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**SYNTHESIS AND CHARACTERIZATION OF LIQUID CRYSTAL
CAPILLARIES FOR USE IN 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

Anjali Katrekar

December 2000

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**APPROVED FOR THE DEPARTMENT OF
CHEMISTRY**

Dr. Joseph Pesek

Dr. Pamela Stacks

Dr. Bradley Stone

APPROVED FOR THE UNIVERSITY

ABSTRACT

SYNTHESIS AND EVALUATION OF LIQUID CRYSTAL MODIFIED CAPILLARIES FOR USE IN CEC

By

Anjali Katrekar

Advisors: Dr. Joseph Pesek, Dr. Maria Matyska-Pesek

The synthesis and evaluation of novel liquid crystalline modified capillaries for use in open tubular capillary electrochromatography (CEC) is described and discussed. CEC in the open tubular mode overcomes many drawbacks associated with the use of packed capillaries. Liquid crystals are a distinct state of matter between the solid crystalline and liquid state. Fused silica capillaries with an i.d. of 50 μm were etched and then chemically modified by the silanization and hydrosilation process to attach two liquid crystal compounds, cholesteryl 10-undecanoate and 4-cyano-4'-pentoxybiphenyl. The inner surface of the capillaries was analyzed by diffuse reflectance Fourier transform (DRIFT) spectroscopy. Electroosmotic flow and velocity measurements done on the capillaries are presented. The capillaries were also evaluated for their performance capabilities by carrying out separations of mixtures of basic drugs, proteins, and small molecules such as nucleotides.

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Anjali Katrekar

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INTRODUCTION

I. Capillary Electrochromatography:

CEC or Capillary Electrochromatography is one of the newer separation techniques that shows great promise in the separation of various analytes, ranging from small ions, to basic compounds, to proteins and peptides. It belongs to the family of techniques that collectively constitute capillary electroseparation. Capillary electroseparation techniques also include capillary electrophoresis (CE), capillary isoelectric focusing (CIEF), and micellar electrokinetic capillary chromatography (MEKC) in addition to CEC [1]. CEC is an electroseparation technique in which an electric field is applied and drives the separation as opposed to a pressure driven separation as in high performance liquid chromatography (HPLC). CEC is considered to be a hybrid between two other separation techniques, HPLC and CE. The basic principles of HPLC and CE apply to CEC as well, and are useful in optimization. The method combines the high selectivity of HPLC and the high efficiency of CE. The separation in CEC is based on the partitioning of the solute between the stationary and the mobile phase for neutral compounds. The stationary phase can be fixed on silica particles packed inside a capillary column, or fixed to the walls of a fused silica capillary. For charged compounds, the electrophoretic mobility of the solutes also contributes to their separation. Since CEC is also a microseparation technique it has various advantages such as:

- reduced solvent and sample consumption

- reduced waste disposal
- reduced operating costs
- increase of mass sensitivity due to high resolution which can be obtained
- trace-level detection of analytes in samples with limited volume
- compatibility to all common detectors and especially mass spectrometry

When an electric field is applied across the separating capillary, a force called the electroosmotic flow (EOF) is generated which drives the solvent and the solutes through the capillary with a flat flow profile that is shown in Figure 1. This reduces band broadening normally associated with the pressure driven parabolic flow. Because flows are driven electroosmotically and not by pressure, especially small particle diameters can be used resulting in very high separation efficiencies. In CEC the efficiency is generally intermediate between HPLC and CE [2].

Figure 1. The flat profile of EOF

A. Electroosmotic flow:

The electroosmotic flow is generated at the solid-liquid interface in the fused silica capillary. The inner wall of the capillary has surface silanol groups that are negatively charged in aqueous solutions due to deprotonation. These ionized silanol groups attract positively charged groups from the buffer and form an electrical double layer. The layer closest to the surface is called the “Inner Helmholtz or the Stern Layer” and the second more diffuse layer is called the “Outer Helmholtz Plane”. When an electrical field is applied, the cations in the outer layer migrate towards the cathode. Since these cations are hydrated, they drag along with them, some of the bulk solution. This flow of the solution in a capillary under the influence of an electric field is called the electroosmotic flow and is responsible for the movement of all the solutes and the solvent through the capillary [2,3]. An illustration of the double layer formed is shown in Figure 2. The zeta potential is formed at the capillary-solution interface and is determined by the nature and concentration of the ions in the diffuse layer at the capillary-solution interface. The electroosmotic flow is an important parameter in the separation of compounds and is affected by various factors including buffer pH and organic additives.

B. CEC Apparatus:

CEC can be carried out using any instrument designed for capillary electrophoresis, commercial or homemade. The essential components consist of a fused silica capillary with an internal diameter of about 50 to 200 μm and length of about 200

Figure 2. The electrical double layer [2]

Figure 3. Schematic of a CEC instrument

to 1000 mm, a high voltage power supply (up to 50 kV), and two reservoirs for the buffers into which the electrodes and the ends of the capillary can be dipped.

An on-column detector, which is usually based on UV/VIS absorbance, measures the concentration of the solutes. A means of thermostating the capillary and pressurizing the inlet and/or outlet can also be provided. Pressurizing reduces the amount of bubble formation in the capillary. There is also a computer attached for data acquisition and analysis [1,2]. A schematic of a CEC instrument is given in Figure 3. To carry out a separation in CEC, first the buffer reservoirs are filled with the appropriate buffer at the appropriate pH and the ends of the capillary are dipped into each of these containers that also contain the electrodes. Several volumes of the buffer are passed through the capillary in order to condition it, thus forming the silanol groups. The sample is then injected into the capillary (injection is generally at the anode) and a voltage of up to 30 kV is applied. This causes the solutes to move through the capillary towards the appropriate electrode and they are separated based on their electrophoretic mobilities and partitioning between the stationary and mobile phase. The solutes pass the detector where data is collected and analyzed by computer software.

C. Separation parameters:

There are various parameters that have to be considered in order to achieve optimum separation. The main parameters include the following:

a) Applied voltage:

Generally a voltage of 10 kV to 30 kV is used for separations. Increasing the voltage leads to faster sample migration and electroosmotic flow and also causes the

peaks to be sharper. However, if the applied voltage is too high, then the Joule heat produced is not efficiently dissipated. This may lead to analyte diffusion and thus band broadening. Decreasing the voltage on the other hand, will cause the solutes to migrate slower and also give higher resolution due to more on-column time.

b) Capillary temperature:

Most separations are usually carried out at room temperature. It is important to have the capillary thermostatted in order to avoid Joule heating. Depending on the sample compounds, the capillary temperature can be increased or decreased to optimize the separations.

c) Capillary i.d. and length:

Increasing the capillary i.d. leads to greater detection sensitivity because of the increased path length. However larger capillaries do not dissipate Joule heat as efficiently and can cause a temperature gradient across the capillary leading to band broadening. Narrower capillaries have a larger surface-to-volume ratio and can dissipate Joule heat more efficiently. Shorter capillaries lead to shorter analysis times, but longer capillaries give better resolution due to more on-column time of solutes [4].

d) Buffers:

It is important to choose the right buffer in order to have optimum separations. The buffer should be compatible with the type of detection used. For UV detection, the buffer components should be low UV absorbing at the detection wavelength used. The buffer pH is very important because it affects the electroosmotic flow. Low pH causes the electroosmotic flow to decrease due to protonation of the silanol groups, and a high

pH leads to an increased electroosmotic flow. At low pH because the surface silanols are protonated, there is less attraction between positively charged solutes and the capillary wall. The reagents used for preparing buffers should be of the highest purity. Buffers should be thoroughly degassed before use and stored at 4°C.

