances using serially-coupled columns; ACE 5 C18 (150 mm x 4.6 mm I.D.) followed by ZIC-HILIC (250 mm x 4.6 mm I.D.); mobile phase – [methanol – water (70:30, v/v)]; flow rate 0.2 mL/min; UV detection at 254 nm.

### 2.3.5 Urine constituents and amitriptyline related substances

It was thought that a similar approach could have been adopted to look at the simultaneous separation of amitriptyline metabolites and endogenous urine constituents. Ultimately it proved more convenient to isocratically resolve the urine constituents on an ACE-5-CN column. Similarly, the amitriptyline metabolites could be separated with a different isocratic mobile phase with the urine-related peaks all eluting close to $t_0$.

As discussed in 2.3.1., it had not proved possible to match the HILIC mode and RP-HPLC for amitriptyline metabolites. Nonetheless, as the work was going on simultaneously, it was decided to continue with a study with the aim of determining both urine metabolites and amitriptyline metabolites in one method. The former should be possible using the HILIC mode and the latter would still be of interest even if the amitriptyline metabolites were not separated from one another, since in metabolomic studies of urine constituents it is useful to be able to ‘remove’ drug-related peaks.

The retention behaviour of urine metabolites on the ZIC-HILIC phase is shown in Fig. 2.13. With an increasing proportion of methanol in the mobile phase there is a discernable ‘switch-over’ of retention order indicating a transition between reversed-phase and HILIC behaviour. However, there is not a 100% reversal of retention order. At the same time there are only a few pairs of compounds (e.g. creatinine and tyrosine) where the retention order stays the same.

Table 2.3 *k* of urinary metabolites on ACE 5 C18, ACE 5 CN and ZIC-HILIC

| **Compound** | ***k* on ACE 5 C18** aqueous - methanol (80:20 v/v); pH-3.0** | ***k* on ACE 5 CN** aqueous - methanol (90:10 v/v); pH-7.0*** | ***k* on ZIC-HILIC** aqueous - methanol (05:95 v/v); pH-3.0** |
|---|---|---|---|
| creatinine | 0.66 | 0.34 | 1.00 |
| pseudouridine | 0.02 | 0.18 | 0.77 |
| hippuric acid | 3.49 | 0.07 | 0.15 |
| phenylalanine | 1.22 | 0.30 | 0.47 |
| tyrosine | 0.27 | 0.25 | 0.76 |
| 4-hydroxy-benzoic acid | 3.85 | 0.01 | 0.35 |
| xanthurenic acid | 3.43 | 0.18 | 0.35 |
| phenyl acetic acid | 3.25 | 0.07 | 0.10 |
| ferulic acid | 3.04 | 0.20 | 0.19 |
| urea | 0.60 | 0.16 | 0.21 |
| IAG* | 0.67 | 0.18 | 0.50 |
| kynurenic acid | 3.10 | 0.20 | 0.27 |

*- indolyl-3-acryloyl glycine (IAG)

**- 0.01 M ammonium formate and formic acid in aqueous - methanol mobile phase; pH adjusted to 3.0 with formic acid

***- 0.01 M ammonium acetate in aqueous - methanol mobile phase; pH-7.0

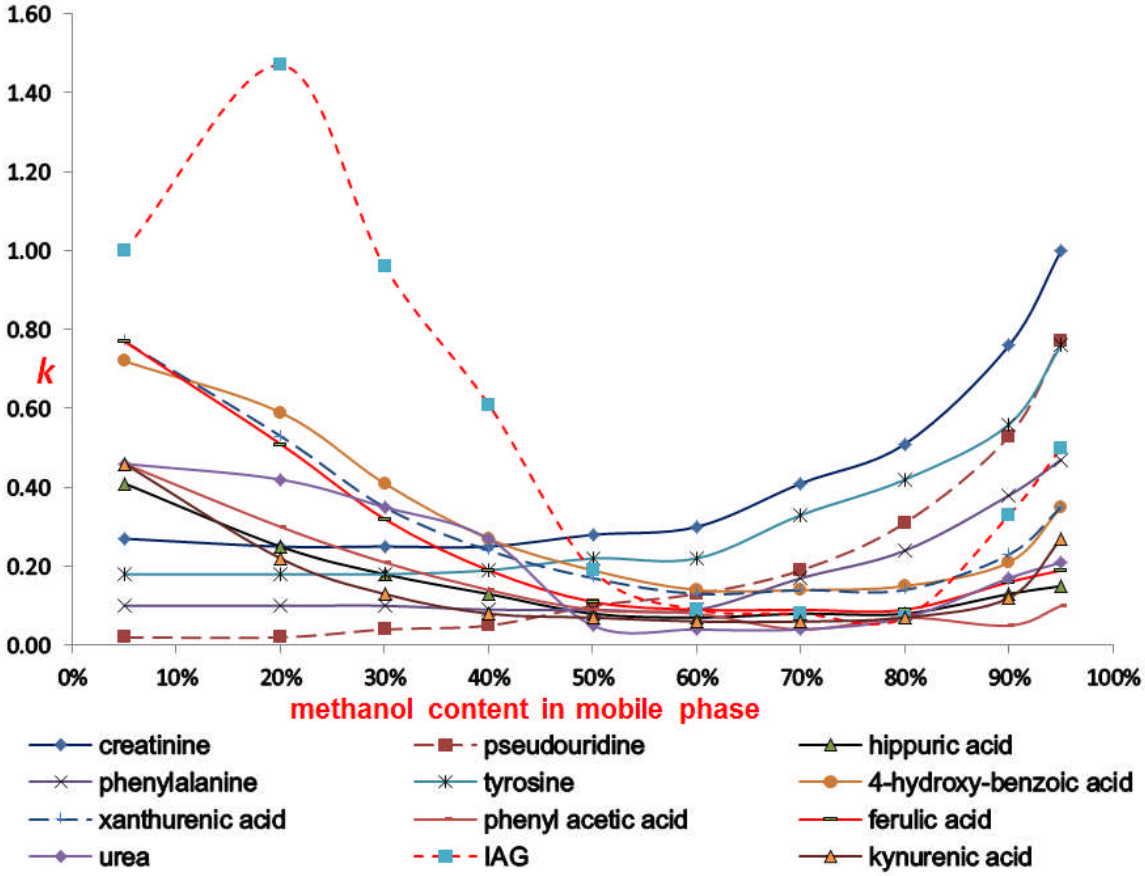



Figure 2.13 Relationship between *k* and % methanol in the mobile phase for urinary metabolites on ZIC-HILIC; mobile phases – [methanol - water], 0.01 M ammonium formate, formic acid at pH-3.0; flow rate – 1.0 ml $min^{-1}$; UV detection at $\lambda_{max}$ for each of the compounds.

So, comparing the behaviour of ZIC-HILIC at high and low proportions of methanol, the two conditions are somewhere between orthogonal to one another and a simple reversal. Having said that, even if the HILIC behaviour was

completely orthogonal to reversed-phase behaviour (where it be on ZIC-HILIC or a C18 phase) it would not be of practical value in most of the applications for which orthogonal selectivity would be exploited (Chapter 2.1) because of it being virtually impossible to match mobile phases. Also, the increase in *k* values at high methanol percentages was limited and would have been of little value in developing methods. In particular, there seemed to be little prospect of using the ZIC-HILIC in HILIC mode in a coupled column system to avoid gradient elution (as mentioned above, no common mobile phase at which there is significant retention on both phases) or to separate urinary metabolites and drug metabolites with one isocratic mobile phase (*k* too low to resolve all urinary metabolites).

Looking at the details of the data (Fig. 2.13), the basic compounds which were more polar in the acidic mobile phase had a much lower retention when low amount of organic component was present in mobile phase and the retention started increasing after 50% methanol and reached the maximum with 95% methanol in mobile phase. In contrast to the basic compounds, the acidic compounds had a high retention with the mobile phase containing low organic component and their retention started decreasing with the gradual increase of organic content in mobile phase. However, their retention started increasing again from 70% organic content until 95%, but to a much lesser extent when compared to basic compounds. This behaviour explains that the basic compounds which were fully or partially unionised in acidic mobile phase were retained with low organic component in mobile phase due to reversed-phase behaviour of ZIC-HILIC and the HILIC behaviour of the phase with mobile phase containing 70-95%

organic component showed only a slight increase of retention for these compounds.

Despite the increased retention of both acidic and basic urine metabolites with high content of organic component in mobile phase on ZIC-HILIC phase, the low retention was not sufficient for the ZIC-HILIC phase to be used in a coupled column system to resolve urine metabolites. Also, as previously explained, similar or only slightly higher retention for these compounds was exhibited (Table 2.3) on ACE 5 C18 and ACE 5 CN phases. The retention on ZIC-HILIC phase could be expected to be increased by replacing methanol in mobile phase with acetonitrile and using a content of it as high as 97% which is the maximum level (McCalley, 2007; ~3% aqueous (McCalley, 2007) needed to form a significant, reproducible water coating of the phase), but such an increase may not be compatible with the use of ZIC-HILIC phase in a coupled column system.

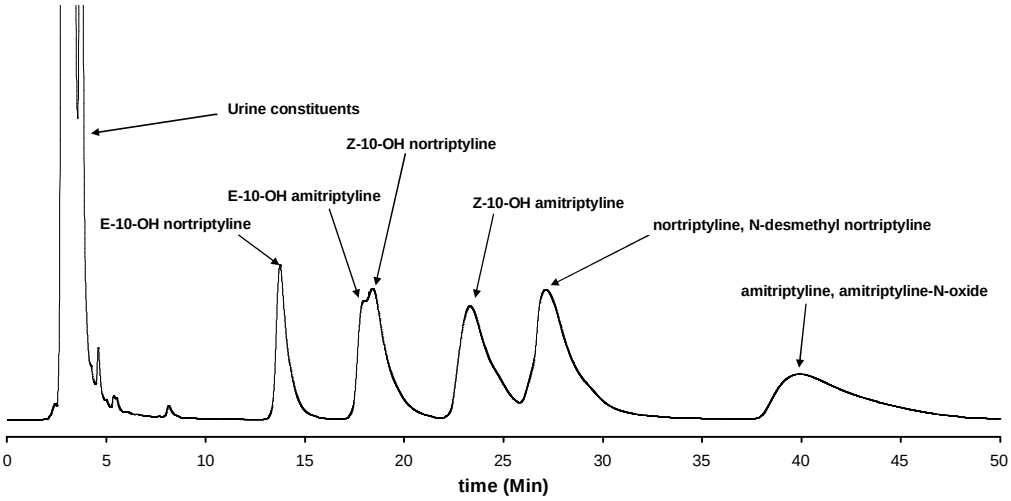



Figure 2.14 RP-HPLC of urine constituents and amitriptyline related substances on ACE-CN (250 mm x 4.6 mm I.D.); mobile phase – [methanol – water (10:90, v/v)], 0.01M ammonium acetate; flow rate 1.0; UV detection at 254 nm.

As for the amitriptyline studies described previously (2.3.1), combining RP-HPLC and HILIC modes proved problematic. However, in assessing different RP-HPLC columns to adjust retentivity it was found that ACE 5 CN was suitable for retaining amitriptyline and its metabolites without the need for gradient conditions. While two of the metabolites were not resolved it would probably still have been possible to distinguish between these isobaric compounds in a LC-MS/MS assay.

In retrospect, looking at the data in Fig. 2.7 the use of non-gradient conditions would also have been possible on ACE 5 C18. However, peak shape on ACE 5

C18 had not been so good, another of the reasons for having switched from ACE 5 C18 to ACE 5 CN.

This feature of the ease with which non-gradient conditions could be used for amitriptyline and its metabolites is probably a feature of amitriptyline itself rather than being a feature of drug metabolites in general. It is a large hydrophobic molecule (log P = 5.04) and clearly metabolic modification at one site is not sufficient to alter the physicochemical properties significantly.

### 2.3.6 Antibacterial Solution Products

A coincidentally occurring need from a regional SME to carry out quality control on its anti-bacterial products provided an opportunity to trial the information acquired on orthogonality (or otherwise as it turned out) of stationary phases and previous studies to assess how well it could be exploited to lead to routine QC methods. This part of the research was carried out, in order to develop suitable analytical methods for two products of AGMA Ltd, a local company. It has been finding that its customers were becoming more interested in the quality of their products (antibacterial solutions) and had therefore decided that it should carry out HPLC analysis of its products so that it could accurately report the composition of each of their products. However, the work completed seemed compatible with the “Evaluation and Application of Stationary Phase Selectivity for Drug Analysis” research programme as the work involved attempting to use some of the systems evaluated, in particular the ZIC-HILIC mode, to solve the method development

problems. Therefore methods were developed for two of AGMA's products, each of which was a three component aqueous solution (Fig. 2.15 and Fig. 2.16), utilising the selectivity information collected on achiral columns in the evaluation stage of the research programme. Given the polar nature of some of the constituents, it was envisaged that some use might need to be made of the ZIC-HILIC column. For example, the use of a designer serially coupled orthogonal columns approach in conjunction with a C-18 column in order to avoid the use of a gradient mobile phase had been a consideration at the outset of this method development exercise which had been requested at the same time as the HILIC evaluation had been being carried out.

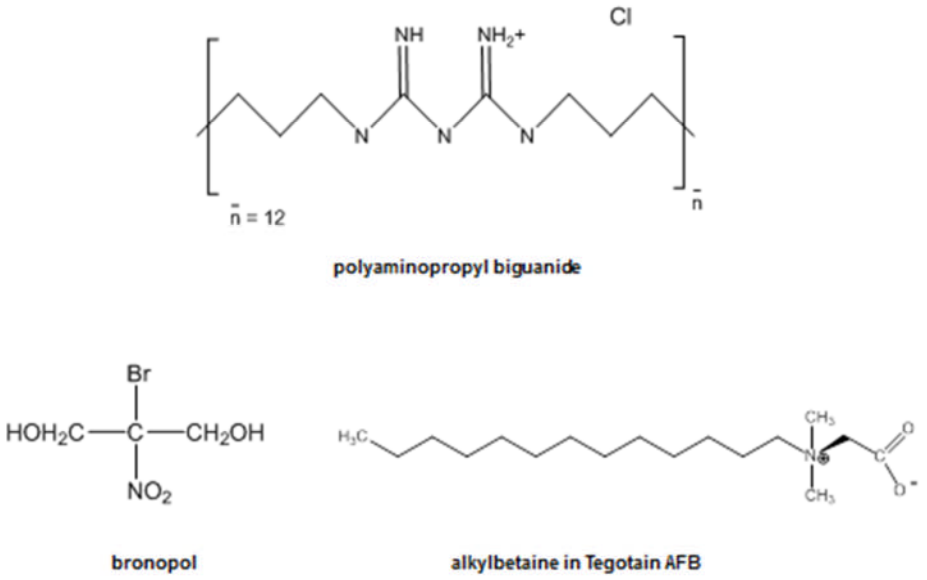
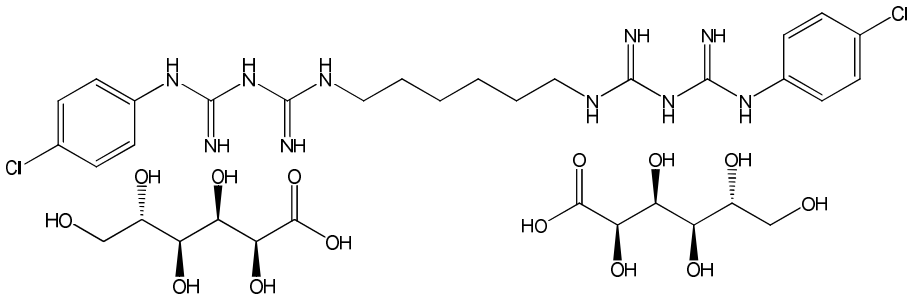
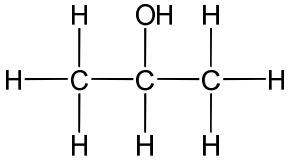



Figure 2.15 Components present in 'Product 1' of antibacterial products

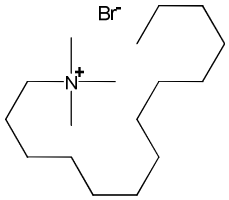
Polyaminopropyl biguanide is a synthetically derived antibacterial preservative which is used to control bacteria, fungi and algae. This was originally developed for use in eye products (http://www.bionity.com/en/encyclopedia/PAPB.html). Bronopol is a preservative which is used in cosmetics, topical medications, paints, textiles, shampoos and industrial water systems. It is a bactericide and fungicide and inhibits dehydrogenase activity by damaging the membrane in microorganisms (Bryce *et al*., 1978). Tegotain AFB is a surface active disinfectant against gram-positive and gram-negative bacteria, yeast and fungi. This is used as a disinfectant in disinfectant sanitizers and as a neutral deodorising agent (Birnie *et al*., 2000; http://www.surfachemgroup.co.uk/tegotain-afb).



**chlorhexidine digluconate**



**isopropyl alcohol**



**cetrimide**

Figure 2.16 Components present in ‘Product 2’ of antibacterial products.

Chlorhexidine digluconate is used as an anti-bacterial agent in formulations for hospitals and veterinary applications and as a preservative in cosmetics and in pharmaceutical preparations such as mouth wash solutions (Persson *et al*., 1991; Adams *et al*., 2005). Isopropyl alcohol is commonly used as a disinfectant in sterilizing pads (Adams *et al*., 2005). It is also used as a cleaner and solvent in industry (http://www.intertronics.co.uk/products/adh1610.htm). The cetrimonium cation is one of the components of the topical antiseptic Cetrimide and which is used against bacteria and fungi (Arias-Moliz *et al*., 2010;http://www.chemicalland21.com).

As things transpired, this work was complicated by the low-UV absorbance and large RMM of some of the compounds. With respect to the latter, it was found that size-exclusion chromatography took place for some of the constituents. It was not possible to obtain a single LC method for the analysis of all components of each three-component product. However, making good use of each of the HILIC modes, ion-exchange LC, reversed-phase LC and size-exclusion LC, suitable LC conditions were found for each of the six constituents.

With initial studies using ACE 5 C18, ACE 5 AQ, Spherisorb 5 SCX and PRP-1 (Polymer Lab) columns and mobile phases containing perchloric acid, it was not possible to develop a suitable single method for any of the two products. Therefore it was decided to use mobile phases containing different additives for both products and also to extend the work using the ZIC-HILIC stationary phase. Much effort was made to develop single methods for each product, which would be suitable for routine quality control procedures. One method for all three components and two methods for only two components in Product 1 were developed. However, in the method for all three components, tegotain AFB was not well retained, eluting soon after the solvent front and another one method was developed for tegotain AFB using ZIC-HILIC column and a mobile phase containing acetonitrile for which the UV absorbance was low, resolving the difficulty of detecting low UV absorbing tegotain AFB while its retentivity on ZIC-HILIC column. In method 4 (Fig. 2.20) for Product 1, the amount of acetonitrile used in mobile phase was 75% which might have been expected to cause

precipitation of phosphate buffer, although such a problem was not experienced at this occasion. The UV absorbance of alkylbetaine which is also tegotain AFB, in Product 1 was very low (190 nm) due to it having a much weaker chromophore and therefore, it was decided to use acetonitrile instead of methanol in this method, in order to facilitate UV detection at lower wave length while achieving longer retention with a higher content of acetonitrile on ZIC-HILIC phase. However, to overcome the problem of possible precipitation of phosphate buffer in higher content of organic solvent, it may be needed to consider a different approach for this method 4 of Product 1.

Two other methods were developed for Product 2. The ability of ACE 5 AQ phase to retain polar compounds with a higher percentage of aqueous component in the mobile phase or even with 100% aqueous mobile phase was taken into consideration and a mobile phase containing both aqueous and methanol components with a much lower concentration of formic acid was used to develop the method 1 of Product 2. Method 2 (Fig. 2.22) was developed, after taking advantage of ZIC-HILIC phase to retain polar compounds with a mobile phase containing a higher (>70%) percentage of organic component. However, method 1 (Fig. 2.21) was quite simple and much more economical as a lesser amount of organic component was used.

Table 2.4 Retention data for method 1 of antibacterial Product 1 (Fig. 2.15)

| Compound | $t_0$ | $t_r$ | *k* |
|---|---|---|---|
| tegotain AFB | 2.70 | 2.75 | 0.02 |
| polyaminopropyl biguanide | 2.70 | 3.72 | 0.38 |
| bronopol | 2.70 | 6.82 | 1.53 |

- experimental conditions of method 1 for Product 1 are given in Fig. 2.17

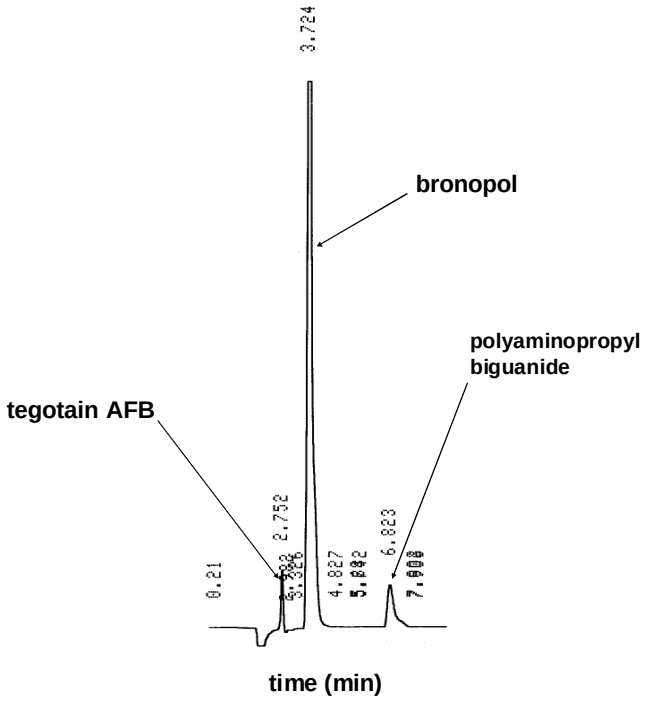



Figure 2.17 RP-HPLC of method 1 for Product 1 (Fig. 2.15) of antibacterial products on H5ODS (250 mm x 4.6 mm I.D.); mobile phase – [methanol – water (30:70 v/v)], 0.03% formic acid; flow rate – 1.0 ml $min^{-1}$; UV detection at 205 nm.

Table 2.5 Retention data for method 2 of antibacterial Product 1 (Fig. 2.15)

| Compound | $t_0$ | $t_r$ | *k* |
|---|---|---|---|
| polyaminopropyl biguanide | 2.10 | 17.8 | 7.49 |
| bronopol | 2.10 | 2.51 | 0.20 |

- experimental conditions of method 2 for Product 1 are given in Fig. 2.18

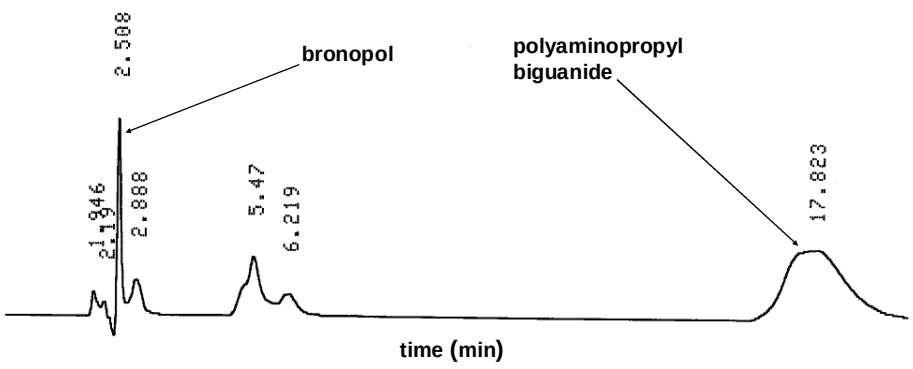



Figure 2.18 LC of method 2 for Product 1 (Fig. 2.15) of antibacterial products on ZIC-HILIC (250 mm x 4.6 mm I.D.); mobile phase – [methanol – water (80:20 v/v)], 0.03% formic acid; flow rate – 1.5 ml $min^{-1}$; UV detection at 229 nm.

Table 2.6 Retention data for method 3 of antibacterial Product 1 (Fig. 2.15).

| Compound | $t_0$ | $t_r$ | *k* |
|---|---|---|---|
| polyaminopropyl biguanide | 2.10 | 4.43 | 1.11 |
| bronopol | 2.10 | 2.88 | 0.37 |

- experimental conditions of method 3 for Product 1 are given in Fig. 2.19

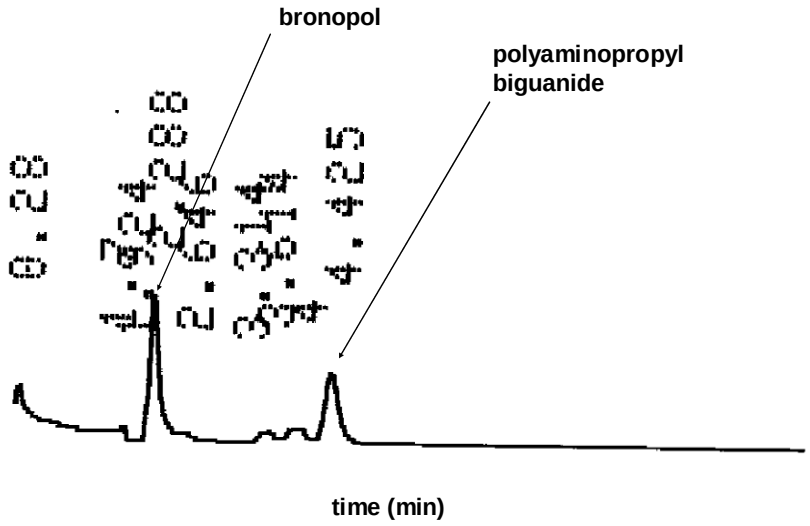



Figure 2.19 LC of method 3 for product 1 (Fig. 2.15) of antibacterial products on ZIC-HILIC (250 mm x 4.6 mm I.D.); mobile phase – [methanol – water (70:30 v/v)], 0.01M ammonium acetate; flow rate – 1.5 ml $min^{-1}$; UV detection at 229 nm.

Table 2.7 Retention data for method 4 of antibacterial Product 1 (Fig. 2.15).

| Compound | $t_0$ | $t_r$ | *k* |
|---|---|---|---|
| tegotain AFB | 3.01 | 7.40 | 1.46 |

- experimental conditions of method 4 for Product 1 are given in Fig. 2.20

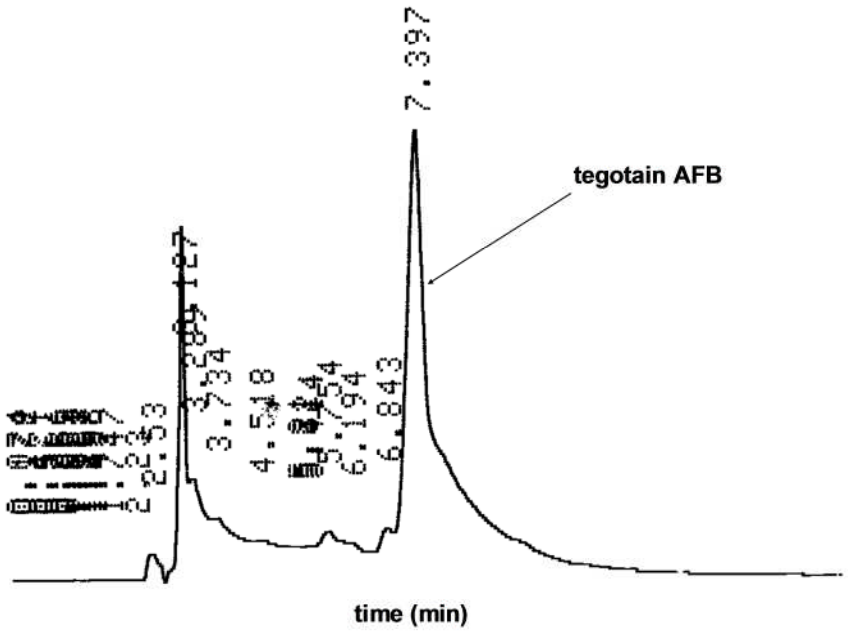



Figure 2.20 LC of method 4 for Product 1 (Fig. 2.15) of antibacterial products on ZIC-HILIC (250 mm x 4.6 mm I.D.); mobile phase – [acetonitrile – aq. 0.02M $KH_2PO_4$ (pH-4.24) (75:25 v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 190 nm.

For all the use of the ZIC-HILIC phase to obtain good resolution of individual components of Product 1 it was probably the simple RP-HPLC method (method 1) that was most useful.

Similarly with Product 2, while attempts were made to make best use of the ZIC-HILIC column, the most suitable method arrived at was based on RP-HPLC.

Table 2.8 Retention data for method 1 of antibacterial Product 2 (Fig. 2.16).

| Compound | $t_0$ | $t_r$ | *k* |
|---|---|---|---|
| Chlorhexidine digluconate | 1.10 | 3.87 | 2.52 |
| cetrimide | 1.10 | 1.89 | 0.72 |

- experimental conditions of method 1 for Product 2 are given in Fig. 2.21

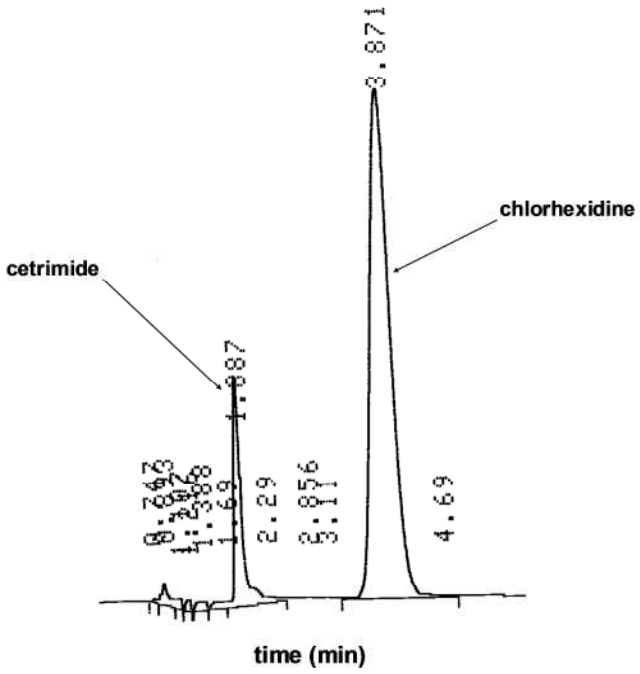



Figure 2.21 RP-HPLC of method 1 for Product 2 (Fig. 2.16) of antibacterial products on ACE 5 AQ (150 mm x 4.6 mm I.D.); mobile phase – [methanol – water (45:55 v/v)], 0.03% formic acid; flow rate – 1.5 ml $min^{-1}$; UV detection at 205 nm.

Table 2.9 Retention data for method 2 of antibacterial Product 2 (Fig. 2.16).

| Compound | $t_0$ | $t_r$ | *k* |
|---|---|---|---|
| Chlorhexidine digluconate | 3.01 | 16.17 | 4.37 |
| cetrimide | 3.01 | 4.59 | 0.53 |

- experimental conditions of method 2 for Product 2 are given in Fig. 2.22

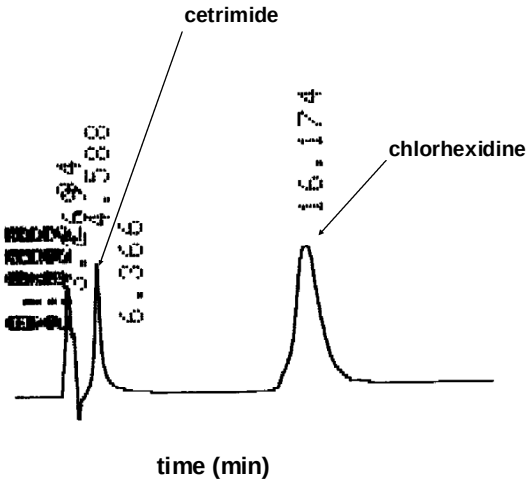



Figure 2.22 LC of method 2 for Product 2 (Fig. 2.16) of antibacterial products on ZIC-HILIC (250 mm x 4.6 mm I.D.); mobile phase – [methanol – water (70:30 v/v)], 0.05% formic acid; flow rate – 1.0 ml $min^{-1}$; UV detection at 205 nm.

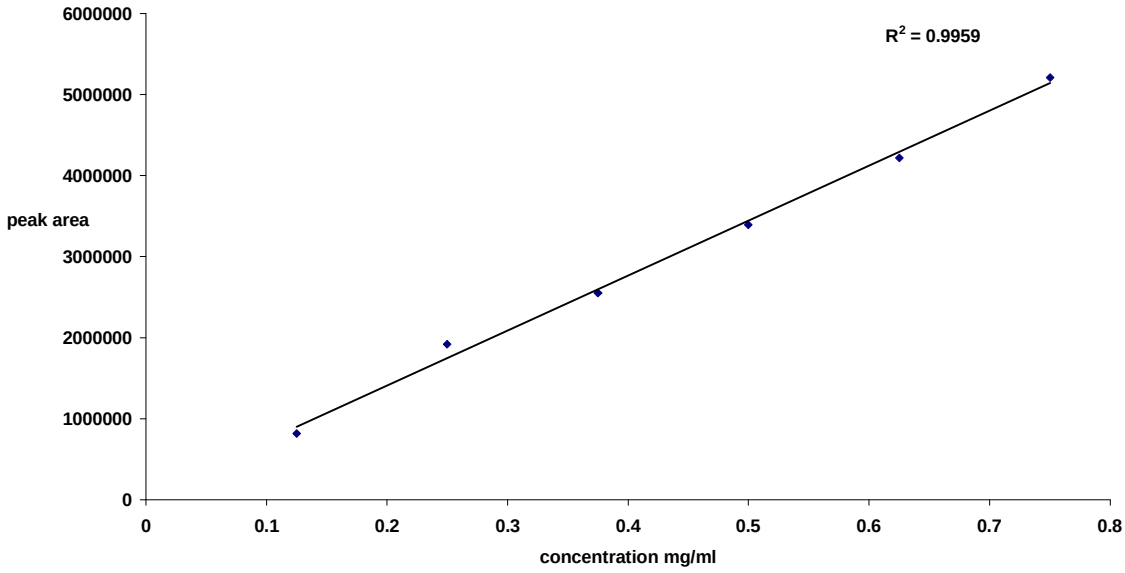



Figure 2.23 Linearity for chlorhexidine (Product 2) peak area *v* concentration on ACE 5 AQ (150 mm x 4.6 mm I.D.); mobile phase – [methanol – water (45:55 v/v)], 0.03% formic acid; flow rate – 1.5 ml $min^{-1}$; UV detection at 205 nm.

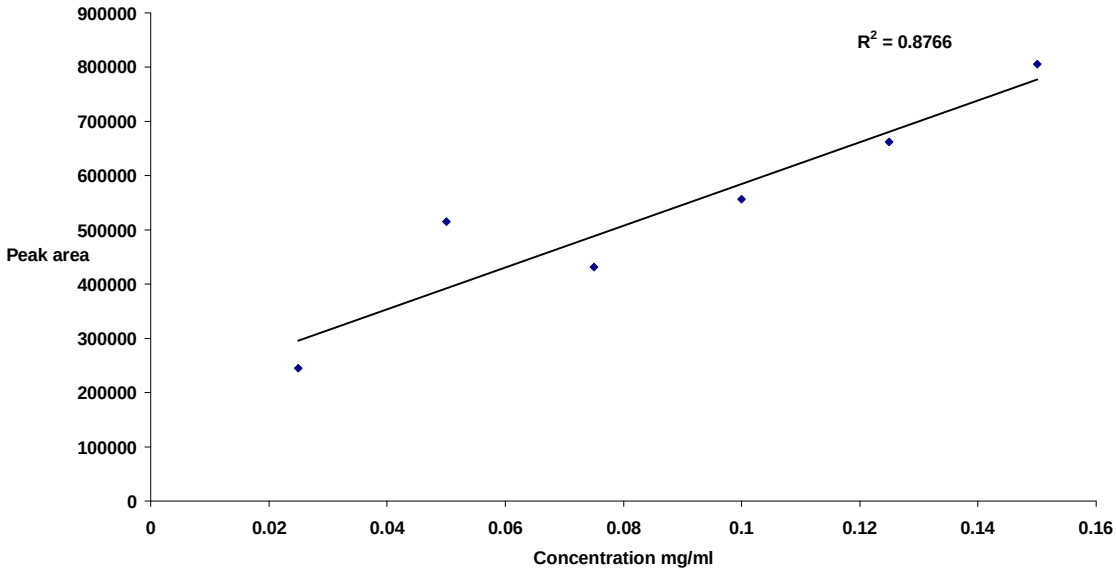



Figure 2.24 Linearity for cetrimide (Product 2) peak area *v* concentration on ACE 5 AQ (150 mm x 4.6 mm I.D.); mobile phase – [methanol – water (45:55 v/v)], 0.03% formic acid; flow rate – 1.5 ml $min^{-1}$; UV detection at 205 nm.

For Product 2, Method 1 was the most economical and simplest method which would be ideal for a routine analysis. While the linearity plots (Fig. 2.23, 2.24) were short of the desired $r^2$ = 0.999, especially for cetrimide, this was probably an instrumental issue or a systematic error relating to the second lowest standard solution.

While it had not been possible to use the ZIC-HILIC column in a combination system, its property of giving retention of polar compounds had proved useful. Similarly, retentivity of C18 AQ proved useful particularly for retaining very polar compounds using water rich or 100% aqueous mobile phase, making the methods much simpler, cost effective and also environment friendly. Also, by unforeseen chance, ion exclusion was involved in the methodology, occurring for polyaminopropyl biguanide, at the initial stage of method development, using mobile phases containing perchloric acid

.

Irrespective of any conclusions on the most suitable method of analysing the products, the most important message to come from this exercise was that it is not always wise to go into a method development exercise with a strongly pre-conceived idea of what the final solution will be. However, if the situation has very obvious applicability to the mixed orthogonal phase approach, then that is a different story, as illustrated below (Sub-Section 2.3.7).

2.3.7 Appropriate application of coupled columns containing orthogonal stationary phases: assay for baclofen products

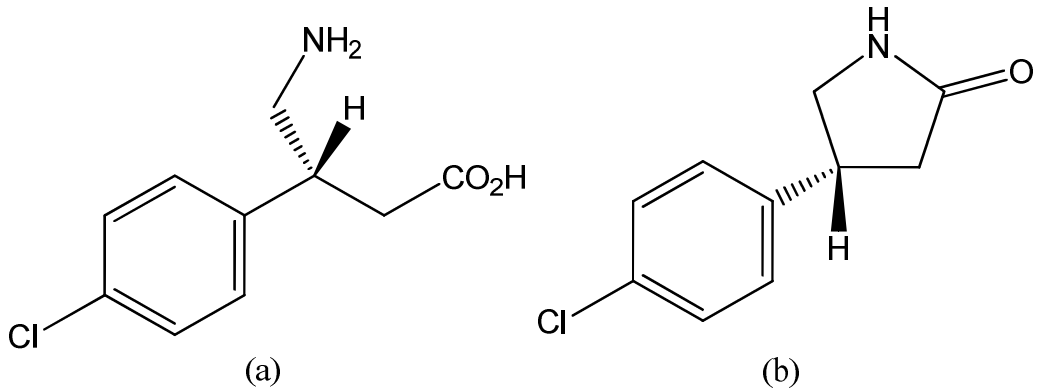



Figure 2.25 Baclofen (a) and its impurity A (b)

In the original BP method, ion-pair LC was used to overcome problems with reversed-phase LC. In reversed-phase LC, impurity A gave a much longer retention time than baclofen and it was not possible to identify any degradents which might have a shorter retention than baclofen. Increasing retention of baclofen would further increase the retention of impurity A and hence total run time would be much longer. Therefore, it was sought to consider a different approach which would not only solve this problem, but also would allow avoiding disadvantages of using an ion-pair method which would cause additional problems due to the coating of ion-pair reagents within the column, producing longer

equilibrium times which would certainly be a disadvantage for routine analysis of the drug product. The initial work in the new approach involved a gradient run (Fig. 2.26) of a mixture of baclofen and its impurity A to illustrate that the long run time of impurity A could be reduced significantly by using a gradient method, and alternatively, a much shorter run times could be achieved through a new approach of using a coupled column system.

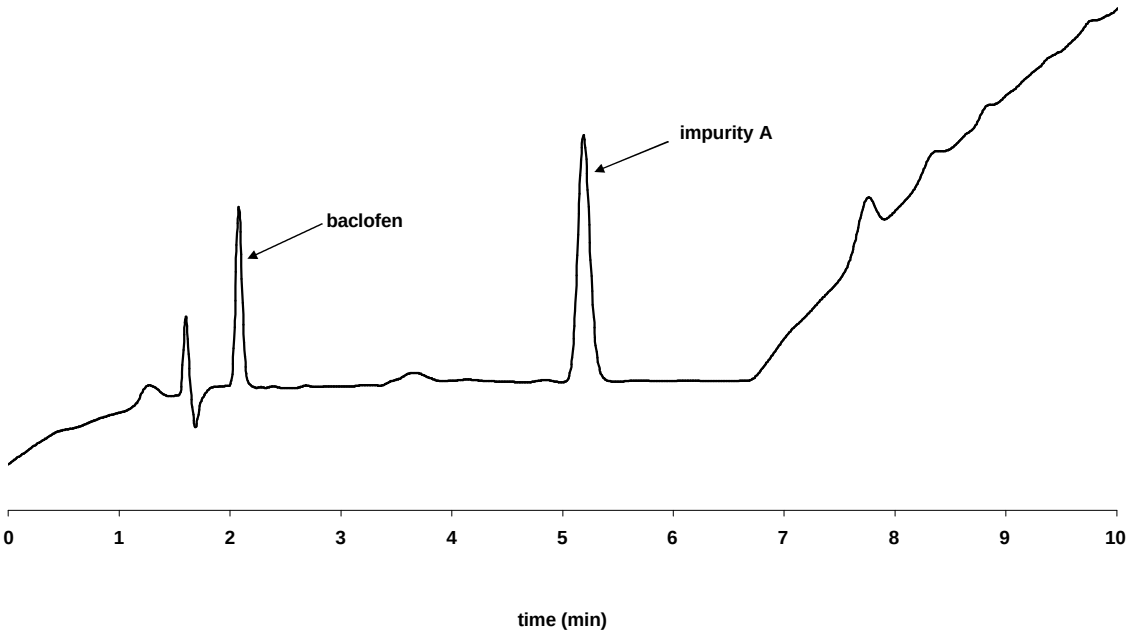



Figure 2.26 HPLC of baclofen and its impurity A on ACE 5 C18 (150 mm x 4.6 mm I.D.) column using a gradient method; mobile phase component A - 0.02 M ammonium formate, 2ml $L^{-1}$ formic acid in water and component B - 0.02M ammonium formate, 2ml $L^{-1}$ formic acid in methanol; gradient method – A 90% for 2 min, A 50% from 2-15 min, A 50% from 15-20 min and A 50% - 90% from 20-22 min. ; flow rate 1.0 ml $min^{-1}$; UV detection 254 nm.

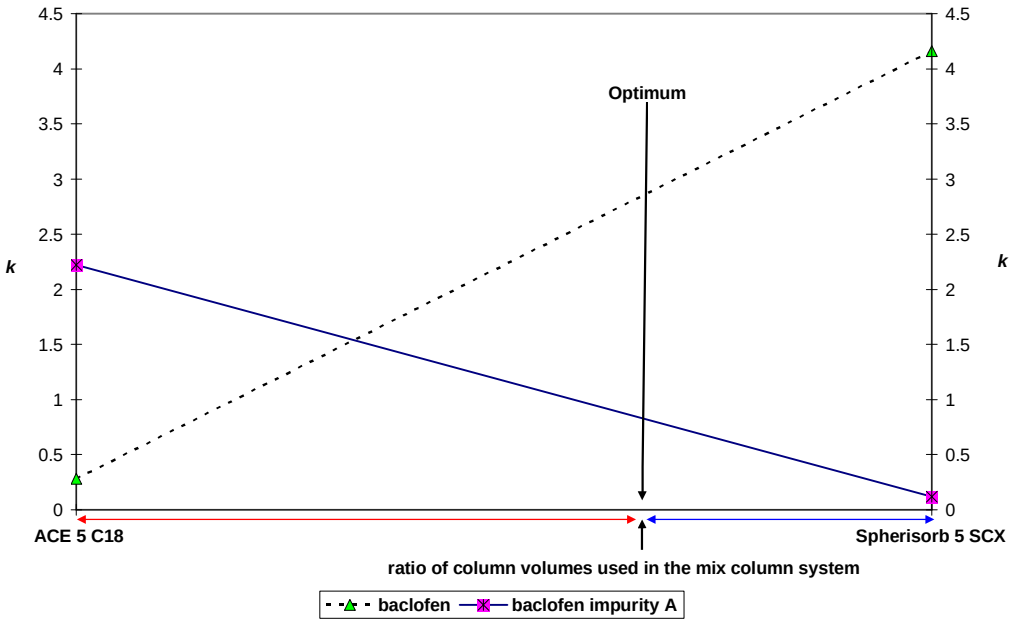



Figure 2.27 *k* values of HPLC for baclofen and its impurity A on ACE 5 C18 and Spherisorb SCX indicating optimum proportions for a coupled column system.

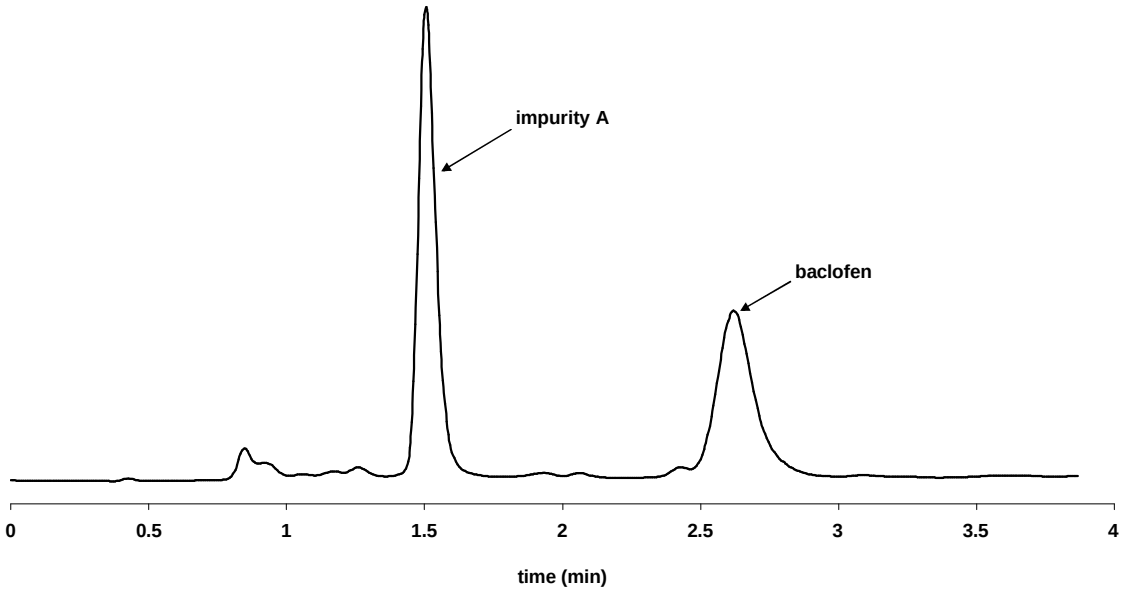



Figure 2.28 HPLC of baclofen and its impurity on a coupled column system - Spherisorb SCX (5 µm) (100 mm x 4.6 mm I.D.) followed by ACE 3 C18 (3 µm) (50 mm x 4.6 mm I.D.); mobile phase – [methanol – water (50:50, v/v)], 0.02 M ammonium formate, formic acid 2 ml/L; flow rate – 2.0ml $min^{-1}$; UV detection at 254 nm.

