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**SYNTHESIS AND EVALUATION OF ETCHED CAPILLARIES FOR CHIRAL
SEPARATION BY CEC**

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Sharel Menezes

August 1999

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ABSTRACT

SYNTHESIS AND EVALUATION OF ETCHED CAPILLARIES FOR CHIRAL SEPARATION BY CEC

by Sharel Menezes

This study presents electrochromatographic separations for etched and chiral bonded stationary phases on quartz capillaries. The chiral selectors used in this study were 2-hydroxy-3-methacryloyloxy-propyl- β -cyclodextrin and (R)-(+)- α -Acryloyloxy- β , β -dimethyl- γ -butyrolactone and (R)-(+)-1-(α -naphthyl)ethylamine. The capillaries undergo silanization and hydrosilation reactions that result in a Si-C linkage. The β -cyclodextrin and the γ -butyrolactone capillaries were then evaluated by conducting electroosmotic flow measurements under the pH range of 2.14 - 6.0. Electric field effects at 30 kV and 5 kV were also compared over the pH range studied. Separation of various tricyclic antidepressants, benzodiazepines, other drugs and derivatized amino acids into their enantiomers were conducted at various pHs on all three chiral capillaries.

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Table of Contents

LIST OF TABLES	xi
LIST OF FIGURES	xii
Chapter I - INTRODUCTION	
1. Stereochemistry	1
2. Significance of Enantiomeric Separation	4
3. History	6
3.1 Chromatography	6
3.2 Electrophoresis	9
3.3 Electrochromatography	12
4. Development of capillary electrochromatography	13
4.1 Capillary electrochromatography apparatus	14
5. Principles underlying Capillary Electrochromatography	14
5.1 High Performance Liquid Chromatography (HPLC)	14
5.2 Capillary electrophoresis (CE)	16
5.3 Capillary electrochromatography (CEC)	16
6. Electrochemical phenomenon involved	17
6.1 The electrical double layer	17
6.2 Electroosmosis	18
6.2.1 Measurements of electroosmotic flow	18
7. Format of Capillaries	20

7.1 Bare capillaries for capillary electrophoresis	20
7.2 Packed capillaries for capillary electrochromatography	21
7.3 Open tubular capillaries for capillary electrochromatography	21
8. Separation of enantiomers	22
8.1 The indirect resolution method	22
8.2 The direct resolution method	23
8.2.1 Ligand exchange mechanism	23
8.2.2 Chiral micelles	24
8.2.3 Inclusion complexation	25
8.2.3.a Cyclodextrins	25
8.2.4 Affinity interactions	29
8.2.5 Combined approaches	29
8.3 Interaction of the chiral compounds on the molecular level	29
9. Goals of the research	32
 Chapter II - EXPERIMENTAL	
 1. Materials	36
2. Instrumentation	38
2.1 Capillaries	38
2.2 Gas chromatography oven	38
2.3 Capillary electrophoresis	39

2.3.1 Analysis	39
2.4 Diffuse Reflectance Infra Red Fourier Transform Spectrometry (DRIFT)	39
2.4.1 Preparation of the samples from etched and modified capillaries for DRIFT	39
3. Methodology	40
3.1 Etching of the capillaries	40
3.2 Formation of the hydride layer	41
3.3 Apparatus for the synthesis of the bonded material	42
3.3.1 Preparation of the β -cyclodextrin mixture	42
3.3.1.a Attachment of 2-hydroxy-3-methacryloyl-oxypropyl- β - cyclodextrin chiral selector.	42
3.3.2 Preparation of the (R)-(+)-acryloxy- β,β -dimethyl- γ - butyrolactone mixture	43
3.3.2.a Attachment of (R)-(+)-acryloxy- β,β -dimethyl- γ - butyrolactone chiral selector.	43
3.3.3 Preparation of the R(+)-1-(α -naphthyl)ethylamine mixture	43
3.3.3.a Attachment of R(+)-1-(α -naphthyl)ethylamine chiral selector	44
3.4 Preparation of buffers	44
3.5 Electrochromatographic analysis	45
3.5.1 Making of a detection window on the capillary	45

3.5.2 Preparation of the samples	45
3.5.3 Effects of the buffers and voltage on enantiomeric separation	49
3.5.4 Measurements for electroosmotic flow	49
 Chapter III - RESULTS AND DISCUSSION	
 1. Etching and modification of the capillaries	50
2. Spectroscopic study of the silica surface	52
3. Bonding of the chiral selectors	52
4. Choice of test solutes	55
4.1 Tricyclic antidepressants	55
4.2 Benzodiazepines	58
4.3 Other drugs	58
4.4 Derivatized amino acids	58
5. Interpretation of chromatographic data	58
5.1 Separation of tricyclic antidepressants	58
5.2 Separation of benzodiazepines	65
5.3 Separation of other drugs	77
5.4 Separation of derivatized amino acids	77
5.5 Effect of pH on separation	82
5.6 Effect of voltage on separation	87
6. Electroosmotic flow measurements	88

6.1 Effect of pH and electric field on electroosmotic flow	88
6.2 Cyclodextrin capillary	88
6.3 Lactone capillary	89
 Chapter IV – CONCLUSION	 95
 Chapter V - REFERENCES	 97

List of Tables

1. Table 1	Comparison of the various cyclodextrins	26
2. Table 2	List of chemicals used in this study along with their chemical abstract service (CAS) registry numbers.	36

List of Figures

Figure 1: Direction of polarized light	2
Figure 2: Rotation of polarized light	2
Figure 3: Mirror images of D- and L- glyceraldehyde	3
Figure 4: Representation of bonds	3
Figure 5: Projection formulas of D- and L- glyceraldehyde	5
Figure 6: R- and S- forms of glyceraldehyde in terms of absolute configuration	5
Figure 7: One enantiomer Darvon is marketed as an analgesic while the other enantiomer Novrad is marketed as an anticough drug	7
Figure 8: Schematic of CEC instrumentation	15
Figure 9: The silanoate groups on the inner wall of the capillary attract the cations forming a fixed layer, while the mobile layer of cations is pulled towards the cathode resulting in electroosmotic flow	19
Figure 10: Cyclodextrins are shaped similar to that of a truncated cone with a hydrophobic interior cavity and a hydrophilic exterior	27
Figure 11: Representation of β-cyclodextrin	27
Figure 12: Three-point interaction between L-arginine and D- and L-dihydroxyphenylalanine	31
Figure 13: Structure of 2-hydroxy-3-methacryloyloxypropyl-β-cyclodextrin	33
Figure 14: Structure of (R)-(+)-acryloxy-β, β-dimethyl-γ-butyrolactone	34
Figure 15: Structure of (R)-(+)-1-(α-naphthyl)ethylamine	35
Figure 16: Structures of the tricyclic antidepressants studied	46
Figure 17: Structures of other drugs used in this study	47

Figure 18: Structures of the benzodiazepines studied	48
Figure 19 a, b: The electronegative fluorine atom causes an electron shift in the neighbouring atoms resulting in the weakening of hydroxyl bonds	51
Figure 20: Diagram of the reactions on the surface of the capillary wall	53
Figure 21: DRIFT spectrum of bonded cyclodextrin on silica hydride surface	54
Figure 22: DRIFT spectrum of bonded lactone on silica hydride surface	56
Figure 23: Structures of the bonded chiral selectors on the silica hydride surface; A: 2-hydroxy-3-methacryloyl-oxypropyl-β-cyclodextrin B: (R)-(+)-acryloxy-β,β-dimethyl-γ-butyrolactone C: (R)-(+)-1-α-naphthylethylamine bonded to 4-allyloxybenzoic acid	57
Figure 24: Separation of D- and L- form of clomipramine on etched and lactone modified capillary. L = 32 cm, I = 24 cm, V= -10 kV, pH =3.0 (1:10)	60
Figure 25: Separation of D- and L- form of nortriptyline on etched and lactone modified capillary. L = 24 cm, I = 16 cm, V= 10 kV, pH =4.41 (1:10)	61
Figure 26: Separation of D- and L- form of doxepin on etched and lactone modified capillary. L = 24 cm, I = 16 cm, V= -20 kV, pH =3.7 (1:10)	62
Figure 27: Separation of D- and L- form of doxepin on etched and lactone modified capillary. L = 24cm, I = 16 cm, V= -20 kV, pH =3.0 (1:10)	63
Figure 28: Interaction between the lactone moiety and clomipramine.	64
Figure 29: Separation of D- and L- form of diazepam on etched and cyclodextrin modified capillary. L = 45 cm, I = 25 cm, V= 30 kV, pH =3.0 (1:10)	66

Figure 30: Separation of D- and L- form of diazepam on etched and cyclodextrin modified capillary. L = 45 cm, I = 25 cm, V= 30 kV, pH =2.14 (1:10)	67
Figure 31: Separation of D- and L- form of diazepam on etched and cyclodextrin modified capillary. L = 45 cm, I = 25 cm, V= 20 kV, pH =2.14 (1:10)	68
Figure 32: Separation of D- and L- form of temazepam on etched and cyclodextrin modified capillary. L = 45 cm, I = 25 cm, V= 30 kV, pH =2.14 (1:10)	69
Figure 33: Separation of D- and L- form of temazepam on etched and cyclodextrin modified capillary. L = 45 cm, I = 25 cm, V= 0 kV, Vacuum = 20", pH =2.14 (1:10)	70
Figure 34: Separation of D- and L- form of temazepam on etched and cyclodextrin modified capillary. L = 45 cm, I = 25 cm, V= 30 kV, pH =3.0 (1:10)	71
Figure 35: Separation of D- and L- form of chlorodiazepoxide on etched and cyclodextrin modified capillary. L = 45 cm, I = 25 cm, V= 30 kV, pH =3.0 (1:10)	72
Figure 36: Separation of D- and L- form of chlorodiazepoxide on etched and cyclodextrin modified capillary. L = 45 cm, I = 25 cm, V= 20 kV, pH =3.0 (1:10)	73
Figure 37: Separation of D- and L- form of oxazepam on etched and cyclodextrin modified capillary. L = 45 cm, I = 25 cm, V= 30 kV, pH =3.0 (1:10)	74
Figure 38: Inclusion-complexation between the cyclodextrin and the benzodiazepines; A- Diazepam B- Temazepam C- Oxazepam D- Chlorodiazepoxide	75
Figure 39: Separation of D- and L- form of diazepam on etched and naphthyl ethylamine modified capillary. L = 50 cm, I = 37 cm, V= 30 kV, pH =2.14 (1:10)	78