D. Columns in CEC

There are two types of columns used in CEC, packed and open tubular columns. Bare capillaries are used for many applications in CE, but the problem with using them is that they have residual silanol groups. These cause a high electroosmotic flow in the capillary and hence cationic solutes moving to the cathode are often not well resolved. Also basic solutes with a positive charge like amines and proteins are attracted to the SiO^- groups and get adsorbed on the walls of the capillary. In order to overcome these problems, capillaries are coated or covalently modified. This diminishes the electroosmotic flow and also reduces analyte adsorption to the walls of the capillary. When a modifier is adsorbed on the walls of the capillary, it is said to be a coated capillary, while if the modifier is covalently attached to the walls of the capillary, it is said to be a covalently modified capillary. Surface coatings and bondings both should be stable over a wide pH range and for many injections. Different types of polymers including polyethers, cross-linked dextrans and polyvinyl alcohol etc. are commonly used as coatings [2,5].

Packed capillaries consist of columns containing silica particles that act as the stationary phase. Since in CEC the flow is electrically and not pressure driven, there is no backpressure during column operation and hence particles smaller than those typical

in HPLC can be used. Particle sizes as low as 1.5 μm have been used with high efficiency. As the particle size decreases, there is less diffusion and resistance to mass transfer which leads to high efficiency [6]. Columns can be packed by various methods including slurry packing and electrokinetic packing. For CEC mostly reversed-phase packing materials with particle diameters of 3-5 μm are commonly used and have a wide range of applications. Chiral separations can also be done by CEC. Monolithic molecularly imprinted flow through polymers can also be used as a stationary phase. This type of separation involves polymerization of a specifically selected monomer around an imprint species. Thus selectivity is predetermined. Baseline chiral separation was achieved by using molecularly imprinted B-adrenergic antagonists [7].

Packed columns are very versatile, though there are some difficulties associated with the packing process. Another problem is that of making the frits to hold the packing in place. The frit should be strong enough to hold the packing in place and porous enough to allow flow of the mobile phase through the column. In general the frit is sintered on a narrow-diameter column, but this is more difficult for a larger diameter column. Also, it is not possible to have identical frits for all columns as the sintering is done in situ. There is also a possibility of bubble formation on the packing and frits leading to unstable operation [8].

Open tubular capillaries are made of fused silica in which the stationary phase is immobilized or bonded onto the walls of the capillary. These capillaries overcome the problems of packing and making frits. However the solutes have to travel to the wall of the capillary in order to interact with the stationary phase. This leads to diffusion of the

solutes and hence broadening of peaks. To avoid this effect, it is necessary to have capillaries with a small internal diameter. In order to reduce the distance the solutes have to travel to reach the stationary phase, and increase the surface area available for modification, the inner surface of the capillary is etched with an agent like ammonium hydrogen difluoride [9]. This procedure increases the surface area by a factor of several 100 to 1000. Radial extensions toward the center of the capillary are produced which decrease the distance the solutes have to travel. This etched surface can then be modified with various organic moieties. A mixture of tetracyclines was separated successfully on a C18 etched and modified column [10]. Similarly diol- and octadecyl-modified etched capillaries were used in the separation of some proteins and peptides [11]. These studies show that the retention characteristics of etched and modified capillaries differ considerably from those of bare capillaries. This is due to solute-bonded phase interactions that occur in the chemically bonded capillaries. It has also been noted that the retention characteristics of the capillary depend on the organic moiety that has been bonded to the inner walls of the capillary. Thus a major part of the research done in CEC is focussed on the stationary phase.

II. Liquid Crystals:

Liquid crystals were first discovered by the Austrian botanist Freidrich Reinitzer in 1888 [12]. While he was studying an organic compound, cholesterol benzoate, he noticed that it had two distinct melting points, one at 145.5°C and the other at 178.5°C. At the first melting point the compound melted into a cloudy liquid and at the second it turned into a clear liquid. A German physicist, Otto Lehmann, who was

studying the effect of temperature on the crystallization of various substances, first coined the term “liquid crystals” [12,13]. The liquid crystal phase is a distinct state of matter and is intermediate between the solid crystalline and the liquid phases. It is however more similar to a liquid than to a solid. It does not possess the positional order that a solid possesses, but it does have some orientational order. This means that the molecules in a liquid crystal are free to move about as in a liquid but for a certain amount of time, orient themselves in a particular direction. This fact has a large effect on the properties of the liquid crystal phase; hence liquid crystals are anisotropic, unlike liquids that are isotropic. Figure 4 illustrates the orientational order present in solids, liquids and liquid crystals [12]. The direction of the preferred orientation is called the director of the liquid crystal.

Figure 4. Orientational order in solids, liquids and gases

In general it has been seen that the type of molecules that can occur as liquid crystals are elongated in shape, there is some rigidity in the central region and the ends are flexible. Figure 5 is an example of a liquid crystal with the common elongated anisotropic geometry allowing the molecule to be aligned preferentially along one spatial direction. The elongated sides of adjacent molecules have stronger attractive forces when they are lined up together and thus stabilize the phase. The flexible ends allow the molecules to move about easily between other molecules as there is constant movement in a liquid crystal. There are many organic compounds that form liquid crystal phases either when the solid crystal is heated above its melting point or when solvents have been added.

Figure 5. Cholesteryl benzoate

A. Types of Liquid Crystals:

Depending on the nature of the transition to the liquid crystal phase, liquid crystals are classified as thermotropic or lyotropic. Thermotropic liquid crystals are

formed due to changes in temperature. There are two types of thermotropic liquid crystals. Enantiomeric liquid crystals are formed due to either an increase in the temperature of a solid or decrease in the temperature of a liquid. Monotropic liquid crystals are formed due to an increase in the temperature of a solid or a decrease in the temperature of a liquid but not both.

Lyotropic liquid crystals are formed due to addition of a solvent and when the concentration of one component of a mixture is varied. Such molecules generally have an amphiphilic structure, i.e. there are two different parts, one part hydrophilic that is in contact with the solvent and the other part hydrophobic that is oriented away from the solvent. Examples of lyotropic liquid crystals are soaps, detergents and phospholipids. Thus lyotropic liquid crystals are important biologically and also as surfactants.

There are three basic types of liquid crystal phases: nematic, cholesteric, and smectic. Though most of the organic compounds form only one of these phases, there are some compounds that form more than one phase [13]. Nematic liquid crystals are those in which the molecules possess intermolecular forces and are aligned parallel to the preferred direction. Examples of nematic liquid crystals include compounds such as azoxy compounds, phenyl benzoates, and the cyano biphenyls.

Cholesteric liquid crystals are those in which the intermolecular forces are aligned at a slight angle to one another. The structure is also described as a twisted nematic phase because the molecules form different nematic-like planes and the director in each plane is twisted with respect to the planes above and below. The director rotates continuously giving rise to a helical structure. The distance it takes the director to rotate

one full turn is called the pitch of the liquid crystal. Many of the compounds that exhibit the cholesteric phase are related to cholesterol and possess optical activity that can be either dextro-rotatory or laevo-rotatory. However it should be noted that not all cholesteric liquid crystals are related to cholesterol and that all cholesteryl compounds do not form cholesteric liquid crystals.

Smectic liquid crystals possess the highest amount of order. The molecules are arranged in layers and possess orientational as well as some positional order. There are two slightly different smectic phases. In the smectic A phase the director is perpendicular to the plane, while in the smectic C phase the director is at a certain angle to the planes. In both, the smectic A and the smectic C phases, the molecules move about randomly within each plane. Many other smectic phases have been discovered in which the molecules are somewhat ordered within each plane.

The three types of liquid crystals are illustrated in Figures 6 A, B and C.

B. Uses:

Liquid crystals have various uses in different fields. They are used as solvents for organic compounds. Liquid crystal displays are used for computers, cameras, watches, clocks, telephones, calculators and many more devices. Thermotropic liquid crystals are used in temperature sensing films and thermometers. Lyotropic liquid crystals are very useful in detergents and surfactants for the oil recovery industry. They are also important in medicine. Knowledge about these materials helps us to understand the physical structure and function of various cellular membranes in our body and some diseases associated with their malfunction [12].