As shown in Figure 2.28, using a mixture of baclofen and its impurity A as an example, it had been successfully demonstrated that the use of coupled column systems could be very conveniently used as an alternative to ion-pair HPLC (EP assay for baclofen) for separations in which neutral and charged species must be eluted with similar retention. Previously, coupled column systems had been shown to be suitable for related substances assays (Soo, 2003; C. Smith, Final Year Project, BSc Chemical and Pharmaceutical Science, University of Sunderland, 2001) C18 - SCX), related substances assays involving a model single enantiomer

drug (Lough, Perera; C18 – Macrobiotic T) and especially for a stability indicating assay involving a basic drug and neutral excipients (Lough, Perera; C18 – SCX) the latter example having the most obvious facile general applicability.

## 2.4 Conclusions and Future Work

More studies beyond the scope of this programme would obviously involve developing similar examples to further demonstrate the effectiveness of the coupled column approach. This would involve full method validation to ICH guidelines in each case. Also, the studies carried out here further emphasised that it is difficult to find achiral phases that are genuinely orthogonal to C18 silicas when using polar organic solvents mixed with aqueous systems. Further work is needed in this area, even if it means drawing on chiral chromatography where distinctive retention mechanisms such as molecular inclusion complexation, charge transfer interaction *etc* are involved. A final consideration is that a coupled column approach runs the risk of becoming outdated with the trend to shorter columns and low dispersion systems. However, previous applications could also be carried out by mixing stationary phases in the appropriate proportion and then packing a column. This could be tried out for the C18 – SCX combination for baclofen with particular emphasis for demonstrating robustness especially with respect to column-to-column reproducibility. Having done this, it would then be possible to move on to using mixed phases in the UPLC (or U-HPLC) systems and fused core shell particles that are coming into vogue.

# 3. Evaluation of Chiral Stationary Phase Enantioselectivity

## 3.1 Introduction

From the previous studies in the University of Sunderland, particulary in relation to a study on paroxetine (R.W.H.Perera and Ng, Final Year Projects, BSc Chemical and Pharmaceutical Science, University of Sunderland, 2005 and 2007) rather than the study on the model N-acetyl-tryptophan system (R.W.H. Perera and W.J. Lough unpublished work on Spherisorb ODS1 / Chirobiotic T coupled systems; also R.W.H. Perera and W.J. Lough, poster presentation, Chirality 2004, New York), it had been shown that C18 phases were not suitable to be used in coupled column systems with chiral stationary phases (CSP) that had commonly used in the reversed-phase mode e.g. CSP like Chiral AGP, cyclodextrins, macrocyclic antibiotics which give significant retention in the RP-HPLC mode only with lower percentages of organic component in the mobile phase as low content of organic component in mobile phase would give much longer retention times. Therefore, as described in Chapter 2, as the first step of the research programme, achiral stationary phases were studied, amongst other things, to try to identify any which might be orthogonal to C18 phases in their selectivity and have similar retention.

From the earlier work by Soo (Soo, 2003) there was a suggestion that chiral stationary phases (CSP) might be worth investigating in the context of orthogonal selectivity to C18 silicas. However in light of the developments taking place on

CSP at the time of this phase of the research programme, there were other aspects of CSP selectivity that needed to be investigated first. For example, screening approaches for chiral LC method development had become firmly established (Perrin *et al*., 2002) but more and more CSP were being produced. Therefore it was felt that before considering achiral selectivity of CSP it would be useful to look at the chiral selectivity of established and emerging CSP in order to determine which products offered something different to justify inclusion in chiral screening exercises and which merely gave enantioseparations for compounds that could already be easily separated on the most common CSP used in chiral screens.

## 3.2 Experimental

### 3.2.1 Instrumentation

The modular HPLC systems used for chiral screening in normal phase employed Spectra Physics (San Jose, CA, USA) SP8810 precision isocratic pump with LDC Analytical (Riviera Beach, USA) spectroMonitor 3100 detector, Shimadzu (Milton Keynes, UK) LC-6A pumps and SPD-6AV detectors. In each case, a manual Rheodyne (Kotati, Ca, USA) 7125 loop injection valve, fitted with a 20 µl loop, was used for loading samples. Data was collected using a Dionex PC-based data system with Automated Computer Interface and *AI* 450 Chromatographic Automation Software Release 3.33 (Leeds, UK). The sonicator used to degas mobile phases was GS Group-ULTRAWAVE Ltd, Cardiff, CF2 1YY.

### 3.2.2 Materials and Methods

#### 3.2.2.1 Evaluation of higher generation of Pirkle-concept CSP.

Whelk-O1 (5 µm) and (10 µm) (250 mm x 4.6 mm I.D.), ULMO (250 mm x 4.6 mm I.D.) and DACH DNB (250 mm x 4.6 mm I.D.) columns used during the course of the study were gifts from Regis Technologies, Inc., Morton Grove, IL 60053, U.S.A.

HPLC grade n-hexane R, propan-2-ol R, triflouroacetic acid and triethylamine used during the course of the study were from Sigma-Aldrich (Poole, Dorset, UK). 50 drug compounds (Fig. 3.1) including examples of acidic, basic, zwitterionic and neutral drug substances, were from Sigma–Aldrich and from a collection of various common drug substances available in Sunderland Pharmacy School.

The mobile phase was prepared pre-mixed using 85 volumes of HPLC grade n-hexane R, 15 volumes of propan-2-ol R, and 0.1 volumes of triflouroacetic acid as an additive in ‘mobile phase 1’ and 0.1 volume of triethylamine (99.5%) as an additive in ‘mobile phase 2’. The sample solutions were prepared at concentrations of 0.2 mg $ml^{-1}$ using the mobile phases. A flow rate of 1.0 ml $min^{-1}$ was used throughout the CSP screening and the sample injection volume was 20 µl. UV detection was used monitoring at $\lambda_{max}$ or wavelengths used for

chromatograms in commercial applications literature. All work was carried out at ambient temperature.

The 50 drug compounds (Fig. 3.1) were run on four Regis Pirkle concept CSP i.e. Whelk-O1 (5 µm) and (10 µm) (250 mm x 4.6 mm I.D.), ULMO (250 mm x 4.6 mm I.D.) and DACH DNB (250 mm x 4.6 mm I.D.) using the two mobile phases.

3.2.2.2 Evaluation of CSP produced by coating derivatized cellulose polymers on silica.

A Chiralcel OD (250 mm x 4.6 mm I.D.) column and columns containing its clones, such as Kromasil CelluCoat (250 mm x 4.6 mm I.D.), QuikPrep CelCoat™ (250 mm x 4.6 mm I.D.) and Nucleocel Delta S (250 mm x 4.6 mm I.D.) were from Chiral Technologies Inc. Europe, Daicel Group (Illkirch, France), Eka Chemicals AB (SE-445 80 Bohus, Sweden), AECS-QUIKPREP™ Ltd (Bridgend, South Wales, UK) and MACHEREY-NAGEL (2850 Emrick Blvd, Bethlehem, PA 18020, USA) respectively and a RegisCell (another Chiralcel OD clone) (250 mm x 4.6 mm I.D.) column was a gift from Regis Technologies, Inc., Morton Grove, IL 60053, U.S.A.

The organic solvents, mobile phase additives and 50 drug compounds (Fig. 3.1) used were as for Section 3.2.2.1.

The method used during the preparation of mobile phases and sample solutions and for running samples was as for Section 3.2.2.1.

The 50 racemic drug compounds (Fig. 3.1) were run on the Chiralcel OD (250 mm x 4.6 mm i.d.), Kromasil CelluCoat (250 mm x 4.6 mm I.D.), QuikPrep CelCoat$^{TM}$ (250 mm x 4.6 mm I.D.) and Nucleocel Delta S (250 mm x 4.6 mm I.D.) columns using the two mobile phases.

3.2.2.3 Evaluation of CSP produced by coating or immobilising derivatized amylose polymers on silica.

Chiralpak AD (250 mm x 4.6 mm I.D.) column, and its clones which are also new arrivals, such as Kromasil AmyCoat (250 mm x 4.6 mm I.D.) and RegisPak (250 mm x 4.6 mm I.D.) used during the course of study were from Chiral Technologies Inc. Europe, Daicel Group (Illkirch, France), Eka Chemicals AB (SE-445 80 Bohus, Sweden), and a gift from Regis Technologies, Inc., Morton Grove, IL 60053, U.S.A, respectively.

The organic solvents, the mobile phase additives used were as for 3.2.2.1. A smaller set of drug compounds (16; a sub-set of the 50) was used.

The method used during the preparation of mobile phases and sample solutions and for running samples was as for Section 3.2.2.1.

The 16 drug compounds were run on Chiralpak AD (250 mm x 4.6 mm I.D.), Kromasil AmyCoat (250 mm x 4.6 mm I.D.) and RegisPak (250 mm x 4.6 mm I.D.) using the two mobile phases.

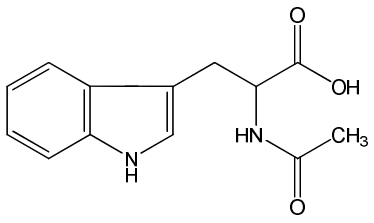
3.2.2.4 Evaluation of CSP which were compared with other CSP.

Chiralpak IA and Chiralcel OJ-H (250 mm x 4.6 mm I.D.) columns used during the course of study were from Chiral Technologies Inc. Europe, Daicel Group (Illkirch, France).

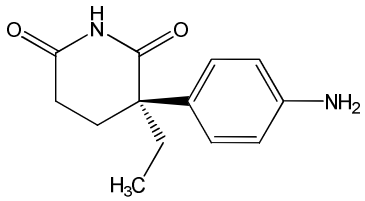
The organic solvents and the mobile phase additives and 16 drug compounds (a sub-set of the 50) used were as for Section 3.2.2.1.

The method used during the preparation of mobile phases and sample solutions and for running samples was as for Section 3.2.2.1.

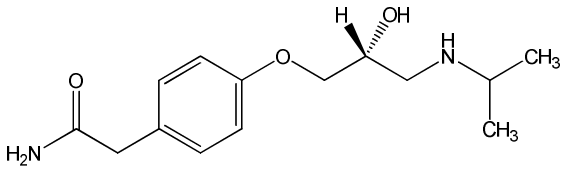
The 16 drug compounds were run on Chiralpak IA and Chiralcel OJ-H using the two mobile phases.



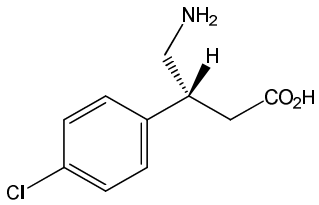
**N-acetyl tryptophan**



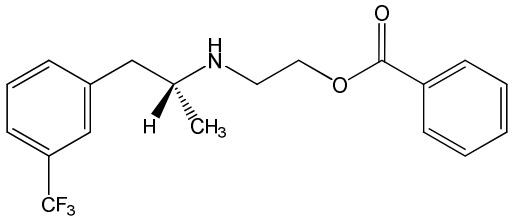
**aminoglutethimide**



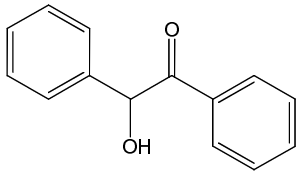
**atenolol**



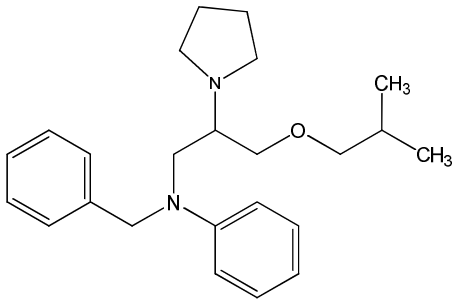
**baclofen**



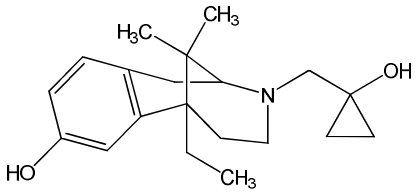
**benflourex**



**benzoin**

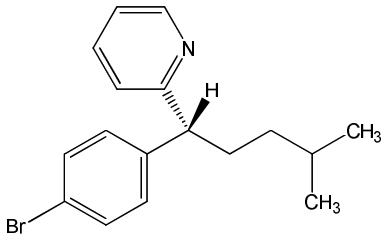


**bepridil**

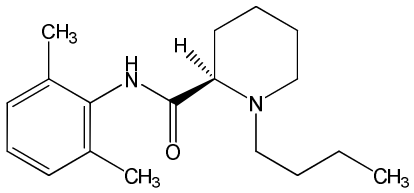


**bremazocine**

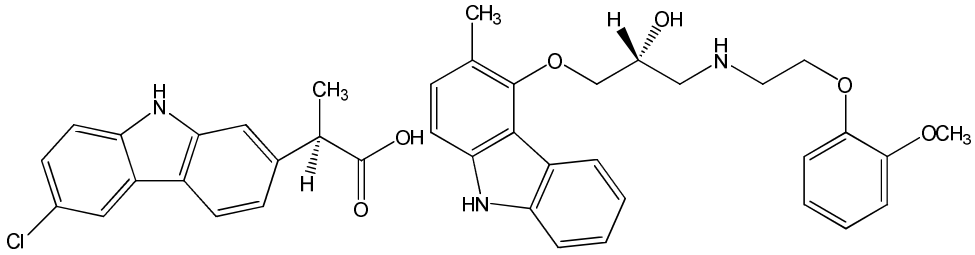
Figure 3.1 An enlarged compound set of chiral drugs (50) that were used during the evaluation of CSP throughout the study. (continued overleaf)




**brompheniramine**

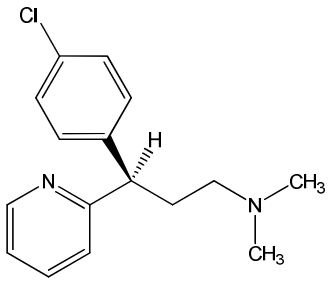



**bupivacaine**

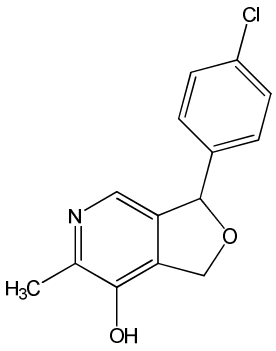



**carprofen**

**carvedilol**

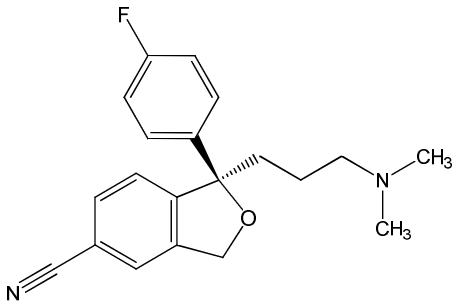



**chlorpheniramine**

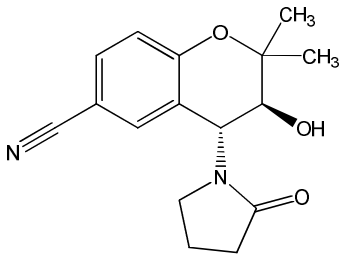



**cicletanine**

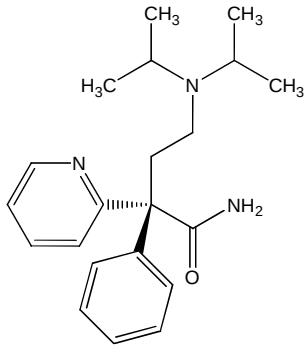
Figure 3.1 An enlarged compound set of chiral drugs (50) that were used during the evaluation of CSP throughout the study. (continued overleaf)



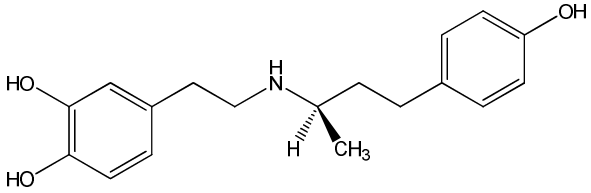

**citalopram**



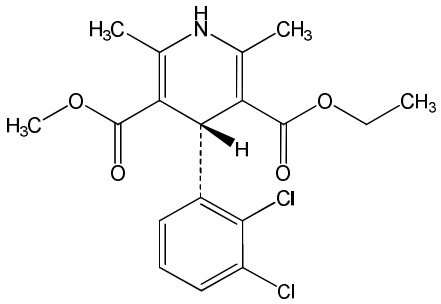

**cromakalim**



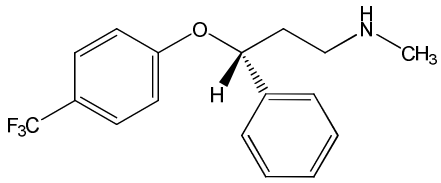

**disopyramide**




**dobutamine**

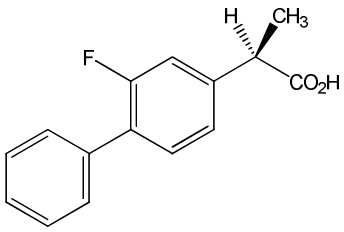



**felodipine**

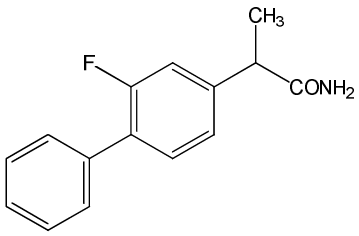



**fluoxetine**

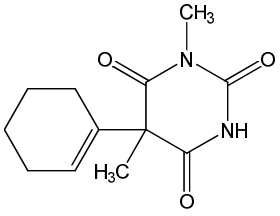
Figure 3.1 An enlarged compound set of chiral drugs (50) that were used during the evaluation of CSP throughout the study. (continued overleaf)



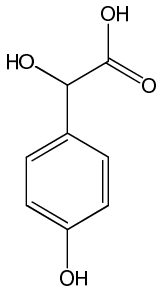

**flurbiprofen**



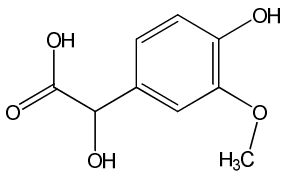

**flurbiprofen amide**



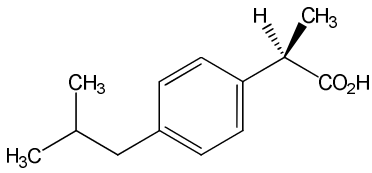

**hexobarbital**




**4-OHmandelic acid**

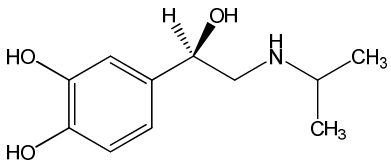



**4-OH, 3-methoxymandelic acid**

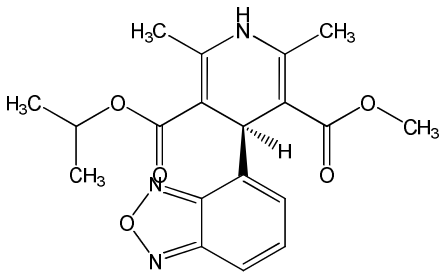



**ibuprofen**

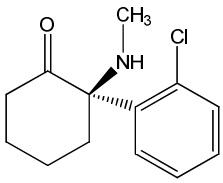
Figure 3.1 An enlarged compound set of chiral drugs (50) that were used during the evaluation of CSP throughout the study. (continued overleaf)



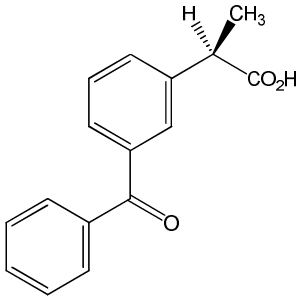

**isoproterenol**



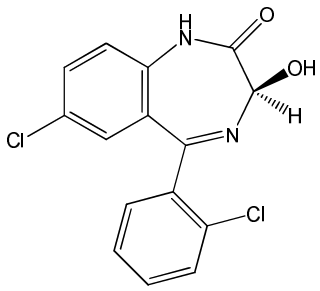

**isradipine**



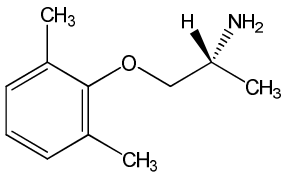

**ketamine**




**ketoprofen**

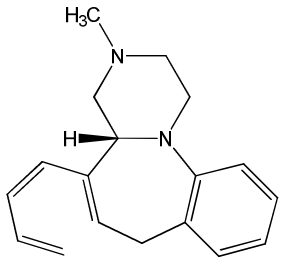



**lorazepam**

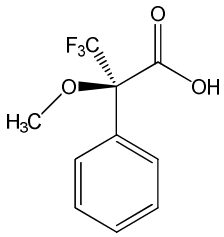



**mexiletine**

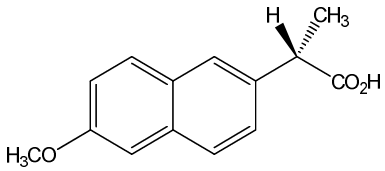
Figure 3.1 An enlarged compound set of chiral drugs (50) that were used during the evaluation of CSP throughout the study. (continued overleaf)



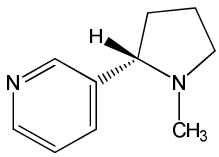

**mianserin**



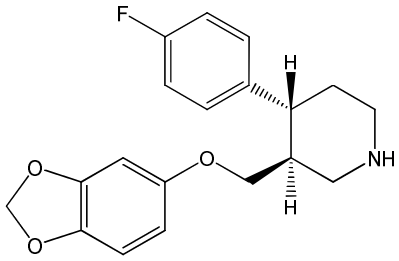

**MTPA***



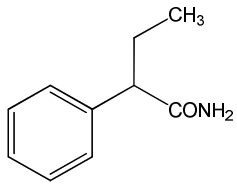

**naproxen**




**nicotine**

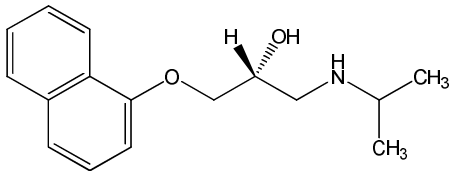



**paroxetine**

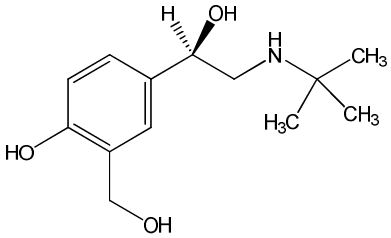



**α-phenyl-butanoicacid carboxymide**

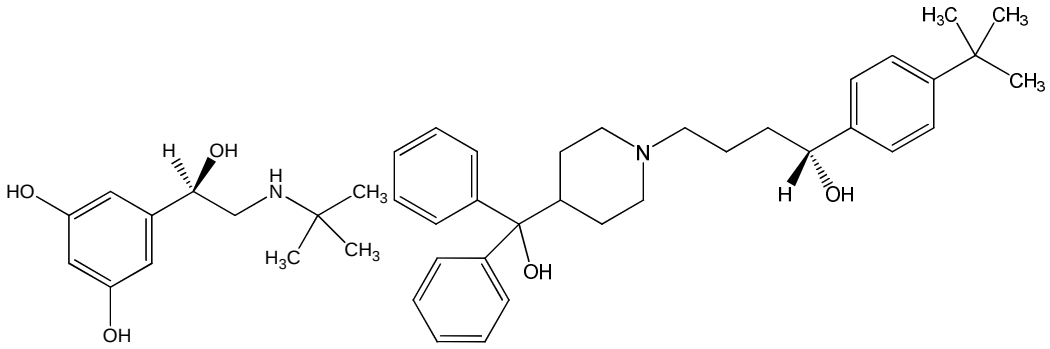
Figure 3.1 An enlarged compound set of chiral drugs (50) that were used during the evaluation of CSP throughout the study. *- α-methoxy- α-(trifluoromethyl)phenylacetic acid (MTPA).(continued overleaf)



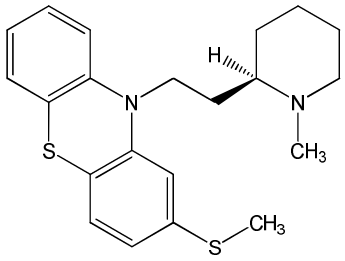

**propranolol**

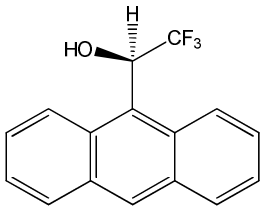



**salbutamol**



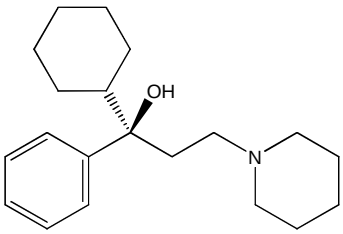

**terbutaline** **terfenadine**



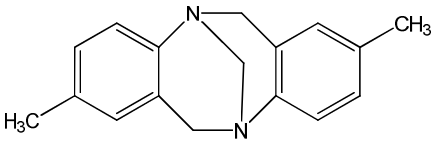




**thioridazine** **2,2,2-triflouro-1-(9-anthryl)-ethanol**

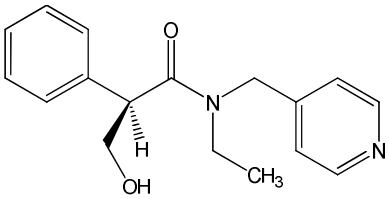
Figure 3.1 An enlarged compound set of chiral drugs (50) that were used during the evaluation of CSP throughout the study. (continued overleaf)



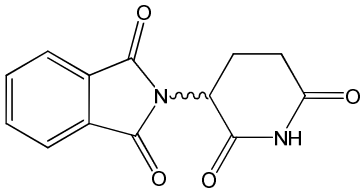

**trihexyphenidyl**



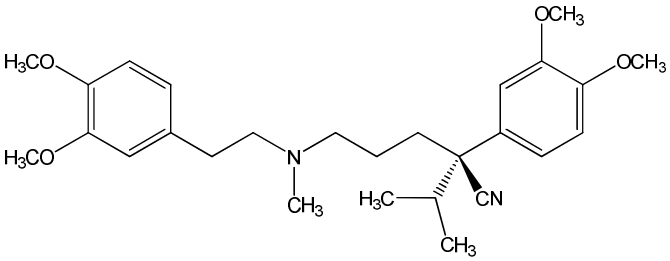

**Troger's base**



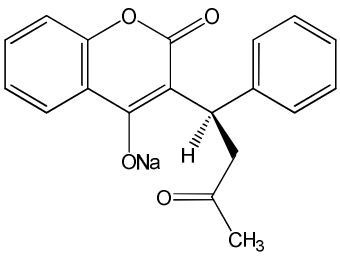

**tropicamide**




**thalidomide**




**verapamil**




**warfarin**

Figure 3.1 An enlarged compound set of chiral drugs (50) that were used during the evaluation of CSP throughout the study. Irrespective of the stereochemistry drawn, in all cases racemates or mixtures of individual enantiomers were used.

## 3.3 Results and Discussion

### 3.3.1 Evaluation of higher generation of Pirkle-concept CSP

As discussed previously, one aspect of considering chiral stationary phase selectivity that needed to be addressed was possible redundancies amongst the large number of CSP that had entered the market. This was particularly the case for higher generation Pirkle-concept CSP where the Whelk-O1 CSP was seemingly much more popular than other similar CSP. It was important to find out whether there was a basis for this in performance and indeed whether other related CSP had anything at all to offer in addition to what could be achieved on the Whelk-O1 CSP. Accordingly a study was set up involving Whelk-O1 (5 µm and 10 µm), ULMO and DACH DNB (Fig. 3.2) to compare and contrast their ‘breadth of spectrum’ with respect to chiral separations.

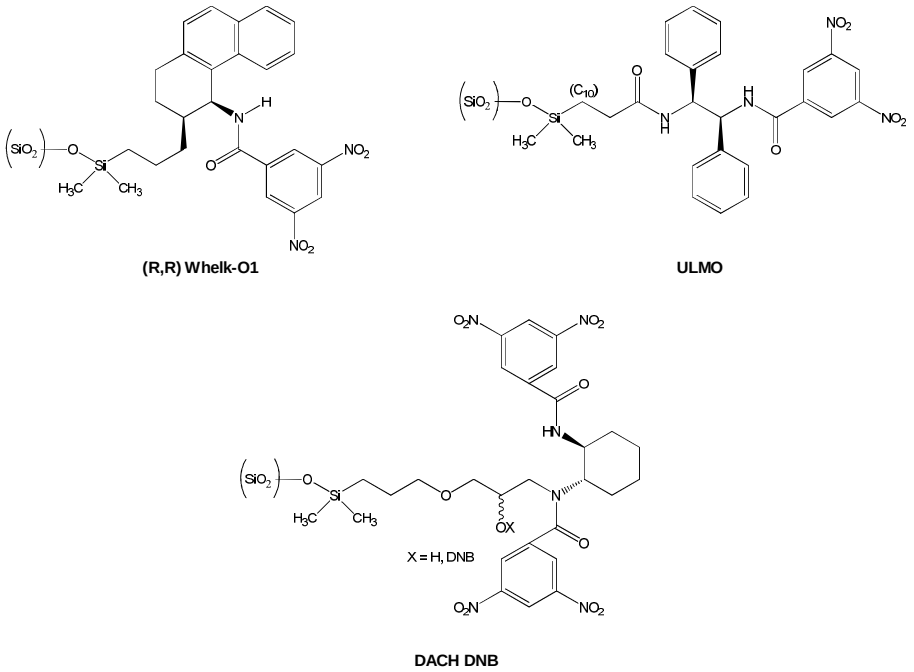



Figure 3.2 Structures of higher generation of Pirkle-concept CSP.

The main feature of the Whelk-O1 phase is that it contains both a $\pi$-acceptor and a $\pi$-donor in addition to two chiral centres and for this reason it can be used in wide range of chiral applications. This was originally designed to resolve the enantiomers of naproxen (Pirkle *et al*., 1992). It can be used under 'reversed-phase' conditions but is mainly operated with 'normal phase' mobile phases under which conditions it has good breadth of spectrum, almost on a par with the best of the carbamate-derivatised polysaccharide phases. It is covalently bonded to the support and therefore the phase is expected to be compatible with all commonly

used mobile phases contributing to the improvement of the column durability to exhibit excellent efficiency and ability to invert elution order (Review of Stereochemistry, http://www.mz-at.de/pdf/RegisChiralReview.pdf, 2000). The ULMO CSP was conceived and first prepared in the laboratories of the group of Wolfgang Lindner in Austria (Uray home page, http://www.uni-graz.at/georg.uray/; Regis Technologies Chiral Application Guide, Vol. 6, http://www.registech.com/Products/ULMO.html). It contains a 3,5-dinitrobenzoyl derivative of diphenylethylendiamine and behaves as both a $\pi$-acceptor and a $\pi$-donor (Review of Stereochemistry, http://www.mz-at.de/pdf/RegisChiralReview.pdf, 2000). Therefore, due to such complementary characters of the ULMO stationary phase it has been claimed to have an ability to resolve the enantiomers of racemic drugs from many classes (Regis Technologies Chiral Application Guide V, page 5).

The DACH-DNB contains a 3,5-dinitrobenzoyl derivative of 1,2-diaminocylohexane. DACH-DNB also has a characteristic of a $\pi$-acceptor (Regis Technologies Chiral Application Guide, Vol. 6). It has been claimed that the DACH-DNB stationary phase has the ability to resolve chiral drugs from racemate classes such as amides, alcohols, esters, ketones, acids, sulfoxides, phosphine oxides, phosphonates and more other compunds (Gasparrini *et al*., 2001; Cancellier *et al*., 1999).

In the study to compare and contrast the higher generation Pirkle-concept CSP a set of 50 chiral compounds was used. Compounds were selected so that, as far as

possible they would be chiral drugs that would be reasonably accessible to other workers (unlike many of the compounds listed by publications on chiral screening by pharmaceutical companies). Also the philosophy was that the balance of basic to acidic to neutral drugs would broadly reflect the balance of drugs on the market. It was thought 50 compounds would be enough to be manageable yet still enough to be representative enough for there not be bias arising from the specific nature of the compounds. In line with previous work in the University (M. Barry, Final Year Project, BSc. Chemical and Pharmaceutical Science, University of Sunderland, 1999), two mobile phases were used, one containing an acidic additive (trifluoroacetic acid) and the other containing a basic additive (triethylamine).

It was obvious immediately that more separations were obtained on the Whelk-O1 CSP than on the ULMO and DACH (Tables 3.1, 3.2). Also the Whelk-O1 CSP proved to be more successful than it had seemed to have been in earlier in-house work (Barry) on 26 compounds. This was not a feature of the difference in the compound sets but arose because of a reduction in the proportion of acidic or basic modifier (0.5 parts down to 0.1 parts to 100) leading to more compounds being eluted from the column.

As would be expected, since it was designed for the purpose, Whelk-O1 was very much the best option for resolving the naproxen enantiomers. The phase based on 5 µm particles gave better resolution than the 10 µm Whelk-O1 material but this was of little consequence for this particular application. The separation on the ULMO column showed a leading edge. Although this did not occur for other

compounds, it would not likely have been caused by the nature of the CSP / naproxen interaction. Regarding the other compounds the data has been summarised in Table 3.3 and Fig. 3.7.

Table 3.1 $k_1$, $k_2$, α and $R_s$ of 50 compounds on Whelk-O1 (5µm) and (10µm), ULMO and DACH DNB columns; mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)] (continued overleaf)

| compound | Whelk-O1 5 µm | | | | Whelk-O1 10 µm | | | | ULMO | | | | DACH DNB | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ |
| N-acetyl tryptophan | 8.74 | 10.10 | 1.16 | 1.89 | 10.06 | 11.35 | 1.13 | 1.52 | 1.55 | -- | -- | -- | -- | -- | -- | -- |
| aminoglutethimide | 8.43 | -- | -- | -- | 10.22 | -- | -- | -- | 1.86 | -- | -- | -- | -- | -- | -- | -- |
| atenolol | -- | -- | -- | -- | -- | -- | -- | -- | 4.54 | 4.71 | 1.04 | 0.14 | -- | -- | -- | -- |
| baclofen | 1.24 | -- | -- | -- | 1.34 | -- | -- | -- | 0.79 | -- | -- | -- | 8.69 | 11.86 | 1.36 | 0.31 |
| benfluorex | 2.71 | -- | -- | -- | 2.93 | -- | -- | -- | 0.85 | -- | -- | -- | -- | -- | -- | -- |
| benzoin | 1.97 | 5.36 | 2.73 | 13.00 | 2.15 | 5.27 | 2.45 | 12.00 | 0.59 | 0.68 | 1.15 | 0.92 | 3.08 | 3.24 | 1.05 | 0.48 |
| bepridil | 5.67 | 6.68 | 1.18 | 1.79 | 10.39 | 12.59 | 1.21 | 2.34 | 0.65 | 0.71 | 1.10 | 0.44 | 1.15 | -- | -- | -- |
| brompheniramine | 2.21 | -- | -- | -- | 3.22 | -- | -- | -- | 17.43 | -- | -- | -- | 2.31 | -- | -- | -- |
| bremazocine | -- | -- | -- | -- | 6.36 | 7.66 | 1.20 | 1.90 | -- | -- | -- | -- | -- | -- | -- | -- |
| bupivacaine | 2.25 | -- | -- | -- | 2.68 | -- | -- | -- | 0.73 | 0.80 | 1.09 | 0.34 | 1.08 | -- | -- | -- |
| carprofen | 3.89 | 7.14 | 1.84 | 8.96 | 4.03 | 6.48 | 1.61 | 5.75 | 0.86 | 1.00 | 1.16 | 1.27 | 6.17 | -- | -- | -- |
| carvedilol | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| chlorpheniramine | 2.15 | -- | -- | -- | 3.17 | -- | -- | -- | 18.87 | -- | -- | -- | 1.06 | -- | -- | -- |
| cicletanine | 14.03 | 23.67 | 1.69 | 4.06 | 13.41 | 21.23 | 1.58 | 3.22 | 0.90 | -- | -- | -- | -- | -- | -- | -- |
| citalopram | -- | -- | -- | -- | -- | -- | -- | -- | 4.18 | -- | -- | -- | -- | -- | -- | -- |
| cromakalim | 6.91 | 8.80 | 1.27 | 2.77 | 8.39 | 10.31 | 1.23 | 2.15 | 1.25 | 1.81 | 1.44 | 3.62 | 20.38 | 25.84 | 1.27 | 2.16 |
| disopyramide | 22.46 | 26.76 | 1.19 | 1.38 | 31.12 | 33.73 | 1.08 | 0.36 | 3.30 | 3.80 | 1.15 | 0.57 | -- | -- | -- | -- |
| dobutamine | 5.15 | 5.46 | 1.06 | 0.42 | 7.29 | 7.67 | 1.05 | 0.27 | 1.71 | -- | -- | -- | -- | -- | -- | -- |
| felodipine | 6.88 | 7.94 | 1.15 | 1.83 | 6.75 | 7.56 | 1.12 | 1.37 | 4.50 | -- | -- | -- | 2.62 | -- | -- | -- |
| fluoxetine | 1.61 | 1.86 | 1.16 | 1.04 | 1.98 | 2.18 | 1.10 | 0.53 | 1.39 | -- | -- | -- | 22.33 | -- | -- | -- |
| flurbiprofen | 0.71 | 0.97 | 1.36 | 2.53 | 0.79 | 1.03 | 1.30 | 2.03 | 0.32 | -- | -- | -- | 0.91 | -- | -- | -- |
| flurbiprofen amide | 3.08 | 3.70 | 1.20 | 2.20 | 3.24 | 3.84 | 1.18 | 1.93 | 1.11 | -- | -- | -- | 5.70 | -- | -- | -- |
| hexobarbital | 1.50 | -- | -- | -- | 1.75 | -- | -- | -- | 0.51 | -- | -- | -- | 2.09 | 2.12 | 1.01 | 0.05 |
| 4-OH mandelic acid | 2.30 | 2.50 | 1.09 | 0.90 | 2.59 | 2.86 | 1.11 | 0.50 | 0.87 | 0.94 | 1.08 | 0.36 | 3.13 | -- | -- | -- |
| 4-OH, 3-methoxymandelic acid | 4.19 | 4.34 | 1.03 | 0.26 | 3.65 | 4.53 | 1.24 | 2.08 | 1.20 | 1.40 | 1.16 | 1.06 | 6.26 | 7.84 | 1.25 | 2.23 |

Table 3.1 $k_1$, $k_2$, α and $R_s$ of 50 compounds on Whelk-O1 (5µm) and (10µm), ULMO and DACH DNB columns; mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)]

| compound | Whelk-O1 5 µm | | | | Whelk-O1 10 µm | | | | ULMO | | | | DACH DNB | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ |
| ibuprofen | 0.38 | 0.52 | 1.38 | 1.71 | 0.38 | 0.46 | 1.23 | 0.34 | 0.19 | -- | -- | -- | 0.43 | -- | -- | -- |
| isoproterenol | 2.22 | 2.44 | 1.10 | 0.75 | 3.32 | -- | -- | -- | 1.09 | -- | -- | -- | 7.11 | -- | -- | -- |
| isradipine | 7.71 | 8.39 | 1.09 | 2.18 | 8.21 | 9.44 | 1.15 | 1.90 | 0.61 | -- | -- | -- | 4.14 | -- | -- | -- |
| ketamine | 2.16 | 2.49 | 1.15 | 1.09 | 2.80 | 3.11 | 1.11 | 0.57 | 0.88 | 0.95 | 1.09 | 0.53 | -- | -- | -- | -- |
| ketoprofen | 3.18 | 3.71 | 1.17 | 2.15 | 3.16 | 3.62 | 1.14 | 1.76 | 0.63 | -- | -- | -- | 3.28 | -- | -- | -- |
| lorezapam | 4.95 | 11.70 | 2.36 | 10.71 | 5.48 | 12.03 | 2.20 | 10.97 | 1.97 | 3.23 | 1.64 | 5.77 | 0.84 | -- | -- | -- |
| mexiletine | 0.64 | 0.69 | 1.08 | 0.24 | 0.79 | -- | -- | -- | 0.51 | -- | -- | -- | 8.92 | -- | -- | -- |
| mianserin | 7.68 | 10.45 | 1.36 | 2.10 | 7.20 | 9.15 | 1.27 | 1.70 | 1.10 | -- | -- | -- | -- | -- | -- | -- |
| MTPA* | 0.24 | -- | -- | -- | 0.23 | -- | -- | -- | 0.19 | -- | -- | -- | 0.59 | -- | -- | -- |
| naproxen | 4.45 | 9.87 | 2.22 | 12.50 | 4.57 | 9.60 | 2.10 | 10.68 | 0.94 | 1.37 | 1.45 | 3.78 | 2.93 | 3.11 | 1.06 | 0.30 |
| nicotine | 22 | 33.5 | 1.52 | 0.42 | -- | -- | -- | -- | 30.8 | -- | -- | -- | -- | -- | -- | -- |
| paroxetine | 4.82 | 5.87 | 1.22 | 1.80 | 5.84 | 6.59 | 1.13 | 1.31 | 1.19 | -- | -- | -- | -- | -- | -- | -- |
| α-phenyl-butanoicacid carboxymide | 1.68 | 2.60 | 1.55 | 5.47 | 1.76 | 2.65 | 1.50 | 4.71 | 0.73 | 0.85 | 1.16 | 1.06 | 3.43 | 3.79 | 1.10 | 0.17 |
| propranalol | 4.57 | 5.76 | 1.26 | 2.33 | 5.18 | 6.28 | 1.21 | 2.08 | 1.41 | -- | -- | -- | -- | -- | -- | -- |
| salbutamol | 3.01 | 3.21 | 1.06 | 0.57 | 3.66 | -- | -- | -- | 1.17 | -- | -- | -- | -- | -- | -- | -- |
| terbutaline | 1.40 | -- | -- | -- | 1.78 | -- | -- | -- | 0.98 | 1.09 | 1.11 | 0.51 | -- | -- | -- | -- |
| terfenadine | 4.68 | 5.75 | 1.23 | 1.88 | 5.54 | 6.73 | 1.22 | 1.88 | 0.67 | -- | -- | -- | 3.06 | 4.90 | 1.60 | 0.75 |
| thalidomide | 16.9 | -- | -- | -- | 21.35 | -- | -- | -- | 2.54 | 2.82 | 1.11 | 1.08 | -- | -- | -- | -- |
| thioridazin | 20.30 | 22.25 | 1.10 | 0.72 | 23.04 | 24.96 | 1.08 | 0.59 | 2.01 | -- | -- | -- | -- | -- | -- | -- |
| 2,2,2,trifluoro-1-(9-anthryl)-ethanol | 0.97 | 1.10 | 1.14 | 0.88 | 1.05 | 1.16 | 1.10 | 0.39 | 0.46 | 0.89 | 1.96 | 2.10 | 2.07 | -- | -- | -- |
| trihexyphenidyl | 1.3 | -- | -- | -- | 1.26 | -- | -- | -- | 0.18 | -- | -- | -- | 1.00 | -- | -- | -- |
| Troger's base | 2.68 | 3.86 | 1.44 | 3.86 | 2.68 | 3.71 | 1.38 | 3.53 | 0.54 | -- | -- | -- | 6.37 | -- | -- | -- |
| tropicamide | 16.00 | 24.21 | 1.51 | 2.51 | 23.60 | 33.06 | 1.40 | 1.76 | 2.86 | -- | -- | -- | -- | -- | -- | -- |
| verapamil | -- | -- | -- | -- | -- | -- | -- | -- | 3.11 | 3.34 | 1.07 | 0.40 | -- | -- | -- | -- |
| warfarin | 9.80 | 25.76 | 2.63 | 9.56 | 9.46 | 21.79 | 2.30 | 10.36 | 1.25 | 1.80 | 1.44 | 4.07 | 13.44 | 14.26 | 1.06 | 0.70 |

*- α-methoxy- α-(trifluoromethyl)phenylacetic acid (MTPA)

Table 3.2 $k_1$, $k_2$, α and $R_s$ of 50 compounds on Whelk-O1 (5µm) and (10µm), ULMO and DACH DNB columns; mobile phase - [hexane – IPA - TEA (85:15:0.1, v/v/v)] (continued overleaf)

| compound | Whelk-O1 5 µm | | | | Whelk-O1 10 µm | | | | ULMO | | | | DACH DNB | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ |
| N-acetyl tryptophan | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| aminoglutethimide | 11.05 | -- | -- | -- | 13.21 | -- | -- | -- | 1.84 | 1.87 | 1.02 | 0.11 | 22.28 | 23.01 | 1.03 | 0.23 |
| atenolol | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| baclofen | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| benfluorex | 1.32 | -- | -- | -- | 1.53 | -- | -- | -- | 0.34 | -- | -- | -- | 1.19 | -- | -- | -- |
| benzoin | 0.83 | 0.98 | 1.17 | 0.71 | 1.97 | 4.78 | 2.42 | 12.57 | 0.62 | 0.76 | 1.22 | 1.03 | 2.59 | 2.74 | 1.05 | 0.42 |
| bepridil | 0.24 | -- | -- | -- | 0.24 | -- | -- | -- | 0.06 | -- | -- | -- | 0.21 | -- | -- | -- |
| brompheniramine | 4.27 | -- | -- | -- | 9.60 | -- | -- | -- | 3.52 | -- | -- | -- | 4.31 | -- | -- | -- |
| bremazocine | 1.07 | -- | -- | -- | 1.17 | -- | -- | -- | 0.35 | -- | -- | -- | -- | -- | -- | -- |
| bupivacaine | 2.97 | 4.27 | 1.44 | 6.83 | 3.48 | 5.06 | 1.46 | 5.69 | 2.95 | -- | -- | -- | 3.20 | -- | -- | -- |
| carprofen | -- | -- | -- | -- | -- | -- | -- | -- | 4.32 | -- | -- | -- | -- | -- | -- | -- |
| carvedilol | -- | -- | -- | -- | -- | -- | -- | -- | 3.93 | -- | -- | -- | -- | -- | -- | -- |
| chlorpheniramine | 4.63 | -- | -- | -- | 10.25 | -- | -- | -- | 3.99 | -- | -- | -- | 4.52 | -- | -- | -- |
| cicletanine | 2.32 | 2.62 | 1.13 | 0.38 | 3.05 | 3.48 | 1.14 | 0.41 | 2.31 | -- | -- | -- | 3.98 | -- | -- | -- |
| citalopram | 3.19 | -- | -- | -- | 5.06 | -- | -- | -- | 1.35 | -- | -- | -- | -- | -- | -- | -- |
| cromakalim | 6.22 | 7.73 | 1.24 | 2.54 | 7.66 | 9.24 | 1.21 | 2.17 | 1.12 | 1.56 | 1.39 | 3.55 | 21.48 | 28.98 | 1.35 | 2.30 |
| disopyramide | 1.17 | 1.39 | 1.19 | 0.86 | 1.68 | 1.85 | 1.11 | 0.46 | 0.71 | -- | -- | -- | 5.36 | -- | -- | -- |
| dobutamine | -- | -- | -- | -- | 19.27 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| felodipine | 5.78 | 6.58 | 1.14 | 1.73 | 7.30 | 8.16 | 1.12 | 1.49 | 0.46 | -- | -- | -- | 2.63 | -- | -- | -- |
| fluoxetine | 2.32 | -- | -- | -- | 4.49 | -- | -- | -- | 3.63 | -- | -- | -- | 4.04 | -- | -- | -- |
| flurbiprofen | 7.95 | 10.18 | 1.28 | 1.23 | 0.79 | 1.03 | 1.30 | 2.03 | 2.21 | -- | -- | -- | -- | -- | -- | -- |
| flurbiprofen amide | 2.92 | 3.56 | 1.22 | 2.39 | 3.55 | 4.20 | 1.18 | 2.20 | 1.04 | -- | -- | -- | 5.43 | -- | -- | -- |
| hexobarbital | 1.60 | -- | -- | -- | 1.75 | -- | -- | -- | 0.48 | -- | -- | -- | 2.31 | 2.45 | 1.06 | 0.39 |
| 4-OH mandelic acid | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| 4-OH, 3-methoxymandelic acid | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |

