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Synthesis and application of steroid bonded stationary phases for HPLC & LC-MS

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SYNTHESIS AND APPLICATION OF
STEROID BONDED STATIONARY PHASES
FOR HPLC & LC-MS

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In partial fulfillment

of the Requirement for the Degree

Master of Science

By

Lopa Dalal

August 2005

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ABSTRACT

SYNTHESIS AND APPLICATION OF STEROID BONDED STATIONARY PHASES FOR HPLC AND LC-MS

by Lopa Dalal

The analysis of bioactive compounds like steroids and catecholamines is considered a challenging problem for separation technology. In this study, new stationary phases were prepared using synthetic steroid molecules to study separation of steroids and catecholamines. Stationary phases were prepared via a silanization/hydrosilation process, in which an organic moiety is attached to the silica hydride surface. Bonded stationary phases were characterised using spectroscopic methods. To compare the separation efficiency of newly synthesised columns, a number of other columns were used. Separation of steroid hormones was studied on all the columns using high performance liquid chromatography.

A mass spectrometer detection system was coupled to HPLC to make it easier to detect steroid solutes, which were difficult to detect solely based on retention time. LC-MS separations of steroids and catecholamines were studied on estradiol-bonded columns as well as on all other columns used for comparative studies.

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I INTRODUCTION

A. Chromatography

Chromatography is one of the most widely used analytical techniques in separation science. It was originally described by the Russian scientist Michael Tswett in 1906 [1]. The components to be separated are distributed between two phases: a stationary phase bed and a mobile phase which percolates through the stationary phase bed. A column holds the stationary phase and the mobile phase carries the sample through it. Sample components that partition strongly into the stationary phase spend a greater amount of time in the column and are separated from components that stay predominantly in the mobile phase and pass through the column faster.

According to the nature of the stationary phase and the mobile phase, chromatography can be mainly divided into two categories, (1) gas chromatography (GC) in which the mobile phase is a gas and the stationary phase is usually a liquid on a solid support or sometimes a solid adsorbent, and (2) liquid chromatography (LC) in which the mobile phase is a solvent and the stationary phase is a liquid on a solid support, a solid, or an ion-exchange resin. Unlike gas chromatography, liquid chromatography provides separation of non-volatile samples and ionic solutes [2]. During the 1970's, most chemical separations were carried out using a variety of techniques including open-column chromatography, paper chromatography, and thin-layer chromatography. However, these chromatographic techniques were inadequate for quantification of compounds and resolution between similar compounds. During this time, high pressure

liquid chromatography (HPLC) began in which separation is carried out at high pressure (up to 6000 psi). HPLC separations quickly improved with the development of column packing materials and the additional convenience of on-line detectors [3].

Depending on the relative polarities of both the stationary phase and the mobile phase, chromatography can be divided into two types: (1) normal phase, where the mobile phase is non-polar and stationary phase is polar and (2) reverse phase, where the mobile phase is polar and the stationary phase is non-polar. In the late 1970's, new methods including reverse phase liquid chromatography allowed for improved separation between compounds of very similar polarities. By the 1980's HPLC was commonly used for the separation of chemical compounds. New techniques improved separation, identification, purification and quantification far above the previous techniques. Computers and automation added to the convenience of HPLC [4].

The two crucial components of HPLC are the stationary phase and the mobile phase. The sample components carried by a mobile phase are retarded by the stationary phase based on various interactions such as: surface adsorption, relative solubility and charge. The composition of the mobile phase can be altered in order to manipulate the interaction of the solute with the stationary phase. The mobile phase composition can be either isocratic or gradient, depending on the demands of the separation. When the solvent composition remains constant throughout the analysis the elution is called isocratic, and when the solvent composition varies during the analysis, the process is termed gradient elution. "Stationary phase" stands for that part of the chromatographic system, which is in equilibrium with the mobile phase. In HPLC, the solid stationary

phase is rigid and impermeable for the analyte molecules so only the surface of the stationary phase actually participates in the chromatographic retention process. In order to increase the variety of interactions between the solutes and the stationary phases, a variety of functional groups have been bonded to the silica surface. The most commonly used stationary phase for liquid chromatographic separation is a hydrocarbon chain bonded on the solid support [2].

B. Silica Surface

Typical HPLC stationary phases are small rigid porous particles with high surface area. Although alumina and zirconia are used as inert supports, silica is the most commonly used base material in both normal- and reverse-phase chromatography. The chemistry of silica is well known and it can be manufactured in many different forms with a variety of different pore sizes and surface areas. The silica used in this research is called AstroSilTM, and it has a high surface area compared to many other types of silica. The chemical composition of porous silica is expressed as $\text{SiO}_2 \times \text{H}_2\text{O}$. 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 maximum concentration of silanol groups on the surface of porous silica is around 5 -OH groups per 100 Å². These silanol groups are weakly acidic and can interact with polar compounds through strong hydrogen bonding and dipole-dipole interactions resulting in peak tailing and loss of chromatographic resolution. Silica bears three kinds of silanols on the surface: isolated, geminal, and vicinal [5]. Each of the silanol species present in the silica structure are illustrated in Figure 1.

Figure 1. The silanol species present on the silica surface.

C. Surface Modification

Although silica supports are still superior to alumina or zirconia supports in terms of efficiency, rigidity, and performance, there are several problems with silica based materials, including severe peak tailing with the use of basic solutes, limited pH stability, and irreproducibility for the same chemistry columns. To overcome these problems, chemically modified versions of silica have received considerable attention. The silanols on the silica surface serve as attachment points that anchor bonded phases to the silica support [5]. There are several types of reactions that can be used to covalently bond organic moieties onto the silica surface. One type is an esterification process, which involves a reaction between an alcohol and the silanol.

This simple coupling reaction results in the formation of a silicon-oxygen-carbon (Si-O-C) linkage between the silica surface and the bonded organic moiety. This linkage is hydrolytically unstable and so this modified surface does not offer many advantages in chromatographic separations [6]. The second process used for silica surface modification is an organosilanization reaction.

This is the most common process used for silica surface modification. This reaction yields a Si-O-Si-C linkage, which is both thermodynamically and hydrolytically stable. A monomeric or polymeric stationary phase can be achieved using different organosilane reagents [6, 7]. A third method involves chlorination of the silica surface. This is a two step process which results in a monomeric bonded material but with a different linkage than the previous reactions.

The first step is the chlorination of silica by reacting the surface with a reagent such as thionyl chloride. The second step involves an organometallic reaction which bonds the desired ligands to the surface. This reaction results in a direct Si-C linkage which is hydrolytically more stable. This process also has some limitations: (1) it produces a chlorinated intermediate which is hydrolytically unstable, and (2) the by-product of the first reaction is a metal salt which can contaminate the surface [6, 7]. Because of these limitations there was a need for a reaction that resulted in a direct Si-C linkage but which was less cumbersome than the chlorination reaction. Silanization/hydrosilation, a two step process, first introduced by Sandoval and Pesek results in the same Si-C linkage but via a simpler synthetic procedure [8]. This process leads to superior coverage of the silica surface, and higher hydrolytic stability of the bonded group.

Silanization/hydrosilation is a novel synthetic approach to produce a chemically bonded silica based chromatographic stationary phase. This process involves preparation of the silica intermediate with a stable silicon hydride linkage which is then reacted with a terminal olefin in the presence of a suitable catalyst. The first reaction is called the silanization process which involves the preparation of the silica hydride intermediate by reacting the surface silanols with triethoxysilane (TES) in the presence of water, an acid catalyst, and an appropriate solvent. This reaction forms an Si-H monolayer on the silica surface. This step is followed by hydrosilation, which involves a reaction between the silica hydride and a terminal olefin. This step results in a direct Si-C linkage [9, 10]. The hydrosilation reaction is carried out in the presence of a transition metal complex. The most commonly used complex is Speier's catalyst, first introduced by Speier to catalyze

this reaction [10]. The catalyst is a solution of chloroplatinic acid hexahydrate ($\text{H}_2\text{PtCl}_6\cdot 6\text{H}_2\text{O}$) dissolved in 2-propanol. For the bonding of an organic group to the silica hydride surface an induction period is often required before the addition of silica hydride. During the induction period platinum forms a complex through the olefin group of the organic moiety. In order to obtain maximum surface coverage, an excess of organic group is usually used with respect to the silica hydride. The complete process of these reactions is shown below.

TES Silanization

Hydrosilation

The silanization/hydrosilation process has a number of advantages over the other synthetic procedures used for the production of bonded phases. The silanization reaction results in greater column efficiency due to a reduction in surface silanols. The other

advantage is that a large number of functional groups can be bonded using a variety of catalysts. In the hydrosilation reaction Speier's catalyst forms a complex with the silica hydride during the intermediate step of the olefin addition. The intermediate species thus formed can be of significant size which may result in lower yield. The second disadvantage of using Speier's catalyst is that the reaction between the hydride and the metal can result in the deposition of Pt (0) on the surface. An alternative catalyst for the hydrosilation process is a free radical initiator compound such as tert-butyl peroxide [11]. In this study the hydrosilation reaction is carried out using both Speier's catalyst and a free radical catalyst. The organic moieties used for bonded phases are 1-octadecyne and estradiol.

D. Alkyne Bonded Phase

The silanization/hydrosilation method allows for the bonding of the organic moiety to the silica hydride surface via a number of different multiple bond functional groups. The most common group used for bonding is an olefin. Studies of attaching different organic moieties showed that there can be number of possible ways for bonding to silica hydride through multiple bond functional groups on the organic moiety. The hydrosilation reaction of the 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 high degree of stability and can be used in both reversed phase and normal phase chromatography. All the possible structures for attachment, when an alkyne organic group is used, are shown in Figure 2 [12]. In this research study all three

organic compounds used for the hydrosilation reaction contain an alkyne terminal functional group.

Figure 2. Alkyne bonded to silica hydride surface.

E. Cholesterol Bonded Phase

Different stationary phases with diverse properties are recommended for the HPLC analysis of biologically active compounds. To achieve the desired results, different functional groups provide a variety of properties for the packing materials. The new type of packing material prepared by bonding of cholesterol compounds on the silica surface leads to a class of stationary phase that is especially of interest to research chromatographers. Previous studies have shown that the cholesterol phase is fundamentally different than common reversed-phase materials and shows very good shape recognition capabilities [13]. This unique separation property of the cholesterol bonded phase can be explained by its liquid crystal properties. A liquid crystal is a state of matter that is intermediate between a solid crystalline phase and an isotropic liquid

phase. The distinctive characteristic of the liquid crystalline state is the tendency of the molecules to point along a common axis, called a director. This phenomenon is in contrast to molecules in the liquid phase which have no intrinsic order and in the solid state where molecules are highly ordered and have little translational freedom. The characteristic orientational order of the liquid crystal state is between the traditional solid and liquid phases [14]. To understand the separation behavior of a bioactive material bonded stationary phases, one of the goals of this study is to synthesize steroid hormone bonded stationary phases via the hydrosilation/silanization process. All of the steroids except retinoic acid are derived from cholesterol and so they are expected to show unique separation properties like cholesterol bonded phases. Two estradiol steroid compounds used for the preparation of the bonded stationary phases are shown in the Figure 3. Estradiols are the most active among all the estrogens in the human body, responsible for stimulating growth of the female reproductive system.

Figure 3. Structures of (A) 17α-ethynylestradiol and (B) ethynylestradiol 3-methylether.

F. Steroids

The analysis of steroids is considered a challenging problem for separation technology. The study of steroid based compounds is rapidly gaining interest because numerous synthetic steroids have been used as therapeutic agents. Due to the metabolic versatility of steroid molecules, extremely complex mixtures are often encountered, necessitating the use of a chromatographic procedure prior to measurement. Gas chromatography and high-performance liquid chromatography are commonly used separation techniques for steroid analysis but GC is limited to volatile compounds, so LC has become the most popular analytical method [15]. A complete separation of five different steroids was achieved by Pesek and coworkers using a cholesterol bonded stationary phase [13]. As mentioned above, estradiol based compounds are derived from cholesterol, and since the cholesterol bonded phase resulted in successful separation of steroids, the focus of this research is to study the analysis of steroids using steroid bonded stationary phases.