Figure 6A. Nematic liquid crystals

Figure 6B. Cholestric liquid crystals

Figure 6C. Smectic liquid crystals

C. Liquid Crystals in Chromatography:

In chromatography, liquid crystals are used as stationary phases. The retention characteristics of these phases are different from the more commonly used C-8 and C-18 phases due to the more ordered nature of the molecules. Liquid crystal stationary phases were first used in gas chromatography (GC). Most of the liquid crystals have high thermal stability and low vapor pressure. These properties make them good stationary phases for GC where high temperatures are often used. Here the liquid crystals are directly coated onto the column; they also can be deposited on the columns from solutions by evaporating the solvents in which they are dissolved [14]. In GC, these phases have been used mainly in the separation of various isomers, including geometric and cis- and trans- isomers.

In HPLC, the liquid crystals cannot be physically coated on the columns because the high pressures used cause the phase to be stripped off and thus shorten the column life. The strong solvents used for the mobile phase also affect the liquid crystal bonded phase. It is important to find a successful bonding method for the preparation of a liquid crystal stationary phase. There are many types of crystals that can be used as bonded phases. Witkiewicz et al. synthesized three liquid crystalline Schiff's bases for the separation of poly aromatic hydrocarbons (PAHs) [15]. Cholesteric phases were also synthesized and tested by Delaurent et al. [16] and Catabay et al. [17]. Jinno et al. evaluated the retention characteristics of PAHs [18] and 1,4-benzodiazepines [19] on a liquid crystal bonded phase. They concluded that the liquid crystal phase could differentiate between planar and non-planar solutes because it formed ordered 'slot like'

structures [18]. Liquid crystal bonded phases have also been used in supercritical fluid chromatography (SFC). Shen et al. prepared a liquid crystal polysiloxane coated stationary phase for the separation of the fat- soluble vitamins E, A, K₁, K₂, D₂ and D₃ [20]. The shape recognition capability of liquid crystal bonded phases was also confirmed in SFC in a study done by Jinno et al. [21].

Considering the unique selectivity of liquid crystal bonded phases in GC, HPLC and SFC, it seems reasonable to extend the use of liquid crystals as bonded phases in CEC. The use of liquid crystal phases in CEC is still relatively new and not many papers have been published in this field. In one study, cholesteryl 10-undecanoate was used as the stationary phase in CEC [22]. This bonded phase had been previously tested in HPLC and was shown to have a different selectivity as compared to the conventional octadecyl silica phase [17].

Liquid crystals show a different selectivity due to the ordered nature of the molecules. Nematic liquid crystals are the least ordered while smectic liquid crystals have the most order. Solid-state NMR studies of liquid crystals show that they have a high degree of association between adjacent bonded moieties [23]. This association leads to an increased shape selectivity that is not even seen in polymeric C-18 phases. The adjacent molecules form ordered 'slots'. Depending on their shape and size, solutes diffuse in and out of these slots and thus get separated. Flat, planar and thin solutes are retained longer than the larger and bulkier solutes [23,24].

D. Liquid Crystal Bonded Phases:

The most commonly used support in chromatography is silica due to its many advantages. It is stable, strong, inexpensive, and is available commercially in different sizes. The bonding of organic moieties to prepare a stationary phase depends on the properties of the support material. In GC, the liquid crystals are physically coated on the silica supports [14], however in HPLC due to the high pressures and solvents used, they have to be chemically bonded. Various methods have been tried out to prepare liquid crystal bonded phases. One of the methods is to use standard organosilane chemistry. First a silanization reagent is synthesized by reacting a liquid crystal compound containing a terminal double bond with an organosilane. The bonded phase is then obtained by reaction of the silanization reagent with the silica support [25]. A liquid crystal compound, [4-(allyloxy) benzoyl]-4-methoxy phenyl was used to synthesize a bonded phase using this reaction. The procedure is shown in Figure 7 [26]. The stationary phase is attached to the silica surface by a siloxane linkage, represented by Si-O-Si-C. However, a direct Si-C linkage is more desirable for a stable bonded phase. This bond is obtained by reacting terminal olefins with silicon hydride present on the silica surface. The silicon hydride intermediate is prepared by chlorination of the silica surface followed by reduction with an inorganic hydride, commonly LiAlH_4 . A bonded phase prepared by Sandoval et al. using this procedure was found to be hydrolytically stable at low pH values [27]. For this reaction to be successful, very dry conditions are needed which increases the amount of time and work required. Another approach to prepare a bonded phase involves the silanization/hyrosilation reaction sequence. In the

silanization step, the silanols on the silica surface are converted to silicon hydrides by reaction with triethoxysilane (TES). A silicon hydride monolayer is produced. The hydrosilation step involves the reaction between silicon hydride and a terminal olefin in the presence of a catalyst. The catalyst used is generally Spiers catalyst, which is hexachloroplatinic acid. This reaction produces a direct Si-C linkage that is very stable [28]. The reaction is simple, easy to carry out and gives good silicon hydride coverage. Thus the number of free silanol groups is decreased. Liquid crystal stationary phases have been synthesized using the silanization/hydrosilation procedure for HPLC [21] as well as CEC [22].

Figure 7. Reaction scheme for the bonding of [4-(allyloxy) benzoyl]-4-methoxyphenyl to silica [26]

III. Characterization Methods:

A. Fourier Transform Infrared Spectroscopy (FTIR):

Infrared spectroscopy is a technique that measures the changes in energy caused by transitions of molecules from one vibrational energy state to another [29]. It has many applications including qualitative and quantitative analysis of complex samples, and structure determination. Infrared spectra can be absorption, emission, or reflection spectra. In Fourier transform spectroscopy, all the resolution elements for a spectrum are measured simultaneously, and this reduces the time required to obtain a spectrum. Fourier transform instruments have other advantages like high throughput and therefore high signal-to-noise ratios, high resolving power, reliability, and convenience. Reflection spectroscopy is generally carried out using Fourier transform instruments and is hence more sensitive than absorbance spectroscopy that uses dispersive instruments. Diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) is a method that is used to obtain infrared spectra of powdered samples. In chromatography, DRIFTS is an important technique to obtain information about bonded organic moieties on a silica surface.

B. Electroosmotic flow measurements:

The EOF, as explained previously, is the flow of the solution in a capillary under the influence of an electric field and is responsible for the movement of all the solutes and the solvent. Measurement of the EOF is an important parameter to characterize the capillary. There are various methods in the literature to measure the EOF. The method of Sandoval and Chen [30] was used to measure the EOF in this study

and is useful for measuring EOF in modified capillaries. The protocol involves injecting a neutral marker into the capillary before and after voltage is applied for a certain period of time. The electrolyte plug that is between the two marker injections is then driven out of the capillary using pressure and detected. The time taken for the markers to be detected is noted. The EOF is then measured using the following equation:

$$\mu_{eo} = Ll / Vt_v(1 - t_1/t_2) \text{ where}$$

μ_{eo} = the electroosmotic velocity of the electrolyte, L = the 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 = migration times of the two marker bands.

IV. Research Goals:

- To etch and chemically modify fused silica capillaries using the silanization / hydrosilation reaction
- To test the following two liquid crystals for use as bonded phases in CEC
  - i) cholesteryl 10-undecenoate (Figure 8)
  - ii) 4-cyano-4'-n-pentoxybiphenyl (Figure 9)
- To carry out FTIR studies to confirm bonding of the liquid crystals to the capillary walls
- To measure the EOF of both the capillaries in order to evaluate their performance
- To carry out separations of various compounds ranging from large proteins, drugs such as benzodiazepines and small molecules such as purines and pyrimidines
- To study the effects of pH and voltage on the separations.

