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Capillary LC and pressurized CEC on a hydride based stationary phase

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CAPILLARY LC AND PRESSURIZED CEC ON A HYDRIDE BASED
STATIONARY PHASE

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

Dipti Sukul

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ABSTRACT

CAPILLARY LC AND PRESSURIZED CEC ON A HYDRIDE BASED STATIONARY PHASE

by Dipti Sukul

The trend of miniaturization is common in all scientific endeavors and certainly apparent in analytical chemistry. Over the past few years HPLC column diameters have decreased from traditional HPLC columns with column internal diameter of 4.5 mm to less than 50 μm . The current study employs capillary columns with a hydride based stationary phase for the analysis of steroid hormones, pharmaceutical drugs, nucleobases as well as nucleosides. The analysis of all these compounds was carried out on a TrisepTM – 2100 capillary system from Unimicro Technologies. This instrument provides the facility of performing capillary LC as well as pressurized CEC. Pressurized CEC is a hybrid form of capillary high performance liquid chromatography and capillary electrochromatography. In pressurized CEC the mobile phase is driven by both pressurized flow and electroosmotic flow. The analysis of the test solutes was carried out both by capillary LC and pressurized CEC using different buffers and varying voltages in order to do a comparative study.

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CHAPTER I

INTRODUCTION AND BACKGROUND

1. Introduction

1.1 Overview of Liquid Chromatography

Chromatography is an essential analytical separation technique used in various fields of science for purification, identification, and quantification of specific analyte from a mixture. The three main essential components of a chromatography system are stationary phase, mobile phase, and analyte of interest. The stationary phase or the adsorbent is the solid medium in which the analytes of interest adhere. The mobile phase is the liquid or gas that flows over the solid stationary phase. The analyte of interest is usually soluble or volatile in the mobile phase.

Separation of the analyte occurs in chromatography due to differences in physical (e.g., size) or chemical properties (i.e., polarity, charge, unique structure or functional groups), which results in different retention times. Retention time is a parameter which indicates the amount of time the analyte takes to elute from the column or how strong the solute adheres to the stationary phase. Thus longer retention times represent stronger retention interaction with the stationary phase. There are different types of chromatography, e.g., thin-layer chromatography (TLC), gas chromatography (GC), and column chromatography [1]. High performance liquid chromatography (HPLC) is a column chromatography which is widely used in industry for research and development.

High performance liquid chromatography (HPLC) has been recognized as superior to gas chromatography since it is not limited to volatile and thermally stable samples. In addition, the choice of mobile and stationary phases is wider in HPLC. The principle of liquid chromatography is that the analytes are separated based on their different distribution in a liquid mobile phase and stationary phase. In high performance liquid chromatography, small particles of uniform size and shape with a narrow pore size distribution are used. In order to use high efficiency packing materials of small particles, high pressure solvent pumping systems are needed so that they can deliver the mobile phase at a uniform flow rate.

1.2 Fundamental Principles

The principle of separation is based upon distribution of solute molecules between two immiscible liquid phases in proportion to their relative solubilities. The separating medium consists of a porous particle support like silica gel, holding a stationary liquid phase. The separation is achieved by passing the mobile phase over the stationary phase. The stationary phase is in the form of a packed column.

Liquid chromatography can be divided into two categories [2]. When the stationary phase is polar and the mobile phase is non-polar it is called normal phase chromatography. In normal phase chromatography (NPC), the solute elution order relies on the principle that non-polar solutes prefer the mobile phase and elute first while polar solutes prefer the stationary phase and elute later. In reversed phase chromatography (RPC), the stationary phase is non-polar and the mobile phase is polar. Here the elution

order is reversed of that in NPC; that is, polar compounds eluting first and the non-polar ones later.

All organic molecules have hydrophobic regions in their structure to some extent; therefore they can interact with the non-polar stationary phase. The interaction is mainly based on adsorption. The longer the molecule is adsorbed on the stationary phase, the longer it is retained on the stationary and it has longer retention time. In reversed phase analysis the most retained compound is the most non-polar [3].

Analysis can also be carried out using a non-polar solvent with a polar stationary phase to separate polar compounds. In this type of analysis the more polar solvent makes the compounds retain longer and the most retained compound is the most polar. This method is referred to as aqueous normal phase analysis (ANP).

2. Capillary Liquid Chromatography

2.1 General Aspects

Today, the trend of miniaturization is common in the field of analytical chemistry and chromatography columns have followed a similar path. Column diameters have decreased from conventional HPLC columns to capillary columns. In normal HPLC, columns of 4.6 mm internal diameter (i.d.) are used, whereas capillary liquid chromatography and capillary electrochromatography employ capillary columns which have an i.d. in the micrometer range [4]. Narrow-bore capillary columns have an i.d. from 20 μm to 100 μm . As the column i.d. decreases the injection volume decreases and so the loading capacity decreases. With the decrease in column i.d. the flow rate decreases and the amount of mobile phase required for the analysis is less compared to

that of conventional HPLC. Capillary columns are suitable for analysis of samples which are costly and are available in small amount.

Liquid chromatography is a versatile tool used for separation of compounds by chemists and biologists. In capillary liquid chromatography, columns are used which have an i.d. smaller than conventional columns. In capillary liquid chromatography systems injection valves with low dead volumes and pumping systems that can provide lower flow rates are used. This lower flow rate is suitable for coupling the capillary LC system with a mass spectrometer. In smaller bore columns the sample gets diluted to a lesser extent than that of conventional HPLC and thus the concentration of the sample eluting is much higher at the detector. For the microbore columns, usually a micro UV detector is used since commercial UV detectors generally have a large cell volume which results in distorted peaks [4].

2.2 Advantages of Capillary Columns

There are many advantages of using micro columns. In recent years the column diameters have decreased from 1-2 mm to micrometer level. The three main advantages of using micrometer diameter capillary columns are: a) minimizing the cost of solvents and reducing waste and thus making it more environmental friendly; b) the lower flow rates used can easily be coupled with a mass spectrometer; and c) resolution. Resolution is defined as the degree of separation between two neighboring peaks. In capillary liquid chromatography the elution volume, a measure of peak width, is much smaller compared to that in standard HPLC. Hence resolution is better in capillary liquid chromatography [4].

2.3 Capillary Columns

There are three different types of capillary columns. These are open tubular, monolithic columns, and packed capillary. This research work was done on packed capillary columns.

2.3.1 Packed Capillary Columns

In the packed capillary column format, the stationary phase is packed into a fused silica capillary. In capillary electrochromatography, flow through the column is generated by electroosmosis in contrast to the high pressure which drives the mobile phase in HPLC. Thus for CEC column packing can be done using smaller particles since there is no backpressure. There are two ways of packing capillary columns; one is the slurry method and the other is electrokinetic packing [5].

In the first method, a slurry of the packing materials is placed in a reservoir and is then pushed into the capillary at high pressure (about 200 to 500 atmosphere). Using this method, columns of fairly good efficiency can be made. However, there are certain limitations of the slurry method. Since higher pressure is required for packing of smaller particles, it is difficult to pack smaller particles using slurry method. It has been shown that the use of smaller particles increase the efficiency of separation. Efficiency, N , is the number of theoretical plates and it can be seen from Table I that smaller size particles have greater number of theoretical plates [6]. However, it is a real challenge to pack columns with smaller particles using the slurry method as extremely high pressures are required to pack the particles. The slurry method of packing particles could be dangerous especially when thin wall capillaries having a wide inner diameter are packed because

higher pressure may lead to the breakage of capillary wall. Moreover, the slurry method is tedious and only one capillary column can be packed at a time. All these problems can be overcome in the electrokinetic method of packing capillaries.