Using conventional detectors (e.g., UV-VIS) in LC analysis there is always some ambiguity as to whether the measured peaks are actually those of the target compounds or (co-eluting) interferants. In such cases, on-line LC-MS (mass spectrometry) is useful in producing positive identifications of components in complex mixtures and an overall reduction in analysis time with respect to off-line techniques, even for unresolved peaks. Some steroids showed more than one peak during HPLC separation and to understand this result the fragmentation pattern of the LC-MS technique is used in this research

study. Numerous reports that deal with LC-MS analyses of hormones, pollutants, drugs, etc., exist [16, 17].

G. Liquid Chromatography Mass Spectrometry (LC-MS)

Mass Spectrometry is an analytical technique that is used to identify unknown compounds, quantify known materials and elucidate the structural and physical properties of ions. The use of the hyphenated technique liquid chromatography–mass spectrometry (LC–MS) in the analysis of biomolecules provides important advantages because of the combination of the separation capabilities of LC and the power of MS as an identification and confirmation method. The principal components of a mass spectrometer are shown in Figure 4.

Figure 4. Components of a mass spectrometer.

A mass spectrometric analysis involves the following steps: (1) injecting a minute amount of sample through inlet system (which in this case is an LC) to the ion source, (2) converting sample components into gaseous ions by thermal or electrical energy, (3) separating ions in the mass analyzer on the basis of their mass-to-charge ratio (m/z), where m is the mass of the ion and z is the charge, and (4) converting the beam of ions to an electrical signal using a transducer which can then be stored in the memory of a computer [2].

Vacuum in the system is generally achieved using a combination of direct drive rotatory and turbo molecular pumps. Previous studies have shown that electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) techniques are effective for the analyses of biomaterials [18]. In this study, APCI is used as an ion source. APCI generally produces protonated or deprotonated molecular ions from the sample via a proton transfer (positive ions) or proton abstraction (negative ions) mechanism. For this study all analyses were carried out in positive ion mode. For the coupling of LC to the APCI system, a heated nebuliser inlet probe is used which is made up of a pneumatic nebuliser and a large heated quartz tube. The sample is vaporized in a heated nebuliser before emerging into a plasma consisting of solvent ions and formed within the atmospheric source by a corona discharge. Proton transfer then takes place between the solvent ions and the sample. Volatile ions are swept to the mass analyzer while non volatile components are swept away easily by the counter-current drying gas [19]. A number of systems are available to detect the mass separated ions. The detection system used in this study is an electronmultiplier with quadrupole mass analyzer.

H. Catecholamines

One of the main advantages of coupling LC and MS is that compounds that are not detected by conventional HPLC detectors such as UV or PDA (photo diode array) can be easily detected by MS. One such class of compounds are catecholamines.

Catecholamines include such compounds as epinephrine, norepinephrine, dopamine, and L- α -methyl-dopa. One of the aims of this work is to study the separation of mixtures of these compounds using a LC-MS system. Catecholamines play a very important role in the control and regulation of a variety of functions in the nervous system. Measurement of catecholamines may be important for monitoring changes in psychiatric disorders [20]. In the past, catecholamine separations were studied using ion pair reversed-phase HPLC with electrochemical detection or ion exchange chromatography with electrochemical detection. These separation techniques were used because catecholamines are polar molecules and therefore their retention on a standard reversed phase is poor. These methods suffered from spurious HPLC peaks, long column equilibration time and poor HPLC peak shape [21]. One of the aims of this work is to study the separation behavior of catecholamines on steroid bonded columns in the reversed phase mode. Methods based on liquid chromatography with mass spectrometry have been successfully used for the determination of neurotransmitter compounds [22, 23].

I. Instrumentation

Diffuse-reflectance infrared Fourier transform spectrometry (DRIFT), nuclear magnetic resonance spectrometry (NMR) and carbon elemental analysis were the

techniques used to evaluate the hydride-modified material and the bonded silica. A general description of these techniques is given below.

1. DRIFT background

Fourier transform infrared spectroscopy provides qualitative information about the structure of a molecular species. The mid-infrared spectrum for organic compounds, 4000-400 cm^{-1} , provides a unique absorption pattern for each compound, and hence it can be used to determine structural information for molecules. The most widespread technique that is used for the analysis of solids is called diffused reflectance infrared Fourier transform spectroscopy (DRIFT). In, this technique, a finely ground solid sample is irradiated with radiation ranging in wavelength from 4000 to 450 cm^{-1} . The radiation excites vibrational modes of the analyte molecule and as a result diffuse reflectance occurs in all directions. The changes in the IR spectrum are very distinct thus revealing the specific surface chemistry. This feature shows that DRIFT is a powerful technique for obtaining qualitative information about different types of materials and it can be used for the study of both native and modified surfaces. DRIFT spectroscopy is used to study formation of the hydride on the silica surface and also to confirm bonding of the organic group on the silica hydride surface [24].

2. NMR background

Nuclear magnetic resonance spectroscopy is one of the most powerful tools available for elucidating the structure of chemical species. It is based on the measurement of electromagnetic radiation in the radio-frequency region of roughly 4 to 900 MHz. In pulse NMR measurements, the nuclei in a strong magnetic field are irradiated

periodically with an intense pulse of radio frequency radiation. The pulse length is usually in the μs range and the delay between pulses is approximately one second. In between the pulses, a time-domain radio frequency signal, called the free induction decay (FID), is emitted as the nuclei begin to relax and return to their equilibrium position. The FID represents all of the frequencies emitted by the nuclei present in the sample and are characteristic of the chemical environment.

In solid-state NMR, lines are much broader than in solution NMR due to faster relaxation and due to the anisotropy of interactions. The situation is worse for ^{13}C nuclei, because of their low natural abundance; the signal-to-noise ratio is often so poor that the spectra are virtually useless. To achieve significant improvements in resolution and sensitivity a technique called magic-angle spinning (MAS) with cross polarization (CP) is used. MAS involves rotating the solid sample rapidly ($>2\text{ KHz}$) in an air-driven rotor at an angle of 54.7° with respect to the applied field. MAS eliminates the line broadening due to chemical shift anisotropy and enhances the resolution. The CP technique transfers energy absorbed by the ^1H nuclei to the nuclei of low natural abundance such as ^{13}C . This increases the signal intensity and also helps to relax the ^{13}C nuclei faster allowing the spectrum to be taken more frequently [24].

3. Carbon elemental analysis background

The CHN elemental analyzer determines the carbon, hydrogen, and nitrogen content of organic compounds. The micro-combustion method is used for determining the carbon percentage of the modified silica surface. The carbon percentage value is then used to determine the surface coverage of silica with organic modifiers.

J. Aims of this Work

The present research has several fundamental objectives. The main goal is to synthesize steroid bonded stationary phases and to test the applicability of these materials for the separation of biochemical compounds. The steroid based compounds used for synthesis are 17 α -ethynylestradiol and ethynylestradiol 3-methyl ether. Bonded stationary phases were prepared via the silanization/hydrosilation method. The reaction was carried out in the presence of both Speier's catalyst and t-butyl peroxide catalyst.

The other goal of this work is to do a comparative study on different columns. For the comparative study, bare silica, endcapped silica, silica hydride, various C8 bonded silicas, some C18 bonded silicas and commercial cholesterol bonded silica columns are used. Also to study the effect of different catalysts, the 1-octadecyne bonded stationary phase was prepared via the silanization/hydrosilation method with a t-butyl peroxide catalyst.

The modified silica was investigated using different characterization techniques. These techniques include DRIFT, ^{13}C CP-MAS NMR, ^1H and ^{13}C NMR, and carbon elemental analysis. These methods were used to determine the success of bonding and the surface coverage of these estradiol based compounds and 1-octadecyne on silica.

Separation of three different sets of solutes was studied on all columns. The first test was performed to prove the character of the bonded stationary phase. A mixture of three solutes with different polarities was used for this test. The second test involved a mixture of six different hormones. For this test, elution conditions were first optimized using reversed phase HPLC on all the columns and then the optimum conditions were

used for a comparative study by LC-MS. For the third test, five catecholamine compounds were used and the separation was studied on all the columns using the same conditions with LC-MS.

II EXPERIMENTAL

A. Materials

All the stationary phases were prepared using AstroSil[®] silica gel. It was obtained from Stellar Phases, Inc. (Langhorne, PA). It has a particle size of 4.2 microns, a pore size of 105 Å and a surface area of 340 m²/g. The other materials used for this research are listed in Tables 1 and 2.

Table 1. Chemicals used in synthesis and characterization.

Chemical name	CAS Registry number	Manufacturing Company
1-octadecyne	629-89-0	GFS Chemicals, Columbus, OH
Ethynylestradiol 3-methyl ether	72-33-3	Aldrich Chemical Co., St Louis, MO
17 α -ethynylestradiol	57-63-6	Aldrich Chemical Co., St Louis, MO
Hexachloroplatinic acid	16941-12-1	Aldrich Chemical Co., Milwaukee, WI
Dioxane	123-91-1	Fisher Chemicals, Fair Lawn, NJ
Triethoxysilane	78-07-9	Aldrich Chemical Co., Milwaukee, WI
Toluene	108-88-3	Fisher Chemicals, Fair Lawn, NJ
Diethyl ether	60-29-7	Fisher Chemicals, Fair Lawn, NJ
Methylene chloride	75-09-2	EM Science, Gibbstown, NJ
t-butyl peroxide	110-05-04	Aldrich Chemical Co., Milwaukee, WI
Chloroform	67-66-3	J.T.Baker Chemical Co., Phillipsburg, NJ

Table 2. Chemicals used in HPLC and LC-MS separation.

A. Chemicals used for separation by standard reference test.

Chemical name	CAS Registry number	Manufacturing Company
Pyridine	110-86-1	Sigma, St Louis, MO
Uracil	66-22-8	Matheson Coleman & Bell, Ohio, NJ
Phenol	108-95-2	Aldrich Chemical Co., Milwaukee, WI
Phosphoric Acid	7664-38-2	Aldrich Chemical Co., Milwaukee, WI
Acetonitrile	75-08-8	Spectrum Mfg, Corp., Gardena, CA

B. Chemicals used for steroid separation.

Chemical name	CAS Registry number	Manufacturing Company
Adrenosterone	382-45-6	Aldrich Chemical Co., Milwaukee, WI
Corticosterone	50-22-6	Aldrich Chemical Co., Milwaukee, WI
11 α -acetoxyprogesterone	2268-98-6	Aldrich Chemical Co., Milwaukee, WI
4-androstene-3,17-dione	63-05-8	Aldrich Chemical Co., Milwaukee, WI
Estrone	53-16-7	Aldrich Chemical Co., Milwaukee, WI
Estradiol	50-28-2	Aldrich Chemical Co., Milwaukee, WI
Methanol	67-56-1	J.T. Baker Chemical Co., Phillipsburg, NJ
Formic acid	64-18-6	Aldrich Chemical Co., Milwaukee, WI

C. Chemicals used for catecholamine separation

Chemical name	CAS Registry number	Manufacturing Company
Dopamine hydrochloride	62-31-7	Sigma Chemical Co., St Louis, MO
(-)-3-(3,4-Dihydroxyphenyl)-2-methyl-L-alanine sesquihydrate	41372-08-1	Aldrich Chemical Co., St Louis, MO
Ephedrine hydrochloride	134-71-4	Sigma Chemical Co., St Louis, MO
(-)-Norepinephrine	51-41-2	Sigma Chemical Co., St Louis, MO
(-)-Epinephrine	51-43-4	Sigma Chemical Co., St Louis, MO
Ammonium Formate	540-69-2	Fisher Chemicals, Fair Lawn, NJ
Acetonitrile	75-05-8	Spectrum Mfg, Corp., Gardena, CA

B. Instrumental Procedures

1. Diffused reflectance Infrared Spectroscopy (DRIFT)

DRIFT spectra were taken using an ATI Mattson Infinity series FTIR spectrometer equipped with a deuterated triglycine sulfate (DTGS) detector and controlled by a digital Venturis Fx-2 series computer with WinFIRSTTM software. An EasiDiff diffuse-reflectance accessory with a 1.6 mm deep sampling cup was used. The samples were prepared by mixing the bonded silica with 5% (by wt) KBr and finely grounding the mixture and filling in the sample cup so as to leave a smooth sample surface. The instrument was purged (55-60 psi) with nitrogen gas from a liquid nitrogen tank to provide a moisture-free sample atmosphere. Spectra were collected at 2 cm⁻¹

resolution in the region of 4000-400 cm⁻¹ and averaged over 100 scans. All sample scans were referenced to KBr.

