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Prediction and Description of Enantioselective Separations on Amylose Based HPLC Chiral Stationary Phases

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May, 1997

A Thesis submitted to the Faculty of
Graduate Studies and Research
in partial fulfilment of the requirements of the degree of
Doctor of Philosophy

PREFACE

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ABSTRACT

Amylose based chromatographic chiral stationary phases (CSPs) are widely used throughout liquid chromatography to separate enantiomers, although little is known about the interaction processes operating on these phases.

This thesis describes a systematic study into the retention and enantioselective separation capabilities of the tris (3,5-dimethylphenylcarbmate), tris (R-phenylethylcarbamate) and tris (S-phenylethylcarbamate) stationary phases.

The underlying aim of this work was to obtain an understanding of the solute-stationary phase interactions which are responsible for the chromatographic retention and separation of enantiomers. By development of quantitative structure-enantioselective retention relationships (QSERR), it was possible to identify the primary components involved in the molecular interaction process and describe those interactions in terms of non-empirical solute descriptors.

Results for several different series of racemic compounds indicate that multiple mechanisms are possible and that retention may be described by combinations of several key classes of interaction including hydrogen bonding, electrostatic, lipophilic, charge transfer and steric interactions. Progressive development of these properties from classical point interactions to molecular interactions, supports a proposed theory of chiral recognition which extends from the basic three-point model to a more complex dynamic molecular model. This new model, derived to explain obtained experimental results, addresses issues in chirality which are not adequately covered by previous theory. It is proposed that the original theory is

a static model which does not authentically reflect real systems and as such, cannot account for all experimentally observed molecular interactions of an enantiospecific nature. A modified and improved theory of chiral recognition has been fashioned, which includes the vital aspects of conformational adjustment and molecular fit.

Finally, the first reported application of artificial neural networks in chiral chromatography is documented. Multi-layer feed forward neural networks, with error back propagation, have been used in combination with QSERR as a basis to development of chiral chromatographic expert systems. Resulting networks contained cross validated predictive ability ranging from 94 to 97%.

RÉSUMÉ

Le travail présenté dans cette thèse concerne l'étude d'une des phases stationnaires chirales (PSC) la plus importante en chromatographie en phase liquide à savoir une phase à base d'amylose. Cette phase a été largement utilisée en chromatographie chirale pour séparer un nombre important d'énantiomères. Toutefois, le processus de séparation et le mécanisme d'interaction sur cette phase ont été très peu étudié.

Cette thèse décrit une étude systématique de la rétention et de l'énantioselectivité des phases stationnaires tris (3,5-dimethylphenylcarbamate), tris (R-phenylethylcarbamate), et tris (S-phenylethylcarbamate).

Le but de ce travail a été de comprendre les interactions soluté-phases stationnaires résponsables de la rétention et de la séparation des énantiomères. En développant des études quantitative structure-énantioselectivité (QSERR), il a été possible d'identifier les composantes principales mises en jeu dans le processus d'interaction moléculaire et de décrire ces interactions à travers des descripteurs non-empiriques des solutés.

L'étude des résultats sur des séries différentes de composés racémiques indique que plusieurs mécanismes sont possible. La rétention peut être ainsi décrite par une combinaison d'interaction-clé comme des interactions de type liaison hydrogène, électrostatique, lipophilique, interaction de type transfert de charge ou encore des interactions stériques. Le fait de considérer ces propriétés comme des interactions moléculaires et non des interactions de contact classiques suggère une théorie de la reconnaissance chirale basée essentiellement sur un modèle dynamique complexe.

Le modèle utilisé jusqu'a présent, connu sous le nom du modèle à trois-point, est un modèle statique. Ce dernier ne tient pas compte de l'état réel du système et donc de la nature énantioselective des interactions moléculaires observées expérimentalement.

Nous avons développé une nouvelle théorie de la reconnaissance chirale basée sur des aspects d'ajustement conformationnelle et d'agencement moléculaire. Ce nouveau modèle a été utilisé pour expliquer des résultats expérimentaux qui sont difficilement explicables par le modèle à trois points.

Finalement, les réseaux de neurones artificiels ont été pour la première fois appliqués en chromatographie chirale. Une combinaison des études QSERR et de la méthode de rétro-propagation de l'erreur utilisée en réseau de neurone a permis de développer un système expert pour prédire la rétention sur ce type de phases stationnaires. Les résultats de cette étude donnent un pouvoir prédictif allant de 94 à 97 %.

ACKNOWLEDGEMENTS

First and foremost I am deeply indebted to my thesis supervisor, Professor Irving W. Wainer, Director of the Pharmacokinetics Division, Department of Oncology. For having faith in my abilities and providing guidence, incentive and opportunity throughout, my sincerest thanks.

Secondly, I wish to express my gratitude to my co-supervisor, Dr. Romas J. Kazlauskas, Department of Chemistry, for help and guidence during my studies at McGill.

My gratitude must also go to Dr. W. John Lough, School of Health Sciences, University of Sunderland, for broadening my horizons and providing the encouragement necessary to pursue my research further afield.

I am most grateful to my colleagues, both past and present, from the Montreal General and Royal Victoria Hospitals whom I have worked with over these past years. I must especially thank Nektaria Markoglou and Karen Fried for friendship and support from my arrival at McGill onwards. Thanks also to Dr. Kamal Azzaoui for invaluable assistance in the latter stages of my research.

Finally I wish to gratefully acknowledge the support and funding of the studies conducted in this thesis, by Chiral Technologies Inc., Exton, PA.

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GLOSSARY

HPLC	High Performance Liquid Chromatography
CSP	Chiral Stationary Phase
AGP	α_1 Acid Glycoprotein
HSA	Human Serum Albumin
LEC	Ligand Exchange Chromatography
CD	Cyclodextrin
AD	Amylose tris (3,5-dimethylphenyl) carbamate
AS	Amylose tris (S-phenylethylcarbamate)
AR	Amylose tris (R-phenylethylcarbamate)
SMB	Simulated Moving Bed
NMR	Nuclear Magnetic Resonance
QSERR	Quantitative Structure-Enantioselective Retention Relationships
QSAR	Quantitative Structure-Activity Relationships
QSRR	Quantitative Structure-Retention Relationships
LFER	Linear Free Energy Relationships
MEP	Molecular Electrostatic Potential
MLP	Molecular Lipophilic Potential
HOMO	Highest Occupied Molecular Orbital
LUMO	Lowest Unoccupied Molecular Orbital
CoMFA	Comparative Molecular Field Analysis
Dip	Total Dipole Moment

To My Parents,

Without your love and encouragement,

none of this would have been possible.

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CHAPTER ONE

1 GENERAL INTRODUCTION

Separation of a racemic compound into its constituent enantiomers by high performance liquid chromatography (HPLC), is most routinely performed by use of chiral stationary phases (CSPs). Formation of a transient diastereomeric complex between an optically pure immobilised selector and a solute containing some form of chirality was first performed on a chromatographic column in 1939 by Henderson and Rule[1]. In this study, a column containing lactose as the adsorbent was successfully employed for the resolution of a racemic camphor derivative. However, it was not until the 1970's when HPLC began to increase in popularity, that significant developments were made in the field of chiral separations[2].

In subsequent years as CSPs became more commercially available, it became apparent that the columns could be categorised by the general type of chiral recognition process believed to be operating on the phase. In 1987 Wainer proposed five types of phase with which to classify the 35 commercially available HPLC-CSPs [3].

Type I: Solute-CSP complexes formed by attractive interactions such as hydrogen bonding, π-π interactions and dipole stacking.

e.g. (R)-*N*-(3,5-dinitrobenzoyl)phenylglycine.

(S)-*N*-(3,5-dinitrobenzoyl)leucine.

D-naphthylalanine.

(R)-α-methylbenzylurea.

1-(α-naphthyl)ethylamine derivative.

tert-butylaminocarbonylvaline

Type II: Solute-CSP interactions formed by attractive interactions and through the inclusion of the solute in a chiral cavity or ravine on the CSP.

e.g. cellulose triacetate.

cellulose tribenzoate.

cellulose trisphenylcarbamate.

cellulose tricinnamate.

cellulose tris(3,5-dimethylphenylcarbamate).

Type III: Primary mechanism involves the formation of inclusion complexes.

e.g. poly(triphenyl methyl methacrylate).

microcrystalline cellulose triacetate.

α-cyclodextrin.

β-cyclodextrin.

Type IV: Solute is part of a diastereomeric metal complex which uses an amino acid bound to the stationary phase as one of the ligands.

e.g. proline.

hydroxyproline.

valine.

Type V: CSP is a protein and the solute-CSP complexes are based upon combinations of hydrophobic and polar interactions.

e.g. α_1-acid glycoprotein.

bovine serum albumin.

Isolating the common structural features of the phases in each category and of the solutes for which successful separations were obtained, gave some direction to the process of CSP selection[4,5]. However, in recent years, the number of commercially available CSPs has increased dramatically. Consequently, an increase in the range of racemic compounds for which enantiomeric separations may be obtained has also taken place. This application diversity could not have been known *a priori*, as many phases were developed on a trial and error basis, the primary exception being the phases developed in the Pirkle laboratories[6].

As columns are now available to cover a broad spectrum of enantiomer separations, the development of new phases for chiral separations has slowed down. With a few notable exceptions such as the immobilised macrocyclic antibiotic phases introduced by Armstrong[7], development has mostly been directed at improving the reliability and robustness of currently

available phases, rather than the appreciably more difficult task of effecting new or improved resolutions. However, slight improvements have been made within series of currently available phases.

Rational design of a new CSP requires comprehensive knowledge of the chiral recognition mechanisms operating on current phases, to enable identification of potential new selector candidates. This however, is the Achilles heel of separation science, due to the inherent difficulties involved with its study. As a result, the question of mechanism has been effectively side-stepped by two rational design alternatives termed the reciprocity route and the CSP-family route[8]. The principle of reciprocity of interaction[9] states that if a CSP based upon a compound (R)-A, resolves the racemic mixture of (S)- and (R)-B, then immobilising compound (R)-B should afford a CSP capable of resolving the racemic mixture of (R)- and (S)-A. Although this technique does not specifically identify an interaction mechanism, it requires the proviso that the immobilisation of (R)-B does not affect the original mechanism.

The family route to CSP development essentially exploits the potential of CSPs based on closely structurally related chiral selectors, already applied in useful stationary phases. This approach may afford a range of novel, interrelated phases, however, an understanding of their primary modes of action at the molecular level would undoubtedly have significant impact on CSP design and on the practice of chiral separations in general.

In order to advance the science of chiral chromatography, in contrast to the art of chiral chromatography, detailed comprehension of current chiral recognition is essential.

2 CHIRAL RECOGNITION IN CHROMATOGRAPHY

2.1 Theory

Chiral recognition is a specific aspect of the much broader area of molecular recognition. In chromatographic terms it usually implies the preferential interaction of one solute enantiomer with a second enantiomer immobilised on an inert support. The three-dimensional structure of the solute requires a complementary three-dimensional structure, with which to form discriminating interactions.

To specify the origin of enantioselective adsorption, one must specify the nature of the various interactions between the species involved[10]. The development and use of structure-retention relationships for this purpose is described in this work and is explained in Chapter Two. It is also necessary to define a model with which to characterise the requirements of enantioselective recognition. This was first proposed by Easson and Stedman (1933)[11] with regard to receptor binding. Easson and Stedman took the view that the differential binding of two enantiomers to a common site (in contrast to a previous study suggesting two enantiomers binding to two sites of the same chirality[12]) was primarily responsible for differences in biological activity. They devised the "three-point contact" model in order to exemplify their concept.

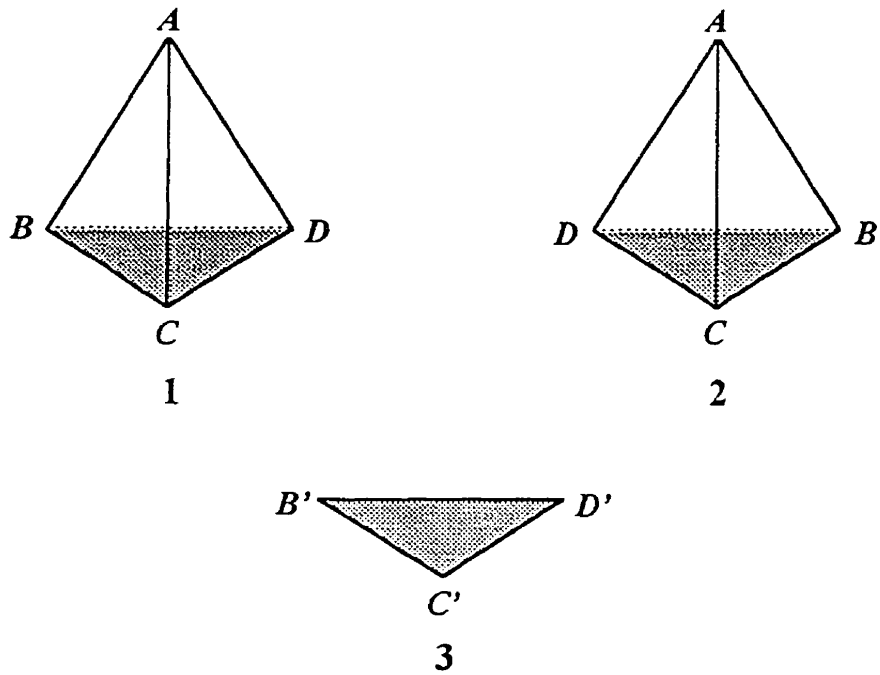


Figure 1. Easson and Stedman's "three-point" interaction model of chiral discrimination.

It was postulated that the more active isomer (1) is the more tightly bound because the sequence of three groups around the chirally substituted carbon atom, BCD, forming a triangular face of the tetrahedral bond array matches a complementary triad of binding sites on the receptor surface, B'C'D' (3), Figure 1. The less active enantiomer (2) is more weakly

complexed as it has a mirror-image sequence of the corresponding three groups, DCB, and so is bound by only two of the three sites on the receptor surface (3), owing to the mismatch.

Ogsten[13] later employed a slight variation of the Easson and Stedman model, to explain the enantiospecific nature of enzymatic reactions. This "three-point binding" theory declared that a substrate must be bound to the enzyme with at least three points in order to ensure enantiospecificity in the enzyme catalysed reaction. The three-point rule differs from Ogsten's theory in that it does not require all three interactions to be attractive.

The first instance of "three-point" theory as applied to chromatography, was reported by Dalgliesh (1952)[14]. Amino acid enantiomers were resolved by paper chromatography and a three-point mechanism postulated for the interaction between the chiral cellulose stationary phase and the solute enantiomers. Amino acid enantiomers had been resolved previously by paper chromatography[15] and the resolution attributed to the chirality of the cellulose.

2.2 Chromatographic Separations

In order for two enantiomers to be separated chromatographically, transient diastereomeric complexes have to be formed between the solute enantiomers and the CSP. There must be a difference in the free energies of binding (ΔG) within the two complexes in order for one enantiomer to be retained more than its opposite counterpart. The energy difference arising from one such interaction would normally be insufficient to permit resolution. However, chromatography is a weighted time-averaged view of many dynamic

adsorption, desorption processes. The sum total of which, through the entire length of a column can be sufficient to allow an observable difference in the retention times of two enantiomers. The difference in free energy ($\Delta\Delta G$), needed for adequate chromatographic separation is influenced by the efficiency of the system employed. Efficiency is related to the process of peak band broadening, and is measured by a theoretical plate count (N).

$$N = \left(\frac{t_r}{\sigma}\right)^2 \qquad (1.1)$$

where t_r is the peak retention time and σ is the peak standard deviation calculated by

$$\sigma = \frac{w_{0.5}}{2.354} \qquad (1.2)$$

and $w_{0.5}$ is the width of the peak at half height.

If the chromatographic system is of high efficiency, so that narrow peaks are observed, relatively small $\Delta\Delta G$ values will afford acceptable analytical scale enantiomer separations.

The distribution of two enantiomerically related analytes between the stationary phase and the mobile phase, are defined by the retention factors k'_1 and k'_2.

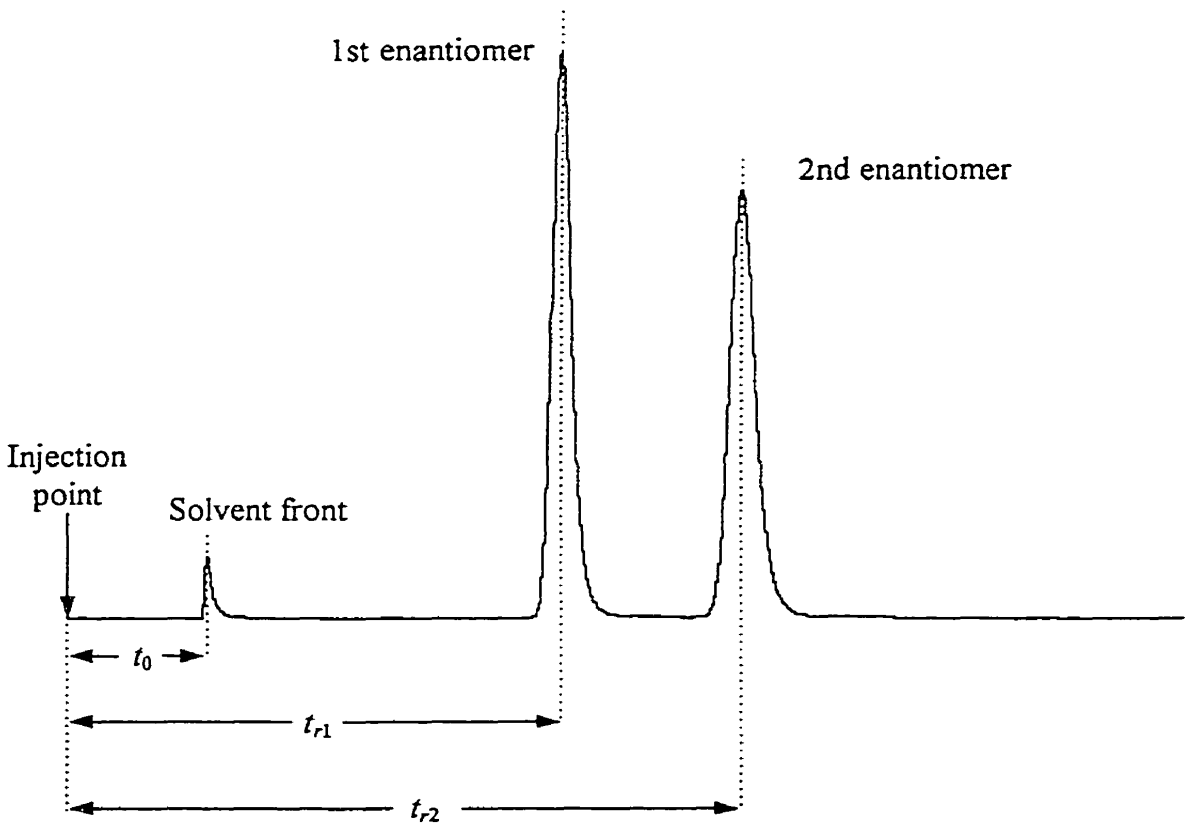


Figure 2. Two enantiomerically related peaks and the measurements required to calculate k'_1, k'_2 and α. t_{r1} is the retention time of the first eluting enantiomer, t_{r2} is the retention time of the second eluting enantiomer and t_0 is the retention time of an unretained solute.

$$k'_1 = \frac{(t_{r1} - t_0)}{t_0} \qquad (1.3)$$

$$k'_2 = \frac{(t_{r2} - t_0)}{t_0} \tag{1.4}$$

$$\alpha = \frac{k'_2}{k'_1} \tag{1.5}$$

$$\Delta G = -RT \ln K \tag{1.6}$$

where K is the equilibrium constant for the distribution of a solute between the stationary phase and the mobile phase

$$\Delta\Delta G = \Delta G_2 - \Delta G_1 \tag{1.7}$$

and ΔG_1 and ΔG_2 are the free energies of binding for the first and second eluting enantiomers respectively

$$\Delta\Delta G = -RT(\ln K_2 - \ln K_1) \tag{1.8}$$

$$\Delta\Delta G = -RT \ln \frac{K_2}{K_1} \tag{1.9}$$

As $k' = \varphi K$ where φ is the column phase ratio, then

$$\Delta\Delta G = -RT \ln \frac{k'_2}{k'_1} \qquad (1.10)$$

$$\Delta\Delta G = -RT \ln \alpha \qquad (1.11)$$

For example, an α value of 1.01 at 25°C, although insufficient for resolution by HPLC, would require a $\Delta\Delta G$ of approximately -25 J mol^{-1}. In practise, complete resolution of two peaks by HPLC requires larger α values.

2.3 Stationary Phase Models

2.3.1 Protein CSPs

Proteins are high molecular weight chiral polymers, and as such, the binding of small enantiomeric molecules is often enantioselective. Charge and hydrophobicity are the two primary properties of proteins which are used to effect retention and resolution in chromatographic systems. Crosslinked proteins are resistant to protein-denaturing organic solvents and thus can be used under conditions similar to those applied in reversed-phase liquid chromatography (LC) on alkylsilica columns. Retention and resolution are regulated via the mobile phase composition, where the pH of the buffer used, the ionic strength and the nature and concentration of the organic modifier are the most important factors.

The two types of protein CSP which have demonstrated the widest application range are the phases based on the serum proteins α_1-acid glycoprotein (AGP) and albumin (SA). Human AGP appears to contain a single drug-binding site with stereoselectivity[16] and human serum albumin (HSA) appears to contain two major drug-binding sites, both with some degree of stereoselectivity[17,18].

The ability of human AGP to bind cationic molecules and the stereoselectivity of this binding is the basis for the chiral resolutions which have been achieved on this phase[19]. The magnitude of the stereochemical resolution of a solute appears to be highly dependent upon molecular structure. Results from AGP studies indicate that the solute should contain at least two bonding groups, for example, an ammonium ion and a hydrogen bonding moiety, in addition to a bulky or rigid structure at or near one of the binding sites. A number of anionic chiral solutes have also been resolved on the AGP-CSP however, these resolutions required the addition of an ion pairing reagent to the mobile phase[20].

Serum albumin phases primarily resolve anionic and neutral enantiomeric solutes. Bovine serum albumin (BSA) appears to require that the solute contain aromatic and polar moieties for successful separation[21]

2.3.2 Ligand-Exchange CSPs

Ligand exchange chromatography (LEC) is separation effected by the preferential mixed chelate complexation of an analyte ligand with a metal ion and a second ligand

introduced into the chromatographic system. The second ligand usually takes the form of an immobilised metal coordinating species[22,23], designed to create a specific solute-stationary phase interaction, or a chiral metal complex mobile phase additive to effect chiral separations on achiral stationary phases[24,25]. This method is typically applicable to the resolution of underivatised α-amino acids.

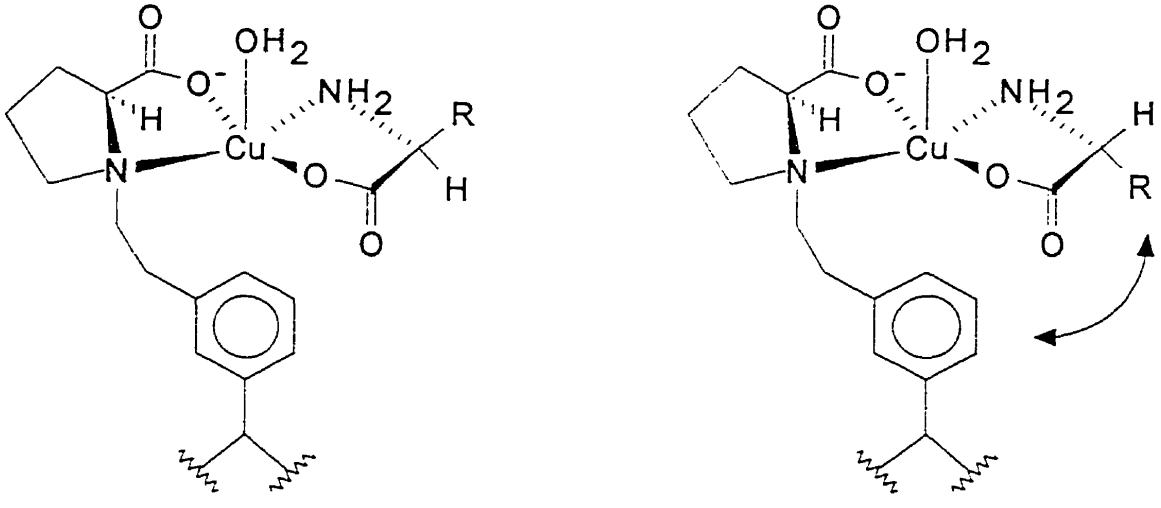


Figure 3. Ligand exchange CSP 1 with a non-polar support matrix (adapted from ref. 10).

CSP 1 prepared with a nonpolar polymer support matrix, selectively retains the analyte enantiomer with the opposite configuration to that of the CSP if R is nonpolar, Figure 3. Favourable hydrophobic interactions between the R group of the analyte and the nonpolar polymer support are believed to increase the stability of heterochiral diastereomeric adsorbate with respect to the homochiral pairing[26,27,28,29].

The polar CSP 2, incorporating a pyridyl ring to provide an axial N ligand for Cu^{2+}, selectively retains the amino acid enantiomer of the same absolute configuration as the CSP, Figure 4.

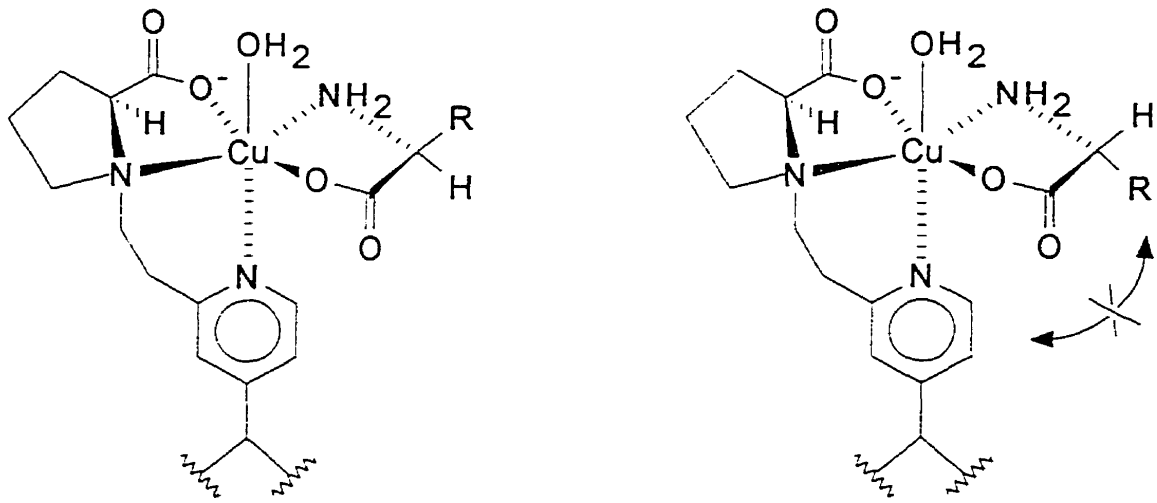


Figure 4. Ligand exchange CSP 2 with a polar support matrix (adapted from ref. 10)

2.3.3 Host-Guest CSPs

Host-guest CSPs are stationary phases which interact with solute molecules via the formation of inclusion complexes. The first example of this type of interaction as applied to chromatography was demonstrated by Cram *et al.*[30,31]. Initial separations of racemic amine salts were obtained using crown ethers such as the binaphthyl derivatives of 22-crown-6

(Figure 5), as mobile phase additives for liquid-liquid chromatography with $NaPF_6$ or water-$LiPF_6$ solutions supported on silica gel.

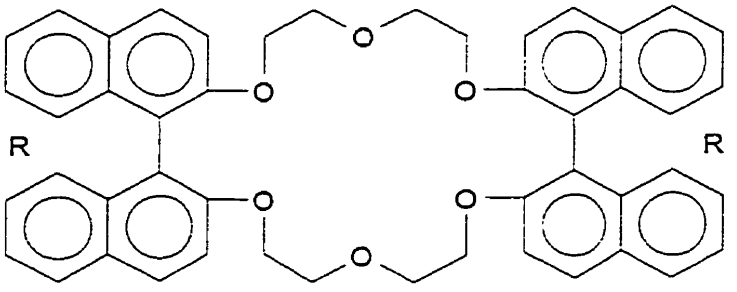


Figure 5. Crown ether selector.

CSPs were later synthesised using covalent attachment of host crown ethers to macroreticular cross-linked polystyrene *p*-divinylbenzene resin. The binaphthalene ring attached to the cyclic ether introduces a chiral barrier and gives the crown ether a rigid structure which also restricts the possibility of reorganisation upon complexation.

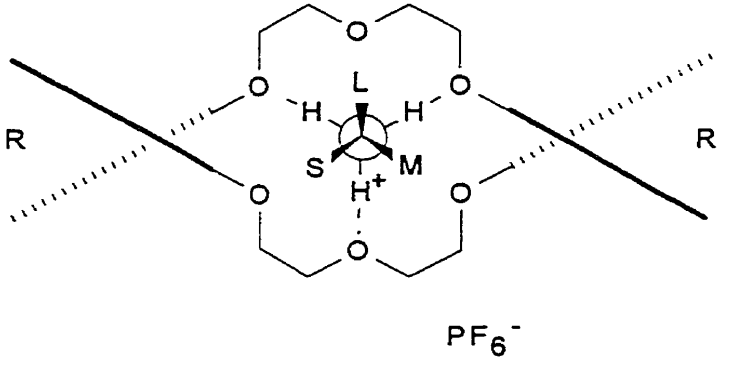


Figure 6. Enantio-discrimination on crown ether selectors (adapted from ref. 30)

The separation of amine derivatives arises from an initial fixing of the NH_3^+ group by hydrogen bonds in a tripole arrangement, to the ether oxygens in the cyclic ring, Figure 6. The chiral cavity formed by the binding of the binaphthalene rings provides an environment in which enantio-discrimination may occur with other groups attached to the solute. Additional interactions such as steric effects, dipole-dipole and π-π interactions and hydrogen bonding between the host and guest, promote a high degree of stereoselectivity in the complexation process.

Cyclodextrins (CDs) are typical 'host' molecules and have been used to resolve a wide variety of racemic compounds[32,33]. α, β and γ cyclodextrins are cyclic oligosaccharides containing 6, 7 and 8 D-glucose units respectively, Figure 7.