Table 3.2 $k_1$, $k_2$, α and $R_s$ of 50 compounds on Whelk-O1 (5µm) and (10µm), ULMO and DACH DNB columns; mobile phase - [hexane – IPA - TEA (85:15:0.1, v/v/v)]

| compound | Whelk-O1 5 µm | | | | Whelk-O1 10 µm | | | | ULMO | | | | DACH DNB | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ |
| ibuprofen | 2.25 | 2.97 | 1.32 | 1.23 | 4.26 | 5.16 | 1.21 | 0.62 | 1.01 | -- | -- | -- | -- | -- | -- | -- |
| isoproterenol | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| isradipine | 3.41 | 3.88 | 1.14 | 1.61 | 3.77 | 4.34 | 1.15 | 1.46 | 0.31 | -- | -- | -- | 2.07 | -- | -- | -- |
| ketamine | 1.70 | 1.86 | 1.06 | 0.89 | 2.01 | 2.20 | 1.09 | 0.91 | 0.55 | -- | -- | -- | 1.84 | 1.93 | 1.05 | 0.28 |
| ketoprofen | 19.77 | 22.58 | 1.14 | 0.56 | 18.76 | 24.17 | 1.29 | 0.45 | 3.39 | -- | -- | -- | -- | -- | -- | -- |
| lorezapam | 3.91 | 8.59 | 2.20 | 10.83 | 5.50 | 11.77 | 2.14 | 9.34 | 20.04 | 21.10 | 1.05 | 0.40 | 2.70 | -- | -- | -- |
| mexiletine | 1.25 | -- | -- | -- | 2.11 | -- | -- | -- | 1.04 | -- | -- | -- | 2.04 | -- | -- | -- |
| mianserin | 1.60 | 4.05 | 2.53 | 13.59 | -- | -- | -- | -- | 0.49 | -- | -- | -- | 0.93 | -- | -- | -- |
| MTPA* | -- | -- | -- | -- | -- | -- | -- | -- | 2.10 | -- | -- | -- | -- | -- | -- | -- |
| naproxen | 20.20 | -- | -- | -- | 5.02 | -- | -- | -- | 3.75 | -- | -- | -- | -- | -- | -- | -- |
| nicotine | 4.74 | -- | -- | -- | 4.47 | -- | -- | -- | 1.53 | -- | -- | -- | 2.30 | -- | -- | -- |
| paroxetine | 4.49 | -- | -- | -- | 8.09 | -- | -- | -- | 2.72 | 3.17 | 1.17 | 0.33 | -- | -- | -- | -- |
| α-phenyl-butanoicacid carboxymide | 1.03 | 1.49 | 1.45 | 6.52 | 1.10 | 1.68 | 1.52 | 4.27 | 0.42 | 0.49 | 1.18 | 0.89 | 1.92 | -- | -- | -- |
| propranalol | 3.41 | 4.05 | 1.19 | 0.89 | 5.78 | 6.65 | 1.15 | 0.49 | 1.88 | -- | -- | -- | 6.88 | -- | -- | -- |
| salbutamol | 5.05 | -- | -- | -- | 6.75 | -- | -- | -- | 2.40 | -- | -- | -- | -- | -- | -- | -- |
| terbutaline | 1.82 | -- | -- | -- | 5.64 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| terfenadine | 0.85 | 0.93 | 1.09 | 0.63 | 0.98 | 1.10 | 1.13 | 0.61 | 0.25 | -- | -- | -- | 1.15 | 1.24 | 1.08 | 0.25 |
| thalidomide | 6.86 | 10.47 | 1.53 | 0.18 | 9.60 | -- | -- | -- | 1.33 | 1.46 | 1.10 | 0.82 | -- | -- | -- | -- |
| thioridazin | 0.81 | -- | -- | -- | 2.83 | -- | -- | -- | 1.00 | -- | -- | -- | 2.89 | -- | -- | -- |
| 2,2,2,trifluoro-1-(9-anthryl)-ethanol | 0.90 | 1.02 | 1.14 | 0.97 | 1.01 | 1.10 | 1.09 | 0.54 | 0.35 | 0.73 | 2.08 | 2.26 | 1.99 | 2.12 | 1.07 | 0.23 |
| trihexyphenidyl | -- | -- | -- | -- | -- | -- | -- | -- | 0.13 | -- | -- | -- | -- | -- | -- | -- |
| Troger's base | 1.03 | 2.18 | 2.12 | 5.96 | 1.17 | 2.24 | 1.91 | 5.74 | 0.45 | -- | -- | -- | 0.92 | -- | -- | -- |
| tropicamide | 9.60 | 15.08 | 1.57 | 4.74 | 12.56 | 18.40 | 1.46 | 2.92 | 1.89 | 1.96 | 1.04 | 0.17 | 10.60 | 11.50 | 1.09 | 0.55 |
| verapamil | 20.42 | -- | -- | -- | 25.46 | -- | -- | -- | 1.34 | -- | -- | -- | 10.53 | -- | -- | -- |
| warfarin | -- | -- | -- | -- | -- | -- | -- | -- | 5.42 | 6.01 | 1.11 | 0.16 | -- | -- | -- | -- |

*- α-methoxy- α-(trifluoromethyl)phenylacetic acid (MTPA)

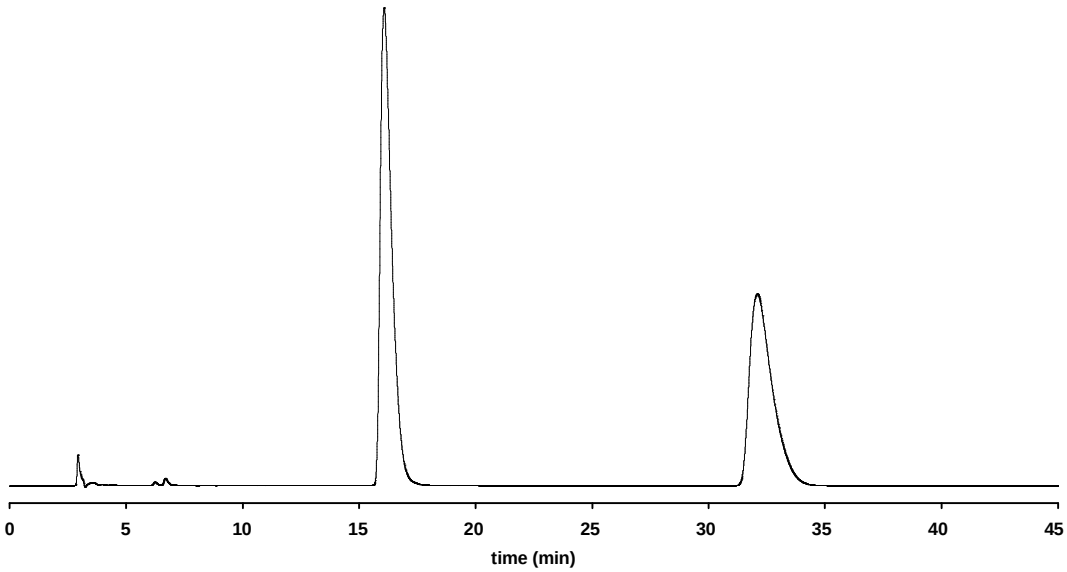



Figure 3.3 NP-HPLC of racemic naproxen on Whelk-O1 (5 µm) (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 260 nm.

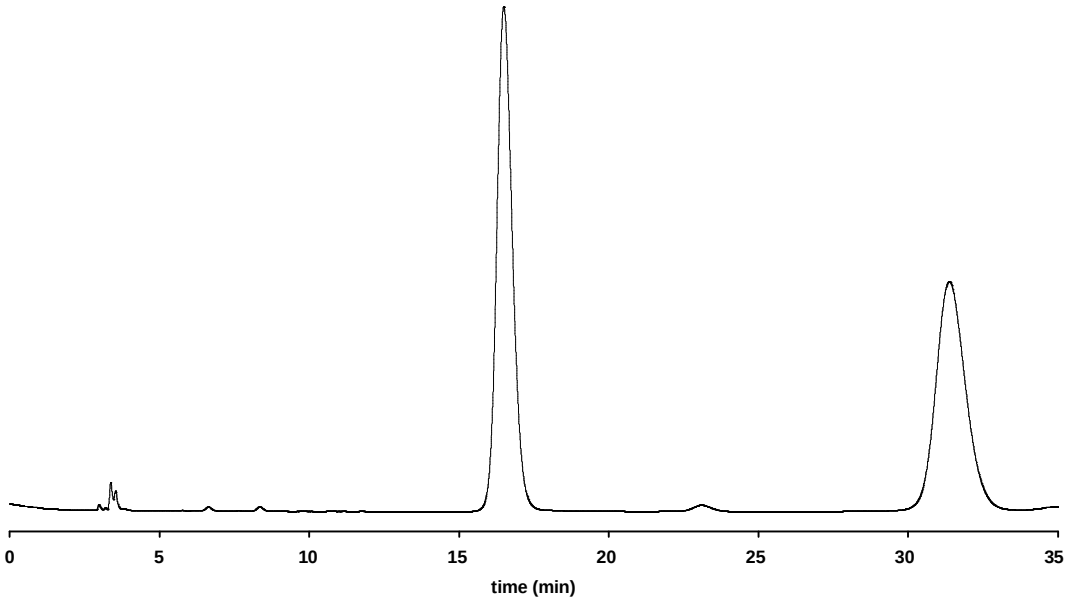



Figure 3.4 NP-HPLC of racemic naproxen on Whelk-O1 (10 µm) (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 260 nm.

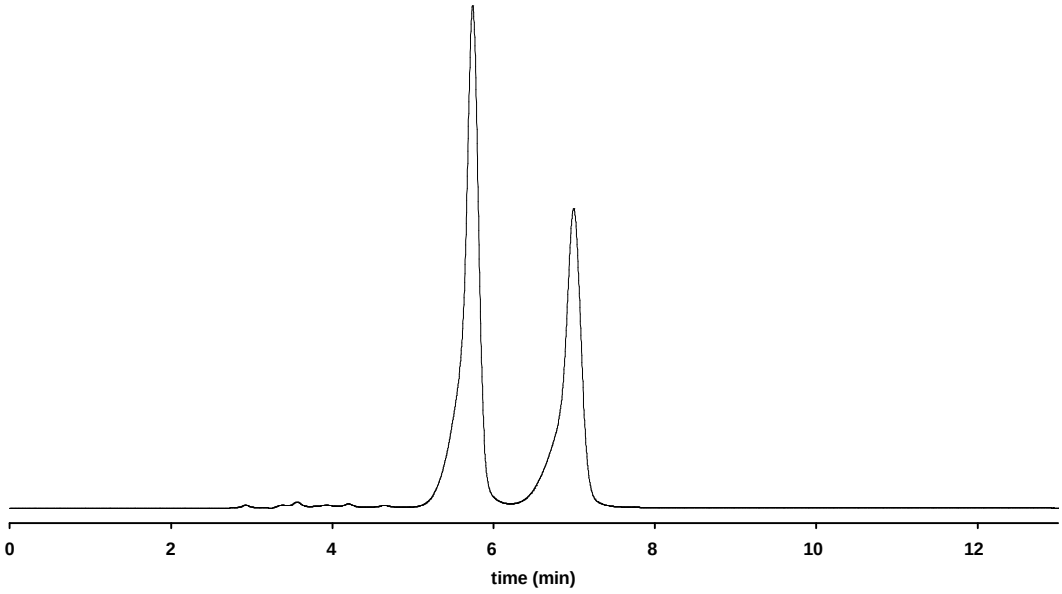



Figure 3.5 NP-HPLC of racemic naproxen on ULMO (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 260 nm.

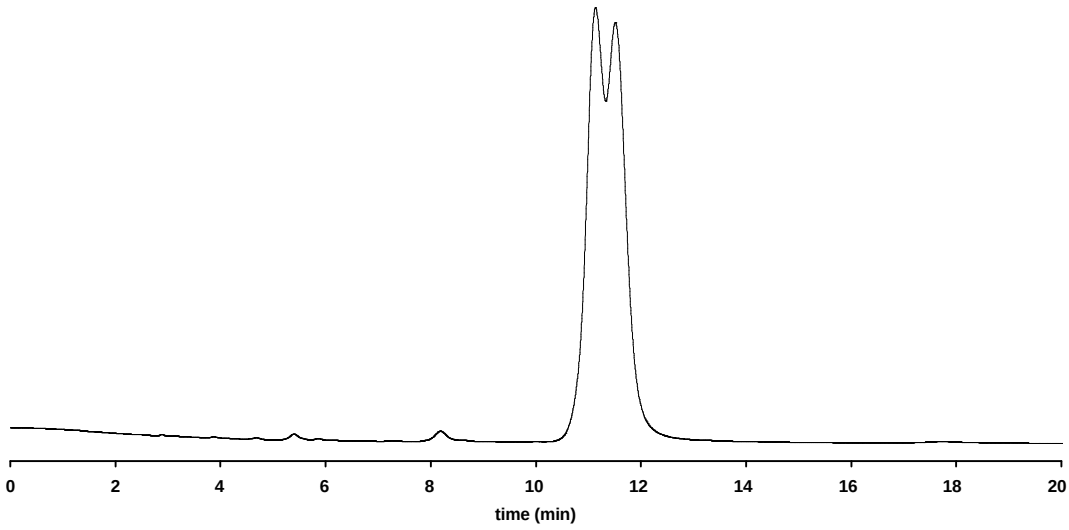



Figure 3.6 NP-HPLC of racemic naproxen on DACH DNB - (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 260 nm.

Table 3.3 'Hit' comparison of all higher generation Pirkle concept CSP with the enlarged compound set (50) of known chiral drugs by normal phase LC, using two mobile phases.(continued overleaf)

| | compound | With Acid Modifier | | | | With Base Modifier | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Whelk-O1 (5 µm) | Whelk-O1 (10 µm) | ULMO | DACH | Whelk-O1 (5 µm) | Whelk-O1 (10 µm) | ULMO | DACH |
| **Acidic** | N-acetyl tryptophan | ✓ | ✓ | | | | | | |
| | carprofen | ✓ | ✓ | ✓ | | | | | |
| | flurbiprofen | ✓ | ✓ | | | ✓ | ✓ | | |
| | hexobarbital | | | | ✓ | | | | ✓ |
| | 4-OH mandelic acid | ✓ | ✓ | ✓ | | | | | |
| | 4-OH, 3-methoxymandelic acid | ✓ | ✓ | ✓ | ✓ | | | | |
| | ibuprofen | ✓ | ✓ | | | ✓ | ✓ | | |
| | ketoprofen | ✓ | ✓ | | | ✓ | ✓ | | |
| | MTPA | | | | | | | | |
| | naproxen | ✓ | ✓ | ✓ | ✓ | | | | |
| | warfarin | ✓ | ✓ | ✓ | ✓ | | | ✓ | |
| | thalidomide | | | ✓ | | ✓ | ✓ | ✓ | |
| **Basic** | aminoglutethimide | | | | | | | ✓ | ✓ |
| | atenolol | | | ✓ | | | | | |
| | benfluorex | | | | | | | | |
| | bepridil | ✓ | ✓ | ✓ | | | | | |
| | bremazocine | | ✓ | | | | | | |
| | brompheniramine | | | | | | | | |
| | bupivacaine | | | ✓ | | ✓ | ✓ | | |
| | carvedilol | | | | | | | | |
| | chlorpheniramine | | | | | | | | |
| | cicletanine | ✓ | ✓ | | | ✓ | ✓ | | |
| | citalopram | | | | | ✓ | | | |
| | disopyramide | ✓ | ✓ | ✓ | | ✓ | ✓ | | |
| | dobutamine | ✓ | ✓ | | | | | | |

Table 3.3 'Hit' comparison of all higher generation Pirkle concept CSP with the enlarged compound set (50) of known chiral drugs by normal phase LC, using two mobile phases.

| | compound | With Acid Modifier | | | | With Base Modifier | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Whelk-O1 (5 µm) | Whelk-O1 (10 µm) | ULMO | DACH | Whelk-O1 (5 µm) | Whelk-O1 (10 µm) | ULMO | DACH |
| | felodipine | ✓ | ✓ | | | ✓ | ✓ | | |
| | fluoxetine | ✓ | ✓ | | | | | | |
| | isoproterenol | ✓ | | | | | | | |
| | isradipine | ✓ | ✓ | | | ✓ | ✓ | | |
| | ketamine | ✓ | ✓ | ✓ | | ✓ | ✓ | | ✓ |
| | mexiletine | ✓ | | | | | | | |
| | mianserin | ✓ | ✓ | | | ✓ | ✓ | | |
| | nicotine | ✓ | | | | | | | |
| | paroxetine | ✓ | ✓ | | | | | ✓ | |
| | propranalol | ✓ | ✓ | | | ✓ | ✓ | | |
| | salbutamol | ✓ | | | | | | | |
| | terbutaline | | | ✓ | | | | | |
| | terfenadine | ✓ | ✓ | | ✓ | ✓ | ✓ | | ✓ |
| | trihexyphenidyl | | | | | | | | |
| | thioridazine | ✓ | ✓ | | | | | | |
| | Troger's base | ✓ | ✓ | | | ✓ | ✓ | | |
| | tropicamide | ✓ | ✓ | | | ✓ | ✓ | ✓ | ✓ |
| | verapamil | | | ✓ | | | | | |
| **Neutral** | benzoin | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| | cromakalim | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| | flurbiprofen amide | ✓ | ✓ | | | ✓ | ✓ | | |
| | lorezapam | ✓ | ✓ | ✓ | | ✓ | ✓ | ✓ | |
| | α-phenyl-butanoicacid carboxymide | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | |
| | 2,2,2,trifluoro-1-(9-anthryl)-ethanol | ✓ | ✓ | ✓ | | ✓ | ✓ | ✓ | ✓ |
| **Zwitterionic** | baclofen | | | | ✓ | | | | |

The Whelk-O1 CSP showed good enantioselectivity for greater than half the common racemic drugs in a set of 50 and was much superior to the related DACH DNB and ULMO CSP. For a newer Whelk-O1 material based on a purer 5-μm silica support, peak shapes were better and resolution slightly improved but no new chiral resolutions were observed compared to the 10 μm version. Comparison with an earlier study carried out on Whelk-O1 using slightly different mobile phases and the original 10 μm Whelk-O1 revealed that the breadth of spectrum of this CSP was much greater than had been imagined since in the earlier work there had been a significant number of racemic compounds that had been totally retained. The ULMO and DACH DNB CSP were much less successful and were better than the Whelk-O1 for only a few of the test drugs.

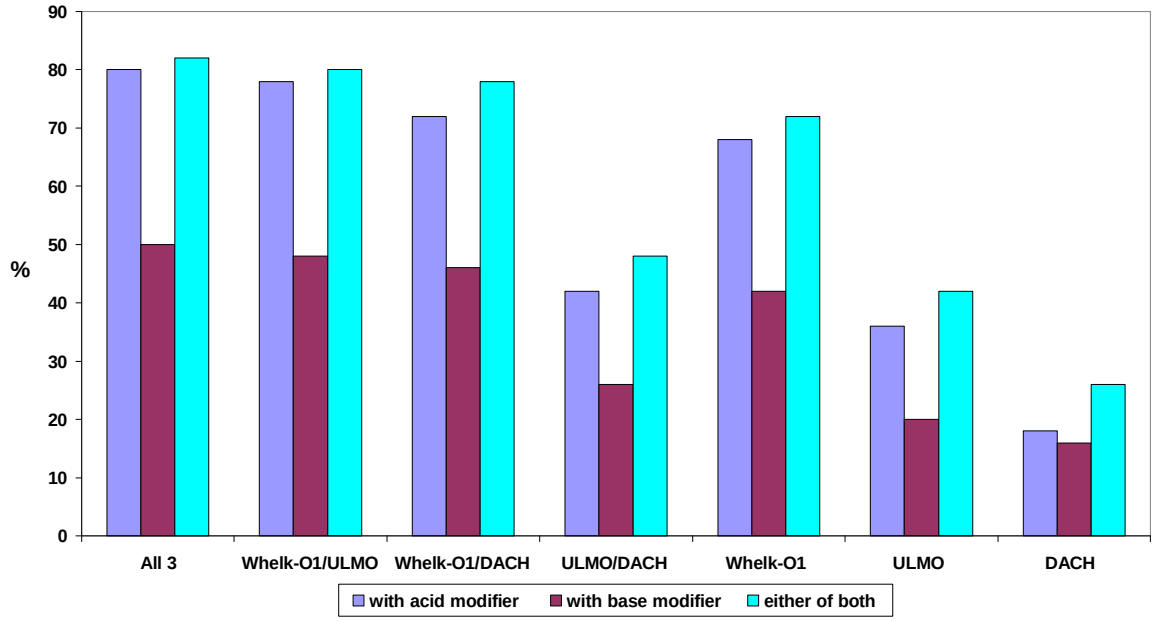



Figure 3.7 'Hit-Rate' of all higher generation Pirkle-concept CSP with the enlarged compound set (50) of known chiral drugs by normal phase LC, using two mobile phases.

Perhaps the most important finding from this study of Pirkle-concept CSP can be observed from Fig. 3.7. The 'hit-rate' for the Whelk-O1 CSP was very respectable, in the order of 70%. However, while this was improved with the addition of one of the other two CSP, the improvement was only marginal and the further improvement was even less when the third CSP was included. This minimal contribution from the ULMO and DACH-DNB is also apparent from Tables 3.1, 3.2 and 3.3 where it can be seen that there were so few compounds that could be resolved on ULMO or DACH-DNB but not on Whelk-O1 (hexobarbital,

aminoglutethimide). Even in those isolated cases the resolution on the other CSP was only partial (e.g. Fig. 3.14; $R_s$ – 0.05).

Another striking feature evident from Fig. 3.7 is that there was a higher success rate when using the acidic modifier, trifluoroacetic acid. This higher success rate was evident most clearly for the acidic drugs. In general, the basic modifier gave higher retention for the acidic drugs (Fig. 3.8) and, less generally, the acidic modifier gave higher retention for the basic drugs (Fig. 3.9). However, there was no clear cut correlation between this and the relative success rate of the two modifiers.

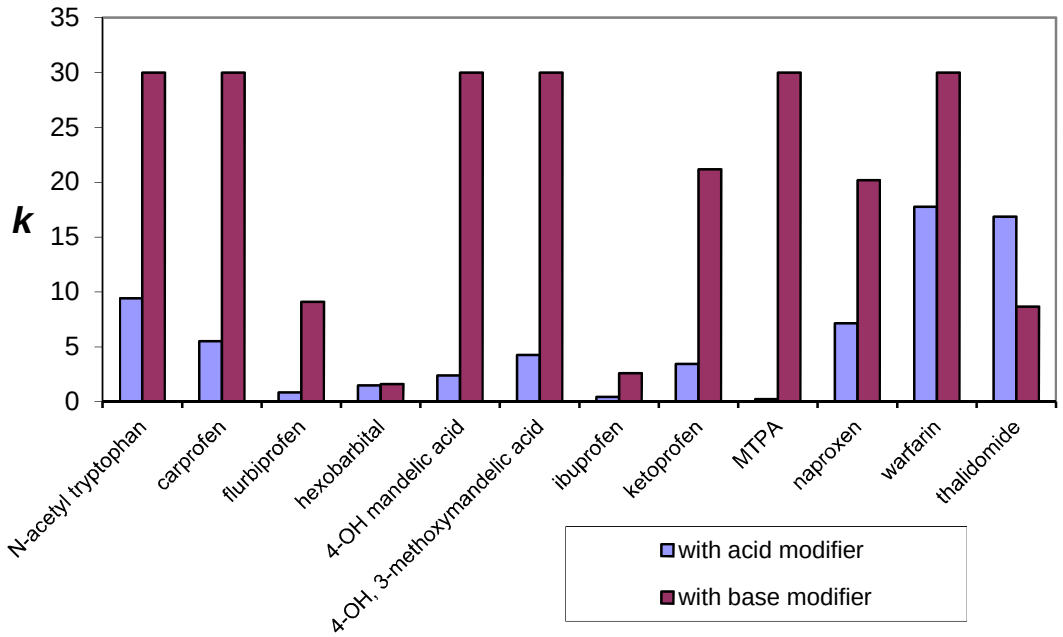



Figure 3.8 *k* of acidic chiral drug compounds with acid and base mobile phase modifiers on Whelk-O1.

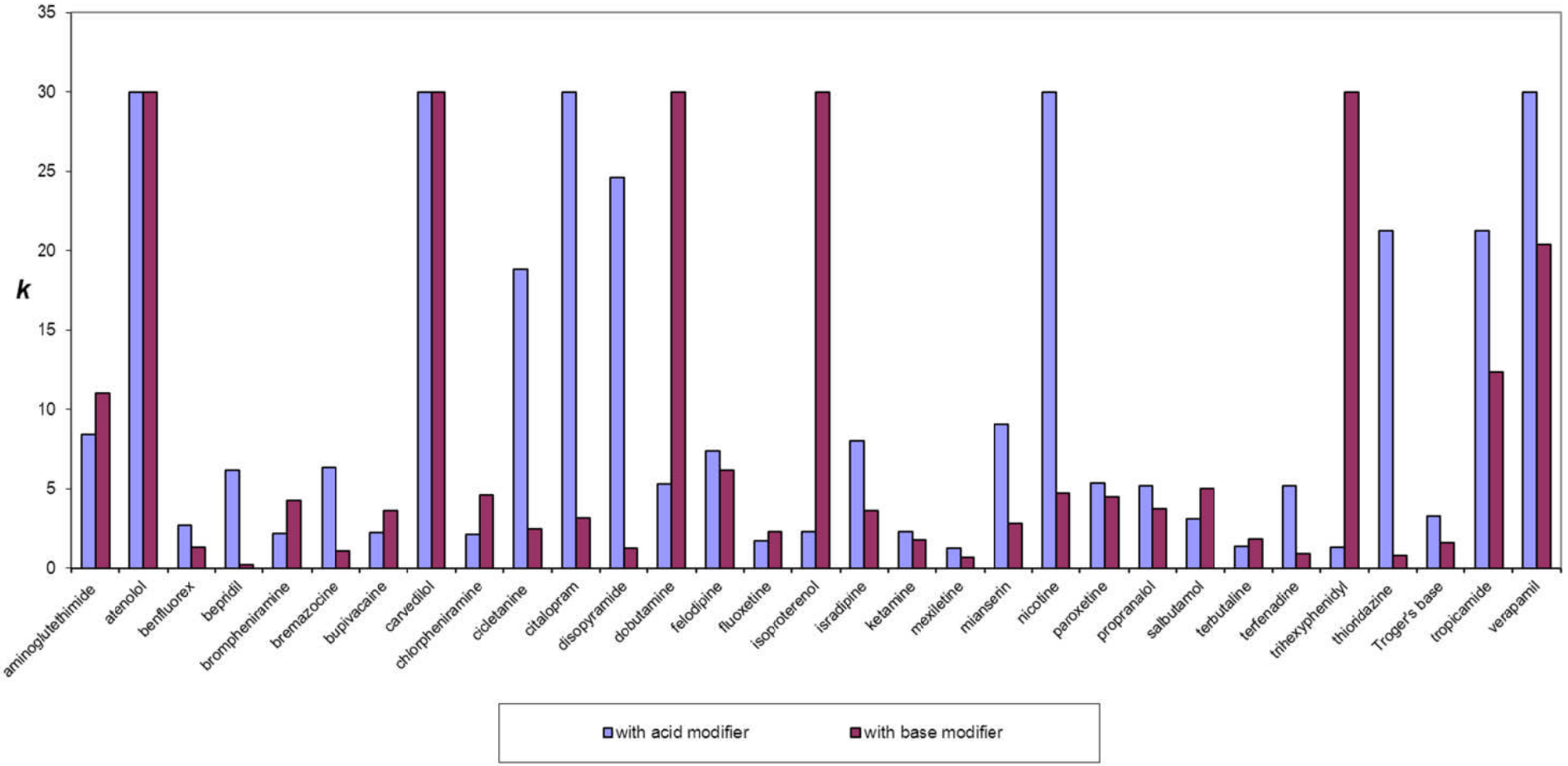



Figure 3.9 *k* of basic chiral drug compounds with acid and base mobile phase modifiers on Whelk-O1.

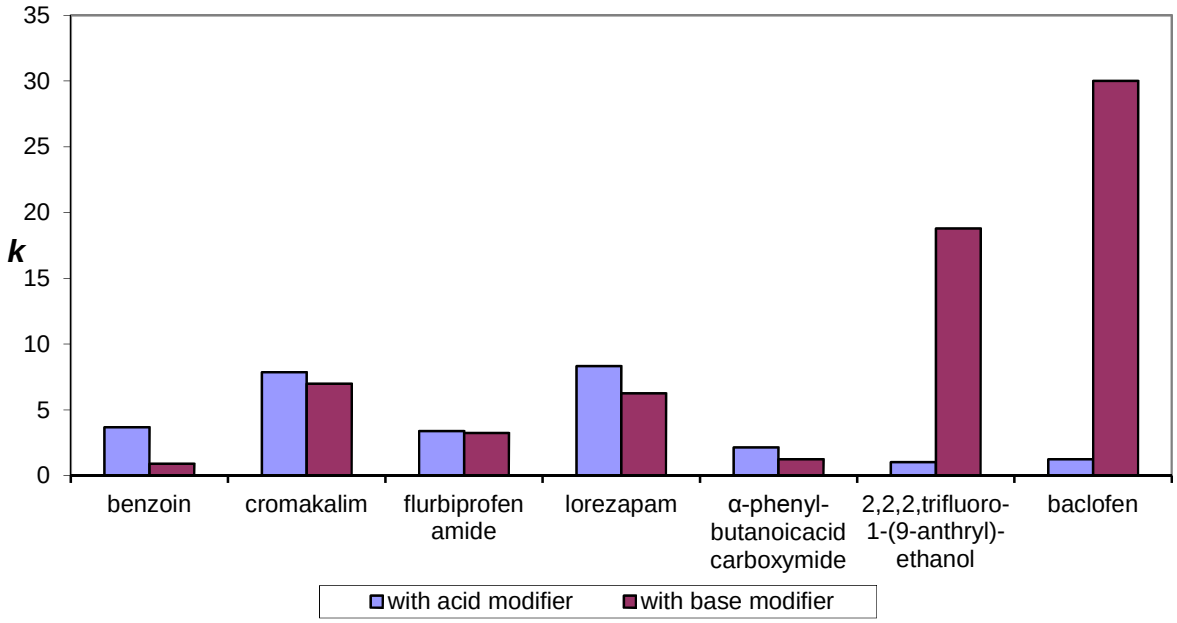



Figure 3.10 *k* of neutral and zwitterionic chiral drug compounds with acid and base mobile phase modifiers on Whelk-O1.

There was a smaller number (6) of neutral compounds but there was enough to observe that the success rate was good and, unsurprisingly, the success rate and *k* values changed little with the nature of the modifier (except for the unusual case of 2,2,2-trifluoro-1-(9-anthryl)-ethanol).

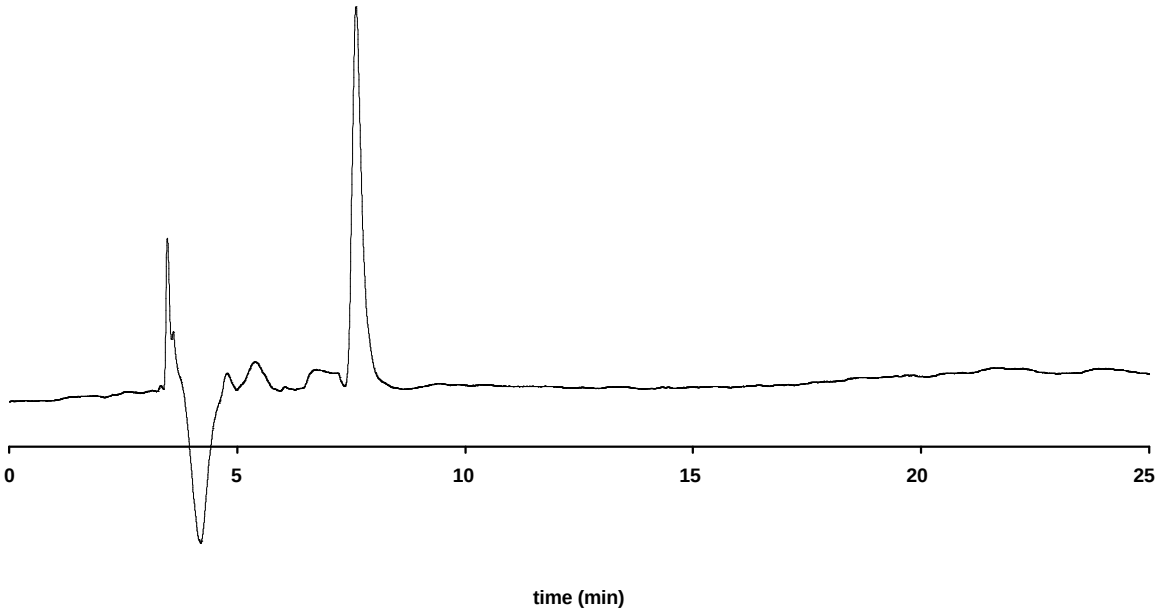



Figure 3.11 NP-HPLC of racemic hexobarbital on Whelk-O1 (5 µm) (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TEA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 254 nm.

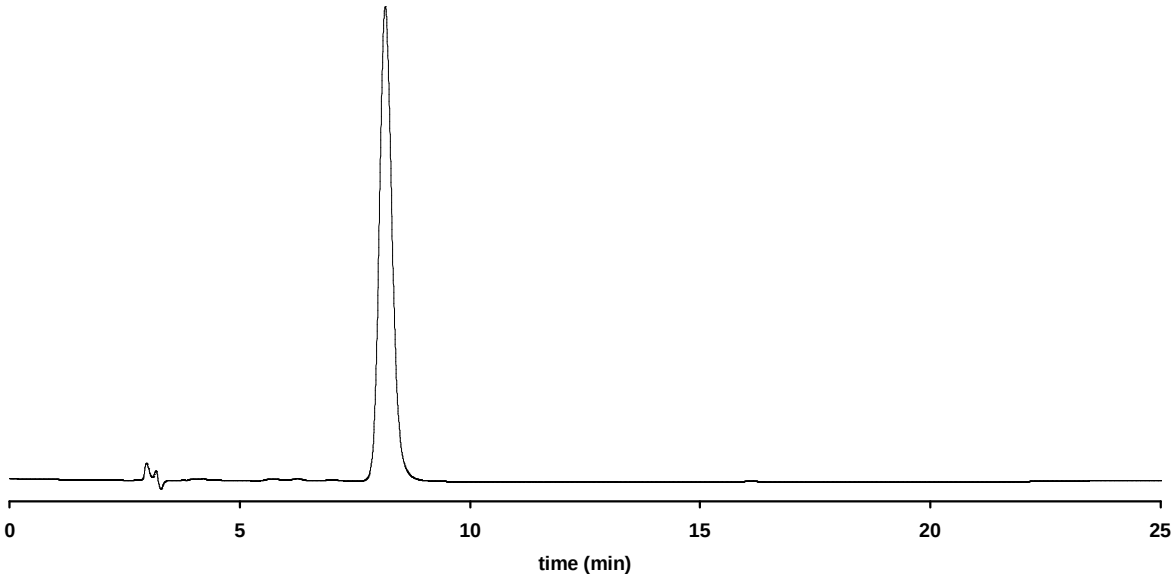



Figure 3.12 NP-HPLC of racemic hexobarbital on Whelk-O1 (10 µm) (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TEA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 254 nm.

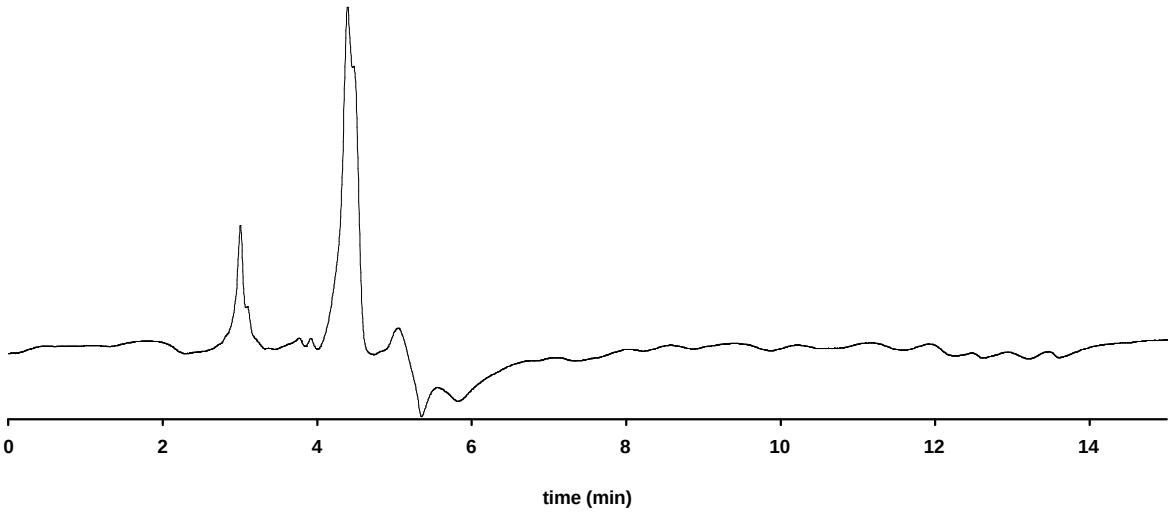



Figure 3.13 NP-HPLC of racemic hexobarbital on ULMO (5 µm) (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TEA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 254 nm.

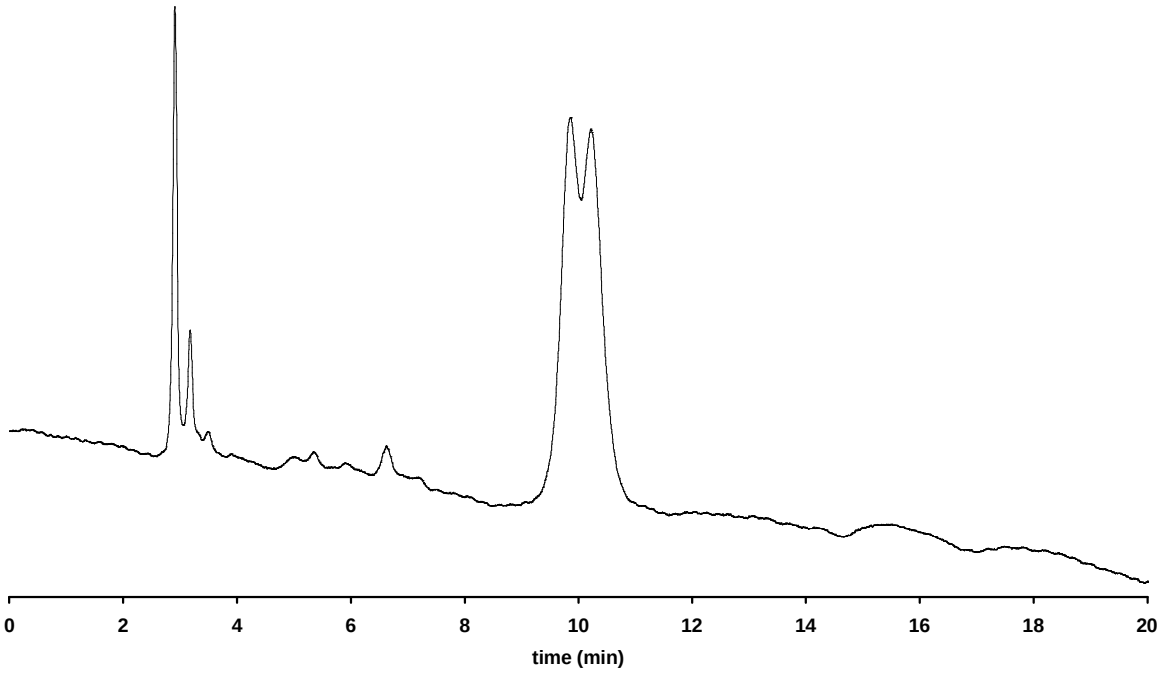



Figure 3.14 NP-HPLC of racemic hexobarbital on DACH DNB (5 µm) (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TEA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 254 nm.

#### 3.3.1.1 Conclusion

The ‘take-home’ message from this study for chiral screening was that if Pirkle-concept CSP were to be included, then it would only be worthwhile including the Whelk-O1 CSP. The few compounds that could be resolved on the ULMO or DACH-DNB but not the Whelk-O1 CSP would most likely be separated by one of the other CSP in the screen. With respect to use of the Whelk-O1 it should be noted that much of the ‘non-hits’ were attributable to non-detection of peaks rather than a failure to be separated. While the Whelk-O1 5 µm version gave rise to an improved peak shape and efficiency, there was no significant impact on ‘hit-rate’ compared to the 10 µm version.

For the manufacturers of these CSP, the message was that for the continuation of the DACH DNB and ULMO CSP more might need to be done to find optimal conditions where these CSP gave improved separations over Whelk-O1 and more could be done to find further compounds where these two CSP are more effective than Whelk-O1 (Most of the β-blockers by DACH-DNB and prilocaine, trichlormethiazide andalthiazide by ULMO; Regis Technologies Chiral Application Guide, Vol. 6).

### 3.3.2 Evaluation of CSP produced by derivatization of cellulose polymers.

A major recent development in the field of chiral separations at the time of the outset of this research programme was the advent of clones of derivatised polysaccharide CSP, the patent (Okamoto, 1997) for the highly successful Diacel Industries' Chiralcel-OD having expired. Chiralcel-OD was already known to exhibit good broad spectrum enantioselectivity. The then new clones of Chiralcel-OD, such as QuikPrep CelCoat, Kromasil CelluCoat, RegisCell and Nucleosil Delta S CSP had presumably been designed to be identical to Chiralcel-OD and would obviously not be orthogonal in any way. However they were worth investigating as it was possible that they might out-perform Chiralcel-OD or at least perform as well as Chiralcel-OD so that they could be used in chiral screening or other work as a cheaper alternative to Chiralcel OD.

Initial work was carried out with 0.5 parts of the additive to 100 parts of propan-2-ol – n-hexane but with one of the clones this led to bleeding of UV-absorbing material from the column. This led to the manufacturer modifying their method for CSP preparation but, nonetheless, it was decided to go with manufacturers' recommendations and use 0.1 parts of the additives.

The outcome of the work, again carried out with the set of 50 racemic drug samples with two mobile phases, is shown in Tables 3.4 and 3.5.Detailed analysis of the data was not needed to arrive at the obvious conclusion that CelluCoat, RegisCell and Nucleosil Delta S showed very similar performance to Chiralcel OD.

Of the clones, only the QuikPrep CelCoat failed to match the performance of Chiralcel OD, giving generally lower retention and resolution. The degree of similarity of the clones to Chiralcel-OD can be seen from the illustrative chromatograms of cromakalim (Fig. 3.15 – 3.18). Apart from the CelCoat, the differences in the CSP were of a magnitude that they may easily have been attributable to minor differences in ambient temperature, mobile phase batches etc rather than differences in the CSP themselves. Despite the similarity of the Chiralcel OD and three OD clones, it was noted from the results shown in Tables 3.4 and 3.5 that some of the OD clones gave a tiny partial separation for certain compounds, while another did not separate them but giving only a single peak (N-acetyl tryptophan, brompheniramine, chlorpheniramine, paroxetine, salbutamol, terbutaline, terfenadine and thioridazine). Accordingly it would not be taking too great a risk to use the CelluCoat, RegisCell and Nucleosil Delta S clones interchangeably with one another and with Chiralcel OD. The slight differences between them may still benefit in chiral screening, if they could be used simultaneously. Whether the price differential is sufficient to prompt users to move away from the tried and trusted Chiralcel OD is another matter.