2. Nuclear Magnetic Resonance Spectroscopy (NMR)

A Varian INOVA 400 MHz NMR spectrometer system with VNMR 6.1B software was used for characterization of the material used for bonding. ¹H and ¹³C NMR spectra for ethynylestradiol 3-methyl ether were performed using a 1 mg sample dissolved in deuterated chloroform (CDCl₃) solvent in a 5 mm o.d glass tube (Wilmad Glass Co., Inc.). ¹H and ¹³C NMR spectra for 17 α -ethynylestradiol were performed using saturated solution of sample dissolved in deuterated chloroform.

Solid state NMR spectra with cross polarization (CP) and magic angle spinning (MAS) were obtained on the same spectrometer. MAS spectra were obtained at a spinning rate of 5 KHz and 300 to 500 mg of the bonded silica sample was placed in a double bearing ZrO₂ rotor.

3. Carbon Elemental Analysis

All the bonded phases were sent to Desert Analytics (Tucson, AZ) for the determination of the carbon content in each sample. A conventional combustion method was used for the analysis. The elemental analysis results were then used to calculate the surface coverage of the bonded phase. The surface coverage was calculated using the equation proposed by Berendsen and De Galan [25].

$$\alpha_R (\mu\text{mol/m}^2) = \frac{10^6 P_c}{(100M_c n_c - P_c M_R) S_{\text{BET}}} \quad (6)$$

Where P_c is the difference in carbon percentage of the modified silica and the silica hydride, n_c is the number of carbon atoms present in the bonded phase, M_c is the atomic weight of the organic compound. BET refers to the nitrogen absorption method to determine the surface area. S_{BET} (m^2/g) is the specific surface area of the silica substrate. The surface coverage values are presented in the Results section.

4. High Performance Liquid Chromatography (HPLC)

The liquid chromatograph used in this work was a Hitachi system equipped with a Hitachi model L-4000 UV detector, a Hitachi model L-6200 gradient pump, a Rheodyne model 7125NS manual injector with 10 μ L loop, and a Hewlett Packard model 3396A integrator. The back pressure was maintained below 1700 psi. The organic solvents and water were degassed using helium gas at a flow rate of 5 mL/min throughout the analysis. For all HPLC separations, a 0.5 mL/min mobile phase flow rate was used. Deionized water was prepared on a Milli-QTM purification system (Milipore Corp., Bedford, MA).

All the columns were packed in-house by a procedure described below. HPLC grade methanol was used as a driving solvent. Approximately 1.75 g of bonded silica was mixed with 90:10 carbon tetrachloride/methanol in a 50 mL beaker followed by 10 minutes sonication. This slurry was added slowly to the reservoir and the remaining volume of the reservoir was filled with filtered methanol. The mixture in the reservoir was packed into the column in the presence of nitrogen under 6000 psi pressure. After completion, the column remained attached to the packing apparatus for about 30 minutes before removal.

Before using the new column for any analysis, it was flushed with methanol for about 20 minutes. The separations were performed under reversed-phase condition. All mobile phases were filtered with a Nylon 66 membrane # 2034 0.2 μm (Alltech Associates, Inc, Deerfield, IL)

All five bonded materials were packed into a stainless steel column (10 cm $\times$ 4.6 mm internal diameter, Alltech Association Inc., Deerfield, IL). Columns used for the comparative study are listed below, some of them were prepared in-house and others were commercial columns. Columns number 1 and 2 were used before for analysis of bromine compounds.

1. UDC cogent cholesterol bonded column, 15 cm $\times$ 4.6 mm ID, Catalog no. 69069-15R, column number M03-CP09-003, particle diameter 5 μm , material lot number 030210-08SJ, (MicroSolv Technology Corporation, NJ).
2. UDC cogent cholesterol bonded column, 15 cm $\times$ 4.6 mm ID, Catalog no. 69069-15R, column number M03-CP09-012, particle diameter 5 μm , material lot number 030210-08SJ, (MicroSolv Technology Corporation, NJ).
3. Bidentate C18 hydride bonded column, 75 $\times$ 4.6 mm, Catalog no. CUSTOM0746, (Higgins Analytical, Inc, CA).
4. Bidentate C18 hydride bonded column, LC-MS format (Sprite), 20 $\times$ 2.1 mm, Catalog no. CUSTOMSPR0221, (Higgins Analytical, Inc, CA).
5. Bidentate C18 (Speier's catalyst), 4.6 $\times$ 75 mm, lot no 11, SP#8-102B, (exposed to pH 8, 10).
6. Bidentate C8, 4.6 $\times$ 75 mm, lot no 040610-03-SJ-C8BDGL, Sp# 8-106A.

7. C8 bonded on hydride (100°A , $5\mu\text{m}$) bonded column $100\times 4.6\text{ mm}$, Lot no 0601169-3, Cat. No. 75008-10R, SN C 0601-130-01, (Cogent)
 8. Bidentate C8 bonded on (Gelest) AstroSil hydride column, $4.6\times 150\text{ mm}$, Lot no- 2-149A.
 9. Endcapped silica hydride, $4.6\times 75\text{ mm}$, lot 030408-11-SJ-HOE2, SP # 8-100A.
 10. AstroSil silica hydride (GELEST HYDRIDE) SP# 8-134a, $4.6\times 75\text{ MM}$.
 11. AstroSil silica (lot 7-122F2, $4\mu\text{m}$) SP # 8-100B.
5. Liquid Chromatography/Mass Spectrometry (LC/MS)

The system consisted of a Waters liquid chromatograph equipped with a Waters 515 pump, a Waters 991 photodiode array detector, a Rheodyne 7725 manual injector with $20\mu\text{L}$ loop, and a computer based data system with Millennium (version 2.1) software to connect with the mass spectrometer. The back Pressure was maintained below 2000 psi. The organic solvents and water were degassed using helium gas at a flow rate of 5 mL/min throughout the analysis.

All LC/MS analyses were carried out using a Micromass Platform LC mass spectrometer system equipped with an Edwards model E2M30 rotary vacuum pump, a Micromass Platform LC model M940150DC1 APCI probe, and a computer based data acquisition system with MassLynx (version 3.4) software. The instrument was purged with high pressure liquid Nitrogen gas (100 psi). The acquisition parameters used for all the separations were as follows: APCI pin 3.20, cone 30, skimmer 2.0, source heater 140°C , APCI probe temperature 600°C , gas flow 250 mL/min , LM resolution 15, HM resolution 15, Ion energy 1.0, multiplier 650. 0.5% formic acid was added to the mobile

phase (methanol:water 50:50) for ionizing the test samples. All LC-MS separations were performed using APCI probe in positive mode.

C. Synthetic Procedures

1. Preparation of silica hydride

The preparation of the silica hydride intermediate was carried out according to a method previously described by Chu et al., [8]. Prior to the synthesis, 15.60 g of silica was weighed and dried overnight at 100°C in a vacuum oven. In a typical procedure, the dried silica was transferred to a 1000 mL, 3-neck round-bottom (RB) flask equipped with a thermometer, a condenser with a drying tube, an addition funnel with an equalizing tube, a stopper, a magnetic stir bar, a heating mantle and a magnetic stirrer. Then 624.0 mL of distilled dioxane was added followed by 7.64 mL of 2.3 M HCl solution. The reaction mixture was heated to about 70°C. Once the temperature was constant, 93.8 mL of dioxane was transferred into the addition funnel equipped with a stopper. Later, under argon, 21.7 mL of triethoxysilane (TES) was added slowly to the addition funnel containing dioxane. The TES/dioxane mixture was then added dropwise into the RB flask over a period of 25-30 minutes with constant stirring. The temperature was raised to 90°C and the mixture was allowed to reflux for 90 min. After a cooling period, the product was carefully transferred into a medium size funnel attached to a filtering flask and the product was filtered using vacuum suction, the upper layer containing the TES/dioxane mixture was filtered leaving the solid silica hydride layer. The product was washed three times with dioxane (50 mL each time), followed by three washings with toluene (50 mL each time) and three times with diethyl ether (50 mL each

time). The final product was dried at room temperature overnight to evaporate the ether and finally dried in a vacuum oven at 110°C for 6 h or more.

2. Hydrosilation using Speier's catalyst

2.1 17 α -ethynylestradiol bonded phase

The bonded phase synthesis was carried out according to a method previously described by Pesek & Matyska [7]. Prior to hydrosilation, all the glassware used was dried in the oven and AstroSil silica hydride was dried overnight in a vacuum oven at 110°C. 2.10 g of 17 α -ethynylestradiol was dissolved in 60 mL of chloroform and was transferred into a 100 mL round bottom flask. Next, 2.08 mL of 10mM hexachloroplatinic acid in 2-propanol (Speier's catalyst) was added. This mixture was then heated at 50°C for 1 hour while being stirred. After this "induction period", when a clear solution was obtained, 2.0 g of hydride silica was added slowly (approximately 15-20 min) to the steroid/catalyst solution. The reaction was allowed to proceed for 96 hours at 58°C with continuous stirring. After cooling, the bonded silica product was transferred into a medium funnel and filtered using vacuum suction. Then, the product was washed with four 30 mL portions of chloroform, followed by similar washings with ethanol and diethyl ether. The final ether solvent was allowed to evaporate at room temperature overnight and the product was then dried under vacuum at 110°C for 6 hours [13].

2.2 Ethynylestradiol 3-methylether bonded phase

A similar procedure as described above was used for ethynylestradiol 3-methyl ether bonded phase synthesis. Here 2.0 g of ethynylestradiol 3-methylether was dissolved in 20 mL of chloroform. Next, 2.08 mL of 10mM Speier's catalyst was added.

After a 1 hour induction period at 50°C, 2.0 g of silica hydride was added slowly to the steroid /catalyst solution. The reaction was allowed to proceed for 96 hrs at 58°C with continuous stirring. After cooling, the washings were done with four 30 mL portions of chloroform, followed by similar washings with ethanol and diethyl ether. The final ether solvent was allowed to evaporate at room temperature overnight and the product was then dried under vacuum at 110°C for 6 hours.

3. Hydrosilation using free radical catalyst

3. 1 1-octadecyne bonded phase

Hydrosilation of the silica hydride surface by free radical addition was carried out according to a method previously described by Pesek et al., [11]. Prior to hydrosilation, silica hydride was dried overnight in a vacuum oven at 110°C. The reaction was carried out in a 100 mL three necked round bottom flask fitted with a thermometer in one neck and a condenser equipped with a drying tube in a second neck. The third neck was closed with a glass stopper. A mixture of 60 mL of distilled toluene, 4 g of 1-octadecyne and 160 μ L of t-butyl peroxide (free-radical catalyst) were added to the flask at the start of the synthesis. This mixture was heated to 60°C and stirred for 1 hour. Then 2.0 g of the silica hydride was added slowly to the flask through the open neck after removal of the glass stopper. The flask was then flushed with nitrogen and all the joints were properly sealed. The flask was then closed and the temperature of the solution with the solid hydride silica was raised and maintained at 100°C with continuous stirring for 100 hours. After this period, the solvent was removed with vacuum suction and the solid was washed with four 30 mL portions of toluene, followed by similar washings with

methylene chloride and diethyl ether. The product was dried overnight at room temperature and then dried overnight at 110°C under vacuum [14].

3. 2 17 α -ethynylestradiol bonded phase

A mixture of 60 mL chloroform, 2.0 g of 17 α -ethynylestradiol and 68 μ l of t-butyl peroxide were added to a 100 mL round bottom flask equipped as described in the above procedure. The mixture was heated at a constant temperature of 50°C for one hour with continuous stirring. Later 2.0 g of dried silica hydride was added slowly through the open neck of the flask. The flask was flushed with nitrogen and the all the joints were sealed. The temperature of the reaction mixture was maintained at 58°C and the reaction was allowed to proceed for 100 hours. The final product was washed with four 30 mL portions of chloroform, followed by ethanol and diethyl ether. The product was first dried at room temperature and later dried overnight in the vacuum oven at 110°C.

3. 3 Ethynylestradiol 3-methylether bonded phase

A mixture of 20 mL chloroform, 2.0 g of ethynylestradiol 3-methylether and 68 μ l of t-butyl peroxide were added to a 100 mL round bottom flask equipped as described in the above procedure. The mixture was heated at a constant temperature of 50°C for one hour with continuous stirring. Later 2.0 g of dried silica hydride was added slowly through the open neck of the flask. The flask was flushed with nitrogen and then all the joints were sealed. The temperature of the reaction mixture was maintained at 58°C and the reaction was allowed to proceed for 100 hours. The final product was washed with four 30 mL portions of chloroform, followed by ethanol and diethyl ether. The product was first dried at room temperature and later dried overnight in the vacuum oven.