Figure 40: Interactions between the R-naphthylethylamine and diazepam.	79
Figure 41: Separation of D- and L- form of terbutaline on etched and lactone modified capillary. L = 32 cm, I = 24 cm, V= -10 kV, pH =3.0 (1:10)	80
Figure 42: Interaction between the lactone moiety and terbutaline.	81
Figure 43: Separation of D- and L- form of 3,5-DNB alanine methyl ester on etched and naphthyl ethylamine modified capillary. L = 50 cm, I = 37 cm, V= 30 kV, pH =2.14 (1:10)	83
Figure 44: Separation of D- and L- form of 3,5-DNB phenylalanine methyl ester on etched and naphthyl ethylamine modified capillary. L = 50 cm, I = 37 cm, V= 30 kV, pH =3.7 (1:10)	84
Figure 45: Separation of D- and L- form of 3,5-DNB phenylalanine methyl ester on etched and naphthyl ethylamine modified capillary. L = 50 cm, I = 37 cm, V= 30 kV, pH =4.41 (1:10)	85
Figure 46: Interactions between the R-naphthylethylamine and A: 3,5-DNB alanine methyl ester B: 3,5-DNB phenylalanine methyl ester	86
Figure 47: Effect of EOF as a function of pH.	91
Figure 48: Plot of EOF as a function of electric field.	92
Figure 49: Effect of EOF as a function of pH	93
Figure 50: Plot of EOF as a function of electric field.	94

Chapter I

INTRODUCTION

1. Stereochemistry

Stereochemical properties influence the activity and interactions of enantiomeric compounds. Two molecules having similar bonds but differing in the arrangement of their atoms in space are referred to as stereoisomers. Normal light consists of electromagnetic radiation in which the electric field oscillates randomly in all directions perpendicular to the direction of the beam.¹ In polarized light, the electric field oscillates in a single plane as seen in Figure 1. When polarized light passes through a sample of an optical isomer, the plane of polarization of the light is rotated as seen in Figure 2.

Chemical compounds that are able to rotate polarized light either to the right or to the left are called chiral compounds. This property is due to the asymmetry in their chemical structure arising from the different spatial arrangement of the groups around the asymmetric center. These compounds are called enantiomers as they contain a single chiral center and can exist in two different forms that are non superimposable mirror images of each other. This study entails enantiomeric separations and interactions and therefore the stereochemistry of chiral compounds is useful. Figure 3 shows the enantiomers of D- and L-glyceraldehyde. Different symbols are used to depict the bonds with asymmetric centers as seen in Figure 4.² The solid line represents a bond lying in the plane of the paper, while a broken line denotes a bond projecting behind this plane. A bond projecting in front of this plane appears wedge shaped. Figure 5 represents projection formulas for D- and L- glyceraldehyde.²

Figure 1: Direction of polarized light¹

Figure 2: Rotation of polarized light¹

Figure 3: Mirror images of D- and L- glyceraldehyde²

Figure 4: Representation of bonds²

The essential physical property that distinguishes optical isomers is the direction in which each rotate plane polarized light. If one isomer turns the plane of polarization in a clockwise (+) manner in the face of the beam, it is described as dextrorotatory (right-handedness). If it alters the passage of light in a counterclockwise manner(-), it is referred to as levorotatory (left-handedness). This property of right- or left-handedness is called chirality.³ The old Fischer system with prefixes D and L and the Cahn-Ingold-Prelog system with the prefixes R and S are two systems that describe the stereoconfiguration on the chiral carbon atom. Figure 6 shows the R- and S- forms of glyceraldehyde in terms of absolute configuration.² Chiral molecules are said to be enantiomers as the two optical isomers of the same compound do not contain a symmetry plane and are nonsuperimposable mirror images of each other. Enantiomers interact differently with other compounds containing chiral centers. There is either attraction or repulsion due to steric hindrance between two interacting compounds due to the orientation of various groups on these enantiomers.

2. Significance of enantiomeric separation

Enantiomeric separations are of key strategic importance in the pharmaceutical, chemical and related industries due to increasing concern for the stereoselective consequences of chiral drugs, food additives and agrochemicals. The activity, pharmacokinetics and metabolism of enantiomers are unique. Two enantiomers may exhibit the same pharmacological activity to different extents or they could have entirely different kinds of chemical or pharmacological activity.⁴

D-glyceraldehyde

L-glyceraldehyde

Figure 5: Projection formulas of D- and L- glyceraldehyde²

Figure 6: R- and S- forms of glyceraldehyde in terms of absolute configuration

For example, dextromethorphan, the D-enantiomer of 3-methoxy-N-methyl-morphinan has low toxic potential and is used in cough medications. The enantiomer, levomethorphan, is a narcotic analgesic with no anti-cough efficacy. The less desirable enantiomer is considered an impurity that must be reduced to low levels in the finished drug material. It is necessary to develop separation techniques so that safety and efficacy of each enantiomeric form may be proven. Another example is dextropropoxyphene. The D-enantiomer is an analgesic and levopropoxyphene is an antitussive drug. Their chemical mirror type relationship is also reflected in their names, Darvon and Novrad (which is Darvon spelled backwards). Their structures are shown in Figure 7.⁵ Optical activity is important in drug analysis and control. Also, the need for obtaining the pure form of an enantiomer has increased in most industries and this has spurred the development of various analytical methods for the separation of enantiomers.

3. History

3.1 Chromatography

Chromatography is a method for separating complex mixtures based on the distribution of molecules between a mobile and stationary phase. The mobile phase may be a liquid, solid or gas and the stationary phase may be a solid or a solid coated with a liquid. The distribution of the molecules between the two phases is governed by various processes such as adsorption, ion-exchange, partitioning and gel filtration. These processes combined with the movement of the mobile phase result in a differential migration or resolution of the molecules.

Figure 7: One enantiomer Darvon is marketed as an analgesic while the other enantiomer Novrad is marketed as an anticough drug⁵

Liquid chromatography is used to separate a mixture in solution into its individual components. In liquid chromatography, separation is based on the differential partitioning of solutes between the mobile phase and stationary phase used. The adsorbent material was first described by Tswett.⁶ He indicated that separation could be achieved when a mobile phase carried solutes over a stationary phase. In 1848, Louis Pasteur performed chiral resolutions by separating mirror-image morphologies.⁷ His conception of dissymmetry was based on the general morphological principle of the crystallographer named Hauy who postulated that a crystal and each constituent space-filling molecule are images of each other from crystal cleavage observations in 1809.⁸ In the 1970's, there were a few reports on low-pressure column liquid chromatographic separation of enantiomers. The 1980's was a turning point for enantiomeric separations because a number of new and improved chiral stationary phases and additives were introduced and also there was an increase in the number of publications. Pirkle et al., used a chiral fluoroalcoholic bonded stationary phase which was capable of separating the enantiomers of a large number of solutes including sulfoxides, lactones and derivatives of alcohols, amines, amino acids, hydroxy acids and mercaptans.⁹ Pietrogrande et al., studied the solvatochromic behavior of benzodiazepines and liquid chromatographic retention behavior by means of linear solvation energy relationships and tested the methodology using reverse phase and normal phase HPLC.¹⁰ Chromatographic techniques including thin layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC) have been employed for chiral separation. Before the 1980's enantiomeric

separations were avoided because they were thought to be tedious. In 1985, Gassman et al., first used capillary electrophoresis (CE) for separation of dansyl amino acid enantiomers.¹¹ Stalcup et al. used a carbohydrate based stationary phase based on malto-oligosaccharides to resolve benzodiazepines by HPLC in 1997.¹² Lurie et al. used HPLC analysis of benzodiazepines using diode array, electrochemical and thermospray mass spectrometric detection methods in 1992.¹³

The use of porous silica-based column packing in HPLC was a major development because these packings have excellent mechanical strength. The silica comes in a variety of particle and pore sizes resulting in higher efficiency of separations. It allows for good bonding chemistry and surface modification and there is good reproducibility and modest cost. However for chiral stationary phases, the columns are relatively expensive, the performance is often much lower than with regular LC columns, and lastly many types of these columns are required in order to cover a relatively narrow range of racemic compounds.

3.2 Electrophoresis

Electrophoresis is the movement of electrically charged particles or molecules in a conductive liquid medium under the influence of an electric field. The electric field applied across the capillary causes the conductive liquid (buffer) to move. This phenomenon is called electroosmotic flow (EOF). The direction of the EOF is towards the negatively charged cathode. Anions and cations in an electrolyte are separated depending on their size and electrophoretic mobilities.

The first successful electrophoretic analyses in free solution were performed by Hjerten in 1958.¹⁴ He used a quartz capillary of internal diameter (i.d.) of 1-3 mm. In 1967, he recognized that carrying out electrophoresis in narrow diameter tubes reduced thermal effects.¹⁵ In 1970, Neuhoff and co-workers used polyacrylamide gel filled glass tubes 0.45 mm i.d. and 3 cm in length to analyze minute quantities of protein.¹⁶ Virtanen and Kivalo used glass capillaries of 0.2-0.5 mm i.d. and 50-100 mm length.¹⁷ Detection was performed by measuring the potential gradient in the separated zones and they also discussed the separation efficiency in detail. In 1975, O' Farrell used flat bed and gel techniques for a two-dimensional method and achieved very high resolution.¹⁸

Castimpoolas, in 1979, used column techniques which are represented by disc electrophoresis in polyacrylamide gel rods for characterization of proteins.¹⁹ The main drawback of this technique was the laborious multi-stage handling of gel beds and insufficient long-term reproducibility of the results. To remove the problem of irreproducibility, the polyacrylamide gels were replaced by the use of capillaries.

The use of thin capillaries showed improvements in stability and reproducibility as undesired dispersions due to hydrodynamic flow were restricted by the small diameter of the tubes. The use of a capillary as a separation column allows a change in the background electrolyte (BGE) and excellent cooling efficiency so that high voltage can be applied, thereby speeding up the analysis time and preventing joule heating effects, discussed in Section 7.1. This in conjunction with on-line detection converts electrophoresis into a dynamic instrumental analytical method. Progress in capillary zone electrophoresis led to the development of capillary isotachophoresis (ITP). ITP is the

the electrophoretic counterpart of displacement chromatography and is performed by sandwiching a sample between a leading and terminating buffer in a capillary with the electric field in a constant current mode. In 1970, Everaets and Hoving-Keulemans performed ITP on their own laboratory-made equipment for the separation of substances using a polytetra-fluoroethylene (PTFE) capillary of 0.5 mm i.d.²⁰ In 1979, Mikkers et al., performed free zone electrophoresis in Teflon tubing of 2 mm i.d. to reduce convection problems.²¹ Here, the molecules migrate as zones and do not undergo zone spreading due to diffusion.