Table 3.4 $k_1$, $k_2$, α and $R_s$ of 50 compounds on Chiralcel OD, RegisCell, Nucleocel, CelluCoat and CelCoat columns; mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)] (continued overleaf)

| Compound | Chiralcel OD | | | | RegisCell | | | | Neucleocel | | | | CelluCoat | | | | CelCoat | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ |
| N-acetyl tryptophan | 3.20 | 3.78 | 1.18 | 0.91 | 5.09 | -- | -- | -- | 6.80 | 7.54 | 1.11 | 0.20 | 3.86 | 4.52 | 1.17 | 1.51 | 6.80 | -- | -- | -- |
| aminoglutethimide | 13.26 | 19.41 | 1.46 | 0.82 | 14.39 | 21.16 | 1.47 | 1.15 | 2.70 | -- | -- | -- | -- | -- | -- | -- | 16.28 | -- | -- | -- |
| atenolol | 9.07 | 10.49 | 1.16 | 0.38 | 20.69 | -- | -- | -- | -- | -- | -- | -- | 17.67 | -- | -- | -- | 6.58 | -- | -- | -- |
| baclofen | 0.08 | -- | -- | -- | 0.39 | -- | -- | -- | 0.83 | -- | -- | -- | 1.37 | -- | -- | -- | 7.34 | -- | -- | -- |
| benfluorex | 1.40 | -- | -- | -- | 1.19 | -- | -- | -- | 2.05 | -- | -- | -- | 1.46 | -- | -- | -- | 1.68 | -- | -- | -- |
| benzoin | 1.76 | 2.73 | 1.55 | 5.21 | 1.62 | 2.49 | 1.54 | 4.56 | 1.95 | 2.91 | 1.49 | 5.86 | 1.95 | 2.91 | 1.49 | 5.82 | 0.88 | -- | -- | -- |
| bepridil | 2.26 | 2.73 | 1.21 | 0.93 | 1.63 | 2.15 | 1.32 | 1.31 | 1.07 | 1.36 | 1.26 | 1.62 | 2.06 | 2.81 | 1.37 | 1.05 | 1.46 | -- | -- | -- |
| brompheniramine | 2.77 | 3.14 | 1.13 | 0.21 | 2.20 | 2.53 | 1.15 | 0.64 | 3.81 | -- | -- | -- | 5.23 | -- | -- | -- | 7.09 | -- | -- | -- |
| bremazocine | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| bupivacaine | 0.88 | 4.72 | 5.36 | 8.62 | 0.67 | 3.21 | 4.80 | 5.63 | 0.84 | 2.99 | 3.56 | 5.18 | 0.84 | 2.99 | 3.56 | 4.50 | 0.92 | 1.40 | 1.53 | 1.58 |
| carprofen | 1.54 | 1.98 | 1.29 | 1.38 | 1.62 | 2.04 | 1.26 | 1.54 | 1.54 | 1.87 | 1.22 | 1.41 | 2.17 | 2.74 | 1.26 | 2.49 | 1.18 | -- | -- | -- |
| carvedilol | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| chlorpheniramine | 2.48 | 2.66 | 1.07 | 0.10 | 2.20 | 2.47 | 1.12 | 0.48 | 4.36 | -- | -- | -- | 5.31 | -- | -- | -- | 7.06 | -- | -- | -- |
| cicletanine | 2.26 | 3.28 | 1.45 | 1.84 | 2.01 | 2.97 | 1.48 | 1.87 | 3.85 | 5.08 | 1.32 | 0.72 | 2.28 | 3.14 | 1.38 | 1.33 | 3.37 | -- | -- | -- |
| citalopram | 1.33 | 1.78 | 1.34 | 2.00 | 5.56 | 6.93 | 1.25 | 0.97 | -- | -- | -- | -- | 13.43 | 17.55 | 1.31 | 0.63 | 1.40 | -- | -- | -- |
| cromakalim | 1.68 | 4.15 | 2.47 | 7.45 | 1.60 | 4.01 | 2.50 | 5.58 | 2.23 | 4.36 | 1.96 | 4.04 | 1.60 | 3.20 | 2.00 | 7.78 | 1.69 | 2.04 | 1.21 | 1.30 |
| disopyramide | 9.92 | -- | -- | -- | 5.77 | 6.21 | 1.08 | -- | -- | -- | -- | -- | 6.35 | -- | -- | -- | 2.62 | -- | -- | -- |

Table 3.4 $k_1$, $k_2$, α and $R_s$ of 50 compounds on Chiralcel OD, RegisCell, Nucleocel, CelluCoat and CelCoat columns; mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)] (continued overleaf)

| Compound | Chiralcel OD | | | | RegisCell | | | | Neucleocel | | | | CelluCoat | | | | CelCoat | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ |
| dobutamine | | | | | | | | | | | | | | | | | | | | |
| felodipine | 0.89 | -- | -- | -- | 0.79 | -- | -- | -- | 1.25 | -- | -- | -- | 1.07 | -- | -- | -- | 0.80 | -- | -- | -- |
| fluoxetine | 1.18 | 1.44 | 1.22 | 0.75 | 1.01 | 1.21 | 1.20 | 0.91 | 2.19 | -- | -- | -- | 1.40 | 1.60 | 1.15 | 0.39 | 0.15 | -- | -- | -- |
| flurbiprofen | 0.38 | -- | -- | -- | 0.32 | 0.36 | 1.12 | 0.34 | 0.48 | 0.53 | 1.09 | 0.32 | 0.48 | 0.54 | 1.11 | 0.64 | 0.41 | -- | -- | -- |
| flurbiprofen amide | 2.65 | -- | -- | -- | 2.62 | -- | -- | -- | 3.78 | -- | -- | -- | 3.11 | -- | -- | -- | 1.54 | -- | -- | -- |
| hexabarbital | 3.13 | 3.85 | 1.23 | 2.22 | 2.71 | 3.22 | 1.19 | 1.83 | 3.28 | 3.65 | 1.11 | 1.00 | 2.80 | 3.19 | 1.14 | 1.85 | 1.06 | -- | -- | -- |
| 4-OH, man.acid..[1] | -- | -- | -- | -- | -- | -- | -- | -- | 1.68 | 4.87 | 2.89 | 8.72 | 2.71 | 3.32 | 1.23 | 2.24 | 7.34 | -- | -- | -- |
| 4-OH, 3-methox..[2] | -- | -- | -- | -- | 12.07 | -- | -- | -- | -- | -- | -- | -- | 4.64 | 5.41 | 1.17 | 1.96 | 14.02 | -- | -- | -- |
| ibuprofen | 0.46 | -- | -- | -- | 0.11 | -- | -- | -- | 0.17 | -- | -- | -- | 0.21 | -- | -- | -- | 0.37 | -- | -- | -- |
| isoproterenol | 1.11 | 2.77 | 2.50 | 2.79 | 1.23 | -- | -- | -- | 2.19 | 9.18 | 4.18 | 1.86 | 4.14 | -- | -- | -- | -- | -- | -- | -- |
| isradipine | 0.89 | -- | -- | -- | 0.76 | -- | -- | -- | 1.25 | -- | -- | -- | 0.99 | -- | -- | -- | 0.83 | -- | -- | -- |
| ketamine | 1.39 | -- | -- | -- | 1.24 | -- | -- | -- | 3.85 | -- | -- | -- | 1.11 | -- | -- | -- | 1.58 | -- | -- | -- |
| ketoprofen | 0.72 | -- | -- | -- | 0.70 | -- | -- | -- | 0.91 | -- | -- | -- | 0.91 | -- | -- | -- | 0.68 | -- | -- | -- |
| lorezapam | 5.40 | 7.47 | 1.38 | 3.12 | 5.89 | 7.84 | 1.33 | 1.68 | 10.00 | 13.08 | 1.31 | 1.38 | 5.72 | 8.28 | 1.45 | 4.40 | 8.93 | -- | -- | -- |
| mexiletine | 1.62 | 2.41 | 1.48 | 1.46 | 1.56 | 2.11 | 1.36 | 1.78 | 2.80 | 3.77 | 1.35 | 0.70 | 1.81 | 2.33 | 1.29 | 1.43 | 1.79 | -- | -- | -- |
| mianserin | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| MTPA[3] | 0.29 | 0.36 | 1.21 | 0.58 | 0.24 | 0.32 | 1.29 | 0.59 | 0.27 | 0.37 | 1.36 | 1.14 | 0.29 | 0.39 | 1.33 | 1.33 | 0.84 | -- | -- | -- |

1. 4-OH, mandelic acid
2. 4-OH, 3-methoxymandelic acid
3. α-methoxy- α-(trifluoromethyl)phenylacetic acid

Table 3.4 $k_1$, $k_2$, α and $R_s$ of 50 compounds on Chiralcel OD, RegisCell, Nucleocel, CelluCoat and CelCoat columns; mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)]

| Compound | Chiralcel OD | | | | RegisCell | | | | Neucleocel | | | | CelluCoat | | | | CelCoat | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ |
| naproxen | 0.72 | 0.85 | 1.19 | 1.00 | 0.69 | 0.85 | 1.22 | 1.35 | 1.07 | 1.36 | 1.26 | 1.62 | 0.91 | 1.13 | 1.24 | 2.14 | 0.67 | -- | -- | -- |
| nicotine | 0.45 | -- | -- | -- | 0.38 | -- | -- | -- | 0.46 | -- | -- | -- | 7.62 | -- | -- | -- | 13.42 | -- | -- | -- |
| paroxetine | 1.06 | -- | -- | -- | 2.12 | -- | -- | -- | 3.23 | -- | -- | -- | 2.75 | 3.81 | 1.39 | 1.26 | 5.51 | 6.49 | 1.18 | 0.32 |
| α-phenyl-but.acid.[4] | 1.31 | 2.63 | 2.01 | 5.73 | 1.34 | 2.01 | 1.51 | 6.67 | 1.90 | 2.70 | 1.42 | 3.33 | 1.64 | 2.43 | 1.48 | 4.78 | 1.09 | 1.25 | 1.14 | 1.35 |
| propranalol | 2.12 | 13.33 | 6.29 | 9.12 | 2.66 | 17.50 | 6.58 | 10.67 | 3.64 | 15.79 | 4.34 | 6.88 | 2.90 | 13.34 | 4.60 | 11.80 | 1.63 | 2.85 | 1.74 | 1.94 |
| salbutamol | -- | -- | -- | -- | 4.19 | 5.69 | 1.36 | 1.04 | -- | -- | -- | -- | 3.29 | 4.00 | 1.22 | 1.91 | -- | -- | -- | -- |
| terbutaline | 2.70 | 3.57 | 1.32 | 1.11 | 4.73 | -- | -- | -- | -- | -- | -- | -- | 4.11 | 4.91 | 1.19 | 0.43 | 3.93 | 4.41 | 1.12 | 0.14 |
| terfenadine | 1.18 | 2.12 | 1.80 | 2.68 | 6.51 | -- | -- | -- | -- | -- | -- | -- | 9.17 | -- | -- | -- | -- | -- | -- | -- |
| thalidomide | 1.11 | -- | -- | -- | 2.55 | -- | -- | -- | 1.23 | -- | -- | -- | 0.86 | -- | -- | -- | 0.50 | 1.02 | 2.04 | 4.68 |
| thioridazine | 3.58 | -- | -- | -- | 2.71 | 2.95 | 1.09 | 0.96 | 7.54 | -- | -- | -- | 3.72 | -- | -- | -- | 3.87 | -- | -- | -- |
| 2,2,2,trifluoro-1..[5] | 1.47 | 4.22 | 2.87 | 9.64 | 1.22 | 3.65 | 3.00 | 6.17 | 1.68 | 4.87 | 2.90 | 8.71 | 1.08 | 1.17 | 1.08 | 0.17 | 0.86 | 1.45 | 1.69 | 1.57 |
| trihexyphenidyl | 1.18 | 2.12 | 1.80 | 2.60 | 0.86 | 1.56 | 1.81 | 2.84 | 2.19 | 2.92 | 1.33 | 0.61 | 1.17 | 1.79 | 1.53 | 1.71 | 1.25 | -- | -- | -- |
| Troger's base | 1.18 | 1.41 | 1.19 | 0.98 | 1.13 | 1.35 | 1.19 | 1.08 | 1.90 | 2.36 | 1.24 | 0.91 | 1.45 | 1.76 | 1.21 | 1.36 | 0.94 | -- | -- | -- |
| tropicamide | 4.87 | 6.24 | 1.28 | 1.68 | 5.02 | 6.80 | 1.35 | 1.11 | -- | -- | -- | -- | 5.62 | 7.57 | 1.35 | 1.77 | 0.32 | -- | -- | -- |
| verapamil | 8.84 | 14.34 | 1.62 | 3.44 | 9.10 | 13.90 | 1.53 | 1.88 | -- | -- | -- | -- | 14.03 | 21.10 | 1.50 | 1.65 | 9.74 | 12.35 | 1.27 | 0.73 |
| warfarin | 2.77 | 6.93 | 2.50 | 1.70 | 2.56 | 6.32 | 2.47 | 2.25 | 3.93 | 9.00 | 2.29 | 1.75 | 3.08 | -- | -- | -- | 1.01 | 1.50 | 1.49 | 1.75 |

1. α-phenyl-butanoicacid carboxymide
2. 2,2,2,trifluoro-1-(9-anthryl)-ethanol

Table 3.5 $k_1$, $k_2$, α and $R_s$ of 50 compounds on Chiralcel OD, RegisCell, Nucleocel, CelluCoat and CelCoat columns; mobile phase - [hexane – IPA - TEA (85:15:0.1, v/v/v)] (continued overleaf)

| Compound | Chiralcel OD | | | | RegisCell | | | | Neucleocel | | | | CelluCoat | | | | CelCoat | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ |
| N-acetyl tryptophan | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| aminoglutethimide | 30.03 | -- | -- | -- | 17.12 | 30.27 | 1.77 | 4.41 | 30.83 | -- | -- | -- | 19.31 | 34.31 | 1.78 | 7.72 | 5.67 | 7.01 | 1.24 | 0.87 |
| atenolol | -- | -- | -- | -- | 10.29 | 17.40 | 1.69 | 2.02 | -- | -- | -- | -- | 9.87 | 15.25 | 1.55 | 2.85 | -- | -- | -- | -- |
| baclofen | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| benfluorex | 0.64 | 0.67 | 1.06 | 0.48 | 0.53 | 0.59 | 1.12 | 0.74 | 0.71 | 0.78 | 1.10 | 0.39 | 0.67 | 0.74 | 1.10 | 0.66 | 0.69 | -- | -- | -- |
| benzoin | 1.78 | 2.81 | 1.58 | 5.15 | 1.89 | 2.93 | 1.55 | 3.70 | 2.66 | 3.83 | 1.44 | 4.14 | 2.08 | 3.13 | 1.50 | 6.32 | 1.40 | -- | -- | -- |
| bepridil | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | 0.16 | -- | -- | -- | 0.81 | -- | -- | -- |
| brompheniramine | 0.46 | -- | -- | -- | 0.31 | -- | -- | -- | 1.03 | -- | -- | -- | 0.52 | -- | -- | -- | 3.67 | -- | -- | -- |
| bremazocine | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| bupivacaine | 0.46 | -- | -- | -- | 0.37 | 0.44 | 1.20 | 0.98 | 0.48 | -- | -- | -- | 0.47 | 0.53 | 1.14 | 0.69 | 0.54 | -- | -- | -- |
| carprofen | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | 4.35 | 4.96 | 1.14 | 0.27 | -- | -- | -- | -- |
| carvedilol | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| chlorpheniramine | 0.46 | -- | -- | -- | 0.28 | -- | -- | -- | 0.89 | -- | -- | -- | 0.50 | -- | -- | -- | 3.76 | -- | -- | -- |
| cicletanine | 1.31 | 1.61 | 1.23 | 1.04 | 1.38 | 1.64 | 1.19 | 0.51 | 3.42 | -- | -- | -- | 1.20 | 1.61 | 1.34 | 2.67 | 0.75 | -- | -- | -- |
| citalopram | 1.33 | 1.78 | 1.34 | 2.00 | 1.27 | 1.37 | 1.08 | 0.24 | 2.55 | -- | -- | -- | 1.58 | 1.71 | 1.08 | 0.70 | 1.40 | -- | -- | -- |
| cromakalim | 1.83 | 5.01 | 2.74 | 8.91 | 1.73 | 3.40 | 1.97 | 4.06 | 2.55 | 4.58 | 1.79 | 3.6 | 1.84 | 3.54 | 1.92 | 7.08 | 1.64 | 2.04 | 1.24 | 1.85 |
| disopyramide | 0.96 | -- | -- | -- | 17.22 | 17.83 | 1.04 | 0.39 | 1.61 | -- | -- | -- | 1.04 | -- | -- | -- | 1.50 | -- | -- | -- |

Table 3.5 $k_1$, $k_2$, α and $R_s$ of 50 compounds on Chiralcel OD, RegisCell, Nucleocel, CelluCoat and CelCoat columns; mobile phase - [hexane – IPA - TEA (85:15:0.1, v/v/v)] (continued overleaf)

| Compound | Chiralcel OD | | | | RegisCell | | | | Neucleocel | | | | CelluCoat | | | | CelCoat | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ |
| dobutamine | -- | -- | -- | -- | 28.00 | -- | -- | -- | -- | -- | -- | -- | 5.11 | 5.74 | 1.12 | 0.41 | 3.02 | -- | -- | -- |
| felodipine | 0.89 | -- | -- | -- | 0.89 | -- | -- | -- | 1.33 | -- | -- | -- | 1.29 | -- | -- | -- | 0.38 | -- | -- | -- |
| fluoxetine | 1.03 | 1.18 | 1.14 | 0.34 | 0.05 | 0.85 | 15.69 | 6.91 | 0.79 | 2.56 | 3.24 | 10.42 | 1.32 | -- | -- | -- | 0.74 | -- | -- | -- |
| flurbiprofen | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| flurbiprofen amide | 2.63 | -- | -- | -- | 2.66 | -- | -- | -- | 3.85 | -- | -- | -- | 3.20 | -- | -- | -- | 0.92 | -- | -- | -- |
| hexabarbital | 3.06 | 3.23 | 1.05 | 1.55 | 2.85 | 3.26 | 1.14 | 1.05 | 3.57 | 4.07 | 1.14 | 1.04 | 3.18 | 3.63 | 1.14 | 1.71 | -- | -- | -- | -- |
| 4-OH, man.acid…[1] | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| 4-OH, 3-methox…[2] | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| ibuprofen | 6.39 | -- | -- | -- | 6.39 | -- | -- | -- | 5.95 | -- | -- | -- | 0.39 | -- | -- | -- | 0.55 | -- | -- | -- |
| isoproterenol | -- | -- | -- | -- | 1.67 | -- | -- | -- | -- | -- | -- | -- | 1.72 | -- | -- | -- | -- | -- | -- | -- |
| isradipine | 0.82 | -- | -- | -- | 0.82 | -- | -- | -- | 1.25 | -- | -- | -- | 1.00 | -- | -- | -- | 0.90 | -- | -- | -- |
| ketamine | 0.82 | 1.03 | 1.27 | 1.87 | 0.72 | 0.92 | 1.28 | 1.87 | 1.11 | 1.31 | 1.18 | 1.63 | 0.89 | 1.08 | 1.21 | 1.88 | 0.87 | -- | -- | -- |
| ketoprofen | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | 3.65 | -- | -- | -- | -- | -- | -- | -- |
| lorezapam | 6.10 | 8.34 | 1.37 | 1.33 | 6.99 | 8.83 | 1.26 | 0.70 | 9.35 | 11.66 | 1.25 | 1.24 | 5.53 | 7.92 | 1.43 | 4.23 | 1.39 | -- | -- | -- |
| mexiletine | 1.18 | -- | -- | -- | 1.14 | 1.28 | 1.12 | 1.05 | 1.68 | -- | -- | -- | 1.24 | 1.33 | 1.07 | 0.64 | 1.99 | 2.36 | 1.18 | 0.48 |
| mianserin | 0.62 | 0.67 | 1.09 | 0.12 | 0.60 | 0.74 | 1.22 | 1.47 | 0.82 | 1.00 | 1.23 | 1.24 | 0.75 | 0.87 | 1.17 | 1.19 | 0.49 | -- | -- | -- |
| MTPA[3] | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | 1.31 | -- | -- | -- | -- | -- | -- | -- |

1. 4-OH, mandelic acid
2. 4-OH, 3-methoxymandelic acid

α-methoxy- α-(trifluoromethyl)phenylacetic acid

Table 3.5 $k_1$, $k_2$, α and $R_s$ of 50 compounds on Chiralcel OD, RegisCell, Nucleocel, CelluCoat and CelCoat columns; mobile phase - [hexane – IPA - TEA (85:15:0.1, v/v/v)]

| Compound | Chiralcel OD | | | | RegisCell | | | | Neucleocel | | | | CelluCoat | | | | CelCoat | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ |
| naproxen | 21.50 | -- | -- | -- | 19.60 | -- | -- | -- | 20.13 | -- | -- | -- | 1.57 | 1.89 | 1.20 | 0.49 | 1.04 | -- | -- | -- |
| nicotine | 0.38 | 0.52 | 1.35 | 0.70 | 2.19 | 2.65 | 1.21 | 0.75 | 0.61 | 0.76 | 1.24 | 1.35 | 1.09 | -- | -- | -- | 0.57 | -- | -- | -- |
| paroxetine | 2.46 | -- | -- | -- | 2.00 | 2.24 | 1.12 | 0.91 | 7.83 | -- | -- | -- | 3.23 | -- | -- | -- | -- | -- | -- | -- |
| α-phenyl-but.acid...[4] | 1.40 | 2.34 | 1.67 | 4.04 | 1.45 | 2.13 | 1.47 | 2.90 | 1.98 | 2.77 | 1.40 | 3.23 | 1.57 | 2.37 | 1.51 | 5.10 | 0.80 | 0.96 | 1.19 | 0.91 |
| propranalol | 2.12 | 13.33 | 6.29 | 5.95 | 2.04 | 4.88 | 2.39 | 7.55 | 3.28 | 5.66 | 1.73 | 3.04 | 2.27 | 4.47 | 1.97 | 6.71 | 1.79 | 2.19 | 1.23 | 0.61 |
| salbutamol | 4.51 | -- | -- | -- | 4.77 | -- | -- | -- | 10.87 | -- | -- | -- | 2.40 | -- | -- | -- | -- | -- | -- | -- |
| terbutaline | 2.34 | -- | -- | -- | 1.73 | -- | -- | -- | 1.25 | -- | -- | -- | 1.45 | -- | -- | -- | 0.92 | -- | -- | -- |
| terfenadine | 0.51 | -- | -- | -- | 0.51 | -- | -- | -- | 0.68 | -- | -- | -- | 0.62 | 0.78 | 1.26 | 1.34 | 0.46 | 0.61 | 1.33 | 1.13 |
| thalidomide | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| thioridazine | 0.60 | -- | -- | -- | 0.41 | -- | -- | -- | 0.99 | -- | -- | -- | 0.59 | -- | -- | -- | 3.08 | -- | -- | -- |
| 2,2,2,trifluoro-1-…..[5] | 1.40 | 4.29 | 3.07 | 7.40 | 1.22 | 3.78 | 3.10 | 7.71 | 1.85 | 4.77 | 2.57 | 7.30 | 1.64 | 4.52 | 2.75 | 10.55 | 5.67 | 7.01 | 1.24 | 1.76 |
| trihexyphenidyl | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Troger's base | 1.18 | 1.34 | 1.14 | 0.95 | 0.82 | 1.03 | 1.26 | 1.25 | 1.10 | 1.44 | 1.31 | 2.20 | 1.02 | 1.29 | 1.27 | 2.53 | 0.25 | -- | -- | -- |
| tropicamide | 2.92 | 3.49 | 1.20 | 1.59 | 3.00 | 3.62 | 1.21 | 1.14 | 6.32 | 6.82 | 1.08 | 0.48 | 3.29 | 3.98 | 1.21 | 2.13 | 3.46 | -- | -- | -- |
| verapamil | 1.98 | -- | -- | -- | 1.89 | -- | -- | -- | 3.64 | -- | -- | -- | 2.39 | -- | -- | -- | 8.81 | 11.00 | 1.25 | 0.74 |
| warfarin | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | 4.82 | 9.02 | 1.87 | 2.69 | -- | -- | -- | -- |

1. α-phenyl-butanoicacid carboxymide
2. 2,2,2,trifluoro-1-(9-anthryl)-ethanol

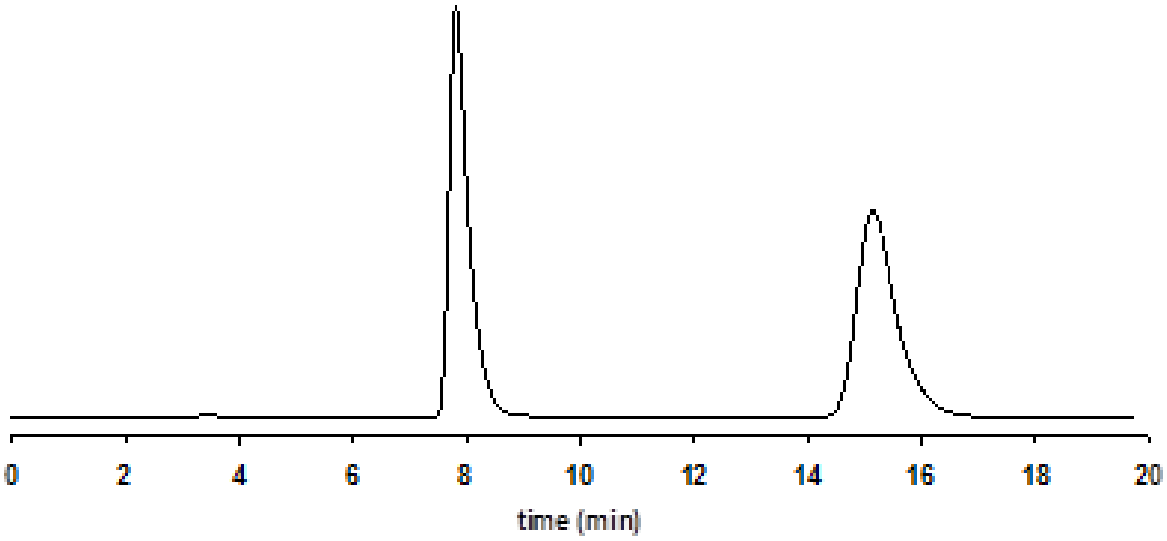



Figure 3.15 NP-HPLC of cromakalim on Chiralcel OD (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 254 nm.

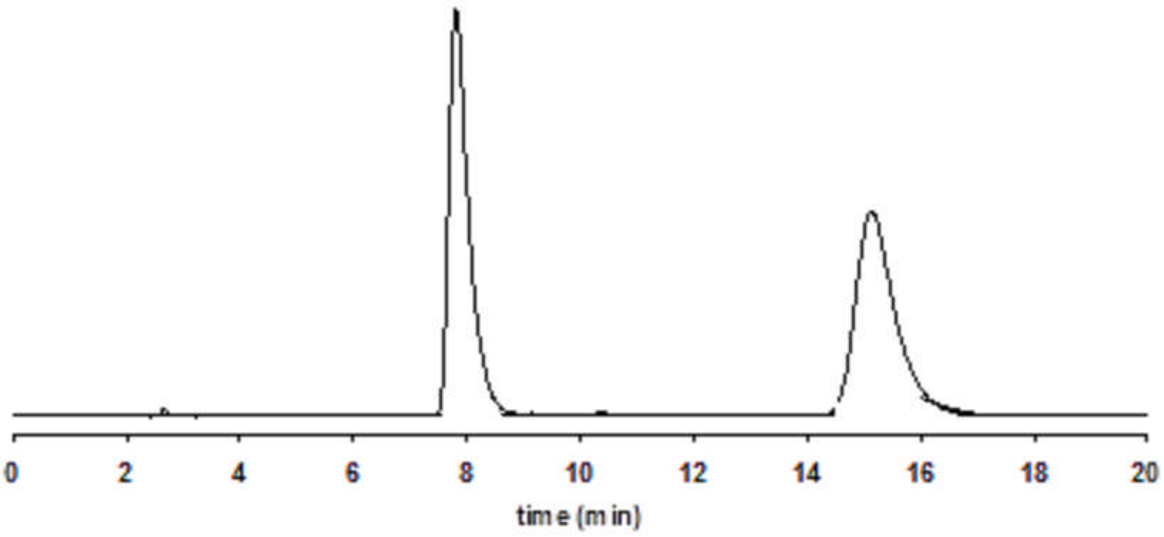



Figure 3.16 NP-HPLC of cromakalim on Nucleocel Delta (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 254 nm.

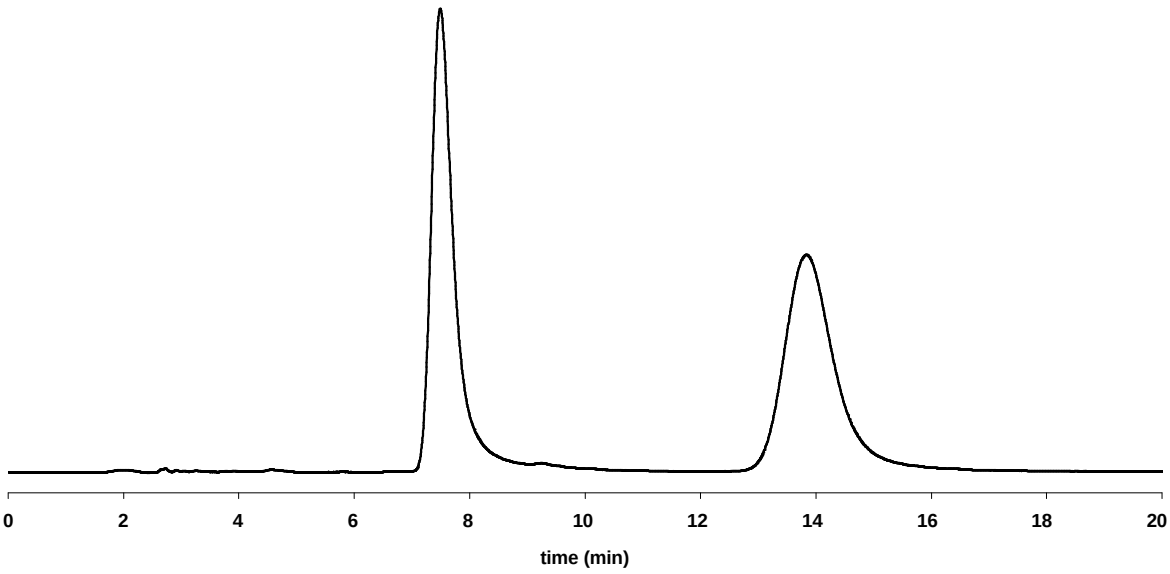



Figure 3.17 NP-HPLC of cromakalim on RegisCell (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 254 nm.

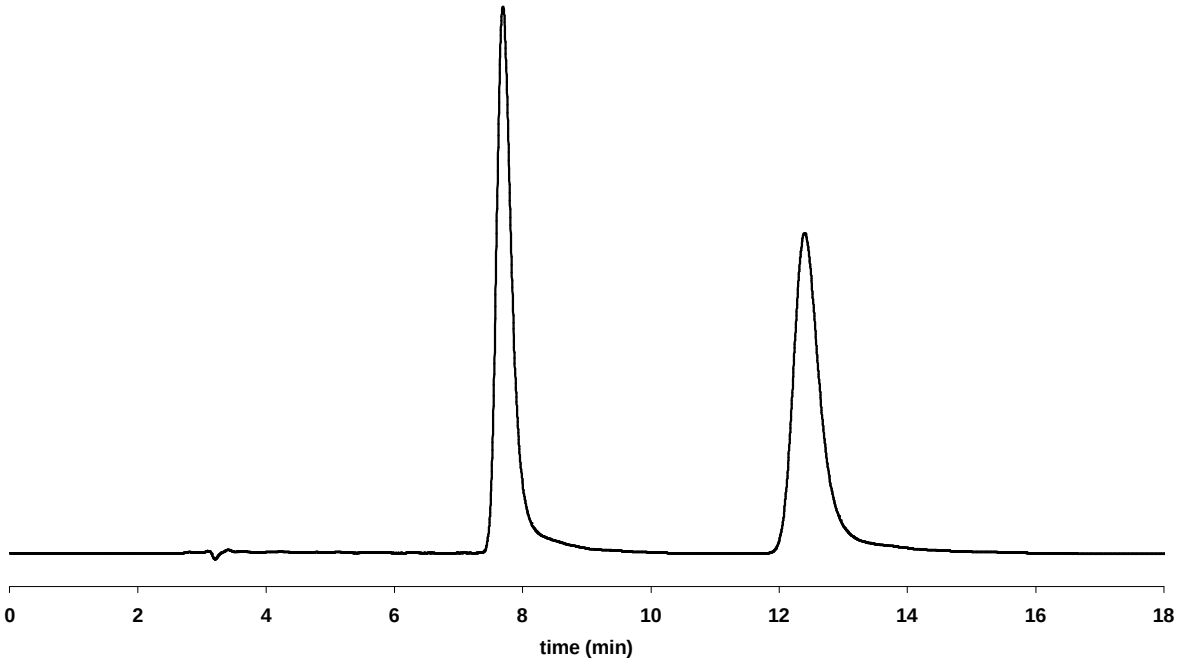



Figure 3.18 NP-HPLC of cromakalim on CelluCoat (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 254 nm.

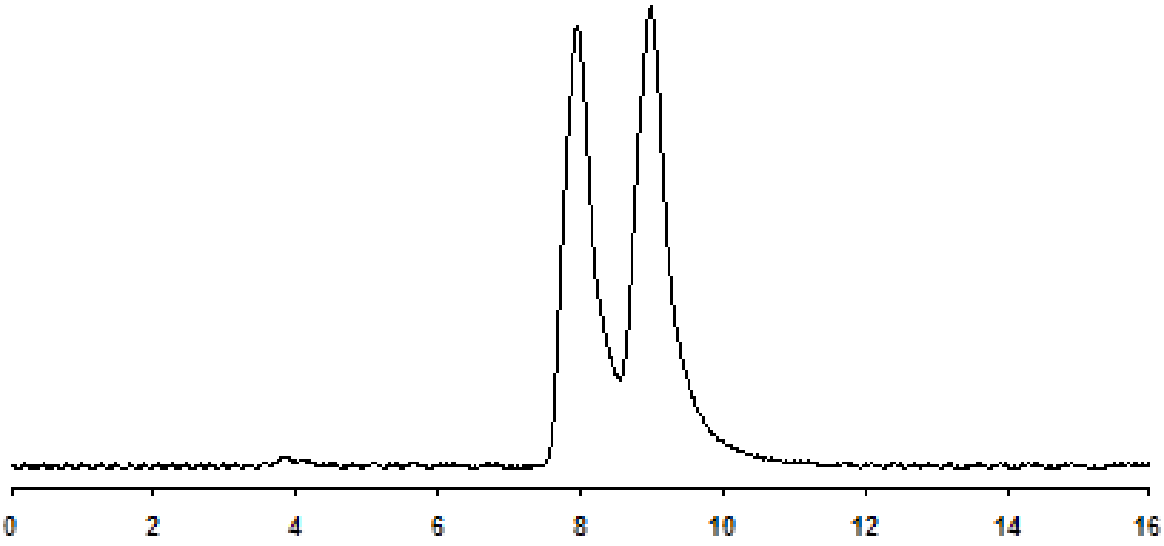



Figure 3.19 NP-HPLC of cromakalim on CelCoat (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 254 nm.

Another benefit of this study was that it also provided more information about Chiralcel OD itself, as for Whelk-O1, the 50 compound screen with additives at 0.1 part giving slightly different information from the earlier in-house screen with 26 compounds and additives at 0.5 parts. The effects of acid and base modifier on 'hit-rate' and *k* for acidic and basic compounds were not as clear-cut as for the Whelk-O1 CSP. Importantly the overall 'hit rate' was improved compared to the earlier work from ~50% to 66% (Table 3.6 and Table 3.7). As with the Whelk-O1 CSP this is mainly attributable to fewer compounds being completely retained. Also the 'hit-rate' that would be obtained from using a screen containing both Chiralcel OD and Whelk-O1 was enhanced from 72% to 96% (Table 3.6 and Table 3.7). Looking at the actual compounds that were separated also allows for an assessment of the degree of 'complementarieness' of Whelk-O1 and Chiralcel OD (Fig. 3.20). Both this and the improvement in 'hit-rate' in going from one of the CSP to both demonstrated that Whelk-O1 and Chiralcel OD are very much worth using together in a screen and do not just give the same range of separations.

Table 3.6 The 'hit rate' of CSP combinations, obtained from the previous study carried out by Barry (M. Barry, Final Year Project, BSc. Chemical and Pharmaceutical Science, University of Sunderland, 1999).

| **CSP combination** | **'hit' rate (%)** |
|---|---|
| Whelk-O1 | 36 |
| Wh + OD | 72 |
| Wh + AS | 76 |
| Wh + AD | 88 |
| Wh + OD, AS | 96 |
| Wh + OD, AD | 96 |
| Wh + AD, AS | 96 |
| Wh + AD, AS, OD | 100 |

Table 3.7 The overall 'hit rate of the Whelk-O1 and OD from the current study.

| **CSP combination** | **'hit' rate (%)** |
|---|---|
| Whelk-O1 | 72 |
| OD | 66 |
| Wh + OD | 96 |

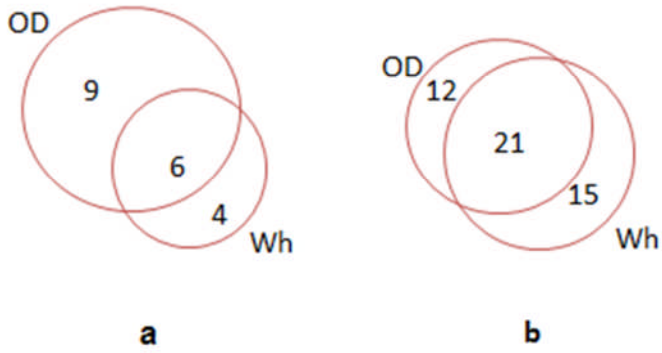



Figure 3.20 Venn diagram showing numbers of hits on Whelk-O1/OD, Whelk-O1 and OD for smaller set of compounds by Barry (a) and for 50 compounds in current study (b).

### 3.3.3 Chiral screen (15) AD, AD clone, OJ and IA

Subsequent to the introduction of Chiralcel OD clones, about one year later, clones became available of the even more successful (commercially and in terms of breadth of spectrum of enantioselectivity) Chiralpak AD. Given how similar the OD clones had been to OD, it was decided to carry out the comparison of AmyCoat and RegisPak with ChiralPak-AD using a reduced set of 16 compounds. Even within the set of 16 compounds though, an effort was made to keep a representative balance between acidic, basic and neutral compounds. Also some compounds were included for which it would be expected that separation would be

easy and also more difficult ones which would be more discriminating. It was also decided to simultaneously evaluate the Daicel ChiralCel OJ, to assess whether it was worthy of consideration alongside the more popular derivatised polysaccharide CSP, and the new Daicel product ChiralPak IA, to establish if any enantioselectivity was lost when using an immobilised rather than a coated derivatised polysaccharide.

As for the Chiralcel OD clones, it was immediately apparent that the manufacturers of the Chiralpak AD clones had done a very good job (Tables 3.8 and 3.9). As expected, Chiralpak AD and its clones showed a high success rate for separating the enantiomers, with very good enantioselectivity being found in some cases (Fig. 3.21 - 3.26)

Table 3.8 *k*, α and $R_s$ for evaluation of CSP Chiralpak AD and its clones;

mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)]

| compound | Chiralpak AD | | | | RegisPak | | | | AmyCoat | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ |
| baclofen | 0.89 | 1.07 | 1.21 | 0.92 | 0.73 | 0.85 | 1.17 | 0.49 | 1.23 | 1.58 | 1.28 | 1.51 |
| brompheniramine | 0.91 | -- | -- | -- | 4.34 | -- | -- | -- | -- | -- | -- | -- |
| chlorpheniramine | 0.98 | 1.09 | 1.12 | 0.72 | 4.38 | -- | -- | -- | 2.33 | -- | -- | -- |
| cicletanine | 1.31 | -- | -- | -- | 0.82 | -- | -- | -- | 1.21 | -- | -- | -- |
| citalopram | 2.59 | 2.92 | 1.13 | 0.98 | 4.97 | 5.22 | 1.05 | 0.43 | 1.46 | -- | -- | -- |
| cromakalim | 4.48 | -- | -- | -- | 3.74 | 3.98 | 1.06 | 0.95 | 5.25 | 5.39 | 1.03 | 0.07 |
| flurbiprofen | 0.62 | 1.02 | 1.65 | 4.65 | 0.31 | 0.62 | 1.99 | 4.40 | 0.68 | 1.25 | 1.83 | 5.79 |
| ketamine | 0.70 | 0.84 | 1.20 | 1.07 | 0.53 | 0.64 | 1.23 | 1.19 | 5.23 | -- | -- | -- |
| mexiletine | 0.31 | -- | -- | -- | 0.08 | -- | -- | -- | 0.42 | -- | -- | -- |
| mianserin | 0.81 | 1.41 | 1.74 | 3.88 | 0.76 | 1.28 | 1.69 | 4.04 | 0.98 | 1.82 | 1.85 | 3.86 |
| naproxen | 1.56 | 1.82 | 1.17 | 1.87 | 1.08 | 1.24 | 1.14 | 1.66 | 1.68 | 2.20 | 1.31 | 3.45 |
| nicotine | 0.99 | -- | -- | -- | 5.81 | -- | -- | -- | 3.24 | -- | -- | -- |
| paroxetine | 1.82 | 2.16 | 1.19 | 1.11 | 1.28 | 1.40 | 1.10 | 0.85 | 2.30 | 2.73 | 1.19 | 1.29 |
| propranalol | 0.60 | -- | -- | -- | 0.53 | -- | -- | -- | 0.82 | -- | -- | -- |
| verapamil | 2.10 | 2.48 | 1.18 | 1.07 | 2.92 | 3.24 | 1.11 | 0.95 | 4.53 | 5.02 | 1.11 | 0.67 |
| warfarin | 1.52 | 6.03 | 3.98 | 11.70 | 1.26 | 5.24 | 4.16 | 17.61 | 1.58 | 6.61 | 4.19 | 10.10 |

Table 3.9 *k*, α and $R_s$ for evaluation of CSP Chiralpak AD and its clones; mobile phase - [hexane – IPA - TEA (85:15:0.1, v/v/v)].

| compound | Chiralpak AD | | | | RegisPak | | | | AmyCoat | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ |
| baclofen | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| brompheniramine | 0.63 | 0.77 | 1.22 | 0.89 | 1.16 | 1.35 | 1.16 | 0.93 | 19.31 | 34.31 | 1.78 | 7.72 |
| chlorpheniramine | 0.56 | 0.72 | 1.29 | 2.12 | 0.94 | 1.06 | 1.13 | 0.63 | 0.58 | 0.69 | 1.20 | 1.33 |
| cicletanine | 1.13 | 1.40 | 1.24 | 1.81 | 0.11 | -- | -- | -- | 1.85 | 2.56 | 1.38 | 1.51 |
| citalopram | 1.18 | 1.26 | 1.07 | 0.65 | 1.65 | 1.74 | 1.05 | 0.25 | 0.93 | 1.03 | 1.10 | 0.82 |
| cromakalim | 4.80 | -- | -- | -- | 4.87 | 5.11 | 1.05 | 0.71 | 4.25 | 5.57 | 1.31 | 3.76 |
| flurbiprofen | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| ketamine | 0.81 | 0.88 | 1.08 | 0.73 | 0.82 | 0.86 | 1.05 | 0.19 | 0.70 | 0.75 | 1.08 | 0.64 |
| mexiletine | 0.31 | -- | -- | -- | 0.45 | -- | -- | -- | 0.69 | -- | -- | -- |
| mianserin | 0.31 | 0.82 | 2.69 | 5.39 | 0.35 | 0.83 | 2.38 | 3.66 | 0.28 | 0.65 | 2.35 | 5.32 |
| naproxen | -- | -- | -- | -- | 18.03 | -- | -- | -- | -- | -- | -- | -- |
| nicotine | 0.41 | -- | -- | -- | 0.54 | -- | -- | -- | 0.41 | -- | -- | -- |
| paroxetine | 1.91 | 2.31 | 1.21 | 1.62 | 5.49 | 5.82 | 1.06 | 0.37 | 0.50 | -- | -- | -- |
| propranalol | 0.69 | -- | -- | -- | 1.21 | -- | -- | -- | 0.97 | -- | -- | -- |
| verapamil | 1.10 | 1.40 | 1.27 | 2.53 | 1.34 | 1.65 | 1.23 | 1.73 | 1.16 | 1.40 | 1.20 | 1.75 |
| warfarin | 9.66 | 13.75 | 1.42 | 0.47 | -- | -- | -- | -- | -- | -- | -- | -- |

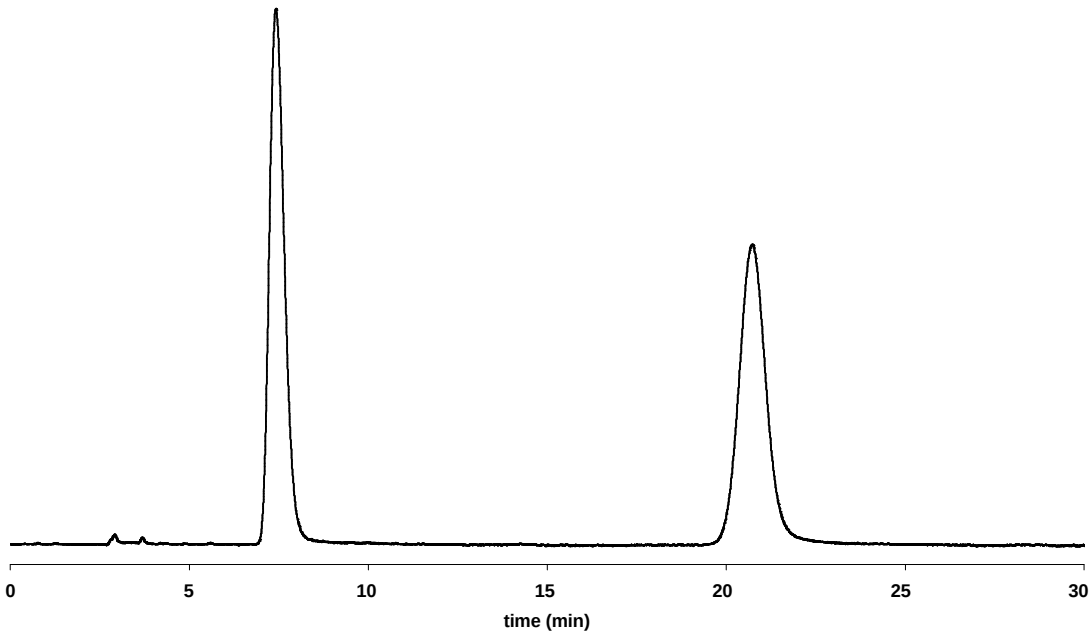



Figure 3.21 NP-HPLC of warfarin on Chiralpak AD (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 308 nm.

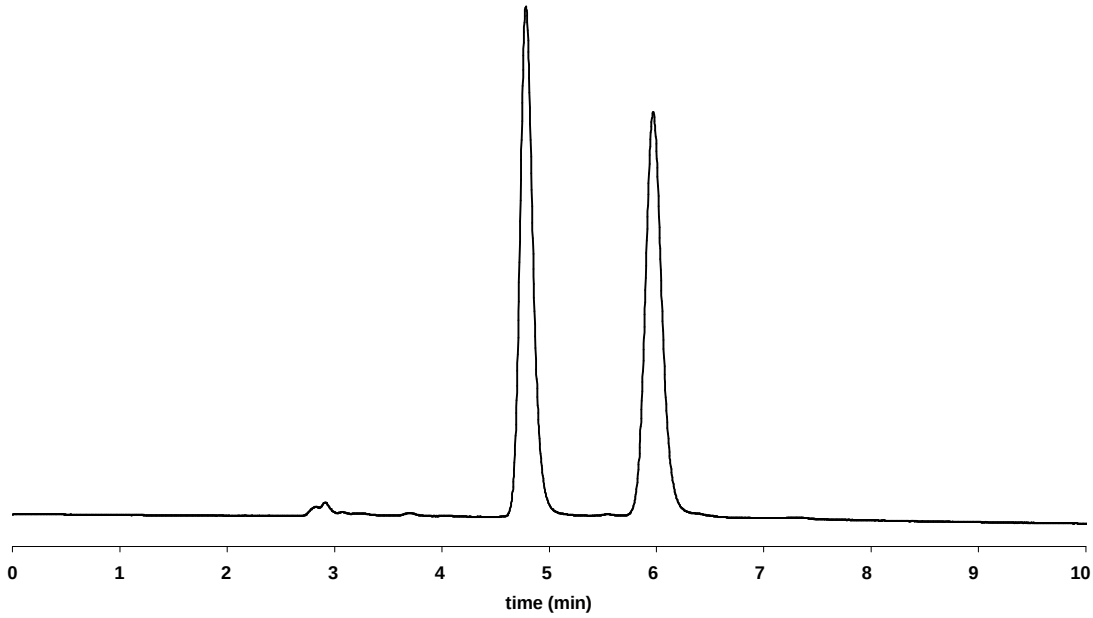



Figure 3.22 NP-HPLC of flurbiprofen on Chiralpak AD (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 254 nm.