III RESULTS AND DISCUSSION

The principal goal of this work was to explore the feasibility of bonding the two steroid compounds to a silica support by means of hydrosilation reaction. Elemental analysis, DRIFT spectra, and CP-MAS NMR spectra were used to monitor success of the bonding process.

A. Elemental Analysis

Table 3 shows the surface coverage values for all the bonded phases that were synthesized. The surface coverage was calculated from the carbon percentage according to equation (6). From the elemental analysis, it appears that each of the three compounds tested in this study was successfully attached to the silica hydride surface. Results show that both types of estradiol bonded silica had higher surface coverage with Speier's catalyst, but had lower surface coverage with the t-butyl peroxide catalyst.

Table 3. Elemental analysis.

Bonded phase	Mw	Catalyst	% C	Surface coverage $\mu\text{mol}/\text{m}^2$
1-octadecyne	250.47	t-butyl peroxide	6.04	0.88
17 α -ethynylestradiol	296.41	hexachloroplatinic acid	6.59	0.88
Ethynylestradiol 3-methyl ether	310.44	hexachloroplatinic acid	3.58	0.44
17 α -ethynylestradiol	296.41	t-butyl peroxide	1.24	0.15
Ethynylestradiol 3-methyl ether	310.44	t-butyl peroxide	1.09	0.13

B. DRIFT Spectroscopic Evaluation

As mentioned above, DRIFT spectra provide a spectroscopic confirmation of the bonding process as well as molecular information about the organic moiety attached to the surface.

1. Silica hydride

The DRIFT spectrum of the silica hydride intermediate is shown in Figure 5. The broad band centered between $3800 - 3000\text{ cm}^{-1}$ region is attributed to H-bonded silanol groups and adsorbed water. Also, the band near 3750 cm^{-1} is assigned to the OH stretch from non-hydrogen bonded silanols. The strong band at 2250 cm^{-1} represents Si-H stretching which confirms the success of the TES silanization reaction. The other peaks in the spectrum are due to various fundamental vibrations of the silica matrix.

Figure 5. DRIFT spectra of AstroSil silica hydride.

2. 1-octadecyne bonded phase

The DRIFT spectrum of 1-octadecyne bonded to silica hydride using t-butyl peroxide is shown in Figure 6. The presence of strong C-H stretching vibration bands in the range of 2800-3000 cm^{-1} indicates the success of bonding the C18 moiety to the silica hydride surface. Also, after the hydrosilylation reaction, the Si-H stretching band was reduced in intensity thus indicating that bonding has taken place.

Figure 6. DRIFT spectra of 1-octadecyne bonded on AstroSil silica hydride using free radical catalyst.

3. 17 α -ethynylestradiol bonded phase

The 17 α -ethynylestradiol bonded phase was prepared using both Speier's catalyst as well as t-butyl peroxide. DRIFT spectra for both the bonded phases are shown in Figure 7. Both spectra show C-H stretching bands in the range of 2800-3000 cm^{-1} which confirms the success of bonding. Both spectra show that after the hydrosilation reaction, the Si-H stretching band is reduced in intensity indicating bonding has taken place with both Speier's catalyst and t-butyl peroxide. Comparison of spectra A and B indicates that the intensity of C-H stretching band is higher for spectrum A than for spectrum B. It can be concluded that with Speier's catalyst, a greater amount of organic moiety is bonded to silica hydride compare to t-butyl peroxide. These results are consistent with the elemental analysis. Also, both spectra show a band at 1600 cm^{-1} , which represents the C=C stretching frequency of benzene ring carbons which confirms the presence of 17 α -ethynylestradiol molecule.

4. Ethynylestradiol 3-methylether bonded phase

The same data were acquired for the ethynylestradiol 3-methylether bonded phase. DRIFT spectra for the ethynylestradiol 3-methylether bonded phase with Pt and free radical catalysts are shown in Figure 8. The success of the bonding was confirmed by the presence of the C-H stretching band in the region between 2800-3000 cm^{-1} . Also like previous results the C-H band is more intense with Speier's catalyst than with the free radical initiator. Also, both spectra show a band at 1600 cm^{-1} , which represents the C=C stretching frequency of benzene ring carbons. Results confirm the presence of ethynylestradiol 3-methylether molecule.

Figure 7. DRIFT spectra of 17 α -ethynylestradiol bonded on AstroSil silica hydride using Speier's catalyst (A) and free radical catalyst (B).

Figure 8. DRIFT spectra of ethynylestradiol 3-methylether bonded on AstroSil silica hydride using Speier's catalyst (A) and free radical catalyst (B).

C. NMR Spectroscopic Evaluation

1. ^1H spectrum of 1-octadecyne

Proton and ^{13}C liquid NMR spectra of 1-octadecyne were obtained from 1mg of the sample dissolved in 0.5 mL deuterated chloroform. Figure 9 shows the ^1H NMR spectrum of 1-octadecyne. The distribution of protons represents a total of five different types of hydrogens. The peak at 2.16 ppm (HA) can be assigned to the proton attached to carbon atom # 2, the peak at 1.93 ppm (HB) can be attributed to the proton attached to carbon atom #1, the peak at 1.44 ppm (HC) is due to the proton attached to the carbon # 4, the peak at 1.26 ppm (HD) is due to 13 equivalent protons attached to carbons # 5 to # 17, and the last peak at 0.89 ppm is due to proton attached to carbon # 18. All the data are presented in Table 4.

2. ^{13}C spectrum of 1-octadecyne

The ^{13}C NMR spectrum of 1-octadecyne is shown in Figure 10. Carbon-13 shifts were referenced to the CDCl_3 solvent. The carbon atoms are numbered according to the decreasing order of their carbon chemical shifts with reference to TMS. 1-octadecyne shows chemical shifts for 12 different carbon atoms. Chemical shifts at 84.67 ppm and 68.04 ppm confirm the presence of alkyne group. The chemical shifts for 1-octadecyne is summarized in Table 5.

3. ^{13}C spectrum of 1-octadecyne bonded phase

The 1-octadecyne hydrosilylation reaction was performed using both a Pt catalyst and a free radical initiator catalyst. Figure 11 shows the solid state ^{13}C CP-MAS NMR spectra for the 1-octadecyne bonded phase with Pt catalyst (A) and free radical initiator

catalyst (B). Both spectra show a similar pattern; a peak at 19.67 ppm (A) and 19.0 (B) represents the second carbon, i.e. adjacent to the carbon which is bonded to silica surface; a large peak at 26.8 ppm (A) and 26.2 (B) represents all methylene carbons, i.e. from carbon # 3 to carbon # 18, except carbon # 16 which has a peak at 33.2 ppm (A) and 33.2 (B). All carbons are numbered starting with the first carbon attached to silica from the alkyne end of the C18 moiety. As mentioned above, the alkyne molecule can be bonded to the silica surface through different structures and all possible structures are shown in Figure 2. A peak observed at 62 ppm can be attributed to a carbon atom attached to the silica surface via structure I. The second possibility for peak at 62 ppm can be a methylene carbon present in structure III, or a third possible explanation is that it can be due to impurity. It is clear from the peak at 124 ppm that some of the bonded moieties contain an olefin group. As discussed before, bonding via structure V is unlikely so the only possible explanation for the olefin peak is bonding via structure II or III [12]. Spectrum B also shows a peak at 9 ppm, which confirms a Si-C linkage through structure I. The results suggest that 1-octadecyne is bonded to silica hydride through a combination of any of the two or three structures out of five possible structures showed in Figure 2. The results also confirm that bonding is successful with both Speier's catalyst and a free radical initiator catalyst.

4. ^1H spectrum of 17α -ethynylestradiol

The ^1H NMR of 17α -ethynylestradiol is shown in Figure 12. A saturated solution of 17α -ethynylestradiol in deuterated chloroform was used for the liquid ^1H and ^{13}C NMR. The peak at 2.6 ppm corresponds to the hydrogen present on the terminal alkyne

carbon. The peaks observed at 6.55, 6.65, 7.15 and 7.25 ppm represent the protons of the benzene ring. All the peaks in range 0 to 1 ppm are the protons labeled as X, Y and Z (refer Figure 12); peaks observed in the range of 1 to 2 ppm represent protons labeled as L, T, N, V, Q, P and U; and all the peaks observed in the range of 2-3 ppm represents protons labeled as G, D F and K.

5. ^{13}C spectrum of 17α -ethynylestradiol

The ^{13}C NMR spectrum of 17α -ethynylestradiol is shown in Figure 13. All the aryl carbons show upfield chemical shifts in the range of 110 to 160 ppm. The two of the three peaks in the chemical shift range of 75 to 90 ppm confirm the presence of terminal alkyne carbons. One peak is due to the carbon attached to both an OH and alkyne group. All the carbons have been assigned chemical shifts, which are summarized in Table 6.

6. ^{13}C spectrum of 17α -ethynylestradiol bonded phase

The 17α -ethynylestradiol moiety was bonded to the silica hydride surface with both a Pt catalyst and a free radical initiator catalyst. Figure 14 shows the solid state ^{13}C CP-MAS NMR spectra for the 17α -ethynylestradiol bonded phases with a Pt catalyst (A) and with a free radical initiator (B). Both spectra give completely different structural information about the bonded moiety. The 17α -ethynylestradiol molecule can bond to the silica hydride surface only through alkyne group. As discussed above bonding through alkyne side chain can result in multiple structures.

Spectrum A shows a peak at 9.3 ppm, which confirms the Si-C linkage, most likely due to structure I (see Figure 2). Three peaks observed in the range of 44 ppm are due to carbons # 10, 11 and 12, a broad peak observed at 37 ppm is due to carbons # 13,

14 and 15, 16 and a second broad peak observed at 25 ppm is due to carbons # 17, 18, 19, 20. Peaks for carbons # 1 to 6 are not observed in the spectrum. These carbons are in the benzene ring, which are expected to give signals in the range of 120 to 180 ppm. One of the possible explanations may be because of π - π interactions between adjacent aromatic rings, which make the relaxation time short. Because of a short relaxation time, peaks become broad and are difficult to observe. The less likely possibility is that the observed peaks are due to an impurity in the bonded phase, but occurrence of all other carbon peaks and the presence of Si-C linkage confirms that 17 α -ethynylestradiol is bonded to silica hydride surface. Also, the presence of benzene ring carbons was confirmed by DRIFT spectra. All the facts suggest that the organic moiety is bonded to the silica hydride surface.

Spectrum B shows completely different behavior than spectrum A. 17 α -ethynylestradiol when bonded to the silica hydride surface using a free radical catalyst shows only two peaks in the ^{13}C NMR spectrum. The first peak appears at 12 ppm and the second peak appears in the range of 55-56 ppm. One of the possible explanations for these two peaks can be either some impurity or the reaction of the free radical initiator with 17 α -ethynylestradiol molecule results into some new compound; also it is possible that the compound is polymerized when reacted with t-butyl peroxide. Spectrum B also shows a low intensity broad peak in the range of 20 to 40 ppm, which is the expected range for the methylene carbons. It can be concluded from the results that a very small amount of organic moiety is attached to the silica hydride surface, which is consistent with the elemental analysis results.

Figure 9. ^1H NMR spectra of 1-octadecyne.

Table 4. Proton chemical shifts for 1-octadecyne.

^1H NMR spectra of 1-octadecyne	
Hydrogens	Chemical shift (ppm)
A	2.16
B	1.93
C	1.44
D	1.26
E	0.89

Figure 10. ^{13}C NMR spectra of 1-octadecyne.

Table 5. Carbon chemical shifts for 1-octadecyne.

^{13}C NMR spectra of 1-octadecyne			
Carbon	Chemical shift (ppm)	Carbon	Chemical shift (ppm)
1	84.67	7	29.21
2	68.04	8	28.86
3	32.02	9	28.61
4	29.78	10	22.77
5	29.61	11	18.46
6	29.46	12	14.13

Figure 11. ^{13}C CP-MAS NMR spectra of 1-octadecyne bonded on AstroSil silica hydride using Speier's catalyst (A) and free radical catalyst (B).