Jorgensen and Lukacs were the first to demonstrate that the use of even narrower capillaries (< 1mm i.d.) produced highly efficient electrophoretic separations.²² An applied field of 30kV and fluorescent detection were used. In 1981, Jorgensen and Lukacs, Stevens and Cortes in 1983 and Knox and Grant in 1991 investigated separations based on electroosmotic flow and demonstrated that it was a good alternative to pressure-driven flows seen in HPLC.^{22,23,24} This was because both charged and uncharged species could be separated according to their differential migration through the capillary.

Electroosmotic flow has a flat profile which is due to the frictional drag. This causes the flow at the wall to be slower (or reduced) than the rest of the tube. Since the area near the wall is small, the reduction in flow from the overall flat profile is very little. Therefore, all solute molecules would experience same velocity and would elute out as narrow bands. HPLC flow is more laminar because the solutes move through a tube under the influence of a pump. The solutes in the center of the tube move faster than those closer to the wall resulting in an uneven flow with broad peaks. Based on the

combination of interactions with the stationary phase and electrophoretic mobilities of the solutes, the method resulted in high efficiency separations. Efficiency (N), is defined as the number of theoretical plates, and is calculated by measuring the migration time (t) and the peak width (w) at the base of the peak. The correlation is $N = 16(t/w)^{1/2}$.

Jorgensen and Lukacs also used CE, which is electrophoresis performed in glass (silica) open tubular capillaries to resolve mixtures of dansyl and fluorescamine derivatives of amino acids, dipeptides, and simple amines. The publications of Jorgensen and Lukacs drew the attention of many scientists. Since then, there has been a rise in the number of publications and reviews on CE published from 1981 to date.²⁵ The introduction of commercial CE instrumentation in late 1988 enhanced the development and application of this technique.

3.3 Electrochromatography

Berraz coined the term electrochromatography in 1943.²⁶ It described a form of paper electrophoresis that was demonstrated earlier for separating dyes using an alumina column by Strain in 1939.²⁷ In 1949, both Tiselius and Synge suggested that electroosmosis be used to move solvent through a porous gel structure so as to avoid hydrostatic pressures.^{28,29} Mould and Synge in 1954 used this approach to separate polysaccharides using collodion membrane strips.³⁰ In 1974, Pretorius et al., were the first to suggest electroosmosis as a pumping technique to drive the solvent through a 1 mm packed column.³¹ This suggested that chromatography could be combined with electroosmotic flow.

Capillary electrochromatography (CEC), shows high potential because of its high efficiency which is due to the flat profile of EOF. Also the speed of analysis, the small volumes and quantities of chiral selector and analytes needed, the simplicity and flexibility of the method and that both charged and neutral compounds can be separated, make CEC a productive technique.

4. Development of capillary electrochromatography

Advances in liquid chromatography and capillary electrophoresis have led to the concept of a hybrid technique called capillary electrochromatography (CEC). This electroseparation technique is based on the partitioning between the stationary and mobile phase and electroosmotic flow that transports the solvent and solute zones through the capillary. CEC can be performed in open tubes where the appropriate moiety depending on the application, is bound to the capillary wall and solute-bonded phase interactions are utilized, or in packed capillaries with conventional micron sized octadecyl silica particles, or in capillaries coated with a layer of material. An advantage of CEC is that the partitioning in HPLC as described in Section 3.1, and the electrophoretic mobility of CE as described in Section 3.2, can be combined to increase separation selectivity.³² Separation of charged and neutral compounds can be carried out. The flat electroosmotic flow profiles combined with small particles provide GC-type efficiency is greater than 10^5 theoretical plates per column. It is also less prone to undesirable joule heating effects.

4.1 Capillary electrochromatography apparatus

CEC is carried out with simple capillary electrophoresis equipment that consists of a fine quartz (silica) capillary (length 200-1000 mm, bore size 20-200 μm) where the separation occurs. There is a high voltage power supply that can deliver 30 kV at 100 μA . The ends of the capillary are placed in electrolyte reservoirs; one of which is grounded and the other is connected to the high voltage supply. At the high voltage end of the capillary, an on-column injection system is present. An on-column detector (either UV or fluorescence) measuring analyte concentration within a short segment of the capillary is attached. Pressure can be applied at the inlet or vacuum at the outlet end of the capillary. Current flowing through the capillary is measured by an ammeter. The instrument is isolated during operation of the high voltage by a suitable Faraday cage. This is a protective casing providing insulation against high voltages. Thermostating can be maintained with forced air or liquid.³³ An example of such instrumentation is shown in Figure 8.

5. Principles underlying capillary electrochromatography

5.1 High performance liquid chromatography (HPLC)

Differences in the distribution ratios of solutes between two phases, the mobile and the stationary phases, cause preferential separation. Molecules are separated based on their partitioning coefficients. The advancement in the packing techniques for packing the columns and pumping systems has allowed particles as small as 5 μm in size to be used. This results in an increased surface area allowing more interactions to occur.

Figure 8: Schematic of CEC instrumentation

Packing of the small sized particles has now been made more uniform. These advancements in liquid chromatography have resulted in greater efficiency of separations. Therefore the term high performance liquid chromatography (HPLC).

5.2 Capillary electrophoresis (CE)

Electrically charged particles in an electrolyte are driven by an electric field when voltage is applied. The positively charged particles migrate toward the cathode and the negatively charged particles migrate towards the anode. Cations move faster than the EOF as they are attracted to the cathode while anions move slower than the EOF as they are attracted to the anode. These charged molecules migrate through a capillary in a particular direction at specific rates depending on their charge to size ratio, therefore, the term capillary electrophoresis. Neutral molecules migrate along with the buffer through the capillary due to electroosmosis. However, there is no separation of neutral compounds.

5.3 Capillary electrochromatography (CEC)

CEC is a hybrid techniques of both HPLC and CE. Electrochromatography has a plug-like profile that is characteristic of a CE profile. The plug-like flow in both open and packed columns is more uniform than the laminar flow of a pressure driven system as seen in HPLC. The same capillary also shows higher efficiency when used in CEC than when used in pressure driven systems. EOF drives the solvent in CEC in contrast to high pressure that is used in HPLC. There are several reasons for the slow development of capillary electrochromatography. It is difficult to construct the capillaries, but if the

technology develops to a point where they can be packed easily and reproducibly, the demand will increase. CEC is an attractive technique because of low eluant consumption and small sample size requirements. It offers the advantage over HPLC because of the increased efficiency, and therefore the possibility of resolving closely related analytes not separable by HPLC.

6. Electrochemical phenomenon involved

6.1 The electrical double layer

The surface of silica contains chemically bound silanoate (Si-O^-) groups at its surface at pH value > 3 and is electrically charged. In contact with an electrolyte, the surface ions are balanced by an excess of positive ions within the electrolyte. Strong electrostatic interactions between the negative ions on the surface and the positive ions in the electrolyte result in a thin layer of excess positive ions. This layer can be separated into two parts and is also referred to as the double layer. Near the surface, the layer of ions which are adsorbed on the surface are fixed. This is called the Stern or Helmholtz layer. The remaining ions form a diffuse layer and are called the Gouy-Chapman layer.³³ The ions in the diffuse layer exchange continuously with those in the solution. The charge density of the excess ions, σ , falls exponentially with distance (z) from the surface and this is denoted in the following equation.

$\sigma = \sigma_0 \exp(-z/\delta)$ where δ is the thickness of the double layer and σ_0 is the charge density of ions at the fixed layer.

6.2 Electroosmosis

When a potential difference is applied to a surface, the ions in the diffuse layer experience a force parallel to the surface. Since there is an excess of positive ions in this layer, the solvent immediately in contact with the surface experiences a net force towards the negative electrode. The viscosity of the liquid resists this effect resulting in a shear within the double layer that is proportional to the excess charge density at any point in the layer. The result is that the liquid at the solution-side boundary of the double layer moves at constant velocity. This process is called electroosmosis and is illustrated in Figure 9.³⁴

6.2.1 Measurements of electroosmotic flow

EOFs can be measured with various techniques. The method adapted from Sandoval and Chen was used in the study.³⁵ Migration times were recorded and the EOF then calculated using the formula:

$$\mu_{eo} = L l / V t_v * (1-t_1/t_2)$$

where μ_{eo} = electroosmotic mobility of the electrolyte, L = total length of the capillary, l = effective length of the capillary, V = applied voltage, t_v = time for which voltage was applied, t_1 and t_2 = the migration times of the two marker bands.

Figure 9: The silanoate groups on the inner wall of the capillary attract the cations forming a fixed layer, while the mobile layer of cations is pulled towards the cathode resulting in electroosmotic flow³⁴

7. Format of capillaries

7.1 Bare capillaries for capillary electrophoresis

An unmodified fused silica capillary is the simplest and most reliable column for various applications. Diameters between 50 to 100 μm are desirable because Joule heat dissipation is efficient even at high voltages. Electric current passes through a conductive solution causing joule heating. This warms the solution and leads to convective diffusion. Molecules in the warmer, center part of the tube migrate faster than those near the cooler wall, leading to zone spreading. Heating can also cause degradation of biological samples. To minimize the amount of heat generated, one approach is to use capillaries of smaller diameter. The separation efficiency can be increased. With bare capillaries, there are residual silanols on the surface of the capillary causing electroosmotic flow (EOF) which cause analyte migration. For positively charged compounds moving towards the cathode, the EOF will diminish the resolution as it contributes to migration in this direction, lowering migration time. Hence, EOF should be diminished to enhance resolution. For negatively charged compounds, EOF is necessary for migration towards the cathode. The silanols are potential sites for adsorption of certain basic solutes and this leads to poor recovery and variable migration times of the analyte due to a decrease in EOF. Proper choice of buffer and control of pH may correct these drawbacks. As pH is lowered, the silanols become protonated causing a reduction in EOF that leads to improved resolution and diminishing the adsorption effects of positively charged compounds.³⁶

7.2 Packed capillaries for capillary electrochromatography

The capillary is similar to an HPLC column where the stationary phase that is chemically bonded to a support particle is packed into a fused silica capillary. High pressure is absent and this allows small sized particles to be used in the column, thus resulting in higher efficiency. This is indicated by narrow peaks obtained in an electropherogram. Endcapping of the bonded material, which is done by treating the packing with a small reactive silane that will react with any remaining unreacted silanolate groups, is not required as EOF is desirable. Columns are packed by using high pressure through a pneumatic amplification pump or the drawn packing method or by electrokinetic packing.³¹

7.3 Open tubular capillaries for capillary electrochromatography

The open tubular (OT) approach can be used to avoid fabrication of frits which are used to hold the packing material in place and to avoid the formation of bubbles due to dissolved gas in the electrolyte under high voltage during an electrochromatographic run. To overcome these drawbacks found in packed capillaries, various (OT) modes of separation have been developed. One method involves binding of the appropriate moiety to the wall of the capillary and the use of solute/bonded phase interactions. Another method is to use a microchip approach where a C-18 moiety is bonded to the channel on the chip and separation is based on differences in electromigration.³⁶ A third method is to enhance solute/bonded phase interactions by increasing the internal surface area of the inner capillary wall. This is done by etching the fused silica surface with NH_4HF_2 .