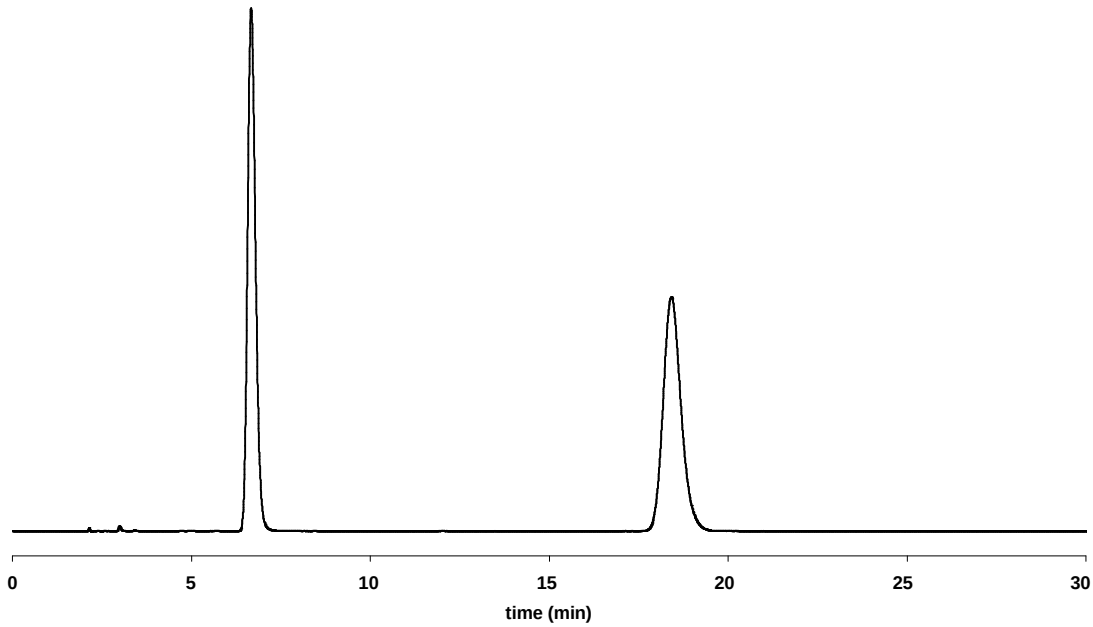



Figure 3.23 NP-HPLC of warfarin on RegisPak (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 308 nm.

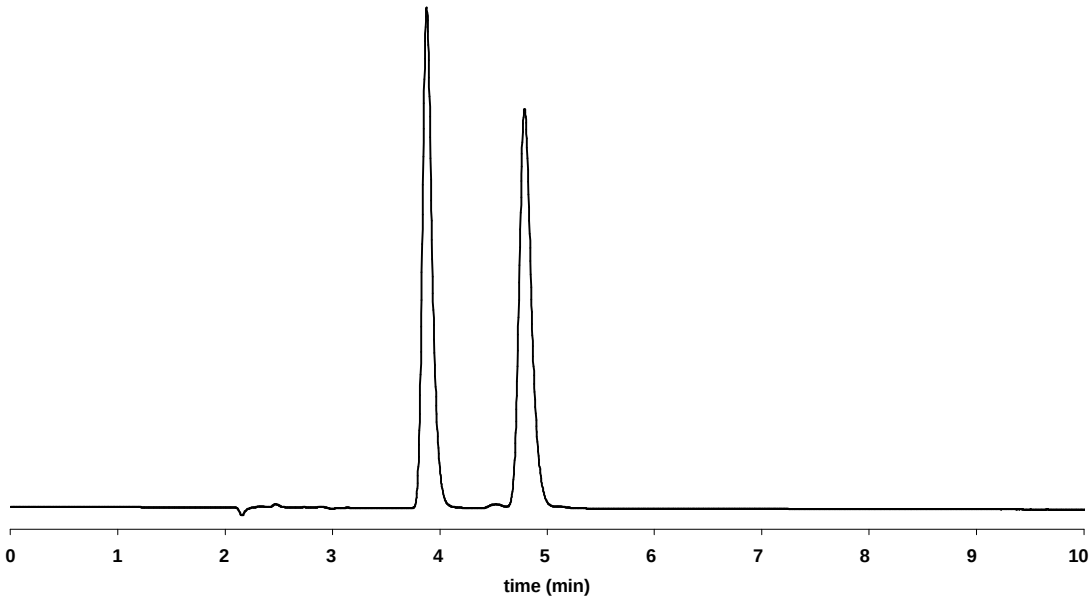



Figure 3.24 NP-HPLC of flurbiprofen on RegisPak (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 254 nm.

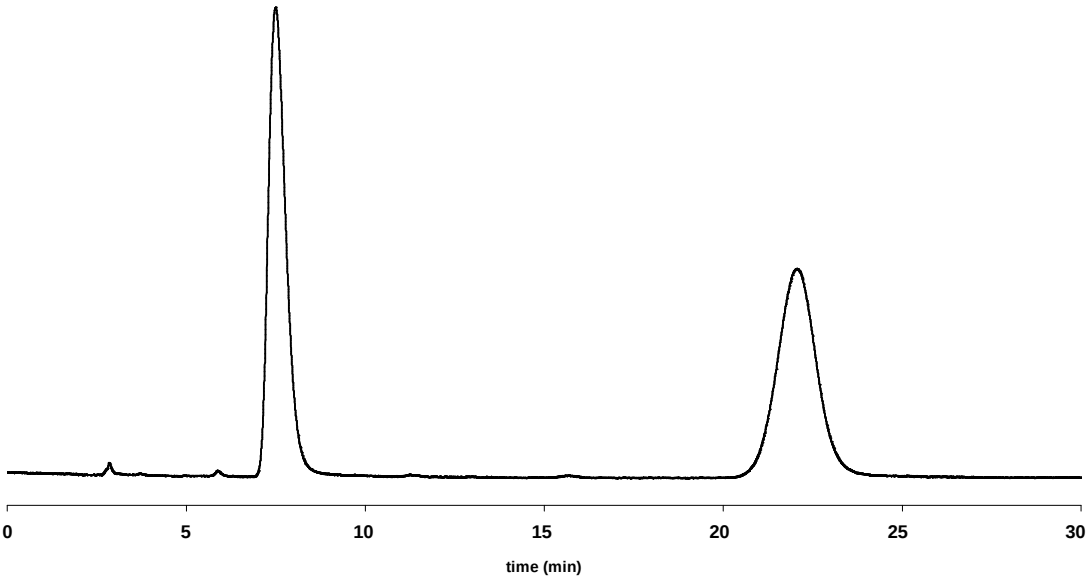



Figure 3.25 NP-HPLC of warfarin on AmyCoat (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 308 nm.

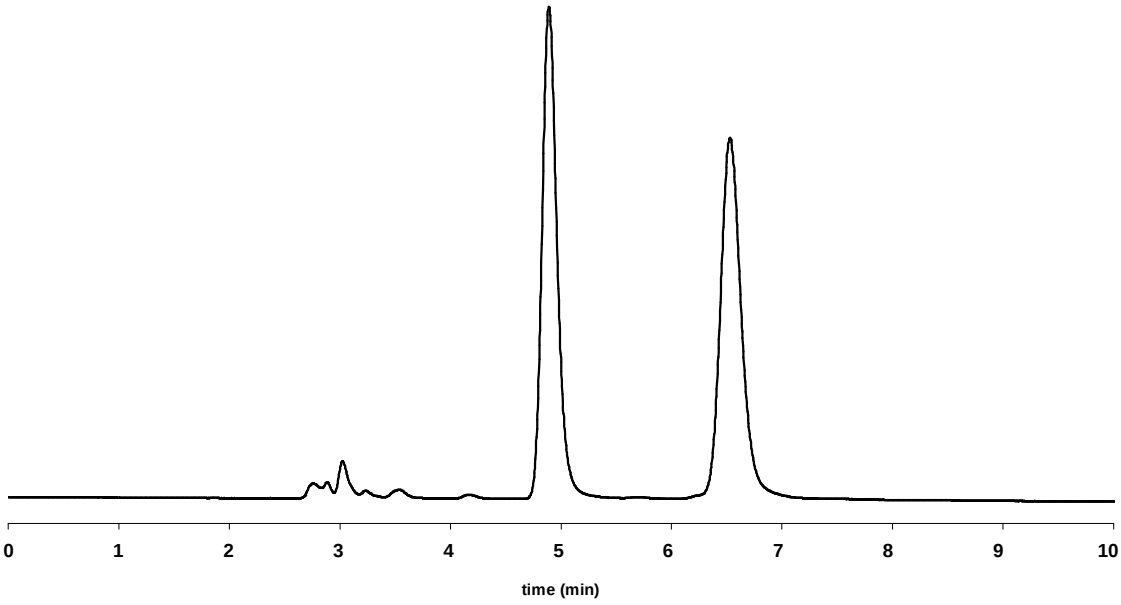



Figure 3.26 NP-HPLC of flurbiprofen on AmyCoat (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TFA (85:15:0.1 v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 254 nm.

In response to the introduction of competitor clones to their coated immobilised derivatised polysaccharide CSP, Daicel introduced versions of their own products in which the derivatised polysaccharide is chemically bonded to the silica packing material. The advantage of these CSP, such as Chiralpak IA which contains the same derivatised polysaccharide as Chiralpak AD is that they can be used with a wider range of mobile phases. Using solvents such as dichloromethane, and chloroform that cannot be used on the coated equivalents and might allow better separations to be developed. However, perhaps the most telling advantage is that they are suitable for production scale chiral LC as there is much less chance of chiral selector leaching into the isolated drug. Although the immobilised CSP may often be used with different mobile phases, it was decided to use the Chiralpak IA with the same mobile phases as had previously been used to evaluate the coated CSP. There had been concern from the column manufacturer about doing this comparison as there had been a suggestion (B. Freer, personal communication) that, since the immobilised CSP were more rigid, they might give lower enantioselectivity. Indeed, this proved to be the case for most of the compounds (Table 3.8). However, while there were three cases (paroxetine, cromakalim and ketamine where the enantioselectivity on Chiralpak AD had been low and there was no enantioselectivity on Chiralpak IA, the drop in performance was not marked and in two cases (chlorpheniramine and brompheniramine) the enantioselectivity was actually slightly higher. As shown in Fig. 3.27 and 3.28 respectively, warfarin and flurbiprofen were still very well separated.

Table 3.10 *k*, α and $R_s$ for evaluation of CSP Chiralpak IA; mobile phases [hexane – IPA – TFA or TEA (85:15:0.1, v/v/v)].

| compound | Mobile Phase 1* | | | | Mobile Phase 2** | | | |
|---|---|---|---|---|---|---|---|---|
| | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ |
| baclofen | 1.96 | 2.14 | 1.09 | 0.19 | -- | -- | -- | -- |
| brompheniramine | 0.23 | 1.07 | 4.63 | 9.87 | 0.75 | 0.84 | 1.12 | 0.20 |
| chlorpheniramine | 0.23 | 1.07 | 4.63 | 10.25 | 0.79 | -- | -- | -- |
| cicletanine | 6.04 | 9.00 | 1.49 | 0.82 | 1.30 | 1.82 | 1.40 | 0.92 |
| citalopram | 0.97 | 1.15 | 1.19 | 1.50 | 1.04 | -- | -- | -- |
| cromakalim | 4.16 | -- | -- | -- | 3.28 | -- | -- | -- |
| flurbiprofen | 0.48 | 0.71 | 1.46 | 3.51 | -- | -- | -- | -- |
| ketamine | 1.90 | -- | -- | -- | 0.55 | -- | -- | -- |
| mexiletine | 0.53 | -- | -- | -- | 0.36 | -- | -- | -- |
| mianserin | 2.38 | 3.06 | 1.28 | 1.01 | 0.24 | 0.45 | 1.86 | 2.76 |
| naproxen | 1.01 | 1.10 | 1.09 | 1.11 | 11.39 | 21.03 | 1.85 | 0.92 |
| nicotine | 0.09 | -- | -- | -- | 0.46 | -- | -- | -- |
| paroxetine | 3.34 | -- | -- | -- | 2.87 | -- | -- | -- |
| propranalol | 1.16 | -- | -- | -- | 0.79 | -- | -- | -- |
| verapamil | 4.74 | -- | -- | -- | 0.79 | 0.91 | 1.15 | 1.23 |
| warfarin | 1.31 | 5.19 | 3.98 | 9.71 | -- | -- | -- | -- |

*- Mobile Phase 1 - [hexane – IPA – TFA (85:15:0.1, v/v/v)]

**- Mobile Phase 2 - [hexane – IPA – TEA (85:15:0.1, v/v/v)]

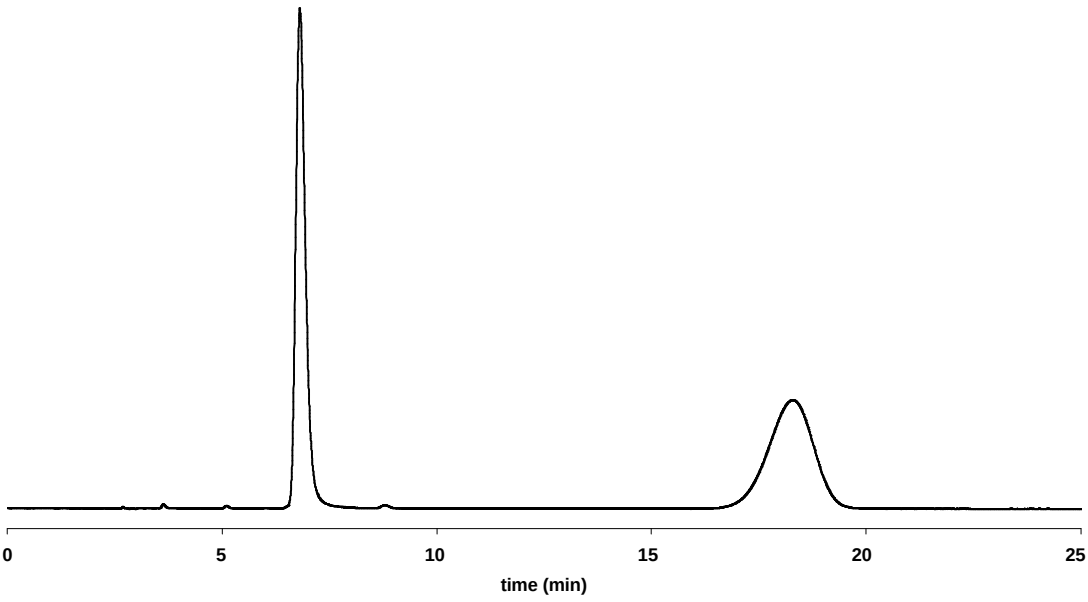



Figure 3.27 NP-HPLC of warfarin on Chiralpak IA (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 308 nm.

.

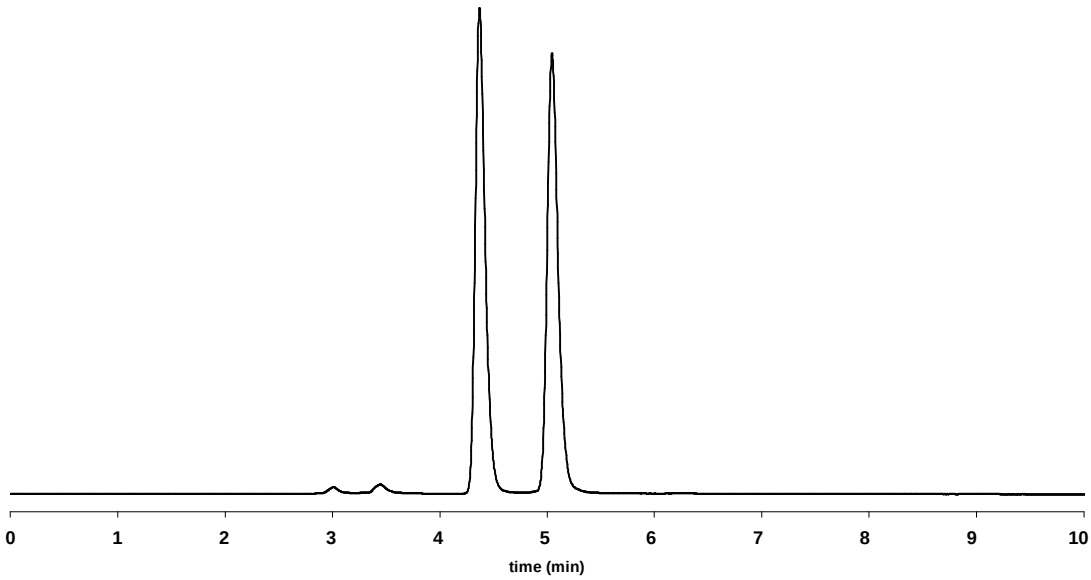



Figure 3.28 NP-HPLC of flurbiprofen on Chiralpak IA (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 254 nm.

As comparisons were being made with Chiralpak AD, perhaps the most commonly used CSP in chiral screening, it was also opportune to do a comparison with another CSP, namely Chiralcel OJ-H, (”H” depicting that it is higher efficiency in that the support silica particles are 5 µm rather than 10 µm) which is less commonly used but still often plays a part in CSP screens. There were several examples where the enantioselectivity was better than Chiralpak AD, especially cicletanine (Fig. 3.30) and paroxetine. The separation of nicotine enantiomers (Fig. 3.29) was unique to the OJ-H and for that degree of complementariness alone might be worth including in a set of columns in a screen.

Table 3.11 *k*, α and $R_s$ for evaluation of CSP Chiralcel OJ-H; mobile phases [hexane – IPA –TFA or TEA (85:15:0.1, v/v/v)].

| compound | mobile Phase 1* | | | | mobile Phase 2** | | | |
|---|---|---|---|---|---|---|---|---|
| | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ |
| baclofen | 0.75 | -- | -- | -- | -- | -- | -- | -- |
| brompheniramine | 2.65 | 2.87 | 1.08 | 0.13 | 0.95 | 1.08 | 1.14 | 1.01 |
| chlorpheniramine | 2.55 | 2.75 | 1.08 | 0.11 | 0.40 | 0.45 | 1.13 | 0.54 |
| cicletanine | 2.27 | 4.78 | 2.10 | 3.71 | 2.62 | 5.85 | 2.23 | 7.41 |
| citalopram | 1.20 | -- | -- | -- | 2.08 | 2.49 | 1.20 | 1.16 |
| cromakalim | 3.37 | 5.17 | 1.53 | 1.00 | 1.50 | 2.47 | 1.65 | 2.55 |
| flurbiprofen | 1.18 | -- | -- | -- | 1.28 | -- | -- | -- |
| ketamine | -- | -- | -- | -- | 2.29 | 6.35 | 2.77 | 13.16 |
| mexiletine | -- | -- | -- | -- | 0.38 | -- | -- | -- |
| mianserin | 3.82 | 6.76 | 1.77 | 1.20 | 0.42 | 0.79 | 1.89 | 3.18 |
| naproxen | 2.98 | 3.45 | 1.16 | 2.50 | 5.79 | 7.31 | 1.26 | 4.04 |
| nicotine | 2.49 | 6.42 | 2.58 | 4.68 | 0.28 | -- | -- | -- |
| paroxetine | 2.86 | 7.20 | 2.52 | 1.12 | 2.91 | 3.22 | 1.11 | 0.50 |
| propranalol | 2.05 | -- | -- | -- | 1.11 | 1.21 | 1.09 | 0.35 |
| verapamil | 19.54 | -- | -- | -- | 6.80 | -- | -- | -- |
| warfarin | 21.68 | -- | -- | -- | -- | -- | -- | -- |

*- Mobile Phase 1 - [hexane – IPA – TFA (85:15:0.1, v/v/v)]

**- Mobile Phase 2 - [hexane – IPA – TEA (85:15:0.1, v/v/v)]

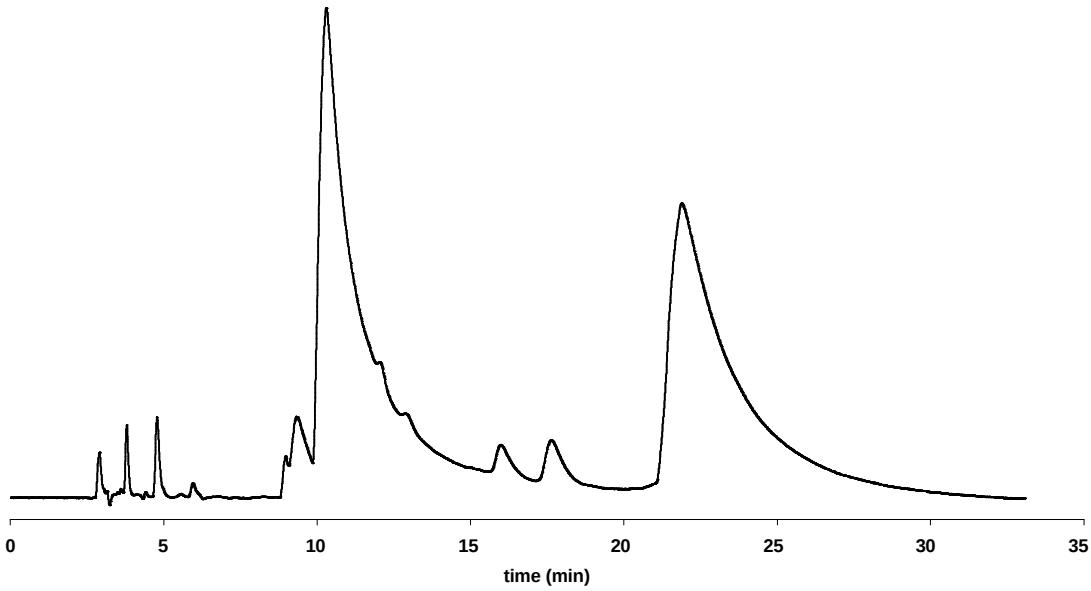



Figure 3.29 NP-HPLC of nicotine on Chiralcel OJ (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 260 nm.

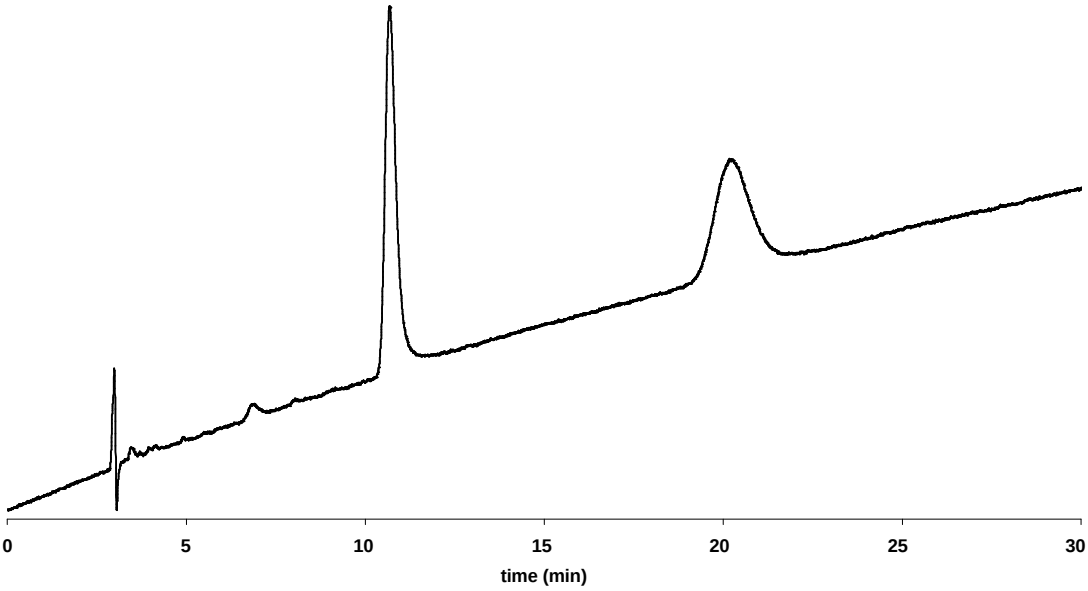



Figure 3.30 NP-HPLC of cicletanine on Chiralcel OJ - H (250 mm x 4.6 mm I.D.); mobile phase - [hexane – IPA - TFA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 254 nm.

Having made a case for the inclusion of Whelk-O1 and Chiralcel OJ-H (alongside the generally employed Chiralcel OD and Chiralpak AD) in a screening approach to chiral method development, it was then instructive to take a look at how successful a screen consisting of Whelk-O1, Chiralcel OJ-H, Chiralcel OD and Chiralpak AD might be (Table 3.12 , Fig. 3.31).

Table 3.12 'Hit' comparison of Whelk-O1, OD, AD and OJ with 16 compounds set of known drugs of normal phase LC, using two mobile phases.

| compound | | With Acid Modifier | | | | With Base Modifier | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Whelk-O1 | OD | AD | OJ | Whelk-O1 | OD | AD | OJ |
| **Acidic** | flurbiprofen | ✓ | | ✓ | | | | | |
| | naproxen | ✓ | ✓ | ✓ | ✓ | | | | ✓ |
| | warfarin | ✓ | ✓ | ✓ | | | ✓ | ✓ | |
| **Basic** | brompheniramine | | ✓ | | ✓ | | | ✓ | ✓ |
| | chlorpheniramine | | ✓ | | ✓ | | | ✓ | ✓ |
| | cicletanine | ✓ | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ |
| | citalopram | | ✓ | ✓ | | ✓ | ✓ | ✓ | |
| | ketamine | ✓ | | ✓ | | ✓ | ✓ | ✓ | ✓ |
| | mexiletine | ✓ | ✓ | | | | ✓ | | |
| | mianserin | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | |
| | nicotine | ✓ | | | ✓ | | | | |
| | paroxetine | ✓ | | ✓ | ✓ | | | ✓ | ✓ |
| | propranalol | ✓ | ✓ | | | ✓ | ✓ | | ✓ |
| | verapamil | | ✓ | | | | | ✓ | |
| **Neutral** | cromakalim | ✓ | ✓ | | ✓ | ✓ | ✓ | | ✓ |
| **Zwitterionic** | baclofen | | | | | | | | |

For the limited range of 16 compounds for which all four CSP were used, the four CSP screen was very successful (Fig. 3.28). In fact three column screens were successful and even a simple Whelk-O1 / Chiralpak AD combination was successful. This perhaps suggests that it should perhaps have been the use of the Chiralcel OD that could have been called into question. While it also suggests that maybe the Chiralcel OJ is not after all worth including, it must be remembered that

the resolution of nicotine enantiomers on Chiralcel OJ was much greater than on Whelk-O1. The ‘Achilles heel’ of the four column screen was baclofen which was by far the most polar compound. This was a clear signpost that for a really wide range of chiral compounds, a screening approach will not be complete without at least some element of ‘reversed-phase’ work.

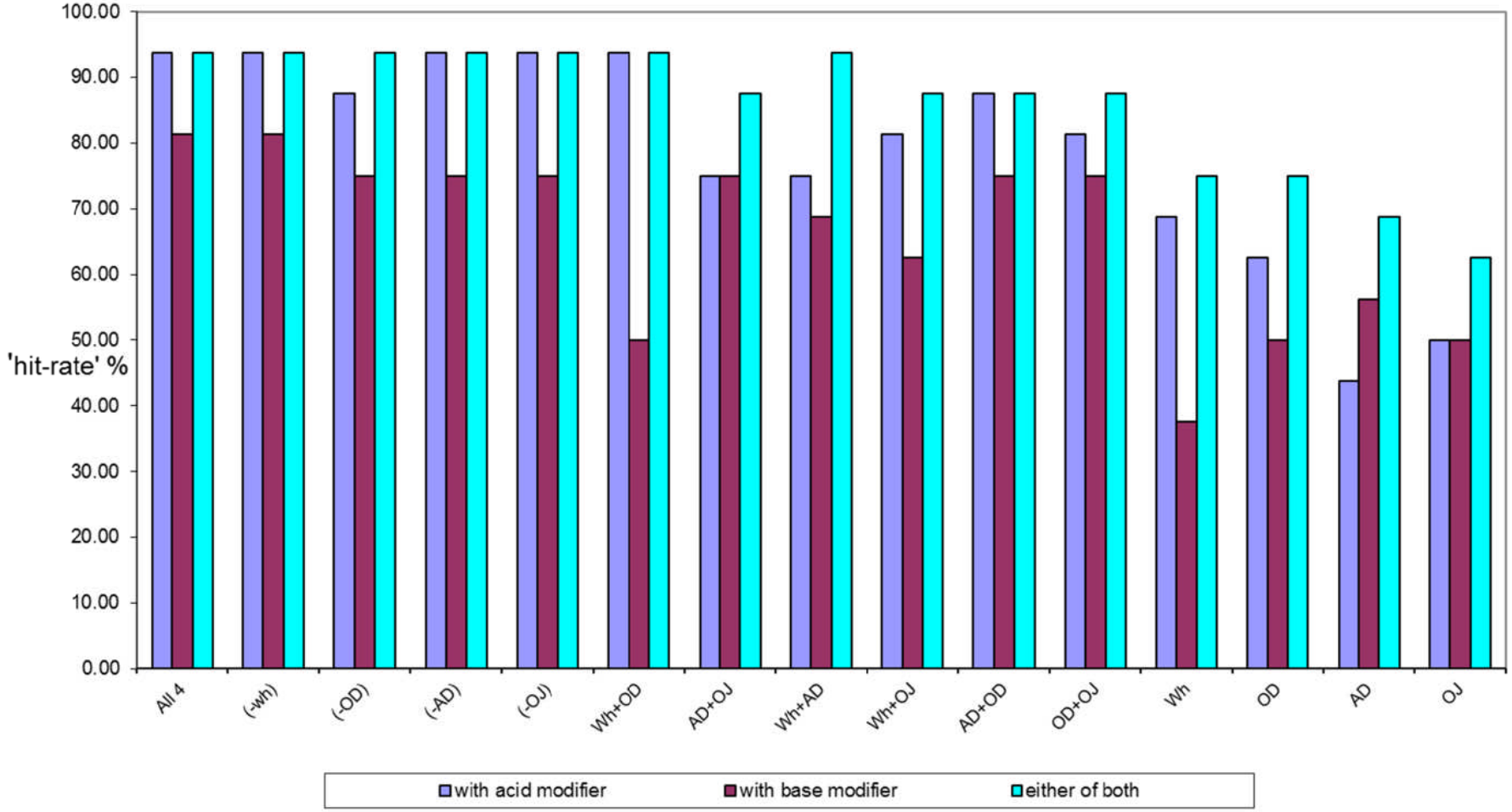



Figure 3.31 'hit-rate' of Whelk-O1, OD, AD and OJ with 16 compounds set of known drugs of normal phase LC, using two mobile phases.

## 3.4 Conclusions

If thinking in terms of enantioselectivity alone, the other Pirkle-concept CSP had little to offer over and above what the Whelk-O1 CSP had to offer. The Chiralcel OD and Chiralpak CSP clones had nothing to offer over and above the originals (except perhaps a slightly lower price). Therefore going forward, for example to look at aspects such as achiral selectivity as well as chiral selectivity in reversed phase, it was decided to restrict use of Pirkle-concept CSP to the Whelk-O1 and to use Chiralcel OD and Chiralpak interchangeably with clones thereof. It could be argued that if studying achiral selectivity of CSP with a view to looking for orthogonal selectivity to alkyl-silicas, this would have most practical value if found in systems also utilising polar organic solvent – aqueous based mobile phases. However, in moving on to study 'reversed-phase' systems it was felt that limited chiral selectivity, albeit in 'normal-phase', was an acceptable means of restricting the number of CSP to be studied. After all, without the operation of very strong interactions (that give chiral recognition) there would be little likelihood of different selectivity to the usually dominant hydrophobic interaction / partition retention mechanism operating in 'reversed-phase' systems. Also, given this dominant mechanism it would not be expected *a priori* that e.g. the ULMO and DACH-DNB CSP would be significantly more effective as chiral selectors in a reversed-phase rather than normal phase system.

# 4 Selectivity, Efficiency and Speed in UPLC™ of Active Pharmaceutical Ingredients

## 4.1 Introduction

In the context of studying stationary phase selectivity it was worthwhile taking into account the overall current thrust in separation science research towards achieving higher column efficiency, e.g. (Cook, 2007). It was therefore worthwhile considering whether selectivity still had a role to play in achieving chromatographic resolution. It was thought therefore that it would be important to demonstrate that even in modern LC systems with the capability of achieving high efficiency obtaining good selectivity is still essential.

To address the role of selectivity and efficiency in obtaining resolution it is necessary to re-visit the Purnell Equation (Smith and Braithwaite, 1996) (Equation 4.1), first introduced in Chapter 1.

*Eqtn. 4.1*

$$R_s = \frac{1}{4}\sqrt{N}.\frac{k}{(1+k)}.\frac{(\alpha - 1)}{\alpha}$$

where $R_S$ = resolution, N = efficiency, *k* = capacity factor, α = selectivity retentivity factor, *k* / (1 + *k*), selectivity factor, (α- 1) / α

However, $N$ is inversely proportional to particle size ($d_p$)

*Eqtn. 4.2* $N \alpha L / d_p$ (L = column length, $d_p$ = particle size)

Therefore, reducing the particle size and the column length by the same factor will enable separations to be achieved in a much reduced run time and, setting aside dead volume issues, without a loss of resolution. However, in studying the effect of efficiency and selectivity on resolution, retentivity must first be considered. In particular it is appropriate to consider the most relevant capacity factor value at which to make the comparison of efficiency and selectivity. From the relationship between the retentivity factor in the Purnell Equation (*Eqtn. 4.*1) and capacity factor there is a sharp increase in resolution with $k$ for $k$<2 but less so for $k$ >2. Therefore, bearing in mind the need to not unnecessarily increase run times, then it best to operate at $k$ ~2 for difficult separations in a mixture and then use either the efficiency or selectivity to pull out the resolution.

The relationship between the Purnell Equation selectivity factor and selectivity, $\alpha$, appears to be similar to the relationship between retentivity factor and capacity factor. However, the message is different in that in instrumental LC difficult separations are usually involved and rarely will $\alpha$ be greater than 2. The fact that the improvement in selectivity factor levels off at high selectivity is not really an issue as, under such circumstances, it will be a simple matter to obtain a separation in any case. Accordingly, in most situations where it is desired to

improve resolution, increasing selectivity will be a highly effective means of achieving this aim. For most analytical situations the objective is to obtain reliable baseline resolution in as short a time as possible. It is therefore instructive to look at the efficiency required to obtain an $R_s$ value of 1.5, when *k* is 2, for a range of selectivity values (Table 4.1).

Table 4.1 Required efficiency for different selectivities (for reliable baseline resolution, $R_s$ = 1.5 and *k* set at 2.0).

It is apparent from these figures that the increase in *N* that may be obtained by using UPLC will only make a difference in resolution within a very limited range ofselectivities.

| α | $N_{required}$ |
|---|---|
| 1 | !!!!!! |
| 1.005 | 3272481 |
| 1.01 | 826281 |
| 1.02 | 210681 |
| 1.03 | 95481 |
| 1.04 | 54756 |
| 1.05 | 35721 |
| 1.1 | 9801 |
| 1.15 | 4761 |
| 1.2 | 2916 |
| 1.25 | 2025 |
| 1.3 | 1521 |
| 1.35 | 1205 |
| 1.4 | 992 |
| 1.45 | 841 |
| 1.5 | 729 |
| 1.55 | 643 |
| 1.6 | 576 |
| 1.65 | 522 |
| 1.7 | 478 |
| 1.75 | 441 |
| 1.8 | 410 |
| 1.85 | 384 |
| 1.9 | 361 |
| 1.95 | 342 |
| 2 | 324 |

However, consideration of van Deemter plots and the flat C-term profile for sub-2 µm particles at high linear velocities (Fig. 4.1) suggests that the most appropriate use of columns packed with sub-2 µm particles would be to achieve good efficiency and resolution with high linear velocities and short run times.

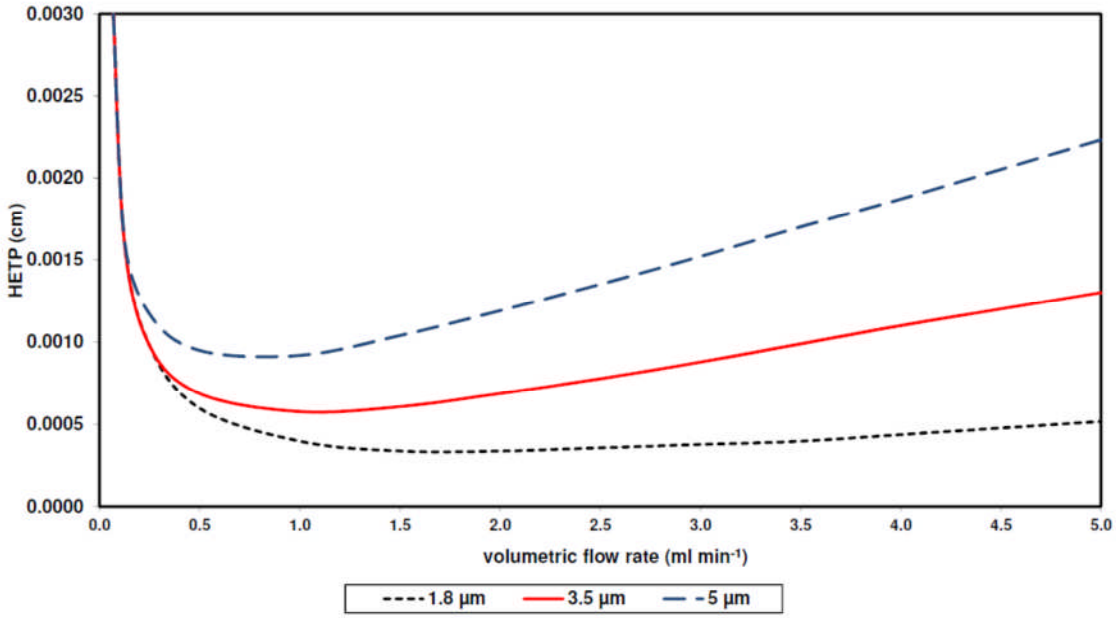



Figure 4.1 van Deemter plots for 5 µm particles to sub-2 µm showing a flatter C term with higher flow rates (adapted from Butchart *et al*., 2007).

Accordingly an exercise was embarked upon to assess the extent to which UPLC™ could be used in the area where it could make most impact i.e. to reduce analysis times for pharmaceutical and biomedical separations for which chromatographic run time was the rate-limiting step (Erni, 1983), in this instance focussing on applications involving active pharmaceutical ingredients (APIs). In so doing it was also hoped to obtain information on the importance or otherwise of the optimisation of selectivity to obtain satisfactory resolution prior to or during the quest for speed. Given the nature of this research programme it was important to establish that it was still an important issue even when working with very modern technology.

## 4.2 Experimental

### 4.2.1 Instrumentation

The automated UHPLC system, Acquity Ultra Performance LC - Waters (Waters Corporation, Milford, MA 01757, USA) used for loading and running samples, consisted of a binary pump (Binary Solvent Manager, Part No.186015001), a sampler (Sample Manager, Part No. 186015006) and a detector (PDA detector – Part No. 186015026). Data was collected using Empower software. Water purifier ELGA Option 3 used was from (ELGA, High Wycombe, Bucks., UK).

### 4.2.2 Materials

A UPLC column (BEH130 C18 1.7 µm, 130 Å, 50 mm × 2.1 mm I.D.), (Waters, Cheshire, UK), was used. For work on benzodiazepines, 100 mm and 150 mm versions of this column were also used. An ACE 3 C18, 50 mm × 2.1 mm I.D. column and a ‘uracil + 4’ column efficiency test solution were supplied by Hichrom Ltd.

Flurbiprofen and its related substances (Fig. 4.2), paroxetine and its related substances (Fig. 4.3) were gifts from Aesica Pharmaceuticals (Cramlington, UK). Cleaning validation ‘worst case’ API’s (cromakalim, hydrocortisone, lormetazapam, phenytoin, thalidomide, nabumetone) (Fig. 4.6) and benzodiazepines (diazepam,

nitrazepam, lorezepam, oxazepam, temazepam) (Fig. 4.8) were from Sigma Aldrich, Tocris or the University of Sunderland's drug collection.

### 4.2.3 Methods

Mobile phases were prepared using HPLC – grade acetonitrile, methanol and tetrahydrofuran (Sigma-Aldrich Poole, Dorset, UK) and distilled and doubly de-ionised water using an ELGA Option 3 Water purifier (ELGA, High Wycombe, Bucks., UK) with glacial acetic acid and trifluoroacetic acid (TFA) (both Sigma-Aldrich Poole, Dorset, UK) as additives in the mobile phases, using gradient UPLC methods. Many gradient profiles and mobile phase compositions were used during the course of the rapid method development. The individual gradient profiles and mobile phase compositions for the final chromatograms are shown in the relevant figures and figure legends. The samples were run at temperatures up to 80 $^{o}$C and pressures up to 12000 psi.

Sample solutions of paroxetine and each of its related substances at 0.1 mg ml$^{-1}$ in tetrahydrofuran – water – trifluoroacetic acid (90:10:0.5, v/v/v) were prepared.

Sample solutions of flurbiprofen and each of its related substances at 1.0 mg ml$^{-1}$ in acetonitrile – water – acetic acid (6:12:0.1, v/v/v) were prepared. Each of these solutions was diluted to 0.1 mg ml$^{-1}$ with the same solvent. A mixture was then prepared by mixing 400 µl of the flurbiprofen solution with 100 µl of each of the related substances solutions.

Sample solutions of cromakalim, hydrocortisone, lormetazepam, phenytoin, thalidomide, nabumetoneat 0.1 mg $ml^{-1}$ in methanol – water (50:50, v/v) were prepared. Further solutions were prepared by diluting to 0. 0.002 mg $ml^{-1}$ (2 µg $ml^{-1}$with methanol – water (50:50, v/v) and then to 0.00004 mg $ml^{-1}$ (0.04 µg $ml^{-1}$, 40 ng $ml^{-1}$) with acetonitrile – water (20:80, v/v).

Sample solutions of benzodiazepines (diazepam, nitrazepam, lorazepam, oxazepam, temazepam) at 0.1 mg $ml^{-1}$ in methanol were prepared by dissolving 1 mg of each compound in 10 ml of methanol. 600 µl of diazepam solution (to account for weaker chromophore) and 200 µl of each of the other solutions were taken to prepare a mixture of benzodiazepines. A fresh solution was prepared for subsequent attempts at developing gradients and using longer columns. This involved a preparation of each solution at 1 mg $ml^{-1}$ in methanol and taking 100 µl of each solution and making up to 1000 µl with methanol. This solution was further diluted by a factor of 10 to give a solution in methanol – water (50:50, v/v) by taking 100 µl of the solution, adding 400 µl methanol and making up to 1.0 ml with water.

## 4.3 Results and Discussion

The original motivation for studying UPLC separations had been to consider whether improvements in efficiency would make the fine-tuning of selectivity, whether it be from the mobile phase or the stationary phase, redundant. However

by the time the study commenced it was becoming clear through the appraisal given above and the emphasis in the literature (Wyndham, 2003), including on kinetic plots, e.g. (Villiers *et al*., 2006), that the generation of high plate numbers would not be one of the main drivers for the use of UPLC. This soon became more apparent. In preliminary work on the Acquity™ instrument it was confirmed that when dealing with short columns with sub-2 µm particles the range of efficiencies obtainable (N 8000 – 9000) was not so much higher than that obtainable with previously existing technology (N ~7700 for an ACE 3 C18 column). This served to illustrate the point that the increase in efficiency obtainable would not be sufficient to bring about a marked increase in obtainable resolution (*cf* Table 1). The key then was to exploit the physical robustness of the UPLC particles (traditional 3 µm RPLC packing materials such as ACE 3 C18 are not recommended for work at 'ultra-pressures' or temperatures significantly above ambient; personal communication, A. Smith, Hichrom Ltd., UK), the high pressure rating of the instrument and the flat mass transfer profile with flow rate in order to obtain speed. In attempting to do so it would be necessary to assess how much the optimisation of selectivity would be needed before increasing temperature and/or flow rate.

The first example to be studied to assess the extent to which selectivity would need to be optimised before resolution and high speed could be obtained involved flurbiprofen.

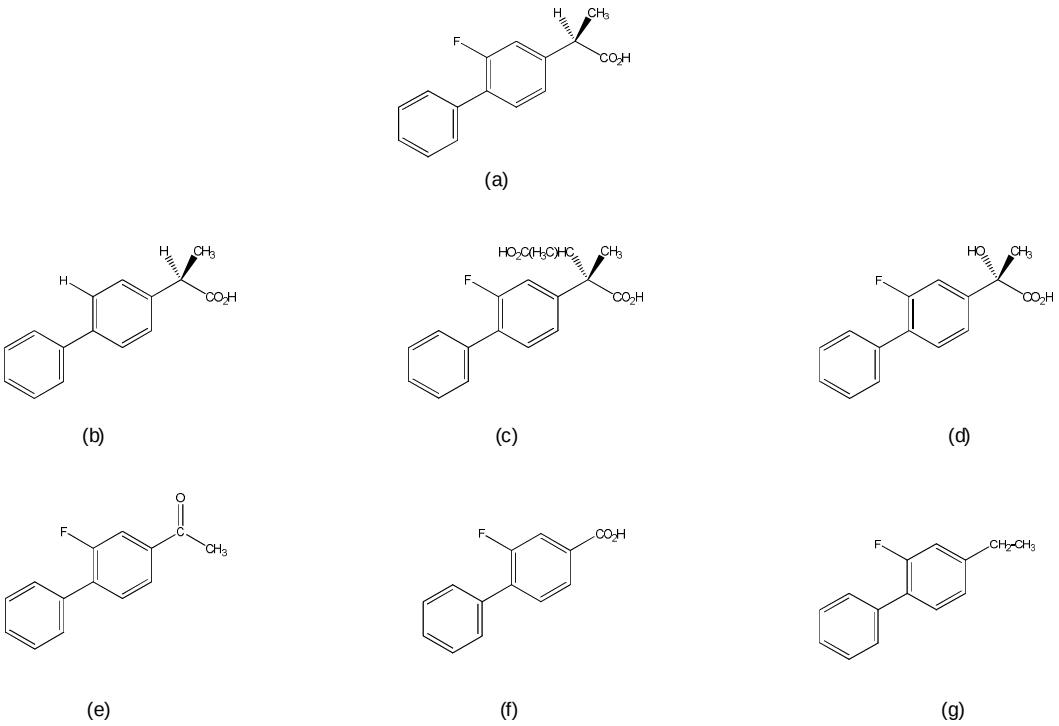



Figure 4.2 Flurbiprofen - a non-steroidal anti-inflammatory drug and its related substances.