In the electrokinetic method, capillaries are packed by applying electric field across them. Packing particles move due to electroosmotic flow, if the particles are charged they also have their electrophoretic mobility. Electrokinetic packing can also be used for packing of uncharged particles. These capillaries can be used for the separation of compounds by micro HPLC as well as by CEC. There is no backpressure generated in this case as the particles migrate under the influence of electric field. In electrokinetic packing, the particles move into the column according to their mass to charge ratio, and therefore the column packed in this way is more uniform. Smaller particles can be easily packed with this method which facilitates higher efficiency and better resolution [5].

3. Column Material

3.1 Stationary Phase

For most materials used in HPLC, silica gel is the main base. Silica gel has the following properties that make it suitable for stationary phases:

- 1) Silica gel is rigid.
- 2) Silica gel is reasonably priced and efficient.
- 3) Silica gel can react with the organic moiety (e.g., C18, C8) as it has reactive surface silanol groups and a relatively high surface area.

Silica gel consists of irregular and spherical shaped particles [3]. It is available in a wide range pore diameters and pore volumes. It can be used in both nonaqueous and

aqueous environments. Due to the presence of silanols which are acidic, it can lead to adsorption of the basic compounds. Due to presence of silica as the support material in a majority of HPLC phases, bonding between organic moieties and the substrate is based on the chemistry of the silanol at the surface.

3.1.1 Silica Surface

Silica is the most commonly used base material in both normal and reversed phase chromatography. The silica used in this research has the commercial name Astrosil. Astrosil silica has a high surface area compared to many other types of silica. Amorphous silica with a porous structure is a three dimensional network of siloxane moieties (Si-O-Si) with silanol groups (-Si-OH) on the surface. The silanol groups are weakly acidic and possess the ability to interact with polar compounds through strong hydrogen bonding and dipole-dipole interactions. There can be three kinds of silanols on the surface: isolated, geminal and vicinal. All three kinds of silanols are shown in Figure 1 [7].

3.1.2 Different Types of Bonded Phase

One of the simplest methods of making chemically bonded stationary phases for HPLC is esterification. In this method, an alcohol is reacted with a silanol to form a Si-O-C linkage. The linkage is not stable hydrolytically and cannot be used for aqueous mobile phases [7].

In another approach known as organosilanization, there are two types of reactions.

Figure 1. Silanol species present on silica surface

The monomeric reaction uses an organosilane with only one leaving group (X), like chlorine or methoxy. The method involves attachment to the silica surface at one point. In a polymeric reaction, an organosilane having either two or three leaving groups is used. The surface chemistry is more complicated due to extensive crosslinking between adjacent organosilanes [8]. A polymeric network is created which extends out from the surface of the silica. More bonded mass is produced but surface coverage is less well defined. This happens because different silanes may anchor to the surface at some distance from the surface.

Organosilanization Reactions

In another approach, chlorination of a silica surface is done with thionyl chloride followed by attachment of organic components through a Grignard reagent. The resulting linkage is a direct Si-C bond which is more stable than the linkage through organosilanization [7, 8].

Yet another approach is silanization / hydrosilation. In this approach, bare silica is reacted with the hydrolysis product of triethoxysilane (TES) to form a monolayer of hydride on the surface. By means of a hydrosilation reaction between the surface hydride and a terminal olefin catalyzed by a transition metal complex like hexachloroplatinic acid, an organic moiety can be attached to silica.

TES Silanization

Polymeric packings, which are available in a variety of particle diameters, porosities, and mechanical strengths, are used in twenty percent of liquid chromatography applications [9]. Polystyrene cross-linked with divinylbenzene is the most popular matrix. One advantage of polymeric packings over silica gel is that they are stable over

Hydrosilation

wider pH range. Some other packing materials are more specialized and are more expensive [10, 11].

3.1.2.1 Alkyne Bonded Phase

Studies of attaching different bonded groups showed that there can be number of possible ways of attaching organic moieties to silica hydride through multiple bond functional groups on the reactant. The hydrosilation reaction of an alkyne compound results in the formation of a double Si-C linkage between the surface and the bonded organic moiety (Figure 2). Such bonded material shows a higher degree of stability and can be utilized in both normal and reversed phase chromatography. Some possible structures for attachment, when an alkyne group is used, are shown in Figure 2 [12, 13]. In this research work, bidentate capillary columns contain an alkyne bonded stationary phase and their structure is represented by structure I of Figure 2.

4. Capillary Formation

4.1 Capillary Cutting and Length Selection

Length is important as too long a capillary will result in longer run times. If the capillary is too short then run time may be inadequate for separation of all the compounds.

Figure 2. Five different ways of bond formation of an alkyne with a silica hydride surface.

The right technique of cutting the capillaries to the appropriate length involves using a fused silica cutter. Failure to have a good square cut may cause distorted peak shapes and irreproducibility. Capillaries can be viewed under a microscope to ensure proper cutting [14].

4.2 Frit Formation

Frit formation is required in packed capillaries to retain the stationary phase. Frits should not be thick otherwise they may cause band broadening. The frit should be made in such a way that it stays in contact with the stationary phase. Several procedures have

been developed for the formation of frits [14]. The most common ones use a paste of silica and sodium silicate that is heated to a temperature of 400 degrees centigrade or above. Another method of making a frit is to heat a wire and thermally fuse the column packing. With the heated wire method frits can be formed at any place in the capillary and is more versatile than the silica and sodium silicate method.

4.3 Window Formation on a Capillary

The capillary column has a polyimide coating which must be removed from the surface in the detection region since methods such as UV, diode array and fluorescence will not work with the polyimide coating in place. The capillary without the polyimide coating is very fragile and can break easily. Hence it should be carefully inserted inside the cassette of a CE or CEC system. The window on the capillary is usually made by burning the polyimide coating with an electrically heated coil or by a flame of a micro torch [14].

5. Chromatographic Efficiency

5.1 Use of Smaller Particles

In order to improve the chromatographic efficiency smaller particles can be used for packing of the column. It was seen that a column packed with 5 μm particles and a length of about 25 cm long can have up to 87,000 plates/m whereas if the particle size is reduced to about 1.5 μm the efficiency can go up to 290,000 plates/m for a similar column. This can be calculated using the Van Deemter equation provided only the particle size changes and other variables remain constant. The increase in number of theoretical plates can in turn decrease the analysis time by a factor of 3. The relation

between theoretical plates, particle diameter and the analysis time can be found out from Table 1 [6].

Table 1. Theoretical relation between theoretical plates, particle diameter and analysis time.

d_p (μm)	Plates/m	t_a (min)
5.0	90,000	50
3.0	150,000	30
1.5	300,000	15
0.5	300,000	5

In the above table, d_p represents the particle diameter and t_a represents the analysis time. According to Table I use of smaller particles is attractive as it decreases the analysis time significantly. However, the smaller diameter columns require greater pressure to drive the mobile phase through the packed bed. The pressure drop in a HPLC system can be calculated using the formula as follows:

$$\Delta P = \frac{u_{\text{opt}} L \eta \phi}{d_p^2} \quad (7)$$

where u_{opt} is the optimum linear velocity, L is the column length, η is the mobile phase viscosity, ϕ is the flow resistance parameter and d_p represents the particle diameter. At the optimum linear velocity the pressure drop is inversely proportional to the diameter of the particle. It is seen that in a conventional HPLC system having a column of 25 cm length and packed with 5.0 μm particles would require an inlet pressure of lower than 20

bar or lower than 300 psi for obtaining an optimum velocity to drive the mobile phase. It is seen that if a smaller particle is used, having a particle diameter of about 1 μm , the column length being the same a higher inlet pressure of above 1030 bar (15,000 psi) is required. Conventional HPLC instruments are incapable of handling this pressure, so an ultra high-pressure instrument is necessary. Smaller particle diameters of about 0.7-1.5 μm can be packed in a capillary column and by employing high pressure of up to 15,000 psi good separation can be achieved. Smaller diameter particles are usually advantageous for packing of smaller inner diameter columns, especially capillary columns due to thermal issues. Capillary columns have a high surface to volume ratio to dissipate the heat whereas the frictional heating is much greater in a regular HPLC column. Due to frictional heating inside a column there can be a non-uniform temperature which may have an adverse effect on the separation of the compounds. The heat produced inside a capillary column was found by Knox and Grant [15]. According to Knox and Grant the heat generated per unit volume of an electrolyte (Q) inside a packed capillary column is given by the following equation

$$Q = E^2 \lambda c \epsilon \quad (8)$$

where E = electric field strength

λ = molar conductivity

c = molar concentration of electrolyte

ϵ = porosity of the packed particles

Since heat generated depends on the concentration of the electrolyte and also on the molar conductivity of the electrolyte, lower concentrations and lower conductivity electrolytes will generate less heat.