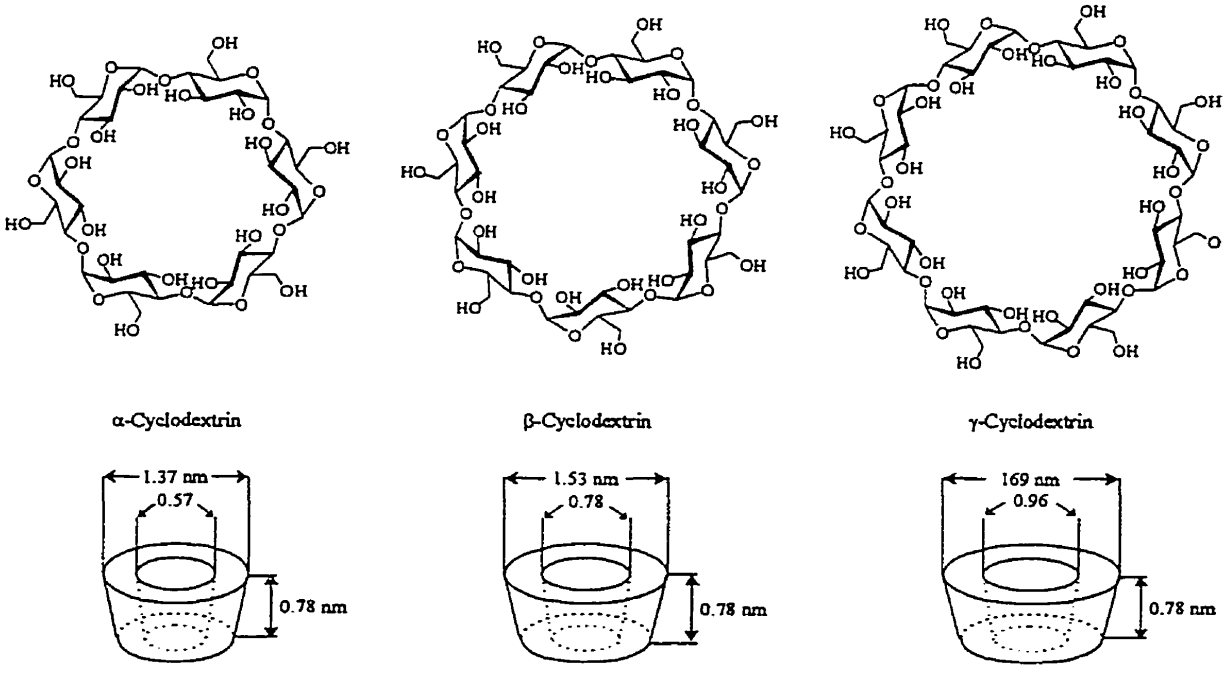


Figure 7. α, β and γ Cyclodextrins.

In an underivatised CD under reversed phase conditions, chiral separations require the solute molecule to enter the hydrophobic cavity in such a way as to place the centre of asymmetry in association with the polar hydroxyl groups at the edge of the cavity. Enantio-discrimination occurs as a result of a difference in stability of the inclusion complex formed for each enantiomer[34]. CDs with different cavity dimensions (α, β and γ) can be used to optimise the fit of the guest solute and thereby maximise molecular interaction. Complex formation with molecules significantly larger than the cavity may also be possible in such a way that only certain groups or side chains penetrate the cavity. As the majority of the chirally discriminating interactions arise from the groups at the edge of the cavity, derivatisation at these positions permits a wider range of interactions whilst at the same time, increasing the depth of the host molecule.

Several theoretical attempts have been made to model retention and separation in CDs. Armstrong and co-workers used molecular graphics representations of β-CD to gain insight into enantiomer separations[32]. The importance of the 2- and 3-hydroxyl groups in resolution was highlighted, which allowed the authors to rationally design CD derivatives and optimise separations.

A statistical thermodynamic theory of chiral solute retention and separation has been formulated by Boehm *et al.*[35], for solutes at infinitely dilute solution. The method relates to the separation of chiral solutes by inclusion in CD cavities and requires the summation of interaction energies for all possible modes of interaction.

By application of the three-point rule, they state that chiral tetrahedral analytes with four non-equivalent groups, are presumed to interact with three separate sites on the CSP by

simultaneous three-point, two-point and one-point interactions. Twelve three-point CSP-solute interactions, 36 two-point and twelve one-point interactions are possible, giving a total of 60 unique interactions which contribute to the total interaction energies for two enantiomers. Of these possible combinations some are enantiomer specific and it is these interaction modes which are deemed responsible for enantio-separation. For the tetrahedral solute with four non-equivalent groups embedded in the interior of a CD with four distinct binding domains, only the four- and three-point interactions are said to be enantio-discriminating. Ultimately, ln α is found to be a linear function of both adsorption energy and the reciprocal of temperature. The slope of ln α against $1/T$ provides the difference between the interaction energies for two enantiomers in their dominant retention mode, with deviation from linearity indicating mixed modes.

Berthod *et al.*[36] devised a scheme for attributing individual substituent contributions to chiral recognition by two enantiomerically related, derivatised CDs. The objective was to predict whether or not a chiral solute would be resolvable on these phases. Chiral interaction contributions were assumed to be independent of one another and additive. The method was able to give reasonable estimations of enantioselectivity.

Atomistic molecular modelling studies where host and guest are considered as transient diastereomeric complexes have been published. Lipkowitz *et al.*[37] used chromatographic data for (R)- and (S)-tryptophan published by Armstrong *et al.*[38] in a molecular modelling study to investigate the fundamental interactions of enantioselective host-guest complexation on CD CSPs. They determined that the more retained (R)-enantiomer was able to make substantially more hydrogen bonds within the interior of the CD

cavity, and that these interactions were usually simultaneous and multiple-contact in nature. Also, the more tightly bound (R)- enantiomer demonstrated lower strain energy in the solute-CSP complex.

The most recent addition to the host-guest family of chiral selectors for liquid chromatography are the macrocyclic antibiotics such as vancomycin, Figure 8. In general, these compounds have molecular weights greater than 600 but less than 2200 and are chemically bonded to silica gel. Multimodal operation and diversity of functionality make this an important new class of CSP, exemplified by the range of racemic analytes already separated. Studies into the mechanisms of enantio-discrimination are still in their infancy however, it is proposed that separation involves π-π complexation, hydrogen bonding, inclusion in a hydrophobic pocket, dipole stacking, steric interactions, or combinations thereof[58]. These phases possess many of the characteristics of protein based CSPs with the distinct advantages of greater stability and much higher capacities.

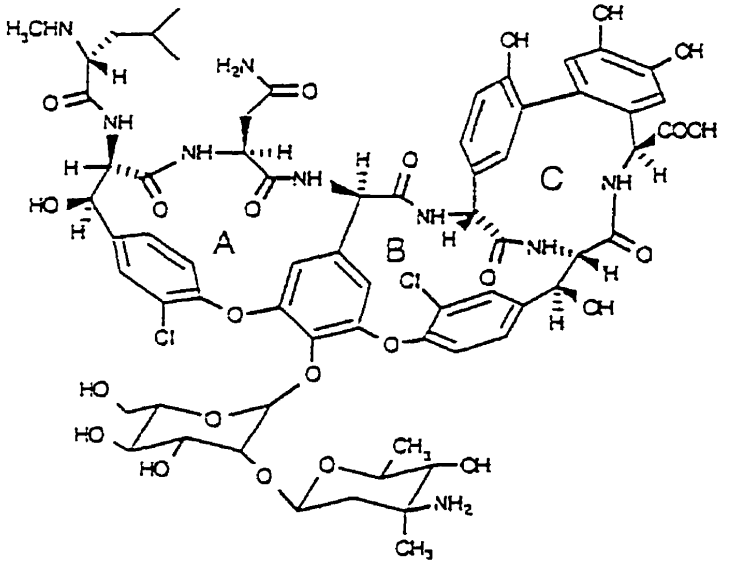


Figure 8. The macrocyclic antibiotic vancomycin

Polymethacrylates are not typical host selectors when compared to the cyclodextrins. However, the retention mechanisms believed to be operating on phases, fall into the same category and involve inclusion complexes. Although many polymethacrylates have been synthesised and tested for potential use as CSPs, only those incorporating very large substituents such as a triphenylmethyl group, afford efficient resolving power[40], Figure 9.

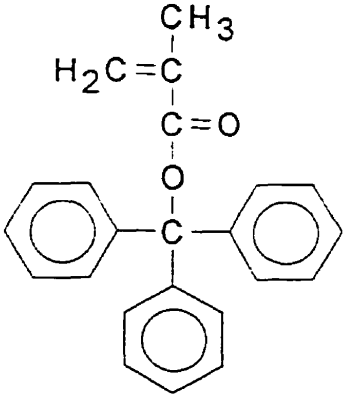


Figure 9. Triphenylmethyl methacrylate

The chiral recognition ability stems from the helical nature[41] of the polymer chain, an effect caused by the incorporation of the bulky subsituent. The result is a stationary phase which has application to solutes devoid of functional groups i.e. compounds containing axial or helical assymetry, and are thus difficult to separate by other methods.

2.3.4 Synthetic Multiple Interaction CSPs

The first examples of this class of CSP were the Type 1 phases utilising attractive interactions between non-ionic functionality. The initial term of donor-acceptor CSPs has given way to the more appropriate synthetic multiple interaction CSPs. Pirkle developed these phases from the premise that the greater the number of specific, discrete, simultaneous interactions between chiral solute molecules and a chiral locus on the stationary phase, then the greater the likelihood of effective chiral discrimination and thus of chromatographic resolution of enantiomeric solutes.

These phases are characterised by at least one each of three types of functional groups, each in proximity to the chiral centre:

1. π-acidic or π-basic aromatic groups, capable of donor-acceptor interaction (charge-transfer complexation)
2. polar hydrogen-bond and/or dipole stacking sites
3. bulky non-polar groups, providing steric repulsion, van der Waals interaction and/or conformational control

Prominent among this class of CSP are the *N*-(3,5-dinitrobenzoyl) derivatives of α-amino acids. Figures 10 and 11 show two commercially available *N*-(3,5-dinitrobenzoyl) phases which have been used to separate many racemic compounds including amines (usually

N-acylated, often with a 1-naphthoyl group)[42,43], alcohols, sulfoxides and sulfoxamides[44,45], epoxides, diols, various heterocyclic compounds[46] and binaphthols[47].

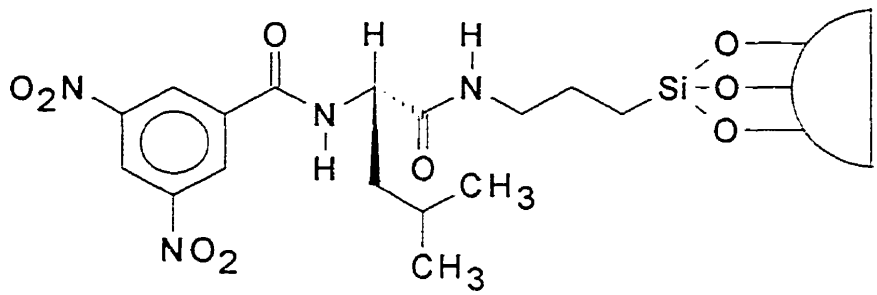


Figure 10. Pirkle CSP 1

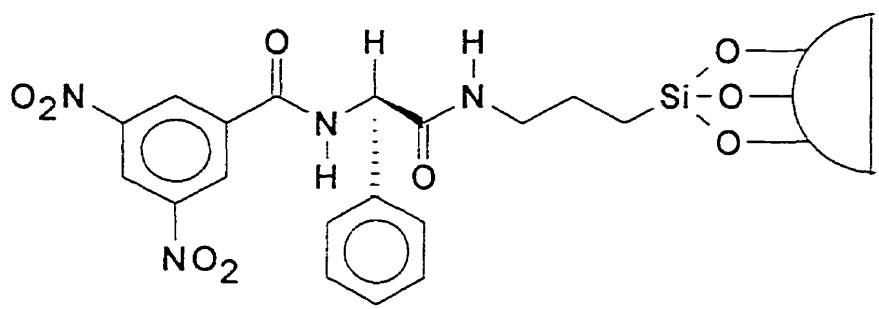


Figure 11. Pirkle CSP 2

The mechanism by which CSPs 1 and 2 operate has been the focus of much attention and somewhat conflicting models have been proposed. Dappen and Pirkle[48] illustrate their

chiral recognition model with the separation of *N*-(2-naphthyl)alanine undecenyl ester on CSP 2, Figure 12.

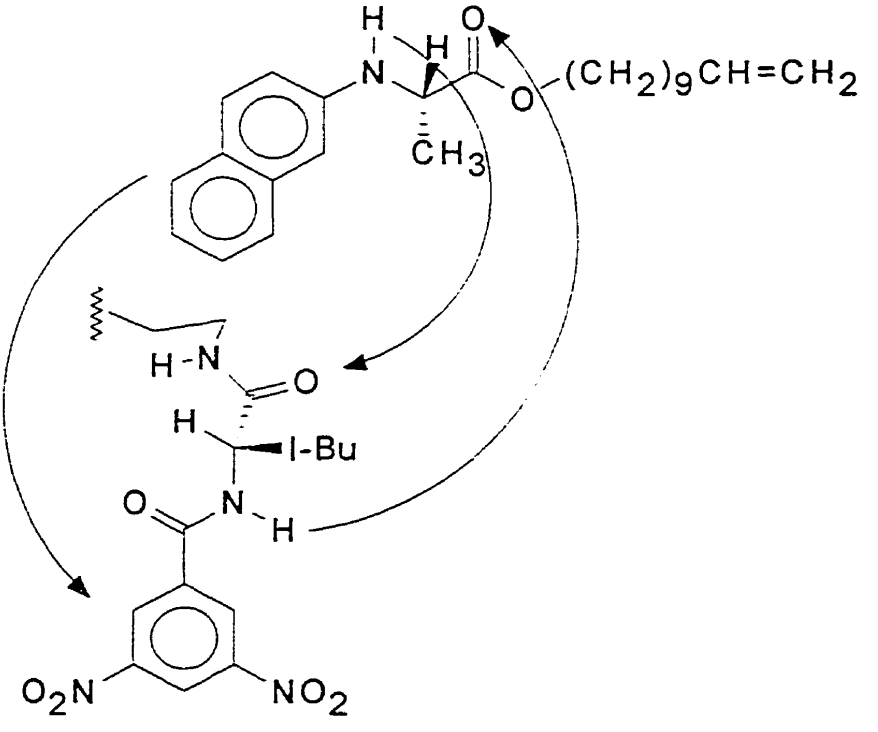


Figure 12. Proposed interaction model for *N*-(2-naphthyl)alanine undecenyl ester on CSP 2.

Multipoint interaction is claimed by π-donor-acceptor overlap between the naphthyl ring of the solute and the dinitrobenzyl ring of the CSP, a hydrogen bond between the dinitrobenzamide NH of the CSP and the carbonyl oxygen of the solute and a second hydrogen bond between the amino NH of the solute and the C-terminal carboxamide oxygen of the CSP. The solute enantiomer which is homochiral with the CSP is selectively retained with a separation factor of 10.2 having been recorded[49].

Computational studies on these CSPs have been performed which predict elution order to be the same as found experimentally. Binding energies were also in agreement with the experimentally observed separation factors. However, the mode of binding with enantiomeric solutes was found to be different to that proposed by Pirkle[50]. The model proposed, claimed that chiral discrimination in these systems (via a "three-point" model) is because the least retained enantiomer cannot obtain the same three interactions as the more stable complex formed with the more retained enantiomer. Topiol *et al.*[51] however showed that both enantiomers can obtain the same number of interactions and suggest that the model posited by Pirkle to explain the mechanism for chiral discrimination, is not 'three-point' as the three interactions described emanate from only two bonds of the stereogenic centre. Thus chromatographic separation may arise from similar interactions in the two competing diasteromeric complexes which differ only in the magnitude of interaction.

A third phase derived from the Pirkle laboratories via the reciprocity route is the Whelk-O 1 CSP, Figure 13. Two naproxen based CSPs were used to screen potential candidate enantioselective naproxen selectors. Immobilisation of the selctor candidate which was resolved to the greatest extent by the naproxen CSP, afforded a new CSP capable of resolving naproxen enantiomers with an α value of 2.25.

The data obtained revealed features presumed significant for the enantioselective recognition of naproxen. The essential feature incorporated into the design of the Whelk-O 1 phase, is a cleft in which face-to-face and face-to-edge π-π interactions can occur simultaneously with an aromatic substituent near the stereogenic centre of the solute. A neighbouring hydrogen bonding site is also required by the chiral recognition rationale.

The significant improvement of the Whelk-O 1 phase over other Pirkle phases, is that solutes can be resolved without any derivatisation.

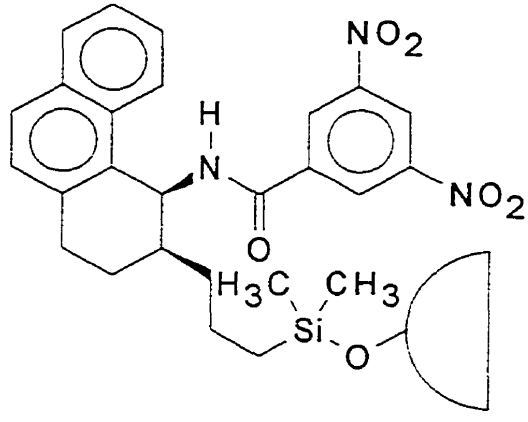


Figure 13. Whelk-O 1 CSP.

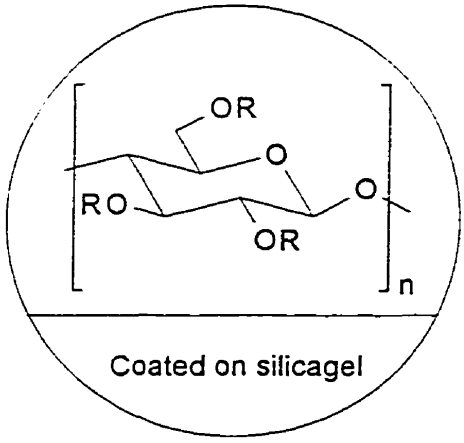
3 POLYSACCHARIDE CHIRAL STATIONARY PHASES

3.1 Development

Polysaccharides such as cellulose and amylose are among the most abundant naturally occurring polymers with optical activity. Although the native polysaccharides, such as microcrystalline cellulose (crystal form I), demonstrate the ability to differentiate between enantiomers[52], their chiral resolving power is not very high and they do not afford practical CSPs. However, cellulose and amylose may be converted into a variety of derivatives such as triesters and tricarbamates by reaction of the active hydroxyl groups.

The most prominent manufacturer of this type of stationary phase is Daicel Chemical Industries Limited. Presently there are thirteen cellulose derivative CSPs and two amylose derivative CSPs marketed by Daicel Chemical Industries Limited, and as such represents the most useful class of stationary phases for chiral separations currently available, Tables 1 and 2.

The chromatographic performance of these stationary phases, demonstrated at the analytical level, is also transferable to larger scale separations such as semi-preparative, preparative and even simulated moving bed (SMB). The facile scale up of most separations permits extremely fast production of single enantiomers of exceedingly high purity.



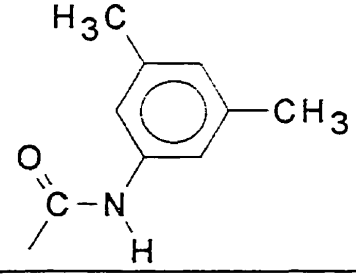
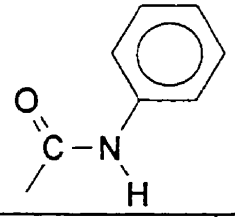
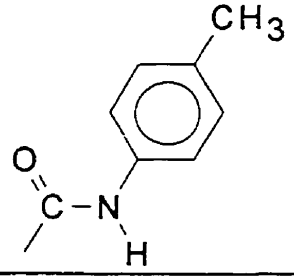
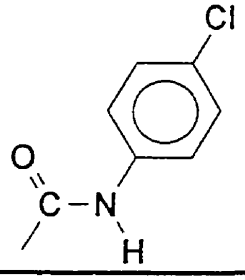
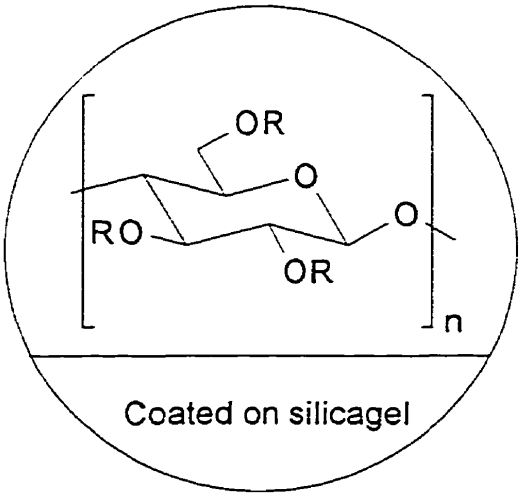
R	Trade Name
	Chiralcel OD
	Chiralcel OC
	Chiralcel OG
	Chiralcel OF

Table 1. Cellulose based CSPs marketed by Daicel Chemical Industries Ltd.



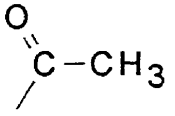
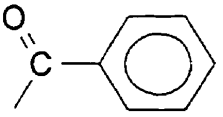
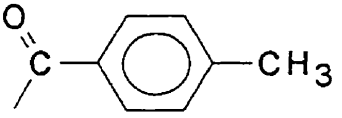
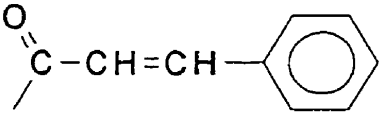
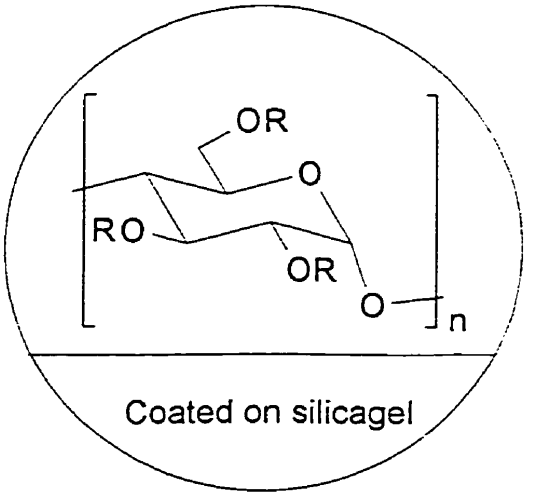
R	Trade Name
	Chiralcel OA
	Chiralcel OB
	Chiralcel OJ
	Chiralcel OK

Table 1 cont.



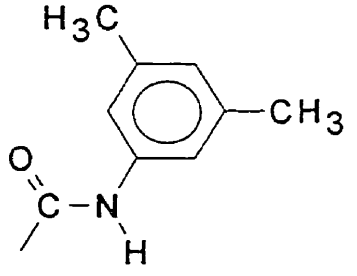
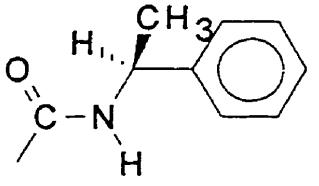
R	Trade Name
	Chiralpak AD
	Chiralpak AS

Table 2. Amylose based CSPs marketed by Daicel Chemical Industries Ltd.

3.2 Cellulose esters

The first widely used CSP derived from cellulose is the microcrystalline cellulose triacetate (CTA-I, Chiralcel CA-I) marketed by Merck, which is prepared by the heterogeneous acetylation of native microcrystalline cellulose in benzene. CTA-I is believed to preserve a structure closely related to native cellulose.

Hesse and Hagel pointed out that the microcrystallinity of CTA-I is essential for chiral recognition, since the resolving ability is substantially reduced, and reversal of the elution order occurs in some cases once the triacetate is dissolved in a solvent[53]. Coating CTA-I onto macroporous silica affords the Chiralcel OA CSP. This phase has a wider application range than the original due to higher column efficiency, durability and the choice of eluents. X-ray crystallographic analysis of the material suggested that it is amorphous rather than crystalline, indicating that microcrystallinity is not essential for chiral recognition[54].

Replacement of the methyl groups in the triacetate with phenyl groups (CTB, Chiralcel OB), allowed for the possibility of π-π and charge transfer interactions to be incorporated into recognition mechanisms. Systematic substitution of the phenyl groups provided information on the relative importance of the type and position of the substituent with regard to resolution ability. Benzoate derivatives having electron donating substituents, such as methyl groups (Chiralcel OJ), showed better chiral recognition ability than those having electron withdrawing groups[55].

The main chiral adsorbing sites of CTB derivatives are considered to be the polar ester carbonyl groups, which can interact with racemates through hydrogen bonding and dipole-dipole interactions. Wainer *et al.* have proposed a similar mechanism[56] based on the chromatographic enantioseparation of a series of chiral aromatic amides[57] and alcohols[58].

Francotte and co-workers[59, 60] have extensively studied the influence of the molecular and supramolecular structure of cellulose based CSPs on their chiral recognition ability. They highlighted the variations in physico-chemical properties arising from different methods of preparation such as precipitation and evaporation. Not only were different retention factors and selectivity values obtained, but also a reversal in elution order was observed in some cases.

3.3 Cellulose carbamates

Cellulose tris(phenylcarbamate) (CTPC) and its derivatives are prepared by the reaction of cellulose with the corresponding phenyl isocyanate derivatives. The optical resolving ability of the phenyl carbamates is also dependent upon the phenyl group substituents. Electron-donating substituents such as alkyl groups and especially *tert*-butyl, or electron-withdrawing substituents such as halogens increase the chiral recognition ability, but methoxy or nitro groups lower chiral recognition. Its is thought that the most important adsorbing site of CTPC for chiral recognition may be the carbamate functionality, whose polarity is influenced by the phenyl group substituents. Electron-donating substituents

increase the electron density of the carbonyl while electron-withdrawing groups increase the acidity of the NH group[61].

Of all the cellulose phenylcarbamates, cellulose tris(3,5-dimethylphenylcarbamate) (Chiralcel OD) demonstrates the greatest optical resolving ability, with the option of running in either normal or reversed phase (see ref. 54 and references therein). The dichloro derivative showed high chiral recognition but was soluble in normal chromatographic solvents. Chemically bonding the phase to silica decreased the solubility, but also reduced the chiral recognition ability[62].

3.4 Amylose carbamates

The amylose tris(3,5-dimethylphenylcarbamate) (Chiralpak AD)[63] and amylose tris(phenylethylcarbamate) (Chiralpak AS)[64], represent the primary CSPs of choice for chiral chromatographic method development on polysaccharide phases. The AD CSP exhibits the highest chiral recognition for most racemates.

The optical resolving capabilities of corresponding amylosic and cellulosic phases such as the AD and OD are quite different. However, no statement can yet be made as to the performance of one phase based on the performance of the other for a particular separation, except that elution orders are often reversed. This must result from the differences in the size of the left-handed helices causing alterations in the higher order structures.

Recently, in an attempt to increase the range of compatible mobile phase solvents, amylose was successfully bonded to silica gel at the reducing terminal residue[65]. This was in order to improve the robustness of the phase and possibly widen the application range even further, by rendering the column stable against previously unusable solvents. The amylose was then allowed to react with 3,5-dimethylpenyl isocyanate to produce a CSP with excellent resolving ability and high durability against solvents such as THF and chloroform.

3.5 Chiral discrimination on polysaccharide phenylcarbamates

Most cellulose tris(phenylcarbamate) derivatives form a lyotropic liquid crystalline phase in highly concentrated solution[66,67], indicating that carbamate derivatives coated on silica gel from solution , will also have an ordered structure. This feature seems important for chiral recognition as the derivatives which did not form a liquid crystalline phase were unable to discriminate between enantiomers. However, the exact nature of the mechanisms involved remain unclear.

Due to the insolubility of these phases in all but the most polar solvents, attempts at NMR studies have been unsuccessful[68]. As a result, computer simulation has recently been used as an alternative method to investigating mechanisms of chiral discrimination. Force-field calculations of the interaction energies between cellulose tris(phenylcarbamate) (CTPC) and *trans*-stilbene oxide have been carried out by Yashima *et al*[69]. CTPC was chosen as its structure had been postulated on the basis of X-ray analysis[70], and it demonstrates high chiral

recognition as a CSP for HPLC. Calculated interaction energies between the (R,R)- and (S,S)-*trans*-stilbene oxide were in good agreement with observed elution orders. However, the difference between the two calculated interaction energies gave a theoretical separation value far higher than experimentally observed.

At this time, the mechanisms of chiral recognition on the amylose based phases, have not been elucidated.

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CHAPTER TWO

1 STRUCTURE-RETENTION RELATIONSHIPS

1.1 Introduction

Quantitative structure-enantioselective retention relationships (QSERR), are relationships between the chromatographic retention of enantiomers and their structures. They are the product of a logical and deliberate extension of studies dating back to 1950. At that time, Martin[1] suggested that a substituent changes the partition coefficient of a solute by a factor that depends on the nature of the substituent and both the mobile and stationary phases employed, but not the remaining part of the molecule. Since that time, chromatographic data has found application in the development of quantitative structure-activity relationships (QSAR), when used as a measure of hydrophobicity for bioactive agents[2, 3, 4, 5].

In 1977, the first reports appeared incorporating the use of QSAR methodology for the analysis of chromatographic retention data[6,7,8]. Soon after, by analogy to QSAR, the term quantitative structure-retention relationships (QSRR) was proposed[9].

QSERR are an extension of QSRR to enantioselective separations. This approach is presently a most useful way to investigate the processes involved in and responsible for the chromatographic separation of enantiomers on CSPs.

1.2 Methodology

There are two principal methods which are used for QSRR studies. In the first method, changes are made to the mobile and/or stationary phases such that information is obtained regarding the thermodynamics of the interaction processes. In the second method, the chromatographic conditions are kept constant and the effect of relative differences between solute structures on k' are investigated. These relationships between chromatographic data and solute structure aim to isolate the governing factors of chromatographic retention and combine detailed models of the process being studied, with specific concepts of thermodynamics[10].

The demonstration of such linear free energy relationships (LFER) in a system, suggests a real connection between the quantities being correlated. In general, the chromatographic data used in QSRR are proportional to the free-energy change associated with the chromatographic distribution process.

$$\Delta G = \Delta H - T\Delta S \qquad (2.1)$$

For LFERs to be found between real and model systems, changes in either enthalpy or entropy must be constant or linearly related[11].

$$\Delta H = \beta\Delta S + \Delta G_{\beta} \quad (\text{at } T = \beta) \qquad (2.2)$$

When enthalpy-entropy compensation is observed within a family of compounds, the values of β and ΔG are invariant and β is the compensation temperature.

At a fixed temperature T, the free energy change is expressed as

$$\Delta G_T = \Delta H\left(1 - \frac{T}{\beta}\right) + \frac{T\Delta G_\beta}{\beta} \qquad (2.3)$$

In chromatography, the retention factor k' is related to the thermodynamic equilibrium constant K for solute binding by $k' = \varphi K$ where φ is the column phase ratio. The free energy change for the chromatographic process is given by

$$\Delta G = -RT\ln K = -RT\ln\left(\frac{k'}{\varphi}\right) \qquad (2.4)$$

where R is the gas constant. Substitution of Eq. (2.4) into Eq. (2.1) yields, for the retention factor

$$\ln k' = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \ln\varphi \qquad (2.5)$$

If the mechanism of the process is invariant over the temperature range studied and the enthalpy is constant, a van 't Hoff plot of $\ln k'$ against 1/T yields a straight line.

Equations (2.3) and (2.4) may be combined to give

$$\ln k'_T = -\frac{\Delta H}{R}\left(\frac{1}{T}-\frac{1}{\beta}\right) - \frac{\Delta G_\beta}{R\beta} + \ln \varphi \qquad (2.6)$$

where $\ln k'_T$ is the retention factor at temperature T. From the slope of a compensation plot of $\ln k'_T$ versus ΔH, the compensation temperature β can be obtained. If values of β for different solutes approach their 95% confidence limits, the individual retention mechanisms are assumed to be identical.