In this case an isocratic production QC method for the determination of its related substances had a 120 min run time, two of the early eluting peaks (arising from 2-fluorobiphenyl-4-carboxylic acid (f) and 2-(4-biphenyl)propionic acid) (b) in particular being very difficult to resolve and one more non-polar compound, 4-ethyl-2-fluorobiphenyl (g), giving rise to a very long-running peak on typical C18 alkyl-silicas. The acetonitrile – water – glacial acetic acid mobile phase used in the original company method used for the first attempt at UPLC on a BEH C18 (1.7 µm) (50 mm x 2.1 mm I.D.) column was modified, replacing glacial acetic acid with trifluoroacetic acid. This brought about an increase in *k* to resolve the critical most

difficult to separate peaks. Thereafter a steep gradient was introduced to elute the last peak. At this point, having achieved the desired separations, the linear velocity was increased to a point where the working pressure was high but was still comfortably below the maximum operating pressure of 16000 psi. This brought about a ten-fold decrease in run time. While this was a comparison of an optimised UPLC method with a non-optimised HPLC method, what was striking was the speed with which changes in mobile phase could be explored.

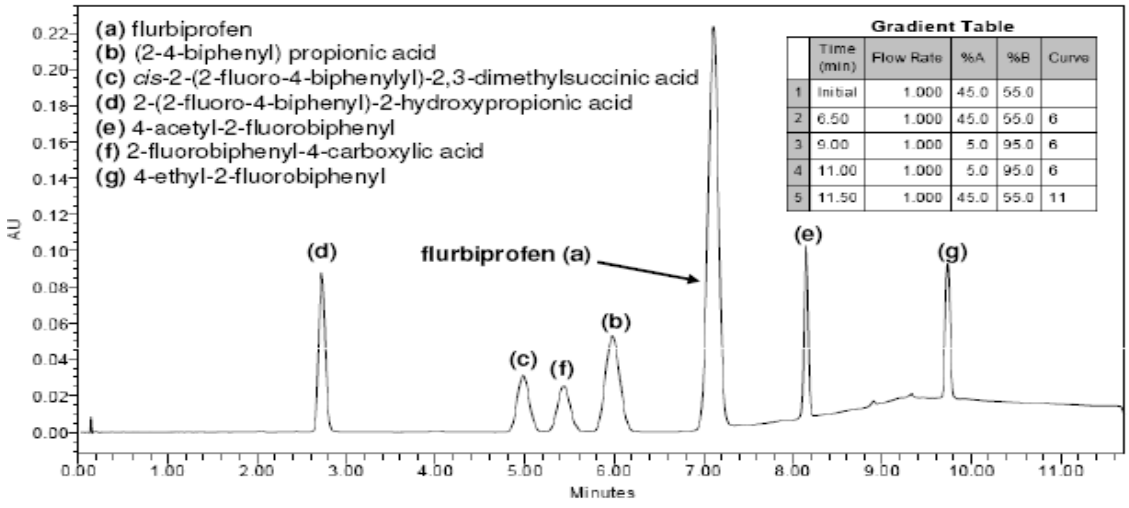



Figure 4.3 UPLC of flurbiprofen and related substances (showing mobile phase gradient table) on BEH C18 (1.7 µm) (50 mm x 2.1 mm I.D.); 10955psi. 0.04 mg $ml^{-1}$ flurbiprofen and related substances ((a) – (f) as per Fig.4.2) at 0.01 mg $ml^{-1}$ Mobile phase component A 0.5% trifluoroacetic acid in methanol, B 0.5% trifluoroacetic acid in water.

The EP method for paroxetine related substances (British Pharmacopoeia, 2010) (Fig. 4.4) involves a gradient mobile phase with a 60 minutes run time. In approaching developing a UPLC method for paroxetine and related substances it was decided to adopt a similar strategy of using selectivity changes to optimise the separation and then increasing the linear velocity until a pressure of ~12000 psi was reached. From previous experience (personal communication, generic LC methods for related substances assays for basic drugs. Lough, W.J.), it had been found that the resolution of paroxetine from its des-fluoro analogue was the most difficult separation to achieve of the compounds that needed to be resolved. However when attempting to resolve this compound it was found that it was the *cis*- isomer that was eluting closest to the main peak. At this point, previous work was further considered and it was apparent that for reversed-phase ion-pair work (as above: personal communication, Lough, W.J.) the des-fluoro analogue had been the closest running related substance and that for reversed-phase LC work which had been the basis of the EP method (British Pharmacopoeia 2010) the cis-isomer had been more of an issue. With further practical work it very soon became apparent that the inclusion of small amounts of tetrahydrofuran (THF) in the mobile phase was the key to obtaining a good separation of paroxetine from its *cis*- isomer. Bearing this in mind and using 10% THF, resolution of all seven peaks was obtained in a much shorter time than that needed for the pharmacopoeial method. Indeed the resolution of des-fluoro-paroxetine, cis-paroxtine and paroxetine in an isocratic portion of the gradient programme was very good, so good that there was clearly scope for sacrificing resolution for speed. Also, an interesting feature of chromatograms during development was a sharply focussed

late-running peak for (-)-*trans*-1-benzyl-4-(4-fluorophenyl)-3-(3,4-methylenedioxyphoxymethyl)-piperidine hydrochloride ('N-benzyl-paroxetine') on a steeply sloping part of the gradient programme. Because of this it was decided to attempt to achieve the separation in a shorter time by using a 4 min linear gradient but, critically, maintaining the 10% THF in the mobile phase. This gave satisfactory resolution and indeed resolution was maintained when the temperature was increased to 80 $^{o}$C and the flow rate was increased to give a pressure of 12000 psi to give a remarkably short run time of 2 min with the actual separation being achieved in 1.2 min (Fig. 4.5), this time taken to elute all peaks of interest being less than a third of that previously achieved by UPLC (Messina *et. al.,* 2007 ).

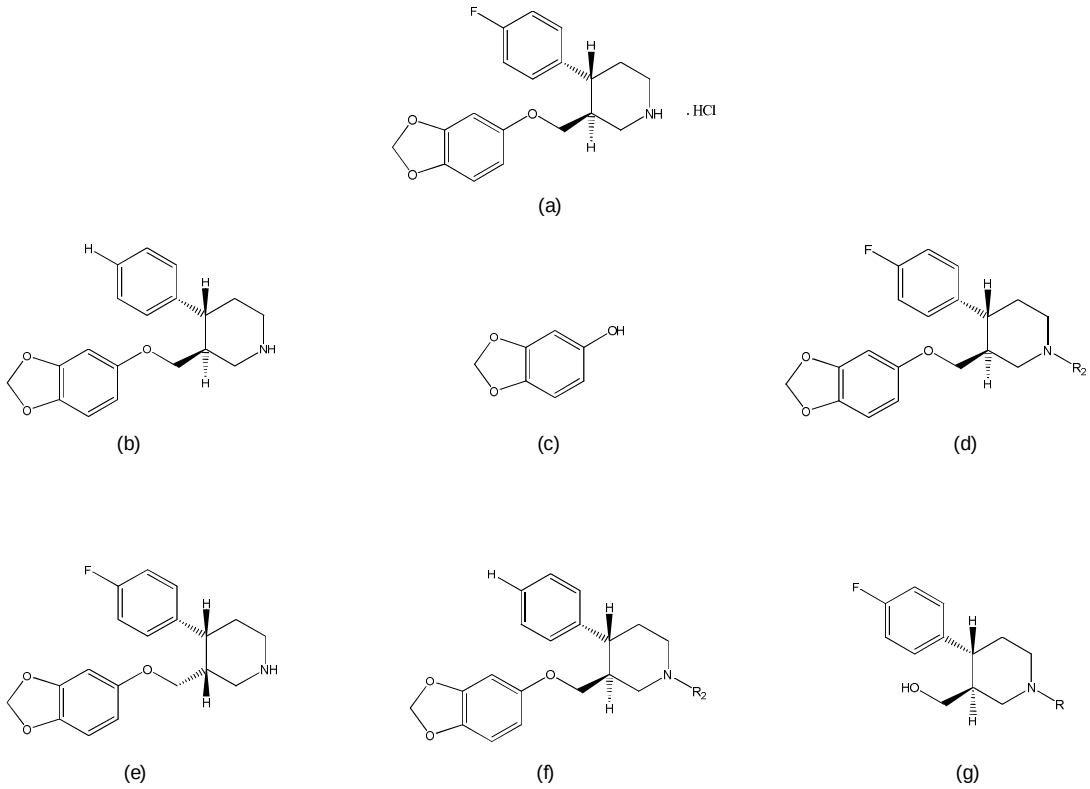



Figure 4.4 Paroxetine and its related substances. (a) (-) paroxetine hydrochloride (b) Impurity A of British Pharmacopoeia (BP); (3S,4R)-3-[(1 -3-benzodioxol-5-yloxy)methyl]-4-phenylpiperidine (c) Impurity B of BP; 1,3-benzodioxol-5-ol (sesamol) (d) Impurity C of BP; (3S,4R)-3-[(1-3-benzodioxol-5-yloxy)methyl]-1-benzyl-4-(4-fluorophenyl)piperidine (e) Impurity E of BP; (3RS,4RS)-3-[(1-3-benzodioxol-5-yloxy)methyl]-4-(4-fluorophenyl)piperidine (f) Impurity H of BP; [(3S,4R)-1-benzyl-4-(4-fluorophenyl)piperidin-3-yl]methanol (g) Impurity I of BP; [(3S,4R)-4-(4-fluorophenyl)piperidin-3-yl]methanol.

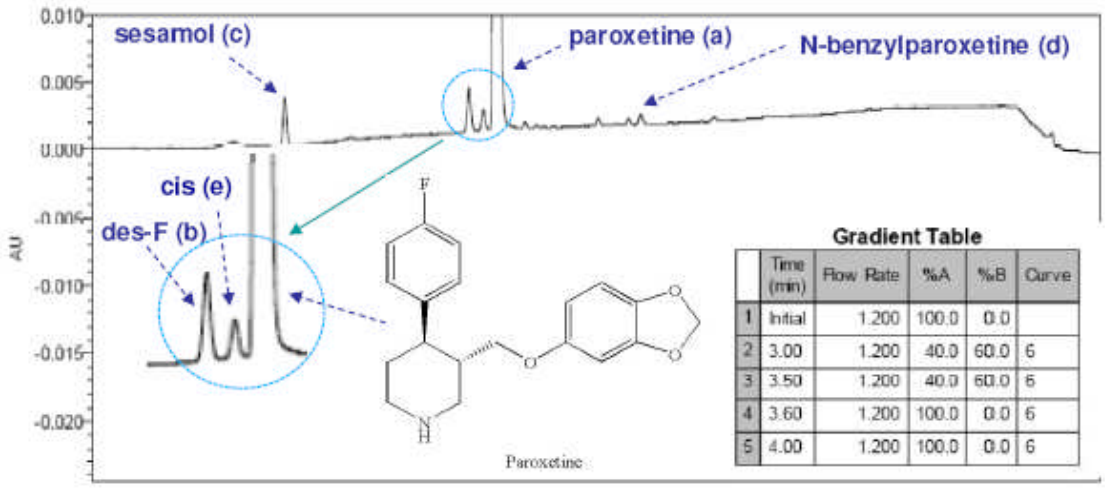



Figure 4.5 UPLC of paroxetine and related substances (showing mobile phase gradient table) on BEH C18 (1.7 µm) (50 mm x 2.1 mm I.D.); 1 mg ml$^{-1}$ paroxetine and related substances at ~ 0.002 mg ml$^{-1}$. UV detection - 295 nm; impurities (f) and (g) which elute just before and just after sesamol respecively are not detected at 295 nm. Mobile phase component A [water – THF – TFA (90:10:0.5, v/v/v)], B [acetonitrile – THF – TFA (90:10:0.5, v/v/v)]. Resolution was maintained when using a steep gradient profile throughout and also when the temperature was raised to 80 $^{o}$C.

Buoyed with this success in speeding up methods once resolution had been achieved with an adapted method by working on selectivity, attempts were then made to assess whether new methods involving APIs could be developed equally quickly. There has been an interest in rapid analytical methods to support cleaning

validation for over a decade now e.g. (Clark*et al*., 2006) and this remains the case. A ‘worst-case scenario’ for cleaning validation is usually regarded to be when the drug being cleaned from machinery is a hydrophobic drug with poor aqueous solubility. However, given the confidential nature of work with such proprietary drugs, it was decided to develop a gradient system with potential for providing the basis of a generic method for ‘worst-case scenario’ (i.e. hydrophobic,drugs by using a selection of such drugs that were freely available. Cromakalim, hydrocortisone, lormetazepam, phenytoin, thalidomide, nabumetone (Fig. 4.6), all from different classes of drugs, were selected. As things transpired minimal method development was required with these APIs being readily resolvable within 1.5 min from one another using a simple acetonitrile – water gradient on a 5 cm BEH130 C18 UPLC column. For this separation to be useful for cleaning validation, it would be necessary for the APIs to be detected at low levels. Using a standard 1.0 µl injection volume of a methanol solution, each compound could easily be detected at a concentration of 0.002 mg $ml^{-1}$(2 µg $ml^{-1}$) (Fig. 4.7a) and there was no deformation of peak shape. However, more impressively, using on column sample focussing (Mills *et al*., 1997) brought about by the use of a poorly eluting solvent for the sample solution to facilitate a 10 µl injection volume, the APIs could comfortably be observed at a concentration as low as 0.00004 mg $ml^{-1}$(40 ng $ml^{-1}$) (Fig. 4.7b).

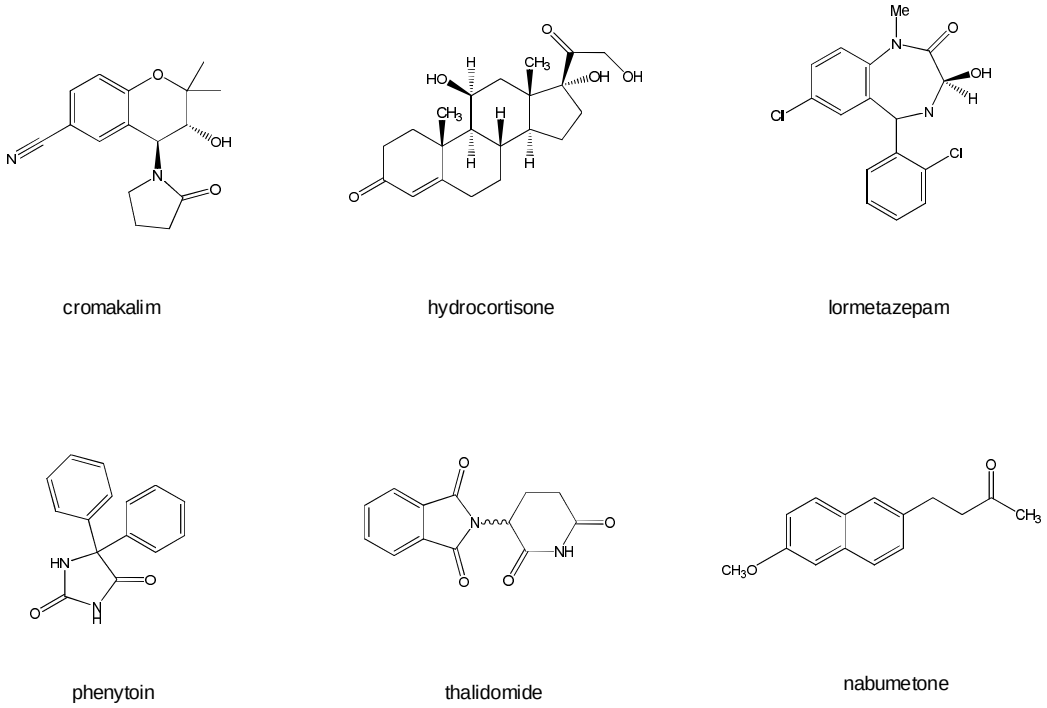



Figure 4.6 Structures of illustrative ‘worst-case scenario’ drugs.

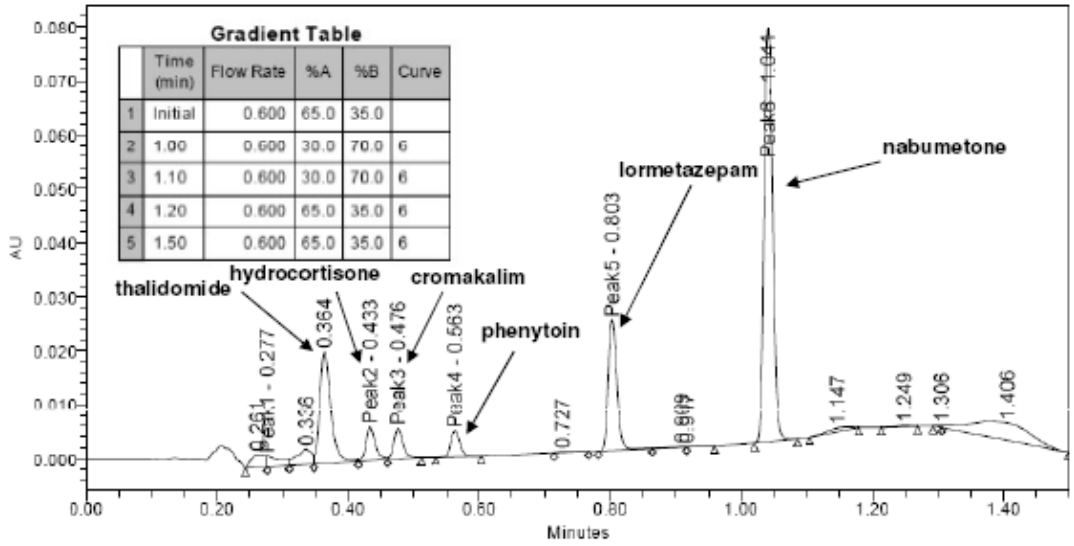



Figure 4.7 a UPLC of cleaning validation 'worst case' API's (showing mobile phase gradient table) on BEH C18 (1.7 µm) (50 mm x 2.1 mm I.D.); initial pressure 11200 psi. All API's present at ~ 0.002 mg $ml^{-1}$ detection 254 nm. Mobile phase component A water, B acetonitrile (1 µl injections from methanol may be made without loss of resolution).

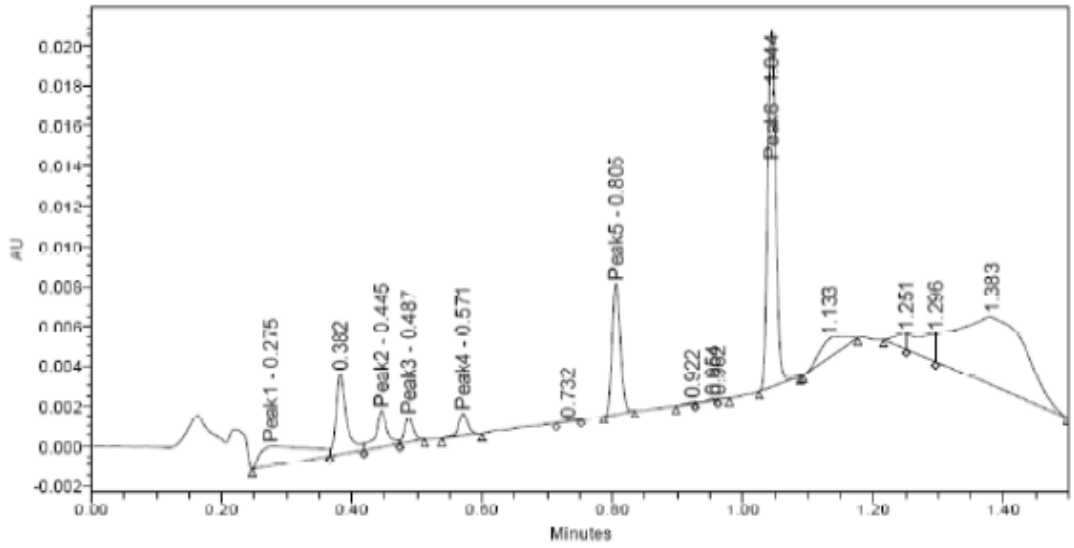



Figure 4.7 b Detection of lower concentrations using on-column sample focussing: 10 µl injection volume of a 0.00004 mg $ml^{-1}$ solution in methanol – water (20:80, v/v); acetonitrile – water gradient; column temperature 80 $^{o}$C; BEH C18. Retention order as in Fig. 4.7a.

A final typical application involving APIs that was studied was the development of a generic identity test for benzodiazepines (Fig. 4.8). On manufacturing sites where several products containing similar APIs are being produced such tests can be very convenient (personal communication, various (GSK, Barnard Castle, UK)). While, as before, it was easy to ring the changes very quickly with mobile phase variables and gradient profiles, unlike the other applications, the desired resolution did not fall into place with a critical change to achieve appropriate selectivity. No matter what the mobile phase and gradient profile, all five benzodiazepines could not be baseline resolved on the 5 cm BEH C18 column. Accordingly, in the

absence of suitable options to significantly vary UPLC stationary phase selectivity at the time, this was one case where using UPLC to gain increased efficiency was an option worth considering. A few mobile phase options were quickly explored using a 10 cm BEH C18 column and it was possible to obtain almost baseline resolution in about 3 min and even better resolution in 5 min. (Fig. 4.9a). However, subsequently, a 15 cm column quickly proved to offer the solution. On a column of this length it was possible to obtain complete baseline resolution in 8 min (Fig. 4.9b).

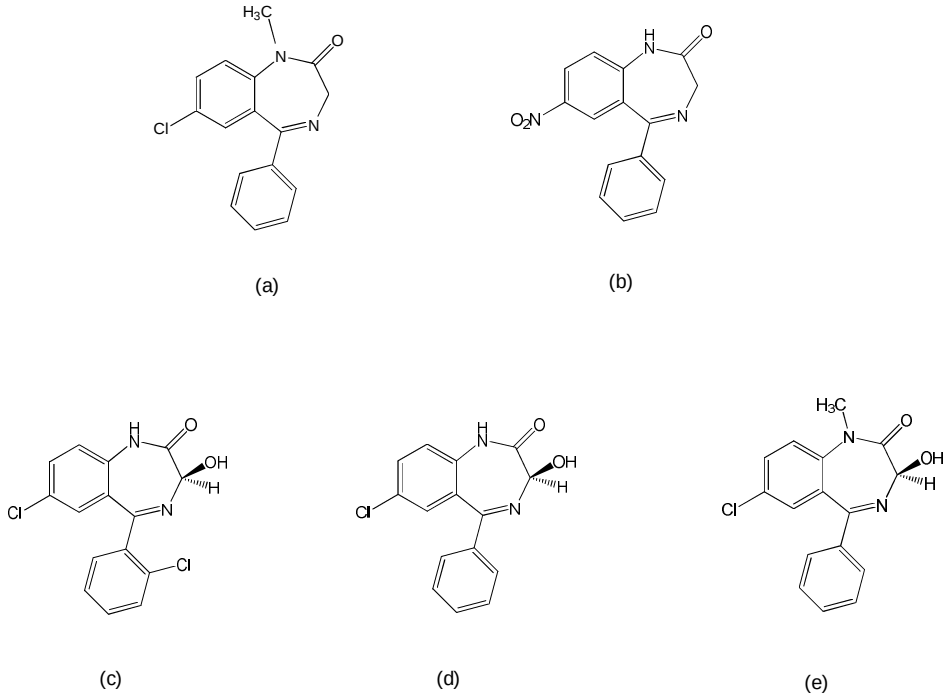



Figure 4.8 Benzodiazepine ((a) diazepam, (b) nitrazepam, (c) lorazepam, (d) oxazepam, (e) temazepam)) structures.

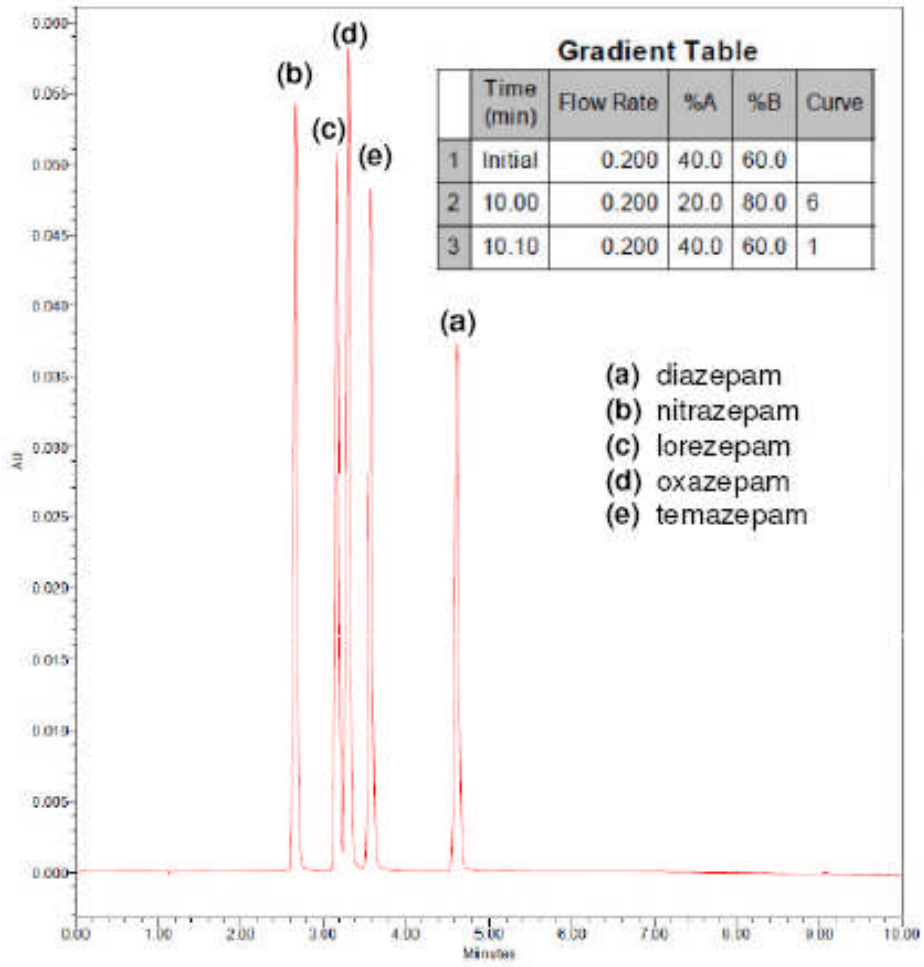



Figure 4.9a UPLC of benzodiazepine identity test (showing mobile phase gradient table) on BEH C18 (1.7 µm) (100 mm x 2.1 mm I.D.); 7040 psi. All compounds present at ~ 0.1 mg $ml^{-1}$ in methanol, detection 254 nm. Mobile phase component A water, B methanol, column temperature 30 $^{o}$C, Retention order – nitrazepam, lorazepam, oxazepam , temazepam, diazepam.

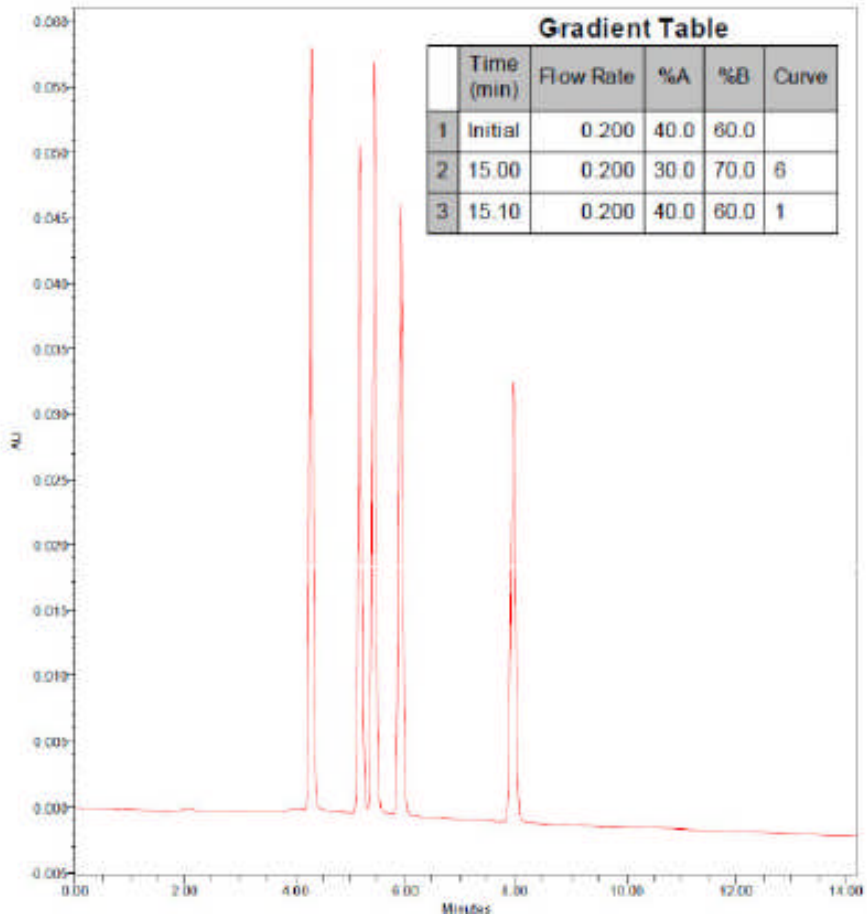



Figure 4.9b UPLC of benzodiazepines on a longer column and optimised mobile phase (showing mobile phase gradient table) on BEH C18 (1.7 µm) (150 mm x 2.1 mm I.D.); 11530 psi. All compounds present at ~ 0.1 mg $ml^{-1}$ detection 254 nm. Mobile phase component A water, B methanol, 30 $^{o}$C, Retention order as per Figure 4.9a.

## 4.4 Conclusions

In all four cases of pharmaceutical separations it was necessary to first address (at speed) the key resolution issue for each mixture by using mobile phase variables to ‘fine-tune’ selectivity. Once this had been done, then and only then it proved very facile to, where appropriate, increase temperature and then simply increase the linear velocity to the point where a comfortable operating pressure of ~ 12000 psi was reached. The speed with which different mobile phase variables were tried out was a feature of the work. This belies the suggestion that speed improvements in comparing HPLC to UPLC should only be made by comparing an optimised HPLC method with an optimised UPLC method. This is because, as was found here that the speed with which optimisation can be carried out in UPLC is one of its important features Al-Sayah (Al-Sayah *et al.,* 2008), compared method development using 15 mm UPLC columns (in theory, N = 35000 achievable) of different selectivity with “laborious trial and error” HPLC mobile phase and stationary phase optimisation. However, experience here suggested that trial and error UPLC mobile phase optimisation using 5 µm columns could be successful and rapid. Only for the benzodiazepine i.d. test would there have been scope for improvement on the trial and error approach by resorting to automated mobile phase optimisation strategies. While there are other emerging technologies which might be used to achieve similar results, the holistic combination (Grumbach*et al*., 2005; Fountain *et al* 2009) of high temperature, high pressure and robust particles, column hardware and instrument design offered by UPLC™ allowed the dramatically shortened run times to be achieved in a very facile manner.

The first two examples in particular served to emphasise the importance of achieving selectivity by mobile phase manipulation even in UPLC. While the benzodiazepine example showed that, contrary to the data for $R_s$ = 1.5 shown in Table 1, high efficiency in UPLC (Al-Sayah *et al.,* 2008; Plumb *et al*., 2007) was useful and achievable at relative speed for increasing $R_s$ from 0 to ~1, it also suggested that having more options for stationary phase selectivity is desirable in UPLC as well as HPLC.

Regarding other examples not involving APIs that were studied i.e. gradient screening of urine extracts and plant extracts on C18 silicas, matters were even simpler. Much shorter run times with similar resolution to the initial situations could be had simply by increasing the temperature and mobile phase linear velocity. An example gradient run of a plant extract is shown in Fig. 4.10. While not directly relevant to the theme of this research programme, these examples served to illustrate the power of UPLC and underscored the point that there is very little difference in selectivity between different C18 silicas. For the urine extract and plant extract complex mixtures, the very short run time was achieved with very little change of selectivity or loss of resolution. Any slight losses in resolution are unlikely to be a problem. If they were then combining UPLC with MS detection would seem to be a more sensible way forward than attempting to obtain very high resolution via alternative chromatographic approaches. UPLC could be used to rapidly monitor the prep-LC fractions as well.

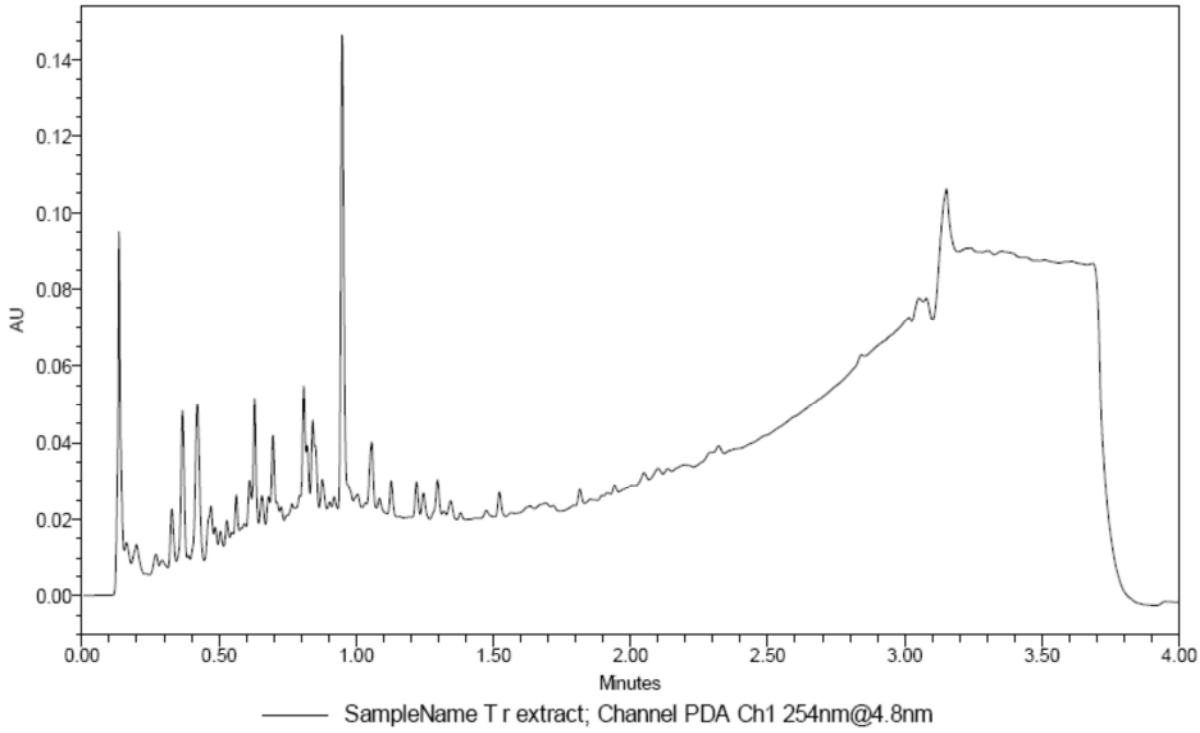



Figure 4.10 UPLC of medicinal plant extract, '*Tetractomia roxburghianna'* (screening crude methanol extract, 1mg $ml^{-1}$) on BDH C 18 (1.7 µm) (100 mm x 2.1 mm I.D); linear gradient, flow 1.2 ml min-1; 30 $^{o}$C; (similar profile to original profile, but much faster than HPLC and 60 min run time was reduced to 4 min).

In the screening examples, the objective was not to change selectivity but to transfer to UPLC keeping the original resolution. In the API cases, the necessary improvements in selectivity were made by addressing mobile phase variables. Importantly though, this work on UPLC demonstrated that this new technique would not bring about a situation where most separations could be achieved by

the use primarily of high efficiency with little consideration of selectivity. Therefore the main thrust of work in this research programme i.e. on stationary phase selectivity, was most certainly not redundant. Unfortunately there are currently very few options in UPLC for utilising stationary phase selectivity as in the other studies in this programme. This is an area which manufacturers of UPLC columns need to address. This seems to be being appreciated by e.g. Restek Inc. (Restek Catalogue) but evidence from work already described in this thesis (Chapter 2) suggests that it will take more than perfluorophenyl- phases to bring about radically different RPLC stationary phase selectivity.

### 4.4.1 Suggestions for Future Work

By considering selectivity, UPLC clearly can be used very effectively in its most appropriate use of reducing run times where chromatographic run time is the rate limiting step. Whether or not this form of LC will go on to make greater impact than it already has done will depend on whether analysts perceive that such dramatic increases in speed are really needed and also that speed increases may also be useful when chromatographic run time is not rate limiting. The use of fused core silica particles (Abrahim *et al.*, 2010) may eventually eclipse UPLC or, more likely, sub-2 μm fused core silica particles may become popular. Either way, studies of achiral and chiral stationary phase selectivity, as carried out in this research programme for HPLC, will still be needed.

# 5 Assessment of Chiral Stationary Phases for Suitability for Combined Enantiomeric Impurity/Related Substances Assays

## 5.1 Introduction

Having explored the selectivity of achiral stationary phases (Chapter 2) and looked at the enantioselectivity of chiral stationary phases (Chapter 3), it was worthwhile recalling the finding of Soo (PhD thesis, University of Sunderland; 2003) that chiral stationary phases (CSP) may show orthogonal selectivity to alkyl-bonded silicas. However, as was apparent from the work described in Chapter 3, much of the work on CSP is carried out with 'normal phase' mobile phases. For any coupling of chiral columns with columns containing alkyl-bonded silicas to be considered though, their performance with the same type of mobile phase needed to be studied. Therefore it was sought to look at the retentivity, selectivity, enantioselectivity and resolution of CSP when using typical 'reversed-phase' mobile phase conditions. While this might give information on useful orthogonal selectivity, perhaps more important might be using this as a lead into achiral and chiral separations on one system.

As has already been stated (Chapter 3), the separation of enantiomers by LC has been a major success story since it became routinely possible in the late 1980's [Lough, 1989]. This is so much so the case that in the field of chiral separations of pharmaceuticals there is little left to do by way of fulfilling genuine unmet needs.

However, when a single enantiomer drug is tested analytically, the trace enantiomeric impurity is generally determined by a chiral LC test which is separate from the related substances test. Clearly it would be more convenient if the enantiomeric impurity and other related substances could be determined simultaneously using one set of LC conditions. This would also give a check on the specificity of the enantiomeric impurity test. Using N-acetyl-L-tryptophan as a model drug it had been demonstrated that this can be achieved by exploiting a specially-tailored combination of achiral and chiral stationary phases (CSP) (R.W.H. Perera, unpublished work on Spherisorb ODS1 / Chirobiotic T coupled systems; also R.W.H. Perera and W.J. Lough, poster presentation, Chirality 2004, New York). However when extending this approach to a real drug example, it was found that when using reversed-phase conditions with the CSP it was necessary to use low percentages of the polar organic solvent in order to achieve the optimum chiral separation (Undergraduate projects, B.Sc. Chemical and Pharmaceutical Science, University of Sunderland). As a consequence, this placed a limitation on the achiral phase that could be used in the combination system with the same mobile phase. At this point it was felt that, despite reversed-phase chiral LC method development by screening CSP having already been carried out [e.g. (Holzheuer *et al.*, 2009); Peng *et al*., 2009)], further retentivity, enantioselectivity and achiral selectivity (i.e. selectivity between different related substances which are not stereoisomers) characterisation of CSP under reversed-phase conditions would be useful in informing attempts at conducting enantiomeric impurity and related substances determinations with one set of conditions. Achiral selectivity was an issue in that a CSP may exhibit orthogonal achiral selectivity to

an achiral phase simply because it has little or no achiral selectivity while the achiral phase does have achiral selectivity. In some cases this may be an advantage but it will be a disadvantage in a combined chiral/achiral system if a contribution from the CSP is needed to bring about resolution of all the related substances from one another. Accordingly it was sought to study those CSP, which might be expected to give retention and enantioselectivity with high amounts of polar organic solvent in the mobile phase, under reversed-phase LC conditions in order to ascertain which of them give large enough enantioresolution and suitable retention to be used in a combination column with an achiral C-18 silica to be able to determine the trace enantiomer and all the other related substances in one test. The use of coupled chiral/achiral columns, often used for drug bioanalysis from the early 1990's onwards (Lough and Noctor, 1994; Angelo *et al*., 1999), was also used by Phinney, (*Phinney et al.*, 1998)] in super-critical fluid chromatography (SFC) work for the separation of different drugs (benzodiazepine and β-blocker sets of compounds) from one another as well for the individual drug enantiomer separations. Lindner and co-workers used a reversed phase column to enhance the "chemical selectivity" of a chiral anion-exchanger used for the separation of the enantiomers of a four-compound test mixture (Sardella *et al*., 2008). However the application in mind in this instance was more challenging, demanding the separation of the drug and enantiomer in a sample containing several closely related substances without heart-cutting the main peak. This has been achieved for voriconazole (Ferretti *et al*., 1998) using normal phase LC and for propionyl L-carnitine, coupling with an ion-exchange column (D'Aquarica *et al*., 2004). However, related substances assays are almost invariably carried out by

reversed-phase LC. Therefore here the principle was that it would help to know more about the retention and enantioselectivity of CSP when using the types of reversed-phase mobile phase with a high proportion of the polar organic solvent component typically used for related substances determinations. A match of retentivity with, say, a C18 silica would be needed to allow the phase or column combination to work effectively.

## 5.2 Experimental

### 5.2.1 Instrumentation

The HPLC systems used for the chiral screening in reversed phase each employed a Shimadzu (Milton Keynes, UK) LC-6A pump and SPD-6AV detector. In each case, a manual Rheodyne (Kotati, Ca, USA) 7125 loop injection valve, fitted with a 20 µl loop, was used for loading samples. Data was collected using a Dionex PC-based data system (Leeds, UK) with an Automated Computer Interface and *AI* 450 Chromatographic Automation Software Release 3.33. The sonicator used to degas mobile phases was from GS Group-ULTRAWAVE Ltd, Cardiff, CF2 1YY.

### 5.2.2 Materials

The ACE 5 C18 (15 cm x 4.6 mm I.D.) column used was a gift from Hichrom Ltd., Theale, Berkshire, UK. The columns containing chiral stationary phases that were

used, Whelk-O1 (5 µm) (250 mm x 4.6 mm I.D.), Cyclobond I 2000 DNP (250 mm x 4.6 mm I.D.) and Chiralpak QD-AX (250 mm x 4.6 mm I.D.) were gifts from Regis Technologies,Inc., Morton Grove, Il., USA, Sigma-Aldrich Chemie, GmbH, Taufkirchen, Germany and Chiral Technologies Europe, Illkirch, France respectively. The particle size for all the stationary phases used was 5 µm. The manufacturers do not disclose the pore size of Chiralpak QD-AX. The pore size for the other stationary phases was 100 Angstrom.

Mobile phases were prepared using HPLC – grade methanol, ammonium formate and formic acid (Sigma-Aldrich, Poole, Dorset, UK). Water was distilled and doubly de-ionised using an ELGA Option 3 Water purifier (ELGA, High Wycombe, Bucks., UK ). All the drug substances used in chiral screening were from Sigma – Aldrich (Poole, Dorset, UK), Tocris (Bristol, UK) or from a collection of pharmaceutical drug substances available within Sunderland Pharmacy School. Flurbiprofen individual enantiomers and related substances were a gift from Aesica Pharmaceuticals Ltd., UK.

### 5.2.3 Methods

Reversed-phase mobile phases were prepared by adding ammonium formate to methanol – water mixtures so that it was present at a 0.02 M concentration. For every 1 L of mobile phase 2 ml of formic acid was added (or *pro rata*) for different volumes. UV detection was used monitoring at $\lambda_{max}$ or wavelengths used for chromatograms in commercial literature applications except for flurbiprofen,

cromakalim, mianserin and ketamine which were monitored at 254 nm. A flow rate of 1.0 ml $min^{-1}$ was used throughout except for the chromatogram of naproxen, its enantiomer and related compounds which was run at 1.5 ml $min^{-1}$, and the sample injection volume was 20 µL.

A sample solution of (*S*)-naproxen, its enantiomer and related compounds at 1.0 mg $ml^{-1}$ in mobile phase with the enantiomer and related compounds present at ~ 10% w/w was prepared by adding (*S*)-naproxen to a solution of racemic naproxen and the related compounds.

Laevokalim was subjected to purposeful degradation by heating a 0.5 mg $ml^{-1}$ solution in mobile phase to 60 $^{O}$C for 7 h. (No significant levels of degradants had been observed after leaving a solution in mobile phase at ambient temperature in natural light for one week.) The original solution had contained ~0.1% w/w of the enantiomer. The resultant solution was spiked with cromakalim to give a level of 0.3% w/w of the enantiomer.

The sample solution of flurbiprofen at 0.2 mg $ml^{-1}$ in mobile phase with its enantiomer and related substances present at 1% w/w was prepared by adding 0.3 mg of (*S*)-flurbiprofen to 300 µl of a mixture of (*R*)-flurbiprofen and the related substances present at 0.01 mg $ml^{-1}$ in mobile phase and then diluting this solution x 5 with mobile phase.

## 5.3 Results and Discussion

The first stage of the study was to select CSP that would be suitable for reversed-phase operation in terms of likely stability and appropriate hydrophobicity of the immobilised moieties. It was already known from previous 'in-house' work (Christopher Edgar, Wimal Perera and Ha Nguyen, B.Sc. Chemical and Pharmaceutical Science, University of Sunderland, final year projects) and the literature (e.g.(http://www.sigmaaldrich.com), in screening kit 30% acetonitrile expected to elute all analytes) that for macrocyclic antibiotic CSP and Cyclobond I 2000 in reversed-phase mode and for Chiral-AGP (Imrie *et al*., 2009) low proportions (usually less than 15 – 20%) of the polar organic component of the mobile phase are usually needed to obtain significant retention. Attempts to couple such CSP to achiral phases of low retentivity such as cyano- or C8- phases had proved to be unsuccessful because there was not a wide enough range of %organic in the mobile phase with which to easily manipulate retention and selectivity. However it was felt that the Whelk-O1 (Fig. 5.1a) and Cyclobond I 2000 DNP CSP (Fig. 5.1b) might be sufficiently hydrophobic in their nature to give similar retentivity to the alkyl-bonded silica stationary phases that are typically used in related substances assays. In a similar vein, it had been found from other previous in-house work involving ion-exchange stationary phases that they could be used with methanol in the mobile phase in the range from 90% to 50% without there being any unacceptably marked increase in *k* values. In the work of Law and Appleby (Law & Appleby, 1998), an isocratic mobile phase of methanol – water – TFA (800:200:2.3, v/v) containing ammonium formate (0.02 M) with an apparent

pH of 2.45 was used for the elution of a wide range of organic bases with varying polarities. Similarly anion exchangers were used with mobile phases with high organic content for the separation of organic acids (Law & Hussain, 1998). Accordingly it was decided to also study the commercially-available chiral ion-exchange CSP, Chiralpak QD-AX (Fig. 5.1c).