Figure 8. Cholesteryl 10-undecenoate

Figure 9. 4-cyano-4'n-pentoxybiphenyl

EXPERIMENTAL

I. Materials:

The liquid crystals used for preparation of the bonded phase were cholesteryl 10-undecenoate purchased from Sigma (St. Louis, MO, USA) and 4-cyano-4'-n-pentoxybiphenyl purchased from EM Industries (Hawthorne, NY, USA). The solutes used in this study were benzodiazepines (temazepam, oxazepam, clonazepam, diazepam and nitrazepam), lysozyme (chicken and turkey egg white), cytochrome c (horse, tuna, chicken and bovine heart), antidepressants (nortriptyline and chlormipramine), proteins (lysozyme, cytochrome, ribonuclease), and purine and pyrimidine bases purchased from Sigma (St. Louis, MO, USA); caffeine, theophylline, serotonin, tryptamine, and 5-hydroxytryptophan purchased from Aldrich (Milwaukee, WI, USA). L-tryptophan was purchased from Difco Laboratories (Detroit, MI, USA). The buffers were: pH = 2.14, 60mM phosphate (Fisher Scientific, Pittsburgh, PA) and 38 mM Tris (Sigma, St Louis, MO, USA); pH = 3.00, 60mM citric acid and 50 mM beta-alanine (Sigma, St. Louis, MO, USA); pH = 3.70, 60 mM beta-alanine and 60 mM lactic acid (Sigma, St. Louis, MO, USA); pH = 4.41, 60 mM acetic acid (Aldrich, Milwaukee, WI, USA) and 60 mM gamma-amino butyric acid (Sigma, St. Louis, MO, USA); pH = 6.00, 60 mM 2-(N-morpholino)ethanesulphonic acid and 45 mM histidine (Sigma, St. Louis, MO, USA); pH = 8.18, 30 mM (N-2[hydroxyethyl]-piperazine-N'-[3-propanesulphonic acid]) and 30 mM Tris (Sigma, St. Louis, MO, USA). Deionized water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA) and was filtered through a 0.20 μ m Nylon 66 membrane filter (Alltech Assoc., Deerfield, IL, USA). The solvents

tetrahydrofuran (J.T. Baker Chemicals, Co., Phillipsburg, NJ, USA) and toluene (EM Industries, NJ, USA) were obtained in reagent grade. Ammonium hydrogendifluoride, NH_4HF_2 , used to etch the capillaries was purchased from Aldrich and triethoxysilane used to prepare the hydride intermediate was purchased from United Chemical Technologies (Bristol, PA, USA). Hexachloroplatinic acid (Speier's catalyst) and t-butyl peroxide were used as catalysts in the hydrosilation reaction and were purchased from Aldrich. The capillary tubing for the columns (375 μm O.D. x 50 μm i.d.) was purchased from Polymicro Technologies, (Phoenix, AZ, USA).

II. Instrumentation

A. Gas chromatography oven:

A GC oven from Hewlett-Packard (Model 5890) was used for the etching and modification procedures. The inlet and outlet ports were modified to allow the insertion of fused silica capillaries. The reagents used were placed in glass test tubes and then inserted in thick walled glass tubes for safety reasons. One end of the capillaries was placed in the reagent tubes and the other end was immersed in small collection tubes also made of glass. Nitrogen gas was used to force reagents through the capillaries and also for drying of the capillaries. The capillaries were coiled up and placed in the GC oven and heated; only the ends remained outside.

B. Capillary Electrochromatography:

For CEC separations and the EOF measurements, an Applied Biosystems Model 270-HT (Foster City, CA, USA) was used. For some of the separations, a homemade machine consisting of a Series 230 high voltage power supply from Bertan Associates,

Inc. (Hicksville, NY, USA); a Spectra 200 programmable wavelength detector from SpectraPhysics (Mountain View, CA, USA); and a ChromJet integrator (SpectraPhysics) was used.

C. Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFT):

A Mattson Instruments-Infinity Series FTIR (Madison, WI, USA) with a mercury cadmium telluride (MCT) detector was used to obtain infrared spectra of the modified capillaries. The instrument was interfaced with a Venturis Fx-2 computer system and an HP 722C printer. All the spectra were obtained in the diffuse reflectance mode using a Pike Technologies accessory (Madison, WI, USA).

The coating from the capillary was first removed using the same method used to make the detector window as described later. The capillary was then crushed, mixed with 5% spectral grade KBr and ground into a fine powder in a mortar and pestle. This powder was placed in a sample cup and inserted into the sample compartment. Nitrogen at 55-60 psi was used to purge the instrument of any moisture present. Pure KBr was used as the reference. Each spectrum was scanned 1000 times in the range of 4000 cm^{-1} - 450 cm^{-1} . A plot of wavenumber versus transmittance was obtained.

III. Modification of the Capillaries

The capillaries were etched and modified according to the procedure by Pesek and Matyska [9]. The steps are described below:

A. Etching:

Two $50\mu\text{m}$ fused silica capillaries were placed in a GC oven. The capillaries were filled with concentrated HCl using nitrogen at about 40 psi and heated overnight at

80°C. The capillaries were washed with distilled water, acetone, diethyl ether and then dried with nitrogen for 1 hour. Next the capillaries were filled with a 5% (w/v) saturated solution of ammonium hydrogen difluoride in methanol and left to stand for 1 hour. The methanol was removed by flowing nitrogen through the capillary. Then the capillaries were sealed at both ends and heated at 300°C for 3 hours and then in the presence of nitrogen at 400°C for 1 hour. The etched capillaries were washed with methanol and dried with nitrogen. They were examined under a light microscope to check the etching and to determine if there was any blockage.

B. Modification of the Etched Surface:

First the capillaries were preconditioned by treating them with a 0.1 M solution of NaOH. They were rinsed with deionized water, flushed with 0.1 M HCl, and again rinsed with deionized water. The water was removed by drying with nitrogen. The capillaries were then ready for modification. This process was done using the silanization/ hydrosilation scheme as shown in Figure 10.

Figure 10. Silanization / Hydrosilation scheme

First the capillaries were washed with dioxane and then treated with a solution of 1.2 mL TES in 8.4 mL dioxane at 90°C for 90 minutes. This step was done in a glove box. This procedure gives rise to the silicon hydride monolayer. The capillaries were washed with dioxane for 2 hours and then with tetrahydrofuran (THF) for 2 hours at room temperature.

i) Bonding of cholesteryl 10-undecenoate bonded phase:

All the glassware was first washed with deionized water and dried in an oven. Distilled toluene (7.5 ml) was placed in a 50 mL three-necked flask. A condensing system was set up with 25 cm condenser, a thermometer, a glass stopper, and a magnetic stir bar and a 50 mL heating mantle. Cholesterol 10-undecenoate (0.836 g) was added to the solution while stirring. After it had dissolved, 0.6 mL of Spier's catalyst (0.04 g hexachloroplatinic acid in 100 mL 2-propanol) was added and the mixture was heated to 70°C for 1 hour. The liquid was passed through the capillary at 100°C, 70psi for 5 days. After 5 days, toluene was run through the capillary overnight and it was dried by the flow of nitrogen. Then 10 cm was cut off from each end of the capillary.

ii) Bonding of the 4-cyano-4'-n-pentoxybiphenyl bonded phase:

4-cyano-4'-n-pentoxybiphenyl (1.15 g) was added to 7.5 mL of toluene along with 40 µL t-butyl peroxide. The apparatus and the procedure for the preparation of this bonded phase was the same as that described above for the cholesteryl bonded phase.