Quantitative structure-enantioselective retention relationships (QSERR) is the term used to when applying QSRR methodology to enantioselective chromatographic systems and solute enantiomers are considered independently. The primary goals of QSERR can be classified as follows:

1. Prediction of retention for new solute enantiomers.
2. Identification of the most important structural features of solutes.
3. Elucidation of interaction mechanisms at the molecular level.

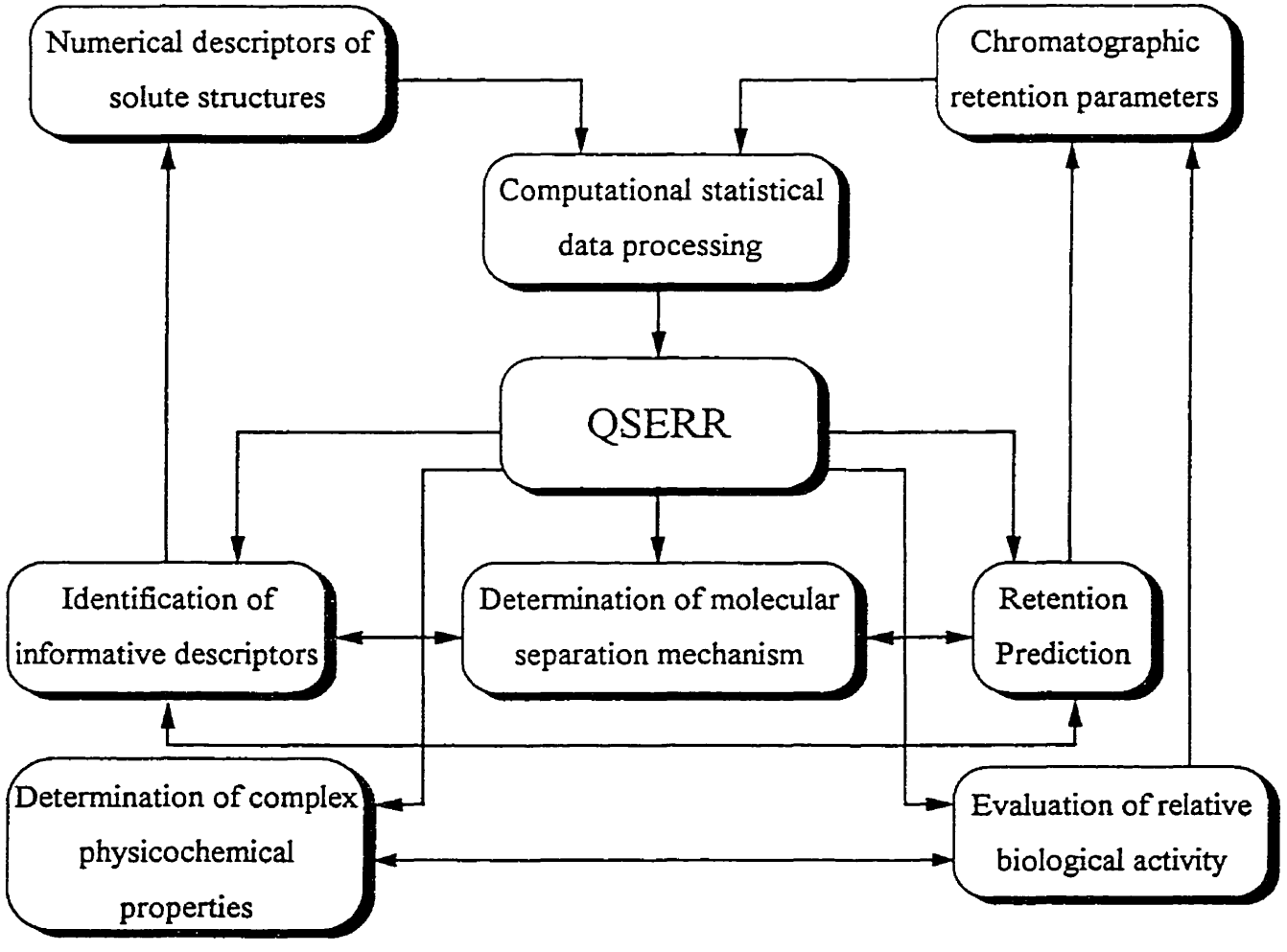
Chromatographic data lends itself to the studies of this kind due to the high reproducibility and precision associated with modern chromatographic systems. Chromatographic retention data are a function of four mutually interacting independent

system variables namely temperature, stationary phase, mobile phase and solute structure. However, no general, canonical mathematical formula exists for the inter-relationship between these four parameters.

Two kinds of input data are required for QSERR studies, a sufficient set of quantitatively comparable retention data (dependent variable) and a set of data reflecting the properties of the various structural features incorporated in the solutes (independent variables). Using chemometric computational techniques, the most significant structural parameters are identified in order to characterise solute retention, Figure 1.

Studies aimed at identifying solute-stationary phase interaction mechanisms and thus describing solute retention, require that all variables be kept constant such that solute structure becomes the only independent variable in the system.

Figure 1. Methodology and goals of QSERR.



2 MOLECULAR DESCRIPTORS

Molecular descriptors are parameters calculated or determined experimentally, which relate directly to the intrinsic properties of a molecule. They describe the type of forces operating between a solute and each of the two chromatographic phases.

Descriptors are used in structure retention relationship studies in order to try and describe interaction mechanisms and predict retention. The presence of a given descriptor in a relationship is an indication of the type of interaction which is part of the overall retention process with the significance level of the term relating to the importance of the interaction. Relationships are usually combinations of several terms which may permit a detailed analysis of molecular interaction processes.

In general the following types of intermolecular interactions are taken into consideration: directional interactions between dipoles, inductive, dispersion, hydrogen bonding, electron pair donor-electron pair acceptor interactions and solvophobic interactions.

For polar molecules, orientation or dipole-dipole interactions (Keesom effect) are characteristic. These interactions concern molecules which possess a permanent dipole moment μ. When two dipolar molecules are oriented towards each other in a head to tail or anti-parallel arrangement, the attraction is proportional to $1/r^3$, where r is the distance between the dipoles. These interactions are strongly temperature dependent as at elevated temperatures the population of unfavourable dipole orientations increases and the overall net attraction is decreased. This has significant consequences in chromatography such that

different parameters may describe retention when measured at different temperatures. Thus it is imperative that temperature is accurately controlled and monitored during chromatographic runs.

Attraction between a molecule possessing a permanent dipole and a non-polar molecule are termed dipole-induced-dipole interactions (Debye effect). The electric dipole in one molecule inducing a dipole in a neighbouring molecule. The induced moment always lies in the direction of the inducing dipole, thus attraction always exists between the two molecules irrelevant of temperature. Induced-dipole moments are proportional to the polarisability of the apolar molecule. Polarisability is defined as the dipole moment induced in a given molecule by an electric field of $1 V\ cm^{-1}$.

A third type of dipolar interaction is known as the London effect, and concerns the attraction of molecules at close distances, which possess no permanent dipole moment. These dispersion interactions result from continuous molecular electron density fluctuations, which at any instant, result in small dipole moments which can polarise the electron systems of neighbouring molecules. Thus, the polarisabilities of involved molecules are critical for dispersion interactions.

The sum total of the previous three types of interactions involving dipoles, form what are generally accepted as van der Waals or non-specific interactions, with the dispersion forces usually predominating at close range and falling off with distance.

From a chromatographic point of view, hydrogen bond interactions and electron pair donor-electron pair acceptor interactions are important due to their strength relative to van der Waals forces. Interactions of this type such as those between Lewis bases and Lewis

acids, are also referred to as charge transfer complexes. These arise if an occupied molecular orbital of sufficiently high energy is present in the electron-pair-donor molecule and a sufficiently low unoccupied orbital is available in the electron-pair-acceptor molecule. Electron transfer is easier the lower the ionisation potential of the donor and the higher the electron affinity of the acceptor.

Many of the descriptors used to represent interactions in studies of this type, may be classified into three basic categories based on the nature of the interactions they describe.

1. Electronic descriptors
2. Lipophilic descriptors
3. Steric descriptors
4. Other descriptors

It is apparent that certain descriptors may be simultaneous members of several classes or that two different descriptors may describe essentially the same property. Therefore, it is imperative for meaningful analysis, that relationships derived make good chemical sense, without mathematically or chemically interrelated variables. Also, for a derived relationship to have predictive potential, it is a necessary prerequisite that all descriptors used be of a non-empirical nature, i.e. not experimentally determined.

In most cases, descriptors are calculated for global low energy conformations, through application of molecular modelling techniques. This is accomplished in several stages beginning with molecule construction followed by geometry optimisation using systematic or random conformational searches. In this manner all conformational space which is accessible to a molecule can be sampled and selected low energy conformations can be used as starting points for further optimisation and calculation using the semi-empirical molecular orbital package, MOPAC.

The systematic conformational search is the most thorough and is used throughout the work presented. The only drawback of using this method is the central processor time required to perform the calculations. For example, to explore the conformational space available to a molecule with 6 rotatable bonds, using bond increments even as large as 30 degrees, requires the sampling of 2, 985, 984 individual conformations. If one conformation is generated per second, then it would take more than 34 days to complete the calculation. Alternatively, crystal structure coordinates may be retrieved and relaxed using similar semi-empirical methods.. This essentially eliminates the risk of calculating properties for molecules in local energy minima which are not significantly populated.

Electronic Descriptors

Descriptors of this general classification essentially describe the electronic properties of molecules with dipole moments, charges, orbital energies and molecular electrostatic potentials (MEP) being among the most commonly incorporated in QSERR. All may be calculated by semi-empirical methods. Dipoles can be total, local or broken down into

contributions along x, y and z axes. Energies of the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) are descriptors relating to charge transfer interactions. The HOMO energy is usually approximated using Koopmans theory[12], to be equal in magnitude to the ionisation potential. Molecular surface potentials are a special case and are discussed under the heading of Other Descriptors.

Lipophilic Descriptors

The most common measure of lipophilicity (or hydrophobicity) is the octanol/water partition coefficient (Log P). Due to the generality of this descriptor (i.e. treatment of the molecule as a whole), it is often necessary to use fragmental contributions or the more realistic calculation of molecular lipophilic potential (MLP).

Steric Descriptors

Steric or bulk descriptors are usually indications of the size and shape of molecules or molecular regions. Included in this classification are descriptors such as molecular volume, molecular surface area and various topological indices. The total energy of molecules reflects differences in the dispersive properties of solutes and therefore falls into the same category as other bulk descriptors. The advantage of this value as a descriptor is that it can distinguish between both different compounds and different conformations of the same compound. Although it has no real chemical meaning, it likely represents non-specific properties indirectly, with other more chemically significant parameters being contained within.

Other Descriptors

MLP and MEP are two very useful descriptors which incorporate aspects of size and shape interactions with lipophilic and electrostatic interactions respectively.

Contour surfaces of electronic charge densities, molecular orbitals, electrostatic potentials, lipophilic potentials, molecular van der Waals surfaces generated by fused atomic spheres, solvent accessible surfaces and various other surfaces surrounding some or all of the nuclei in a molecule are collectively referred to as molecular surfaces.

It is well known that the electrostatic potential generated by a molecule has a strong influence on molecular interaction. MEPs may be calculated and used for the representation of molecular shapes and steric interactions between charged regions of molecules. Likewise, MLPs may be calculated and used for representation of molecular shapes and steric interactions between lipophilic regions of molecules.

3 DATA ANALYSIS

3.1 Multivariate Regression Analysis

Multiple linear regression was the first and most widely used statistical method applied in structure-activity relationship studies. The method was popularised by Hansch in relating bioactivity data to the measures of lipophilic, electronic and steric properties of a series of derivatives.

After the formalism of linear free energy relationships (LFER), a chromatographic retention factor k' may be quantitatively described by a set of experimentally or theoretically derived molecular parameters x_i such that

$$k' = ax_1 + bx_2 + cx_3 + d \qquad (2.7)$$

A combination of the following statistics are usually reported in conjunction with such a calculation to permit the significance of the resulting calculation to be assessed.

1. The correlation coefficient R, or the coefficient of determination or percentage of data variance accounted for by the regression equation, R^2.

2. The standard error of the estimate or the standard deviation from regression, s.

3. The F value for assessing the overall significance of the derived equation, calculated by the formula

$$F = \frac{(n-k-1)R^2}{k(1-R^2)} \qquad (2.8)$$

where R is the correlation coefficient, n is the number of data points (retention data) for the dependent variable and k is the number of independent variables (structural parameters) in the regression equation. The F values are verified by comparison with tabulated statistical data.

4. Confidence intervals (usually 95%) for the individual regression coefficients in the equation or the t-test values.

5. The cross-correlation coefficients r_{ij} between the independent variables i and j. Low values are important to ensure true independence between variables. Orthogonality of variables is a necessary condition for meaningful results.

In order to prevent overfitting of data, it is often necessary to verify whether the introduction of additional term in an equation is justified. This is done by application of the sequential F-test.

$$F^* = \frac{(R_1^2 - R_2^2)/p}{(1-R_1^2)/[n-(k+p)-1} \qquad (2.9)$$

If R_1 is the multiple correlation obtained when $k+p$ independent variables are used and R_2 is the multiple correlation found when only k independent variables are used, the Eq. (3.3) provides a test of whether R_1 is significantly greater than R_2. This F^* has p and $n-(k+p)-1$ degrees of freedom. When $p=1$, this tests whether the addition of a single variable provides a significant increase in the multiple correlation coefficient. The F^* values are usually compared with statistics tables for a given significance level, usually $\alpha = 0.05$ or $\alpha = 0.01$.

In studies of this kind, at least four or five empirical data points should fall per independent variable to minimise the potential for chance correlation. According to Goodford[13] the final number of degrees of freedom ($n-k-1$) should be at least 10.

3.2 Neural Networks

Neural networks arose from attempts to model the functioning of the human brain. In the human cortex, signals enter a neuron via the dendrites. If the sum of these signals at a given time is greater than a certain threshold value, the cell body generates an output signal which then travels along the axon and is passed on to other neurons. The magnitude of the influence of this signal on the next neuron is modulated by the efficiency of the intervening synapse (synaptic strength). In the artificial or computer simulated neuron, signals entering are referred to as *input*, signals exiting are referred to as *output*, the synapse is referred to as a *connection* and the synaptic strength is represented by a *weight* associated with each connection.

The architecture of an artificial neural network is determined by the way in which the outputs of the neurons are connected to the other neurons. In the standard architecture, the neurons of a network are divided into several layers. Both single and multi layer architectures are possible. A three layer feed forward network is illustrated in Figure 2. The first layer of the network is called the input layer, on which the neurons perform no special processing. The second and third layers are referred to as the hidden and output layers respectively. The neurons in the hidden and output layers pass on the weighted sum of their inputs via transfer functions. In general, the output Out_i^n of the i-th neuron in layer n is given by

$$Out_i^n = f(Net_i^n) = f(\sum W_{ij} Out_j^{n-1} + \Theta_i) \qquad (2.10)$$

where W_{ij} are the weights connecting neuron i with each neuron j in the previous layer, Out_i^n is the output of neuron i on the layer n, Net_i^n is the linear activation of the neuron (the weighted linear combination of the output of the neurons on the previous layer, Out_j^{n-1}) and Θ_i is the bias of neuron i which is usually introduced as a separate weight[14], Figure 3. Each neuron needs a bias to adjust the overall input so that it falls into the region where it can be used by the transfer function to the greatest benefit of the overall performance of the network.

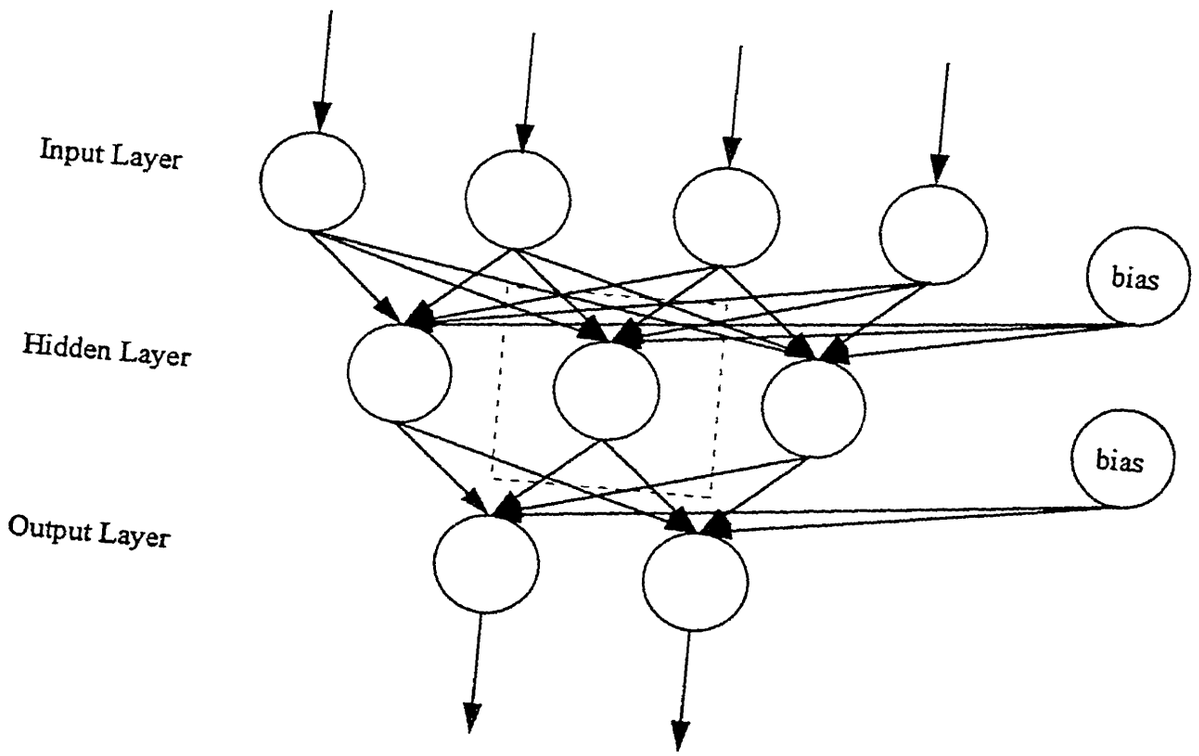


Figure 2. A three-layer feed-forward network

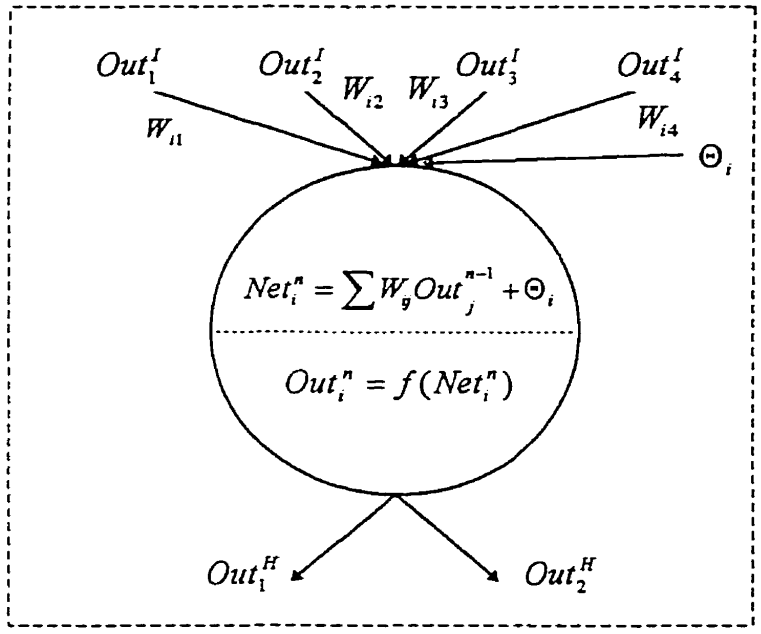


Figure 3. Schematic of a neuron.

The function by which the output signal is calculated from the Net_i^n, is referred to as a transfer or squashing function. In applications of neural networks, the most common transfer function is the sigmoidal function

$$Out_i^n = \frac{1}{1+e^{(-Net_i^n)}} \qquad (2.11)$$

Training of neural networks consists of presenting pairs of input/output data to the system in an iterative fashion. If the final output of the network is in discrepancy with the expected output, the weights and biases are adjusted to minimise the error and another iteration is performed. The average square output error can be expressed by

$$E^n = \frac{1}{2}\sum_i (t_i - Out_i^n)^2 \qquad (2.12)$$

where t_i is the expected output for the training pair and Out_i^n is the corresponding actual output from the network at neuron I. The gradient descent method is used with error back propagation[15] such that

$$\Delta W_{ij}^n = \eta \delta_i^n Out_j^{n-1} \qquad (2.13)$$

Where η is called the learning rate, δ_i^n is the error term on the output neuron i, and Out_j^{n-1} is the signal from neuron j on the previous layer.

The error terms from the output and hidden layers are back propagated through the network by adjusting the weights of the corresponding layers according to Eq. 2.13. In order to achieve faster convergence, this equation is usually augmented by an additional term

$$\Delta W_{ij}^{n} = \eta \delta_{i}^{n} Out_{j}^{n-1} + \mu \Delta W_{ij}^{n(previous)} \qquad (2.14)$$

where μ is called the momentum term and $\Delta W_{ij}^{n(previous)}$ is the improvement of weight W_{ij}^{n} in the previous iteration.

Andrea and Kalayah[16] developed an expression for ρ as an aid to obtaining optimal neural networks

$$\rho = \frac{n}{P} \qquad (2.15)$$

where n is the number of data sets and P is the number of connections in the network, given by

$$P = \left[(I+1)H + (H+1)O\right] \qquad (2.16)$$

I, H and O are the number of neurons in the input, hidden and output layers respectively. Ideally ρ should fall between 1.8 and 2.2.

4 PRINCIPAL STUDIES IN STRUCTURE-RETENTION RELATIONSHIPS

The overwhelming majority of work in this area concerns the development of equations relating the retention factors for congeneric series of achiral solutes to combinations of empirical and non-empirical solute descriptors. However, the primary focus of this thesis concerns chirality and its consequences on chromatographic retention.

Although structure retention relationships are a source of important and otherwise unobtainable information on the basic interactions between molecules, chirality introduces an extra dimension which has rendered the subject more difficult and in general, less successful than its predecessors. As such, QSERR studies are somewhat less common than QSRR studies.

One of the earliest and most successful studies to date is the work of Kaliszan *et al.*[17] on the separation of 1,4-Benzodiazepines on an HSA CSP. Retention factors (log k') were correlated with molecular descriptors derived from computational chemistry methods using multiparameter regression analysis. Regression equations describing the retentions of both enantiomers of solutes in terms of non-empirical solute descriptors were identified.

In the QSRR analysis, the logarithms of the retention factors corresponding to the first and second eluting peaks were considered as two sets of mutually independent variables.

Equation 1

$$\log k'_1 = -1.7497 + (\pm 0.0751) \log f_y - 1.8392\ (\pm 0.5020)\ C_3 - 0.1609\ (\pm 0.0485)\ W$$

$$+ 0.0354\ (\pm 0.0150)\ \beta_{CNN} + 0.1736\ (\pm 0.0939)\ f_x$$

$$n = 21,\ R = 0.8814,\ F = 10.5,\ p < 2\times 10^{-4}$$

Equation 2

$$\log k'_2 = 1.9922 + 0.8926\ (\pm 0.1147)\ P_{SM} + 0.4830\ (\pm 0.0751)\ f_y$$

$$- 4.1482\ (\pm 0.7367)\ C_3 - 0.1197\ (\pm 0.0544)\ W + 0.1324\ (\pm 0.0814)\ f_x$$

$$n = 16,\ R = 0.9702,\ F = 32,\ p < 8\times 10^{-6}$$

where f_y is the hydrophobicity of the substituent at position 7 in the fused benzene ring, C_3 is the excess electron charge on carbon C(3) of the 1,4-diazepine system, W is the width of the molecule as measured from the extremity of the phenyl substituent, P_{SM} is the submolecular polarity parameter, β_{CNN} is the angle formed by atoms C(2)-C(3)-N(4) of the diazepine ring and f_x is the hydrophobicity of the 2' substituent of the phenyl system, Figure 4.

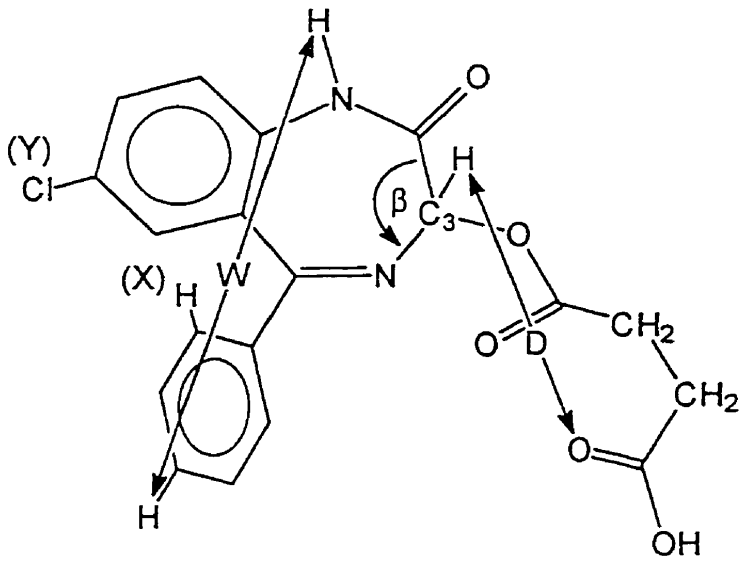


Figure 4. Structure of the benzodiazepines studied by Kaliszan *et al.*[17]

Analysis of the two equations allowed for a proposal on the nature and number of the benzodiazepine binding sites on HSA. The results also indicated that QSERR can be used to calculate retention and separation of chiral benzodiazepines on the HSA CSP, prior to chromatography.

Wolf *et al.*[18] studied the quantitative correlation between calculated molecular properties and retention of a series of structurally related racemates on cellulose triacetate. Racemic compounds consisting of an alicyclic six-membered ring with a phenyl group attached to the stereogenic centre were investigated. The alicyclic moiety was varied systematically in order to study the consequences on separation. Two major factors were

found to govern the interaction between the solutes and the cellulose triacetate CSP. The relative ability of the compounds to assume the flattest possible conformation, determined by the rotational freedom of the bond to the phenyl group and degree of negative charge in proximity to the stereogenic centre. Using a linear combination of these two terms in relationship for $\ln k'_2$, retention factors were calculated and plotted against the experimentally determined values. The obtained correlation coefficient of 0.96 suggests that the two parameters reflect the major contributions to the retention mechanism and may satisfactorily be used to predict retention.

Altomare *et al.*[19] used a QSERR approach in an investigation into the enatiomeric resolution of sulfoxides on an *N,N'*-(3,5-dinitrobenzoyl)-*trans*-1,2-diaminocyclohexane (DACH-DNB) CSP.

Initially, two equations were derived for a subset of eleven compounds, relating the logarithms of the (R)- and (S)- enantiomers retention factors, to the Hammett electronic constant (σ) and the Hansch hydrophobic constant (π).

Equation 3

$$\log k'_S = -0.67(\pm 0.14)\,\sigma - 0.40(\pm 0.12)\,\pi + 1.50(\pm 0.06)$$

$$n = 11,\ s = 0.070,\ R^2 = 0.951$$

Equation 4

$$\log k'_R = -0.74(\pm 0.14)\,\sigma - 0.38(\pm 0.12)\,\pi + 1.60(\pm 0.06)$$

$$n = 11,\ s = 0.070,\ R^2 = 0.956$$

However, the inability of these equations to incorporate an extended series of compounds, including bulky alkyl phenyl sulfoxides, resulted in diminished correlation coefficients. In order to accommodate the more diverse nature of the whole series, theoretical electronic parameters were calculated for the compounds and the equations re-developed. The final relationships identify log P, HOMO energy and the net charge on the sulfoxide oxygen, as the most important structural descriptors involved in retention for this series of compounds. Also, using comparative molecular field analysis (CoMFA), selectivity was determined to be primarily related to steric factors, as well as the electrostatic properties of the sulfoxide group.

5 STUDY OBJECTIVES

There are essentially three primary objectives underlying the work presented in this thesis. All three are equally important and thus are not presented in any specific order.

1. To apply quantitative structure-enantioselective retention relationship methodology, to the separation of chiral solutes chromatographed on amylosic CSPs.

2. To use the relationships to describe the mechanisms of enantioselective recognition operating on amylosic CSPs.

3. To develop systems with the inherent ability to predict retention, separation and elution order.

The use of chromatographic stationary phases to effect separations of racemic compounds, has reached the point where it has become necessary to try and understand the fundamental processes behind those separations. To interpret and rationalise a separation, then use that information to predict other separations, has both academic and commercial value. Academic in the sense of exploring mechanisms of interaction between molecules. Commercial in the sense of speeding up analytical method development and designing customised chiral separations, especially as a precursor to preparative scale separations.

For this study it was decided to use a class of chiral selectors, which is possibly the most frequently applied in chiral separations today. Thus, maximising the potential market for future expert separation system technology.

The first aim of this project was to establish the application of quantitative structure-enantioselective retention relationships to amylose based CSPs, and to build the foundation for further investigation. The robustness of the column packing material and the reproducibility of retention times over long periods of time and wide temperature ranges, may render this type of study inappropriate for some phases.

Once, it had been verified that the methods and materials were compatible, the study was expanded to a diverse, yet congeneric series of compounds which included the therapeutically important class of non steroidal anti-inflammatory agents. The goal of this study was an extension of the previous work, with more emphasis on using molecular modelling resources to try and gain further insight into the specific details of the solute-stationary phase interaction processes. The simulated docking experiments, guided by the molecular descriptors included in the multiparameter regression equations, identified a mechanism for chiral recognition which was not fully explained according to prior theory. A modified theory was proposed to account for the observed results, prompting re-evaluation and debate into the 'three-point' model of chiral recognition.

By introducing two other related amylose based stationary phases (the AS and AR) the changes in chromatographic retention, selectivity and elution order could be investigated as a function of both column and solute.

In order to apply and use the information obtained regarding the chromatographic separation of chiral solutes on amylosic CSPs, a new method of data analysis was required, which was able to overcome the limitations of the more commonly used methods.

Neural network methods were investigated, with the objective of developing truly predictive working models using identified chromatographic descriptors, which have the potential to be used as the basis of a chiral expert systems.

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CHAPTER THREE

The following chapter comprises of a series of five sequential and interrelated manuscripts written by the candidate. In all cases, without exception, the experiments were designed, conducted and interpreted by the candidate, with collaboration from co-authors primarily serving to verify the validity and accuracy of results and interpretation.

PAPER I

Mechanistic investigation into the enantioselective separation of mexiletine and related compounds, chromatographed on an amylose tris(3,5dimethylphenylcarbamate) chiral stationary phase.

This initial paper reports the findings from the first QSERR investigation using an amylose based CSP. The aim of the study was first to verify the applicability of structure-retention methodology to polymeric stationary phases used under normal phase conditions. The second aspect of the study was to probe the mechanisms by which solute enantiomers were retained and separated on the column. The final aspect of this preliminary study was to investigate the potential for development of equations which could serve as predictive indicators of the ability of the column to separate enantiomers of a given type.