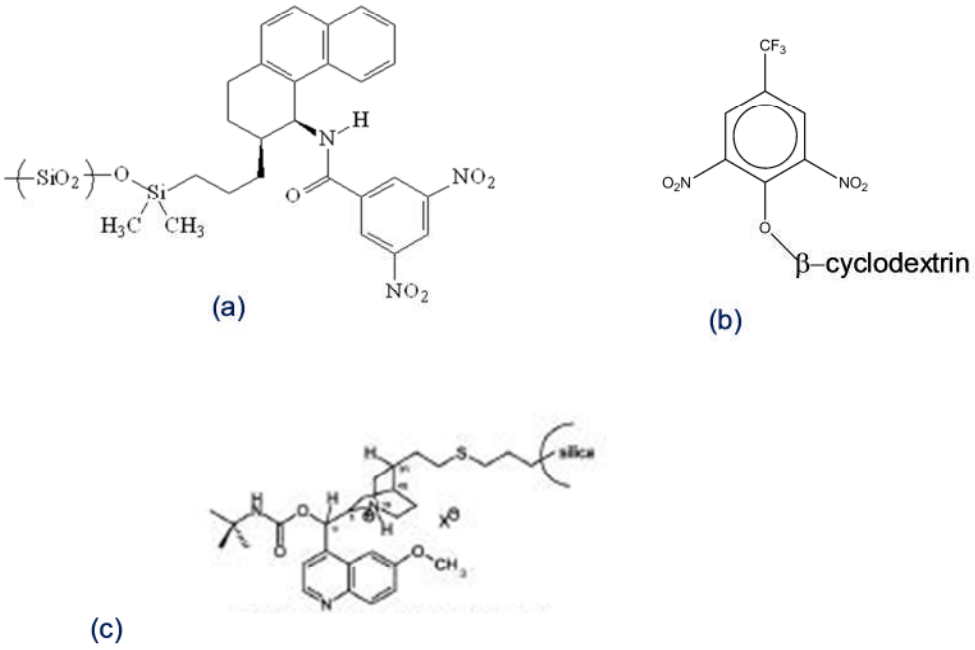



Figure 5.1 Structures of chiral stationary phases (a) Whelk-O1 (b) Cyclobond I 2000 DNP (c) Chiralpak QD-AX.

The intention was to use a reduced set (50 down to 16) of chiral drugs from that that had been used in earlier normal-phase screening work (Chapter 3). However, clearly only a few of this set were acidic so, in the evaluation of Chiralpak QD-AX only, additional acidic chiral drugs were used. These are shown in Table 5.1 along

with the retention, enantioselectivity and resolution data found when using a mobile phase consisting of [methanol – water (90:10, v/v)] 0.02M ammonium formate, formic acid 2ml/L. With this mobile phase containing 90% organic component there was significant retention for all of the carboxylic acids other than the polar amino acid, baclofen. For the 8 (out of 12) carboxylic acids for which there was some resolution, the enantioselectivity was low, with the notable exception of N-acetyl-D,L- tryptophan. As had been anticipated, the change in retention with reducing percentage of organic component in the mobile phase was not marked (Fig. 5.3a, b) and with 70% methanol in the mobile phase the retention was comparable to that of ACE 5 C18. However, only for N-acetyl-D,L- tryptophan (Fig. 5.2), and to a lesser extent carprofen, was there sufficient enantioresolution to suggest that it would be possible to 'trade-off' some enantioresolution in a coupled chiral – achiral system designed to separate both trace enantiomer and all related substances. This possibility was not pursued as this example had already been used as a model 'proof-of-concept' example using a Chirobiotic T – Spherisorb ODS1 system (R.W.H. Perera and W.J. Lough, poster presentation, Chirality 2004, New York) for N-acetyl-L-tryptophan, N-acetyl-D-tryptophan, L-tryptophan, L-tryptophanamide, L-tryptophan methyl ester, 5-hydroxy-L-tryptophan and kynurenic acid, all having been resolved from one another. Also, in general, this Chiralpak QD-AX CSP had been shown to be well suited to derivatised amino acids (Oberleitner *et al*., 2002) and would probably be less well suited to more 'drug-like' carboxylic acids. However, it was noted for future reference that this anion exchanger might give better enantioselectivity at pH values higher than the pH 3.0 chosen here for related substances work.

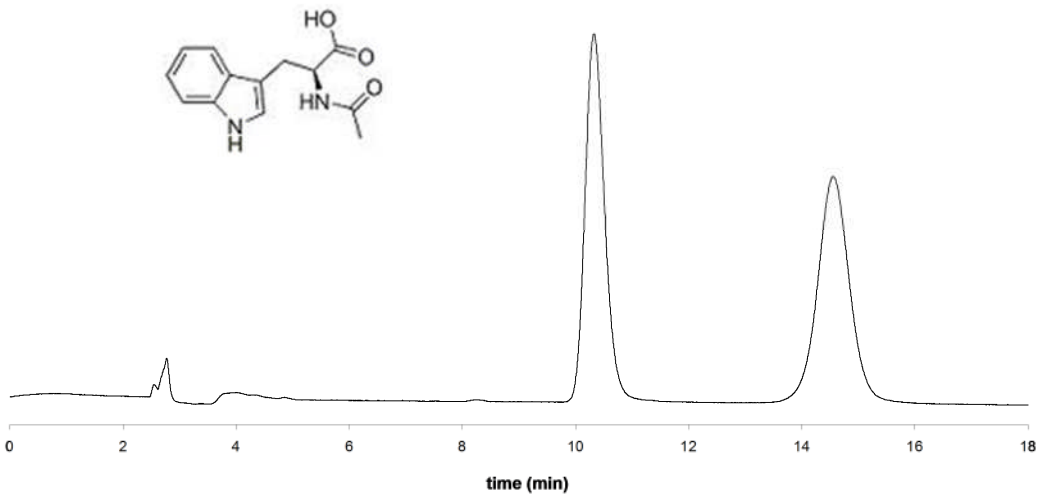



Figure 5.2 RPLC of Chiral separation of enantiomers of N-acetyl-tryptophan on Chiralpak QD-AX (250 x 4.6 mm I.D.); Mobile phase - [methanol - water (90:10 v/v)], 0.02 M ammonium formate, formic acid 2 ml/L; flow rate – 1.0 ml $min^{-1}$; UV detection at 254 nm.

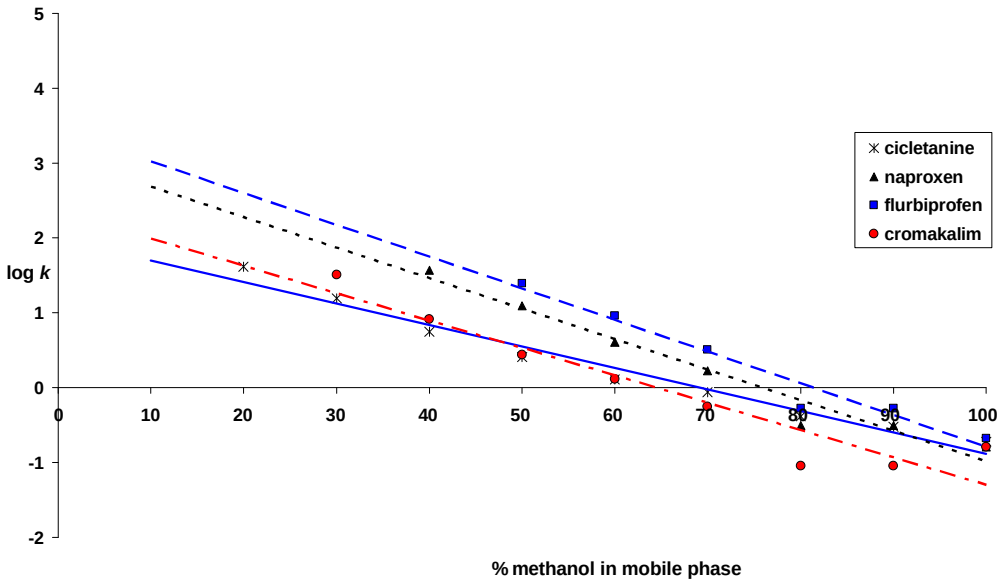



Figure 5.3a Relationship between log *k* and % methanol in the mobile phase for cicletanine, naproxen, flurbiprofen and cromakalim on ACE 5 C18 Mobile phases – [methanol - water], 0.02 M ammonium formate, formic acid 2 ml/L; flow rate – 1.0 ml $min^{-1}$; UV detection at $\lambda_{max}$ for each of the drugs.

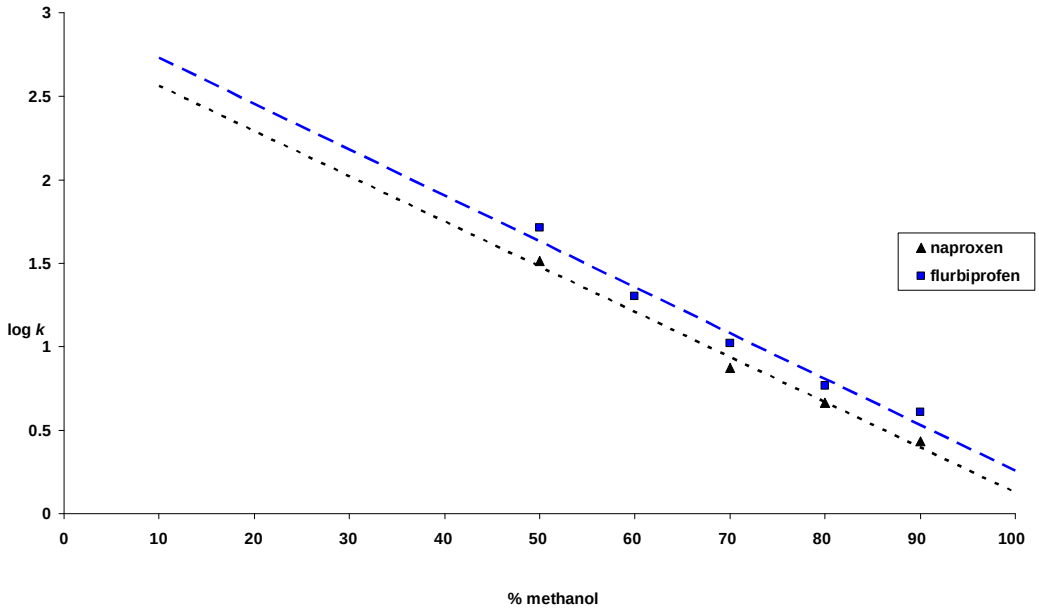



Figure 5.3b Relationship between log *k* and % methanol in the mobile phase for naproxen and flurbiprofen on Chiralpak QD-AX; Mobile phases - [methanol – water], 0.02 M ammonium formate, formic acid 2 ml/L; flow rate – 1.0 ml $min^{-1}$; UV detection detection at $\lambda_{max}$ for each of the drugs.

Table 5.1 Retention, selectivity and resolution data for RPLC of racemic organic acids on Chiralpak QD-AX (250 x 4.6 mm I.D.); Mobile phase - [methanol - water (90:10 v/v)], 0.02 M ammonium formate, formic acid 2ml/L; flow rate – 1.0 ml $min^{-1}$; UV detection at $\lambda_{max}$ for each of the drugs.

| Drug Compounds | $k_1$ | $k_2$ | α | $R_S$ |
|---|---|---|---|---|
| N-acetyl-tryptophan | 1.54 | 2.66 | 1.73 | 5.98 |
| baclofen | 0.10 | -- | -- | -- |
| carprofen | 4.61 | 5.33 | 1.16 | 2.26 |
| cicletanine | 1.88 | -- | -- | -- |
| 4-OH mandelicacid | 1.68 | 1.75 | 1.04 | 0.14 |
| 4-OH, 3-methoxy-mandelic acid | 1.58 | 1.72 | 1.09 | 0.77 |
| 3-OH, 4-methoxy-mandelic acid | 1.76 | -- | -- | -- |
| ibuprofen | 1.75 | 1.89 | 1.08 | 0.94 |
| 3-indole lacticacid | 3.68 | 4.26 | 1.16 | 1.88 |
| flurbiprofen | 2.63 | 2.88 | 1.10 | 1.35 |
| MTPA[b] | 1.39 | -- | -- | -- |
| naproxen | 2.58 | 2.83 | 1.09 | 1.16 |
| warfarin | 1.97 | -- | -- | -- |

[a] mobile phase - ([methanol - water (90:10, v/v)], 0.02 M ammonium formate, formic acid 2 ml/L)

[b] α - methoxy - α - (trifluoromethyl)phenylacetic acid (MTPA)

The retention characteristics of Cyclobond I 2000 DNP may be observed in Fig. 5.3a with comparison with Fig. 5.3c showing that it was clearly less retentive than ACE-5-C18. Furthermore, it can be seen from Table 2 that good chiral resolution was only obtained (for warfarin) when the %polar organic component of the mobile phase was reduced to 40% v/v. Having said that, one of the reasons for having opted to select the Cyclobond I 2000 DNP was that it had been one of the more recently introduced modified-cyclodextrin CSP and accordingly had been less extensively studied. In retrospect the Cyclobond 1 2000 SN CSP would clearly have been likely to be more hydrophobic (more hydrophobic substituent group, more groups on the ring secondary alcohols) and might have had the greater degree of retentivity that would be needed for a good match with ACE-5-C18. However, this product is now no longer commercially available. A compromise, if looking to use serially-coupled columns, would be to match the Cyclobond I 2000 DNP with less retentive C18 phases (various characterisations available e.g. (Euerby & Petersson, 2003)) or C8 phases and use a mobile phase containing methanol in the range of 20-40% v/v, even although, as already indicated, the use of these less retentive achiral phases had previously proved unsuccessful.

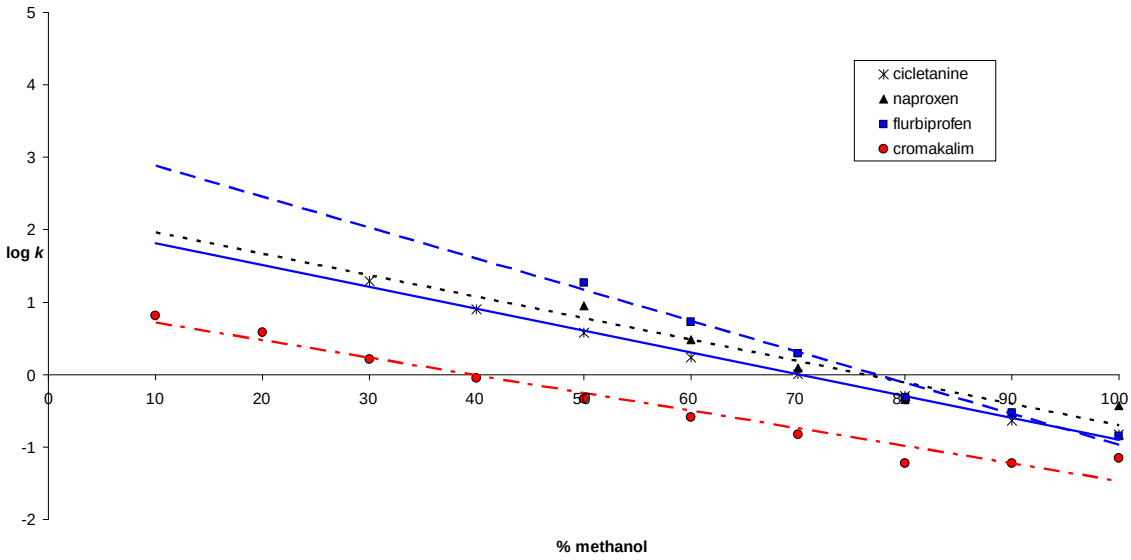



Figure 5.3c Relationship between log *k* and % methanol in the mobile phase for cicletanine, naproxen, flurbiprofen and cromakalim on Cyclobond I 2000 DNP. Mobile phases - [methanol - water], 0.02 M ammonium formate, formic acid 2 ml/L; flow rate – 1.0 ml $min^{-1}$; UV detection at $\lambda_{max}$ for each of the drugs.

Table 5.2 Retention, selectivity and resolution data for RPLC of set of 16 racemic drugs on Cyclobond I 2000 (250 mm x 4.6 mm I.D.).

| **compounds** | **methanol - aqeous[a]** | | | | **methanol - aqeous[b]** | | | |
|---|---|---|---|---|---|---|---|---|
| | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ |
| baclofen | 0.21 | -- | -- | -- | 0.14 | -- | -- | -- |
| brompheniramine | 1.48 | 1.55 | 1.05 | 0.18 | 0.73 | -- | -- | -- |
| chlorpheniramine | 0.88 | 0.99 | 1.12 | 0.68 | 0.47 | -- | -- | -- |
| cicletanine | 3.86 | -- | -- | -- | 1.73 | -- | -- | -- |
| citalopram | 1.83 | -- | -- | -- | 0.80 | -- | -- | -- |
| cromakalim | 0.85 | 0.98 | 1.15 | 1.26 | 0.45 | 0.50 | 1.11 | 0.50 |
| flurbiprofen | >37 | >37 | -- | -- | 18.67 | 19.03 | 1.02 | 0.04 |
| ketamine | 0.03 | -- | -- | -- | 0.02 | -- | -- | -- |
| mexiletine | 0.22 | -- | -- | -- | 0.08 | -- | -- | -- |
| mianserin | 0.90 | 1.07 | 1.19 | 1.18 | 0.43 | 0.50 | 1.16 | 0.68 |
| naproxen | >30 | >30 | -- | -- | 8.95 | -- | -- | -- |
| nicotine | 0.02 | -- | -- | -- | 0.02 | -- | -- | -- |
| paroxetine | 7.76 | 8.18 | 1.05 | 0.54 | 3.20 | 3.34 | 1.04 | 0.23 |
| propranolol | 3.40 | -- | -- | -- | 1.44 | -- | -- | -- |
| verapamil | 1.71 | -- | -- | -- | 0.77 | -- | -- | -- |
| warfarin | 15.57 | 22.22 | 1.43 | 3.12 | 4.53 | 6.09 | 1.34 | 2.42 |

[a] mobile phase - [methanol - water (40:60, v/v)], 0.02 M ammonium formate, formic acid 2 ml/L

[b] mobile phases - [methanol - water (50:50, v/v)], 0.02 M ammonium formate, formic acid 2 ml/L

[c] flow rate – 1.0 ml $min^{-1}$; UV detection at $\lambda_{max}$ for each of the drugs

In comparing Whelk-O1 CSP and ACE-5-C18 retention data (Fig. 5.3d) (Table 5.3, 4) it appeared at first that the achiral selectivity of the two phases was orthogonal (Fig. 5.4) ($r^2$=0.3967 for *k v k*). However on closer inspection it was clear that there was a suggestion of linearity in the majority of the data points. The exact degree of linearity is dependent on the data points included in the computation. The most optimistic interpretation ($r^2$ = 0.9672 for 12 data points) is also shown in Figure 5.4 If considering the data point for naproxen which is the most obvious outlier, a bimodal retention mechanism for the Whelk-O1 CSP could be suggested whereby the fastest eluting enantiomer is retained by a general hydrophobic interaction mechanism similar to that that is taking place on ACE 5 C18 while the slowest eluting enantiomer is interacting significantly with the chiral recognition site on the CSP. This is not inconceivable given that this CSP was originally designed to separate the enantiomers of naproxen (Pirkle*et al*., 1992). However this would not explain the other outliers. For flurbiprofen, for which there is good enantioresolution, retention is actually reduced on the Whelk O1 CSP compared to what would be expected for general hydrophobic interaction retention. For the other outliers, propranolol and verapamil, there was no discernable resolution but it could be postulated that in these cases both enantiomers interacted significantly with the chiral recognition site.

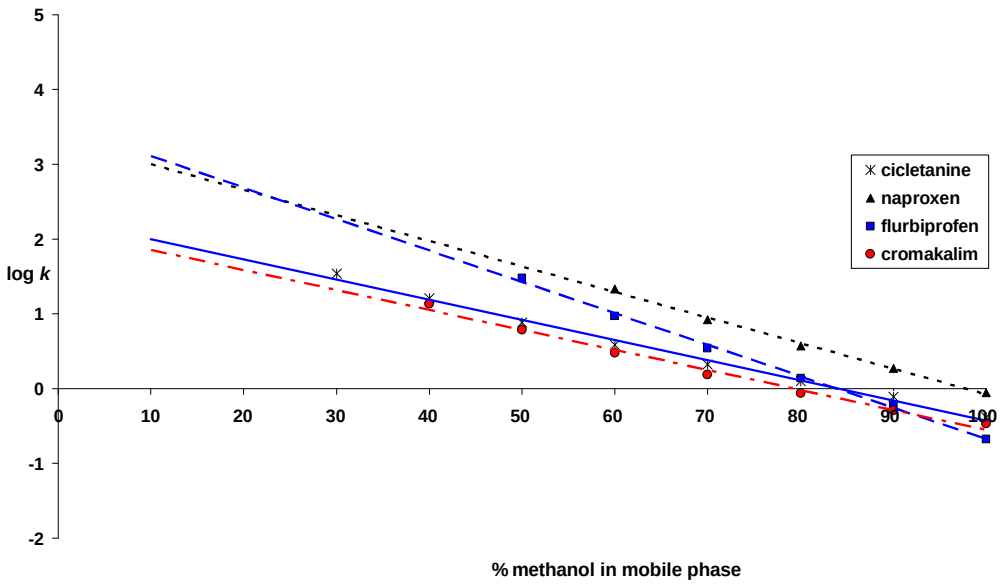



Figure 5.3d Relationship between log *k* and % methanol in the mobile phase for cicletanine, naproxen, flurbiprofen and cromakalim on Whelk-O1; Mobile phases – [methanol - water], 0.02 M ammonium formate, formic acid 2 ml/L; flow rate – 1.0 ml $min^{-1}$; UV detection at $\lambda_{max}$ for each of the drugs.

Table 5.3 Retention data for RPLC of set of 16 racemic drugs on ACE 5 C18 (150 mm x 4.6 mm I.D.) and Whelk-O1 columns (250 mm x 4.6 mm I.D.).

| **compounds** | ***k* (ACE 5 C18)** | ***k* (Whelk-O1)** |
|---|---|---|
| baclofen | 0.14 | 0.38 |
| brompheniramine | 0.45 | 1.48 |
| chlorpheniramine | 0.37 | 1.27 |
| cicletanine | 0.88 | 2.07 |
| citalopram | 0.34 | 1.21 |
| cromakalim | 0.57 | 1.52 |
| flurbiprofen | 3.22 | 3.40 |
| ketamine | 0.13 | 0.38 |
| mexiletine | 0.33 | 0.46 |
| mianserin | 0.54 | 1.46 |
| naproxen | 1.68 | 8.32 |
| nicotine | 0.06 | 0.22 |
| paroxetine | 0.79 | 2.06 |
| propranolol | 0.47 | 2.50 |
| verapamil | 0.43 | 4.27 |
| warfarin | 1.87 | 5.68 |

[a] mobile phase - [methanol - water (70:30 v/v)], 0.02 M ammonium formate, formic acid 2 ml/L

[b] mean *k* for two resolved or partially resolved peaks

[c] flow rate – 1.0 ml min-1; UV detection at $\lambda_{max}$ for each of the drugs

Table 5.4 Retention, selectivity and resolution data for RPLC of 16 racemic drugs on Whelk-O1 (250 mm x 4.6 mm I.D.).

| compounds | $k_1$ | $k_2$ | A | $R_s$ |
|---|---|---|---|---|
| baclofen | 0.38 | -- | -- | -- |
| brompheniramine | 1.48 | -- | -- | -- |
| chlorpheniramine | 1.27 | -- | -- | -- |
| cicletanine | 1.86 | 2.14 | 1.15 | 1.59 |
| citalopram | 1.21 | -- | -- | -- |
| cromakalim | 1.34 | 1.57 | 1.17 | 1.53 |
| flurbiprofen | 3.15 | 3.45 | 1.10 | 1.24 |
| ketamine | 0.38 | -- | -- | -- |
| mexiletine | 0.46 | -- | -- | -- |
| mianserin | 1.38 | 1.43 | 1.04 | 0.07 |
| naproxen | 5.85 | 10.37 | 1.77 | 8.86 |
| nicotine | 0.22 | -- | -- | -- |
| paroxetine | 2.06 | -- | -- | -- |
| propranolol | 2.50 | -- | -- | -- |
| verapamil | 4.27 | -- | -- | -- |
| warfarin | 4.25 | 6.80 | 1.60 | 6.92 |

[a] mobile phase - [methanol - water (70:30 v/v)], 0.02 M ammonium formate, formic acid 2 ml/L

[b] flow rate – 1.0 ml $min^{-1}$; UV detection at $\lambda_{max}$ for each of the drugs

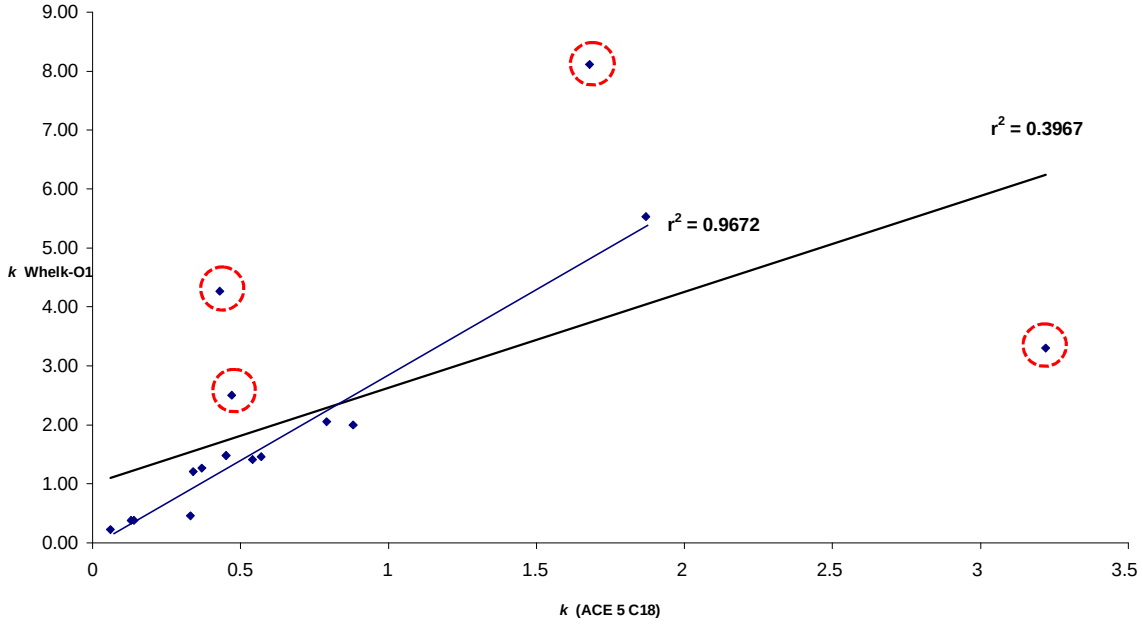



Figure 5.4 *k* (Whelk-O1) *v k* (ACE 5 C18) for RPLC of set of 16 racemic drugs with mobile phase - [methanol - water (70:30 v/v)], 0.02 M ammonium formate, formic acid 2 ml/L; flow rate – 1.0 ml $min^{-1}$; UV detection at $\lambda_{max}$ for each of the drugs. The encircled data points represent those not taken into account when determining $r^2 = 0.9672$ for 12 compounds.

Irrespective of the details of the analysis, there was certainly enough to suggest that the Whelk O1 CSP had very good achiral selectivity. Also, while not being not so efficient as would be expected for an alkyl - silica phase column of the same dimensions (N~6400, *cf* N ~9950 for ACE 5 C18; (for Chiralpak QD-AX N was 4700 – 5600 and for Cyclobond 1 2000 DNP N was ~2000)), the Whelk O1 CSP was clearly efficient enough to give peak capacities sufficient for related

substances work. This begged the question that if the Whelk-O1 CSP had similar retentivity and achiral selectivity to a typical achiral C-18 silica and only differed when there was a stereoselective interaction, then perhaps it might be possible that it could be used on its own for the RPLC simultaneous determination of enantiomeric impurity and related substances rather than needing to be used in combination with an achiral column. This indeed proved to be the case. The most difficult part of demonstrating such a possibility was actually acquiring full sets of structurally related impurities of chiral drugs. Therefore initially it was demonstrated that the drug (*S*)-naproxen could be resolved from (*R*)-naproxen and a set of structurally similar naphthalene-containing compounds on the Whelk-O1 CSP (Fig. 5.5). Subsequently the anti-hypertensive agent, laevokalim, was subjected to forced degradation and it was demonstrated (Fig. 5.6) that the drug, its enantiomer and the degradants could be separated from one another. Flurbiprofen is generally administered as a racemate but consideration is being given to marketing the S-enantiomer as a single enantiomer drug in some markets, e.g. (http:www.biospectrumasia.com), using a process of preparing the racemate and then carrying out a resolution. Indeed even the R- enantiomer might have potential as a single enantiomer drug given the interest in activity related to prostate cancer (Quann *et al*., 2007) and Alzheimer's disease (Geerts, 2007). It was therefore appropriate to demonstrate a separation on Whelk-O1 of (*S*)-flurbiprofen from (*R*)-flurbiprofen from all the structurally-related impurities of racemic flurbiprofen (Fig. 5.7), This separation of all related substances checked by the manufacturer represents a significant achievement compared to simply analysing actual production samples (in which not all the related substances might

be present). Also, it was noteworthy that the racemic related substances were not only resolved from one another but also were resolved into their individual enantiomers. For all three of these applications, the mobile phase optimisation was of a fairly routine nature. This suggested that using this approach might be generally facile and not cause difficulties which are not counterbalanced by the efficiencies gained.

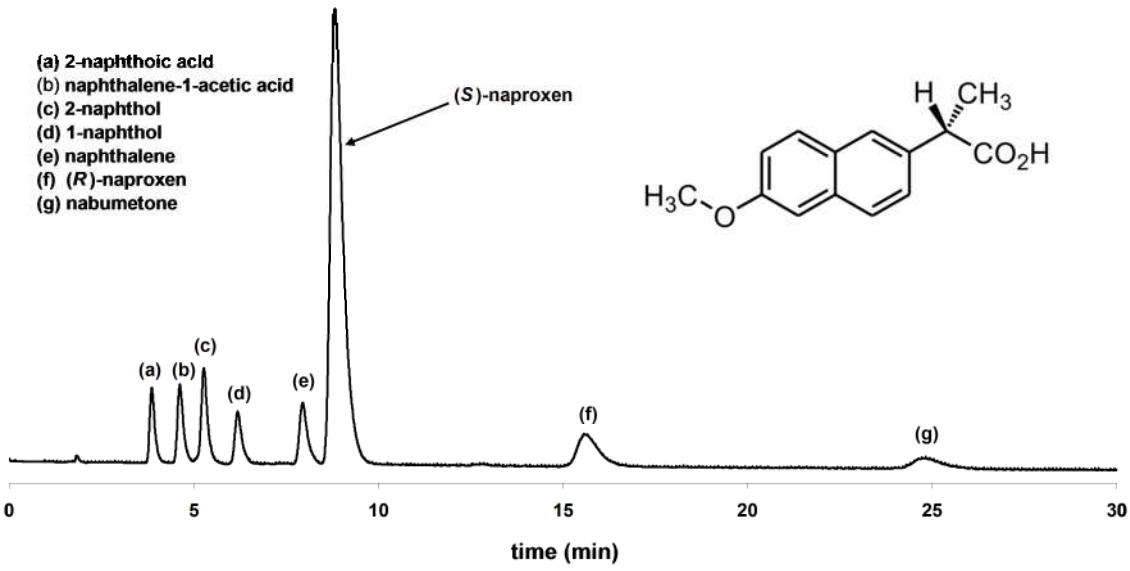



Figure 5.5 RPLC of naproxen, its enantiomer and related compounds on Whelk-O1 (250 mm x 4.6 mm I.D.). 1.0 mg $ml^{-1}$ in mobile phase, enantiomer and related compounds present at ~ 10% w/w; Mobile phase - [methanol - water (65:35 v/v)], 0.02 M ammonium acetate, acetic acid 1 ml/L; flow rate – 1.5 ml $min^{-1}$; UV detection at 254 nm.

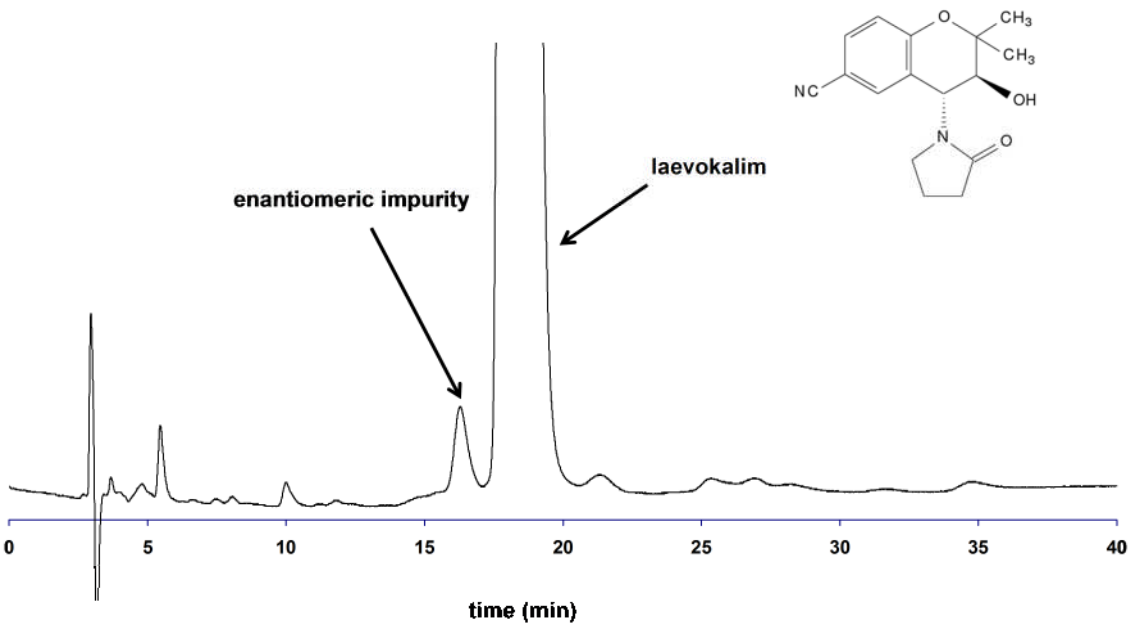



Figure 5.6 RPLC of laevokalim, enantiomer and degradants on Whelk-O1 (250 x 4.6 mm I.D.). 0.5 mg $ml^{-1}$ in mobile phase, enantiomer present at 0.3% w/w; Mobile phase - [methanol - water (45:55 v/v)], 0.02 M ammonium formate, formic acid 2 ml/L; flow rate – 1.0 ml $min^{-1}$; UV detection at 254 nm.

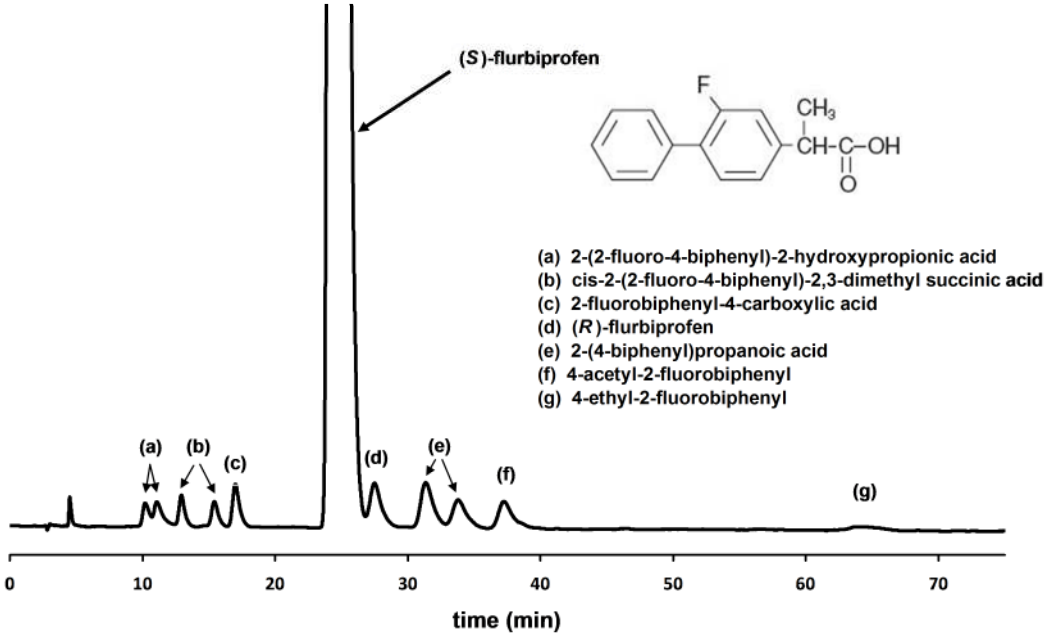



Figure 5.7 RPLC of flurbiprofen, its enantiomer and related substances on Whelk-O1 (250 x 4.6 mm I.D.). 0.2 mg $ml^{-1}$ in mobile phase, enantiomer and related substances present at 1% w/w; Mobile phase - [methanol - water (55:45 v/v)], 0.04 M ammonium formate, formic acid 0.5 ml/L; flow rate – 1.0 ml $min^{-1}$; UV detection at 254 nm.

## 5.4 Conclusions

Overall, the impression gained from the studies carried out was that the success rate of obtaining chiral separations using polar organic solvent – aqueous mobile phases rich in the organic component was greater than might have been imagined. This encouraged the notion that columns containing CSP could be used in series with achiral C-18 silica columns using this type of mobile phase to develop separations of active pharmaceutical ingredient, enantiomeric impurity and related substances. Having discounted other CSP in the initial selection process for this type of application, it was possible to demonstrate that, using mobile phases containing a high proportion of a polar organic solvent, Chiralpak QD-AX and Whelk-O1 CSP both exhibited similar retentivity to C-18 silicas and that significant chiral resolution was obtained on the Whelk-O1 for a wider range of compounds. However, since the achiral or compound selectivity of the Whelk-O1 in the absence of a stereoselective interaction was good and similar to that of ACE-5-C18, rather than being orthogonal, under reversed-phase LC conditions when using a high proportion of polar organic solvent, it could be used directly for the simultaneous determination of active pharmaceutical ingredient, enantiomeric impurity and related substances rather than having to resort to its use in coupled column systems. This possibility was demonstrated for (*S*)-naproxen, laevokalim and (*S*)-flurbiprofen. While Bicker (Bicker *et al*., 2004) exploited the achiral selectivity of a CSP to separate all stereoisomers of a diastereomer, this is the first time a single CSP has been used to separate a drug enantiomer from its antipode and from a series of related substances.

Further work could now be carried out with a wider range of compounds and mobile phases to confirm that the good achiral selectivity of Whelk-O1 has genuine promise for this type of application and that the success obtained here was not just a function of the nature of the compound set used. For example, as already intimated, the Whelk O1 CSP was designed to separate the enantiomers of naproxen (Pirkle*et al*., 1992), so there might be a skew in a small set of compounds containing an unrepresentative number of compounds with structural similarity to naproxen. It would also be worthwhile to ascertain if any CSP other than the Whelk O1 CSP exhibited good achiral and chiral selectivity with similar retentivity to C18 silicas under reversed-phase LC conditions. This could include further study of the Chiralpak QD-AX (exploring its use with higher pH mobile phases) and the Cyclobond 1 2000 DNP CSP (exploring coupling with less retentive achiral RPLC stationary phases). Such studies are worth pursuing not just on the basis of providing savings through a reduced number of assays being used on drug substances but also because it underscores the need to ensure that all assays developed to determine enantiomeric impurity need to be specific not just with respect to the enantiomeric impurity peak being resolved from the main peak but with respect to other related substances being resolved from the enantiomeric impurity peak. Little was done by the way of evaluating the orthogonal selectivity of Chiralpak QD-AX. However, judging from previous in-house work on anion-exchangers (Watson, Perera, 2004) Chiralpak QD-AX would be expected to be orthogonal by virtue of having very little achiral selectivity for anions. Also, by virtue of its use of an element, at least, of an inclusion

complexation retention mechanism, Cyclobond 1 2000 DNP would be expected to show different achiral selectivity from alkyl-bonded silica phases. Both these likely differences might prove useful in developing achiral or achiral-chiral separations.

## 6a RP-HPLC and NP-HPLC Chiral Stationary Phase Optimisation: nicotine

### 6a.1 Introduction

In the final stages of the research programme not only had orthogonal stationary phase selectivity been considered but also a considerable amount of data had been gathered on the enantioselectivity of a range of CSP in both normal phase and reversed-phase modes of LC. In other words things were in place to be able to conduct chiral screening in a better informed manner. Accordingly it was decided to take advantage of this.

To reiterate, the separation of drug enantiomers by LC using chiral stationary phases had been relatively common place since the late 1980's (Lough, 1989) and, accordingly, with the wide range of more advanced CSP available today (Lough, 2003) it was rare to find a chiral organic compound which still presented a significant challenge in terms of being able to achieve reliable enantioresolution with at least one set of conditions. Often success could be had simply by resorting to one of the so-called 'gold standard' CSP such as Chiralcel OD and Chiralpak AD without having to consider a range of complementary CSP. However this proved not to be the case for nicotine (Fig. 6a.1) for which a simple method was required to determine trace levels of the unnatural enantiomer. Nicotine, which is present in tobacco products, exists naturally as the (S)-enantiomer with only trace

levels of the (R)-enantiomer but recent studies (Perfetti *et al*., 1998) had shown that about 2-4% of (*R*)-nicotine is present in tobacco smoke. Clearly it would have been useful to have methodology available to be able to carry out further studies to establish whether this ratio is constant or is affected by various variables during processing and the smoking process (*e.g.* high temperature during deep inhalation). Therefore, it was sought to develop a method for the determination of two enantiomers qualitatively and quantitatively, to enable analysis of (*R*)-nicotine present in a variety of nicotine samples from different sources.

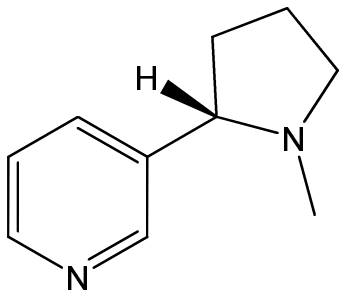



Figure 6a.1 (*S*)-nicotine (3-(1-methyl-2-pyrrolidinyl) pyridine), the naturally-occurring enantiomer.

## 6a.2 Experimental

### 6a.2.1 Instrumentation

The HPLC systems used for the chiral separation of nicotine in normal phase employed a Spectra-Physics (San Jose, CA, USA) SP8810 precision isocratic pump with LDC Analytical (Riviera Beach, USA) spectroMonitor 3100 detector and a Shimadzu (Milton Keynes, UK) LC-6A pump and Shimadzu SPD-6AV UV-vis spectrophotometric detectors. In each case, a manual Rheodyne (Kotati, Ca, USA) 7125 loop injection valve, fitted with a 20 µl loop, was used for loading samples. Data was collected using a Dionex (Leeds, UK) PC-based data system with Automated Computer Interface and *AI* 450 Chromatographic Automation Software Release 3.33. The sonicator used to degas mobile phases was from GS Group-ULTRAWAVE Ltd, Cardiff, CF2 1YY. Berkshire, UK.

### 6a.2.2 Materials

Mobile phases were prepared using HPLC – grade hexane, propan-2-ol, methanol, trifluoroacetic acid and triethylamine (Sigma-Aldrich, Poole, Dorset, UK). The columns containing chiral stationary phases that were used were Chiralcel OJ (10 µm) (250 mm x 4.6 mm I.D.) a gift from Pfizer Global Reseach (Sandwich, Kent, UK) and Chiralcel OJ-H (5 µm) (250 mm x 4.6 mm I.D.) was from Chiral Technologies Europe, (Illkirch, France). The ACE 5 CN (150 mm x 4.6 mm I.D.) column used was a gift from Hichrom Ltd., (Theale, UK). The column block

heater used was from Jones Chromatography Ltd, Henged, Wales, UK. Racemic nicotine and (*S*)-nicotine were from Sigma – Aldrich (Poole, Dorset, UK). The samples collected from various smoking processes that were analysed in the study were provided by an anonymous donor.

### 6a.2.3 Methods

Initial experiments involved a Chiralcel OJ (10 µm) (250 mm x 4.6 mm I.D.) column and a mobile phase with hexane – IPA - TFA, 85:15:0.1, v/v/v using a 20 µl injection volume of racemic nicotine. The TFA in mobile phase was replaced with TEA and later both TFA and TEA were added at a concentration of 0.1 in 100.1 v/v in total (*e.g*. TFA – TEA, 0.08:0.02, 0.06:0.04, 0.05:0.05, v/v/). The flow rate used was 1 ml $min^{-1}$ and the work was carried out at ambient temperature. The next stage of the experiment involved a Chiralcel OJ (10 µm) (250 mm x 4.6 mm I.D.) column and a mobile phase with hexane – IPA - TFA – TEA, 85:15:0.05:0.05, v/v/v/v using a 20 µl and 10 µl injection volumes of racemic nicotine at ambient and higher temperatures (30 and 35 $^{o}$C respectively) with a flow rate of 0.5 ml $min^{-1}$. The subsequent studies involved a Chiralcel OJ-H (5 µm) (250 mm x 4.6 mm I.D.) column and a mobile phase with hexane – IPA - TFA – TEA, 85:15:0.05:0.05, v/v/v/v using a 20 µl injection volumes of racemic nicotine with a flow rate of 1 ml $min^{-1}$ at 35 $^{o}$C.

### 6a.3 Results and Discussion

A review of the literature revealed that while nicotine enantiomers could be separated using the Chiral AGP CSP, the method was not simple and involved carrying out LC at 15 $^{o}$C to get sufficient resolution (Demetriou *et al*, 1993). There was also reference to the use of a Chirex 1034 CSP in some commercial literature (Tang *et al.*, 1998). However, the only other viable alternative appeared to be was to use the Chiralcel-OJ CSP, interestingly demonstrating the value of complementary CSP and not relying entirely on the most commonly used CSP such as Chiralpak AD and Chiralcel OD. The initial conditions involved a Chiralcel OJ (10 µm) (250 mm x 4.6 mm, I.D.) column and a mobile phase of hexane – IPA – TFA (85:15:0.1, v/v/v). Although baseline resolution was obtained, the peak shape was not ideal (Fig. 6a.2), certainly not good enough to allow high loadings to be able to observe low levels of trace enantiomer, even when working with low concentrations and/or low flow rates. The use of triethylamine instead of triflouroacetic acid to improve peak shape resulted in complete loss of resolution. As the next stage of this experiment, both TFA and TEA were used to improve the peak shape while retaining a satisfactory resolution of both enantiomers. The method was optimised firstly to find the ratio of the TEA and TFA additives, maintaining the additive concentration at 0.1% that gave the best resolution. Subsequent improvements were made by using a much lower sample concentration and increasing the column temperature to 35 $^{o}$C. Finally the OJ column was replaced with an equivalent OJ-H column (i.e. containing 5 µm particles). Using the final mobile phase of hexane – IPA – TFA - TEA

(85:15:0.05:0.05, v/v/v/v) (*R*)-nicotine eluted after the natural (*S*)-nicotine ($k_R$ 1.74, $k_S$ 4.77, $\alpha$ 2.74, $R_S$ 8.96) (Fig. 6a.3).