Radial extensions of several microns in length from the surface are formed which reduce the distance that the solute must travel so as to interact with the stationary phase. After etching, the appropriate organic moiety can be attached to the surface by standard bonding chemistry. The separation is due to differences in electrophoretic mobility and solute/bonded phase interactions.

8. Separation of enantiomers

Chiral chromatographic systems have been classified in accordance with the single, most distinct type of intermolecular interactions such as hydrogen-bonding, ligand-exchange and charge-transfer phases.³⁷ The following scheme depicts the interaction between a racemic compound (En) and a chiral selector (Sel):²

The separation methods are categorized into the indirect and direct resolution methods which are discussed below.

8.1 The indirect resolution method

Here the chiral compound reacts with the two enantiomers to produce stable diastereoisomers due to the formation of strong covalent bonds.² The electrophoretic analysis of these diastereoisomers is carried out in an achiral background electrolyte. The major advantage of this method is there is flexibility in selecting the composition of the electrolyte. This method can be applied only if the enantiomers bear easily derivatizable groups such as amino and hydroxyl groups. However, the chemical

reaction is time consuming and the sample may be affected. A very high chiral purity of the reagent is required.

8.2 The direct resolution method

Here the chiral reagent is either added to the background electrolyte (BGE) or bound to the capillary wall. Labile diastereoisomeric complexes of the enantiomers with the chiral selector differing in stability are formed during the electrophoretic run, resulting in different effective mobilities of the two compounds in the system.² Weak bonds (hydrogen, π - π , dipole-dipole, hydrophobic) are formed between the members of the enantiomeric pair and the chiral environment. The chiral purity of the environment will influence the resolution only. This method eliminates all drawbacks of the indirect separations. It also prevents the sample from being destroyed due to a derivatization reaction. This method is rapid and the purity of the chiral selector is not important. However, the appropriate chiral selector must be established for each particular application concerned. The direct resolution method is broadly divided into five categories based on the mechanisms: ligand exchange, chiral micelles, inclusion complexation, affinity interactions and combined approach mechanisms

8.2.1 Ligand exchange mechanism

This mechanism is based on the interaction of a coordination complex with the two enantiomers of a racemic mixture forming labile complexes with different stability constants.² Coordination complexes are obtained by reaction of transition metals such as Cu(II) or Ni(II) with a suitable chiral ligand and are characterized by a defined

geometry with a certain orientation of the ligand. The ligands contain aminocarboxylic or hydrocarboxylic groups. This approach was introduced first by Davankov and Rogozhin.³⁸ The metal-ion and the chiral-ligand complexes, their concentration and ratio, the pH, the ionic strength and the organic solvent of the environment have to be controlled. The resolution mechanism can be denoted by the following equation:

where A represents a six-membered ring, B represents a five-membered chelate ring and M is a metal. B has replaced one A molecule to afford a more stable ternary complex than the six-membered one of the A moiety. The two enantiomers can be separated because the two corresponding diastereoisomeric complexes possess different formation constants.

8.2.2 Chiral micelles

Terabe introduced chiral surfactants like bile acid salts (e.g. taurodeoxycholate) for the resolution of several racemic mixtures into their enantiomers.³⁹ The analyzed compounds are zwitterions that can be positively or negatively charged at pHs either lower or higher than their isoelectric point. The structure of bile salts is characterized by a steroid portion and a side chain with a carboxylic group that can be conjugated with a glycine or taurine unit thus allowing the salts of bile acids to be both hydrophobic and hydrophilic. At neutral pH 7, unsatisfactory results were obtained. At pH 3, the best resolution of the enantiomers of zwitterionic form of dansyl-amino acids was due to the suppression of EOF and by use of taurodeoxycholate. The interactions between the

hydrophobic part (dansyl group) of the amino acid and the steroid moiety of the bile salt as well as between the charged amino group and the charged portion of the micelle cause a strong influence on the resolution mechanism.

8.2.3 Inclusion complexation

This is a widely used method for improving selectivity of the separation of isomeric compounds such as structural and positional isomers and enantiomers. Structural isomers have the same molecular formula but differ in their structural arrangement of atoms. Positional isomers such as 1-butene and 2-butene have the same molecular formula but differ in the position of the double bond. In the case of enantiomeric resolution, the two analytes (guest) interact with the chiral environment (host) resulting in the formation of host-guest complexes. Separation occurs if labile inclusion complexes possess different association constants. Two classes of guest compounds, cyclodextrins and crown ethers, have been used in capillary electrophoretic techniques for inclusion complexation.

8.2.3.a Cyclodextrins

Cyclodextrins (CD) are neutral, natural cyclic oligosaccharides obtained by enzymatic cleavage of starch. Starch when treated with glycosyltransferases, undergoes hydrolysis furnishing a mixture of oligosaccharides consisting of 6 up to 13 glucose units. CDs have been separated into six (α), seven (β), eight (γ) and nine (δ) glucose units by selective precipitation. Table 1 shows a comparison between the various cyclodextrins.⁴⁰ They are shaped similar to that of a truncated cone with a hydrophobic interior cavity and

a hydrophilic outside as seen in Figure 10.⁴⁰ The wider side of the CD cavity contains secondary hydroxyl (2- and 3-hydroxyl) groups, while the opposite smaller opening is occupied by primary hydroxyl (6-hydroxyl) groups as shown in Figure 11.⁴⁰ The rigid structure of the CD is due to the formation of intramolecular hydrogen bonds between the 2- and 3-hydroxyl groups of adjacent glucose units. Since CDs are neutral, they do not influence the conductivity of the BGE when they are used as additives to these electrolytes.

Table 1: Comparison of the various cyclodextrins

	d ₁ [nm]	d ₂ [nm]	Depth [nm]	Glucopyranose rings	Water molecules included	M _r	Solubility in water [g/100mL]
α-CD	1.37	0.57	0.79	6	6	972	14.5
β-CD	1.53	0.78	0.79	7	11	1135	18.5
γ-CD	1.69	0.78	0.79	8	17	1297	23.3

d₁ = outer ring diameter, d₂ = inner ring diameter, M_r = molecular weight (See Figure 10)

Chiral recognition arises from the asymmetric carbons of the glucose units with their hydroxyl groups present on the outside surface. The bonds between these groups and the analytes influence the stability of the two labile diastereoisomers formed. Weak interactions such as hydrogen bonding, π - π and dipole-dipole are involved.

CDs can be used as chiral additives to the electrolyte. The type of CD used as a chiral additive in the BGE is an important parameter for the chiral resolution of racemic mixtures into their enantiomers. The analyte should fit into the cavity to form inclusion complexes. The stability of the complex increases with the formation of bonds with the

Figure 10: Cyclodextrins are shaped similar to that of a truncated cone with a hydrophobic interior cavity and a hydrophilic exterior³⁵

Figure 11: Representation of β -cyclodextrin³⁵

groups located on the outside of the CD. The appropriate type of CD must be chosen taking in account the depth, width, type of substituents and the dimensions of the analytes. The resolution and the migration time of analytes are influenced by the CD concentration and they increase with an increase in concentration. The selectivity of an enantiomeric separation with a CD is affected by organic modifiers in the BGE, ionic strength, pH, buffer composition and temperature.

When cyclodextrins are a part of the stationary phase, enantiomeric separation occurs by the formation of transient diastereomeric complexes involving the selector (host) and enantiomers (guest). The retention times of enantiomers depend on the differences in the stabilities of the complexes formed. The less stable complex elutes earlier than the more stable complex.

One or more of the primary and secondary hydroxyls present on the cyclodextrin can be replaced by organic moieties. This allows modification of the properties of the parent cyclodextrin.⁴¹ Derivatization introduces interaction sites and this alters the charge, solubility and enantioselectivity of the parent cyclodextrin.

In CE, Mayer and Schurig in 1992 and 1994 reported the use of a CD-coated capillary (Chrasil -Dex)- a commercially available methylated β -cyclodextrin, to resolve enantiomers of binaphthyl derivatives and 1-phenylethanol.⁴² Nielen, in 1993, separated a few basic chiral drugs such as norephedrine and ephedrine.⁴³ In 1997, Skanchy and colleagues were able to resolve dipeptides using sulfobutylether derivatized β -cyclodextrin.⁴⁴

8.2.4 Affinity interactions

In addition to chiral selectors, polymeric substances of biological origin consisting of chiral monosaccharides (linear saccharides), or amino acid units (proteins) or a combination of both units can result in the separation of enantiomeric pairs of compounds. These substances behave as ligands attached to the chiral selector and they have strong binding affinity for one specific form of the enantiomer. Only a few compounds of this type have been used.

8.2.5 Combined approaches

Chiral resolution has been achieved by adding β -CD to a gel matrix containing a BGE and urea.² CD-modified micellar electrokinetic chromatography (CD-MEKC) has been used for the resolution of DL-amino acid enantiomers.² Here the CDs are added to a micellar solution which moves with the electroosmotic flow because of its net zero charge. For quantitative analysis, baseline resolution is important especially if the concentration of one enantiomer is higher than the other. If the minor enantiomer moves at a lower velocity, resolution is lost. So inversion of the migration order of the two analyzed enantiomers can be obtained by reversing the electroosmotic flow.

8.3 Interaction of the chiral compounds on the molecular level

For the interaction of chiral compounds on the molecular level, the three-point interaction rule established by Dalglish in 1952 for the resolution of the enantiomers of amino acids by paper chromatography can be utilized.⁴⁵ Amino, carboxylic and other

substituents at the asymmetric center are considered in predicting the probable interactions or steric repulsions with the chiral environment.