The stationary phase was found to be particularly well suited to the methodology and further studies therefore became viable.

Analysis of the results led to the identification of two separate retention mechanisms, based on either the presence or absence of secondary hydrogen bonding groups in the solutes. QSERR were successfully developed, incorporating non-empirical solute descriptors, thus giving the relationships valuable predictive potential.

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Journal of Chromatography A, 741 (1996) 205–211

JOURNAL OF CHROMATOGRAPHY A

Mechanistic investigation into the enantioselective separation of mexiletine and related compounds, chromatographed on an amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phase

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Received 2 January 1996; revised 27 February 1996; accepted 28 February 1996

Abstract

Mexiletine and a series of structurally related compounds have been chromatographed on an amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phase. By application of quantitative structure–enantioselective retention relationship and thermodynamic approaches, two separate retention mechanisms were identified. These mechanisms are based on either the presence or absence of secondary hydrogen-bonding groups. Highly statistically significant regression equations have been derived which describe the retentions of the first and second eluting enantiomers in terms of non-empirical molecular descriptors.

Keywords: Chiral stationary phases, LC; Quantitative structure–retention relationships; Retention mechanisms; Enantiomer separation; Amylose tris(3,5-dimethylphenylcarbamate); Mexiletine

1. Introduction

Mexiletine [1-(2,6-dimethylphenoxy)-2-aminopropane] is an oral antiarrhythmic drug, similar to lidocaine in structure and physiological effects [1]. It has been shown to be effective in the treatment of neuropathic pain of different aetiologies.

In this paper we report the findings of a study aimed at identifying the relationships between mexiletine and its analogues (at the enantiomeric level), and their respective affinities for a given receptor site. This is most conveniently achieved through extrathermodynamic linear free-energy relationships (LFERs). An extrathermodynamic approach to isolating the governing factors of chromatographic retention and enantioselectivity entails the combination of detailed models of the process being studied, with specific concepts of thermodynamics [2]. The demonstration of LFERs in a system insinuates a genuine link between the quantities being correlated, and means that the form of the underlying connection is probably identifiable.

Quantitative structure–enantioselective retention relationships (QSERRs) require reliable input data to ensure validity of the resultant equations. Chromatographic data lends itself to studies of this kind due to ready reproducibility and high precision. However, intercolumn variability introduces the potential for

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0021-9673/96/$15.00
PII S0021-9673(96)00208-7

less universally meaningful relationships. The Chiralpak AD chiral stationary phase (CSP) (Daicel Chemical Industries, Tokyo, Japan) column was chosen for this study as it is representative of a class of stationary phases which are uniform, reliable and robust. These CSPs are widely used for the separation of many diverse racemic compounds [3–14], but little is actually known about the mechanisms of enantioseparation, operating on any of the amylosic phases. However, recently our group identified evidence for a conformationally driven chiral recognition mechanism operating on the Chiralpak AD CSP [15]. It is believed that by attempting to study specific molecular interactions operating during the chromatographic process, the information obtained may be exploited to gain the ability to predict enantioseparations and also to further development of highly efficient and even customised CSPs.

2. Experimental

2.1. Chemicals

Compounds 1–12 were all gifts from Boehringer Ingelheim (Burlington, Canada). HPLC-grade ethanol and hexane were obtained from Anachemia Science (Montreal, Canada). Diethylamine was purchased from Aldrich (Milwaukee, WI, USA).

2.2. Chromatography

The chromatographic system was composed of a Spectra-Physics P1500 binary pump, a Spectra-Physics UV1000 variable-wavelength detector and a Spectra-Physics SP8875 autosampler equipped with a 20-μl loop (Thermo Separation Products, Toronto, Canada). Separation was performed on a Chiralpak AD chiral column, 250×4.6 mm I.D. (Chiral Technologies, Exton, PA, USA). A Spectra-Physics Datajet integrator acted as an interface for electronic data collection using Winner on Windows software run on a 386 personal computer.

Column temperature regulation was achieved by using a Haake D1-G refrigerated bath/circulator (Fischer Scientific, Montreal, Canada) and a column water jacket.

The mobile phase consisted of hexane–ethanol (95:5)+0.1% diethylamine, filtered and degassed. All samples were prepared in mobile phase.

2.3. Computational chemistry

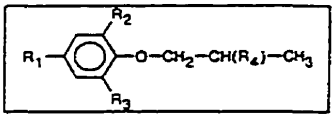
Molecular models were created using Insight II release 230 (Biosym, San Diego, CA, USA) run on an IBM RS6000 RISC workstation (IBM Corporation, Austin, TX, USA). All energy minimisations were performed using Discover ver. 2.9 (Biosym), running within Insight II

3. Results

A series of 12 mexiletine-related compounds were chromatographed on an amylose tris(3,5-dimethylphenylcarbamate) (AD) CSP, using a hexane–ethanol (95:5, v/v)+0.1% diethylamine mobile phase. Retention data were collected over the temperature range 0 to 30°C, and the results interpreted from both QSERR and thermodynamic aspects. The structures of the compounds studied are presented in Fig. 1.

The compounds were chosen in order that the small structural variations could be investigated in terms of their physico-chemical significance towards retention and enantioselectivity. Table 1 lists the relative retention (k') and enantioseparation (α) values of the twelve sample compounds, chromatographed at the highest and lowest limits of the temperature range.

The retention data were subjected to multiparame-



COMPOUND	R_1	R_2	R_3	R_4
1.	OH	CH_3	CH_3	OH
2.	H	CH_3	CH_2OH	OH
3.	H	CH_3	CH_2OH	NH_2
4.	CH_3	CH_3	CH_2OH	NH_2
5.	CH_3	CH_3	H	NH_2
6.	CH_3	CH_3	CH_3	NH_2
7.	NO_2	CH_3	CH_3	NH_2
8.	NH_2	CH_3	CH_3	NH_2
9.	OH	CH_3	CH_3	NH_2
10.	H	CH_3	CH_3	NH_2
11.	H	OCH_3	OCH_3	NH_2
12.	H	CH_2CHCH_2	CH_2CHCH_2	NH_2

Fig. 1. Structures of the compounds used in this study.

Table 1
Retention and selectivity data at 0°C and at 30°C, for compounds 1–12 chromatographed on a Chiralpak AD CSP

Compound	ln k'_1 at 0°C	ln k'_2 at 0°C	α at 0°C	ln k'_1 at 30°C	ln k'_2 at 30°C	α at 30°C
1	1.42	1.58	1.17	1.36	1.45	1.12
2	1.72	2.27	1.73	1.49	1.74	1.29
3	2.39	2.56	1.18	1.84	1.88	1.05
4	2.46	2.77	1.36	1.83	1.96	1.14
5	0.58	0.66	1.08	−0.01	−0.01	1.00
6	−0.48	−0.48	1.00	−0.80	−0.80	1.00
7	1.51	1.57	1.06	0.87	0.87	1.00
8	2.38	2.65	1.31	1.65	1.92	1.19
9	1.75	2.02	1.31	1.42	1.59	1.19
10	−0.16	−0.16	1.00	−0.67	−0.67	1.00
11	1.60	1.60	1.00	0.91	0.91	1.00
12	−1.24	−1.24	1.00	−1.43	−1.43	1.00

See Section 2.2 for chromatographic conditions.

ter regression analysis against various non-empirical molecular descriptors (Table 2), intuitively chosen for their suspected role in solute–stationary phase interactions (Table 1). The simplest possible relationships between the ln k' values at 0°C and these descriptors were established and are presented in Eq. (1) and Eq. (2) (see Table 3). Relationships were derived for retention at 0°C, as this temperature enabled the largest number of compounds to be separated.

In view of these two equations, it would appear that the major contribution to retention arises from the hydrophobicity of the substituents at R_1 and R_3.

Table 2
Structural descriptors used in the final QSERR

Compound	π_{R1}	π_{R3}	A_c	S_d
1	−0.67	0.56	−0.332	−2.742
2	0.00	−1.03	−0.505	0.187
3	0.00	−1.03	−0.498	0.134
4	0.56	−1.03	−0.436	−2.433
5	0.56	0.00	−0.470	−2.413
6	0.56	0.56	−0.406	−2.417
7	−0.28	0.56	−0.195	−2.840
8	−1.23	0.56	−0.389	−4.190
9	−0.67	0.56	−0.335	−3.003
10	0.00	0.56	−0.471	0.102
11	0.00	−0.02	−0.278	0.141
12	0.00	1.10	−0.458	0.118

π_{R1}=fragmental hydrophobicity of substituent R_1. π_{R3}=fragmental hydrophobicity of substituent R_3. A_c=total aromatic excess charge. S_d=substructure dipole. (Descriptors calculated according to Kaliszan et al. [20]).

With the electronic charge and dipole terms added, more differentiation between the first and second eluting enantiomers becomes possible. The hydrophobicity terms are common for both ln k'_1 and ln k'_2, and, being negative, indicate that retention increases with increasing hydrophilicity. This is to be expected for chromatographic systems operating under normal-phase conditions, due to solute solubility in the mobile phase. However, previous studies with aromatic acids [15] have shown that hydrogen-bonding ability can have a higher correlation to retention than hydrophobicity when a large series of compounds is investigated. Within this series, the term π not only relates to hydrophobicity, but also hydrogen-bond donor ability through an interrelation between the two descriptors of −0.8669 (n=12) at R_1 and −0.9234 (n=12) at R_3. Hence the model developed for the aromatic acids is comparable to the present series of mexiletine analogues. The incorporation of more specific descriptors is unfortunately not facilitated by the small sample set, therefore π is adopted with its inherent duality.

The inclusion of fragmental hydrophobicity, as compared to total molecular hydrophobicity, is believed to emphasise solute orientation during the partitioning process between mobile and stationary phases. Fragmental hydrophobicity is therefore more useful in describing aspects of specific retention, in contrast to general retention. The initial interaction is thus assumed to take place between the polar functional group at R_2 and a hydrogen-bond acceptor

Table 3
Equations (1) and (2)

$$\ln k' = 3.1791 - 1.3383(\pm 0.5547)\pi_{R_2} - 1.6451(\pm 0.4157)\pi_{R_3} - 4.9370(\pm 3.2306)A_v$$

$t=5.31,\ p=0.0002 \qquad t=8.71,\ p<0.0001 \qquad t=3.36,\ p=0.0063$

$n=12,\ R=0.9580,\ F_{3,8}=29.74,\ F_{3,8,0.005}=4.07,\ p<0.0001$ (1)

$$\ln k'_2 = 0.9128 - 1.4554(\pm 0.5214)\pi_{R_2} - 1.7345(\pm 0.3696)\pi_{R_3} - 0.2834(\pm 0.1718)S_d$$

$t=6.14,\ p<0.0001 \qquad t=10.33,\ p<0.0001 \qquad t=3.63,\ p=0.0040$

$n=12,\ R=0.9702,\ F_{3,8}=42.71,\ F_{3,8,0.005}=4.07,\ p<0.00009$ (2)

π_{R_2}: Fragmental hydrophobicity at position R_2;
π_{R_3}: Fragmental hydrophobicity at position R_3;
A_v: Total aromatic excess electronic charge;
S_d: Substructure dipole.

The figures in parentheses are the standard deviations of the regression coefficients. n is the number of data points used to derive the regression. R is the correlation coefficient. F is the f-test value. t is the t-test value and p is the significance level of the individual variables and of the whole equation.

situated in the outside of the helical stationary phase cavity. Examination of the retention of compounds devoid of any further functional group suggests that this initial interaction is relatively weak, leading to low retention and practically no enantioselectivity. An increase in the stabilisation of the diastereomeric complex, sufficient to allow observable discrimination between enantiomers at high temperature, is only achieved via further hydrogen-bonding interactions. At low temperature, stabilisation, and consequently discrimination, is far more significant for those compounds containing secondary hydrogen-bonding interactions. Computer docking interactions employing the functional group at R_4 as the primary tethering interaction, followed by conformational adjustment and energy minimisation, enabled favourable stabilising interactions between the stationary phase and solute aromatic substituents to take place. Bulky substituents at both R_2 and R_3 seemed to sterically hinder the ability of the molecule to completely enter the stationary phase cavity, which is consistent with the chromatographic results.

A complimentary approach to investigating chromatographic retention mechanisms involves enthalpy–entropy compensation [16]. Analogously to QSERRs, enthalpy–entropy compensation manifests itself in a linear dependence of the overall free energy changes on the corresponding enthalpy change for intrinsically similar physico-chemical phenomena. By application of the Gibbs-Helmholtz equation ($\Delta G=\Delta H - T\Delta S$), it can be deduced that, in the vicinity of T, changes in ΔH are offset by changes in ΔS so that the free energy change is practically independent of temperature.

The capacity factor k', is the dimensionless unit employed to measure chromatographic retention, such that $k'=(t_R - t_0)/t_0=KV_s/V_m$, where t_R is the retention time of the solute, t_0 is the retention time of an unretained solute, K is the thermodynamic equilibrium constant for solute binding, V_s is the volume of stationary phase in the column and V_m is the volume of mobile phase in the column. V_s/V_m is also referred to as the column phase ratio, φ. The free energy change for the chromatographic process is expressed by $\Delta G=-RT \ln K=-RT \ln (k'/\varphi)$. Substitution into the Gibbs–Helmholtz equation for the capacity factor yields the final relationship $\ln k'=-\Delta H/RT+\Delta S/R+\ln \varphi$. If the mechanism of the chromatographic recognition process is invariant over the temperature range studied and the enthalpy is constant, then a linear dependence between $\ln k'$ and $1/T$ (Van't Hoff plot) is exhibited. Non-linearity within these plots is attributed to the presence of multiple retention mechanisms or distinct shape

Table 4
Van't Hoff plot data obtained for k'_1

Compound	Gradient	Intercept	Correlation
1	0.381	0.037	0.9090
2	0.722	−0.919	0.9780
3	1.590	−3.438	0.9966
4	1.866	−4.355	0.9966
5	1.511	−4.936	0.9872
6	0.991	−4.061	0.9516
7	1.845	−5.220	0.9936
8	2.171	−5.541	0.9957
9	0.936	−1.684	0.9980
10	1.399	−5.311	0.9939
11	1.865	−5.221	0.9984
12	0.743	−3.928	0.7120

Table 5
Van't Hoff plot data obtained for k'_2

Compound	Gradient	Intercept	Correlation
1	0.493	−0.216	0.9380
2	1.541	−3.375	0.9963
3	1.978	−4.689	0.9969
4	2.332	−5.757	0.9988
5	1.671	−5.469	0.9906
6	0.991	−4.061	0.9516
7	1.965	−5.620	0.9969
8	2.443	−6.263	0.9964
9	1.225	−2.471	0.9986
10	1.399	−5.311	0.9939
11	1.865	−5.221	0.9984
12	0.743	−3.928	0.7120

differences between molecules with similar retention [17].

In order to evaluate the effect of temperature on enantiomer retention, capacity factors for the twelve mexiletine analogues were measured over the temperature range 0–30°C. Table 4 and Table 5 list data obtained from Van't Hoff plots constructed for k'_1 and k'_2. The data for compounds 6, 10, 11 and 12 are the same for k'_1 and k'_2 as no separation was achieved at any temperature. Compound 12 clearly demonstrates non-linear behaviour suggesting multiple recognition mechanisms. This may be an artefact of this compound's very low retention in this chromatographic system and was subsequently removed from any further analysis. For the remaining eleven compounds, all plots are linear with positive slopes, thus the enthalpies of association are constant and negative over the temperature range studied.

Compensation temperatures may be used to determine whether or not all the solutes display similar retention mechanisms in a given system. A close correspondence of the compensation temperatures may be accepted as proof that the mechanisms are essentially identical [18]. Linearity in plots of ln k' vs. ΔH are indicative of compensation due to similar solute–stationary phase recognition mechanisms. Compensation plots for compounds 1–11 are shown in Fig. 2 and Fig. 3. Values for the capacity factors recorded near the harmonic mean of the temperature range were used in the plots to enhance accuracy

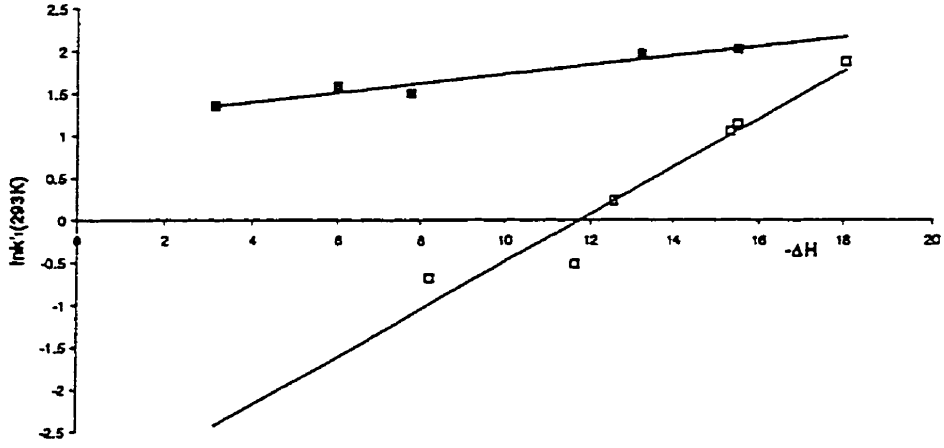


Fig. 2. Compensation plot for the first eluting enantiomers.

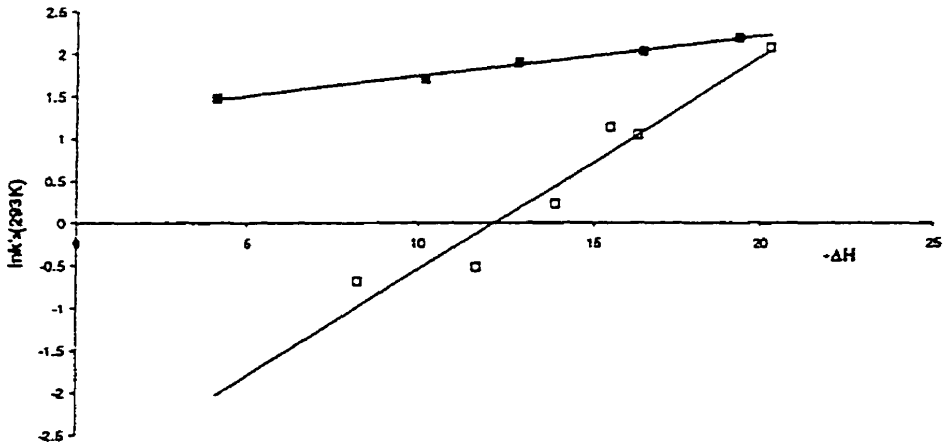


Fig. 3. Compensation plot for the second eluting enantiomers.

[19]. It is observed that in both plots the data fall into two distinct groups, with linearity being observed in both groups. All the compounds containing hydroxyl groups form the upper set, while the remaining compounds form the lower set. Although each set is linear, demonstrating enthalpy–entropy compensation within each set, the slopes and intercepts differ greatly. For ln k'_1 upper set, R=0.9736, slope=0.056 and intercept=1.1677. For ln k'_1 lower set, R=0.9651, slope=0.283 and intercept=−3.3238. For ln k'_2 upper set, R=0.9941, slope=0.0476 and intercept=1.2646. For ln k'_2 lower set, R=0.9665, slope=0.251 and intercept=−3.047. This indicates that the upper and lower sets have different compensation temperatures and therefore different retention mechanisms. The negligible differences between the two upper sets and also between the two lower sets indicates that, within each pair of separated enantiomers, the retention mechanisms are essentially the same, with only the free energy of the binding interactions differing slightly.

4. Discussion

Retention for this series of mexiletine analogues, chromatographed on a Chiralpak AD CSP, under the present conditions, has been shown to conform to two different mechanisms, primarily based upon substituent characteristics. The overwhelming ability of solute hydrogen-bond donor groups to influence retention and enantioselectivity has been demonstrated by the division in the compensation plots. The data for k'_1 and k'_2 are grouped into two sets, distinguished by their respective retention mechanisms. The first set contains solutes with secondary hydrogen-bonding sites in the form of hydroxyl groups and the second set contains the remainder of the compounds. Compound 8 is observed to be capable of being a member of both sets. It can be present in the first set due to the hydrogen-bonding capacity of the para amino group, but as this interaction is weaker than with a para hydroxyl group (as demonstrated by compound 1), it may also be grouped with the second set. These observations conform to the initial retention model proposed via computer docking simulations. Retention is believed to be a two-step process, the first interaction being a hydrogen bond, formed between the outer edge of the CSP helical cavity and the functional group at the solute stereogenic centre. This is followed by conformational adjustment and, steric interactions permitting, stabilisation of the diastereomeric complex via any remaining hydrogen-bonding groups.

Enantioselectivity arises mainly from increasing the stability of the diastereomeric complex via multiple hydrogen-bonding interactions. This forces a reduction in the separation between the solute stereogenic centre and the CSP, thus enhancing any discriminating steric interactions.

Further studies into the recognition mechanisms operating on amylosic CSPs have been conducted.

The results of these studies will be published elsewhere.

Acknowledgments

The authors wish to thank Chiral Technologies Inc. Exton. PA. USA. for advice and financial assistance.

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PAPER II

Investigation of the enantioselective separations of α-alkylarylcarboxylic acids on an amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phase using quantitative structure-enantioselective retention relationships.

Identification of a conformationally driven chiral recognition mechanism.

Based on encouraging results from the initial study, the project was expanded to further probe the mechanisms of enantioselective recognition operating on the same, amylose tris (3,5-dimethylphenylcarbamate) chiral stationary phase. A series of compounds based on 2-aryl propionic acid was chosen due to the inclusion of the therapeutically important class of non steroidal anti inflammatory compounds (NSAIDs).

A larger molecular modelling component was included in an attempt to gain a deeper understanding of the processes involved in chiral recognition. These studies being guided by the interactions identified from the QSERR.

A chiral recognition mechanism which included a conformational adjustment component, was proposed to explain the observed results. This mechanism deviates from the generally accepted theory of chiral recognition and questions the universal validity of the Easson and Stedman model.

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Journal of Chromatography A, 737 (1996) 157–169

JOURNAL OF CHROMATOGRAPHY A

Investigation of the enantioselective separations of α-alkylarylcarboxylic acids on an amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phase using quantitative structure–enantioselective retention relationships Identification of a conformationally driven chiral recognition mechanism

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Received 25 October 1995; revised 29 December 1995; accepted 3 January 1996

Abstract

A series of 28 chiral α-alkyl arylcarboxylic acids were chromatographed on an amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phase (AD-CSP). The retention data were correlated to a series of molecular descriptors to produce quantitative structure–enantioselective retention relationships (QSERR) incorporating the hydrogen bonding ability and aromaticity of the solutes.

The QSERR equations were used to guide molecular modelling experiments designed to investigate the chiral recognition mechanism responsible for the observed enantioselective separations. The results of the study indicate that unlike the standard "three-point interaction" model of chiral recognition, enantioselectivity was due to a "conformationally driven" chiral recognition process.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Quantitative structure–retention relationships; Molecular modelling; Enantioselectivity; Benoxaprofen; Amylose tris(3,5-dimethylphenylcarbamate)

1. Introduction

A wide variety of chiral stationary phases (CSPs) have been developed for the enantioselective separation of chiral substances. The majority of the chiral separations achieved on CSPs are obtained based upon the accumulated trial and error knowledge of the analyst, and often simply by chance. In order to improve the utility of a number of the CSPs, some laboratories have compiled data banks containing reported enantioselective separations [1,2]. Others have developed predictive empirical rules based upon molecular structure [3–5]. A comprehensive quantitative approach to chiral separations has not been developed, although theoretical and chemometric attempts at understanding CSP structure and

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0021-9673/96/$15.00
PII S0021-9673(96)00011-8

recognition mechanisms have been receiving increased attention. This has been recently reviewed by Lipkowitz [6].

One strategy for the development of chemometrically driven predictions of retention and enantioselectivity is the construction of quantitative structure–enantioselective retention relationships (QSERR) [7]. Two types of data are needed for QSERR studies: (1) quantitatively comparable retention data for a set of solutes; (2) molecular descriptors reflecting the structural features of the solutes. Through the use of multiparameter regression analysis or factor analysis, the HPLC capacity factors and molecular descriptors are mutually related. The resulting relationships can then be used to predict retention and enantioselective separations based on the structures of the solute and HPLC-CSP.

In addition to predicting retention and enantioselectivity, QSERR correlations can also be used to describe the interactions which produce the observed chromatographic results [7,8]. Kaliszan and co-workers [9–11] have successfully used this approach to study the retention and chiral recognition mechanisms for a series of benzodiazepines chromatographed on a human serum albumin (HSA)-based CSP. The QSERR analysis also provided information on the structure of the HSA-benzodiazepine binding site [11], which was consistent with the structure derived from X-ray crystallographic studies. Analogous studies have been performed on a variety of other phases [12–15].

A key class of HPLC-CSPs are those based upon the derivatized polysaccharides, cellulose and amylose. While phases based upon both of these backbones are widely used in analytical and preparative enantioselective separations, little is known about the chiral recognition mechanisms operating on the amylose-based CSPs.

In contrast, a number of studies have investigated the foundations of the chiral recognition mechanisms on the cellulose-based CSPs. Francotte and co-workers [16–18] have utilised the cellulose triacetate (CTA I-CSP) and tribenzoate CSP to investigate the relationships between solute structure and enantioselective resolutions, as well as the effect of cellulose supramolecular structure on these separations [19]. Roussel et al. [20] and Isaksson et al. [21] have also carried out computational studies of enantioselective separations on the CTA I-CSP.

Wainer and co-workers [22,23] have investigated the chiral recognition mechanisms responsible for enantioselective resolutions on a CSP composed of cellulose tribenzoate coated upon macroporous silica gel (OB-CSP). When a series of 29 related chiral amides were chromatographed on the OB-CSP, the data suggested the existence of an "attractive interaction-steric fit" chiral recognition mechanism involving the amide dipole on the solute and the ester-moiety on the CSP [22]. When the solutes were chiral aromatic alcohols, a similar mechanism was proposed where the first attractive interaction was a hydrogen bonding interaction between the alcohol moiety on the solute and the carbonyl oxygen on the ester moiety of the CSP [23].

Yashima et al. [24] recently calculated interaction energies between cellulose trisphenylcarbamate (CTPC) and (*R,R*)- and (*S,S*)-*trans*-stilbene oxide, as well as between CTPC and (*R,R*)- and (*S,S*)-1,2-diphenylcyclopropane. The results were more qualitative than quantitative but, when compared to HPLC retention data, demonstrated the potential of this approach. ^{1}H NMR spectroscopy has also been used to observe the chiral discrimination of *trans*-stilbene oxide and 2-butanol enantiomers by cellulose tris-4-methylsilylphenyl carbamate (CTSP) [25]. More information on the actual mechanism of recognition will possibly be obtained by the observation of nuclear Overhauser effect enhancements.

In this paper, we report the results of a series of studies into the chiral recognition mechanism responsible for the enantioselective resolutions of chiral α-alkyl aromatic carboxylic acids on a CSP based upon amylose tris(3,5-dimethylphenylcarbamate), the AD-CSP. The AD-CSP was chosen due to the numerous reported applications using this CSP, c.f. [26–37], and the lack of a clear description of the chromatographic chiral recognition processes operating on this phase.

In the reported studies, QSERR equations were developed by comparing the retention and enantioselectivity data from 28 solutes with a variety of molecular descriptors. Molecular modelling studies were then carried out using the QSERR results to guide the docking of the solutes with a representative

structure of the AD-CSP. The results of these studies indicate that the chiral recognition process involves initial hydrogen bonding interactions between the solute and CSP, insertion of the solute into a ravine on the surface of the CSP, and stabilisation of the solute–CSP complex by formation of an additional hydrogen bond within the ravine. Both enantiomers of the solute can create the same hydrogen bonding interactions with the CSP although one of the enantiomers must expend a greater amount of energy to produce the conformation required for this interaction. Thus, unlike the standard "three-point interaction" model of chiral recognition, enantioselectivity in the system investigated in this study is due to a "conformationally driven" chiral recognition process.

2. Experimental

2.1. Compounds

Compounds 1, 2, 3, 9, 10, 13, 17–21, 27 and 28 were gifts from Upjohn (Kalamazoo, MI, USA). Compounds 4 (Ketoprofen) and 12 (suprofen) were obtained from Sigma (St. Louis, MO, USA). 8 (Benoxaprofen) was obtained from Eli Lilly (Indianapolis, IN, USA). Compounds 5, 6, 11, 14, 16, 22–26, benzoic acid and phenylacetic acid were obtained from Aldrich (Milwaukee, WI, USA). Compounds 7 and 15 were kindly provided by Dr. W.J. Lough (University of Sunderland, Tyne and Wear, UK).

2.2. Preparation of esters

Esters were prepared by dissolving 100 mg of acid in 25 ml dry methanol. A few drops of concentrated hydrochloric acid were added and the reaction mixture left to reflux. All reactions were complete in under 3 h as determined by TLC. The methanol was then removed under vacuum and the ester reconstituted in 25 ml dichloromethane. The organic layer was then washed, 3×30 ml water, 3×30 ml saturated sodium bicarbonate solution and 3×30 ml saturated sodium chloride solution. The organic layer was collected, dried over magnesium sulphate and evaporated to dryness. All compounds were identified by GC–MS.

2.3. Chemicals

HPLC-grade methanol, hexane and isopropanol were obtained from Anachemia Science (Montreal, Canada). Trifluoroacetic acid was obtained from Aldrich.

2.4. Chromatography

The chromatographic system was composed of a Spectra-Physics P1500 binary pump, a Spectra-Physics UV100 variable-wavelength detector and a Spectra-Physics SP8875 autosampler equipped with a 20-μl loop (Thermo Separation Products, Toronto, Canada). Separation was performed on a Chiralpak AD chiral column, 250×4.6 mm I.D. (Chiral Technologies, Exton, PA, USA). Elution order was determined on a Chiramonitor 2000 optical rotation detector (Interscience, Markham, Canada). A Spectra-Physics Datajet integrator acted as an interface for electronic data collection using Winner on Windows software run on a 386 personal computer.

Column temperature regulation was achieved by using a Haake D1-G refrigerated bath/circulator (Fischer Scientific, Montreal, Canada) and a column water jacket.

The mobile phase consisted of hexane–isopropanol (95:5, v/v) plus 1% trifluoroacetic acid, filtered and degassed. All samples were prepared in mobile phase.

Column performance was monitored daily by injecting compound 9 (flurbiprofen). Capacity factors and α values were compared to those obtained when the column was first used, to ensure no variation in column performance over the period of the study.

2.5. Computational chemistry

Molecular models were created using Insight II release 235 (Biosym, San Diego, CA, USA) run on an IBM RS6000 RISC workstation (IBM, Austin, TX, USA). Conformational searching was performed using Search-Compare ver. 2.3 (Biosym) and all energy minimisations were performed using Discov-

er ver. 2.9.5 (Biosym), both running within Insight II.

Statistical analysis was performed using CSS (Statsoft, Tulsa, OK, USA) run on a 486 personal computer.