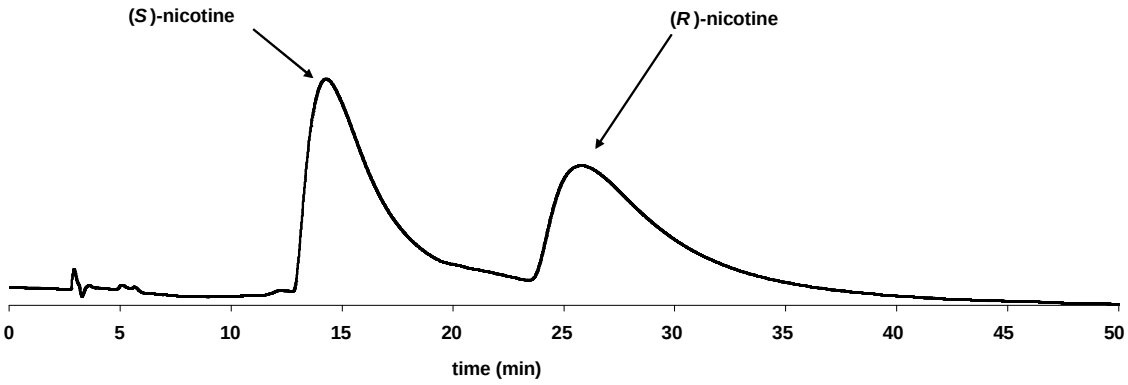



Figure 6a.2 HPLC of nicotine and its enantiomer on Chiralcel OJ (10 µm) (250 mm x 4.6 mm, I.D.); Mobile phase – [hexane – IPA – TFA (85:15:0.1, v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 260 nm, ambient temp (22-25 $^oC$).

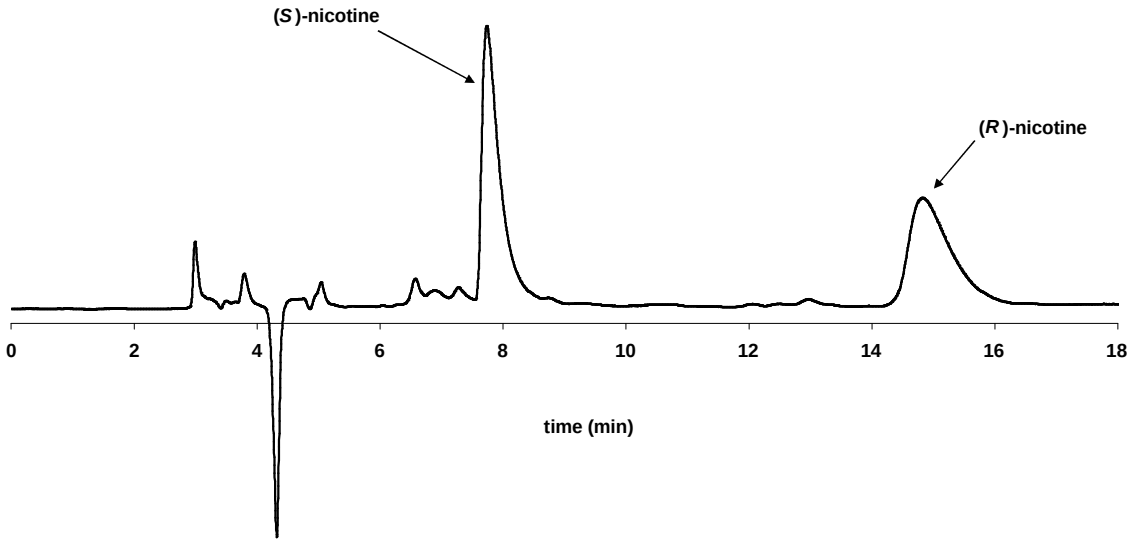



Figure 6a.3 HPLC of nicotine and its enantiomer on Chiralcel OJ-H (5 µm) (250 mm x 4.6 mm, I.D.); mobile phase – [hexane – IPA – TFA –TEA (85:15:0.05:0.05, v/v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 260 nm, 35 $^{o}$C.

On using these conditions to analyse nicotine samples from smoking experiments it was found that there were a large number of interfering peaks (Fig. 6a.5). On considering the options for sample pre-treatment it was decided that the simplest approach most likely to be successful quickly would be to collect nicotine peaks separated out in a normal phase LC system. Doing this off-line using an ACE 5 CN (150 mm 4.6 mm I.D.) with methanol as mobile phase, evaporating off methanol and reconstituting in mobile phase, it was possible to demonstrate that the low level of (*R*)- in (*S*)-nicotine had not been changed by the smoking process (Fig. 6a.6). The methodology developed would clearly be suitable for the study of

the ratio of nicotine enantiomers in a number of different situations and has the potential for being converted to a rapid on-line method if that was required.

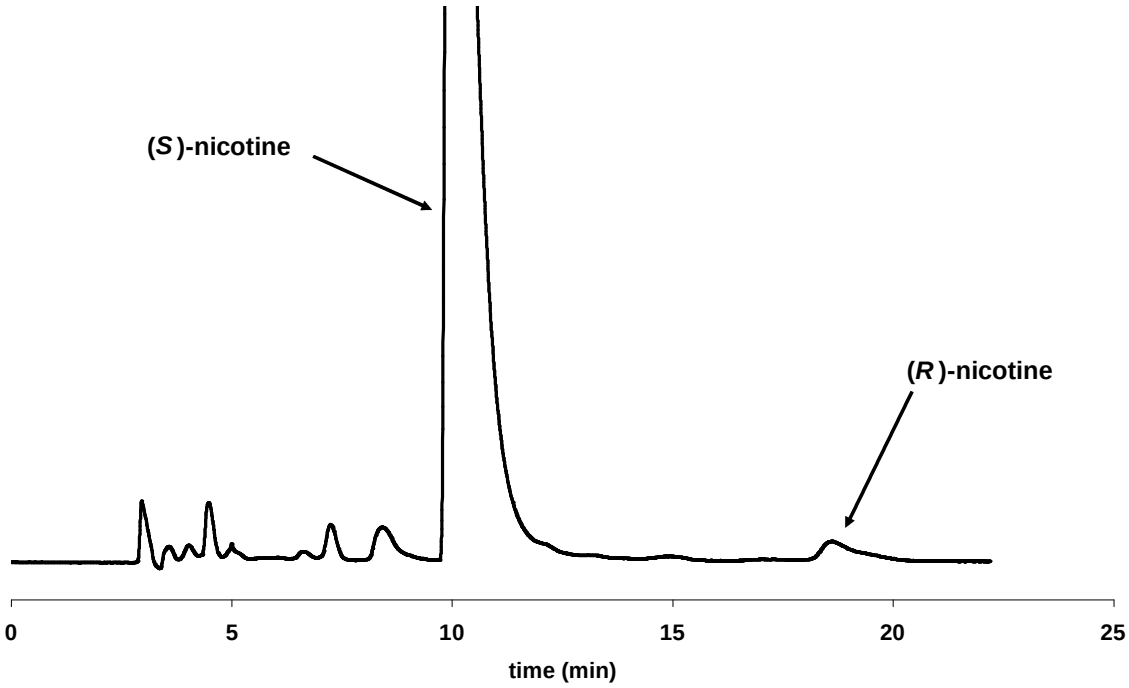



Figure 6a.4 HPLC of (*S*)-nicotine on Chiralcel OJ-H (5 µm) (250 mm x 4.6 mm, I.D.); mobile phase – [hexane – IPA – TFA –TEA (85:15:0.05:0.05, v/v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 260 nm, 35 $^{o}$C.

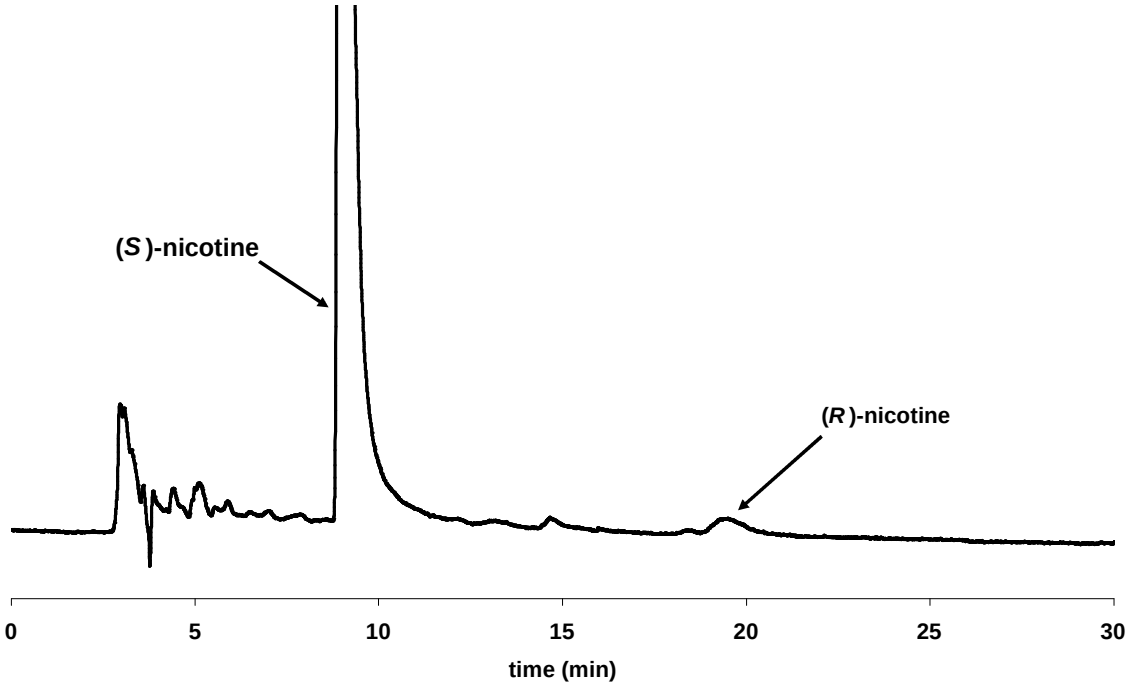



Figure 6a.5 HPLC of nicotine after smoking process, reconstituted in mobile phase, on Chiralcel OJ-H (5 µm) (250 mm x 4.6 mm, I.D.); mobile phase – [hexane – IPA – TFA –TEA (85:15:0.05:0.05, v/v/v/v)]; flow rate – 1.0 ml $min^{-1}$; UV detection at 260 nm, 35 $^{o}$C.

## 6a.4 Conclusion

The resolution and peak shape finally obtained for the separation of nicotine enantiomers along with the achiral LC clean-up was suitable for the determination of (*R*)-nicotine in tobacco-related samples containing nicotine. The methodology developed would clearly be suitable for the study of the ratio of nicotine enantiomers in a number of different situations and has the potential for being converted to a rapid on-line method if that was required.

In terms of chiral LC method development using screening approaches, this example of nicotine constitutes a good example of the valuable contribution that can be made by one of the slightly less commonly used CSP.

## 6b RPLC & NPLC Chiral Stationary Phase Optimisation: mephedrone

### 6b.1 Introduction

As has already been discussed in Chapter 3, chiral LC screening for the development of methods for the separation of drug enantiomers had already been well established and more recent work on such screens was related to achieving even higher ‘hit-rates’ (Anderson, 2003), substituting with new CSP (Dossou, 2010; Peng, 2010; Younes, 2011; Younes, 2011) or obtaining greater efficiency. In the context of the latter, from the work described in Chapter 3, more was now known about redundancies with respect to selectivity amongst CSP, especially in the context of clones, and about which CSP are likely to be complementary. Accordingly things were well placed to put this knowledge to good use. The racemic, former ‘legal-high’ drug, mephedrone (Fig. 6b.1) (Garnett, 2011) was an ideal example for employing a screen based on the CSP that had already been studied (Chapter 3). Also, for completeness some reversed-phase work was carried out in the screening along with ongoing developments in exploring the retentivity and selectivity of CSP in the reversed-phase mode (Chapter 5).

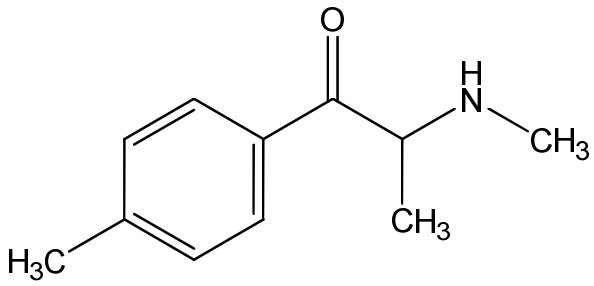



Figure 6b.1 A former ‘legal-high’ drug, mephedrone.

Being sold widely under the guise of being a plant food, there had been much interest in mephedrone in the media and popular press e.g. (Garnett, 2011) after a number of adverse effects had been reported (Torrance and Cooper, 2010) and ultimately it had been banned in the UK, being designated as a Class B drug (Garnett, 2011). Surprisingly, despite the continuing interest in mephedrone and related cathinone (De Paoli *et al*., 2011) and other drugs-of-abuse (Abdel-Hay *et al*., 2010), little or no attention seemed to have been paid to the chiral nature of the molecule. While the cathinones were named after the original, natural source, the African plant Khat (Chappell, 2010; BBC-Health, 2012) they were prepared synthetically in clandestine laboratories as racemates. However, especially in light of possible adverse effects, it is important that such drugs were studied as the individual enantiomers whether this be to isolate samples of the individual enantiomers for subsequent pharmacological or toxicological testing or to monitor the *in vivo* fate of the drug. The importance of looking at the individual enantiomers was best expressed by Ariens who talked of the “scientific non-sense” of trying to draw conclusions based on data derived from studies using the racemate. To fully appreciate the implications of the stereochemistry of these new drugs it was not

sufficient just to draw comparisons with older, well-studied drugs (Maickel *et al*., 1982). It was decided to embark on a chiral LC screening exercise using CSP that had previously been studied in order to arrive at conditions that could be optimised to give a method for the determination of the individual mephedrone enantiomers and/or be used for scale-up to allow the preparative isolation of the enantiomers.

## 6b.2 Experimental

### 6b.2.1 Instrumentation

The HPLC systems used for the chiral separation of mephedrone employed Spectra-Physics (San Jose, CA, USA) SP8810 precision isocratic pump, connected to LDC Analytical Spectromonitor and Shimadzu (Milton Keynes, UK) LC-6A pump connected to Shimadzu SPD-6AV UV-vis spectrophotometric. In each case, a manual Rheodyne (Kotati, Ca, USA) 7125 loop injection valve, fitted with a 20 µl loop, was used for loading samples. Data was collected using a Dionex (Leeds, UK) data system. Water was distilled and doubly de-ionised using an ELGA Option 3 Water purifier (ELGA, High Wycombe, Bucks., UK). The sonicator used to degas mobile phases was from GS Group-ULTRAWAVE Ltd, Cardiff, CF2 1YY. Berkshire, UK.

### 6b.2.2 Materials

Mobile phases were prepared using HPLC – grade hexane, propan-2-ol, trifluoroacetic acid and triethylamine (Sigma-Aldrich, Poole, Dorset, UK). Of the columns containing chiral stationary phases that were used in normal phase chiral screening, Chiralcel OJ-H (5 µm) (250 mm x 4.6 mm I.D.) was from Chiral Technologies Europe, (Illkirch, France) and RegisCell (5 µm) (250 mm x 4.6 mm I.D.), RegisPak (5 µm) (250 mm x 4.6 mm I.D.), Whelk-O1 (older) (5 µm) (250 mm x 4.6 mm I.D.), Whelk-O1 (10 µm) (250 mm x 4.6 mm I.D.) and Whelk-O1 (newer) (5 µm) (250 mm x 4.6 mm I.D.) were from Regis Technologies, Inc., Morton Grove, IL 60053, USA). Also other columns containing chiral stationary phases that were used such as Whelk-O1 (5 µm) (250 mm x 4.6 mm I.D.), Cyclobond I 2000 DNP (250 mm x 4.6 mm I.D.) and Chirobiotic V (250 mm x 4.6 mm I.D.) were gifts from Regis Technologies, Inc., Morton Grove, IL., USA and Sigma-Aldrich Chemie, GmbH, Taufkirchen, Germany respectively. The mephedrone (designated M4 to distinguish it from other such samples) was purchased on the Internet (www.mrmeph.com) prior to mephedrone being designated as a classified drug.

### 6b.2.3 Methods

Initial chiral screening of mephedrone involved Chiralcel OJ-H (5 µm) (250 mm x 4.6 mm I.D.), RegisCell (5 µm) (250 mm x 4.6 mm I.D.), RegisPak (5µm) (250 mm x 4.6 mm I.D.), Whelk-O1 (older) (5 µm) (250 mm x 4.6 mm I.D.), Whelk-O1 (10 µm) (250 mm x 4.6 mm I.D.) columns and two mobile phases of hexane - IPA –

TFA (85:15, v/v) with TFA and TEA as additives at a concentration of 0.1 using a 20 µl injection volumes of racemic mephedrone. Later both TFA and TEA were added at a concentration of 0.1 in 100.1, v/v in total (*e.g*. TFA – TEA, 0.075:0.025, 0.05:0.05, v/v). The mobile phase used to run mephedrone on Whelk-O1 (5 µm) (250 mm x 4.6 mm I.D.) column was hexane – IPA – TFA – TEA, (90:10:0.05:0.05, v/v/v/v).

10.3 mg of mephedrone was dissolved in a 20 ml volume of 0.05 M aq. sodium hydroxide and then mephedrone was extracted into two 20 ml volumes of dichloromethane in a 50 ml separative funnel. Two dichloromethane layers were collected and a 20 µl volume of it was injected to HPLC system with Whelk-O1 5 µm column using 100% dichloromethane as the mobile phase. As a next step, 20 µl of mephedrone was injected to the system with well conditioned 100% chloroform as the mobile phase. In another step, dichloromethane in extracted mephedrone solution was evaporated and mephedrone residue was dissolved in chloroform and run with 100% chloroform as a mobile phase.

### 6b.3 Results and Discussion

In embarking on a screening approach to method development to obtain a separation of the enantiomers of mephedrone, CSP to be used were selected to a limited extent on the basis of availability but primarily on the basis of success rates and extent to which the CSP might be complementary in enantioselectivity, as apparent from the literature and previous work (Chapter 3). Both normal and

reversed-phase mobile phase conditions were used on the basis that normal phase conditions might be preferable for using a separation to isolate enantiomers and reversed-phase conditions might be preferable for a method for determining the drug in biological fluids. Having said that, a normal phase method could easily be used for drug bioanalysis and a reversed-phase method could be used for preparative work. For normal phase work, Whelk O1, Chiralcel OJ-H, RegisCell, and RegisPack CSP were used, the latter two being clones of Chiralcel OD and Chiralpak AD respectively and having been shown to behave in an almost identical fashion to these two Daicel counterparts (Chapter 3). For reversed-phase work, Whelk O1 and AmyCoat were used, the latter being a clone of the derivatised polysaccharide CSP, Chiralpak AD. Rather than using other derivatised polysaccharide CSP, Chirobiotic V and Cyclobond DNP were used since they were designed for and have been successful in reversed-phase use. The results for a total of sixteen different sets of conditions are shown in Table 6b.1.

Table 6b.1 Retention, selectivity and resolution data obtained with different chiral columns and mobile phases in normal phase HPLC screening method development aimed at achieving a separation of the enantiomers of mephedrone.

| Mobile Phase | **Chiralcel OJ-H** 250 mm X 4.6 mm i.d. | | | | **RegisCell** 250 mm X 4.6 mm i.d. | | | | **RegisPack** 250 mm X 4.6 mm i.d. | | | | **Old Whelk - O 1** 250 mm X 4.6 mm i.d. | | | | **Whelk - O 1, 10 µm** 250 mm X 4.6 mm i.d. | | | | **Whelk-O 1, 5 µm** 250 mm X 4.6 mm i.d. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ |
| 1 | 0.54 | -- | -- | -- | 0.53 | 0.59 | 1.11 | 0.59 | 0.27 | -- | -- | -- | 0.67 | 0.94 | 1.41 | 1.93 | 1.01 | 1.25 | 1.23 | 0.89 | -- | -- | -- | -- |
| 2 | 0.24 | -- | -- | -- | 0.06 | 0.09 | 1.71 | 0.43 | 0.35 | 0.45 | 1.30 | 1.43 | 1.97 | -- | -- | -- | 2.48 | -- | -- | -- | -- | -- | -- | -- |
| 3 | -- | -- | -- | -- | 0.42 | 0.54 | 1.27 | 0.92 | -- | -- | -- | -- | 0.94 | 1.33 | 1.42 | 2.92 | -- | -- | -- | -- | -- | -- | -- | -- |
| 4 | -- | -- | -- | -- | 0.23 | -- | -- | -- | -- | -- | -- | -- | 0.63 | 0.87 | 1.39 | 1.99 | 0.75 | 0.95 | 1.28 | 1.51 | -- | -- | -- | -- |
| 5 | -- | -- | -- | -- | 0.80 | 1.01 | 1.27 | 1.30 | -- | -- | -- | -- | 1.60 | 2.53 | 1.58 | 4.73 | 1.90 | 2.84 | 1.49 | 3.94 | 1.74 | 2.75 | 1.59 | 5.90 |

Mobile Phase 1: hexane - IPA - TFA, (85:15:0.1 v/v/v)

Mobile Phase 2: hexane - IPA - TEA, (85:15:0.1 v/v/v)

Mobile Phase 3: hexane - IPA - TFA - TEA, (85:15:0.05:0.05 v/v/v/v)

Mobile Phase 4: hexane - IPA - TFA - TEA, (85:15:0.075:0.025 v/v/v/v)

Mobile Phase 5: hexane - IPA - TFA - TEA, (90:10:0.05:0.05 v/v/v/v)

Table 6b.2 Retention, selectivity and resolution data obtained with different chiral columns and mobile phases in reversed phase HPLC screening method development aimed at achieving a separation of the enantiomers of mephedrone.

| Mobile Phase | **Chirobiotic V** 250 mm X 4.6 mm i.d. | | | | **Cyclobond I 2000 DNP** 250 mm X 4.6 mm i.d. | | | | **AmyCoat** 250 mm X 4.6 mm i.d. | | | | **Whelk - O 1** 250 mm X 4.6 mm i.d. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ | $k_1$ | $k_2$ | α | $R_s$ |
| 1 | -- | -- | -- | -- | 0.37 | -- | -- | -- | 0.47 | -- | -- | -- | 3.25 | -- | -- | -- |
| 2 | 0.00 | -- | -- | -- | 0.00 | -- | -- | -- | 0.00 | -- | -- | -- | 0.27 | -- | -- | -- |

Mobile Phase 1: [methanol - water, (70:30 v/v)], 0.02 M ammonium formate, formic acid 2 ml/L

Mobile Phase 2: [methanol - water, (70:30 v/v)], 0.02 M ammonium acetate

No chiral resolution was obtained in the reversed-phase work. Drawing comparisons with normal phase work and successful reversed-phase work on acidic and neutral chiral drugs [chapter 3], it may be that for on the Whelk O1 and Cyclobond DNP CSP in reversed phase, the mobile phase needs to be more basic than used here to obtain successful enantioresolution. The best 'hit' was obtained on the Whelk O1 CSP under normal phase conditions (Fig. 6b.2).

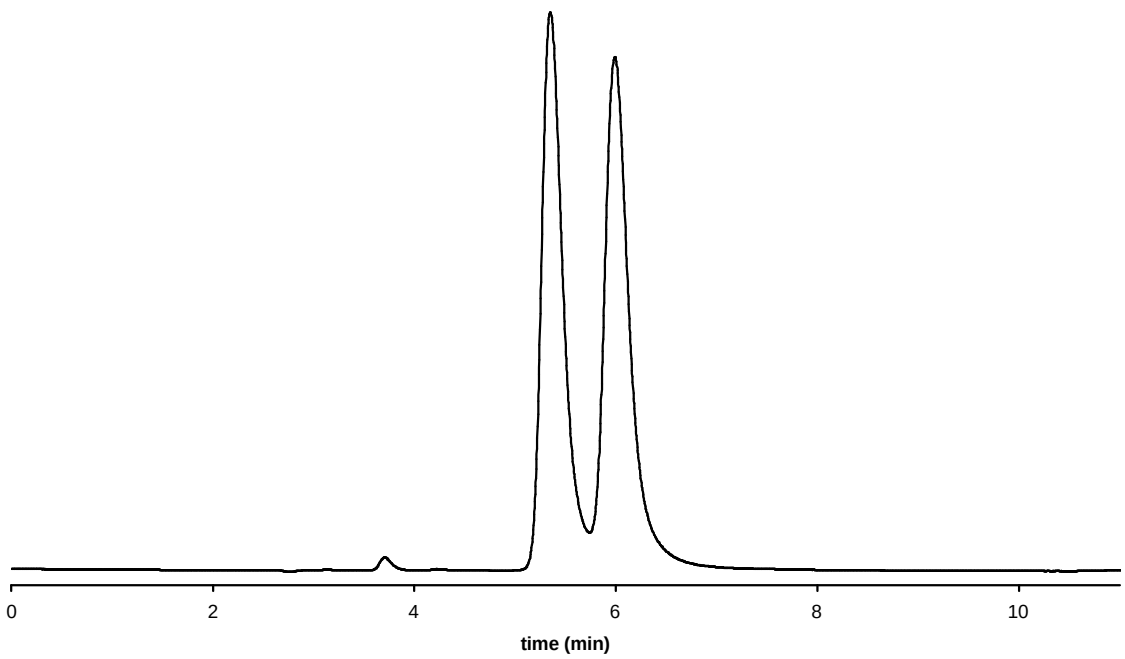



Figure 6b.2 HPLC of mephedrone on Whelk - O1 (10 µm) (250 mm x 4.6 mm, I.D.); mobile phase - [hexane - IPA - TFA -TEA (85:15:0.075:0.025, v/v/v/v)]; flow rate - 1 ml $min^{-1}$; UV detection at 260 nm.

The optimisation process from this point onwards was quite simple and involved switching to a newer version of the Whelk O1 material which used a 5 µm rather than a 10 µm particle and Exsil Silica rather than Kromasil Silica, changing the ratio of isopropanol to n-hexane and adjusting the ratio of acidic to basic mobile phase additive. The optimised separation is shown in Fig. 6b.3

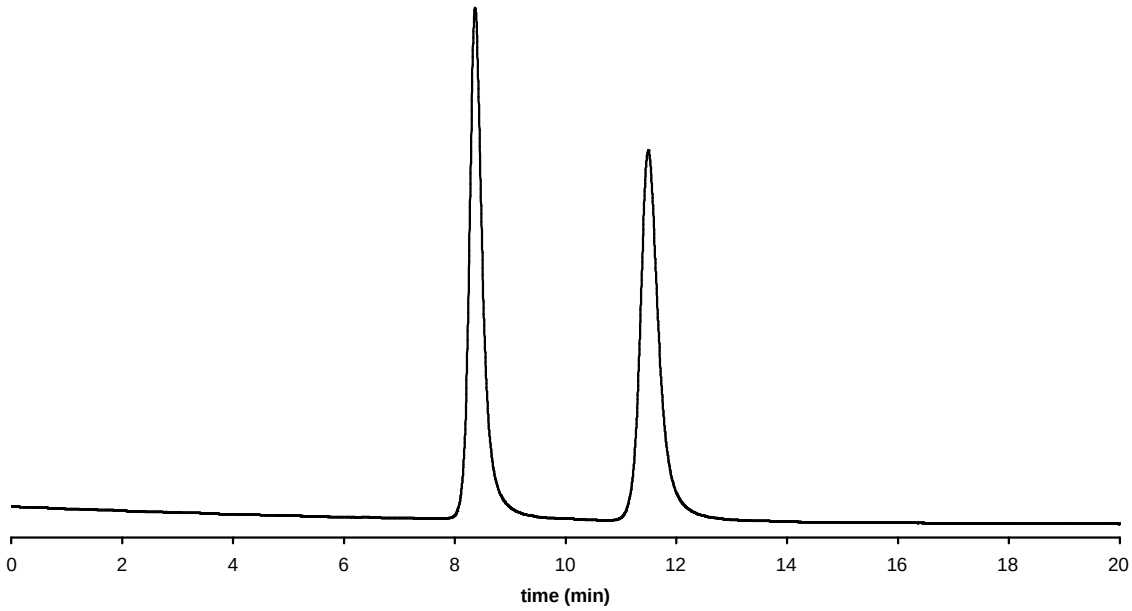



Figure 6b.3 HPLC of mephedrone on Whelk - O1 (5 µm) (250 mm x 4.6 mm, I.D.); mobile phase - [hexane - IPA - TFA -TEA (90:10:0.05:0.05, v/v/v/v)]; flow rate - 1 ml $min^{-1}$; UV detection at 260 nm.

Clearly the resolution obtained was suitable to be the basis of a quantitative method. Also, the resolution was such that there would be scope for losing resolution during scale-up but still having reliable baseline resolution. However, before progressing to this and considering the scale of the resolution on the Whelk O1 CSP and previous success in resolving underivatised drugs during achiral derivatisation studies using dichloromethane as a mobile phase (Project work, BSc Chemical and Pharmaceutical Science, University of Sunderland, UK), an attempt was made to separate the enantiomers of mephedrone free base using on Whelk O1 using dichloromethane as mobile phase. This was successful (Fig. 6b.4) but during subsequent scale-up work, peak shape and stability in solution problems were encountered.

Mephedrone extracted in dichloromethane was run on Whelk-O1 5 µm column using 100% dichloromethane as the mobile phase and only one peak was obtained. However, mephedrone extracted in dichloromethane was injected to the system with 100% chloroform it was, surprisingly, separated within 8-15 min time. The separation, retention and the stability in dichloromethane was confirmed as it showed a similar separation when the run was repeated several times. At the next stage of the study, mephedrone residue in extracted mephedrone solution was dissolved in chloroform and run with 100% chloroform as the mobile phase, but no clear separation of two peaks were observed, instead showed several peaks which gave an indication that mephedrone may not be stable in chloroform.

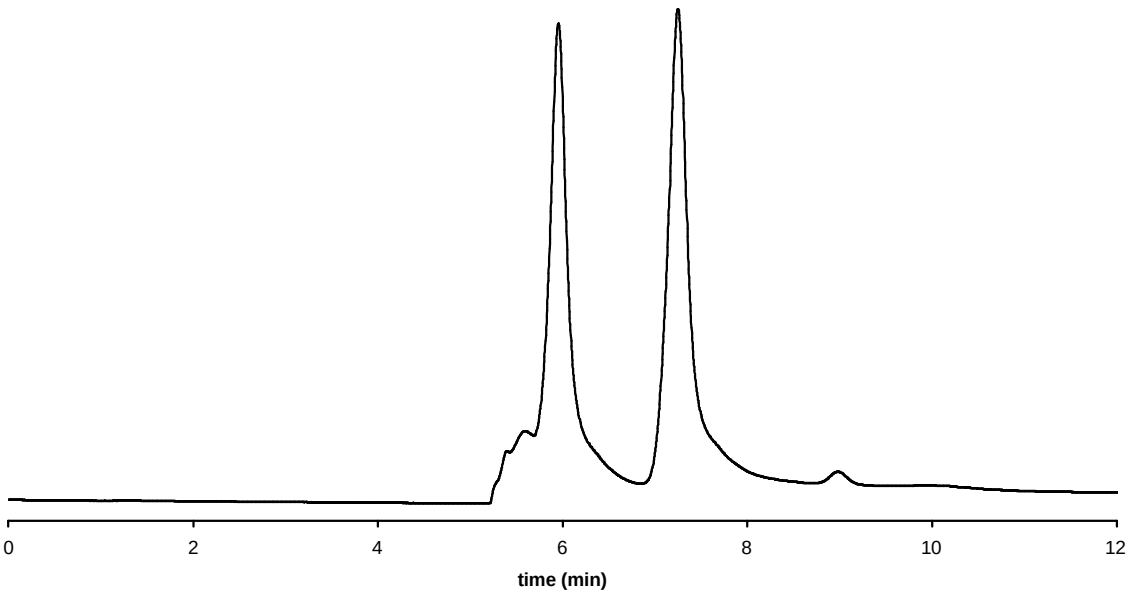



Figure 6b.4 HPLC of mephedrone on Whelk - O1 (5 µm) (250 mm x 4.6 mm, I.D.); mobile phase - chloroform; flow rate - 1 ml $min^{-1}$; UV detection at 260 nm.

It was then decided to proceed to scale-up using the mobile phase / stationary phase combination used to obtain the chromatogram shown in Fig. 6b.5. Because of the availability of automated injection systems and automated fraction collectors in modern laboratories, it was decided to restrict the scale up study to more concentrated solutions of drug in mobile phase rather than to increase sample

solubility by using an alternative sample solvent. Also it was decided to look for the point to which reliable baseline resolution was just maintained rather than to consider overload and peak-shaving options (discussed in) (Cox, 2012). This exercise proved to be extremely facile as evidenced by the chromatogram shown in Fig. 6b.5, starting with an injection volume of 100 µl.

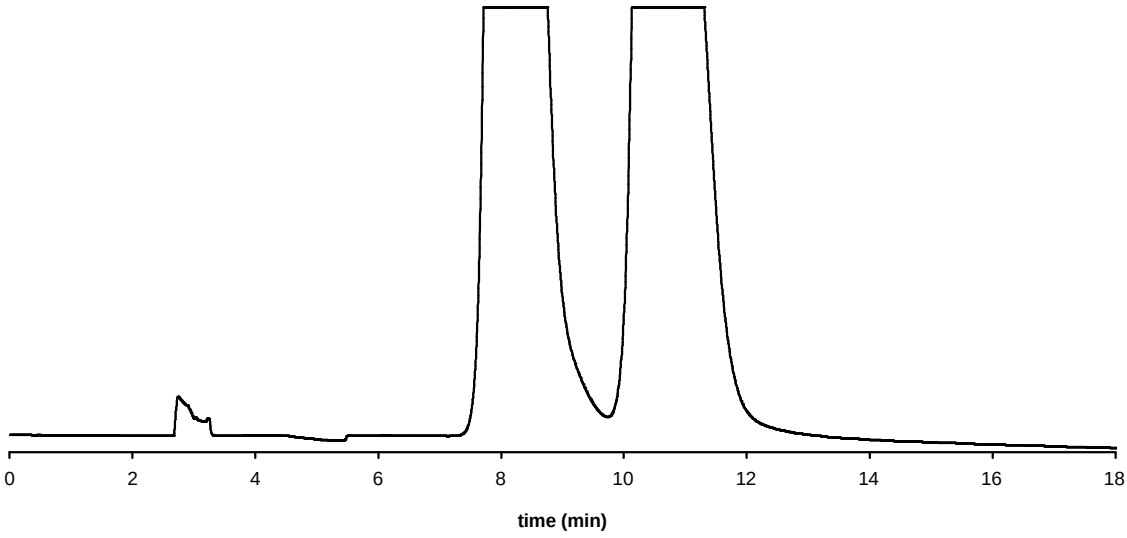



Figure 6b.5 HPLC of mephedrone on Whelk - O1 (5 µm) (250 mm x 4.6 mm, I.D.); mobile phase - [hexane - IPA - TFA -TEA (90:10:0.05:0.05, v/v/v/v)]; flow rate - 1 ml $min^{-1}$; Inj.100 µl of 2.5 mg $ml^{-1}$; UV detection at 260 nm.

Further work on throughput, using high concentration and larger injection volumes was subsequently performed by another student (S. Gupta, Project of MSc.Drug Discovery and Development, University of Sunderland, 2010-11). Also calculations of throughput on automated injections and/or larger columns, which may be carried out in the future, were made, the work not being extended to a very large scale because of compound availability.

### 6b.4 Conclusions

This example on mephedrone constituted another good illustration of the success of the screening approach to chiral LC method development using the CSP that had been studied earlier in the research programme. Like the case of nicotine, there was little success with the reversed-phase element of the screen. However, again like nicotine, this may be a feature of the drug being basic.

Obvious further work on this would be to establish whether the Whelk O1 CSP was suitable for all the cathinone drugs or whether it would be necessary to carry out chiral screening method development for every individual cathinone.

## 7. General Conclusions and Suggestions for Future Work

Conclusions from the individual studies involved in this research programme are many and these have been indicated at the end of each chapter in this thesis. While it could be said that the major achievement of the research programme was generating a large volume of information on stationary phase retentivity and selectivity, this is not necessarily important in itself. More significant is that it helps regarding the general understanding of stationary phase selectivity and retentivity and that the information can be applied to develop better separations.

With respect to the selectivity of achiral stationary phases it was found that, contrary to manufacturer claims (HPLC Columns RESTEK), the Ultra PFP phase was not different in selectivity to C18 phases, at least not for two sets of compounds containing a drug and structurally-related compounds i.e. amitriptytline metabolites and acemetacin related substances. Having said this, there is evidence (HPLC Columns RESTEK) from the manufacturer of some difference in selectivity in certain (specially selected?) cases. There might be a case for future work studying compound sets not quite so challenging as drug related substances. In this way it might be possible to assess the extent to which PFP-like phases only show different selectivity for specially designed ‘probes’.

At the outset of the work on ZIC-HILIC it had been sought to establish whether it had orthogonal selectivity or reversed selectivity to C18 phases. The retention plots for amitriptyline metabolites (Fig. 2.6) and for endogenous metabolites (Fig.

2.13) shed much light on the retention properties of the ZIC-HILIC column and, in fact, it was found that, depending on the mobile phase conditions, it could have the same selectivity, orthogonal selectivity or reversed selectivity! Unfortunately, because of mobile phase considerations, none of these make it useful for use in combination with C18 phases. This was achieved in the case of acemetacin (Fig. 2.11, 2.12) but generally this can only be done when *k* is low and selectivity relative to C18 in reversed-phase is difficult to predict, especially with additional complications arising from possible ion-exchange interactions. Having said this, interest in the HILIC mode (normal phase with polar mobile phases) will continue because it can be used to achieve retention of very polar compounds and is very compatible with LC-MS.

Importantly, in terms of selectivity that is reliably orthogonal to C18 in reversed-phase, it was confirmed that in the absence of any other promising phases Hypercarb and ion-exchange phases remain the best options. This was exemplified by the coupling of specified Spherisorb 5 SCX and ACE 5 C18 column lengths to give conditions suitable for the separation (Fig. 2.28) of baclofen from its much more hydrophobic related substance, impurity A which was rapid, robust and avoided the use of ion-pairing agents.

The studies on chiral stationary phases (described in Chapter 3) generated much data, were highly informative and gave very clear cut useful messages. The comparison of higher generation Pirkle-type CSP demonstrated (Tables 3.1, 3.2 and 3.3) very clearly that the Whelk-O1 CSP was far superior to ULMO and DACH

DNB CSP the Whelk-O1 giving enantioseparation for a very high proportion of the set of 50chiral compounds and the others giving very few with even fewer being better than what was achieved on the Whelk-O1. The Whelk-O1 CSP is therefore most definitely the Pirkle-type CSP of choice for inclusion in a chiral method development screen. If Regis Technologies Inc., the manufacturers of ULMO and DACH wish to continue to sell these CSP they will need to look for mobile phases with which they perform much better or else specifically search for compounds for which ULMO and DACH outperform Whelk-O1. As discussed previously (Chapter 3), they seem, at least partially, to be addressing this.

The message from the study of Chiralcel OD and clones thereof was equally clear. All the clones (CulluCoat, RegisCell, Nucleocell Delta S), with the exception of CelCoat, were very similar to the original Chiralcel OD from Daicel and could be used almost interchangeably with it. CelCoat gave lower degrees of chiral resolution and was less retentive. So, moving forward to a chiral method development screen scenario, any one of Chiralcel OD, CelluCoat, RegisCell and Nucleocell Delta S could be used. There would be no point in using more than one.

Similarly, the RegisPak and AmyCoat clones were very similar to ChiralPak AD. For the Daicel immobilised version, Chiralpak IA, the loss in enantioresolution due to immobilisation was not too marked. The performance of Chiralcel OJ relative to Chiralpak AD and Chiralcel OD was much better than that of DACH and ULMO

relative to Whelk-O1 but still there were not too many examples (nicotine, cicletanine and ketamine) where it would be the first choice CSP.

All-in-all, the large volume of data on retention, selectivity and resolution generated on Whelk-O1, ULMO, DACH DNB, Chiralcel OD, Chiralpak AD, Chiralcel OJ-H, will be useful not only in chiral method development screens but also in parallel analysis, 2D-LC and in coupled column systems in method development in drug analysis. In fact, within the scope of the research programme, it was possible to develop some good illustrative applications of the knowledge gained.

A very good example was the use of Whelk-O1 for both chiral and achiral separations. It was found that even a CSP with a relatively complex structure such as Whelk O1 was very similar to alkyl-silicas in selectivity (and retentivity). However, this applied to compounds, mainly bases, which were not separated into their enantiomers. It could be that it could still serve as an orthogonal stationary phase to alkyl-silicas for compounds that are separated into their enantiomers by interacting in an appropriate manner with the chiral selector. This would include single enantiomer acidic and neutral drugs of which there are many. Going with this line of thinking, it might be worthwhile revisiting CSP such as cyclodextrins and macrocyclic antibiotics despite their less retentive nature than C18 silicas. Measures could be taken to increase their retentivity while still maintaining their enantioselectivity. As was found in this research programme, CSP and screening approaches to chiral method development in normal phase LC are highly effective

but, for CSP used in reversed phase with similar retentivity to C18 silicas, there is not the same breadth of spectrum of enantioselectivity.

In terms of chiral LC method development using screening approaches, the example of nicotine constitutes a good example of the valuable contribution that can be made by one of the slightly less commonly used CSP, Chiralcel OJ-H. The example on mephedrone constituted another good illustration of the success of the screening approach to chiral LC method development and the case of optimisation. Like the case of nicotine, there was little success with the reversed-phase element of the screen. However, again like nicotine, this may be a feature of the drug being basic. The mephedrone success highlighted the point that chiral screening even with two or three CSP can be highly effective. The case of nicotine where Chiralcel OJ was needed was perhaps an exception to the rule.

Going back to taking the body of work as a whole and setting it in a more general context, it is possible to identify several overriding themes. From the work on achiral stationary phases, and indeed chiral stationary phases, using polar organic solvents with aqueous buffers as mobile phases, the findings from previous work that a hydrophobic interaction mechanism dominates and that it is very difficult to find stationary phases that are genuinely orthogonal to typical modern C-18 silicas such as ACE-C18 were reaffirmed. For orthogonality of selectivity it is necessary to look for stationary phases that operate through a completely different retention mechanism. In this context and as already stated, the use of ion-exchange phases such as Luna-SCX (Soo, 2003) remains one of the most useful options. Hypercarb

(Soo, 2003) remains an interesting possibility despite inherent practical idiosyncracies such as the development of high back pressures after storage and changes in retention properties after use with totally retained compounds. Accordingly, rather than continuing to look for new materials, it might be better to look at the newer 3 μm version of Hypercarb or related materials such as the ZirChrom-CARB (ZirChrom products catalogue 2006, http:www.zirchrom.com) used by Carr and Stoll (Stoll *et al*., 2006). The latter might be easier to use in routine work but it remains to be seen just how much of the intriguing selectivity features of Hypercarb are found in this alternative carbonaceous material. With respect to ion-exchange phases, the baclofen application could be taken further to demonstrate that it is stability-indicating. More importantly, a phase mixture could be used instead of coupling columns. This would be an excellent opportunity to demonstrate that this highly versatile (phase mixing) approach is robust and compatible with use in short columns.

Although the important point from the study of the achiral phases ZIC-HILIC and PFP was the further underlining that when using polar organic solvent – aqueous buffer mobile phases it is very difficult to find stationary phases that give significantly different retention orders to ODS silicas, several lessons were learned from the individual studies. The most telling lesson though from the applications work was that while an approach of coupling columns containing orthogonal stationary phases may be useful, it is best used when the obvious occasion arises. The case of baclofen was a very good example and, as indicated already, has the potential to be taken through to a fully validated stability-indicating assay suitable

for pharmacopoeial use and would be a suitable vehicle for demonstrating the robustness of conducting coupled systems through mixed phase packed columns. This mixed phase approach could even include UPLC (UHPLC), while it might be fair to say that, as yet, the range of stationary phases developed for UPLC does not differ sufficiently in selectivity, this is changing rapidly and it would be a fruitful area of future research to develop very fast, selective methods using the mixed phase approach in UHPLC and also when using fused core shell technology materials.

Overall then, more still needs to be done to identify more stationary phases with orthogonal selectivity to alkyl-silicas. Beyond that, the best way forward would be to take the most interesting findings of this programme and consider where they might be optimally applied in the general area of where selective stationary phases can be exploited; to reiterate i.e. difficult (isomer) separations, screening approaches in method development, coupled column or phase systems or in 2D-LC. With respect to the latter area, the instrumentation is now becoming more available so the use of truly orthogonal phases in each dimension should see wider application for this approach in complex mixture analysis including applications of interest at the University of Sunderland such as screening for drugs of abuse and the analysis of plant extracts.

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## Appendix

## Summary of Research Training

- "The Future of Preparation, Detection and Data Processing for Chromatography",The Chromatographic Society Spring Symposium, Shimadzu UK, Milton Keynes, 24th May 2006.
- "Exploiting Instrumentation and Automation to Support Pharma R and D",The Chromatographic Society Golden Jubilee "Triad" Meeting, GSK Medicines Research Centre, Stevenage, Herts, 21st & 22nd November 2006.
- "Advances in Liquid Separations and Hyphenated Techniques"& Chromatographic Society AGM, The Natural History Museum, London, 16th May 2007.
- Research Student Training Programme - Generic Core Course 2, Friday 18th January 2008.
- Visiting Lecturer's Training Course, 20th September, 2007 onwards.
- Autumn Sunderland Meeting and Big Prep 4, Stadium of Light, Sunderland, 23rd August 2007
- Waters North East Technology Seminar on UPLC, University of Sunderland, 12th March 2008.
- "New Development in Column and stationary Phase Technology", Chromatographic Society Spring Meeting & AGM, 21st-22nd May 2008, Madejski Stadium, Reading, UK.
- Research Student Training Programme – Generic Core Course 3, Friday 20th June 2008.

- Sigma-Aldrich/Supelco Seminar - “Chiral Chromatography Training Day”, Theatre 068, Lecture Centre, Brunel University, Kingston Lane, Uxbridge, London, UB8 3PH, 1st July 2008.
- Analytical Research Forum 2008, University of Hull, 21st – 23rd July 2008.
- “Desty Memorial Lecture for Innovation in Separation Science”, The Royal Institution of Great Britain, 21 Albemarle St. London, 8th October 2008.
- Regular research group seminars, presentations and workshops of the Sunderland Pharmacy School
- Familiarisation visit to ONEX laboratory, January 2008.
- Visiting AGMA Ltd. – To demonstrate and train staff of the analytical department on the methods for assay of their anti-bacterial product using HPLC.
- Laboratory demonstration – Undergraduate Lab classes from, 2007 to 2009.
- Assisting several number of final year students regularly in their project work.
- Advances in High Resolution and High Speed Separations, Chromatographic Society 1-day meeting, Thursday 18th March 2010, AstraZeneca R&D, Alderley Park, Cheshire, UK.
- Current Method Development Srategies in Separation Science, Chromatographic Society Spring Symposium & AGM, Wednesday and Thursday 19th-20th May 2010, Merck Sharpe & Dohme, Hoddesdon, UK.
- “Desty Memorial Lecture for Innovation in Separation Science”, The Royal Institution of Great Britain, 21 Albemarle St. London, 17th September 2010.
- Laboratory Demonstration – Projects of MSc. Drug Discovery and Development 2009/10.

- Chirality 2011, University of Liverpool, 10th to 13th July 2011.
- 'Desty Memorial Lecture for Innovation in Separation Science', The Royal Institution of Great Britain, 21 Albemarle St. London, 5th October 2011.