In capillary electrochromatography a packed capillary columns is used for analysis. The separation of compounds using capillary electrochromatography is independent of the particle size of the column. In capillary electrochromatography, electroosmotic flow is mainly responsible for transporting the mobile phase. Electroosmotic flow has a plug like flow profile. Due to the plug like flow profile in a capillary electrochromatography system there is less band broadening compared to that of a HPLC system [14].

6. Principles of CEC

6.1 Capillary Electrochromatography

Capillary electrochromatography is a combination of CE and HPLC. Like CE an electric field is applied across the capillary for the separation of the compounds and like HPLC CEC employs packed columns for the separation of the compounds. There is no backpressure in capillary electrochromatographic systems and smaller diameter particles can be used for the separation of the compounds. The flow profile in a HPLC system is parabolic which can cause band broadening, whereas in a capillary electrochromatographic system the flow profile is plug-like due to the electroosmotic flow produced by the charged inner walls of the capillaries.

6.2 Electrophoresis

In capillary electrophoresis, separation occurs due to differences in solute velocity in an electric field. Velocity of an ion in an electric field is given by the following equation.

$$v = \mu_e E \quad (9)$$

where v is the velocity of the ion, μ_e is the electrophoretic mobility and E is the applied electric field. Mobility of an ion or molecule under the influence of an electrical field is determined by the electrical force that the molecule experiences, balanced by the frictional force of the medium. Electrical force that the ion experiences is given by the following equation

$$F_E = qE \quad (10)$$

where q is the charge of the ion and E is the electric field. Frictional force of the ion is given by the following equation

$$F_F = -6\pi\eta r v \quad (11)$$

where η is the viscosity of the medium, r is the radius of the ion and v is the velocity of the ion. The balance between the frictional force and the electrical force that a molecule or ion experiences is known as the electrophoretic mobility. Electrophoretic mobility of an ion or molecule can be represented by the following equation

$$\mu_e = \frac{q}{6\pi\eta r} \quad (12)$$

where q is the charge of the ion, η is the viscosity of the medium and r is the radius of the ion [15].

6.3 Electroosmosis

Electroosmotic flow is generated by the propagation of charges along the interface between a liquid and solid. Many solids have fixed charges along their surface. In silica the charged group is Si-O^- . In order to maintain charge neutrality there is a cation like Na^+ associated with the surface charge. This cation is mobile and can be replaced by another cation. Cations are attracted to the opposite charge electrode (cathode) in the presence of an electric field. During their movement towards the cathode they drag the solvent causing a net liquid flow (electroosmotic flow). Electroosmotic flow is a function of the applied electric field E , permittivity of vacuum ϵ_0 , the dielectric constant of the mobile phase ϵ_r , the zeta potential ζ (it is the potential difference between the Stern layer and the shear layer of movement) and the viscosity η . The equation is shown below.

$$u_{eo} = \frac{E\epsilon_0\epsilon_r\zeta}{L\eta} = \mu_{eo}E \quad (13)$$

where E is in Vcm^{-1} and μ_{eo} is the electroosmotic mobility in $\text{cm}^2 \text{V}^{-1} \text{s}^{-1}$ and thus electroosmotic velocity has a unit of cm s^{-1} [16].

6.4 Double Layer

Inside the capillary wall under alkaline conditions the silanol groups are ionized and are negatively charged. To maintain the charge neutrality the positively charged ions from the buffer are attracted to the negatively charged ions and form a low mobility layer called the Stern layer. The remainder of the excess charge is distributed beyond the Stern layer and constitutes the Gouy or diffuse layer. Most of the ions in this region are fully solvated and extend into the bulk liquid. This region is referred to as the diffuse double

layer. The concentration of the excess ions is lower in the diffuse double layer compared to the compact (Stern) layer. According to the theory the concentration of the excess ions falls off quasi exponentially from the surface into the bulk of the liquid. The electrical potential drop across the double-layer is known as zeta potential (ζ). The zeta potential is proportional to the excess charge density. As the distance from the Stern layer increases the excess charge density falls off and so does the zeta potential. From the surface to the point where the value of zeta potential is $1/e$ times than it is at the surface is the known as the double-layer thickness or Debye-length. The double layer thickness δ may be approximately calculated with the following [17].

$$\text{The equation for } \delta = [\epsilon_0 \epsilon_r RT / 2cF^2]^{1/2} \quad (14)$$

ϵ_0 = dielectric constant

ϵ_r = permittivity of vacuum

R = universal gas constant

T = absolute temperature

c = molar concentration

F = Faraday constant

Under the influence of the electric field electroosmotic flow arises because the ions from the double layer move toward the cathode. The zeta potential can be calculated with the following equation:

$$\zeta = \delta \sigma / \epsilon_0 \epsilon_r, \text{ where } \sigma \text{ is the average excess charge density in double layer} \quad (15)$$

From the above equation it can be seen that the zeta potential is directly proportional to the excess charge density of the medium. The zeta potential is again related to linear

velocity of the ions according to equation 13. Thus it can be seen that the velocity of the ion in a capillary electrochromatographic system depends on the surface charge density, the thickness of the double layer and the viscosity of the medium.

6.5 Electroosmotic Flow in Packed Capillary Columns

In capillary electrochromatography, electroosmotic flow drives the mobile phase from the inlet side to the outlet of the capillary. It was found by Knox and Grant that EOF is lower in packed capillary columns than in the open capillary format as in packed columns the EOF resides only on the outside of the particles [15]. After studying the properties of different C18 stationary phases it was found by Dittman and Rozing that in capillary electrochromatography the EOF does not reside on the inner walls of the capillary [17]. In reversed phase capillary electrochromatography the silica particles are modified with long chain hydrocarbons which decreases the density of free silanol groups on the surface of the silica particles and hence the EOF decreases whereas the unmodified capillaries have only free silanol groups and the EOF is greater in the unpacked capillaries.

6.6 Reversed Electroosmosis Direction

The direction of the electroosmotic flow in a capillary electrophoresis system is from anode to cathode. Thus the analyte is usually detected at the cathodic end and so the cathode must be located at the outlet. The conventional CE system contains an anode at the inlet end and cathode at the outlet. In a pressurized electrochromatography system the sample can be introduced inside the capillary even though the electroosmotic flow is reversed due to the existence of pressurized flow. The speed of the migration of the

molecule depends on three factors in a pressurized CEC system: the electroosmotic flow u_{EOF} , electrophoretic mobility u_{mob} and the pressurized flow u_{pre} . In the TrisepTM -2100 system used in the study, the electrode at the splitting junction is grounded and the outlet end can be either negative or positive polarity as the solute velocity is supplemented by pressurized flow along with the electroosmotic flow and electrophoretic mobility. Thus as long as $|u_{EOF} + u_{mob} + u_{pre}| > 0$ the overall flow is from the inlet end to the outlet end [18]. If the voltage applied is zero then the molecule moves to the detector end with the same velocity as that of the velocity of the pressurized flow. In this case the sample gets separated on the retention mechanism based on the interaction of the stationary phase. As the molecules are detected at the cathodic end the positively charged molecules are detected first, then the neutral molecules and finally the negatively charged species.