Figure 13. ^{13}C NMR spectrum of 17 α -ethynylestradiol.

Table 6. Carbon chemical shifts for 17 α -ethynylestradiol.

Carbon	ppm	Carbon	ppm
1	153.37	11	47.15
2	138.28	12	43.53
3	132.63	13	39.43
4	126.57	14	38.98
5	115.27	15	32.76
6	112.73	16	29.64
7	87.50	17	27.20
8	79.98	18	26.42
9	74.10	19	22.83
10	49.48	20	12.70

Figure 14. ^{13}C CP MAS-NMR spectra of 17 α -ethynylestradiol bonded to silica hydride using Speier's catalyst (A) and free radical catalyst (B).

7. ^1H spectrum of ethynylestradiol 3-methylether

The ^1H NMR of ethynylestradiol 3-methylether is shown in Figure 15. Proton and ^{13}C liquid NMR spectra of ethynylestradiol 3-methylether were obtained from 1mg of the sample dissolved in 1 mL of deuterated chloroform. The peak at 2.6 ppm corresponds to the hydrogen present on the terminal alkyne carbon. The peak observed at 3.75 ppm represents the methoxy group attached to the benzene ring. Peaks observed in the range of 0 to 1 ppm presents protons labeled as X, Y and Z (refer Figure 14), peaks observed in the range of 1 to 2 ppm represents protons labeled as L, T, N, V, Q, P and U, and the peaks observed in the range of 2-3 ppm represents protons labeled as G, D F and K.

8. ^{13}C spectrum of ethynylestradiol 3-methylether

The ^{13}C NMR spectrum of ethynylestradiol 3-methylether is shown in Figure 16. All of the aryl carbons have chemical shifts in the range of 110 to 160 ppm. The carbon in the methoxy group is confirmed by a peak at 55.18 pm. Also three peaks in the range of 75 to 90 ppm confirm the presence of terminal alkyne carbons. All of the carbons have been assigned chemical shifts, which are summarized in Table 7.

9. ^{13}C spectrum of ethynylestradiol 3-methylether bonded phase

Ethynylestradiol 3-methylether was bonded to silica hydride surface with both a Pt catalyst and a free radical catalyst. Figure 17 shows the solid state ^{13}C CP-MAS NMR spectra for ethynylestradiol 3-methylether bonded phase with Pt catalyst (A) and with a free radical initiator catalyst (B). The results are the same as the 17α -ethynylestradiol bonded phase. Both spectra give completely different structural

information about the bonded moiety. The ethynylestradiol 3-methylether molecule can bond to the silica hydride surface only through the alkyne group. As discussed above bonding through alkyne side chain can result in multiple structures.

A very small peak at 9.3 ppm in spectrum A is due to the Si-C linkage, most likely as in structure I (see Figure 2). A peak observed at 13 ppm may be due to carbon # 8 which is bonded to the OH group or due to carbon # 17. Peaks observed near 40 ppm are due to carbons # 10, 11 and 12, a broad peak observed at 37 ppm is due to carbons # 13, 14 and 15, 16 and a broad peak observed at 24 ppm is due to carbons # 17, 18, 19, 20. As observed for the 17 α -ethynylestradiol bonded phase, the ethynylestradiol 3-methylether bonded phase also does not show any of the peaks from carbons # 1 to 6 in the spectrum. These carbons are benzene ring carbon atoms, which are expected to give signals in the range of 120 to 180 ppm. As explained before it may be because of π - π interactions between adjacent aromatic rings, which makes the relaxation time short. Because of a shorter relaxation time, peaks become broad and are difficult to observe. The interesting point is the peak due to the methoxy carbon, which is attached to the benzene ring is observed in the spectrum near 50 ppm. The occurrence of all other carbon peaks, the presence of the Si-C linkage and the presence of the methoxy carbon confirm that ethynylestradiol 3-methylether is bonded to the silica hydride surface. The results confirm that bonding of ethynylestradiol 3-methylether is successful with the Pt catalyst.

Spectrum B shows a completely different behavior than spectrum A and is similar to the 17 α -ethynylestradiol bonded with a free radical catalyst. When ethynylestradiol 3-

methylether was bonded to silica hydride surface using a free radical catalyst, it showed only two peaks in the ^{13}C NMR spectrum. The first peak appears at 12 ppm and the second peak appears in the range of 55-56 ppm. As explained before, it may be because of some impurity or the reaction of the free radical initiator with ethynylestradiol 3-methylether results in a new compound. Another possibility is that the compound is polymerized when reacted with t-butyl peroxide. Spectrum B also shows a broad peak in the range of 20 to 40 ppm, which is the expected range for all the methylene carbons. It can be concluded from these results that a very small amount of organic moiety is attached to the silica hydride surface, which is consistent with the elemental analysis results.

Figure 15. ^1H NMR of ethynylestradiol 3-methylether.

Figure 16. ^{13}C NMR spectra of ethynylestradiol 3-methylether.

Table 7. Carbon chemical shifts for ethynylestradiol 3-methylether.

Carbon	ppm	Carbon	ppm
1	157.49	12	47.14
2	137.95	13	43.56
3	132.57	14	39.47
4	126.35	15	39.00
5	113.84	16	32.79
6	111.54	17	29.83
7	87.60	18	27.27
8	79.89	19	26.43
9	74.03	20	22.82
10	55.18	21	12.70
11	49.50

Figure 17. ^{13}C CP-MAS NMR spectra of ethynylestradiol 3-methylether bonded to silica hydride using Speier's catalyst (A) and a free radical catalyst (B).

D. Chromatographic Studies

1. Separation of standard reference mixture

The reference test is used to define the silanol activity and its effect on the resulting chromatography. A mixture of uracil, pyridine and phenol is used as the test mixture. This test is an industrial standard method used by column manufacturers to define the amount of residual silanols and also the separation of this mixture makes a good test for studying hydrophobic retention of reversed phase packings. The structures of the solutes used for this test are shown in Figure 18.

Figure 18. Structure of solutes used for standard reference test.

The separation of this mixture was carried out in the isocratic mode using 0.05% phosphoric acid:acetonitrile (90:10) as the mobile phase. The flow rate was set as 0.5 mL/min at ambient temperature with an injection volume of 10 μ L. UV detections was set at 254 nm. A typical reversed phase order for any mixture is from the most polar to the least polar, and for this mixture the elution order should be uracil < pyridine < phenol. As discussed before the hydrosilation reaction cannot remove all the silanol species

attached to the silica surface. If large amounts of silanols are present on the silica surface, it will affect the chromatographic separation and can change the elution order of analytes. So from the elution order of these three compounds, the presence of residual silanol groups on the silica surface can be inferred and accordingly the chromatographic behavior of a bonded stationary phase can be evaluated. This test was performed on all the estradiol bonded columns as well as all the columns used for the comparative study, which are listed in Chapter II (B.4). According to the elution order of these compounds, all the columns are divided into two categories and their retention time and elution order are listed in Table 8.

The AstroSil silica column, the C18 bonded on Vydac silica, the 17 α -ethynylestradiol (t-butyl peroxide) bonded column, and the ethynylestradiol 3-methylether (t-butyl peroxide) bonded column showed the same elution order. As shown in Figure 19, on all three columns uracil and phenol elute together followed by pyridine. The AstroSil silica contains a large number of silanol groups and due to electrostatic interactions with a basic solute such as pyridine results in larger retention. Both of the estradiol bonded stationary phases prepared with t-butyl peroxide show the same elution order. It was determined by DRIFT and elemental analysis that a very small amount of organic group was attached to the silica hydride surface, so these columns should behave like silica hydride columns. However they unexpectedly give results similar to a bare silica column. It may be that during the hydrosilation reaction, silanol groups left on silica hydride were exposed leading to behavior like silica columns or reaction of t-butyl peroxide with estradiol resulted into some polar compound.

Figure 19. Separation of reference mixture on AstroSil silica column (A), C18 bonded Vydac silica column (B), ethynylestradiol 3-methylether (t-butyl peroxide catalyst) column (C), and 17 α -ethynylestradiol bonded (t-butyl peroxide catalyst) column (D), solutes: 1 = uracil, 2 = phenol, 3 = pyridine.

C8 bonded columns showed different behavior than all other columns. Two C8 bonded columns were used for the comparative study, one of them was prepared in-house via a hydride intermediate and the other one was a commercial column (MicroSolv) prepared via organosilanization. Figure 20 shows the separation of the standard test mixture on both the columns, and the elution order was pyridine < uracil < phenol. As phenol was eluted after uracil and pyridine we can conclude that there are less silanol groups present on the bonded phase compared to bare silica. Also, the long retention time for phenol shows the hydrophobic character of the C8 bonded material. The elution of the basic pyridine molecule before uracil and phenol suggests that the C8 bonded stationary phases are more hydrophobic in nature compared to other bonded stationary phases used in this study.

Figure 20. Separation of reference mixture on C8 bonded silica hydride column (A) and C8 bonded (via organosilanization) column (B), solutes: 1 = pyridine, 2 = uracil, 3 = phenol.

The second type of elution behavior, which is generally expected for the reversed phase material, was observed on all the other columns including the AstroSil silica hydride column, the endcapped silica hydride column, the C18 bonded column (with Speier's catalyst), the C18 bonded column (with t-butyl peroxide catalyst), the C18 bonded column (LC-MS format, Higgins), the C18 bonded column (75mm length, Higgins), the 17 α -ethynylestradiol bonded column (with Speier's catalyst), the ethynylestradiol 3-methylether bonded column (with Speier's catalyst) and the cholesterol columns. It was observed that the cholesterol columns showed the longest retention time for phenol compared to all other columns and so it can be concluded that bonding of cholesterol moiety shields the silanol groups left on the silica hydride surface. Also the results confirm that the bonded group made with Speier's catalyst behaves differently than bonded groups made with the t-butyl peroxide catalyst. The C18 bonded group made with Speier's catalyst showed baseline separation for uracil and pyridine whereas the C18 bonded group made with t-butyl peroxide did not show baseline separation for uracil and pyridine. Also the retention time for phenol was longer on the C18 bonded with Speier's catalyst compared to the C18 bonded with t-butyl peroxide catalyst. In the case of estradiol bonded columns, a totally different behavior was observed with Speier's catalyst and the t-butyl peroxide catalyst.

From the retention time and elution order of the standard reference test mixture it can be conclude that both the 17 α -ethynylestradiol, and ethynylestradiol 3-methylether bonded columns with t-butyl peroxide behave like a bare silica column and so will not be useful for further characterization. The C8 bonded columns show a different retention

order than any other columns. Columns made with Speier's catalyst behave differently than columns made with the free radical catalyst. All other columns used for the comparative study showed the same elution order so it should be useful to undertake a comparative study of bioactive solutes.

Figure 21. Separation of reference mixture on an endcapped silica
hydride column (A) and a UDC cholesterol column (B), solutes:
1 = uracil, 2 = pyridine, 3 = phenol.

Figure 22. Separation of reference mixture on AstroSil silica hydride column (A), 17 α -ethynylestradiol (Pt) column (B), ethynylestradiol 3-methylether (Pt) column (C), C18 (Pt) column (D), C18 (free radical) column (E), C18 short length column (Higgins) (F), C18 LC-MS format column (Higgins) (G), solutes: 1= uracil, 2 = pyridine, 3 = phenol.

Table 8. Retention times and elution order for standard reference mixture.

Column	AstroSil Silica	C18 bonded on Vydac silica	17 α -ethynylestradiol (free radical)	ethynylestradiol 3-methylether (free radical)
Solutes	t_R	t_R	t_R	t_R
Uracil	0.79	1.09	2.63	2.68
Phenol	0.79	1.51	2.67	2.70
Pyridine	1.00	1.79	3.23	3.24

Column	C8 Hydride	C8 cogent
Solutes	t_R	t_R
Pyridine	1.67	2.32
Uracil	1.89	2.70
Phenol	7.72	12.51

Column	C18 (Pt)	C18 (free radical)	C18 (short)	C18 (Sprite)	UDC	17 α -ethynyl estradiol (Pt)	Ethynyl estradiol 3-methylether (Pt)
Solutes	t_R	t_R	t_R	t_R	t_R	t_R	t_R
Uracil	1.53	2.85	1.10	0.35	4.01	3.91	3.64
Pyridine	1.98	3.21	1.96	0.52	4.10	4.27	4.38
Phenol	8.42	5.36	5.58	1.09	17.7	5.81	5.46

2. HPLC separation of steroid hormones

For steroid separation a mixture of adrenosterone, corticosterone, estradiol, estrone, 4-androstene-3,17-dione, 11- α acetoxypregesterone was used as the test solutes. Figure 23 shows the structures of the steroids listed above. As mentioned above, the analysis of steroids has and continues to be a challenging separation problem. Most successful steroid separations have been done in the normal phase mode. Also, gradients are normally required for complex mixtures. Steroid mixtures have been successfully separated on the diol bonded column in the normal-phase mode with a gradient mobile phase [26]. The main problem with the gradient method is the long column equilibration time. The cholesterol column has successfully separated the same steroid mixture in the reversed phase mode by an isocratic method [13].