In 1989, Pirkle and Pochapsky revised this rule by stating that the three interactions were necessary and one of them must depend on stereochemistry.⁴⁶ According to this model, any combination of three attractive or repulsive interactions in the vicinity of the chiral center of the molecule could prove productive for the chiral recognition of its enantiomers. Possible interactions with the sorbent surface or matrix should also be considered. All types of close contacts between basic sites, acidic sites, steric interaction sites, electrostatic interaction sites, π -stacking interactions, π -acidic and π -basic aromatic rings on the chiral selector of the chromatographic system and the enantiomers can be important for chiral discrimination.

Aromatic rings result in π - π interactions, while sites due to an acidic hydrogen and the basic sites due to hydroxyl groups, ether oxygens or amino groups participate in intermolecular hydrogen bonding. Steric interactions come from large bulky groups present on the molecule. Electrostatic interactions are due to charged groups or permanent and induced dipoles. Figure 12 shows an example of 3-point interaction between L-arginine and D- and L- hydroxyphenyl alanine.² Compounds such as (R)-(+)-acryloxy- β , β -dimethyl- γ -butyrolactone, (R)-(+)-limonene and (8S,9R)-(-)-N-benzylcinchonidium chloride are some examples of Pirkle-type chiral selectors which show three point interactions and their role in enantiomeric separation has been investigated by HPLC.⁴⁷

Figure 12: Three-point interaction between L-arginine and D- and L-dihydroxyphenylalanine²

9. Goals of the research

The investigations carried out were preliminary studies for chemically bonded chiral stationary phases on etched capillaries. The study involved using the open tubular (OT) approach to CEC where the capillaries were etched, then modified by silanization with TES (triethoxy silane) followed by hydrosilation where the chiral selectors were attached to the inner wall in presence of a catalyst. The chiral selectors used in this study were (R)-(+)-acryloxy- β,β -dimethyl- γ -butyrolactone, 2-hydroxy-3-methacryloyl-oxypropyl- β -cyclodextrin and (R)-(+)- α -(naphthyl)ethylamine. Figures 13, 14 and 15 show the structures of these chiral selectors. These chiral selectors have been successfully tried on HPLC.^{47,48} Effects of various buffers and voltage, temperature, modifiers and many other parameters could improve resolution of the enantiomers giving two well defined peaks with baseline separation and good peak symmetry which are characteristics of good chiral separations. Only effects of pH and voltage on separations were studied. Enantiomeric separation of various chiral compounds such as tricyclic antidepressants, benzodiazepines and other drugs were carried out.

The performance of the capillaries was estimated by measuring electroosmotic flow (EOF) under these conditions. Electric field changes were compared in the various pH ranges used in this study. Characterization of the modified silica surface was carried out by diffuse reflectance infrared fourier transform (DRIFT) spectrometry.

Figure 13: Structure of 2-hydroxy-3-methacryloyloxypropyl- β -cyclodextrin⁴⁷
Average molecular weight = 1400

Figure 14: Structure of (R)-(+)-acryloxy-β, β-dimethyl-γ-butyrolactone⁴⁷

Figure 15: Structure of (R)-(+)-1-(α-naphthyl)ethanamine⁴⁸

Chapter II

EXPERIMENTAL

1. Materials

Table 2: List of chemicals used in this study along with their chemical abstract service (CAS) registry numbers.

Name	Source	CAS Registry Number
β -Alanine	Sigma	[107-95-9]
γ -aminobutyric acid (GABA)	Sigma	[56-12-2]
(R)-(+)-acryloxy- β , β -dimethyl- γ -butyrolactone	Aldrich Chemicals	[1020-96-60-6]
1,3-dicyclohexylcarbodiimide	Aldrich Chemicals	[538-75-0]
2-(N-morpholino)ethanesulfonic acid	Sigma	not available
4-Alloxybenzoic acid	prepared in the laboratory ⁴⁹	not available
Acetic acid	Aldrich chemicals	[64-19-7]
Acetone	Fischer Scientific	[67-64-1]
Ammonium hydrogen difluoride	Aldrich Chemicals	[1341-49-7]
Beta W7 MAHP 0.1/0.4 Lodextrin (β -CD)	Wacker Chemical	unassigned
Chloroplatinic acid hexahydrate	Aldrich Chemicals	[16941-12-1]
Citric acid	Sigma	[77-92-9]
Dichloromethane	Fischer Scientific	[75-09-2]
Diethyether	Fischer Scientific	[60-29-7]
Dimethylsulfoxide (DMSO)	Cambridge Labs	[67-68-5]

Dioxane	Aldrich Chemicals	[1764-74-4]
DL-Chlorodiazepoxide	Sigma	[58-25-3]
DL-Clenbuterol	Sigma	[21898-19-1]
DL-Clomipramine	Sigma	[303-49-1]
DL-Desipramine	Sigma	[50-47-5]
DL-Diazepam	Sigma	[439-14-5]
DL-Doxepin	Sigma	[1668-19-5]
DL-Histidine	Sigma	[4998-57-6]
DL-Homatropine	Sigma	[51-56-9]
DL-Immipramine	Sigma	[50-49-7]
DL-Oxazepam	Sigma	[604-75-1]
DL-Propanalol	Sigma	[525-66-6]
DL-Temazepam	Sigma	[849-50-4]
DL-Terbutaline	Sigma	[23031-32-5]
DL-Tropicamide	Sigma	[1508-75-4]
Ethanol	Shield Chemicals	[64-17-5]
Hydrochloric acid	Fischer Scientific	[7647-01-0]
Lactic Acid	Sigma	[50-21-5]
Methanol	General Chemicals	[67-56-1]
N-hydroxysucciniimide	Aldrich Chemicals	[6066-82-6]
Phosphoric acid	Fischer Scientific	[7664-38-2]

Potassium Bromide	Aldrich Chemicals	[7758-02-3]
R(+)-1-(α -naphthyl)ethylamine	Aldrich Chemicals	[3886-70-2]
Sodium hydroxide	Fischer Scientific	[1370-73-2]
Tetrahydrofuran (THF)	J. T. Baker	[109-99-8]
Toluene	Fischer Scientific	[108-88-33]
Triethoxysilane (TES)	Aldrich Chemicals	[998-30-1]
Tris [(hydroxymethyl) aminomethane]	Sigma	[77-86-1]

2. Instrumentation

2.1 Capillaries

The capillary tubing used was 375 μm o.d. x 50 μm i.d. (Polymicro Technologies, Phoenix, AZ). The capillary was made of quartz (silica) and therefore silanol groups were present on the inner wall. The capillary was coated on the outside with polyimmine giving it flexibility.

2.2 Gas chromatography oven

The etching and modification of the capillaries were carried out in a Hewlett-Packard Model 5890 gas chromatographic oven. The GC was modified to allow placement of several capillaries at both the inlet and outlet. Temperatures in the range of 300 $^{\circ}\text{C}$ to 400 $^{\circ}\text{C}$ were used for the etching process. Thick glass walled tubes were used as reservoirs and were placed in by plastic tubes for safety reasons. The reservoirs were held in metal containers. The reagents were added in vials that were either plastic or

glass depending on the corrosivity of the reagent and then placed in the thick-walled glass tubes. The tubes had swagelok fittings so that nitrogen gas could be used to force the reagents from the capillary inlet that was immersed in the reagent tube to the outlet end of the capillary placed in a collection vial.

2.3 Capillary electrophoresis

2.3.1 Analysis

CEC separations were carried out on the Spectra PHORESIS 500[™] system and on the Applied Biosystems Model 270A-HT (Foster City, CA) capillary electrophoresis systems. Measurements of EOF and electric field were carried out on the Applied Biosystems 270A-HT system.

2.4 Diffuse Reflectance Infra Red Fourier Transform Spectrometry (DRIFT)

The organic moiety attached to the capillary was investigated by a Mattson Instruments-Infinity Series FTIR[™] (Madison, WI, USA). The spectral range was between 4000 cm⁻¹ to 450 cm⁻¹ using 400 scans or 1000 scans with an MCT (HgCdTe) detector. The data were analyzed by the Venturis FX-2 Computer. The spectra were plotted by an HP 722C Printer (Palo Alto, CA).

2.4.1 Preparation of the samples from etched and modified capillaries for DRIFT

Parts of the capillaries are stripped of the coating by the method described in section 3.5.1. The capillaries are then crushed and mixed with 5% spectral grade KBr. This mixture is ground into fine powder using a mortar and pestle. A pure sample of KBr

is run as a reference background signal. The sample is placed in a holder, the surface is made uniform and then inserted in a compartment in the FTIR instrument. The instrument is then purged with nitrogen at 55-60 psi for about 30 minutes depending on the humidity in the laboratory. The spectra for both the β -CD and the γ -butyrolactone were scanned 400 times and 1000 times at a resolution of 4 cm^{-1} and 8 cm^{-1} , respectively. The spectral range was from 4000 cm^{-1} to 450 cm^{-1} . A plot of transmittance (%) versus wavenumber was obtained.

3. Methodology

3.1 Etching of the capillaries

Four $50\text{ }\mu\text{m}$ bare capillaries were connected in the GC oven as described previously. About 1 mL of concentrated HCl was placed in the inlet plastic vial. With a nitrogen pressure maintained at 40 psi, the capillaries were filled with concentrated HCl and heated overnight at $80\text{ }^{\circ}\text{C}$. The capillaries were then washed with distilled water, acetone and diethylether successively. The capillaries were dried with nitrogen gas for an hour.

A 5% (w/v) saturated solution of ammonium hydrogen difluoride was prepared in methanol and was used to etch the capillaries. About 1 mL of this solution was placed in the inlet plastic vial. The capillary was filled with this solution and allowed to stand for an hour. The methanol was then removed by uniform nitrogen flow through the capillary for 5 minutes. The capillaries were sealed at both ends using rubber corks while the remainder of the capillaries were coiled neatly, wrapped in aluminum foil and placed

inside the GC oven. The capillaries were then heated at 300 °C for two hours and then in the presence of nitrogen at 400 °C for one hour. The capillaries were washed with methanol once using a syringe and then dried for two hours with nitrogen pressure. Using a light microscope, any blockage in the inner walls of the capillaries could be observed.