7. Pressurized CEC

7.1 Theory and Mechanism of Pressurized CEC

In the capillary LC system there is no electric field so the equation for the capacity factor is the same as that of HPLC and is represented by the following equation:

$$k_0' = \frac{t_R - t_0}{t_0} \quad (16)$$

where t_R is the retention time of the solute and t_0 is the void time of a molecule which is not retained in the column [19]. The pressurized CEC system has a different retention

mechanism than that of either HPLC or CEC and the velocity at which the molecule travels is determined by the following equation:

$$v = v_p + v_{eo} + v_{ep} \quad (17)$$

where v_p , v_{eo} and v_{ep} are the linear velocity of pressurized, electroosmotic and electrophoretic flow respectively of the solute. The electroosmotic velocity and the electrophoretic velocity can be written as follows:

$$v_{eo} = \mu_{eo}E \quad (18)$$

$$v_{ep} = \mu_{ep}E \quad (19)$$

where μ_{eo} and μ_{ep} are the electroosmotic flow and electrophoretic velocity respectively and E is the electric field strength. The velocity of the mobile phase is given by the following equation:

$$v_m = v_{p,m} + v_{eo,m} \quad (20)$$

where v_m , $v_{p,m}$ and $v_{eo,m}$ are the overall linear velocity of the mobile phase, the linear velocity of the pressurized flow of the mobile phase and the linear velocity of the electroosmotic flow of the mobile phase respectively.

8. Goals of this Research Work

The major goal of this research work was to compare the two techniques capillary LC and pressurized CEC (PCEC) on hydride based bidentate C18 columns of different particle size. Test solutes used for characterization of the columns were steroid hormones, drugs, nucleobases as well as nucleosides. All of these compounds were analyzed in capillary LC as well as the pressurized CEC mode. Analysis of steroids was also carried out on a commercial capillary column. The studies were done on a Trisep-

2100 in which analysis could be done by capillary LC as well as pressurized CEC. A comparison of the separations on capillary LC and pressurized CEC using two different bidentate C18 columns having different particle sizes were made.

CHAPTER II

EXPERIMENTAL

1. MATERIALS

The following table shows a list of chemicals used in this study along with their chemical abstract service (CAS) registry numbers.

Table 2. CAS registry number of chemicals

Name	Source	CAS Registry Number
Acetonitrile	Omnisolv	75-05-8
Adenine	Baker Analyzed Biochemical	73-24-5
Adenosine	Calbichem	58-61-7
Amphotericin	USP Rockville	1397-89-3
Aniline	Fischer Scientific	62-53-3
Corticosterone	Sigma Aldrich	50-22-6
Cytidine	Pharmaceuticals, Inc.	65-46-3
Cytosine	Nutritional Biochemical Corporation	71-30-7
Formic acid	Mallinckrodt	64-18-6
Guanine	Baker Analyzed Biochemical	73-40-5
Guanosine	Sigma Aldrich	118-00-3
Metformin	Sigma Aldrich	657-24-9
L-Norgestrel	Sigma Aldrich	797-63-7

Progesterone	Sigma Aldrich	57-83-0
Sulfonamide	Unknown	63-74-1
Sodium acetate	Fischer Scientific	127-09-3
Thiamine hydrochloride	Baker Analyzed Chemical	67-03-8
Thymine	Baker Analyzed Biochemical	65-71-4

2. Instrumentation

2.1 Details of Instrumentation

The TrisepTM -2100 is an instrument from Unimicro Technologies (Pleasanton, CA, USA) in which both capillary HPLC and pressurized electrochromatography can be performed. A schematic diagram of the instrument is shown in Figure 3 [20]. As seen from the figure it consists of two mobile phase reservoirs. The two reservoirs are connected to microbore pumps producing flow rate in microliters and this liquid then goes to the mixer. The mixed solution then flows to a splitting tee junction. The grounded electrode is placed at the tee junction. One part of the tee junction is connected to the flow restrictor which in turn is connected to the back pressure regulator and finally goes to the waste reservoir. The flow restrictor controls the flow velocity of the mobile phase and the back pressure regulator maintains the appropriate pressure and prevents bubble formation. The tee junction is then connected to the injector. The injector is a 6-port nano volume sample device. The capillary column is inserted into the injector port and then connected to the UV detector near the end of the column. The end of the column is then connected to another electrode having negative polarity and finally the

capillary goes to the waste reservoir. This instrument can be operated using two modes: the capillary LC mode and the pressurized CEC mode. The high voltage is not turned on when the instrument is operated in the capillary LC mode and the high voltage is switched on when the instrument is operated in the pressurized CEC mode.

Figure 3. Schematic diagram of Trisep 2100 pCEC system. 1. Mobile phase reservoir, 2. pump one, 3. pump two, 4. micromixer, 5. tee, 6. low dead-volume connector, 7. injector, 8. column, 9. detector, 10. interlocked safety box, 11. micro-splitter valve, 12. back pressure regulator, 13. cross, 14. electrode, 15. high-voltage power supply, 16. waste.

2.2 Capillaries

The packed capillaries used for this research work were obtained from Unimicro Technologies, Pleasanton, California. Three different types of packed capillaries were used for this research work. The three capillaries were as follow:

- a) Packed commercial C18 capillary: This capillary consisted of 5 micron silica particles having a 300 Å pore size bonded with a C18 organic moiety via organosilanization.
- b) Packed Bidentate C18 capillary: This capillary consisted of 4 micron silica particles having a 100 Å pore size bonded with bidentate C18 through silanization and hydrosilation.
- c) Packed Bidentate C18 capillary: This capillary consisted of 1.8 micron silica particles having a 100 Å pore size bonded with bidentate C18 through silanization and hydrosilation.

3. Reagents and Solutions

3.1 Preparation of Samples

Steroid hormone samples were prepared in 50:50 acetonitrile/water. The concentration of the samples was 1 mg/mL. Thus the concentration of the samples was 1000 ppm. Drugs samples were also prepared in the same way except amphotericin was prepared by dissolving 100 µg of the compound in 1 mL of acetonitrile. All the nucleobases and nucleotides were prepared in 50:50 acetonitrile / water. Among the nucleobases guanine is the only base which was not soluble in acetonitrile: water. Guanine was dissolved by adding a drop of concentrated sulphuric acid and diluting it with 2 mL of water. All the sample solutions were filtered using 25 mm syringe filters containing nylon, PTFE and regenerated cellulose membranes having a retention volume of 90 µL. These filters were obtained from Microsolv Technology, New Jersey, USA.

The filters have a maximum operating pressure of 110 psi and a maximum operating temperature of 100 °C.

3.2 Preparation of Mobile Phase

The mobile phase used consisted of 100% acetonitrile and 0.1% formic acid. 0.1% formic acid was prepared by dissolving 1 mL of concentrated formic acid in 999 mL of D.I. water. The 0.1% formic acid prepared was then filtered using a 0.20 µm Nylon 66 membrane filter (Altech Assoc., Deerfield, IL). The acetonitrile concentration was varied from 90% to 10%.