2.6. Construction of the AD-CSP

A model of the amylose tris(3,5-dimethylphenylcarbamate) stationary phase was constructed following an approach described by Francotte and Wolf [18]. A molecule of α-D-glucose was retrieved from the molecular fragment library within Insight II. This was appropriately derivatised with pre-geometry optimised 3,5-dimethylphenyl groups. Geometry optimisation was performed in three steps: steepest descents followed by conjugate gradients and then the Broyden-Fletcher-Goldfarb-Shanno (BFGS) algorithm. All steps used the constant valence force field (cvff). A convergence criterion of 0.001 kcal mol^{-1} RMS deriv. was used for the final step. All three derivatives were subjected to a systematic conformational search (e.g. for two rotatable bonds using a 1° interval, 129 600 conformations would be sampled), followed by geometry optimisation to give the lowest energy conformation. This monomer was duplicated and the two glucose units joined via the oxygen at C_1 and C_4. The dimer was relaxed and a second conformational search performed to find the values of ϕ and ψ (torsion angles in the linkage between two glucose units) corresponding to the lowest energy structure. These were found to be $\phi = -28.0°$ and $\psi = -13.8°$. Using these values and the previous convergence criterion, a highly relaxed polymer consisting of 20 glucose units was created.

The low energy conformations of (*R*)- and (*S*)-benoxaprofen were also created by systematic searches and geometry optimisation. Both enantiomers obtained exactly the same total energy (E_T) value of 96.078 kcal mol^{-1}.

An 8 Å radius subset of the stationary phase was created. (*R*)- and (*S*)-benoxaprofen enantiomers were docked into the subset in real time whilst monitoring the interaction energies and hydrogen bonds. A molecule was assumed to be optimally docked when the maximum number of possible hydrogen bonds had formed, combined with the lowest achievable interaction energy.

3. Results

A series of 28 α-alkyl arylcarboxylic acids and related compounds were chromatographed on an amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phase: the structures of these compounds are presented in Fig. 1. The contributions of the substituents at the stereogenic centre i.e. the aromatic, alkyl, and acidic moieties, to relative retention (k') and enantioselectivity (α) were investigated by systematically varying these groups. The results are presented below.

3.1. Carboxylic acid moiety

In order to study the contribution of the carboxylic acid moiety, selected compounds were converted into their methyl ester derivatives. Table 1 lists the retention data for the acids and their corresponding methyl esters. In all cases a substantial reduction in retention was observed for the ester as compared to the acid. Separation was only achieved for two of the esters (compound 8, benoxaprofen methyl ester, and compound 25, methyl mandelate) as compared to separation for all of the acids.

A decrease in retention is to be expected in normal-phase chromatography when the hydrophobicity of a solute is increased, thus increasing solute solubility in the mobile phase. In addition, reduction of non-specific interactions between the solute and the more polar stationary phase will also decrease retention. In this case, replacement of the acidic proton with a methyl group reduced the ability of the solute to act as a hydrogen bond donor. The formation of hydrogen bonds between the solute and stationary phase appears to be a key factor in the general retention, as is demonstrated by the drastic reductions in k' following conversion from acid to ester.

Although separation was not obtained for most of the esters, this does not necessarily indicate that the structural requirements for enantiorecognition are no longer present. Rather, solute stationary phase interactions governing general retention, were insufficient to permit noticeable discrimination between enantiomers. Benoxaprofen methyl ester and methyl mandelate both retain a higher degree of retention (k'_2 = 2.09 and 3.18, respectively) thus allowing observable

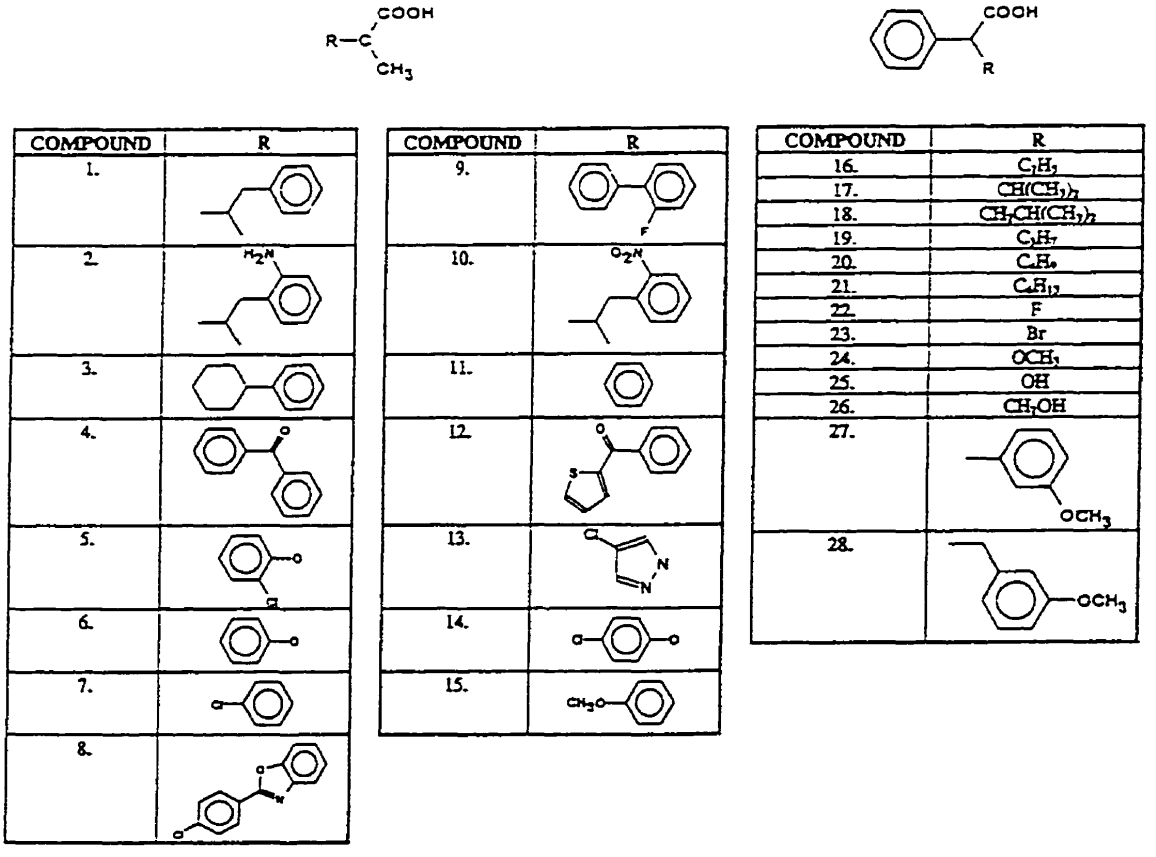

Fig. 1. Structures of the compounds used in this study.

separation. This indicates that other factors can play a significant role in the determination of k' and α.

3.2. α-Alkyl moiety

The effects of variations in the alkyl substituent on k' and α are presented in Table 2. The observed retention values can be grouped into three distinct categories, $k'<4$, $4<k'<8$ and $k'>8$. The compounds whose k' values were less than 4 contained only a straight chained or branched alkyl moiety. The compounds which lay within the $4< k'<8$ group contained *meta* methoxy phenyl moieties and the compounds which comprised the $k'>8$ group

Table 1
Effect on k' and α following conversion from acid to methyl ester

Compoundmethyl ester	k'_2	Decrease in k'_2 (%) on conversion from acid to ester	α Ester	α Acid
5	0.41	78.9	1.00	1.23
6	0.38	83.2	1.00	1.33
8	2.09	91.6	1.17	1.82
9	0.28	92.4	1.00	1.48
11	0.27	86.9	1.00	1.10
14	0.48	83.5	1.00	1.27
25	3.18	68.7	1.09	1.17

See Experimental for chromatographic conditions.

Table 2
Effect of variations in the α-alkyl moiety on k' and α

Compound	k'_1	k'_2	α	Elution
11	1.88	2.06	1.10	R(−):S(−)
16	1.80	2.04	1.14	R(−):S(−)
17	1.55	1.75	1.13	(−):(−)
18	1.36	1.52	1.12	(−):(−)
19	1.66	1.82	1.10	(−):(−)
20	1.56	1.78	1.14	(−):(−)
21	1.42	1.71	1.21	(−):(−)
22	2.31	2.51	1.09	S(−):R(−)
23	2.84	3.20	1.13	(−):(−)
24	2.74	3.01	1.10	R(−):S(−)
25	8.68	10.17	1.17	(−):(−)
26	10.06	12.25	1.22	(−):(−)
27	6.11	7.28	1.19	(−):(−)
28	5.44	5.53	1.02	(−):(−)

See Experimental for chromatographic conditions.

contained hydroxyl moieties. This suggests that additional hydrogen bonding interactions are taking place between the oxygen and hydroxyl moieties in the solute and CSP.

When the correlation between hydrophobicity (log P) of these substituents and their associated k' values was tested, a correlation factor of $r=-0.5941$ ($p=0.03$) was determined. This indicates that a correlation exists between the structure of the side chain and retention: an increase in hydrophobicity causing a decrease in retention. Similar effects have been identified on Pirkle-type [38] and cellulosic [22] CSPs.

Although enantioselective separations were achieved for all compounds, α values were low and fell within a fairly narrow range (1.02–1.22). Previous studies [22,38] have indicated that the size of an alkyl substituent at the stereogenic centre affects α, the α value increasing with increasing substituent size. In this study, when the correlation between alkyl substituent molecular volumes and their associated α values was tested, a correlation factor of $r=-0.1443$ (p=not significant) was determined. This indicates that in the whole series, molecular volume does not appear to be correlated with enantioselectivity.

However, when only the unsubstituted alkyl side chains (compounds 11, 16–21) were considered, a statistically significant correlation, $r=0.7842$ ($p=0.04$), was observed. Further analysis of this subset, indicated that removal of compound 21 from the analysis reduced the correlation factor to $r=0.3938$ (p=not significant) and the comparison lost significance. These results, coupled with observation that compound 28 has the α-substituent with the largest molecular volume but the lowest enantioselectivity ($\alpha=1.02$), suggests that steric limitations exist within the interaction site on the AD-CSP.

3.3. Aromatic moiety

The effect of the structure of the aromatic substituent on k' and α is presented in Table 3. From this data, it is evident that this moiety plays a key factor in the determination of k' and α. The magnitude of this effect is dependent upon the position, nature and number of hydrogen bonding groups within the substituent. Compounds 2, 8 and 9 which contain *meta* acceptors, exhibit the highest enantioselectivities (1.48–1.82), and compounds with acceptors α to the stereogenic centre have enantioselectivities in the 1.23–1.33 range.

For those compounds where absolute configuration is known, the elution order *R*:*S* is prevalent. Compounds 5, 6 and 14 elute *S*:*R* only as a result of a change in priority of the Cahn, Ingold, Prelog

Table 3
Effect of variations in the aromatic moiety on k' and α

Compound	k'_1	k'_2	α	Elution
1	1.48	1.48	1.00	R(−):S(−)
2	10.48	16.99	1.62	(−):(−)
3	1.26	1.65	1.31	(−):(−)
4	8.51	9.52	1.12	R(−):S(−)
5	1.58	1.94	1.23	S(−):R(−)
6	1.70	2.26	1.33	S(−):R(−)
7	2.22	2.46	1.11	(−):(−)
8	13.71	24.89	1.82	R(−):S(−)
9	2.48	3.67	1.48	R(−):S(−)
10	3.06	3.37	1.10	(−):(−)
11	1.88	2.06	1.10	R(−):S(−)
12	12.11	14.01	1.16	S(−):R(−)
13	3.42	4.30	1.26	(−):(−)
14	2.29	2.91	1.27	S(−):R(−)
15	3.42	4.17	1.22	(−):(−)

See Experimental for chromatographic conditions.

naming system for chiral molecules. Column efficiency for compound 1, was too low to directly observe the enantioselective separation by UV however, determination of the enantiomeric elution order was possible using optical rotatory dispersion (ORD).

3.4. QSERR analysis

The retention data were subjected to multiparameter regression analysis against various non-empirical molecular descriptors. The simplest possible relationships between these descriptors were established and are presented in Eq. 1 and Eq. 2.

$$\ln k'_1 = -2.499 + 1.369X(\pm 0.177) \quad p = 0.00000$$
$$+ 0.791Y(\pm 0.121) + 0.415Z(\pm 0.094) \quad (1)$$
$$p = 0.00000 \qquad p = 0.00022$$

$n = 26$, Multiple $R = 0.9469$, $p < 0.0000$.

$F_{(3,22)} = 63.63$, $F_{(3,22),\alpha=0.01} = 4.82$

$$\ln k'_2 = -2.659 + 1.498X(\pm 0.151) \quad p = 0.00000$$
$$+ 0.896Y(\pm 0.103) + 0.439Z(\pm 0.080) \quad (2)$$
$$p = 0.00000 \qquad p = 0.00002$$

$n = 26$, Multiple $R = 0.9670$, $p < 0.0000$,

$F_{(3,22)} = 105.58$, $F_{(3,22),\alpha=0.01} = 4.82$

X and Y are the numbers of hydrogen bond donors and acceptors, respectively. Z is the degree of aromaticity in the molecule.

3.5. Molecular modelling studies

Directed by the initial QSERR equations, molecular modelling was used to either expand or reject the hypotheses proposed via the equations. Benoxaprofen (BP, compound 8), was chosen as the test molecule as it demonstrated the highest retention and enantioselectivity of the series. A conformation for the amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phase (AD-CSP) has been proposed (Fig. 2). From this model it would appear that while the helix is much larger than those proposed for related cellulosic phases [39], it is consistent with other reports [40]. It is recognised that this may not be a true representation of the actual stationary phase, however, in the absence of a crystal structure, this simulation was accepted as the working model.

The model AD-CSP also emphasises the number of possible hydrogen bonding sites covering the surface of the helical cavity. The modelling study was not aimed at trying to reproduce the energies of interaction between the two enantiomers of BP and the stationary phase, but rather probe the possible modes of binding. It is clear that there are many sites for acid interactions of a non-enantiospecific nature thus contributing to general retention.

3.6. Chiral recognition process

A site was located within the helical ravine (Fig. 3) at which it is believed that enantioselective discrimination occurs. When BP is docked in this ravine, there is a potential for three simultaneous hydrogen bonding interactions (Fig. 4). Two of these interactions can occur between the carbonyl oxygen and acid hydroxyl proton on the acid moiety of BP and the amide proton and ether oxygen on the CSP. A third hydrogen bond can be formed between an amide proton on the CSP and the oxygen and/or nitrogen atom in the five-membered oxazole ring.

The first two interactions are presumably the initial solute–stationary phase interactions and serve to tether BP to the CSP. These interactions constitute a major portion of the chromatographic retention (see Table 1), but are not the primary driving force behind the chiral recognition process. The third hydrogen bonding interaction also contributes to the chromatographic retention. In the case of BP, complementary distances between interacting groups in BP and CSP and the ability of the solute to fit the contour of the stationary phase contribute to the high retention of this solute. This is suggested by the relatively high retention of BP after the primary hydrogen bonding interactions are reduced by conversion of the acid to the methyl ester (Table 1).

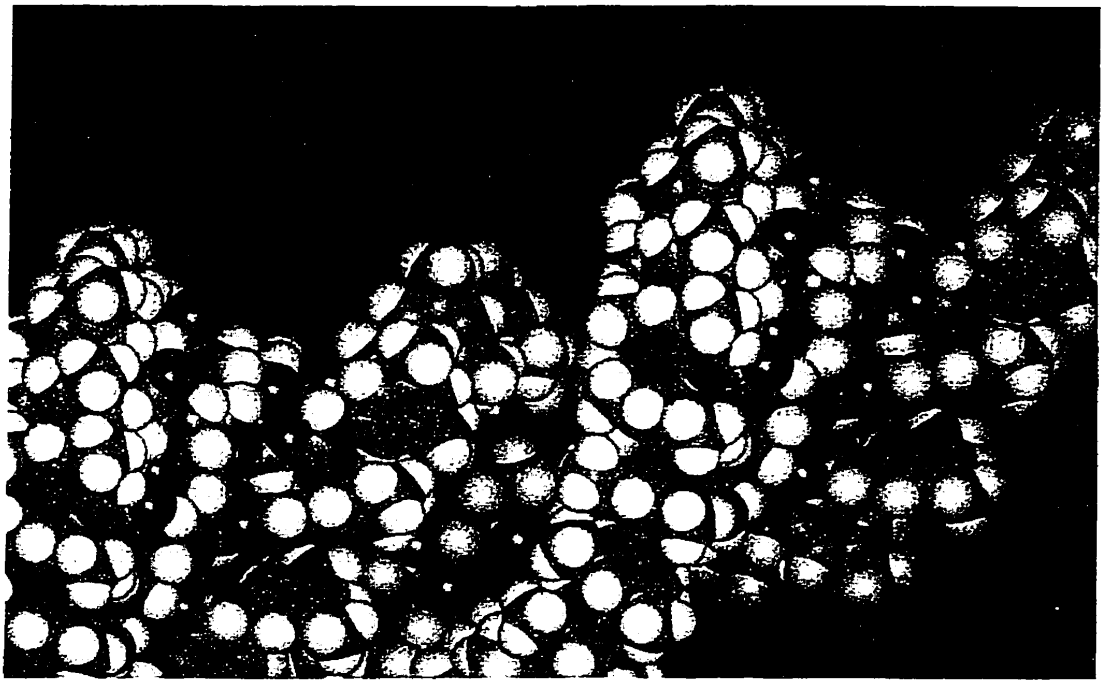

Fig. 2. Model of the proposed amylose tris(3,5-dimethylphenylcarbamate) chiral selector.

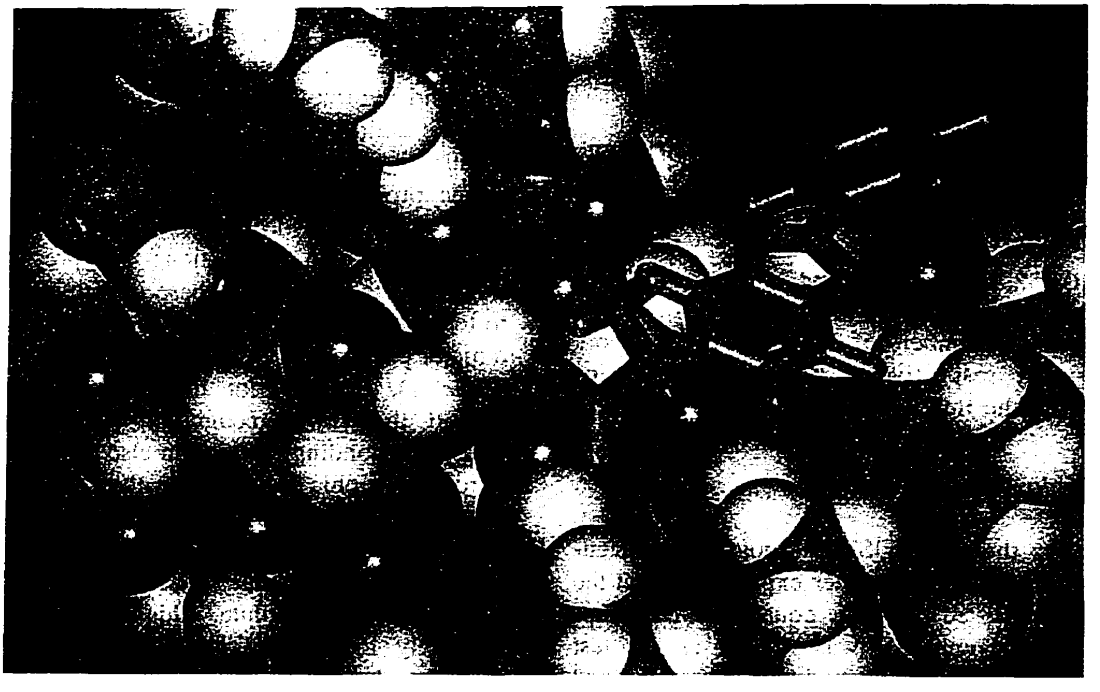

Fig. 3. Model showing *R*(−)-benoxaprofen docked into the helical cavity within the chiral selector.

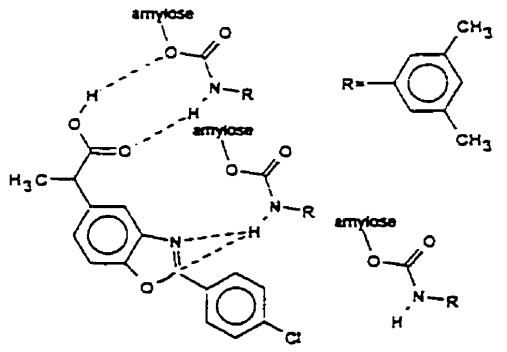

Fig. 4. Proposed mechanism of binding showing hydrogen bonding sites for benoxaprofen.

When (*S*)-BP was docked in the ravine in its lowest energy conformation, all three simultaneous hydrogen bonding interactions were formed without significant molecular deformations. (*R*)-BP has a mirror image lowest energy conformation. The docking of this conformation in the ravine produced significant steric interactions between the solute and stationary phase and the same hydrogen bonding interactions could not be obtained. However, a slight rotational change in the conformation of (*R*)-BP produced a structure which was capable of forming the identical solute–CSP interactions obtained by (*S*)-BP.

Even though the two enantiomers form identical hydrogen bonding interactions, and presumably the same hydrophobic interactions as well, the diastereomeric BP–CSP complexes differ in their stabilities leading to chiral discrimination. The difference arises from the internal energies of the two enantiomer conformations. The bonding conformation of (*R*)-BP is approximately 250 cal mol^{-1} higher in energy than that of (*S*)-BP. The theoretical enantioselectivity arising from this energy difference can be estimated using the equation $\Delta\Delta G = -RT\ln\alpha$ [41], and the calculated α (1.52) is consistent with the observed α (1.82).

The proposed conformationally driven chiral recognition mechanism is also consistent with the observed minimal effect of the steric bulk of the α-substituent (Table 2). When the solutes are docked in the ravine, the α-alkyl substituents are directed away from the stationary phase, out of the ravine and into the mobile phase (Fig. 4). Since differences in molecular conformational energy and not steric interactions appear to be the key driving forces behind chiral discrimination of the majority of the solutes, it would be assumed that in a series of 14 α-alkyl phenylacetic acids, there would be relatively little variation in the magnitude of the observed enantioselectivities. This is indeed the case.

In the proposed chiral recognition mechanism, the stronger the bonding interactions between the solute and CSP, the larger are the associated decreases in the entropic freedoms of the two entities, together with a greater increase in the conformational (and therefore, energetic) demands. In the system studied, the key determinant of the strength of the solute–CSP complex is the third hydrogen bonding interaction between an amide hydrogen on the CSP and a hydrogen bond acceptor in or on the aromatic moieties. The importance of this interaction is illustrated by the variation in the observed enantioselectivities for the series of 15 α-methyl arylcarboxylic acids (Table 3).

It should be noted that the chromatographic process utilised in this study involves distribution from a non-polar mobile phase to a polar stationary phase. Thus, general molecular hydrophobicity should reduce chromatographic retention and not play a major role in the chiral recognition process. However, once the solute has been tethered to the CSP via the hydrogen bonding interactions between the carbonyl oxygen and acid hydroxyl proton on the acid moiety of BP and the amide proton and ether oxygen on the CSP, the hydrophobicity of the aromatic portion of the solute may play a role in the insertion of the solute into the ravine in the CSP. Thus the enantioselective separations of the α-alkylphenyl acetic acids are a result of either the hydrophobicity of the phenyl ring, or π-facial hydrogen bonding interactions [42] between the aromatic ring on the solute and an amide hydrogen on the CSP. In either case, the interactions are weak and the resultant enantioselectivity relatively low (Table 2 and Table 3).

The computer-simulated docking of (*S*)-BP and (*R*)-BP suggested that the interaction of (*S*)-BP with the CSP takes place via a concerted mechanism while the (*R*)-BP–CSP interaction involves a two-step process. In order to investigate this possibility, a

study was conducted to determine the effect of temperature on the chromatographic retentions of the enantiomers of BP, BP methyl ester and for the achiral phenylacetic and benzoic acids. Retention data were obtained at 10, 15, 20, 25, 30, 35 and 40°C, and Van 't Hoff plots created for the 6 compounds, Fig. 5.

From the Van 't Hoff plots it can be seen that the two enantiomers of BP exhibit different behaviour. (*S*)-BP displayed linear behaviour while distinct curvature was observed for (*R*)-BP. Linearity within these graphs is attributed to enthalpy–entropy compensation with an invariant retention mechanism over the temperature range studied [43]. Non linearity is attributed to multiple retention mechanisms. Curvatures in Van 't Hoff plots have been observed in achiral RPLC systems [44], and attributed to transition within the stationary phase, from a less ordered to a more ordered state at lower temperatures. In this system, since a curvature is only noted for one enantiomer, then it is the solute not the CSP which is undergoing a temperature-dependent transition. This is consistent with the results of the docking interactions and is supported by the Van 't Hoff plots for the achiral phenylacetic and benzoic acids which are both linear over the temperature range studied.

It is interesting to note that both enantiomers of BP methyl ester exhibit the same characteristics as (*R*)-BP, but with a substantial reduction in retention. The absence of two simultaneous hydrogen bonding interactions between the acid moiety of the solute and the CSP obviously changes the relative initial positions of the two entities and reduces the stability of the initial complex. Both the (*S*)-BP methyl ester and (*R*)-BP methyl ester must undergo conformational changes to produce the additional hydrogen bond involving the aromatic moiety. Thus, the retention mechanisms of both enantiomers should show temperature-dependent transitions and lower relative energy differences for the resultant diastereomeric complexes, i.e. lower enantioselectivities.

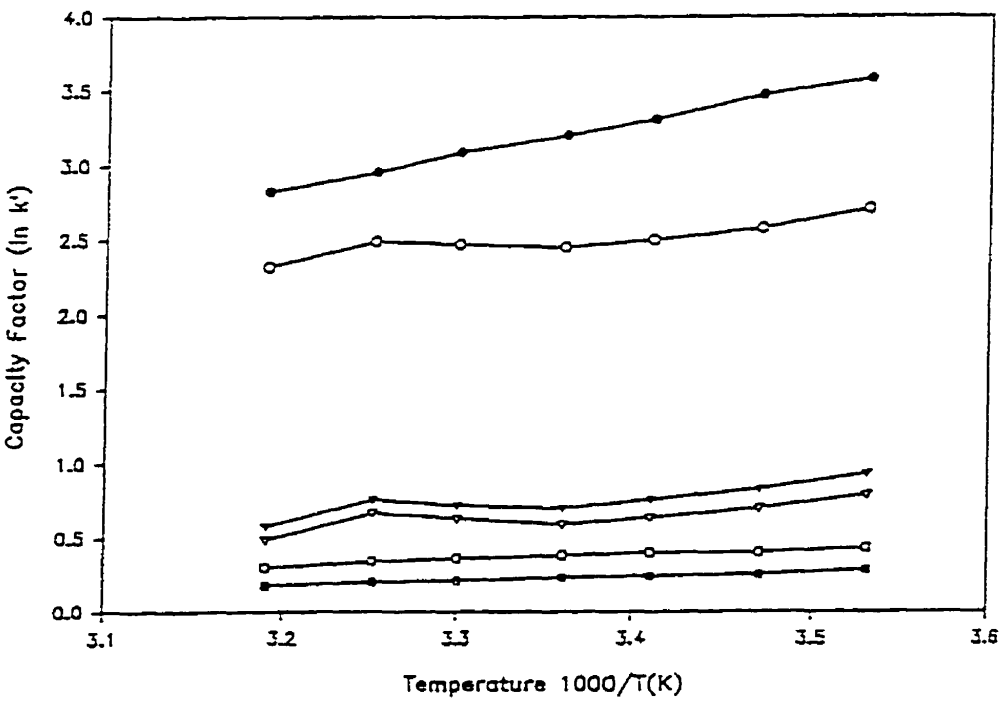

Fig. 5. Van 't Hoff plots for benoxaprofen, benoxaprofen methyl ester, benzoic acid and phenylacetic acid. (●) *S*(−)-benoxaprofen, (○) *R*(−)benoxaprofen, (■) benzoic acid, (□) phenylacetic acid, (▼) benoxaprofen methylester k'_1, (▽) benoxaprofen methylester k'_2.

4. Conclusions

4.1. Conformationally driven chiral recognition process

The chiral recognition process suggested by the results from this study is diagrammed in Fig. 6. In this scheme, step 1 is the distribution of the solute from the mobile phase to the CSP. This distribution is driven by hydrogen bonding interactions between the carbonyl oxygen and acid hydroxyl proton at the acid moiety of the solute and the amide proton and ether oxygen on the CSP. The tethering of the solute to the CSP results in the formation of two diastereomeric complexes [(*R*)-BP–CSP and (*S*)-BP–CSP] of equivalent stabilities. This is a retention step, not a chiral discrimination step. However, it is evident that without formation of the solute–CSP complexes, chiral recognition could not occur, and thus, these interactions are a key first step towards enantioselective separation.

Once the complex has been formed, the solutes and CSP form a third hydrogen bonding interaction produced after insertion of the solute into a ravine on the CSP. In this step the solutes and the CSP conformationally adjust to each other to maximise the interactions and, thereby, the stabilities of the diastereomeric complexes. It is this step that produces the different stabilities of the (*R*)-BP–CSP and (*S*)-BP–CSP complexes and ultimately the observed enantioselective separation.

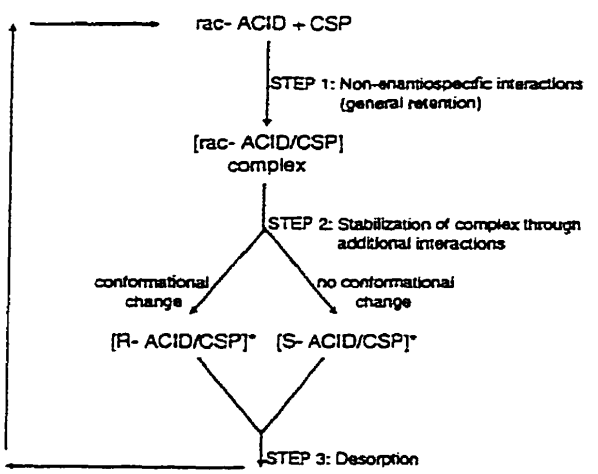

Fig. 6. Proposed mechanism of chiral discrimination.

Since three hydrogen bonding interactions are apparently involved in chiral discrimination, it is tempting to describe this process in terms of the "three-point" interaction model delineated by Pirkle et al. [45], for chiral chromatographic recognition. In this model, there must be at least three simultaneous interactions between the CSP and solute enantiomers, where at least one of the interactions is stereochemically dependent. Is this the case for the enantioselective resolutions achieved on the AD-CSP with this series of compounds?

(a) While three simultaneous hydrogen bonding interactions produced the largest enantioselectivities, the observed chiral resolution of (*RS*)-BP methyl ester indicates that in this series, two simultaneous hydrogen bonding interactions were sufficient.

(b) One could also view the "three interactions" as involving three separate bonds to the stereogenic centre. In this case, the hydrogen bonding interactions involving the carboxylic and aromatic moieties of the solute would position the solutes relative to the CSP and the substituents on one or both of the remaining bonds to the stereogenic centre would provide the stereochemically dependent interaction(s). In the standard "three-point" interaction model, the third and defining factor is often due to steric interactions: this is illustrated by studies demonstrating a correlation between α and the size of alkyl side chains at the stereogenic centre [22,38,46]. This was not the case with this series of solutes on the AD-CSP, although steric structure of the interaction site on the CSP does play a role.