In this study, all HPLC separations were performed isocratically in the reverse-phase mode using the following four different mobile phase compositions with methanol/water ratios: 70:30, 60:40, 50:50, and 40:60. The flow rate was set at 0.5 mL/min, at ambient column temperature and with an injection volume of 10 μ L. UV detection was set at 254 nm. First the retention times for all the steroids were determined individually and finally mixtures of the steroids were run isocratically. Steroid samples were prepared by dissolving 1mg of sample into 10 mL of methanol. Considering all the columns showing reversed phase behavior, an elution order of corticosterone < estradiol < estrone < adrenosterone < 4-androstene-3,17-dione < 11- α acetoxypregesterone is expected with the highly polar solutes eluting first.

Compound	Structure
Adrenosterone
Corticosterone
11 α -acetoxyprogesterone
4-androstene-3,17-dione
Estrone
Estradiol

Figure 23. Structures of solutes used for separation of steroids.

The main goal of this work is to study separation of steroids on estradiol bonded columns and on other columns for the comparative study. For each of these compounds, the capacity factor k' , $\{k' = (t_r - t_0)/t_0\}$ was determined from their retention times. The retention time is given by t_r and t_0 is the retention time of an unretained compound. The retention time and capacity factor for each of these compounds are listed in Tables 9-20.

As expected, the bare silica column did not show separation for any of the four compositions of mobile phase. The AstroSil silica hydride column showed partial separation with all four compositions of methanol and water and as the water content increases, retention time increases. Six solutes were partially separated in three peaks with the 40:60 methanol:water mobile phase composition (Figure 24-A). On this column estrone, estradiol and corticosterone eluted first as a single peak, followed by adrenosterone, and finally 11α -acetoxyprogesterone and 4-androstene-3,17-dione eluted as one peak. Total elution time was around 10 minutes.

The endcapped silica hydride column showed a different elution order than the silica hydride column. As endcapping removes a considerable amount of silanols on the silica hydride surface, it shows different chromatographic behavior than the silica hydride column. Separation was performed using the four mobile phase compositions and the best separation was obtained with a methanol:water 50:50 ratio. Estrone, adrenosterone and corticosterone eluted first as a single peak, followed by estradiol + 4-androstene-3,17-dione and finally 11α -acetoxyprogesterone. The total elution time was around 10 minutes (figure 24-B). All the compounds gave baseline separation with methanol:water ratio 40:60, but total elution time increased to 60 minutes.

Figure 24. Separation of steroids on AstroSil silica hydride column (A), mobile phase methanol:water 40:60, and endcapped silica hydride column (B), mobile phase methanol:water 50:50, solutes: 1 = adrenosterone, 2 = corticosterone, 3 = 4 androstene-3,17-dione, 4 = 11 α -acetoxyprogesterone, 5 = estrone, 6 = estradiol, isocratic separation, flow rate 0.5 mL/min, detection at 254 nm.

Two C8 bonded columns were used for the comparative study; one was prepared via a hydride intermediate and the second one was a commercial column, which was prepared via organosilanization. Separation of a mixture of six hormones on both columns is shown in Figure 25. The C8 hydride bonded column showed partial separation in 18 minutes with methanol:water ratio 50:50 whereas other C8 (MicroSolv) column showed partial separation in 16 minutes with methanol:water ratio 60:40. Both the columns showed the same elution pattern. Compared to silica hydride and endcapped silica hydride columns, C8 bonded columns showed better resolution. It can be concluded that attachment of an organic moiety to the silica surface increases the separation capabilities.

A unique observation for both the columns was the occurrence of multiple peaks for estrone and estradiol solutes. It was confirmed with individual injection that estrone shows four peaks on these columns where as estradiol shows three peaks. Estrone and estradiol showed very small peaks compared to other solutes so it was very difficult to separate solute peaks from a peak due to unretained compound. The experiment was repeated with concentrated freshly prepared solutions and the same multiple peaks were observed. Possible explanations for multiple peaks are: (1) it may be because of impurities; or (2) it may be because of decomposition of the compound. Based on the retention time alone it was difficult to find out which peak corresponded to the solute molecule and which peaks are due to impurities or decomposition. This problem was overcome by coupling a mass spectrometer to the LC. The LC-MS study of steroids is discussed later which focuses on the various peaks of estrone and estradiol.

Figure 25. Separation of hormones on C8 column (MicroSolv) (A), mobile phase methanol:water 60:40, and C8 hydride column (B), mobile phase methanol:water 50:50. Other conditions same as Figure 24.

Two other columns used for comparative studies were C18 bonded hydride columns. One was prepared using Speier's catalyst and the other was prepared using a free radical catalyst. Separation of a mixture of hormones was performed on both the columns using all four mobile phases and the best separations on both the columns are showed in Figure 26. The elution orders of these columns were found to be different. The C18 column prepared with the Pt catalyst showed baseline separation for adrenosterone and corticosterone whereas the C18 column with a free radical catalyst did not give baseline separation for adrenosterone and corticosterone. The other interesting feature observed was that the C18 (free radical catalyst) column showed two peaks for estrone and two peaks for estradiol. The same effect was observed in the C18 (Pt catalyst) column when 40:60 methanol:water was used as the mobile phase. The separations shown below are with methanol:water 50:50 ratio for C18 (Pt catalyst) and 60:40 ratio for C18 (free radical catalyst) column. For both of the columns, the retention time increases as the water content increases in the mobile phase. The results of the C8 bonded column and the C18 bonded columns showed that elution order of the four solute molecules adrenosterone < corticosterone < 4 androstene-3,17-dione < 11 α -acetoxypregesterone is expected on reversed phase material where as for estrone and estradiol it was difficult to identify the solute molecule peak as there were multiple peaks. As explained above, it may be because of an impurity or because of decomposition of the solute but based on only retention time it is difficult to distinguish the solute peak from any impurity peak. Further studies were done using LC-MS to get positive identification of the solutes.

Figure 26. Separation of hormones on C18 (Pt) column (A), mobile phase methanol:water 50:50, and C18 (free radical) column (B), mobile phase methanol:water 40:60. Other conditions same as Figure 24.

As described previously, four columns were prepared via estradiol bonding on a silica hydride surface. Two columns were prepared using 17 α -ethynylestradiol, one with a Pt catalyst and the other with a free radical catalyst. The other two columns were prepared using ethynylestradiol 3-methylether, and bonded the same way on silica hydride using both a Pt catalyst and a free radical catalyst. Separations of hormones were studied on all four columns using all four mobile phase compositions. The ethynylestradiol 3-methylether bonded column with free radical catalyst did not show any separation with methanol:water 70:30, 60:40 and 50:50 ratios and showed partial separation with two peaks for all the six compounds with a methanol:water 40:60 ratio. Similar results were obtained with the 17 α -ethynylestradiol bonded column with a free radical catalyst. It showed no separation at 70:30 and 60:40 mobile phase compositions and showed partial separation with two peaks at 50:50 methanol:water composition and showed partial separation with three peaks with 40:60 methanol water. Separations on both of these columns are showed in Figure 27. On both of these columns the total elution time was around 6 minutes which is shorter when compared to the C8 and C18 bonded columns. Also the elution order pattern is totally different on these columns when compared to the C8 and C18 bonded columns and the elution order more closely matches that of the silica column. These results are consistent with the results from Test 1 (separation of the reference mixture). Another reason for this behavior is that a very small amount of organic moiety was attached to the silica hydride surface using the free radical initiator catalyst.

Figure 27. Separation of hormones on 17α -ethynylestradiol (free radical catalyst) column (A), mobile phase methanol:water 40:60, ethynylestradiol 3-methylether (free radical catalyst) column (B), mobile phase methanol:water 40:60. Other conditions same as Figure 24.

Separation on both estradiol bonded columns with Pt catalyst is showed in Figure 28. 17 α -ethynylestradiol bonded column showed elution in 16 minutes with a methanol:water 60:40 mobile phase where as the ethynylestradiol 3-methylether bonded column showed partial separation in same time with a methanol:water composition of 40:60 in the mobile phase. These results can be explained by elemental analysis that showed almost double the amount of organic moiety was bonded to the silica hydride surface when 17 α -ethynylestradiol was used for hydrosilation compared to ethynylestradiol 3-methylether hydrosilation. The 17 α -ethynylestradiol column showed the expected elution pattern starting with a single peak for adrenosterone + estrone + estradiol followed by corticosterone, then 4-androstene-3,17-dione and finally 11 α -acetoxyprogesterone. The ethynylestradiol 3-methylether bonded column showed a different elution pattern than the 17 α -ethynylestradiol column, with multiple peaks from estrone and estradiol. Estrone and estradiol gave three peaks; the first peak for both the solutes coeluted with adrenosterone, the second peak coeluted with 4-androstene-3,17-dione and the third peak coeluted with 11 α -acetoxyprogesterone. The estrone and estradiol multiple peaks were confirmed with individual injection of both the solutes which again may be attributed to an impurity or decomposition of the solute molecule.

The other column used for the comparative study was a UDC cholesterol column packed by MicroSolv. As mentioned above, separation of hormones was successfully achieved on cholesterol columns. Two UDC cholesterol columns were tested, both of these columns were identical, but they were used before by other chromatographers.

Figure 28. Separation of hormones on 17α -ethynylestradiol (Pt) column (A), mobile phase methanol:water 60:40, and ethynylestradiol 3-methylether (Pt) column (B), mobile phase methanol:water 40:60. Other conditions same as Figure 24.

Separations of hormones were examined on both cholesterol columns using all four mobile phase compositions. Both columns showed identical separation of hormones, an example on one of the columns is showed in Figure 29. The same elution order, adrenosterone < corticosterone < 4 androstene-3,17-dione < 11 α -acetoxyprogesterone was observed on both columns like the other bonded columns. The cholesterol column showed a very similar separation to the estradiol bonded columns prepared with a Pt catalyst. Also, multiple peaks for estrone and estradiol were observed when a 70:30 methanol:water ratio was used as the mobile phase.

Figure 29. Separation of hormones on UDC cholesterol column, mobile phase methanol:water 60:40. Other conditions same as Figure 24.

Figure 30 shows a comparison of the retention behavior of all the columns using adrenosterone as the reference at four different mobile phase compositions. All of the columns showed the reversed-phase characteristic of an increase in retention time with an increase in mobile phase polarity. Also it can be seen that bonding of the organic group on the silica surface enhances elution.

Figure 30. Comparison of retention behavior on all the columns using adrenosterone as a reference in reversed-phase mode.

Table 9. Retention times and capacity factors for AstroSil silica hydride column.

Mobile Phase	Methanol:Water 70:30		Methanol:Water 60:40	
Solute	Retention time (t_R)	Capacity factor k'	Retention time (t_R)	Capacity factor k'
Corticosterone	1.98	0.86	2.19	0.82
11 α -acetoxyprogesterone	2.21	0.12	1.93	0.83
Adrenosterone	2.23	0.17	2.44	0.26
4-androstene-3,17-dione	2.38	0.18	2.67	0.36
Estrone	1.99	0.87	1.92	0.82
Estradiol	1.91	0.80	1.92	0.82

Mobile Phase	Methanol:Water 50:50		Methanol:Water 40:60	
Solute	Retention time (t_R)	Capacity factor k'	Retention time (t_R)	Capacity factor k'
Corticosterone	2.55	0.39	4.25	2.93
Adrenosterone	3.02	0.59	5.49	1.84
11 α -acetoxyprogesterone	3.52	1.10	8.95	3.63
4-androstene-3,17-dione	3.71	0.96	8.34	3.32
Estrone	1.87	0.66	3.8	0.86
Estradiol	1.99	0.76	3.5	0.79

Table 10. Retention times and capacity factors for endcapped silica hydride column.