IV. Electrochromatography:

The modified capillaries were cut to the appropriate length with a capillary cutter. The detection window was then made by the following method. The position of

the window was marked. A glass block with grooves was heated in a fume hood and 1-2 drops of sulphuric acid were dropped on the grooves. The marked area of the capillary was placed in the groove and held there for a few seconds. This process removes the coating. The capillary was then wiped with methanol and deionized water, and inserted in the CE instrument. The capillaries were equilibrated by forcing buffer through them with a syringe for 10 minutes. The buffers were degassed by purging with helium for 30 minutes. The samples were injected hydrodynamically using vacuum. The separations were carried out at various pH values of 2.14, 3.0, 3.7, 4.41, and 6.0. For some samples, pH 8.19 was also used. The effects of different applied voltage (30kV, 25kV, 20kV, 15kV, and 10kV) were also investigated. Electroosmotic mobility values were determined as explained previously in section III B.

V. Sample Preparation:

All the samples tested were water-soluble. Generally 1 mg of the sample was dissolved in 100 mL of deionized water and then diluted as required. A previous graduate student had prepared the lysozyme and the cytochrome samples. The benzodiazepine samples were prepared from tablets. The tablets (10 mg) were crushed and ground to a powder, dissolved in methanol and filtered through a Millipore filter. The methanol was evaporated and the resulting residue was dissolved in a solution of methanol : water (1:1). All of the samples were stored in the refrigerator.

VI. Data Analysis:

After the capillaries were installed in the CE instrument, the data was acquired. For the separations, the retention times of the samples to be tested were measured

individually. Each sample was run through the capillaries three times and an average of the three values was taken. Data was shown in the form of an electrochromatogram, which is a plot of the detector response versus time. Next, a mixture of the samples was made and run through the capillaries. The retention time of each of the components of the mixture was obtained, again by taking an average of three values. Since the compounds present in the mixture were known but their order of migration was unknown, peak assignments were made by comparing the migration times of the unknowns and standards. Thus the order of migration of the compounds present in a mixture was obtained.

The runs were made on an Applied Biosystems Capillary Electrophoresis System, Model 270A. The software used for the data analysis was HPLC ChemStation, version A.00.33 (Hewlett-Packard, Palo Alto, CA, USA). The CE system was connected to the computer by a Hewlett Packard Interface, Model 35900. Printouts of the chromatograms were obtained using an InkJet printer (Hewlett Packard). The data on the homemade machine was obtained by using a ChromJet integrator (SpectraPhysics). Voltage measurements as well as Electroosmotic flow data were obtained using the same software. Quantitative analysis, or determination of concentrations of the compounds using peak height or peak area was not carried out.

RESULTS AND DISCUSSION

I. Capillary Modification:

A. Etching:

The etching process is carried out to increase the surface area of the inner walls of the capillary. When the etching agent, ammonium hydrogen difluoride is passed through the capillary, it dissolves the silica and redeposits it on the inner surface of the capillary. This redeposition of the silica on the capillary walls is not uniform and forms a rough surface with a highly increased surface area. The etching process gives rise to certain structures or formations on the capillary walls. These can be clearly seen in the scanning electron micrographs of the inner surface of the capillary shown in Figure 11 [31]. The surface morphology is dependent on the temperature and the time during which the etching is carried out. When the capillary is heated at 300°C for 3 hours (Figure 11A.), sharp extensions are formed which protrude from the capillary walls. However when the capillaries are further heated at 400°C for 1 hour (Figure 11B.), a more uniform and wavy structure is seen, which is also more stable. The etching process, and thus the surface morphology can be made reproducible by controlling the reaction conditions.

B. Synthesis of the bonded layer:

The silanization / hydrosilation scheme for the synthesis of the cholesteryl and the cyano pentoxy bonded phases is shown in Figures 12 and 13 respectively.

Figure 11. The etched capillary [31]

A. 300° for 3 hours

B. 300° for 3 hours ;
 400° for 1 hour

Probable Mechanism

Figure 13. Reaction scheme for the synthesis of cyano bonded phase using free radical initiation

In the silanization process, when reacted with TES, the silanol groups on the silica surface are converted to a silicon hydride intermediate. The hydride layer is then modified with the required organic moiety using the appropriate catalyst, which is Spier's catalyst for the cholesteryl capillary and tertiary butyl peroxide for the cyano pentoxy capillary. Cholesteryl bonded phases have been successfully prepared using Spier's catalyst. This catalyst contains the transition metal platinum. The cyano group of the 4-cyano-4'-n-pentoxybiphenyl liquid crystal has a very strong affinity for platinum; hence the hydrosilation reaction is not very successful. Therefore free radical initiation using tertiary butyl peroxide was used for the preparation of the cyanopentoxy-bonded phase.

II. DRIFT Spectroscopy Analysis:

DRIFT spectroscopy reveals whether the bonded liquid crystal phase has been successfully synthesized. The capillaries were stripped of their coating, crushed to a powder, mixed with 5% spectral grade KBr and the spectrum was taken. The features in the spectrum are small since a low surface area in comparison to the total mass of material was available. Figure 14 is a DRIFT spectrum of the cholesteryl-modified capillary. The C-H stretching between 2800 and 3000 cm^{-1} proves that the cholesteryl liquid crystal has been attached to silica via the silanization reaction. The presence of Si-H stretching at 2259 wavenumbers confirms that the silicon hydride monolayer was formed. Figure 15 compares the DRIFT spectra of a bare etched capillary with the cholesteryl-modified capillary. It is evident that both, the C-H and the Si-H stretches that show the bonding of an organic moiety to the silica surface are absent in the bare etched capillary.

Figure 14. DRIFT spectrum of the cholesteryl modified capillary

Figure 15. DRIFT spectrum of bare etched and cholesteryl modified capillary

DRIFT spectroscopy of the cyano pentoxy stationary phase was not done due to an insufficient amount of this capillary. However, the bonding of this phase was confirmed by electroosmotic flow measurements as well as chromatographic separations.

III. Electroosmotic flow measurements:

The EOF for the cholesteryl and the cyano pentoxy capillary was measured using the procedure described previously. Dimethylsulfoxide (DMSO) was used as the neutral marker and the EOF was measured at several pH values: 2.14, 3.00, 3.70, 4.41, 6.00, and 8.19. The pH of the buffer has an effect on the electroosmotic flow of the capillary. As the pH increases, the electroosmotic flow also increases because there is more dissociation of SiOH groups on the inner wall of the capillary to SiO⁻. The increase in overall negative charge on the inner walls of the capillary leads to an increase in the zeta potential which in turn causes the electroosmotic flow to increase. Figures 16 and 17 show the effect of pH on the EOF for the cholesteryl and the cyano pentoxy capillaries respectively. It can be seen that as the pH is increased, the EOF also increases. For both the capillaries, the EOF is anodic at a low pH (between pH 3 and 4). It then changes to cathodic above pH 4. These results indicate that there are some other species besides the silanol groups on the inner walls of the capillary which are responsible for the EOF. The etching process leads to the deposition of these species on the capillary walls. Photoelectron spectra of fused silica capillaries etched according to the same procedure as the cholesteryl and cyano pentoxy capillaries were taken [32]. The spectrum revealed fluorine and nitrogen peaks in addition to the Si and O peaks from the silanol groups. The F and N species are due to the etching agent ammonium hydrogen difluoride and

have been incorporated into the silica matrix as a result of the etching procedure. These species are responsible for the anodic EOF. At low pH, the lone pair of electrons on N accepts hydrogen ions from the buffer and becomes protonated. The inner surface of the capillary gets a positive charge leading to an anodic EOF. As the pH is increased the N and the silanols get deprotonated, the capillary surface has a net negative charge and the EOF becomes cathodic.

Figure 16. Effect of pH on the EOF for the cholesteryl modified capillary

Figure 17. Effect of pH on the EOF for the cyano modified capillary

The cholesteryl capillary has a higher electroosmotic flow above pH 4 as compared to the cyano pentoxy capillary. A higher EOF means that more of the deprotonated silanols are exposed and the capillary surface has a higher negative charge. The cholesteryl moiety is larger than the cyano pentoxy moiety. Thus the number of bonded cholesteryl groups is smaller than the number of bonded cyano pentoxy groups. This causes less shielding of the free silanols in the cholesteryl capillary. As a result the EOF in the cholesteryl capillary is greater than in the cyano pentoxy capillary.