The results of this study suggest that another chiral recognition mechanism is responsible for the enantioselective separation of this series of solutes on the AD-CSP. It appears that the key factor in the chiral recognition mechanism is that both enantiomers can and do make the same hydrogen bonding interactions with the CSP. To make these interactions, the solute enantiomers must undergo conformational changes: the extent and energetic consequences of these changes are dictated by the chirality of the CSP. Thus, in this case, chiral recognition is not due to "point" interactions but "molecular" interactions and the process is conformationally driven.

This is an interesting direction for the study of

chiral recognition mechanisms, which will necessitate further investigations. A key aspect which is not yet available, is the elucidation of the CSP molecular conformation via such methods as NMR. Yashima et al. [47] has reported some initial NMR studies using a cellulose tris(4-trimethylsilylphenylcarbamate) CSP, and a few studies have been attempted on various amylose derivatives [48]. However, direct studies with the AD-CSP are difficult due to the insolubility of the phase [48].

Further studies with the AD-CSP and related phases are underway and will be reported elsewhere. A combination of the QSERR and molecular modelling results from this and the following studies, should help clarify the chiral recognition mechanism.

Acknowledgments

The support and funding of this work by Chiral Technologies Inc. is greatly appreciated. The authors would also like to thank Dr. Michael Trani (BRI, Montreal, Canada).

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2.1 Appendix

The chromatographic conditions for this study were selected on the basis that most of the compounds obtained some degree of separation without unacceptably long retention times. Five representative compounds were chosen to investigate the effects of mobile phase composition. Although mobile phase composition is necessarily a constant in this work, it was important to verify the nature of the constant, in order to rationalise the contributions to retention and selectivity.

The addition of TFA to the mobile phase in concentrations ranging from 0.1 - 1% has no observable effect on retention or selectivity. Removal of TFA from the mobile phase causes considerable peak tailing and therefore was included to improve chromatographic peak symmetry. The five test compounds were chromatographed in mobile phase containing isopropanol in concentrations ranging from 0.4 - 1.5 M. In all cases, reducing the molar concentration of alcohol caused an increase in retention of both enantiomers of each solute. No reversal in elution order was observed, Figures 1-5. Compounds 1, 2 and 9 all demonstrate slightly increased selectivity at low alcohol concentrations. Compound 10 shows no variation in selectivity and selectivity for compound 11 is lost at very low alcohol concentration. The curves represent the ability of the solute enantiomers to displace the competing alcohol molecules from hydrogen bonding sites on the stationary phase. It must be noted that the alcohol is not a prerequisite for chiral separation, and the discriminating properties of the stationary phase remain constant or are enhanced at lower concentrations of alcoholic modifier.

Compound 1

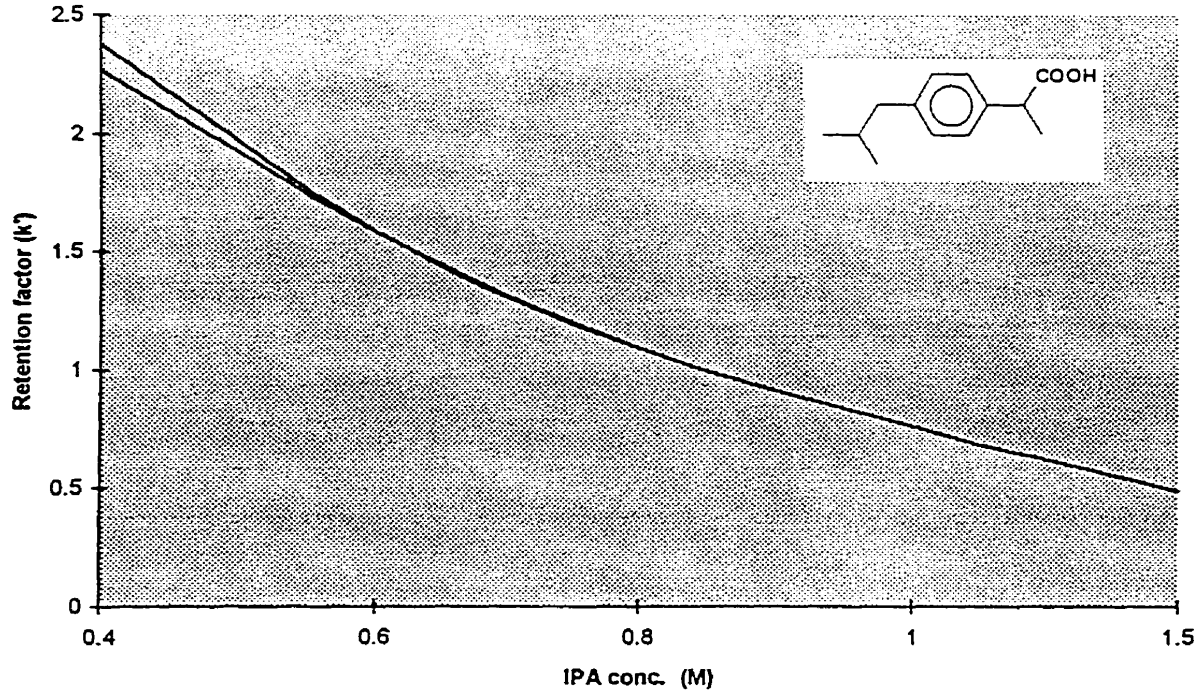

Figure 1. Retention of ibuprofen enantiomers *vs.* mobile phase IPA concentration.

Compound 2

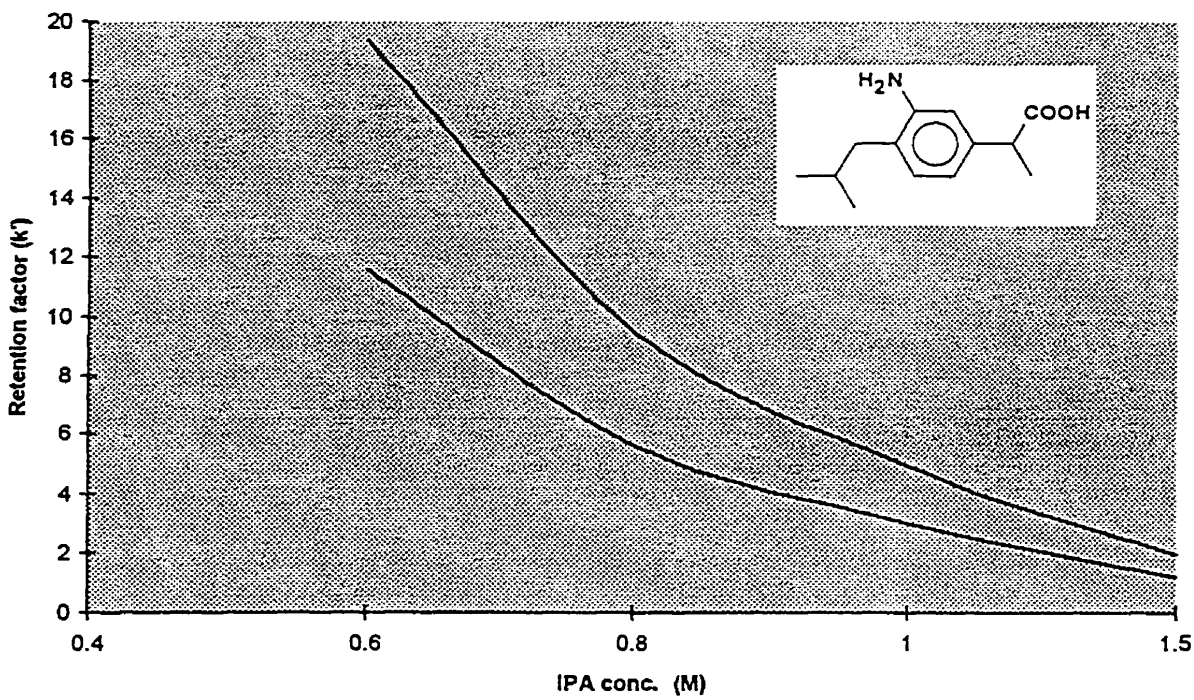

Figure 2. Retention of meta-amino ibuprofen enantiomers *vs.* mobile phase IPA concentration.

Compound 9

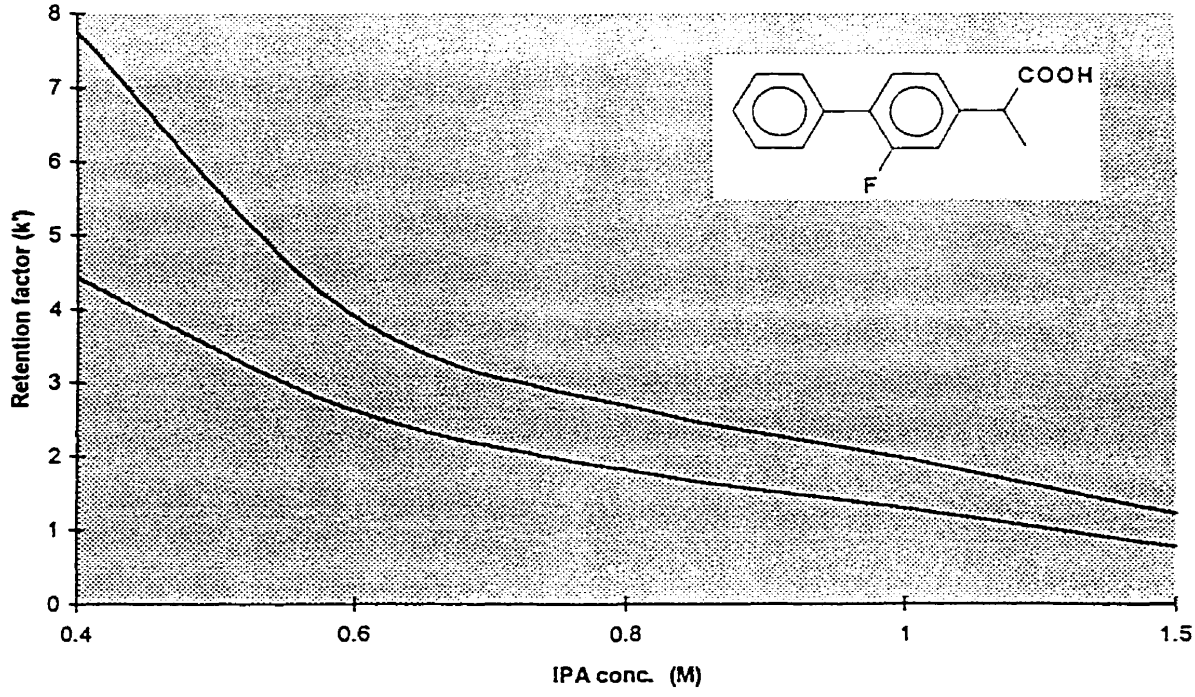

Figure 3. Retention of flurbiprofen enantiomers *vs.* mobile phase IPA concentration.

Compound 10

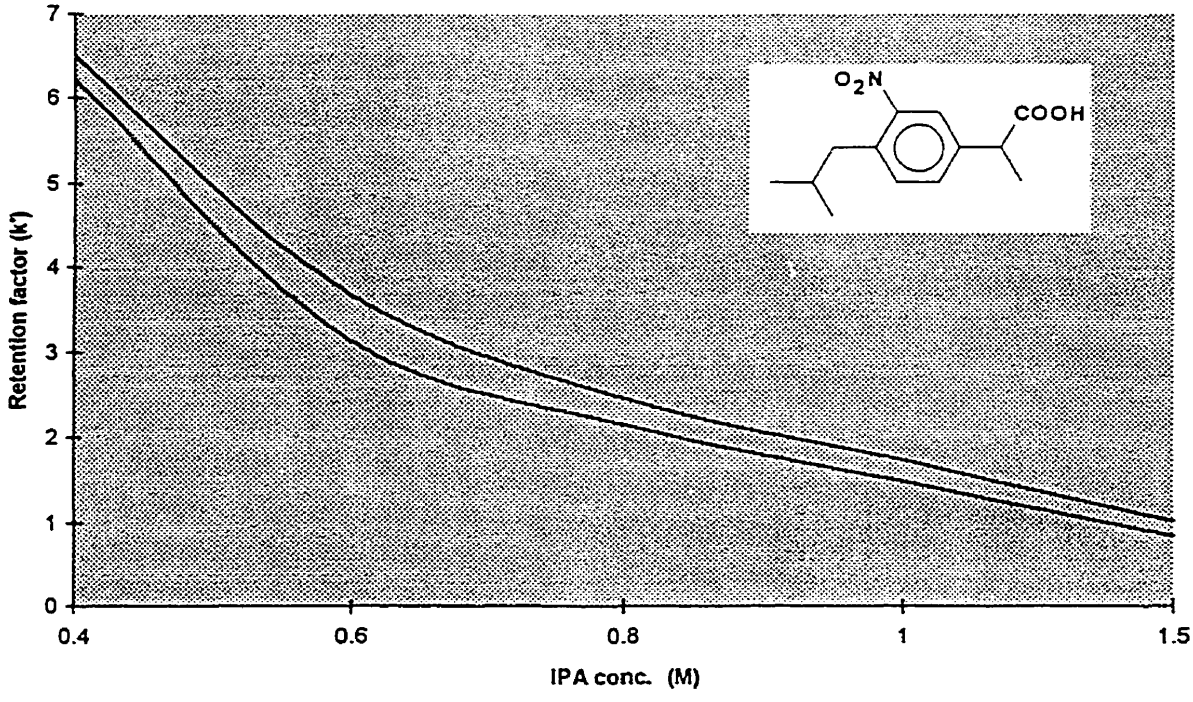

Figure 4. Retention of meta-nitro ibuprofen enantiomers *vs.* mobile phase IPA concentration

Compound 11

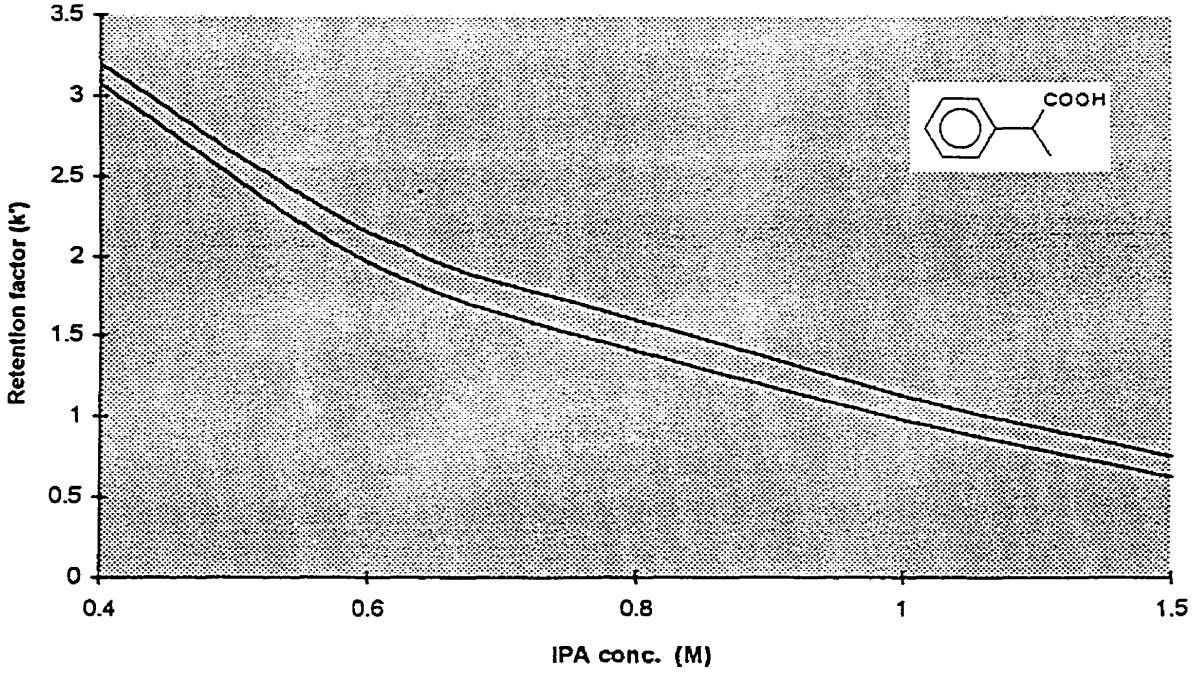

Figure 5. Retention of 2-phenylpropionic acid enantiomers *vs.* mobile phase IPA concentration.

PAPER III

Enantioselective separation of enantiomeric amides on three amylose-based chiral stationary phases.

Effects of backbone and carbamate side chain chiralities.

At this point, insight had been gained into the operation of the most commonly used amylose based chiral column. A logical expansion of the study was to move from a purely intracolumn investigation to an intercolumn investigation. This allowed for the structural contributions of both solute and stationary phase to be related to retention and enantio-discrimination.

The recent commercialisation of an amylose tris (S-phenylethylcarbamate) and preparation of an amylose tris (R-phenylethylcarbamate) by Prof. Y. Okamoto, enabled the characteristics of three closely related columns to be studied.

It was determined that the stacking of the stationary phase side chain derivative is critical in determining the size and shape of the binding cavities. This has a profound effect on the ability of a particular phase to separate enantiomers, due to differences in the type and extent of possible molecular interaction. Elution order is entirely determined by the position of the solute stereogenic centre, in relation to the direction of the solute amide moiety.

THE ENANTIOSELECTIVE SEPARATION OF ENANTIOMERIC AMIDES ON THREE AMYLOSE-BASED CHIRAL STATIONARY PHASES: THE EFFECTS OF BACKBONE AND CARBAMATE SIDE CHAIN CHIRALITIES

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ABSTRACT

A series of enantiomeric amides have been chromatographed on three amylose-based chiral stationary phases (CSPs): amylose tris (3,5-dimethylphenylcarbamate) (AD-CSP), amylose tris (S-phenylethylcarbamate) (AS-CSP) and amylose tris (R-phenylethylcarbamate) (AR-CSP). The relative retentions and enantioselectivities of the solutes on the three CSPs were compared and basic structure-retention relationships developed to describe the chromatographic results. The data indicate that for these solutes, the observed elution order was a function of the chirality of the amylose backbone, while the magnitude of the enantioselective separations was affected by the chirality of the carbamate side chain.

INTRODUCTION

Derivatised polysaccharide-based HPLC stationary phases are a key class of chiral stationary phases (CSPs) owing to their wide versatility and robustness[1]. These phases are built upon cellulose and amylose backbones and created through derivatisation of the free hydroxyl groups on the glucose components of the biopolymers. Both ester and carbamate derivatives have been reported and successfully used to resolve enantiomeric compounds.

The CSP based upon amylose tris (3,5-dimethylphenylcarbamate) has been a particularly successful member of the polysaccharide-based class of CSPs [2-13] . The optimal use of the AD-CSP requires an understanding of how chiral recognition occurs on this CSP. This subject has been the topic of two recent studies[14,15], but has yet to be definitively described. This study is a part of this process and investigates the role of the carbamate derivative.

Since the introduction of the first Pirkle-type HPLC-CSP based upon a 3,5-dinitrobenzoyl derivative of R-phenylglycine[16], amide and carbamate moieties have played a key role in the design of CSPs. These functional groups are primarily used to facilitate the distribution of the solute to the CSP through hydrogen bonding and dipole-dipole interactions. This is obviously a key aspect of the chiral recognition process, since the formation of the solute-CSP complex is a necessary prerequisite to enantioselectivity and the strength of this complex affects the magnitude of the observed enantioselectivity.

In most CSPs, the amide and carbamate derivatising moieties are achiral and any observed enantioselectivity arises from the properties of the derivatised backbone, i.e. amylose or R-phenylglycine, as cited above. However, if the derivatising agent possesses its own chirality, enantioselectivity can arise from the chirality of the backbone, the side chain or a combination of the two. Using R- and S-naphthylethylcarbamate derivatives of β-cyclodextrin, Armstrong[17] demonstrated that depending upon solute structure and mobile phase conditions, enantioselective separations occurred on these CSPs due to either the chirality of the cyclodextrin molecule itself, to a combination of the chiralities of the cyclodextrin and the side chain, or solely on the basis of the stereochemical structure of the carbamate moieties.

This approach has been adapted to the study of the carbamate moiety on the AD-CSP by comparing the chromatographic properties of the AD-CSP to two related CSPs, previously described by Okamoto, which are based upon the tris (S-phenylethylcarbamate) and tris (R-phenylethylcarbamate) derivatives of amylose, the AS-CSP and AR-CSP, respectively[7]. The structures of the CSPs are presented in Figure 1. A series of enantiomeric amides were chromatographed on each CSP and the relative retentions and enantioselectivities compared. Basic structure-retention relationships were developed for the separation of compounds on the three phases, in order to facilitate both intra- and inter-phase comparisons in terms of retention, selectivity and elution order. The results indicate that for these solutes, the observed elution order was a function of the chirality of the amylose backbone, while the magnitude of the enantioselective separations was affected by the chirality of the carbamate side chain.

MATERIALS AND METHODS

Compounds

Compounds 1-5 were prepared according to a previously reported procedure involving conversion of the appropriate aliphatic acids into acid chlorides followed by condensation with aniline[18]. Compounds 6-11 were gifts from M. Wang (University of Sunderland). Compound 12 (flurbiprofen) was a gift from Upjohn Co. (Kalamazoo, MI, USA). Compounds 13 was prepared by refluxing flurbiprofen in methanol.

Chemicals

HPLC grade hexane and isopropanol were obtained from Anachemia Science (Montreal, Que., CA). Trifluoroacetic acid was obtained from Aldrich (Milwaukee, WI, USA).

Chromatography

The chromatographic system was composed of a Spectra-Physics P1500 binary pump, a Spectra-Physics UV1000 variable wavelength detector and a Spectra-Physics SP8875 autosampler equipped with a 20 µl loop (Thermo Separation Products, Toronto, Ont., CA). Separations were performed on Chiralpak AD and Chiralpak AS chiral columns, 250mm x 4.6mm i.d. (Chiral Technologies Inc., Exton, PA, USA), and an amylose tris (R-phenylethylcarbamate) chiral column (AR), 250mm x 4.6mm i.d. kindly provided by Prof. Y. Okamoto. Elution order was determined on a Chiramonitor 2000 optical rotation detector (Interscience, Markham, Ont., CA). A Spectra-Physics Datajet integrator acted as an

interface for electronic data collection using Winner on Windows software run on a 386 personal computer.

Column temperature regulation was achieved by using a Haake D1-G refrigerated bath/circulator (Fischer Scientific Limited, Montreal, Que., CA) and a column water jacket.

The mobile phase consisted of [hexane - isopropanol (95 : 5, v/v)] + 1% trifluoroacetic acid, filtered and degassed. All samples were prepared in mobile phase.

To ensure no variation in column performance over the period of the study, column performance was monitored daily by injecting compound 12 (flurbiprofen). Capacity factors and α values were compared to those obtained when the column was first used.

Computational chemistry

Molecular models were created using Insight II release 235 (MSI/Biosym, San Diego, CA, USA) run on an IBM RS6000 RISC workstation (IBM Corporation, Austin, TX, USA). Conformational searching was performed using Search-Compare ver. 2.3 (MSI/Biosym) and all energy minimisations were performed using Discover ver. 2.9.5 and MOPAC ver. 6 (MSI/Biosym), both running within Insight II. Molecular surface properties were calculated using MOLCAD run on a Silicon Graphics Indigo Workstation (Silicon Graphics Computer Systems, Saint-Laurent, Que., CA).

Statistical analysis was performed using TSAR ver 2.41 (Oxford Molecular Ltd., Oxford, UK) run on an IBM RS6000 RISC workstation.

RESULTS

The 17 compounds used within this study were chromatographed under identical conditions on the AD, AS and AR CSPs, so that all results would be directly comparable. The chromatographic conditions were the same as those used in a previous study into the enantioselective separations of α-alkyl arylcarboxylic acids on an AD CSP[14].

The amide solutes fall into two distinct groups, compounds 1-5 and compounds 6-11. Compounds 1-5 are benzoyl amides prepared from α-methyl alkylcarboxylic acids of varying length (C_2-C_6). These compounds were chosen in order to study the contribution of the alkyl chain to retention factor (k'), enantioselectivity (α) and elution order. Compounds 6-11 are α-methyl arylamides, chosen in order to study the contribution of the aromatic group to retention factor (k'), enantioselectivity (α) and elution order. As these compounds form two different groups, each group was considered separately. Since the groups were of limited size, the chromatographic results could not be subjected to multiple regression analysis. It was possible however, to develop single term linear regression equations utilising the most significant molecular descriptor in each case. The terms used in these equations give an indication as to the nature and extent of the interaction primarily responsible for the observed chromatographic property.

Chromatographic results for compounds 1-5 are given in Table 1 with the associated structure-retention relationships given in Table 2. The primary factor governing retention on all three columns is the size of the aliphatic carbon side chain. This general bulk descriptor

being negative in all cases accounts for the decreasing retention with increasing solute hydrophobicity. Slightly reduced correlation coefficients were obtained for the AR regression due to retention being fairly constant until the side chain length reaches 5. This indicates that at least a second factor is required to account for the majority of the retention. Selectivity on the AD and AS are somewhat comparable such that values do not change as a function of side chain length, with α being invariant at around 1.10 on the AD and 1.00 on the AS. Selectivity on the AR phase increases with increasing side chain steric bulk. Elution order on the three columns is invariant with the (+) enantiomer eluting first in each case.

Chromatographic results for compounds 6-11 are given in Table 3 with the associated structure-retention relationships given in Tables 4 and 5. The negative dipole terms incorporated in the retention equations for the AD and AS phases suggest that electrostatic interactions play a more important role than molecular hydrophobicity for this series of compounds. This is also reasonable as the three amylose derivatives contain complimentary CONH sites capable of forming various types of significant attractive electrostatic interactions with solutes. Electrostatic interactions are able to play different roles in the retention of the two sets of solutes due to the inherent differences in the basic structures of the sets. The two types of amide are non equivalent and will thus be affected to a lesser or greater extent by external forces.

A different key variable was observed for the retention of solutes 6-11 on the AR phase. In this series, the connectivity descriptor Kier ChiV3 adequately accounts for the majority of retention. This suggests that solute molecular shape plays an important role with

this CSP. Kier ChiV3 is a topological descriptor used to characterise the constitution and configuration of a molecule by a single number.[19,20]

The selectivity on the three phases for the chromatographic separation of compounds 6-11 is illustrated in Table 5. Although the terms used in the equations are highly correlated to the observed chromatographic separations, selectivity amongst the phases is obviously dependent upon factors peculiar to each phase, thus the three descriptors highlight the apparent structural differences between the phases.

Elution order on the three phases remains constant, where determinable, with the (+) enantiomer eluting first in each case.

The third group of compounds address the significance of solute functional group by systematically varying this moiety. Flurbiprofen was changed from acid to ester and amide. The chromatographic results for these compounds are given in Table 6. The amides demonstrate by far the highest retention of the four functional groups with AS retention > AR retention > AD retention. The esters show no retention on any phase and consequently no enantioselectivity.

DISCUSSION

From the tables of chromatographic results, it can be seen that both groups of amides elute from all three columns with the (+) enantiomer being the least retained. Therefore, chirality of the carbamate side chain has no effect on enantiomeric elution order. This suggests that the chiral discriminating interaction arises from the inherent chirality of the amylose helix. Although the order of elution is invariant, the magnitude of the observed selectivity differs greatly. For the first group of amides (compounds 1-5), the AD and AS columns seem unable to effectively differentiate between enantiomers. For the AR column however, the size of the solute alkyl side chain is highly correlated to α.

It is believed that the amylose derivative dictates the shape of the binding cavity in the stationary phase. This is especially true for the AS and AR phases which only differ in the chirality of this derivative. Molecular modelling simulations have shown the AD phase as being a highly ordered helical polymer[14]. The same simulations were undertaken with the AS and AR phases. The results suggest that addition of chirality to this model completely disrupted the order in this highly organised system. This disorganisation creates a new range of binding sites for solute-CSP interaction. The size and shape of these sites dictate the type and extent of molecular interplay, permitting stronger interactions and thus longer retention times. For the second group of amides (compounds 6-11), retention on the AD and AS phases is described by electrostatic parameters. Shape differences in the AR phase do not

permit the same electronic interactions to the same extent and solute topology i.e. the Kier ChiV3 term in the regression equation becomes the principal retention factor.

The magnitudes of the observed separations for the second group of amides are adequately described by the equations in table 5. Combined with the retention equations, they give a reasonable account of the possible recognition processes. On the AD phase, retention is primarily governed by electrostatic interactions, with selectivity arising from the molecular connectivity or topology of the solutes. On the AR phase however, retention is primarily governed by molecular connectivity with selectivity arising from electrostatic interactions. The AS phase is similar to the AD phase in this respect.

The nature of the solute-CSP interactions, as expected, are similar for all three phases, however, it is the contributions and order of these interactions which varies from phase to phase. The solutes all contain a stereogenic centre attached to the amide carbonyl carbon. It is proposed that elution order is governed by the positioning of the solutes in proximity to the amylose backbone by the direction of the amide.

The three compounds in Table 6 further illustrate the role of hydrogen bonding as described previously. Removal of the acidic proton in changing from an acid to a methyl ester causes a complete loss of retention (and consequently selectivity) on all three phases. Addition of an extra hydrogen bond donor causes a significant increase in retention on all three phases. The observed effects are also enhanced by changes in functional group hydrophobicity. The amide being the least hydrophobic and therefore the least soluble in the non-polar mobile phase. As far as can be determined, there are no inter-column changes in elution order.

ACKNOWLEDGMENTS

The support and funding of this work by Chiral Technologies Inc. (Exton, PA, USA) is gratefully acknowledged. The authors also wish to thank Prof. Y. Okamoto for the loan of the AR column used in this study. This work is supported in part by an NSERC grant awarded to I.W.W.