Mobile Phase	Methanol:Water 70:30		Methanol:Water 60:40	
Solute	Retention time (t_R)	Capacity factor k'	Retention time (t_R)	Capacity factor k'
Corticosterone	2.29	0.21	2.73	1.43
Adrenosterone	2.35	0.21	2.77	0.49
4-androstene-3,17-dione	2.72	0.44	3.23	0.79
11 α -acetoxyprogesterone	2.75	0.51	3.89	1.13
Estrone	1.98/3.62	0.83/2.34	2.22/3.1	0.76/1.45
Estradiol	1.98/3.62	0.83/2.34	2.4/3	1.21/2.34

Mobile Phase	Methanol:Water 50:50		Methanol:Water 40:60	
Solute	Retention time (t_R)	Capacity factor k'	Retention time (t_R)	Capacity factor k'
Corticosterone	4.29	2.50	11.70	5.24
Adrenosterone	4.89	2.86	13.47	6.18
4-androstene-3,17-dione	7.44	3.08	34.47	17.37
11 α -acetoxyprogesterone	9.37	3.96	61.5	31.78
Estrone	3.9	2.42	5.15	3.65
Estradiol	5.05	3.43	4.6	3.03

Table 11. Retention times and capacity factors for bidentate C8 hydride column.

Mobile Phase	Methanol:Water 70:30		Methanol:Water 60:40	
Solute	Retention time (t_R)	Capacity factor k'	Retention time (t_R)	Capacity factor k'
Adrenosterone	2.61/5.43	1.24/3.67	3.22	1.71
Corticosterone	2.73/5.47	1.17/3.33	3.22	1.71
4-androstene-3,17-dione	3.45/5.43	1.69/2.45	5.51	2.38
11 α -acetoxyprogesterone	3.52/5.47	1.51/1.03	5.51	2.38
Estrone	2.67/3.51/5.5	1.02/1.7/3.18	2.25/7.1	0.5/3.68
Estradiol	2.68/3.53/5.5	1/1.62/3.06	2.12/8.45	0.55/5.16

Mobile Phase	Methanol:Water 50:50		Methanol:Water 40:60	
Solute	Retention time (t_R)	Capacity factor k'	Retention time (t_R)	Capacity factor k'
Adrenosterone	6.18	2.69	13.53	7.32
Corticosterone	7.67	4.11	18.4	10.32
4-androstene-3,17-dione	14.13	9.09	45.5	27
11 α -acetoxyprogesterone	18.2	11.83	74.72	44.98
Estrone	6.3/7.76/14.5/18.5	3.4/4.4/9.1/11.8	13.2/19	7.1/10.7
Estradiol	6.6/8/14.53/18	3.6/4.55/9.1/11.5	13.3/19.1	7.2/10.7

Table 12. Retention times and capacity factors for bidentate C8 (MicroSolv) column.

Mobile Phase	Methanol:Water 70:30		Methanol:Water 60:40	
Solute	Retention time (t_R)	Capacity factor k'	Retention time (t_R)	Capacity factor k'
Adrenosterone	3.81	0.63	6.22	1.75
Corticosterone	5.08	1.18	10.26	3.61
4-androstene-3,17-dione	6.40	1.71	16.08	5.09
11 α acetoxypregesterone	6.23	1.66	13.89	6.03
Estrone	6.4	1.80	4.6/6/10/13.6/15.7	1.2/3.7/5.5/6.4
Estradiol	8.7/10.9	2.87/3.8	6/9.9/13.6/15.7	1.6/3.3/4.9/5.8

Mobile Phase	Methanol:Water 50:50		Methanol:Water 40:60	
Solute	Retention time (t_R)	Capacity factor k'	Retention time (t_R)	Capacity factor k'
Adrenosterone	10.59	2.75	27.1	3.93
Corticosterone	21.24	4.11	68.4	11.43
4-androstene-3,17-dione	31.5	12.73	80.63	13.66
11 α acetoxypregesterone	42.6	11.83	109.04	18.82
Estrone	10.77/22/32.5/35	6.5/14.3/21.5/23.3	5.5/11.35	1.174/3.48
Estradiol	11.5/24/36	6.97/15.64/23.96	5.5/11.35	1.174/3.48

Table 13. Retention times and capacity factors for bidentate C18 (Pt) column.

Mobile Phase	Methanol:Water 70:30		Methanol:Water 60:40	
Solute	Retention time (t_R)	Capacity factor k'	Retention time (t_R)	Capacity factor k'
Adrenosterone	2.38/5.22	1.5/2.2	3.35	1.18
Corticosterone	2.65/5.22	2.1/2.34	4.14	1.70
4-androstene-3,17-dione	3.13/5.22	2.23/2.43	5.94	3.01
11 α -acetoxyprogesterone	3.27/5.21	2.41/2.38	6.02	4.56
Estrone	3.2/5.191	2.37/2.45	3.4/6.1	1.16/2.88
Estradiol	3.2/5.209	2.35/2.43	3.4/6.4	1.49/3.69

Mobile Phase	Methanol:Water 50:50		Methanol:Water 40:60	
Solute	Retention time (t_R)	Capacity factor k'	Retention time (t_R)	Capacity factor k'
Adrenosterone	5.07	2.4	10.26	5.21
Corticosterone	7.0	3.64	17.29	9.46
4-androstene-3,17-dione	11.39	6.35	30.0	17.15
11 α -acetoxyprogesterone	12.75	7.72	40.75	23.65
Estrone	9.22	5.31	10.66	5.45
Estradiol	9.67	5.62	16.43	8.94

Table 14. Retention times and capacity factors for bidentate C18 (free radical) column.

Mobile Phase	Methanol:Water 70:30		Methanol:Water 60:40	
Solute	Retention time (t_R)	Capacity factor k'	Retention time (t_R)	Capacity factor k'
Adrenosterone	3.58/4.48	0.47/0.84	5.34	1.2
Corticosterone	3.56/4.96	0.47/1.04	5.59/13.5	1.3
4-androstene-3,17-dione	3.35/4.45/5.15	0.4/0.8/1.1	8.69/13	2.57
11 α acetoxypregesterone	3.35/4.42/4.96	0.4/0.8/1	9.29/13	2.82
Estrone	3.35/4.96	0.4/1.01	4.2/13	0.72/4.345
Estradiol	3.35/6	0.4/1.01	4.1/13	0.72/4.345

Mobile Phase	Methanol:Water 50:50		Methanol:Water 40:60	
Solute	Retention time (t_R)	Capacity factor k'	Retention time (t_R)	Capacity factor k'
Adrenosterone	8.010	2.58	13.2	4.5
Corticosterone	8.79	2.93	16.5	5.87
4-androstene-3,17-dione	16.15	6.21	33.3	12.87
11 α -acetoxypregesterone	19.4	7.67	46	18.17
Estrone	4.3/10.0	0.92/3.47	12.85	4.35
Estradiol	4.3/11	0.92/3.91	15.61	5.51

Table 15. Retention times and capacity factors for 17 α -ethynylestradiol (Pt) column.

Mobile Phase	Methanol:Water 70:30		Methanol:Water 60:40	
Solute	Retention time (t _R)	Capacity factor k'	Retention time (t _R)	Capacity factor k'
Adrenosterone	6.24	1.03	9.22	2.01
Corticosterone	6.30	1.06	9.96	2.25
4-androstene-3,17-dione	7.46	1.46	13.42	3.38
11 α -acetoxyprogesterone	7.97	1.64	15.37	4.02
Estrone	4.97	0.62	9.01	1.94
Estradiol	4.97	0.62	8.86	1.89

Mobile Phase	Methanol:Water 50:50		Methanol:Water 40:60	
Solute	Retention time (t _R)	Capacity factor k'	Retention time (t _R)	Capacity factor k'
Adrenosterone	14.89	3.80	24.48	6.79
Corticosterone	17.44	4.62	31	8.86
4-androstene-3,17-dione	32.33	9.43	50.28	14.99
11 α -acetoxyprogesterone	34.35	10.08	71.8	21.84
Estrone	17.02	4.49	27.98	7.9
Estradiol	19.0	5.13	31.23	8.93

Table 16. Retention times and capacity factors for ethynylestradiol 3-methyl ether (Pt) column.

Mobile Phase	Methanol:Water 70:30		Methanol:Water 60:40	
Solute	Retention time (t_R)	Capacity factor k'	Retention time (t_R)	Capacity factor k'
Adrenosterone	3.27	1.56	3.52	0.49
Corticosterone	3.08	1.41	3.59	0.53
4-androstene-3,17-dione	3.37	0.43	4.21	0.78
11 α -acetoxyprogesterone	3.39	0.43	4.41	0.86
Estrone	3.37	0.46	4.25	0.80
Estradiol	2.97	0.15	3.84	0.63

Mobile Phase	Methanol:Water 50:50		Methanol:Water 40:60	
Solute	Retention time (t_R)	Capacity factor k'	Retention time (t_R)	Capacity factor k'
Adrenosterone	4.53	0.79	6.83	1.68
Corticosterone	5.18	1.19	8.16	2.20
4-androstene-3,17-dione	6.23	1.48	15.26	4.99
11 α -acetoxyprogesterone	6.82	1.83	12.28	3.82
Estrone	6.5	1.73	6.7/11.3/16	1.63/3.4/5.3
Estradiol	6.8	1.88	6.7/11.0/22	1.63/3.3/7.6

Table 17. Retention times and capacity factors for 17 α -ethynylestradiol (free radical) column.

Mobile Phase	Methanol:Water 70:30		Methanol:Water 60:40	
Solute	Retention time (t _R)	Capacity factor k'	Retention time (t _R)	Capacity factor k'
Corticosterone	2.79	0.69	3.27	0.70
Adrenosterone	3.02	0.83	3.30	0.71
11 α -acetoxyprogesterone	3.08	0.87	3.69	0.92
4-androstene-3,17-dione	3.13	0.9	3.44	0.79
Estrone	2.98	0.81	3.22	0.67
Estradiol	2.97	0.80	3.21	0.67

Mobile Phase	Methanol:Water 50:50		Methanol:Water 40:60	
Solute	Retention time (t _R)	Capacity factor k'	Retention time (t _R)	Capacity factor k'
Corticosterone	3.46	1.07	3.78	0.89
Adrenosterone	3.83	1.3	4.48	1.24
11 α -acetoxyprogesterone	3.68	1.21	4.46	1.23
4-androstene-3,17-dione	3.93	1.36	4.75	1.37
Estrone	3.63	1.18	4.75	1.37
Estradiol	3.63	1.17	4.76	1.38

Table 18. Retention times and capacity factors for ethynylestradiol 3-methyl ether (free radical) column.

Mobile Phase	Methanol:Water 70:30		Methanol:Water 60:40	
Solute	Retention time (t_R)	Capacity factor k'	Retention time (t_R)	Capacity factor k'
Corticosterone	2.51	0.76	2.70	0.25
Adrenosterone	2.59	0.80	2.82	0.34
11 α -acetoxyprogesterone	2.55	0.79	2.57	0.80
4-androstene-3,17-dione	2.57	0.80	2.58	0.81
Estrone	2.51	0.75	2.53	0.77
Estradiol	2.49	0.74	2.52	0.76

Mobile Phase	Methanol:Water 50:50		Methanol:Water 40:60	
Solute	Retention time (t_R)	Capacity factor k'	Retention time (t_R)	Capacity factor k'
Corticosterone	2.58	0.73	3.08	0.92
Adrenosterone	3.10	1.07	3.63	1.26
11 α -acetoxyprogesterone	3.03	1.03	3.79	1.36
4-androstene-3,17-dione	3.18	1.13	4.05	1.52
Estrone	2.59	0.73	2.61/4.23	0.63/1.64
Estradiol	2.59	0.73	2.61/4.33	0.63/1.70

Table 19. Retention times and capacity factors for UDC cholesterol column (1).