3.3 Preparation of Buffers

Sodium acetate (pH = 4.41) and sodium formate buffers (pH = 8.5) were prepared using de-ionized water and the concentration of each was 20 mM. The deionized water was obtained from a Milli-Q purified water system (Millipore Corp., Bedford, MA). The buffers were diluted with the water obtained from Milli-Q water system in appropriate proportion to obtain the desired concentration.

4. Test solutes Used in This Research

Test solutes used in this research work consist of steroid hormones, drugs, nucleobases, and nucleosides. The name and the structures of all the test solutes are shown in Table 3, 4, 5, 6, and 7.

Table 3. Structure of steroid hormones used for analysis

Prednisolone
Corticosterone
Levo Norgestrel
Progesterone

Table 4. Structure of drugs

Metformin
Sulfonamide
Amphotericin

Table 5. Structure of nucleobases

Adenine	<p>The chemical structure of Adenine is a purine base, consisting of a fused pyrimidine and imidazole ring system. It features an amino group (NH_2) at the 6-position and a hydrogen atom on the nitrogen at the 9-position.</p> <chem>Nc1ncnc2[nH]cnc12</chem>
Guanine	<p>The chemical structure of Guanine is a purine base, consisting of a fused pyrimidine and imidazole ring system. It features a carbonyl group (C=O) at the 6-position, an amino group (NH_2) at the 2-position, and a hydrogen atom on the nitrogen at the 1-position.</p> <chem>Nc1nc2[nH]cnc2c(=O)[nH]1</chem>
Thymine	<p>The chemical structure of Thymine is a pyrimidine base, consisting of a single six-membered ring. It features carbonyl groups (C=O) at the 2 and 4 positions, a methyl group (CH_3) at the 5-position, and a hydrogen atom on the nitrogen at the 1-position.</p> <chem>CC1=CNC(=O)NC1=O</chem>
Cytosine	<p>The chemical structure of Cytosine is a pyrimidine base, consisting of a single six-membered ring. It features an amino group (NH_2) at the 4-position, a carbonyl group (C=O) at the 2-position, and a hydrogen atom on the nitrogen at the 1-position.</p> <chem>Nc1cc[nH]c(=O)n1</chem>

Table 6. Structure of nucleosides

<p>Adenosine</p>
<p>Guanosine</p>
<p>Cytidine</p>

Table 7. Structure of basic compounds

<p>Thiamine hydrochloride</p>
<p>Aniline</p>
<p>Pyrrole</p>

CHAPTER III

RESULTS AND DISCUSSIONS

1. Analysis and Results

This study consisted of analyzing some steroid hormones, drugs, nucleobases, nucleosides, as well as a few basic compounds on three capillary columns. The three capillary columns used for the analysis are a 5 micron commercial C18; a C18 capillary in which the stationary phase was prepared by silanization / hydrosilation procedure using 4 micron Astrosil silica; and a third capillary also prepared by the silanization / hydrosilation procedure using 1.8 micron Astrosil silica. Capillary LC of the steroid hormones was done on the commercial C18. Both capillary LC and pressurized CEC analysis were done on the hydride based stationary phase columns for all the analytes.

1.1 Analysis of Steroid Hormones on Commercial C18

Figure 4 shows a chromatogram for four steroid hormones analyzed on the commercial C18 packed capillary column. They were retained in ninety percent acetonitrile. Among the four steroid hormones prednisolone showed the lowest retention time and progesterone had the highest retention in ninety percent acetonitrile. The analysis was done in reversed phase using aqueous/organic solvents. It can be seen from Figure 4 that all four steroid hormones were separated within about six minutes. The first test was done using 90:10 acetonitrile / 0.1% formic acid. The concentration of acetonitrile was then reduced and the mixture of the four steroid hormones was run using 80:20 acetonitrile / 0.1% formic acid. It can be seen from Figure 5 that all four hormones were eluted within 8.8 minutes. As the concentration of acetonitrile was reduced the

steroid hormones were more strongly retained. Figure 6 shows the chromatogram obtained using 70:30 acetonitrile / 0.1% formic acid. The retention map of the steroid hormones was then plotted and it can be seen from Figure 7 that as the percentage of acetonitrile is reduced the steroid hormones are more retained. The elution order of the compounds is as follows: Prednisolone < Corticosterone < Levo Norgestrel < Progesterone. Since in a reversed phase analysis the compound that has the greatest retention is most non-polar, it can be concluded that progesterone is the most non-polar compound among all the steroid hormones and prednisolone is the most polar compound among all the steroid hormones. It can be confirmed from the retention map that all these compounds show reversed phase behavior.

1.2 Analysis of Steroid Hormones on Bidentate C18 (4 micron)

The steroid hormones were analyzed on a Bidentate C18 packed capillary column containing four micron Astrosil silica. It was seen that the steroid hormones showed reversed phase behavior on this column too. The retention time was somewhat longer than the commercial C18 packed capillary column. Bidentate C18 columns contain a stationary phase that was prepared by the silanization / hydrosilation procedure and formation of a hydride layer minimizes the number of hydroxyl groups on the surface of silica making it more non-polar than the commercial column. It can be seen from the chromatograms obtained on the Bidentate C18 stationary phase that the steroid hormones were retained for a longer time than on the commercial C18 capillary column. Figures 8, 9, and 10 show the chromatograms obtained after analyzing the steroid hormones using reversed phase solvents consisting of ninety, eighty, and seventy percent acetonitrile

Figure 4. Capillary LC analysis of steroid hormones using 90:10 acetonitrile / 0.1 formic acid. Flow rate-70 microliters per minute, pressure-1022 psi [A] = Prednisolone, [B] = Corticosterone, [C] = Norgestrel, [D] = Progesterone.

Figure 5. Capillary LC analysis of a mixture of steroid hormones in 80:20 Acetonitrile / (0.1% formic acid). Flow rate- 70 microliters per min, pressure-1022 psi
[A] = Prednisolone, [B] = Corticosterone, [C] = Norgestrel, [D] = Progesterone.

Figure 6. Capillary LC analysis of mixture of steroid hormones in 70:30 acetonitrile / 0.1% formic acid. Flow rate- 70 microliters per minute, pressure- 1022 psi
[A] = Prednisolone, [B] = Corticosterone, [C] = Norgestrel, [D] = Progesterone.

Figure 7. Plot of retention time vs percentage of acetonitrile used for the analysis of steroid hormones.

respectively. It can be seen from Figure 10 that all four steroid hormones were eluted within twenty two minutes using seventy percent acetonitrile. As the retention time was longer on the Bidentate C18 the lines in the retention map obtained were much steeper than the lines in the retention map of commercial C18. Figure 11 shows the retention map of the steroid hormones for the four compounds on the Bidentate C18 column.

1.3 Pressurized CEC Analysis of Steroid Hormones on Bidentate C18

In pressurized capillary electrochromatography, compounds are transported through the column by electroosmotic flow as well as by pressure flow. The result of these two mechanisms is that the compounds separate faster than in capillary liquid chromatography which is based only on pressurized flow. Analysis of steroid compounds were done in the pressurized CEC mode at different concentrations of acetonitrile. Figure 12 shows the pressurized CEC analysis of a mixture of four hormones using 80:20 acetonitrile / 0.1% formic acid at 20 kilovolts. It can be seen that when the steroid hormones were analyzed in the capillary mode at eighty percent acetonitrile all four compounds were eluted within eleven minutes whereas in pressurized CEC analysis all the compounds were eluted within four minutes. This shows that pressurized CEC analysis is faster than capillary LC analysis. Figure 13 shows the pressurized CEC analysis of the steroid hormones using 70:30 acetonitrile / 0.1% formic acid. All four compounds were eluted within nine and a half minutes using seventy percentage acetonitrile whereas in capillary LC the elution time of the four compounds is around twenty two minutes. Figure 14 demonstrates that as the voltage increases the migration of the analyte becomes faster and the analysis time is shorter. Two important conclusions

Figure 8. Capillary LC analysis of mixture of steroid hormones on Bidentate C18, 90:10acetonitrile/ 0.1% formic cid. Flow rate- 70 microliters per minute, pressure-1022 psi, [A] = Prednisolone, [B} = Corticosterone, [C] = Levo Norgestrel, [D] = Progesterone.