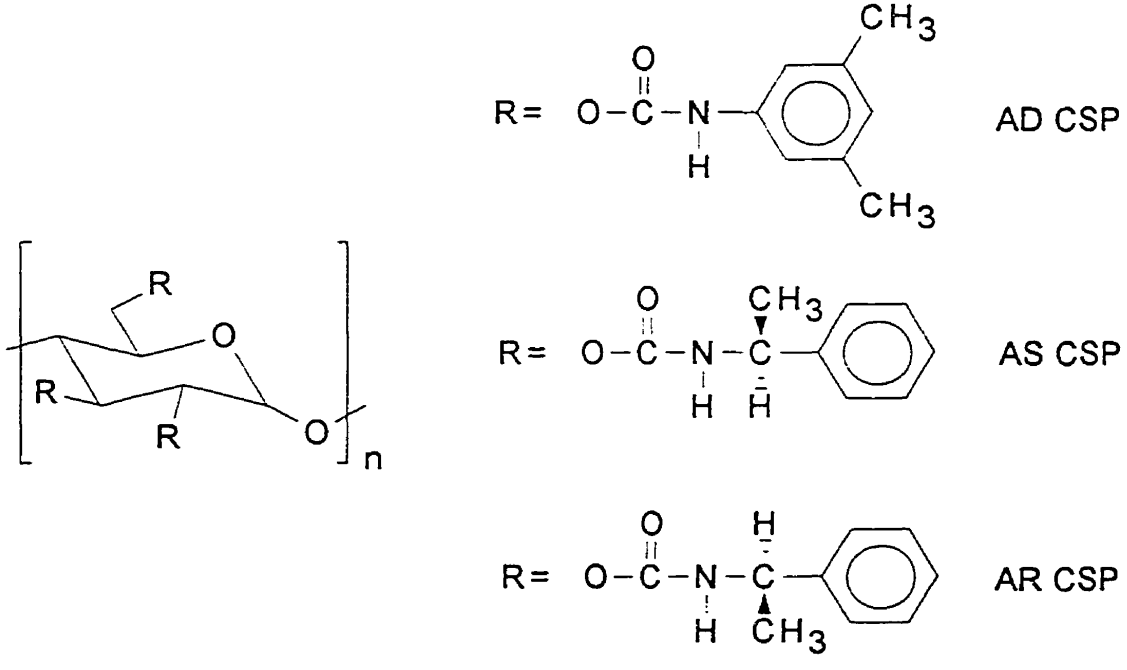


Figure 1. Structures of the amylose backbone and stationary phase derivatives.

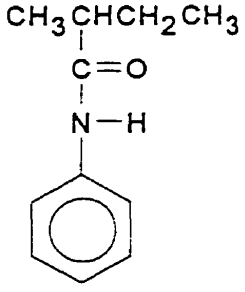
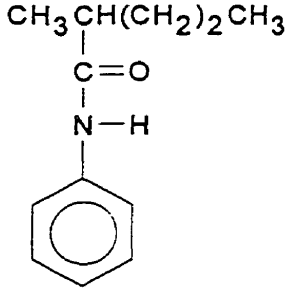
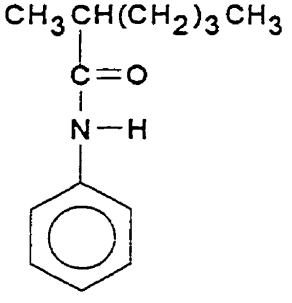
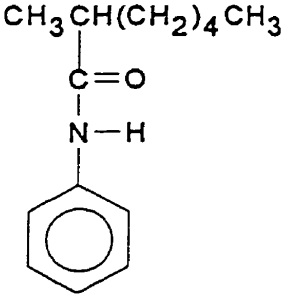
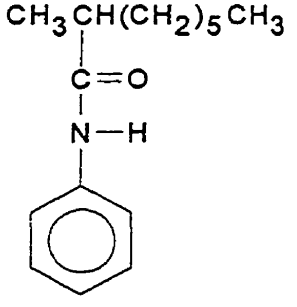
COMPOUND	ID #		AD	AS	AR
	1	k'_1	2.51	1.97	3.23
		k'_2	2.69	1.97	3.23
		1st eluting	(+)	(+)	(+)
		α	1.07	1.00	1.00
	2	k'_1	2.26	1.63	3.32
		k'_2	2.60	1.63	3.65
		1st eluting	(+)	(+)	(+)
		α	1.15	1.00	1.10
	3	k'_1	2.14	1.38	3.30
		k'_2	2.42	1.38	3.41
		1st eluting	(+)	(+)	(+)
		α	1.13	1.00	1.03
	4	k'_1	2.05	1.17	1.43
		k'_2	2.28	1.17	1.89
		1st eluting	(+)	(+)	(+)
		α	1.11	1.00	1.32
	5	k'_1	1.88	0.99	1.52
		k'_2	2.04	0.99	2.32
		1st eluting	(+)	(+)	(+)
		α	1.09	1.00	1.53

Table 1. Retention factors (k'_1 and k'_2), elution orders (+/-) and selectivity values (α).

Column	Retention equation	Correlation	n
AD	$\ln k'_1 = -0.147\ \#C + 3.050$	0.984	5
	$\ln k'_2 = -0.162\ \#C + 3.378$	0.989	5
AS	$\ln k' = -0.242\ \#C + 2.880$	0.992	5
AR	$\ln k'_1 = -0.531\ \#C + 5.746$	0.847	5
	$\ln k'_2 = -0.358\ \#C + 5.048$	0.748	5

Table 2. Structure-retention relationships for amides 1-5. (#C: length of alkyl sidechain)

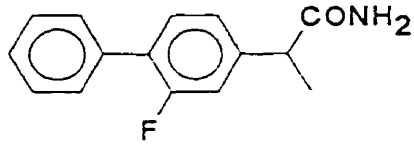
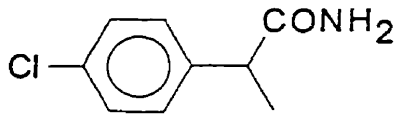
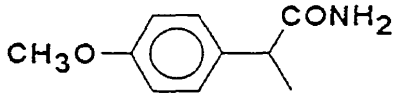
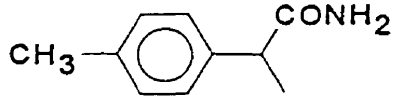
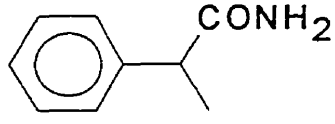
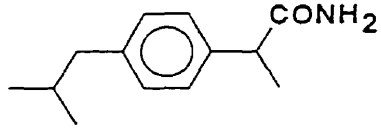
COMPOUND	ID #		AD	AS	AR
	6	k'_1	4.61	16.75	8.21
		k'_2	6.25	19.52	10.15
		1st eluting	(+)	------	(+)
		α	1.36	1.17	1.24
	7	k'_1	4.07	14.93	5.55
		k'_2	4.53	17.75	6.65
		1st eluting	(+)	------	------
		α	1.11	1.19	1.20
	8	k'_1	5.38	16.97	13.41
		k'_2	6.76	23.79	15.39
		1st eluting	(+)	------	(+)
		α	1.26	1.40	1.15
	9	k'_1	3.32	8.66	6.46
		k'_2	3.92	10.16	7.24
		1st eluting	(+)	------	(+)
		α	1.18	1.17	1.12
	10	k'_1	ND	10.30	7.90
		k'_2	ND	13.36	8.81
		1st eluting	------	------	(+)
		α	------	1.30	1.12
	11	k'_1	2.58	4.99	2.24
		k'_2	4.50	6.15	2.51
		1st eluting	(+)	(+)	(+)
		α	1.74	1.25	1.12

Table 3. Retention factors (k'_1 and k'_2), elution orders (+/-) and selectivity values (α) for amides 6-11. (ND: Not Determined)

Column	Retention equation	Correlation	n
AD	$\ln k'_1 = -0.336$ Dip(x) + 2.192	0.852	6
	$\ln k'_2 = -0.313$ Dip(tot) + 2.531	0.941	6
AS	$\ln k'_1 = -0.646$ Dip(x) + 4.039	0.885	6
	$\ln k'_2 = -0.661$ Dip(x) + 4.285	0.871	6
AR	$\ln k'_1 = -3.616$ Kier ChiV3 (cluster) + 3.782	0.950	6
	$\ln k'_2 = -3.684$ Kier ChiV3 (cluster) + 3.967	0.946	6

Table 4. Structure-retention relationships for amides 6-11. (Dip(x): x component of dipole moment, Dip(tot): total dipole moment, Kier ChiV3 (cluster): molecular connectivity)

Column	Selectivity equation	Correlation	n
AD	$\alpha = 0.461\ K_{\alpha}3 + 0.134$	0.976	6
AS	$\alpha = -0.007\ Hf + 0.919$	0.917	6
AR	$\alpha = 0.029\ MEP_{min} + 2.636$	0.977	6

Table 5. Selectivity equations for amides 6-11. ($K_{\alpha}3$: molecular connectivity, Hf: heat of formation, MEP_{min}: minimum molecular electrostatic potential)

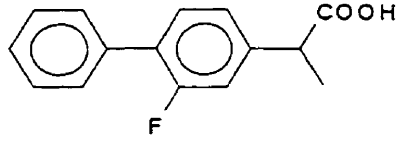
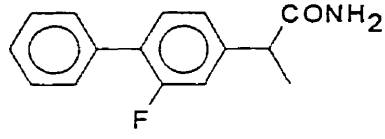
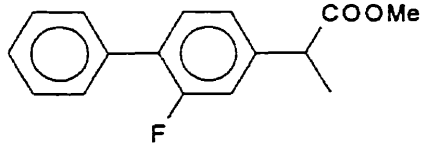
COMPOUND	ID #		AD	AS	AR
	12	k'_1	2.48	1.67	1.99
		k'_2	3.67	1.78	1.99
		1st eluting	(-)	(-)	(-)
		α	1.48	1.07	1.00
	6	k'_1	4.61	16.75	8.21
		k'_2	6.25	19.52	10.15
		1st eluting	(+)	------	(+)
		α	1.36	1.17	1.24
	13	k'_1	0.28	0.35	0.37
		k'_2	0.28	0.35	0.37
		1st eluting	------	------	------
		α	1.00	1.00	1.00

Table 6. Retention factors (k'_1 and k'_2), elution orders (+/-) and selectivity values (α) for flurbiprofen and derivatives.

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3.1 Appendix

To further the investigation into the role of the carbamate side chain, a third class of amides were chromatographed. Structures for a representative set of three solutes and associated chromatographic data are presented in Table 7. The basic distinction between these compounds and those previously tested, lies in the direction of the amide dipole, Figure 1.

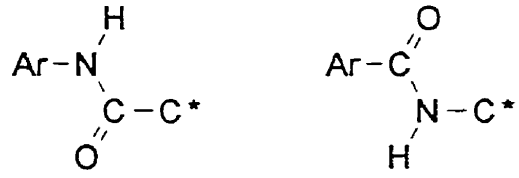


Figure 1. Dipole direction reversal in test solutes.

It was observed that the order of elution was now consistently reversed between the AS and AR phases. This indicated that the carbamate side chain was definitely directing the solute stationary phase interaction. This can be explained with a head to tail dipole arrangement, causing the stereogenic centre of the solutes to be either directed into the amylose helix or away from the amylose helix and towards the aromatic group in the side chain. For these compounds, in the case of the AS and AR phases, this would position the solute stereogenic centre in proximity to the side chain stereogenic centre. Thus the chirality of the stationary phase derivative can now influence the binding interactions with the solutes and consequently a reversal in elution order is observed.

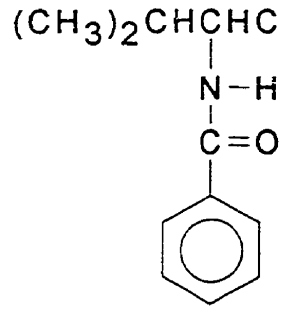
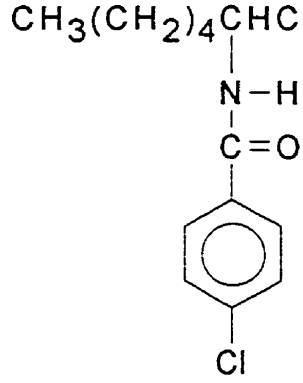
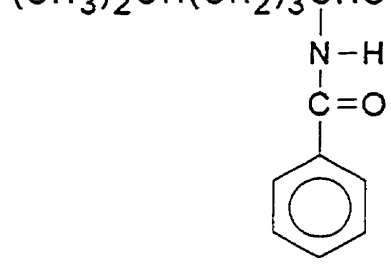
COMPOUND	ID #		AD	AS	AR
	14	k'1	1.39	1.82	5.88
		k'2	1.60	1.82	11.10
		1st eluting	(-)	(-)	(+)
		α	1.15	1.00	1.89
	15	k'1	1.93	2.16	3.21
		k'2	2.40	3.18	6.30
		1st eluting	(-)	(-)	(+)
		α	1.24	1.47	1.96
	16	k'1	1.35	1.37	7.86
		k'2	1.35	1.69	12.36
		1st eluting	(-)	(-)	(+)
		α	1.00	1.23	1.57

Table 7. Retention factors, (k'_1 and k'_2), elution orders (+/-) and selectivity values (α) for the additional series of amides.

PAPER IV

Prediction of chiral chromatographic separations using combined multivariate regression and neural networks.

An important feature of the previous papers is the use of point descriptors in the regression equations resulting from general acceptance of the Easson and Stedman model incorporating point interactions. However, the modeling studies initiated by the equations resulted in a description of the recognition process which could not be completely explained by the three point model or indeed only point interactions. Molecular interactions are examined as a more realistic reflection of components of the recognition process. Also the use of multiparameter regression, although allowing identification of molecular descriptors and development of acceptable correlations, did not produce truly predictive relationships with potential for development into an analytical tool. With this purpose in mind, the first application of neural networks in chiral chromatography was attempted and achieved. Using trained networks, elution orders and separations for previously unseen test solutes were predicted with a high degree of accuracy.

This combined multivariate regression/neural network approach has demonstrated itself to be a key step in the goal to achieve expert systems capable of prediction and description of enantioselective separations.

PREDICTION OF CHIRAL CHROMATOGRAPHIC SEPARATIONS USING COMBINED MULTIVARIATE REGRESSION AND NEURAL NETWORKS

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ABSTRACT

A new method for the prediction and description of enantioselective separations on HPLC chiral stationary phases (CSPs) is described. Based on the combination of multivariate regression and neural networks, the method was successfully applied to the separation of a series of 29 aromatic acids and amides, chromatographed on three amylosic CSPs. Combinations of charge transfer, electrostatic, lipophilic and dipole interactions, identified by multivariate regression were found to describe retention and enantioselectivity, with highly predictive models being generated by the training of back propagation neural networks.

INTRODUCTION

HPLC chiral stationary phases (CSPs), are now routinely used in enantiomeric separations. With over 100 commercially available CSPs, most racemates can be separated. However, at the present time, there is no reliable way to determine which CSP should be applied to a particular separation. Therefore, development of a system which is able to determine whether or not an enantioselective separation will be achieved on a particular CSP, would be of immense benefit.

Development of predictive models for empirical systems such as chromatography are often attempted, due to the high reproducibility of the results obtained from the technique. However, the models are rarely successful outside the realms of the initial data set and are therefore not truly predictive. One strategy for the development of chemometrically driven predictions of retention and enantioselectivity, is the construction of quantitative structure-enantioselective retention relationships (QSERR). These relationships take an extrathermodynamic approach to identification and isolation of the most important structural characteristics within series of racemic solutes, which are responsible for the observed chromatographic retention. These descriptors are usually used as the independent variables in multivariate regression analysis and correlated against the experimental ln k' data.

Development of statistically significant equations allows for the extraction of physically meaningful information relating to the fundamental solute-stationary phase interactions. Kaliszan *et al.*[1,2] successfully used this approach to investigate chiral recognition

on a human serum albumin CSP and consequently provide information on the HSA-benzodiazepine binding site[3]. Similar studies have also been performed on CSPs such as (S,S)-*N,N'*-(3,5-dinitrobenzoyl)-*trans*-1,2-diaminocyclohexane[4], cellulose triacetate[5] and amylose tris(3,5-dimethylphenylcarbamate) (Chiralpak AD)[6,7].

Neural networks[8,9,10] have recently found application for solving different chemical problems. This results from the development of nonlinear transfer functions and feedback coupling[11], which gave new flexibility to old networks. They have successfully been applied to various areas of chromatography and have demonstrated considerable potential for retention prediction in achiral systems[12,13,14,15,16].

Networks are made up of several layers of interconnected neurons, to which pairs of input/output data are presented. The input data consists of a set of pattern vectors, each describing a given solute in terms of non-empirical solute descriptors. The output data are the empirically determined retention factors for the respective solutes. During the training phase, neuron weights are adjusted by repeatedly presenting input/output pairs to the network, until the network has been optimized to give an acceptable final output for a given input set. The network may then be tested with previously unseen input data and the network output compared to the empirically determined results to assess the predictive power of the system.

In this paper we report the application of multivariate regression analysis, combined with multi-layer feed forward neural networks trained with error back-propagation, to model the enantioselective chromatographic retention behavior of a series of aromatic acids and amides, as a function of non-empirical solute descriptors. The solutes were chromatographed on three amylose based CSPs, the commercially available Chiralpak AD and amylose (S)-

phenylethylcarbamate (Chiralpak AS), and the experimental amylose (R)-phenylethylcarbamate (AR). Solute descriptors were first identified using classical QSERR and then transferred to the input layer of the networks. QSERR was able to provide information relating to the fundamental mechanistic interactions, incorporated in the retention and separation of solutes on the three CSPs. However, due to the low predictive ability obtained with multivariate regression, neural networks were applied with the aim of developing enantioselective expert systems.

EXPERIMENTAL SECTION

Chemicals. Solute formulas are given in Table 1. Solutes 1, 4, 11-15, were obtained from Upjohn (Kalamazoo, MI, USA). Solutes 3 and 4 from Sigma (St. Louis, MO, USA). Solute 5 from Eli Lilly (Indianapolis, IN, USA). Solutes 6-10 and 16-20 from Aldrich (Milwaukee, WI, USA) and solutes 21 and 22 from Dr. W. J. Lough (University of Sunderland, Tyne and Wear, UK). All of the amides, solutes 23-29, were prepared by previously reported procedures[17].

Chromatography. Separations were performed on 250×4.6mm I.D. columns packed with Chiralpak AD and AS, (Chiral Technologies, Exton, PA, USA). The AR CSP packed in a 250×4.6mm I.D. column was kindly provided by Prof. Y. Okamoto (University of Nagoya, Japan). Elution orders were determined on a Chiramonitor 2000 optical rotation detector

(Interscience, Markham, Ont., Canada). Column temperature regulation was achieved using a Haake D1-G refrigerated bath/circulator (Fischer Scientific Ltd., Montreal, Que., Canada) and a column water jacket.

All injections were made using a mobile phase of [hexane - isopropanol (95:5, $^{v}/_{v}$)] plus 1% trifluoroacetic acid, filtered and degassed. All samples were prepared in mobile phase and run at 27°C, with a flow rate of 1ml min^{-1}. Column performance was monitored daily to ensure no variation in column properties.

Computational chemistry. Molecular structures were created with Insight II ver. 235 (Molecular Simulation Inc., San Diego, CA, USA) on an IBM RS6000 RISC. Complete conformational searches were performed on all structures using Search/Compare ver. 2.3 (Molecular Simulation Inc.) and molecular geometries were optimized using the semi-empirical molecular orbital program MOPAC 6 (keywords: aml, precise), this program was used also for the calculation of charges, dipole moment and LUMO energy.

The Connolly surfaces[18] were calculated using MOLCAD (Tripos Associates Inc., St. Louis, MO, USA) run on a Silicon Graphics Indigo. Specific properties such as molecular electrostatic potential (MEP)[19] or molecular lipophilic potential (MLP)[20] may be mapped onto the Connolly surface. Software developed at the University of Orleans, France, was used to calculate the maximum, minimum and average values of MEP and MLP[21].

Statistical analysis and neural networks. Statistical analysis and neural network training was performed using TSAR ver. 2.41 (Oxford Molecular Ltd., Oxford, UK).

Multivariate regression analyses were run using the non-standard deviation method with stepwise regression. The one leave-out cross validation method was used to estimate the predictive power of regression models.

All the data presented to the neural networks were automatically scaled to fall between 0.1 and 1. An initial a weighting value of 1.0 was applied to all connections. Starting weights in the range of -0.03 to +0.03 and -1 to +1 for the initial node biases were selected using the Monte Carlo algorithm.

In order to prevent networks becoming trapped in local minima and to promote faster convergence, the general equation for the correction of weights[22] (with momentum) was used. Weights and bias terms were adjusted during training. Parameters start at the maximum values of 0.025 and 0.9 for the learning rate and momentum respectively, and decay to the minimum values of 0.0001 and 0.0009.

RESULTS AND DISCUSSION

Retention and solute descriptor data for the 29 racemic compounds chromatographed in this study, are presented in Table 2. The most significant molecular descriptors were identified using multivariate regression analysis and a summary of the best equations obtained for the retention of each solute enantiomer on the AD, AS and AR CSPs are presented in Table 3. Combinations of the same four descriptors were used to describe enantiomer retention on all three CSPs. Five of the resulting equations contained three descriptors while the (S)- enantiomers on the AD phase required a four term equation. Good correlation coefficients ranging from 0.938 to 0.979 were obtained for the AS and AR phases, but only borderline values, 0.874 and 0.916, were obtained for the AD phase. This may be associated with a larger variation in retention factors being observed on the AD phase which necessitated a more detailed model for adequate description of the empirical data. Most of the terms in the equations carry significance levels in the range 3.1×10^{-4} to 6×10^{-10}. Only the MEP and DIPOLE terms in the AD phase regressions have higher, although still acceptable values.

The complexity of a QSERR model is entirely governed by the size of the initial data set, with the predictive power of the model being determined by cross validation. Results of leave-one-out cross validations for each of the six derived relationships are presented in Table 3.

Due to the similarities between the three CSPs, it is not surprising that a common set of descriptors was isolated. The differences between the phases and indeed between

enantiomers thus being highlighted by varying combinations and weights of the individual terms.

The f_1 (LUMO) descriptor is an indication of the presence of charge transfer interactions between the solute and CSP[2]. It has previously been demonstrated that the LUMO descriptor can also be used as a reflection of hydrogen bond donor ability in liquid chromatography[23]. The f_2 (MEP) and f_3 (MLP) descriptors represent a combination of bulk steric effects with electrostatic and lipophilic[24] properties respectively. The f_4 (DIPOLE) descriptor indicates the possibility of dipole-dipole or dipole stacking interactions between the solute and CSP.

Terms for which there is very little difference between the coefficients for (R)- and (S)- enantiomers on a CSP, indicate interactions for which the two enantiomers are equally affected, i.e. the interactions are not entantio-discriminating and may be considered as contributing to general retention. Conversely, any difference in the magnitude of the terms for two enantiomers suggests that the interaction is dependent upon the absolute configuration of the enantiomers. Thus it is possible to ascertain the major differences in the enantiomer-CSP interactions, for a series of CSPs, which give rise to the chromatographic separation. These differences thus being related to the structure of the stationary phase. The primary enantio-discriminating interactions on the AS CSP would appear to be the charge transfer or hydrogen bonding interactions, as indicated by a 5% difference in the f_1 (LUMO) terms. A 6% change in the f_3 (MLP) terms for the AR CSP indicates that lipophilic and steric interactions are a significant source of enantio-discrimination on this phase. Enantio-discrimination on the AD CSP appears to be more of a cumulative effect of all descriptors as the f_2 and f_3 terms differ by

similar amounts but in opposite directions, effectively reducing the overall contribution to discrimination.

The structure of the AD CSP has been proposed as being a regular ordered helical polymer. Attempts in this group to model representative structures for the AS and AR phases failed, primarily as a result of the non-linearity of the chiral phenylethyl derivatives. Although the two derivatives differ only in configuration at the stereogenic center, it is believed that the disorder within the two CSPs (relative to the AD CSP), creates chiral environments responsible for the individual discriminating abilities of each phase.

In five out of the six regression equations, cross-validation results indicate that the predictive ability of the models are low. Only the equation derived for (R)-enantiomers on the AS CSP demonstrated any degree of predictive power.

Back-propagation neural networks were trained using data from the same compounds as used for the multivariate regression. Optimal network architecture was determined using a limit of four neurons in the input layer, corresponding to the set of common solute descriptors.

Networks were trained for the retention of (S)- and (R)-enantiomers on all three CSPs. A summary of the final optimized networks is given in Table 4. All networks were trained in under 3000 epochs, with the optimal number of iterations per epoch ranging from 500 to 2500. Training was considered to be completed when no improvement in the RMS output was observed over 400 epochs.

A test set of previously unseen acids and amides, approximately equal in size to 25% of the initial sample set, was chosen to probe the predictive properties of the trained networks.

The appropriate descriptor data for the test compounds were calculated and presented to the networks. Results for retention factor prediction of test solutes are given in Tables 5 and 6.

Linear regression was performed between the experimental retention factors and the network training outputs (including predicted values from the test compounds), results are shown in Table 7. Leave-one-out cross validation results indicate that the networks seem to be quite effective in modeling the structure-retention relationships, with a view to predicting enantioselective chromatographic retention.

Table 8 gives a comparison of the experimental and predicted separation values, along with the associated elution orders. For 11 out of the 16 test compounds (all three CSPs), predicted alpha values were quite close to the experimentally determined values. In all cases where no separation was predicted, none was found experimentally. Separation was only predicted once (solute 27 on the AS CSP) where none was found experimentally, however the actual value of the predicted separation was very low. The predicted elution orders were only incorrect in two cases (solute 8 on the AD and AR CSPs).

CONCLUSIONS

A combined multivariate regression/neural network approach has been successfully used to develop expert systems capable of prediction and description of enantioselective separations on three amylosic CSPs. Neural networks have been shown to be capable of much higher predictive power than multivariate regression, although the descriptive power of

the networks is limited and must be compensated by the regression equations. Further application of this combined approach should generate a better understanding of enantioselective processes, as well as provide a valuable chromatographic tool.

ACKNOWLEDGMENTS

The authors wish to thank Chiral Technologies Inc., Exton, PA, for financial support. This work is also supported in part by an NSERC grant awarded to I.W.W.

No.	Empirical formula	Compound
1.	$C_{13}H_{18}O_2$	α-Methyl-4-(2-methylpropyl)benzeneacetic acid
2.	$C_{16}H_{14}O_3$	3-Benzoyl-α-methylbenzeneacetic acid
3.	$C_{14}H_{12}O_3S$	α-Methyl-4-(thienylcarbonyl)benzeneacetic acid
4.	$C_{16}H_{12}ClNO_3$	2-(4-Chlorophenyl)- α-methyl-5-benoxazoleacetic acid
5.	$C_{15}H_{13}FO_2$	2-Fluoro-α-methyl-4-biphenylacetic acid
6.	$C_9H_9ClO_3$	2-(2-Chlorophenoxy)propionic acid
7.	$C_9H_{10}O_3$	2-Phenoxypropionic acid
8.	$C_9H_9ClO_3$	2-(4-Chlorophenoxy)propionic acid
9.	$C_9H_{10}O_2$	2-Phenylpropionic acid
10.	$C_{10}H_{12}O_2$	2-Phenylbutyric acid
11.	$C_{11}H_{14}O_2$	2-Phenyl-3-methylbutyric acid
12.	$C_{11}H_{14}O_2$	2-Phenylpentanoic acid
13.	$C_{12}H_{16}O_2$	2-Phenyl-4-methylpentanoic acid
14.	$C_{12}H_{16}O_2$	2-Phenylhexanoic acid
15.	$C_{14}H_{20}O_2$	2-Phenyloctanoic acid
16.	$C_8H_7FO_2$	α-Fluorophenylacetic acid
17.	$C_9H_{10}O_3$	α-Methoxyphenylacetic acid
18.	$C_8H_8O_3$	α-Hydroxyphenylacetic acid
19.	$C_9H_{10}O_3$	2-Hydoxy-3-phenylpropionic acid
20.	$C_9H_{10}O_3$	2-Phenyl-3-hydroxypropionic acid
21.	$C_9H_9ClO_2$	2-(4-Chlorophenyl)propionic acid
22.	$C_{10}H_{12}O_3$	2-(4-Methoxyphenyl)propionic acid
23.	$C_{11}H_{15}NO$	*N*-Benzyl-2-methylbutyl amide
24.	$C_{12}H_{17}NO$	*N*-Benzyl-2-methylpentyl amide
25.	$C_{13}H_{19}NO$	*N*-Benzyl-2-methylhexyl amide
26.	$C_{14}H_{21}NO$	*N*-Benzyl-2-methylheptyl amide
27.	$C_{15}H_{23}NO$	*N*-Benzyl-2-methyloctyl amide
28.	$C_{14}H_{20}ClNO$	*N*-(1-methylhexyl)-4-chlorobenzamide
29.	$C_{15}H_{23}NO$	*N*-(1,5-dimethylhexyl)-benzamide

Table 1. Chiral acids and amides chromatographed on the three amylose based CSPs.

No.	AD		AS		AR		Solute Descriptors			
	ln k'_R	ln k'_S	ln k'_R	ln k'_S	ln k'_R	ln k'_S	1	2	3	4
1.	0.39	0.39	-0.37	-0.36	nd	nd	0.198	-11.41	0.103	1.739
2.	2.14	2.25	1.78	1.78	2.06	2.06	-0.513	-8.30	0.098	2.884
3.	2.64	2.49	2.49	2.65	2.85	2.85	-0.637	-0.71	0.110	2.681
4.	2.62	3.21	1.96	1.64	1.64	1.75	-1.015	-7.64	0.129	0.770
5.	0.91	1.30	0.51	0.58	0.69	0.69	-0.442	-8.83	0.119	0.807
6.*	0.46	0.66	0.45	0.75	0.58	0.79	-0.063	-8.35	0.107	3.288
7.*	0.53	0.82	0.45	0.71	0.48	0.62	0.315	-10.06	0.078	2.457
8.*	0.83	1.07	nd	nd	0.48	0.57	-0.028	-8.08	0.116	2.296
9.	0.63	0.72	nd	nd	0.22	0.22	0.213	-10.95	0.096	1.690
10.	0.59	0.71	0.02	-0.04	0.03	0.03	0.216	-11.33	0.100	1.695
11.	0.44	0.56	-0.27	-0.39	-0.26	-0.26	0.219	-11.86	0.100	1.725
12.	0.51	0.60	-0.06	0.05	-0.02	-0.02	0.219	-11.37	0.104	1.679
13.	0.31	0.42	-0.33	-0.33	-0.24	-0.24	0.231	-11.39	0.102	1.691
14.	0.44	0.58	-0.07	-0.07	-0.22	-0.22	0.218	-11.40	0.103	1.673
15.	0.35	0.54	-0.29	-0.29	-0.14	-0.14	0.217	-11.35	0.106	1.666
16.	0.92	0.84	1.42	1.18	0.99	1.50	-0.088	-7.72	0.091	2.764
17.	1.01	1.10	nd	nd	1.47	1.15	0.010	-8.42	0.067	1.817
18.	2.32	2.16	2.16	2.12	2.20	2.13	-0.035	-7.01	0.061	2.228
19.	1.88	1.68	1.82	1.82	1.81	1.67	0.193	-9.81	0.060	1.858
20.*	2.51	2.31	2.01	2.01	2.22	2.22	0.152	-8.35	0.050	2.17
21.	0.80	0.90	0.09	0.09	-0.11	-0.11	-0.144	-8.58	0.135	1.872
22.	1.43	1.23	0.85	0.85	0.90	0.90	0.242	-11.18	0.073	0.952
23.	0.99	0.92	0.68	0.68	1.17	1.17	0.360	-6.79	0.082	3.311
24.	0.96	0.82	0.49	0.49	1.29	1.20	0.359	-7.12	0.084	3.297
25.	0.88	0.76	0.32	0.32	1.23	1.19	0.360	-7.17	0.087	3.273
26.	0.82	0.72	0.16	0.16	0.64	0.36	0.359	-7.18	0.089	3.271
27.	0.71	0.63	-0.01	-0.01	0.84	0.42	0.358	-7.08	0.092	3.257
28.	0.66	0.88	0.77	1.16	nd	nd	-0.070	-13.28	0.085	3.299
29.	0.30	0.30	0.31	0.52	2.51	2.06	-0.067	-13.38	0.086	3.303

Table 2. Solute retention data and descriptor values. $k'=(t_r-t_0)/t_0$ where k' is the enantiomer retention factor, t_r is the enantiomer retention time and t_o is the retention time of an unretained solute or solvent front.