3.2 Formation of the hydride layer

The capillaries were then treated with 0.1 M NaOH at room temperature for 20 hours under a nitrogen pressure maintained at 40 psi. They were then rinsed with deionized water for an hour. The capillary was flushed with 0.1 M HCl for 4 hours and rinsed again with deionized water for 2 hours. The capillaries were dried under a nitrogen pressure of 10 psi at 100 °C for 20 hours. This process was used to precondition the capillaries.

The capillaries were ready to undergo silanization. They were washed with dioxane for 10 minutes and then treated at 90 °C for 90 minutes with 1.2 mL of a 1M TES solution in 8.4 mL of dioxane which were mixed in a nitrogen glove box. A plastic vial was used for TES at the inlet. The hydride layer was formed in this step. The capillaries were then washed for two hours with dioxane and then washed again for two hours with THF at room temperature.

3.3 Apparatus for the synthesis of the bonded material

One opening of a 50 mL three-neck round bottom flask was connected to a thermometer and sealed with parafilm. Another neck opening was closed with a stopper and sealed with parafilm. A magnetic stirrer bar was placed inside the flask along with the reagents that are described in the methodology section below. A water condenser was then connected to the middle neck. All glassware was washed thoroughly and rinsed with distilled deionized water and dried in an oven. Cooling water circulated through the condenser. The flask was then placed on a heating mantle that in turn was placed on a magnetic stirrer. The heating mantle was connected to an electric heating device for temperature regulation. The reagents inside the flask were refluxed for 30 minutes.

3.3.1 Preparation of the β -cyclodextrin mixture

The cyclodextrin mixture was prepared by adding 100.5 mg of 2-hydroxy-3-methacryloyl-oxypropyl- β -cyclodextrin to 5 mL of a 75% : 25% (v/v) methanol : ethanol solution and 40 μ L of 10mM Speier's catalyst. The experimental set up is described in Section 3.3. This mixture was refluxed at 68 °C for 30 minutes. Speier's catalyst consists of 0.04 g of chloroplatinic acid hexahydrate dissolved in 100 mL of 2-propanol and stored in the freezer.

3.3.1.a Attachment of 2-hydroxy-3-methacryloyl-oxypropyl- β -cyclodextrin chiral selector.

Two of the hydride-modified capillaries were washed with a 75: 25 (v/v)

methanol : ethanol solution. The β -cyclodextrin solution was then flushed through the capillaries at 68 °C for 5 days. The capillaries were then washed with a 75 : 25 (v/v) methanol : ethanol solution and dried under nitrogen at 100 °C overnight.

3.3.2 Preparation of the (R)-(+)-acryloxy- β,β -dimethyl- γ -butyrolactone mixture

Two mL of toluene, 150 μ L of (R)-(+)-acryloxy- β,β -dimethyl- γ -butyrolactone and 40 μ L of 10 mM Speier's catalyst were mixed together. The experimental set up is described in 3.3. This mixture was refluxed at 82 °C for 1 hour.

3.3.2.a Attachment of (R)-(+)-acryloxy- β,β -dimethyl- γ -butyrolactone chiral selector.

One hydride-modified capillary was washed with dry toluene. The (R)-(+)-acryloxy- β,β -dimethyl- γ -butyrolactone mixture was flushed through the capillary at 82°C for 5 days. The capillary was then washed with toluene and THF twice. Further washing was then done with THF : water 1:1 (v/v) and diethylether. The capillary was then dried under nitrogen at 100 °C overnight.

3.3.3 Preparation of the R(+)-1-(α -naphthyl)ethylamine mixture

To a dry reaction flask, 0.39 g of 4-allyloxybenzoic acid (ABA), 0.39 g of N-hydroxysuccinimide, 0.41 g of 1,3-Dicyclohexylcarbodiimide (DCC) and 10 mL of dichloromethane were added. The flask was kept in ice to maintain a temperature of 0 °C and then 0.69 g of R(+)-1-(α -naphthyl)ethylamine was added to the flask. Nitrogen was flushed through the system for several minutes to maintain an inert atmosphere and

then the outlets were sealed. The reaction proceeded overnight in ice. The assembly was kept in a freezer to avoid melting of the ice. The mixture was then kept at room temperature for 30 minutes and the supernatant then discarded.

To the solid product obtained above, 20 mL of toluene and 300 μ L of 10 mM Speier's catalyst were added. The temperature was increased to 60 °C and the mixture was stirred for an hour.

3.3.3.a Attachment of R(+)-1-(α -naphthyl)ethylamine chiral selector

One hydride-modified capillary was washed with dry toluene. The R(+)-1-(α -naphthyl)ethylamine mixture was flushed through the capillary at 100 °C for 5 days. The capillary was washed with toluene, dichloromethane and diethylether. The capillary was then dried under nitrogen at 100 °C overnight.

3.4 Preparation of buffers

The buffers used in this study were: pH = 2.14, 0.3M phosphate (phosphate was from 85% H₃PO₄) and 0.19 M Tris; pH = 3.00, 0.3 M citric acid and 0.25 M β -alanine; pH = 3.70, 60 mM β -alanine and 60 mM lactic acid; pH = 4.41, 300 mM acetic acid and 375 mM γ -amino butyric acid; pH = 6.00, 60 mM 2-(N-morpholino) ethanesulfonic acid and 45 mM histidine. Deionized water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA) and was filtered through a 0.20 μ m Nylon 66 membrane filter (Altech Assoc., Deerfield, IL). The buffers were diluted with deionized water in the ratio of 1 : 10 and degassed with helium for 15 minutes before use.

3.5 Electrochromatographic analysis

3.5.1 Making of a detection window on the capillary

The capillary was shortened to the appropriate length with a capillary cutter. The appropriate position for the detection window was measured and marked. A glass block with tiny grooves was heated on a heating mantle in the fume hood until hot, and then a few drops of concentrated sulfuric acid were added in these grooves. When hot, the marked area for the window on the capillary was placed in the groove, and the coating was removed exposing the quartz glass. The capillary was then wiped gently with methanol and deionized water and was ready to be inserted in the electrophoresis instrument.

3.5.2 Preparation of the samples

Most of the drugs used were water-soluble. Typically 1 mg of the sample was dissolved in 1 mL of deionized water (0.1 %) and stored in vials in a refrigerator. Figure 16 shows structures of the tricyclic antidepressants.⁵⁰ Figure 17 shows the structures of other compounds studied.⁴⁷

Benzodiazepine samples were obtained from tablets. A 10 mg tablet was crushed, dissolved in methanol and filtered through a nylon membrane on a Millipore filtration unit. The filtrate was then evaporated to give the resulting benzodiazepine. A solution was then made by dissolving the dried benzodiazepine in methanol : water (1 : 1) and gently evaporating the methanol. These samples were also stored in vials in a refrigerator. Figure 18 shows the structures of the benzodiazepines used in this study.¹²

Figure 16: Structures of the tricyclic antidepressants studied⁵¹

Figure 17: Structures of other drugs used in this study⁴⁷

DIAZEPAM

TEMAZEPAM

OXAZEPAM

CHLORODIAZEPOXIDE

Figure 18: Structures of the benzodiazepines studied¹²

3.5.3 Effects of the buffers and voltage on enantiomeric separation

Each of the samples were investigated for chiral separation with the various buffers prepared (Section 3.4) at the following voltages: 30 kV, 20 kV, 10 kV and 5 kV. The experimental set up is described in Chapter I, section 4.1. Once in the instrument, the capillary was equilibrated with the running buffer for 10 minutes. The injections were done hydrodynamically using a vacuum at 5" of Hg. After every run, the capillary was flushed with the running buffer for 2 minutes.

3.5.4 Measurements for electroosmotic flow

The capillary was first flushed with the running buffer for 2 minutes. About 1 mg of DMSO, a neutral analyte, was dissolved in 1 mL of the running buffer (0.1 %). This marker was injected into the capillary under a vacuum of 5" of Hg. Voltage was applied to the capillary for a specific period of time. This caused the marker and the electrolyte to move electroosmotically. A second injection of the same marker was made into the capillary. A vacuum of 5" of Hg was then applied and the two bands were detected at 211 nm. The marker, due to the second injection, moved under the influence of the applied vacuum. This cycle was repeated for two more measurements under the same conditions.³⁵ The EOF for all buffer pH and voltage conditions used in the study, was then calculated as discussed in Chapter I, Section 6.2.1.

Chapter III

RESULTS AND DISCUSSION

1. Etching and modification of the capillaries

Various chemical reactions have been used to modify silica surfaces.⁵¹ The capillary consists of the weakly acidic surface hydroxyl groups (silanols) and when treated with the etching agent made up of a saturated solution of 5% ammonium hydrogen difluoride followed by heating at high temperatures, strong protonic sites are produced on the glass by partial replacement of the -OH groups by -F and the subsequent surface exhibits high catalytic activity.⁵² The electronegative fluorine atom causes an electron shift in the neighboring atoms which would result in the weakening of the hydroxyl bond in the silanol group and increase in surface acidity. The acid groups produced are of the Bronsted type as seen in Figure 19.⁵² The surface now consists of silanol and Si-F and has a high surface area.

The fused silica capillaries first dissolve non-uniformly in the etching agent in the above reaction. They then precipitate on the surface and are observed as radial extensions from the surface. The surface area increases up to 1000-fold, thus opening up sites for bonding of stationary phase material.⁵³ On heating at 300 °C for 2 hours and 400 °C for 1 hour, the surface formed is more uniform and well defined. The mechanical stability of these surface structures were investigated earlier by vibration of the capillaries for 30 minutes.⁵³ It was found that the surfaces produced under the above condition do not sustain any damage and can withstand mechanical stress from vibration.

Figure 19 a,b: The electronegative fluorine atom causes an electron shift in the neighboring atoms resulting in the weakening of hydroxyl bonds⁵²

The surface of the capillary is then treated with TES. This removes the silanol groups that were opened up by the etching agent and a monolayer of silica hydride is formed. This process is the silanization reaction.

The chiral selectors are then treated with Speier's catalyst, which promotes an efficient attachment of the chiral selector onto the silica hydride monolayer. This step is referred to as the hydrosilation reaction. Figure 20 shows the process described above.

2. Spectroscopic study of the silica surface

Spectroscopic studies are carried out to confirm the success in etching and modification procedures of the capillary. In previous experiments carried out by Pesek et al. in 1997, scanning electron microscopy (SEM) studies on the bare, etched and hydride capillaries were performed.⁵⁴ The SEMs obtained confirmed the success of the etching process. DRIFT spectroscopy was used in this study to further characterize the surface of the capillaries.