IV. Chromatographic Separations:

Various solutes were tested on the cholesteryl and the cyano pentoxy capillaries to evaluate their separation capabilities. Some of the solutes were also tested on a bare capillary to compare the separation capability of the three capillaries. The comparisons were done under identical experimental conditions.

i) Separation of benzodiazepines:

Benzodiazepines are one of the most commonly prescribed anti- anxiety, anti- convulsing, sedative and hypnotic drugs. Benzodiazepines have been successfully separated using the cholesteryl 10-undecenoate liquid crystal phase in microcolumn liquid chromatography [19] as well as in packed capillary CEC [22]. The structures of the 5 benzodiazepines tested in this study are shown in Figure 18. The parent structure of benzodiazepines is a 5-aryl-1, 4-benzodiazepine nucleus. The 5 test solutes are all 2-keto derivatives with a C=O at position 2 and the benzene ring at position 5.

DIAZEPAM

TEMAZEPAM

NITRAZEPAM

OXAZEPAM

CLONAZEPAM

CHLORDIAZEPOXIDE

Figure 18. Structures of benzodiazepines studied

The cholesteryl bonded phase was able to separate all five components of the benzodiazepine mixture. Figure 19 shows the elution order of the benzodiazepines on the cholesteryl bonded phase at a pH of 2.14 and a voltage of 25 kV. Temazepam was the least retained, followed by oxazepam, clonazepam, diazepam and nitrazepam. Nitrazepam, clonazepam and oxazepam have polar functional groups while temazepam and diazepam are nonpolar. Since the cholesteryl stationary phase is hydrophobic, the nonpolar solutes should interact with it to a greater extent than the polar mobile phase and elute after the polar solutes. However separation is not based only on polarity and the retention mechanism also depends on the shape and molecular structures of the solutes. The structure of the liquid crystal also influences the separation, and this in turn is dependent on the solvent composition, temperature, and applied field.

The cyano pentoxy capillary could separate a mixture of only two benzodiazepines chlordiazepoxide and diazepam. Chlordiazepoxide has a secondary amine at position 2 and a methyl group attached to it. Chlordiazepoxide elutes first followed by diazepam as is shown in Figure 20.

ii) Separation of cytochromes:

Figures 21, 22 and 23 show the separation of a cytochrome c mixture on a cholesteryl, cyano pentoxy and a bare capillary respectively. It is clear that while the two liquid crystal capillaries could separate all the four components of the mixture, the bare capillary could not, and all the solutes eluted as a single peak. The order of elution of the four solutes on both the liquid crystal capillaries is the same. This is because the cytochromes are relatively large biomolecules.

Figure 19. Separation of a benzodiazepine mixture on a 50 μm i.d. etched cholesteryl modified capillary. Conditions: $V = 25$ kV, $\text{pH} = 2.14$, detection at 254 nm, manual injection for 30 s at 10 cm. $L = 70$ cm, $l = 45$ cm. Solutes: 1 = temazepam, 2 = oxazepam, 3 = clonazepam, 4 = diazepam, 5 = nitrazepam

Figure 20. Separation of a benzodiazepine mixture on a 50 μ m i.d. etched cyano pentoxy modified capillary. Conditions: $V = 25$ kV, $pH = 2.14$, detection at 254 nm, injection for 3s at 5". $L = 50$ cm, $I = 25$ cm. Solutes: 1 = chlordiazepoxide, 2 = diazepam.

Figure 21. Separation of a cytochrome c mixture on a 50 μm i.d. etched cholesteryl modified capillary. Conditions: $V = 15 \text{ kV}$, $\text{pH} = 3.0$, detection at 211 nm, manual injection for 15 s at 10 cm, $L = 70 \text{ cm}$, $I = 45 \text{ cm}$. Solutes: 1 = chicken, 2 = tuna, 3 = horse, 4 = bovine.

Figure 22. Separation of a cytochrome c mixture on a 50 μm i.d. etched cyano pentoxy modified capillary. Conditions: $V = 25 \text{ kV}$, $\text{pH} = 3.0$, detection at 211 nm, injection for 3 s at 5". $L = 50 \text{ cm}$, $l = 25 \text{ cm}$. Solutes: 1 = chicken, 2 = tuna, 3 = horse, 4 = bovine.

Figure 23. Separation of a cytochrome c mixture on a 50 μm i.d. bare capillary. Conditions: $V = 25 \text{ kV}$, $\text{pH} = 3.0$, detection at 211 nm, injection for 3s at 5". $L = 44.7 \text{ cm}$, $l = 23 \text{ cm}$. Solutes: 1 = chicken + tuna + horse + bovine.

Cytochrome c is a protein that is present in all eukaryotic organisms. It is an essential part of the electron-transport chain whose function is to generate energy. Cytochrome c is an evolutionary conserved protein and there are not many sequence differences between cytochromes from various sources. The liquid crystal moieties are closely spaced on the silica surface and thus cannot differentiate between the large proteins. Smaller solutes that can pass into the slot-like structures present in the liquid crystal bonded phase are more easily differentiated based on their shape and planarity by these phases. It is assumed that even at pH 3 these proteins are not completely denatured. This is seen in the sharp peak shapes. The resolution is good for both the liquid crystal capillaries though the retention time on the cholesteryl capillary is longer due to its longer length.

iii) Separation of lysozymes:

A mixture of chicken and turkey lysozymes was separated on the cholesteryl, cyano pentoxy and bare capillary. This mixture was best separated on the cholesteryl capillary as seen in Figure 24. There is baseline resolution and even some impurities in the chicken lysozyme could be detected. The long length of the cholesteryl capillary leads to more interaction with the stationary phase and thus the solutes are retained longer. The cyano pentoxy capillary could also separate the lysozyme mixture, however, the resolution is not as good as the cholesteryl capillary. This result is shown in Figure 25. The bare capillary could not separate the two components of the mixture and they eluted as a single peak (Figure 26). Lysozyme is an enzyme that destroys bacterial cell walls and is present in the cells and secretions of vertebrates. The order of elution on

Figure 24. Separation of a lysozyme mixture on a 50 μm i.d. etched cholesteryl modified capillary. Conditions: $V = 15$ kV, $\text{pH} = 2.14$, detection at 211 nm, manual injection for 15 s at 10 cm, $L = 70$ cm, $l = 45$ cm. Solutes: 1 = turkey, 2 = chicken.

Figure 25. Separation of a lysozyme mixture on a 50 μm i.d. etched cyano pentoxy modified capillary. Conditions: $V = 25\text{ kV}$, $\text{pH} = 3.0$, detection at 211 nm, injection for 3s at 5". $L = 50\text{ cm}$, $l = 25\text{ cm}$. Solutes: 1 = turkey, 2 = chicken.