* Due to apparent (R)-/(S)- retention inversion (relative to the rest of the series) arising only from the Cahn-Ingold-Prelog naming convention for chiral compounds, enantiomeric retention factors for these compounds were reversed.

Solute descriptors: 1-LUMO (energy of the lowest unoccupied molecular orbital), 2-MEP (average molecular electrostatic potential), 3-MLP (average molecular lipohillic potential), 4-DIP (total dipole moment). nd: not determined.

	AD CSP		AS CSP		AR CSP	
	R	**S**	**R**	**S**	**R**	**S**
f_1	**-1.639**	**-1.624**	**-2.062**	**-1.955**	**-2.554**	**-2.563**
t	-5.71	-5.66	-11.3	-11.02	-10.1	-11.88
p	5.5×10^{-5}	2.3×10^{-5}	2.5×10^{-9}	3.7×10^{-9}	7.6×10^{-9}	6.0×10^{-10}
f_2	**0.098**	**0.112**	**0.114**	**0.119**	0	0
t	2.64	3.12	5.42	5.82		
p	0.016	0.006	4.5×10^{-5}	2.0×10^{-5}		
f_3	**-21.84**	**-18.57**	**-33.18**	**-33.20**	**-36.43**	**-34.21**
t	-4.93	-4.38	-11.1	-11.41	-8.26	-9.08
p	3.1×10^{-4}	3.6×10^{-5}	3.4×10^{-9}	2.2×10^{-9}	1.6×10^{-7}	3.8×10^{-8}
f_4	0	**-0.279**	0	0	**0.417**	**0.412**
t		-2.65			4.33	5.02
p		0.016			4.0×10^{-4}	8.8×10^{-5}
f_5	**4.057**	**4.539**	**4.933**	**4.997**	**3.510**	**3.299**
R	0.874	0.916	0.971	0.972	0.938	0.952
R_{CV}	0.166	0.386	0.926	0.777	0.832	0.817
s	0.400	0.339	0.226	0.219	0.352	0.301
F	20.5	23.4	95	96.8	43.7	57.8
n	23	23	21	21	22	22

Table 3. Summary of results obtained from multivariate regression analysis.

General equation: $\ln k' = f_1\,\mathrm{LUMO} + f_2\,\mathrm{MEP} + f_3\,\mathrm{MLP} + f_4\,\mathrm{DIP} + f_5$

t: t-test, *p*: significance level, R: correlation coefficient, R_{cv}: cross validation, s: standard error of the estimate, F: Fischer test, n: number of solutes.

		AD CSP		AS CSP		AR CSP	
		R	S	R	S	R	S
Input Variables	1	LUMO	LUMO	LUMO	LUMO	LUMO	LUMO
	2	MLP	MLP	MLP	MLP	MLP	MLP
	3	MEP	MEP	MEP	MEP	MEP	MEP
	4	------	DIPOLE	DIPOLE	DIPOLE	DIPOLE	DIPOLE
Configuration		3-3-1	4-3-1	4-2-1	4-2-1	4-2-1	4-3-1
No. of Epochs		1600	1991	2460	1981	2482	2988
Iterations per Epoch		2500	500	2500	2500	1000	2500
Training RMS		0.160	0.127	0.143	0.173	0.232	0.164
Test RMS		0.182	0.246	0.153	0.192	0.140	0.178

Table 4. Summary of the final optimised neural networks.

	AD CSP				AS CSP				AR CSP			
	ln *k'* R		ln *k'* S		ln *k'* R		ln *k'* S		ln *k'* R		ln *k'* S	
No.	**exp**	**pred**	**exp**	**pred**	**exp**	**pred**	**exp**	**pred**	**exp**	**pred**	**exp**	**pred**
8.	0.83	0.73	1.07	0.71	nd	nd	nd	nd	0.48	0.19	0.57	0.16
13.	0.31	0.46	0.42	0.60	-0.33	-0.18	-0.33	-0.17	-0.24	-0.09	-0.24	-0.09
18.	2.32	2.11	2.16	1.66	2.16	2.12	2.12	2.09	2.20	2.37	2.13	1.76
23.	0.96	0.86	0.82	0.80	0.49	0.47	0.49	0.48	1.30	1.05	1.20	0.99
27.	0.71	0.92	0.63	0.74	-0.01	0.19	-0.01	0.15	0.84	0.64	0.42	0.49
28.	0.66	0.35	0.88	0.42	0.77	0.35	1.16	0.55	nd	nd	nd	nd

Table 5. Experimental and network predicted retention factors for test solutes chromatographed on the AD, AS and AR CSPs.

	AD CSP		AS CSP		AR CSP	
	R Δln *k'*	S Δln *k'*	R Δln *k'*	S Δln *k'*	R Δln *k'*	S Δln *k'*
mean	0.06	0.17	0.03	0.06	0.09	0.15
standard deviation	0.20	0.30	0.24	0.32	0.22	0.25
mean absolute deviation	0.16	0.27	0.16	0.22	0.19	0.21
n	6	6	5	5	5	5

Table 6. Results summary for the ability of trained networks to predict output from previously unseen data. $\Delta \ln k' = \ln k'_{experimental} - \ln k'_{predicted}$

	AD CSP		AS CSP		AR CSP	
	R	**S**	**R**	**S**	**R**	**S**
R	0.988	0.974	0.988	0.980	0.980	0.984
R_{CV}	0.973	0.942	0.969	0.958	0.956	0.964
s	0.119	0.168	0.136	0.176	0.186	0.164
F	1060	494	1011	575	605	741
n	29	29	26	26	27	27
test n	6	6	5	5	5	5
train n	23	23	21	21	22	22

Table 7. Results of experimental *vs.* network output linear regression.

R: correlation coefficient, R_{cv}: cross validation, standard error of the estimate, F: Fischer test, n: total number of solutes, test n: number of solutes in the test set, training n: number of solutes used for training the networks.

	AD CSP				AS CSP				AR CSP			
	exp		pred		exp		pred		exp		pred	
No.	α	*	α	*	α	*	α	*	α	*	α	*
8.	1.27	R	1.02	S	nd	nd	nd	nd	1.09	R	1.03	S
13.	1.12	R	1.15	R	1.00	na	1.00	na	1.00	na	1.00	na
18.	1.17	S	1.57	S	1.04	S	1.03	S	1.07	S	1.84	S
23.	1.15	S	1.06	S	1.00	na	1.01	na	1.10	S	1.06	S
27.	1.09	S	1.20	S	1.00	na	1.04	S	1.53	S	1.17	S
28.	1.24	R	1.07	R	1.47	R	1.22	R	nd	nd	nd	nd

Table 8. Experimental and predicted separation values and first eluted enantiomer configurations for test solutes chromatographed on the AD, AS and AR CSPs.

α: chromatographic separation. *: configuration of the first eluting enantiomer. na : not applicable due to no separation. nd : not determined.

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PAPER V

Is chiral recognition a three-point process ?

One of the issues raised in this work is the validity and applicability of the concept of chiral recognition via three points of interaction. This subject is of fundamental importance in many areas of science, and is all too often used as the law to which data must fit. Examples have appeared for which the model does not sufficiently incorporate the observed results, necessitating a re-examination and perhaps modification of the theory.

This issue prompted a debate on chiral recognition at the 8th International Symposium on Chiral Discrimination in Edinburgh, Scotland. This paper formed part of the proceedings.

IS CHIRAL RECOGNITION A THREE-POINT PROCESS?

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Chirality, 9 (1997) 96

In 1858, Pasteur reported that the *dextro* form of ammonium tartrate was more rapidly destroyed by the mold *Penicillium glaucum* than the *levo* isomer[1]. This was the initial observation of the biological differences between enantiomorphs and led Pasteur to the recognition of the role stereochemistry plays in the basic mechanisms of life. He wrote:

> Most natural organic products, the essential products of life, are asymmetric and possess such asymmetry that they are not superimposable on their images...This establishes perhaps the only well marked line of demarcation that can at present be drawn between the chemistry of dead matter and the chemistry of living matter[2].

During the next 50 years, there were a number of additional reports of stereochemical differences in biochemical properties. The initial pharmacological observations are credited to Abderhalde and Müeller, who, in 1908, described the differential pressor effects of (-)- and (+)-epinephrine[1]. With this report, chirality entered mainstream pharmacological research and by 1933, Easson and Stedman[3] had laid the basis for the initial theoretical understanding of stereochemical differences in pharmacological activity.

In the Easson and Stedman model, stereochemical differences in pharmacological activities were due to the differential binding of enantiomers to a common site on an enzyme or receptor surface, Figure 1. In order to distinguish between enantiomers, the receptor/enzyme had to possess three non-equivalent binding sites. Discrimination occurred when one isomer could simultaneously interact with all of the sites while its enantiomorph could only bind at one; chiral recognition based upon a "three-point" interaction.

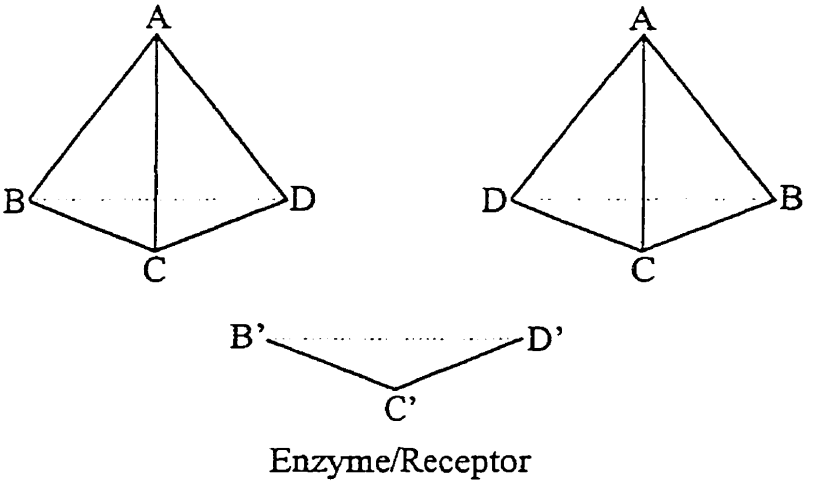

Figure 1. The "three-point" interaction model of chiral recognition proposed by Easson and Stedman.

The "three-point" interaction model was ignored for 15 years until Ogston resurrected it to explain the enzymatic conversion of L-serine to glycine[4]. The pivotal step in this conversion was the stereoselective decarboxylation of the prochiral intermediate metabolite aminomalonic acid. In Ogston's model, the carboxylic moieties on the aminomalonic acid become inequivalent due to the existence of three nonequivalent binding sites on the enzyme, one of which was responsible for the decarboxylation. Figure 2A.

Ogston published his work in 1948 and by the mid-1950s, the "three-point" interaction model was generally accepted as the source of biological enantioselectivity and it quickly spread to other disciplines. In medicinal chemistry, the model became a key element in quantitative structure-activity, relationship studies[5]. In chromatographic science, Dalgliesh invoked a "three-point" interaction to explain the enantioselective separation of amino acids

by chromatography on cellulose paper[6]. Indeed, the "three-point" model has been the basis of the rational design of a number of chromatographic chiral stationary phases and the rationalization of how these phases work[7].

It is clear that the "three-point" interaction model works in a number of situations. However, is it an accurate description of how the dissymmetry of one molecule is perceived by a second or of how stereochemically equivalent moieties are distinguished from each other? In principle, the model is a static picture of bimolecular process, essentially the "lock and key" model of enzymatic activity[8]. But the "lock and key" model has been superseded by the understanding that a key step in enzymatic conversions involves mutually induced conformational adjustments of the substrate and enzyme[9]. Perhaps it is now time to extend this dynamic understanding to the process of chiral recognition.

The best place to begin is with a reexamination of Ogston's model. The first assumption which needs to be clarified is the assignment of three distinct "binding sites" on the enzyme. Surely, the catalytic area of an enzyme is best described as a spatial environment or a cavity, not as a point or single interaction site. Binding sites which position and constrain the substrate relative to the enzyme do not necessarily have to lie within the catalytic site; for example, the hydrophobic binding site of chymotrypsin. If this is the case, then Ogston's model contains only two interaction sites which produce binding interactions between the substrate and enzyme. Since the binding sites are not equivalent, the bound substrate is presented to the enzyme in two distinct spatial configurations, Figures 2A, 2B. The chirality of the enzyme places the catalytic site in such a position where only one of the configurations

can be decarboxylated {Figure 2A}, leading to the production of a single enantiomeric product.

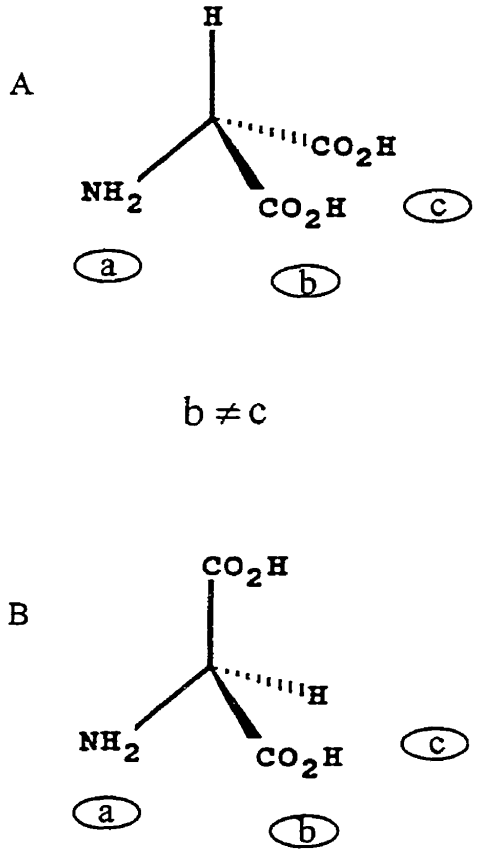

Figure 2. A. The "three-point" interaction model for the enzymatic transformation of aminomalonic acid as proposed by Ogston. B. A second possible interaction between substrate and enzyme. Where a, b and c are three non-equivalent areas on the enzyme and c is designated as the catalytic site.

In this case, enantioselectivity is due to a two-point directional interaction and to the chirality of the enzyme. This model is similar to the one previously proposed by Sokolov and Zefirov which is described by the authors as the "rocking tetrahedron" [10], Figure 3. In this

approach to chiral recognition, the substrate is secured to the enzyme by two binding interactions. These interactions must be non-equivalent and directional so that only one orientation is possible. The tethered substrate still has conformational mobility and the two hydrogen atoms sweep out overlapping but not identical steric volumes. Where and to what extent the active site of the enzyme interacts with these steric volumes, determines the enantioselectivity of the process. If the chirality of the enzymes places the interaction perpendicular to the plane of the substrate, no enantioselectivity is observed. As the deviation from the perpendicular increases, so does the enantioselectivity.

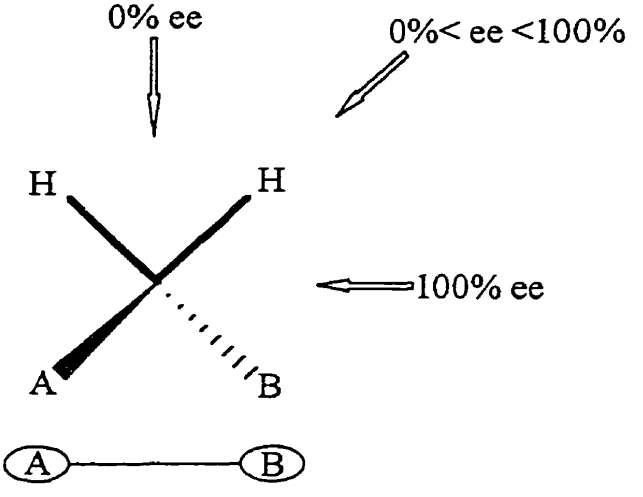

Figure 3. The "rocking tetrahedron" model of chiral discrimination. Where the arrows reflect the direction of enzymatic interaction with the substrate and *ee* represents the enantiomeric excess produced by the enzymatic conversion.

In the first model, the conformational mobility of the substrate before the completion of the directional binding leads to two non-equivalent configurations. In the second model, the conformational mobility of the bound substrate is the source of the asymmetric interactions. These are "conformationally driven" chiral recognition mechanisms.

A "conformationally driven" chiral recognition mechanism has also been described for the separation of α-alkyl arylacetic acids on an amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phase {the AD-CSP}[11]. The results from quantitative structure-enantioselective retention relationships and molecular modeling studies, indicate that the enantioselective discrimination of the α-alkyl arylacetic acids on the AD-CSP is a three-step process.

These steps are:

Step 1. Distribution of the solute to the stationary phase through hydrogen bonding interactions between the acid moiety on the solute and amine moieties on the CSP.

Step 2. Conformational adjustments of the solutes and insertion of the aromatic portion of the solute into a ravine on the CSP.

Step 3. Stabilization of the solute/CSP complex through electrostatic and hydrogen bonding interactions within the ravine.

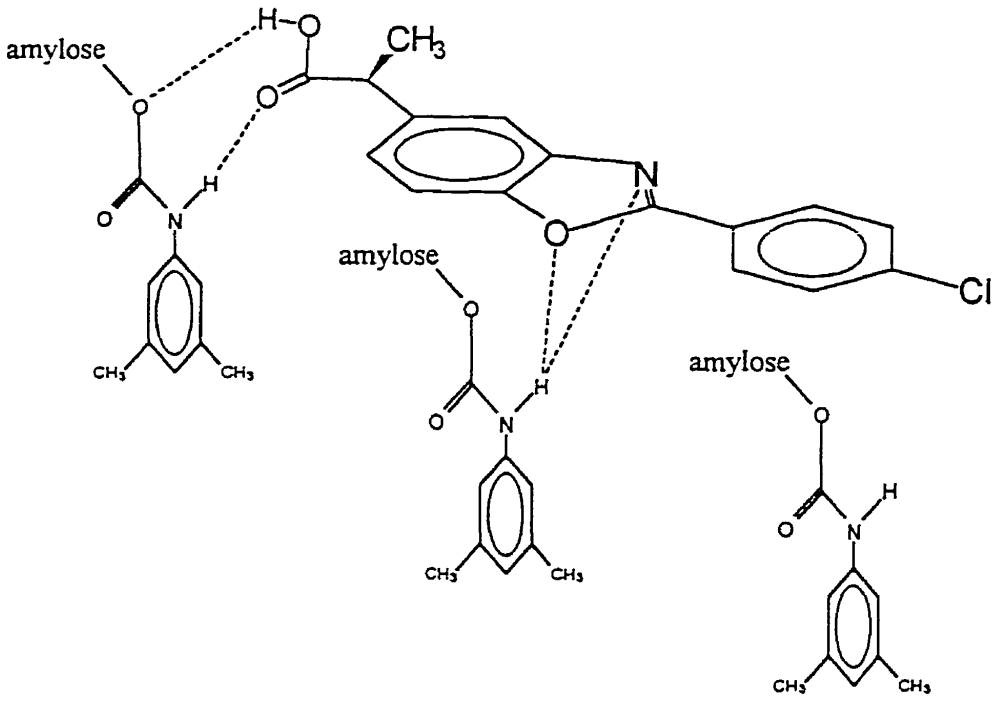

Figure 4. Representative interaction between S-benoxaprofen and the amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phase representing a "conformationally driven" chiral recognition mechanism.

Both enantiomers of the solute form identical hydrogen bonding interactions, and presumably the same hydrophobic interactions as well, Figure 4. The diastereomeric solute-CSP complexes differ in their stabilities leading to chiral discrimination. The differences arise from the internal energies of the two enantiomer conformations which were required to achieve the optimum interactions.

The determining factor in these process is the molecular chirality of the biopolymer. Enzymes, amylose, *etc.* are large chiral biopolymers with distinct three-dimensional structures. While it is possible to assign specific electrostatic or hydrogen bonding sites within these

molecules, most interactions take place within cavities or ravines. Thus, a more accurate description of the chiral recognition process would be to replace the "three-point" interaction model with one based upon molecular chiralities.

A general chiral recognition process based upon this strategy is presented in Figure 5. This process involves the initial formation of the complex, followed by conformational adjustment of the two elements, activation of the complex through additional binding interactions and expression of the molecular chiralities of the two elements in the complex. This process describes enantioselective discrimination by all classes of chiral selectors from biopolymers to derivatised amino acids.

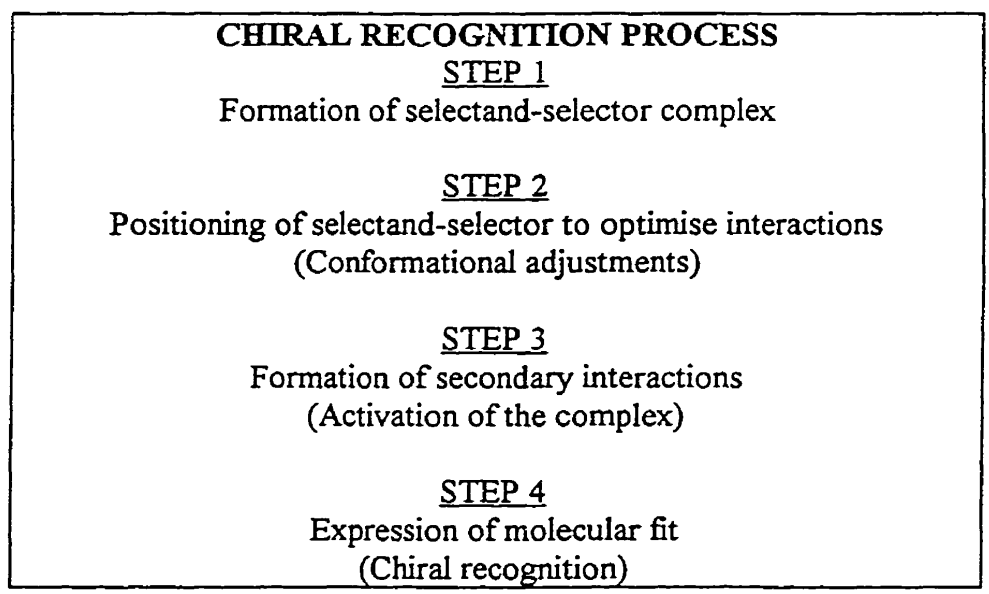

Figure 5. A proposed model of the chiral recognition process.

It is hoped that this proposal will initiate an extensive reexamination of chiral recognition. Within the advance from a static to a dynamic understanding of this process will come a deeper understanding of this key biological and chemical process.

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6 SUMMARY

The work presented in this thesis represents an examination of the processes involved in the chromatographic separation of enantiomers and the general theory of enantio-discrimination. In particular, it addresses the issues of prediction and description of enantioselective separations on amylose based CSPs. Prior to this work, no qualitative or quantitative attempt had been reported, which investigated these important areas of chiral chromatography in the test system.

Successful application and development of QSERR models have allowed for identification of important aspects of the interaction mechanisms on amylosic stationary phases, which may be projected to other chromatographic separations. Unlike many previous structure-retention relationship studies, the classical descriptors which were isolated and incorporated into the initial models, such as number of hydrogen bonds, fragmental hydrophobicity and degree of aromaticity, enable the separate interactions to be rationalised chemically, rather than just applied as terms in an equation. These same interactions were also present in the descriptors which were calculated by more advanced quantum chemical approaches, thus reiterating and highlighting their importance as fundamental aspects of the solute stationary phase interaction.

The progressive development of descriptors is perhaps of more significance when considering interactions of a specifically chiral nature. The classical descriptors basically represent point interactions, a consequence of the Easson and Stedman model. However, it

has been demonstrated that it is possible for chiral recognition to take place in a manner which does not totally conform to this theory. The enantioselective separation of benoxaprofen on the Chiralpak AD CSP occurs as a result of a change in conformation of the (R)-enantiomer leading to differences in internal energy of the two enantiomers and consequently the two diastereomeric complexes.

The acknowledgment and inclusion of the binding environment is important, as is the recognition that point interactions are far less likely to occur in real systems as compared to site or molecular interactions. It is believed that descriptors which are derived from the calculation of molecular properties and/or molecular surface properties, as compared to point properties, are more representative of actual molecules and the interactions thereof. Suitable computer hardware will also allow these properties to be visualised in three-dimensions and manipulated in real time. These three-dimensional representations permit studies of reactive sites, conformational rearrangements, molecular size and volume, in which molecular surfaces play a dominant role.

The initial studies were performed entirely on the Chiralpak AD CSP, as this is frequently the first column of choice in obtaining a new separation. Extension of the investigation to two other enantiomerically related amylosic phases, allowed for intercolumn effects on retention and separation to be studied also. Results for a series of amides verify the directional binding effects of the carbamate side chain dipole. Results also highlight differences in the shape of the binding regions or cavities, as a consequence of the amylose derivative.

It has been shown that the power of QSERR lies in the area of identifying and isolating the primary features of the interaction mechanisms. Prediction of chromatographic retention, separation and elution maybe somewhat limited. The need for an alternative method of data analysis, led to the evaluation of artificial neural networks for the development of models with high predictive ability. This was the first reported example of neural network application in chiral chromtography. Using descriptors isolated from QSERR, multi-layer feed forward neural networks trained with error back propagation, were used to model the enantioselective chromatographic retention behaviour of a diverse series of acids and amides. The resulting trained networks showed impressive predictive powers, although with limited descriptive ability. The combined approach of multiparameter regression and neural networks has the desired objective of possessing the inherent ability to be developed into a highly sought after, chromatographic prediction tool.

CONTRIBUTIONS TO KNOWLEDGE

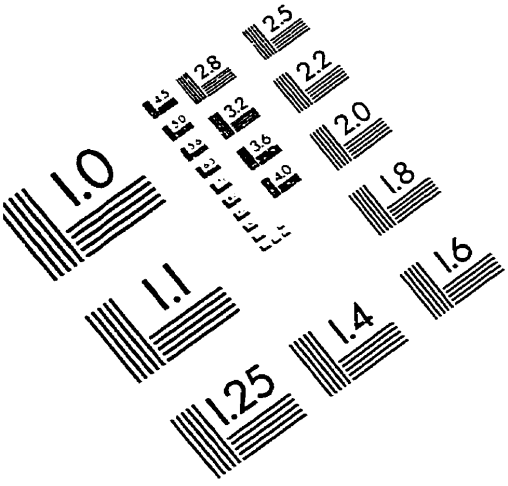
The research documented in this thesis has resulted in the publication four papers plus one further paper accepted for publication.

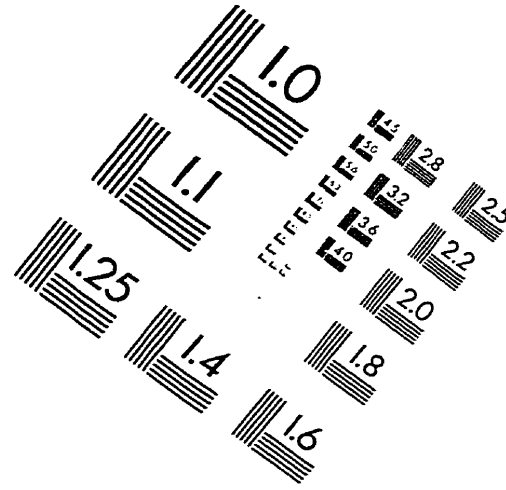
Demonstration of the first application of neural networks to the field of chiral chromatography has constructed a backbone for the development of a chiral expert system, for the intelligent prediction of enantioselective separations. Chiral Technologies Inc. has acknowledged the potential of the method and is currently investigating possible applications in the field of preparative separations. This work has also promoted new research incorporating the use of genetic algorithms as a possible replacement for multivariate regression in the combined regression-network method.

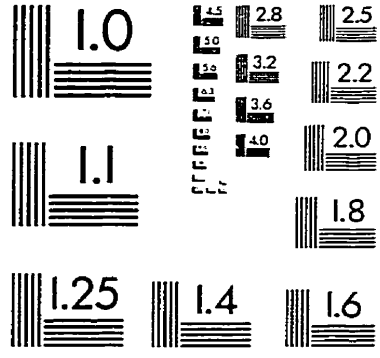
The first study into the operation of an important class of CSP is described. QSERR have been developed and applied to enantioselective separations on three related amylose based CSPs. The recognition mechanisms have been elucidated and described in terms of non-empirical descriptors, with predictive ability inherent in the final relationships. The derived relationships give detailed insight into the processes operating during retention and enantioselective recognition.

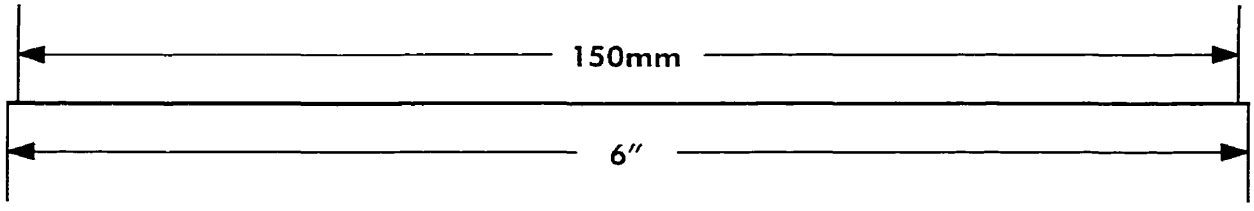
In order to further understand results obtained, a close examination of the "three-point" interaction model of chiral discrimination was required. A modified theory has been postulated to incorporate the dynamic nature of conformational adjustment and molecular fit, to help explain experimental results.

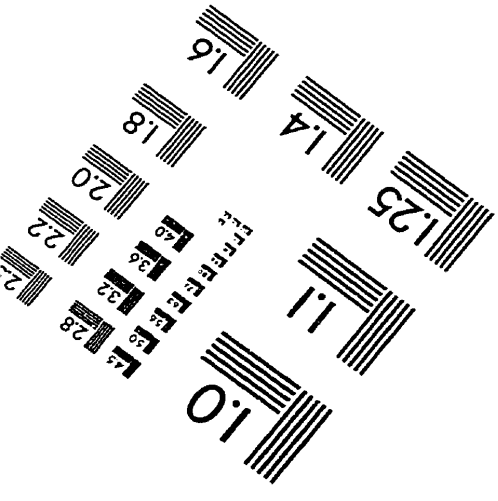
IMAGE EVALUATION
TEST TARGET (QA-3)

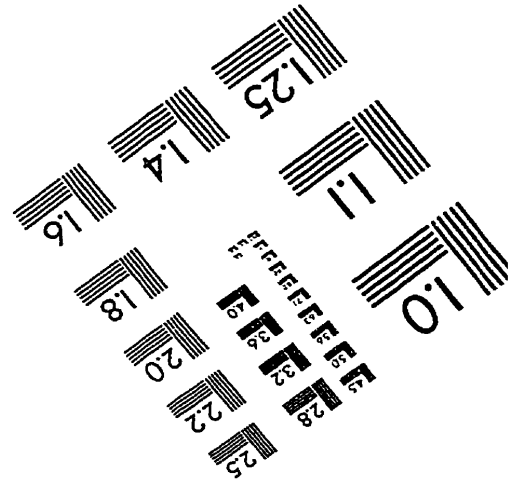












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