3. Bonding of the chiral selectors

Attachment of 2-hydroxy-3-methacryloyl-oxypropyl- β -cyclodextrin to silica hydride results in a stable Si-C bond. In Figure 21, the DRIFT spectra reveals the appearance of C-H stretching between 2800 cm^{-1} and 3000 cm^{-1} and silica hydride stretching at 2270 cm^{-1} . This confirms bonding of the cyclodextrin to the silica surface and the presence of residual Si-H groups that have not reacted with the chiral selector. The peak has low intensity and this indicates a very small quantity of measurable cyclodextrin. This is because cyclodextrin is bulky and the ratio of the number of the

Figure 20: Diagram of the reactions on the surface of the capillary wall.

Cyclodextrin modified etched capillary ID = 50 μm
Resolution : 8; Scans = 1000

Figure 21: DRIFT spectrum of bonded cyclodextrin on silica hydride surface.

bulky cyclodextrin to the inner wall surface is high. It is only a result of the etching process that this ratio is diminished enough to allow spectroscopic data to be obtained.

On attachment of the (R)-(+)-acryloxy- β,β -dimethyl- γ -butyrolactone, the silica hydride reacts with the lactone and a stable Si-C bond is formed. In Figure 22, the DRIFT spectra reveals the absence of free SiOH stretching at $\sim 3750\text{ cm}^{-1}$. The C-H stretching between 2800 cm^{-1} and 3000 cm^{-1} is very faint. But the absence of silica hydride stretching at 2270 cm^{-1} confirms that the hydride has been replaced due to bonding of the lactone to the silica surface.

The DRIFT spectrum of the R(+)-1- α -naphthylethylamine bonded stationary phase on silica for HPLC obtained in 1997 confirmed bonding of the chiral selector.⁵⁵ DRIFT spectroscopy was not carried out on this stationary phase for the capillary with the α -naphthylethylamine chiral selector. Figures 23a,b,c show the structures of the bonded chiral selectors on the silica surface.

4. Choice of test solutes

4.1 Tricyclic Antidepressants

These drugs have been developed by modification of the central ring of the phenothiazine molecule. Imipramine, desipramine and clomipramine are iminodibenzyl derivatives that have a -C-C- bridge. Doxepin is a dibenzoxepine derivative and nortriptyline is a dibenzocycloheptene derivative which has the -C-C- bridge and the N atom of the phenothiazine ring is replaced by a doubly-bonded carbon.

Figure 22: DRIFT spectrum of bonded lactone on silica hydride surface.

Figure 23: Structures of the bonded chiral selectors on the silica hydride surface;
A: 2-hydroxy-3-methacryloyl-oxypropyl- β -cyclodextrin
B: (R)-(+)-acryloxy- β , β -dimethyl- γ -butyrolactone
C: (R)-(+)-1- α -naphthylethylamine bonded to 4-allyloxybenzoic acid

4.2 Benzodiazepines

They are an important class of pharmaceutically active compounds encountered in clinical and forensic applications. Benzodiazepines are variations of the 5-aryl-1, 4-benzodiazepine nucleus. Oxazepam, chlorodiazepoxide and temazepam contain -OH at the 3-position of the benzodiazepine ring that constitutes the chiral center. Diazepam has a non-planar ring system¹² which forms a barrier to inversion so that the enantiomers do not interconvert.

4.3 Other drugs

Terbutaline is a sympathomimetic drug that simulates the sympathetic nervous action in physiological conditions, clenbuterol is a beta-2 agonist that combines with a beta receptor on a cell and initiates a reaction. Homatropine and tropicamide are mydriatics that dilate the pupil of the eyes. There is one chiral center in each structure. They have the attributes for inclusion- complexation and three-point interactions.

4.4 Derivatized amino acids

The amino acids used were 3,5-dinitrobenzoyl DL-leucine, DL-valine, DL-alanine and DL-phenylalanine. This created the three point interaction necessary for separation.⁴⁸

5. Interpretation of Chromatographic Data

5.1 Separation of tricyclic antidepressants

The cyclodextrin capillary and the R-naphthylethylamine capillary could not resolve the tricyclic antidepressant compounds into their optical isomers. Only one peak

was obtained. However, with the lactone capillary, some enantiomeric discrimination (separation) was obtained. Figures 24 to 27 show separations of tricyclic antidepressants (TCAs) obtained with the lactone capillary. With variations in buffer pH and voltage, separations could be improved. These results are discussed in Sections 5.5 and 5.6. The enantiomers of clomipramine could be resolved at -10 kV and pH = 3.0. The enantiomers of nortryptiline were resolved at 10kV and pH = 4.41. The enantiomers of doxepin were resolved at -20kV and pH = 3.0 and pH = 3.7.

As seen in Figure 28, the interactions proposed for complex formation are either:

- 1) A weak hydrophobic interactions between the dimethyl group on the lactone moiety and the aromatic rings of clomipramine;
- 2) weak charge transfer interaction between the ester group in the lactone moiety and the aromatic ring attached to the chlorine atom of clomipramine and
- 3) steric hindrance due to the orientation of the bulky R group on clomipramine on one of the enantiomers that would prevent interaction with the lactone moiety.

The interactions proposed are transient with weak diastereomeric complexes being formed. Future studies could involve computer simulations of the molecules in order to verify that the interactions are feasible. The isomers were not baseline separated and the resolution is considered marginal. Therefore the proposed interactions are not sufficient to provide for the separation of the two enantiomers. In Figure 25, nortryptiline was barely resolved into two peaks. The same is observed for doxepin. Therefore, for these compounds, the lactone bonded phase may not be the best chiral selector.

Figure 24: Separation of D- and L- form of clomipramine on etched and lactone modified capillary. L = 32 cm, I = 24 cm, V = -10 kV, pH = 3.0 (1:10)

Figure 25: Separation of D- and L- form of nortriptyline on etched and lactone modified capillary. L = 24 cm, I = 16 cm, V= 10 kV, pH =4.41 (1:10)

Figure 26: Separation of D- and L- form of doxepin on etched and lactone modified capillary. L = 24 cm, I = 16 cm, V= -20 kV, pH =3.7 (1:10)

Figure 27: Separation of D- and L- form of doxepin on etched and lactone modified capillary. L = 24cm, I = 16 cm, V= -20 kV, pH =3.0 (1:10)

Figure 28: Interaction between the lactone moiety and clomipramine.

5.2 Separation of benzodiazepines

The lactone capillary did not resolve the benzodiazepine compounds into their enantiomers. The benzodiazepines are structurally bigger than lactone so there may be insufficient interactions to give enantiomeric separation.

The cyclodextrin capillary was able to resolve all four compounds to different extents. Figures 29 to 37 show separations of benzodiazepines obtained with the cyclodextrin capillary. With variations in buffer pH and voltage, separations could be improved. These factors are discussed in Sections 5.5 and 5.6. The enantiomers of diazepam were resolved at 30 kV and pH = 3.0. The enantiomers of temazepam were resolved at 30 kV and pH = 2.14. In Figure 32 and Figure 34, there is a large difference in retention time. This may be due to the strong complexes formed between the cyclodextrin and temazepam at 30kV and also due to the effects at pH 2.14 on electroosmotic flow. The enantiomers of chlorodiazepoxide showed better resolution at pH = 3.0, at 30 kV and 20 kV. The enantiomers of oxazepam showed very little resolution at 30 kV and pH = 3.0.

As shown in Figures 38 A, B, C, D, the separation mechanism proposed for the cyclodextrin selector is based on inclusion-complexation. The weakly bound enantiomer eluted out first while the most strongly bound enantiomer eluted out last. Also, EOF may have some effect on the mobilities of these compounds. The effects of electric field and pH on EOF are described in Section 6.1.

The benzodiazepines were extracted from tablets and so impurities could be present. With this technique, it was possible to separate an impurity as well as the

Figure 29: Separation of D- and L- form of diazepam on etched and cyclodextrin modified capillary. L = 45 cm, I = 25 cm, V= 30 kV, pH =3.0 (1:10)

Figure 30: Separation of D- and L- form of diazepam on etched and cyclodextrin modified capillary. L = 45 cm, I = 25 cm, V= 30 kV, pH =2.14 (1:10)

Figure 31: Separation of D- and L- form of diazepam on etched and cyclodextrin modified capillary. $L = 45$ cm, $I = 25$ cm, $V = 20$ kV, $pH = 2.14$ (1:10)

Figure 32: Separation of D- and L- form of temazepam on etched and cyclodextrin modified capillary. L = 45 cm, I = 25 cm, V= 30 kV, pH =2.14 (1:10)

Figure 33: Separation of D- and L- form of temazepam on etched and cyclodextrin modified capillary. L = 45 cm, I = 25 cm, V= 0 kV, Vacuum = 20", pH =2.14 (1:10)

Figure 34: Separation of D- and L- form of temazepam on etched and cyclodextrin modified capillary. L = 45 cm, I = 25 cm, V= 30 kV, pH =3.0 (1:10)

Figure 35: Separation of D- and L- form of chlorodiazepoxide on etched and cyclodextrin modified capillary. L = 45 cm, I = 25 cm, V = 30kV, pH = 3.0 (1:10), X = impurity

Figure 36: Separation of D- and L- form of chlorodiazepoxide on etched and cyclodextrin modified capillary. L = 45 cm, I = 25 cm, V = 20kV, pH = 3.0 (1:10), X = impurity

Figure 37: Separation of D- and L- form of oxazepam on etched and cyclodextrin modified capillary. L = 45 cm, I = 25 cm, V= 30 kV, pH =3.0 (1:10)

Figure 38: Inclusion-complexation between the cyclodextrin and the benzodiazepines; A- Diazepam, B- Temazepam, C- Oxazepam, D- Chlorodiazepoxide

C

OXAZEPAM

D

CHLORODIAZEPOXIDE

Figure 38: (contd.)

enantiomers of chlorodiazepoxide as shown in Figures 35 and 36.

The R-naphthylethylamine capillary resolved diazepam as shown in Figure 39. This was the only benzodiazepine separated by this capillary. Diazepam does not have an -OH group at the 3-position. This may cause the molecule to orient itself so as to have (1) π - π interactions between the aryl group of diazepam and the naphthyl group of the chiral selector; (2) hydrogen bonding interaction between the carbonyl oxygen on diazepam and the amino hydrogen on the chiral selector. Figure 40 shows the postulated interactions between diazepam and R-naphthylethylamine.