Figure 9. Capillary LC analysis of mixture of steroid hormones in 80:20 acetonitrile / 0.1% formic acid. Flow rate-70 microliters per minute, [A] = Prednisolone, [B] = Corticosterone, [C] = Norgestrel, [D] = Progesterone, pressure-1022 psi.

Figure 10. Capillary LC of a mixture of steroid hormones using 70:30 acetonitrile / 0.1% formic acid. Flow rate- 70 microliters per minute, pressure-1022 psi.

Figure 11. Plot of retention time vs percentage of acetonitrile used for the analysis of steroid hormones on Bidentate C18 capillary (4 micron).

can be drawn from these data: analysis time becomes shorter at higher percentage of acetonitrile and at higher voltages in PCEC.

1.4 Analysis of Drugs on Bidentate C18

Metformin, amphotericin, and sulfonamide were analyzed using an acetonitrile/water mobile phase on the Bidentate C18 column. Metformin and amphotericin showed aqueous normal phase (ANP) behavior and were retained to a greater extent at higher concentrations of acetonitrile. Figure 15 shows the retention map of the three drugs and it can be seen that metformin and amphotericin developed ANP retention whereas sulfonamide was retained more at lower concentrations of acetonitrile. Thus it can be concluded that metformin and amphotericin are more polar than sulfonamide. In chromatography, like adsorbs like, so metformin and amphotericin are eluted at a longer time when higher concentrations of acetonitrile are used since water is the more polar solvent and both metformin and amphotericin prefer to stay in water rather than in acetonitrile. Sulfonamide showed reversed phase behavior and it can be seen from the retention map that as the percentage of acetonitrile increases the retention time decreases. Hence it can be concluded that sulfonamide is more non-polar than metformin and amphotericin.

Pressurized CEC analysis was done on metformin and amphotericin at fifty percent acetonitrile to determine the effect of voltage on the polar compounds. Figure 16 shows a graph of migration time versus the applied field in kilovolts. It can be seen that the analysis time was much shorter in pressurized CEC.

Figure 12. Pressurized CEC analysis of mixture of hormones in 80:20 acetonitrile / (0.1% formic acid). Voltage: 20kv, Pressure-1022 psi, flow rate- 70 microliters per minute, [A] = Prednisolone, [B] = Corticosterone, [C] = Norgestrel, [D] = Progesterone.

Figure 13. Pressurized CEC of mixture of steroid hormones 70:30 acetonitrile / 0.1% formic acid. Voltage applied: 20 kv, pressure-1022 psi , flow rate-70 microliters per minute, [A] = Prednisolone, [B] = Corticosterone, [C] = Norgestrel, [D] = Progesterone.

Figure 14. Plot of migration time vs voltage using 70:30 acetonitrile / 0.1% formic acid.

Figure 15. Plot of retention time vs percentage of acetonitrile used for the analysis of drugs.

1.5 Analysis of Nucleosides and Nucleobases on Bidentate C18

Nucleosides and nucleobases were analyzed and all of them showed reversed phase behavior. Figures 17 and 18 show the retention maps for all nucleobases and nucleosides. It can be seen from the retention map that as the concentration of acetonitrile increases in the mobile phase the retention time decreases. Thiamine hydrochloride, aniline, and pyrrole also showed reversed phase behavior on this column. Figure 19 shows the retention map for aniline and pyrrole. All of the above compounds showed reversed phase behavior. All these compounds have very close retention times and the mixtures were not separated at higher concentrations of acetonitrile. Some good

Figure 16. Plot of migration time vs voltage using mobile phase- 50:50 acetonitrile / 0.1% formic acid, acetic acid buffer pH - 4.41.

separations of these compounds were obtained at fifty and sixty percentage of acetonitrile in capillary LC. Figures 20, 21, and 22 show the separation of some of the compounds in the capillary LC mode.

Pressurized CEC analysis of the basic compounds showed that the elution time is much shorter compared to capillary LC. It can be seen from Figure 23 that analysis time decreased when voltage was applied. As the voltage is increased from 5 to 20 kilovolts the analysis time became shorter. As the electroosmotic flow increases with an increase in voltage, this effect results in a shorter analysis time. The movement of the ions also depends on their electrophoretic mobility. Electrophoretic mobility varies for different ions and for this reason the elution order of thymine has changed in pressurized CEC analysis as can be seen in Figures 24, 25, 26, and 27 respectively. The effect of voltage on the migration time of the test solutes can be seen from the graphs in Figures 28 and 29. The graphs plotted in Figures 30, 31, 32, and 33 also show the effect of voltage on migration time, but instead of acetate buffer, formate buffer was used. There was not much difference when formate buffer was used.

1.6 Effect of Voltage in Pressurized CEC Analysis

It was seen in pressurized CEC that analysis time decreased with increase in voltage. An increase in voltage leads to an increase in electroosmotic flow due to the higher electric field. The relationship between electric field and voltage is based on the following equation:

$$E = V / L \quad (21)$$

where E = electric field, V = voltage and L = total capillary length

Increased electroosmotic flow leads to faster migration of solutes which reduces the analysis time. For example, in the current study involving steroids, it was seen that as voltage increased from 5 kV to 20 kV, the analysis time was reduced considerably. However, higher voltages lead to higher currents and an increase in Joule heating. In general, the heat produced is proportional to power and is given by $P = VI$

where P = power in watts, V = voltage, I = current

If the heat is not dissipated quickly, it will lead to increase in the capillary temperature [14]. The higher temperature will lead to a decrease in buffer viscosity allowing more current to flow through the capillary. Increased heat production within a capillary will lead to broader peaks and non-reproducible migration or retention times. Increased heat also causes sample decomposition and boiling of buffer may lead to bubble formation which can create problems in the experimental process such as noisy baseline etc.

The effect of decreasing voltage is that better resolution can be obtained. The electroosmotic flow is much lower at these voltages and hence there is time for the solutes to interact with the bonded C18 leading to better separation.

1.7 Analysis of Steroid hormones on Bidentate C18 (1.8micron)

Steroid hormones were also analyzed on a 1.8 micron particle size Bidentate C18 column. It was found the compounds were retained for a longer time than on the other two capillary columns. Even at ninety percent acetonitrile concentration the elution time of the mixture of hormones was around twenty six minutes. Figure 34 shows the analysis

Figure 17. Plot of retention time vs percentage of acetonitrile of nucleobases.

of a mixture of steroid hormones at ninety percent of acetonitrile. It was observed that the pressure was almost same as it was in the 4 micron Bidentate C18. As discussed before in liquid chromatography there is a large pressure drop for smaller particles as the pressure change is inversely related to the square of the diameter of the particles and thus it can be concluded that higher pressure is needed for smaller particle diameter columns. The reason for longer retention time on this column is due to lower pressure. It has been estimated that such smaller diameter particles need a pressure of up to 15,000 psi which can be achieved only in UPLC (ultra high pressure liquid chromatography) systems. Figure 35 shows the separation of the mixture of steroid hormones using 70:30 acetonitrile / 0.1%formic acid in water. The elution time of the mixture was more than an hour for this mixture. Figure 36 shows the retention map of steroid hormones on the 1.8 micron particle size bidentate C18 column.