Figure 26. Separation of a lysozyme mixture on a 50 μm i.d. bare capillary. Conditions: $V = 15 \text{ kV}$, $\text{pH} = 3.0$, detection at 211 nm, injection for 3s at 5". $L = 44.7 \text{ cm}$, $l = 23 \text{ cm}$. Solutes: 1 = turkey + chicken.

both the liquid crystal capillaries is the same with turkey lysozyme eluting first followed by chicken lysozyme.

iv) Separation of proteins:

A mixture of three proteins, cytochrome c, lysozyme, and ribonuclease was separated on the three capillaries. The cholesteryl and the cyano pentoxy capillaries could resolve the mixture into the three components. The solutes were well separated and minor impurities were also detected. As in the previous two separations of biomolecules, even though the resolution is good there is not much difference with regard to the separation for the two liquid crystal capillaries. The separations on the liquid crystal capillaries are shown in Figures 27 and 28. Figure 29 shows the separation of the protein mixture on a bare capillary. This capillary could separate the mixture to some extent. The cytochrome c is not baseline resolved and elutes out along with lysozyme. Ribonuclease is well separated. As in the previous separations, the retention times on the bare capillary are short compared to those on the liquid crystal capillaries. This is because the bare capillary does not have any bonded layer for increased interaction with the solutes.

v) Separation of antidepressants:

A mixture of two antidepressants, nortriptyline and chlormipramine was separated on the two liquid crystal capillaries. Figure 30 shows the separation on a cholesteryl capillary while Figure 31 shows the separation on a cyano pentoxy capillary. Both components could be separated on the two capillaries though the cholesteryl capillary gave a better separation. The bare capillary could not fully separate the mixture

Time in minutes

Figure 27. Separation of a protein mixture on a 50 μm i.d. etched cholesterol modified capillary. Conditions: $V = 25\text{ kV}$, $\text{pH} = 3.0$, detection at 211 nm, manual injection for 15 s at 10 cm, $L = 70\text{ cm}$, $l = 45\text{ cm}$. Solutes: 1 = cytochrome c, 2 = lysozyme, 3 = ribonuclease.

Figure 28. Separation of a protein mixture on a 50 μm i.d. etched cyano pentoxy modified capillary. Conditions: $V = 25\text{ kV}$, $\text{pH} = 3.0$, detection at 211 nm, injection for 3s at 5". $L_1 = 50\text{ cm}$, $L_2 = 25\text{ cm}$. Solutes: 1 = cytochrome c, 2 = lysozyme, 3 = ribonuclease.

Figure 29. Separation of a protein mixture on a 50 μm i.d. bare capillary. Conditions: $V = 15\text{ kV}$, $\text{pH} = 3.0$, detection at 211 nm, injection for 3s at 5". $L = 44.7\text{ cm}$, $l = 23\text{ cm}$. Solutes: 1 = cytochrome c, 2 = lysozyme, 3 = ribonuclease.

Figure 3Q Separation of an antidepressant mixture on a 50 μ m i.d. etched cholesteryl modified capillary. $V = 25$ kV, pH = 2.14, detection at 254 nm, manual injection for 15 s at 10 cm, $L = 70$ cm, $l = 45$ cm. 1 = nortriptyline, 2 = chlorimipramine.

Figure 31. Separation of an antidepressant mixture on a 50 μ m i.d. etched cyano pentoxy modified capillary. Conditions: $V = 25$ kV, $pH = 3.0$, detection at 254 nm, injection for 5s at 10kV. $L = 50$ cm, $l = 25$ cm. Solutes: 1 = nortriptyline, 2 = chlorimipramine.

Figure 32. Separation of an antidepressant mixture on a 50 μm i.d. bare capillary. Conditions: $V = 25$ kV, $\text{pH} = 2.14$, detection at 254 nm, injection for 4s at 5". $L = 44.7$ cm, $l = 23$ cm. Solutes: 1 = nortriptyline, 2 = chlomipramine.

Figure 33. Separation of purine and pyrimidine residues and their derivatives on a 50 μm i.d. etched cholesteryl modified capillary. Conditions: $V = 25\text{ kV}$, $\text{pH} = 2.14$, detection at 260 nm, injection for 15s at 10 cm. $L = 70\text{ cm}$, $l = 45\text{ cm}$. Solutes: 1 = thymine hydrochloride, 2 = cytosine, 3 = adenine, 4 = guanine hydrochloride, 5 = adenosine.

and the two components are only just visible in a single peak. This result can be seen in Figure 32.

vi) Separation of purines and pyrimidines and their derivatives:

Some small molecules such as purine and pyrimidine residues were also separated on the cholesteryl and cyano pentoxy capillaries. Figure 33 shows the separation of such a mixture on a cholesteryl modified capillary. All five components were well separated. The cyano pentoxy capillary could also separate the same mixture but the order of elution of the components is different as is seen in Figure 34. It should be noted that for the separation of other biomolecules like the proteins, cytochromes and lysozymes described previously the order of elution on both the capillaries was similar. However the smaller purine and pyrimidine residues eluted differently on the two capillaries. This could be due to the effects of the liquid crystal bonded phase that is used. Cholesteryl 10-undecenoate is a cholesteric liquid crystal while 4-cyano-4'-n-pentoxybiphenyl is a nematic liquid crystal. These are two different classes of liquid crystals that differ in structure and therefore could have different selectivities. Also since the size of the purine and pyrimidine residues is small in comparison to the proteins, it could be easier for them to interact with the closely associated liquid crystal moieties. This could cause them to elute differently on the two capillaries.

vii) Separation of xanthines:

Caffeine and theophylline are two xanthine derivatives that were separated by the cholesteryl (Figure 35) and the cyano pentoxy (Figure 36) capillaries. These compounds were well separated on both the capillaries. The order of elution of the

Figure 34. Separation of purine and pyrimidine residues and their derivatives on a 50 μm i.d. etched cyano pentoxy modified capillary. Conditions: $V = 25\text{ kV}$, $\text{pH} = 2.14$, detection at 260 nm, injection for 5 s at 5". $L = 50\text{ cm}$, $l = 25\text{ cm}$. Solutes: 1 = thymine HCl, 2 = guanine HCl, 3 = cytosine, 4 = adenine, 5 = adenosine

Figure 35. Separation of caffeine and theophylline on a 50 μm i.d. etched cholesteryl modified capillary. Conditions: $V = 25$ kV, $\text{pH} = 8.19$, detection at 272 nm, manual injection for 15 s at 10 cm. $L = 70$ cm, $l = 45$ cm. Solutes: 1 = caffeine, 2 = theophylline

Figure 36. Separation of caffeine and theophylline on a 50 μ m i.d. etched cyano pentoxy modified capillary. Conditions: $V = 25$ kV, $pH = 8.19$, detection at 272 nm, injection for 3s at 5". $L = 50$ cm, $l = 25$ cm. Solutes: 1 = caffeine, 2 = theophylline.

Figure 37. Separation of caffeine and theophylline on a 50 μm i.d. bare capillary. Conditions: $V = 30 \text{ kV}$, $\text{pH} = 8.19$, detection at 272 nm, injection for 4s at 5". $L = 44.7 \text{ cm}$, $l = 23 \text{ cm}$. Solutes: 1 = caffeine, 2 = theophylline.

solutes on both the capillaries is the same. It can be observed that the elution time on both the capillaries is almost the same even though the cholesteryl capillary is much longer. Also, the resolution for the compounds on the cyano pentoxy capillary is greater. This indicates that there is more interaction of the solutes with the cyano pentoxy bonded phase leading to longer retention and thus better separation. Figure 37 shows the same separation on a bare capillary. The separation on this capillary is not very good and one of the peaks is very broad.

viii) Separation of serotonin and its metabolites:

Serotonin is an important neurotransmitter that is present in the neurons in the brain. It is responsible for controlling fundamental physiological aspects of the body including motor activity, cardiovascular activity, respiration, and control of body temperature. It is also involved in behaviors like eating, sleeping and aggression. Low levels of serotonin are implicated in many disorders such as depression, schizophrenia, and Parkinson's disease. Serotonin is synthesized in the body by the hydroxylation and decarboxylation of the amino acid tryptophan. The main metabolite of serotonin is 5-hydroxyindoleacetic acid (5-HIAA). The synthesis and metabolism of serotonin is shown in Figure 38. The cyano pentoxy capillary was used for the separations of these compounds. Figure 39 shows the separation of serotonin and tryptamine (serotonin is the hydroxy derivative of tryptamine). Figure 40 shows the separation of serotonin and its metabolites. Two different wavelengths were used to separate the four compounds as this gave better sensitivity. An unidentified peak was also detected which could be an impurity.