5.3 Separation of other drugs

Homatropine, clenbuterol and tropicamide could not be resolved with any of the capillaries since the isomer did not have sufficiently different interactions with the chiral selectors for enantiomeric separation to occur. Terbutaline could be partially resolved with the lactone capillary as seen in Figure 41. The interactions proposed are between the basic sites (carbonyl groups) on the lactone moiety and acidic sites on terbutaline as shown in Figure 42. There could also be interactions between the carbonyl groups on the lactone and the acidic hydrogen on the amine group and the hydroxy group linked to the aliphatic chain in terbutaline. These interactions form weak diastereomeric complexes and are not strong enough to give any separation of the enantiomers.

5.4 Separation of derivatized amino acids

The R-naphthylethylamine capillary was able to resolve the derivatized alanine and phenylalanine methyl esters. Figures 43 to 45 show the separations obtained with

Figure 39: Separation of D- and L- form of diazepam on etched and naphthyl ethylamine modified capillary. L = 50 cm, I = 37 cm, V= 30 kV, pH =2.14 (1:10)

Figure 40: Interactions between the R-naphthylethyamine and diazepam.

Figure 41: Separation of D- and L- form of terbutaline on etched and lactone modified capillary. L = 32 cm, I = 24 cm, V= -10 kV, pH =3.0 (1:10)

BUTYROLACTONE

BUTYROLACTONE

Figure 42: Interaction between the lactone moiety and terbutaline.

this capillary. Separation was presumed to be due to the three-point interaction between the amino acid and the chiral selector. The postulated interactions shown in Figure 46 are as follows: (1) π - π interactions between the 3,5-DNB group of the analyte and the naphthyl group of the chiral selector; (2) acidic-basic group interactions between carbonyl oxygens and amide hydrogens and (3) electrostatic hydrogen bonding interactions between the carbonyl oxygen and the hydroxyl group. It is presumed that there was a reversal in the elution order for 3,5-DNB phenylalanine methyl ester at pH = 4.41 when compared to pH = 3.7. A nitrogen containing species from the etching agent may be attached to the silica surface. At this point it is not known in what form this nitrogen species exists and this entity could be affected by the buffer pH. Such a structure on the surface could reverse the order of elution. Or inversion of the migration order of the two analyzed enantiomers may be due to reversal of the electroosmotic flow as described in Chapter I, Sections 8.2.5. Another possibility is that there could be impurities that are causing interference as these derivatized amino acid methyl esters were synthesized in our laboratory.

5.5 Effect of pH on separation

Figures 26 and 27 show the difference between pH 3.0 and pH 3.7 buffers on the enantiomeric separation of doxepin at an applied voltage of -20 kV on the lactone capillary. At pH 3.7 and pH 3.0, the two enantiomers of doxepin are partially resolved. Changes in the buffer pH cause basic and acidic groups in the enantiomers to be ionized to different extents. Each enantiomer thus interacts differently with the chiral selector.

Figure 43: Separation of D- and L- form of 3,5-DNB alanine methyl ester on etched and naphthyl ethylamine modified capillary. L = 50 cm, I = 37 cm, V = 30 kV, pH = 2.14 (1:10)

Figure 44: Separation of D- and L- form of 3,5-DNB phenylalanine methyl ester on etched and naphthyl ethylamine modified capillary. L = 50 cm, I = 37 cm, V= 30 kV, pH =3.7 (1:10)

Figure 45: Separation of D- and L- form of 3,5-DNB phenylalanine methyl ester on etched and naphthyl ethylamine modified capillary. L = 50 cm, I = 37 cm, V= 30 kV, pH =4.41 (1:10)

* Y = NO₂ and A: R = Alanine, B: R = Phenylalanine

Figure 46: Interactions between the R-naphthylethylamine and A: 3,5-DNB alanine methyl ester; B: 3,5-DNB phenylalanine methyl ester

This results in optimum peak symmetry and discrimination between the enantiomers. The stronger the interactions, the greater the time the analyte is retained in the capillary. At pH 3.7, these conditions allowed stronger interaction between one form of the enantiomer to the chiral selector and therefore there was better resolution than the separation carried out at pH 3.0. Figures 29 and 30 show the difference between pH 2.14 and 3.0 buffers on the enantiomeric separation of diazepam at an applied voltage of 30 kV. At pH 3.0, the enantiomers had defined symmetric peaks although they were not baseline resolved unlike the separation at pH 2.14 which did not give symmetric peaks. This is also due to the changes in the buffer pH that cause basic and acidic groups in the enantiomers to be ionized to different extents. Each enantiomer thus interacts differently with the chiral selector resulting in optimum peak symmetry and discrimination between the enantiomers. The stronger the interactions, the greater the time the analyte is retained in the capillary. At pH 3.0, these conditions allowed stronger interaction between one form of the enantiomer with the chiral selector and therefore there was better resolution than the separation carried out at pH 2.14.

5.6 Effect of Voltage on separation

Figures 30 and 31 show the effect of voltage (30 kV and 20 kV) at pH 2.14 on the enantiomeric separation of diazepam on the cyclodextrin capillary. A decrease in voltage decreases electroosmotic flow and increases migration time. This causes the enantiomers to be in the capillary longer thus allowing for more interaction time between the strongly

bound enantiomer and the chiral selector. Therefore at 30 kV, the enantiomers elute within 4 minutes and at 20 kV, the enantiomers elute within 8 minutes.

6. Electroosmotic flow measurements

Dimethylsulfoxide (DMSO) was used as a neutral marker and the effect of pH = 2.14, 3.0, 3.7, 4.41 and 6.0 at different voltages on EOF was measured. From the data obtained it was certain that other species besides silanols and siloxanes are present on the surface of the capillary. The observation and interpretation of data are discussed with respect to each capillary.

6.1 Effect of pH and electric field on electroosmotic flow

An increase in the pH of the buffer causes more dissociation of the silanols to negatively charged silanoate ions on the capillary surface. A zeta potential develops on the surface of the capillary wall and is proportional to the surface charge. This results in an increase in the electroosmotic velocity. An increase in pH would thus lead to an increase in EOF. Figures 47 to 50 show the effect of EOF as a function of pH and electric field.

6.2 Cyclodextrin capillary

In the case of the cyclodextrin capillary, EOF was positive throughout. With an increase in pH, an increase in EOF was observed as shown in Figure 47. Figure 48 shows the plot of EOF as a function of electric field. It was observed that electroosmotic flow was cathodic at all pH ranges studied. This phenomenon may be due to etching of the

silica surface with ammonium hydrogen difluoride. A nitrogen species from the etching agent may be attached on the capillary wall and would form a continuum in the silica matrix. It is not possible to specify the surface nitrogen species. It is likely that this entity is positively charged at low pH because hydrogen ion is attached to the lone pair of electrons. Cyclodextrin is a bulky molecule with a large number of hydroxyl groups present and therefore could shield or block the surface protonated nitrogen species from the buffer ions so that, in effect, the positive charges get masked. Similar shielding effects are observed when polymers are attached to or adsorbed on the silica surface. Another explanation might be some kind of chemical interaction such as hydrogen bonding between the cyclodextrin and the surface nitrogen species that would also neutralize the positive charges. In either case, a cathodic EOF would be observed.

6.3 Lactone Capillary

In the case of the lactone capillary, an anodic EOF is observed at pH less than 4.5. With an increase in pH, an increase in EOF was observed as shown in Figure 49. Figure 50 shows the plot of EOF as a function of electric field. The anodic EOF is probably due to the etching with ammonium hydrogen difluoride of the silica surface containing the silanol groups. A nitrogen containing species from the etching agent may be attached to the silica surface forming a continuum in the silica matrix. This entity would be affected by the pH of the buffer used. The lactone molecule is small compared to cyclodextrin. Since the nitrogen species has a lone pair of electrons, it would accept a hydrogen ion from the buffer. Therefore, at a pH below 4.41, the nitrogen is probably

protonated and the EOF is observed to be anodic. Above $\text{pH} = 4.41$, the EOF switches to the cathodic direction because the nitrogen as well as the silanol becomes deprotonated giving the surface a net negative charge.

Figure 47: Effect of EOF as a function of pH

Cyclodextrin modified etched capillary ID = 50 μ m

Figure 48: Plot of EOF as a function of electric field.

Lactone modified etched capillary ID = 50 μm

Figure 49: Effect of EOF as a function of pH

Figure 50: Plot of EOF as a function of electric field.

Chapter IV

Conclusion

The modification of capillaries by etching, silanization and hydrosilation reactions were successful. Etching of the capillary not only increased surface area, but in some cases, after chemical modification, generated selective interaction between the enantiomers and the chiral selector. DRIFT was the only spectroscopic tool used for confirmation of attachment of the bonded organic moiety on the capillary wall. The quantities were so small that 1000 scans were necessary to observe any significant changes. C-H stretching bands were observed indicating that some of the chiral selector was present on the surface. Surface coverage could not be estimated. It could be assumed from the fact that cyclodextrin being bulky, surface coverage would be less in terms of the number of cyclodextrin molecules per m^2 when compared to the lactone bonded phase. EOF was cathodic throughout the buffer pH ranges studied for the cyclodextrin capillary. In the case of the lactone capillary, EOF was anodic below $\text{pH} = 4.41$ and cathodic above it. This was due to the nitrogen entity in the etching agent, ammonium hydrogen difluoride which probably affected the EOF thus affecting enantiomeric separation.

The CD capillary could resolve benzodiazepines due to inclusion-complexation interactions and the hydroxyl group at position 3 could have enhanced resolution. With the lactone capillary, separation between lactone and clomipramine enantiomers were not sufficiently resolved. Nor was terbutaline separated by the bonded lactone selector. Cyclodextrin was found to be a better chiral selector than the lactone based on the

resolution of the two enantiomers and peak symmetry. Naphthylethylamine could separate diazepam, the only benzodiazepine, probably because the absence of the hydroxyl group at position 3 on diazepam did not create any steric hindrance between the two molecules. It could also separate 3,5-DNB phenylalanine methyl ester and 3,5-DNB alanine methyl ester based on the three point interactions as discussed earlier.

Since the mechanisms of these interactions are probable, future studies could involve computer modeling of these molecules. These studies would give more insight to the basis of separation. Different chemical modifications could be attached to the enantiomeric compounds and their effect on separation of the enantiomers could be studied. These studies would help elucidate the mechanism of interactions between the solute and the chiral selector. A few examples of modifications would be varying chain length or adding functional groups.

Chapter V

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