Figure 18. Plot of retention time vs percentage of acetonitrile used for the analysis of nucleobases.

Figure 19. Plot of retention time vs percentage of acetonitrile.

Figure 20. Capillary LC analysis of a mixture of guanosine (8.77 mins) , thymine (9.959 mins) and cytidine (11.677 mins) using 50:50 acetonitrile / 0.1% formic acid. Flow rate- 70 microliters per minute, pressure-1036 psi.

Figure 21. Capillary LC analysis of a mixture of adenosine (10.236 mins) & adenine (13.514 mins) using 50:50 acetonitrile / 0.1% formic acid. Flow rate-70 microliters per minute, pressure-1036 psi.

Figure 22. Capillary LC analysis of a mixture of cytosine (10.355 mins) and guanine (12.653 mins) using 50:50 acetonitrile / 0.1% formic acid. Flow rate-70 microliters per minute, pressure-1036 psi.

Figure 23. Pressurized CEC analysis of a mixture of guanosine (6.105 mins), cytidine (7.238 mins) and thymine (8.415 mins) using 50:50 acetonitrile / 0.1% formic acid.

Voltage-5kv, flow rate-70 microliters per minute, pressure-1022 psi.

Figure 24. Pressurized CEC analysis of a mixture of guanosine (4.258 mins), cytidine (4.612 mins), thymine (5.313 mins) using 50:50 acetonitrile / 0.1% formic acid. Voltage applied -10 kv, pressure- 1022 psi, flow rate- 70 microliters per minute.

Figure 25. Pressurized CEC analysis of a mixture of guanosine (3.203 mins), cytidine (3.896 mins) and thymine (4.498 mins) using 50:50 acetonitrile / (0.1% formic acid). Voltage-15 kv, pressure-1022 psi, flow rate-70 microliters per minute.

Figure 26. Pressurized CEC of a mixture of guanosine (2.678 mins), cytidine (3.502 mins), thymine (4.059 mins) using 50:50, acetonitrile / 0.1% formic acid. Voltage applied: 20kv, flow rate-70 microliters per minute.

Figure 27. Pressurized CEC of a mixture of adenosine (2.538 mins) & adenine (3.308 mins) using 50:50 acetonitrile / (0.1% formic acid). Flow rate- 70 microliters per minute, pressure-1008 psi, 20kv.

Figure 28. Pressurized CEC analysis of a mixture of guanosine (4.640 mins) and thymine (5.297 mins) using 60:40 acetonitrile / (0.1% formic acid). Voltage used -5kv, flow rate-70 microliters per minute, pressure-1022 psi.

Figure 29. Pressurized CEC on Bidentate C18 using 70:30 acetonitrile / (0.1% formic acid), pH-4.41, flow rate-70 microliters per minute, pressure-1022 psi.

Figure 30. Pressurized CEC analysis of basic compounds using 70:30 acetonitrile / (0.1% formic acid), buffer pH-4.41 flow rate-70 microliters per minute, pressure-1022 psi.

Figure 31. Pressurized CEC on Bidentate C18 using 50:50 acetonitrile / 0.1% formic acid. Pressure- 1036 psi.

Figure 32. Pressurized CEC analysis using 70:30 acetonitrile / 0.1 % formic acid, formate buffer pH-8.5, pressure-1022 psi.

Figure 33. Pressurized CEC analysis using 70:30 acetonitrile / (0.1% formic acid). Flow rate- 70 microliters per minute, pressure-1022 psi.

Pressurized CEC analysis was also done on this column, and Figure 37 shows that in this mode the analysis time is shorter due to the presence of electroosmotic flow along with the pressurized flow using 70:30 acetonitrile / 0.1% formic acid in water the analysis time is almost half of that in capillary LC. Figure 38 shows the voltage vs migration time of the compounds. It can be seen from this figure that as the voltage increases the analysis time of the compounds becomes shorter. Capillary LC analysis of guanosine and thymine is seen in Figure 39 and Figures 40 to 45 show the pressurized CEC analysis of the nucleobases and nucleosides. The increase of electroosmotic flow with an increase in voltage is shown in Figure 46. Steroid hormones are not totally hydrophobic compounds and thus separation at higher concentrations of acetonitrile in capillary LC was unusual. To confirm this observation, analyses were carried out on a regular HPLC column.

1.8 Analysis of steroid hormones using standard/normal analytical HPLC

In order to verify that the retention behavior of steroid hormones is reproducible on a standard HPLC column, analysis of steroid hormones were carried out using Microsolv Technology Bidentate C18 HPLC column (i.d. 4 micron).

Isocratic analyses of steroid hormones were carried out on a Waters HPLC instrument with autosampler and photodiode array detector. Figure 47 shows the separation of the four steroid compounds using ninety percent acetonitrile. It can be seen from Figures 47 to 50 that as the acetonitrile concentration decreases the compounds are retained for a longer time on the column. Thus the compounds showed reversed phase behavior and in spite of having polar groups all four compounds behaved the same way as that in capillary liquid chromatography. Figure 51 shows the retention map of the

Figure 34. Capillary LC analysis of a mixture of hormones using 90:10 acetonitrile / 0.1% formic acid. Flow rate-70 microliters per minute, pressure-1022 psi, prednisolone (12.4 mins), corticosterone (15.2 mins), levo norgestrel (17.3 mins), progesterone (25.8 mins).

Figure 35. Capillary LC of a mixture of hormones, prednisolone (18.7 mins), corticosterone (25.1 mins), levo norgestrel (38.2 mins), progesterone (68.6 mins) using 70:30 acetonitrile / 0.1% formic acid. Flow rate – 70 microliters per minute, pressure – 1022 psi.

Figure 36. Retention map of steroid hormones, prednisolone, corticosterone, norgestrel and progesterone on bidentate C18 (i.d. 1.8 micron) in capillary LC.

Figure 37. Pressurized CEC of a mixture of hormones; prednisolone (6.811 mins), corticosterone (9.064 mins), norgestrel (13.733 mins) and progesterone (24.398 mins) using 70:30 acetonitrile / 0.1% formic acid. Voltage-20kv, flow rate-70 microliters per minute, pressure-1022 psi.

Figure 38. Plot of migration time vs voltage of steroid hormones using 70:30 acetonitrile / 0.1% formic acid.

Figure 39. Capillary LC of a mixture of guanosine (13.357 mins) and thymine (14.648 mins) using 70:30 acetonitrile / 0.1% formic acid. Flow rate-70 microliters per minute, pressure-1022 psi.

Figure 40. Pressurized CEC of mixture of guanosine (6.0 mins) and thymine (7.1 mins) using 70:30 acetonitrile / 0.1% formic acid. Voltage-15kv, pressure- 1022 psi, flow rate- 70 microliters per minute.

Figure 41. Pressurized CEC of a mixture of guanosine (7.117 mins) and thymine (7.958 mins) using 70:30 acetonitrile / 0.1% formic acid. Voltage-10kv, flow rate- 70 microliters per minute, pressure- 1022 psi.

Figure 42. Pressurized CEC analysis of a mixture of guanosine (4.819) and adenosine (6.315) using 80:20 acetonitrile / 0.1% formic acid. Voltage-20 kv, flow rate - 70 microliters per minute, pressure- 1022 psi.

Figure 43. Pressurized CEC of a mixture of thymine (5.094 min) and adenosine (6.315 min) using 80:20 acetonitrile / (0.1% formic acid). Voltage-20 kv, pressure- 1022 psi, flow rate-70 microliters per minute.