V. Effect of Voltage on Separation:

Runs were also carried out at different voltages to see the effect on separation. Voltage values of 30 kV, 25 kV, 20 kV, 15 kV, 10 kV and 5 kV were used. Figure 41 is a plot of voltage versus the migration time for chicken lysozyme. At higher voltages the migration of the compounds is faster. When the compounds spend more time in the capillary there is more interaction between the solutes and the bonded phase. The solutes are better separated at lower voltages, however the peak shape is broader. This is shown in Figure 42, which is a separation of proteins at 25 kV and 10 kV. At 25 kV there is less resolution but the peaks are sharper

Figure 38. Synthesis and metabolism of serotonin

Figure 39. Separation of serotonin and tryptamine on a 50 μm i.d. etched cyano pentoxy modified capillary. Conditions: $V = 25$ kV, $\text{pH} = 2.14$, detection at 223 nm, injection for 3s at 5". $L = 50$ cm, $l = 25$ cm. Solutes: 1 = tryptamine, 2 = serotonin.

Figure 40. Separation of serotonin and its metabolites on a 50 μm i.d. etched cyano pentoxy modified capillary. Conditions: $V = 25 \text{ kV}$, $\text{pH} = 2.14$, detection at 211 nm and 254 nm, injection for 3s at 5". $L = 50 \text{ cm}$, $l = 25 \text{ cm}$.

Cholesteryl modified capillary ID = 50 μ m

Figure 41. Plot of voltage versus migration time

Figure 42. Separation of a protein mixture on a 50 μm i.d. etched cholesterol modified capillary. Conditions: A; $V = 25$ kV, B; $V = 10$ kV, $\text{pH} = 3.0$, detection at 211 nm, injection for 15s at 10 cm. $L = 70$ cm, $I = 45$ cm. Solutes: 1 = cytochrome c, 2 = lysozyme, 3 = ribonuclease.

VI. Effect of pH on Separation:

In order to achieve optimum separation it is necessary to control the buffer pH. Figures 43 to 48 show the effect of pH on the separation. Figure 43 is the separation of a mixture of cytochromes at pH 2.14 and an applied voltage of 25 kV on a cholesteryl modified capillary. At this pH the resolution is very poor and the four components are barely distinguishable. The separation is much better at pH 3.0 (Figure 44) and is further improved by decreasing the voltage to 15 kV as shown in Figure 45. As the pH is increased to 4.41 the resolution is not complete and two of the components elute together (Figure 46). Thus the optimum pH for the separation of cytochromes using the cholesteryl modified capillary was found to be 3.0. The pI of cytochromes is high (9-11), and the pH values tested are much lower. Thus the cytochromes are positively charged at all the pH values and the analysis time at the various pH values tested also does not differ much. Another example of the effects of pH is seen in the separation of chicken and turkey lysozyme. Figure 47 shows the separation of the mixture of lysozymes at pH 2.14 at an applied voltage of 25 kV on the cholesteryl modified capillary. The two components are separated though the resolution is not very good. It can be seen that when the pH is increased to 3.0 (Figure 48) the resolution is greater. The impurities are also detected. The analysis time is only slightly longer but still acceptable.

Figure 4J. Separation of a cytochrome c mixture on a 50 μm i.d. etched cholesteryl modified capillary. Conditions: $V = 25$ kV, $\text{pH} = 2.14$, detection at 211 nm, injection for 15s at 10 cm. $L = 70$ cm, $l = 45$ cm. Solutes: 1 = chicken, 2 = tuna, 3 = horse, 4 = bovine.

Figure 44. Separation of a cytochrome c mixture on a 50 μm i.d. etched cholesteryl modified capillary. Conditions: $V = 25 \text{ kV}$, $\text{pH} = 3.0$, detection at 211 nm, injection for 15s at 10 cm. $L = 70 \text{ cm}$, $l = 45 \text{ cm}$. Solutes: 1 = chicken, 2 = tuna, 3 = horse, 4 = bovine.

Figure 45 Separation of a cytochrome c mixture on a 50 μ m i.d. etched cholesteryl modified capillary. Conditions: $V = 15$ kV, $pH = 3.0$, detection at 211 nm, manual injection for 15 s at 10 cm, $L = 70$ cm, $t = 45$ cm. Solutes: 1 = chicken, 2 = tuna, 3 = horse, 4 = bovine.

Figure 46. Separation of a cytochrome c mixture on a 50 μm i.d. etched cholesteryl modified capillary. Conditions: $V = 25$ kV, $\text{pH} = 4.41$, detection at 211 nm, injection for 15s at 10 cm. $L = 70$ cm, $l = 45$ cm. Solutes: 1 = chicken, 2 = tuna, 3 = horse, 4 = bovine.

Figure 47. Separation of a lysozyme mixture on a 50 μm i.d. etched cholesterol modified capillary. Conditions: $V = 25$ kV, $\text{pH} = 2.14$, detection at 211 nm, injection for 15s at 10 cm. $L = 70$ cm, $l = 45$ cm. Solutes: 1 = turkey, 2 = chicken.

Figure 48. Separation of a lysozyme mixture on a 50 μm i.d. etched cholesteryl modified capillary. Conditions: $V = 25\text{ kV}$, $\text{pH} = 3.0$, detection at 211 nm, injection for 15s at 10 cm. $L = 70\text{ cm}$, $l = 45\text{ cm}$. Solutes: 1 = turkey, 2 = chicken.

CONCLUSION

The goal of this research study was to synthesize and evaluate two liquid crystal capillaries using the silanization/hydrosilation scheme. The etching and modification of the capillaries using TES silanization was successful. The etching procedure increased the surface area greatly and led to more solute-bonded phase interactions. This was confirmed by comparing the separations done on liquid crystal capillaries with those done on a bare unetched and unmodified capillary. The synthesized liquid crystal capillaries were characterized by FTIR studies and EOF measurements. The C-H stretching bands observed in the FTIR spectra confirmed the bonding of the liquid crystal phase to the capillary walls. The surface coverage of the bonded phases could not be not ascertained. Since the cholesteryl moiety is much larger than the cyano pentoxy moiety, it is assumed that there would be fewer bonded cholesteryl groups per unit area as compared to the bonded cyano pentoxy groups. The electroosmotic flow for both the capillaries was anodic between pH 3-4 and became cathodic above pH 4. The anodic EOF could be attributed to the nitrogen species in the etching agent ammonium hydrogen difluoride. The EOF depends on the pH of the buffer, which can be used to optimize separations. Voltage studies done showed that a lower voltage gave better separation but led to increased retention time. Separations were done on both the liquid crystal capillaries. Some of the separations were also carried out on a bare capillary to compare the performance with a liquid crystal capillary. The retention times on a bare capillary were shorter than those on a liquid crystal capillary because of less interaction between the solute and stationary phase. The bare capillary could not resolve all the mixtures

completely. The performance of both the liquid crystal capillaries was excellent. The individual components of mixtures were well separated with a good resolution. The peaks were symmetrical and narrow. There was hardly any peak tailing even for the strongly basic compounds. This meant that these compounds were not adsorbed to the inner walls of the capillary. For large biomolecules the selectivity of both the liquid crystal capillaries was similar. In the case of smaller molecules such as purine and pyrimidine bases and their derivatives, the retention order was different for the cholesteryl and the cyano pentoxy capillaries. Since these liquid crystals are of different types, they have a dissimilar molecular structure that appears to be responsible for the retention order. However this process is not fully understood and more detailed studies need to be done using cholesteric and nematic liquid crystals. Temperature studies can also be carried out as changes in temperature causes changes in the molecular orientation of the liquid crystals. Thus one can control selectivity. Another extension of this research work would be to carry out separations of water insoluble compounds such as tocopherols, carotenes and fat-soluble vitamins.

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