Figure 44. Pressurized CEC of a mixture of adenosine (9.37 min) & adenine(10.30 min) using 90:10 acetonitrile / 0.1%formic acid. Flow rate-70 microliters per minute, voltage-10 kv.

Figure 45. Pressurized CEC of a mixture of guanosine (5.822 min) and thymine (6.462 min) using 70:30 acetonitrile / (0.1% formic acid). Voltage-15 kv, pressure-1022 psi, flow rate-70 microliters per minute.

Figure 46. Plot of migration time vs voltage of nucleosides using 80:20 acetonitrile / 0.1% formic acid, pH 4.41 acetic acid buffer.

Figure 47. HPLC analysis of steroid hormones. A = Prednisolone. B= Corticosterone, C= Levo Norgestrel, D= Pregesterone. mobile Phase – 90:10 acetonitrile / (0.1% formic acid). Flow rate - 0.5ml per minute, pressure-380 psi.

Figure 48. Analysis of steroid hormones. A – Prednisolone, B- Corticosterone, C- Levo Norgestrel, D- Progesterone. Mobile phase – 80:20 acetonitrile (0.1% formic acid). Flow rate - 0.5 ml per minute.

Figure 49. Analysis of steroid hormones. A – Progesterone, B-Corticosterone, C- Levo Norgestrel, D- Progesterone, mobile phase- 60:40 acetonitrile / 0.1% formic acid. Flow rate - 0.5 ml per minute.

Figure 50. Analysis of steroid hormones. A – Progesterone, B-Corticosterone, C- Levo Norgestrel, D- Progesterone, mobile phase- 50:50 acetonitrile / 0.1% formic acid. Flow rate - 0.5 ml per minute.

Figure 51. Plot of retention time vs percentage of acetonitrile used for the analysis of steroid hormones, prednisolone, corticosterone, norgestrel and progesterone on standard HPLC column (i.d. 4 micron).

steroid hormones and it can be confirmed from the retention map that the steroid hormones showed reversed phase behavior.

2.0 Conclusions

2.1 Summary of Results

The main goal of the research was to compare the two techniques, capillary LC and pressurized CEC, using two different types of bidentate C18 packed capillary columns, a 4 and 1.8 μm form. A commercial C18 capillary column was used in a subset of experiments for comparison. Table 8 lists the types of experiments conducted and summarizes the overall changes in performance.

2.2 Organosilane versus Bidentate Stationary Phases

A baseline for gauging the performance of the bidentate systems was made by analyzing a mixture of steroids (prednisolone, corticosterone, levo-norgestrel, and progesterone) on the commercial and bidentate columns. As might be expected these steroid hormones showed reversed phase behavior and were well separated on these columns. The retention order of the four compounds was found to be as follows:

Prednisolone < corticosterone < levo-norgestrel < progesterone. Table 9 summarizes the results of steroid obtained in all the three capillary columns. The bidentate columns exhibited less peak tailing (e.g. progesterone), higher efficiency and greater retention (20 versus 14 minutes at 70% CH_3CN) implying a likely larger volume and perhaps more uniformly hydrophobic phase for the bidentate system relative to the commercial organosilane one.

2.3 HPLC vs PCEC Operation

The overall comparison between purely pressure driven and electroosmotically assisted pressure driven flow was instructive as well. In general, and as expected, it was

Table 8. Comparison of pumped flow vs pumped plus electroosmotic flow

	Pumped flow: HPLC mode		Pumped plus Electroosmotic: PCEC mode:		Differences observed for PCEC relative to HPLC:	
					Retention time (s)	Efficiency (plates):
C18 commercial 5 micron	Steroids	✓	Steroids	✗		
	Drugs	✗	Drugs	✗		
	Nucleobases	✗	Nucleobases	✗		
	Nucleosides	✗	Nucleosides	✗		
C18 bidentate 4 micron	Steroids	✓	Steroids	✓	decreased	decreased
	Drugs	✓	Drugs	✓		
	Nucleobases	✓	Nucleobases	✓		
	Nucleosides	✓	Nucleosides	✓		
C18 bidentate 1.8 micron	Steroids	✓	Steroids	✓	decreased	decreased
	Drugs	✗	Drugs	✗		
	Nucleobases	✓	Nucleobases	✓	decreased	decreased
	Nucleosides	✓	Nucleosides	✓		
✓ indicates experiments done, ✗ indicates not done						

Table 9. Summary of results of steroid experiments in all three packed capillaries

Steroid HPLC experiments			
	C18 commercial	C18 bidentate 4 micron	C18 bidentate 1.8 micron
Efficiency (plates)	700,000	800,000	13,000,000
Elution order RP/NP/ANP	RP	RP	RP
Mixture elution time.	6.0	6.4	25.8

found that the pressure driven flow was slower than electroosmotically assisted flow yielding uniformly faster analysis for PCEC when comparing the same SP particle sizes (4 μm). For example the mixture of steroid hormones eluted in approximately nine minutes using PCEC at 20 kV (70% CH_3CN / 0.1% formic acid) but took twenty two minutes when eluted using pressure only. Table 8 shows the comparison of pumped flow vs pumped plus electroosmotic flow. The comparison was even more striking for the 1.8 μm stationary phase – 24 versus 68 minutes for PCEC relative to HPLC modes. Increasing the driving voltages for PCEC separation (5, 10, 15, and 20 kV) yielded shorter analysis times but at the expense of resolution.

2.4 Effect of SP Particle Size

All the test solutes including steroid hormones, drugs, nucleobases, and nucleosides were analyzed on both the 4 and 1.8 μm columns and using both capillary LC and pressurized CEC separation mode so a good basis for comparison between them exists. Overall, the 1.8 μm system showed yielded higher efficiencies but at the expense of longer analysis times. PCEC hybrid pumping compensated for this, but generally at the expense of some loss in efficiency.

2.5 General Performance Characteristics of the 4 μm Bidentate C18 Capillary Column

The bidentate stationary phases are prepared by a hydrosilation route using an alkyne (1-octadecyne) that upon reaction makes mainly two Si-C bonds at the bound terminus. As noted above, this column yielded the same elution order (and reverse phase

behavior) but greater overall efficiency when compared to the commercial organosilanized C18 capillary column.

Among the three drugs, metformin and amphotericin showed aqueous normal phase behavior and sulfonamide showed reversed phase behavior. These drugs were not analyzed on the commercial C18 column above, but literature reports indicate that when metformin and amphotericin were analyzed on C18 HPLC column it showed aqueous normal phase behavior [21]. Hence, the aqueous normal behavior of metformin and amphotericin was expected. All the nucleobases and nucleosides showed reversed phase behavior in capillary LC. Because nucleobases and nucleosides are quite polar, aqueous normal phase behavior might be expected under these conditions but elution was found to be universally consistent with reverse phase retention. The best separation conditions for that were found in this study of these compounds were at 50:50 = acetonitrile: 0.1% formic acid in water (v/v). This composition was able to resolve adenine, guanine, and cytosine but did not resolve the mixtures including all nucleobases or nucleosides. For further study these compounds were injected individually.

Since sample requirement is low and both pressure and voltage can be adjusted to elute a compound in Trisep-2100, future work should focus on separation of some challenging compounds like proteins using a pressurized CEC system. For improvements in mass detection sensitivity, column efficiency, and peak resolution, further developments in capillary LC separation methods are quite crucial. The lower flow rates of the capillary columns are ideal for coupling the system with a mass spectrometer. Reduction of mobile phase consumption makes it more environment friendly and

reduction of the amount of stationary phase facilitates the use of expensive materials for the synthesis of separation materials. Development of multifunctional systems that allow switching between CE, CEC, pressurized CEC and LC modes can be anticipated and conditions customized for each separation can be selected.

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