Mobile Phase	Methanol:Water 70:30		Methanol:Water 60:40	
Solute	Retention time (t_R)	Capacity factor k'	Retention time (t_R)	Capacity factor k'
Adrenosterone	5.91	0.89	8.21	1.51
Corticosterone	6.87	1.20	11.09	2.40
4-androstene-3,17-dione	8.60	1.75	14.74	3.52
11 α -acetoxyprogesterone	8.39	1.69	16.82	4.15
Estrone	7.02/11.6	1.24/2.71	11.66	2.57
Estradiol	7.26/11.6	1.32/2.71	11.13	2.41

Mobile Phase	Methanol:Water 50:50		Methanol:Water 40:60	
Solute	Retention time (t_R)	Capacity factor k'	Retention time (t_R)	Capacity factor k'
Adrenosterone	18.57	4.54	32.90	8.67
Corticosterone	30.76	8.17	54.50	15.02
4-androstene-3,17-dione	47.86	13.27	84.81	23.93
11 α -acetoxyprogesterone	68.21	19.34	121.53	34.57
Estrone	47.12	13.05	82.69	23.30
Estradiol	55.87	15.66	95.95	27.20

Table 20. Retention times and capacity factors for UDC cholesterol column (2).

Mobile Phase	Methanol:Water 70:30		Methanol:Water 60:40	
	Retention time (t_R)	Capacity factor k'	Retention time (t_R)	Capacity factor k'
Adrenosterone	8.60	1.16	8.03	1.41
Corticosterone	10.41	1.61	10.32	2.09
4-androstene-3,17-dione	13.26	2.33	13.82	3.14
11 α acetoxypregesterone	14.02	2.52	15.33	3.59
Estrone	8.6/10.4/13/14/15	1.2/1.6/2.3/2.7	11.66	2.49
Estradiol	8.5/10.3/13.1/14/15	1.2/1.6/2.3/2.7	11.13	2.33

Mobile Phase	Methanol:Water 50:50		Methanol:Water 40:60	
	Retention time (t_R)	Capacity factor k'	Retention time (t_R)	Capacity factor k'
Adrenosterone	16.61	3.96	29.12	7.7
Corticosterone	25.15	6.51	45.32	12.54
4-androstene-3,17-dione	39.73	1.91	71.58	20.38
11 α -acetoxypregesterone	49.37	13.75	88.86	25.55
Estrone	35.17	9.51	63.38	17.93
Estradiol	45.10	15.44	81.27	23.28

3. LC-MS analysis of steroid hormones

Coupling of liquid chromatography with mass spectrometry allows one to combine a modern powerful separation method with a sensitive and selective detection technique. The LC-MS technique was chosen for two reasons: (1) not all compounds of interest have acceptable UV properties (e.g., catecholamines); (2) sometimes it is difficult to determine the identity of a solute molecule in a mixture solely based on retention time. Coupling of HPLC with MS and post-separation analysis of the mass spectra allows determination and quantification of steroids that are not well resolved [27].

Different ionizing agents have been used in mass spectrometry, including formic acid and acetic acid, to produce ions of the solute molecules; they can be added either in the mobile phase or in the sample solution. In the current study, formic acid was added to the mobile phase for ionization of the solute molecules. Reversed phase HPLC separations showed that the optimum mobile phase for steroid separation was methanol:water 50:50. The mobile phase used for LC-MS separation was 0.5% formic acid in 50:50 methanol:water. Ionization and MS acquisition conditions are listed in Chapter II (B.5). The LC pump flow rate was set at 0.5 mL/min at ambient column temperature with an injection volume of 10 μ l. The steroid sample mixture used for HPLC separation was also used for LC-MS analysis. Considering all the columns showing reversed phase behavior, an elution order of corticosterone < estradiol < estrone < adrenosterone < 4-androstene-3,17-dione < 11 α -acetoxyprogesterone is expected with the highly polar solutes eluting first. HPLC separations confirmed the reversed phase elution order for corticosterone, adrenosterone, 4-androstene-3,17-dione, and 11 α -

acetoxypregesterone. Also, it was observed that some columns showed more than one peak for the estrone and estradiol solutes. This behavior could not be understood based on retention time alone from HPLC chromatograms. However, coupling of a mass spectrometer to LC solved this problem. The mass spectrometer identifies a solute based on its molecular weight. The ion source used in this study was an APCI probe in positive mode which produces protonated ion from the sample via a proton transfer mechanism. Also, unlike ESI (electrospray ionization), the APCI ion source produces singly charged ions. To identify ions produced by each sample, all the samples were injected individually and then as a mixture in MS. Analyses were first performed without any column and then with a column to identify the solute in the mixture. Identification of solutes based on the parent ion and other ions produced in the mass spectra are discussed below.

All the steroids used in this study formed ions in the presence of methanol and formic acid. Most of them exhibited $[M+H]^+$ ions as the most intense peak in the spectrum. Other ions observed in the spectrum were $[M+H - H_2O]^+$ and $[M+H - 2H_2O]^+$. A mass spectrum for each solute is shown in Figures 31, 33 and 35. The molecular weight of adrenosterone is 300. The most intense peak observed in the adrenosterone mass spectrum (Figure 31-A) was for ion 301 m/z with 100% relative intensity. Another peak in the adrenosterone mass spectrum was observed for ion 283 m/z, which was due to the loss of a water molecule. The mass spectrum also showed a peak at m/z 333, which may be due to the oxygenated form of the molecule. As the ion source was used at atmospheric pressure, the sample might be forming an adduct with an oxygen. A mass

spectrum for corticosterone is shown in Figure 31-B. The molecular weight of corticosterone is 346 and the most intense peak observed at m/z 347 with 100% relative intensity. Another peak observed for ion 329 m/z was obtained by the loss of a water molecule. A third peak obtained for ion 311 m/z indicated the loss of a second water molecule. A small peak at m/z 379 was due to the oxygenated form of the molecule. Figure 31-C shows the mass spectrum for 4androstene-3,17-dione. The molecular weight of this solute is 286 and the mass spectrum showed the most intense peak for ion 287 m/z with 100% relative intensity. As the molecule does not contain any OH group, the loss of a water molecule did not result in any peak at a lower m/z than the parent ion. The only additional peak was observed for ion 319 m/z which was due to the oxygenated form of the molecule. 11 α -acetoxyprogesterone showed a different fragmentation pattern. The mass spectrum for this molecule is shown in Figure 31-D. The molecular weight of this solute is 372 and the most intense peak was observed for ion 373 m/z . A second peak was observed with 50% relative intensity for ion 313 m/z , and was obtained by loss of the acetate molecule, $[M+H - CH_3COOH]^+$. As HPLC chromatograms showed more than one peak for estrone and estradiol samples, the LC-MS spectra were used to understand this. The C8 bonded columns, the C18 columns bonded with Pt catalyst and a free radical catalyst, the AstroSil silica hydride column and the endcapped silica hydride column showed multiple peaks for estrone and estradiol which were detected with UV detector. Estrone and estradiol gave small peaks compared to other solutes at equivalent concentration, so in some chromatograms it was difficult to find the various peaks for these solutes as they coelute with other solutes and the large peaks of the coeluting

solutes obscure the peaks of estrone and estradiol. The multiple peaks for estrone and estradiol were confirmed by injecting them individually. The total ion chromatograms of estrone and estradiol on the endcapped silica hydride column are shown in Figures 32 and 34 and mass spectra for corresponding peaks are shown in Figures 33 and 35.

Estrone has a molecular weight of 270. Some of the columns showed one peak for estrone and some showed two peaks. When the estrone total ion chromatogram showed only one peak, the corresponding mass spectrum contains a number of ion peaks which are listed below: the most intense peak was observed for ion 271 m/z. Another peak was observed for ion 253 m/z, which can be obtained by loss of water molecule. A couple of other peaks observed were for ions 295 m/z, 163 m/z, 181 m/z and 205 m/z. The peak for ion 295 m/z may be because of impurity or due to the formation of an adduct and other peaks with small intensities may be because of fragmentation of the molecule. When estrone showed two peaks on some of the columns (refer Figure 32), the first peak was due to ions 271 m/z and 253 m/z and the second peak was due to ions 295 m/z, 163 m/z, 181 m/z and 205 m/z with highest relative intensity for ion 295 m/z (refer Figure 33). It can be concluded that as all the stationary phases have different hydrophobicity, on some of the columns, solute peak and impurity peak split up into two peaks and in other columns solute and impurity showed a single peak. Similar behavior was observed for estradiol which has a molecular weight of 272. The most intense peak observed in the spectrum was for ion 272 m/z, with another peak for ion 255 m/z due to the loss of a water molecule. Other peaks in the mass spectrum of estradiol were observed for ions 163 m/z, 181 m/z, 205 m/z, and 295 m/z. When the chromatogram

showed only one peak for estradiol, all the ions were observed in the single peak.

However when the chromatogram showed two peaks (refer to Figure 34), the first peak was due to ions 272 m/z and 255 m/z and the second peak was due to ions 163 m/z, 181 m/z, 205 m/z and 295 m/z (refer Figure 35). For the mass spectrum of the second peak, the most intense peak was for ion 295 m/z. This may be due to impurity or formation of an adduct and other ions might be due to fragmentation of the estradiol molecule.

Further study is required to understand the fragmentation pattern of these two molecules. The use of a more concentrated solution might help to understand the pattern more clearly as in the present data estrone and estradiol gave very noisy peaks which made it difficult to distinguish the signal from background noise. All the ions observed by LC-MS separation of the steroid solutes are listed in Table 21.

The mass spectrometer was coupled to LC to get more accurate information about the presence of solute molecules in the mixture. Study of individual steroid ions using LC-MS made it easier to find each solute in the mixture. Also, the estrone and estradiol mass spectra helped distinguish between the parent ion peak and ion peaks from impurities or adducts. All the columns were studied using the same LC and MS acquisition conditions and using the same mobile phase composition (0.05% formic acid in methanol:water 50:50). The LC-MS separation of steroid mixtures on each of these columns used for comparative studies is discussed below. To make it easy to understand various peaks of estrone and estradiol, in each chromatogram the parent ion peak for estrone is labeled with a 5 and the second peak due to an impurity or adduct is labeled

with a 5'. Similarly the parent ion peak for estradiol is labeled with a 6 and the second peak is labeled with a 6'.

As expected, the AstroSil silica column did not give any separation and all the solutes eluted as a single peak at 2 minutes. The AstroSil silica hydride column showed partial separation in 6 minutes and the solutes elution order are labeled in Figure 36-A. Estrone and estradiol gave two peaks on the AstroSil silica hydride column, and as confirmed by mass spectra the first peak for both the solutes at 2 minutes was due to the parent compound and the second peak at 2.6 minutes was due to either impurity or adduct. Considering only the parent ion peak for estrone and estradiol, the elution order was estrone + estradiol < adrenosterone < corticosterone < 4 androstene-3,17-dione + 11 α -acetoxyprogesterone. The chromatogram for endcapped silica hydride column is shown in Figure 36-B. The endcapped silica hydride column showed better resolution and a different elution order compared to the silica hydride column. Estrone and estradiol gave two peaks on this column. The first peak was due to the parent compound and the second peak was due to impurity or adduct. Considering only the parent ion peak for both the solutes, the observed elution order was adrenosterone + corticosterone < estrone + estradiol < 4 androstene-3,17-dione < 11 α -acetoxyprogesterone. It was also observed that compared to HPLC, the LC-MS separations gave sharp peaks with higher signal to noise ratio.

The LC-MS separations on both C8 bonded columns are shown in Figure 37. As mentioned above, one column was prepared in-house via a hydride intermediate and the other obtained from MicroSolv was prepared via organosilanization. The C8 hydride

column took 20 minutes for sample elution but did not show baseline separation for all six solutes (Figure 37-A). Estrone showed two peaks, considering the second peak was due to the parent compound, the observed elution order was adrenosterone < corticosterone < 4 androstene-3,17-dione + estradiol < 11 α -acetoxyprogesterone + estrone. The separation on the C8 commercial column is shown in Figure 37-B. This column took 45 minutes for sample elution and it showed baseline separation for all six solutes with the elution order as adrenosterone < corticosterone < 4 androstene-3,17-dione < estradiol < estrone < 11 α -acetoxyprogesterone.

Two bidentate C18 columns were evaluated. One was prepared with Speier's catalyst and the other one was prepared with a free radical catalyst. Both columns showed different separation and elution patterns. Figure 38 shows the separation on the C18 column prepared with Speier's catalyst and Figure 39 shows the separation on the C18 column prepared with a free radical catalyst. The C18 (Pt) column showed elution in 22 minutes. Estrone and estradiol both showed two peaks; parent peak and a peak due to an impurity or adduct which were further confirmed with mass spectra. All the ions are labeled according to m/z and shown in Figure 38. The C18 (free radical) column took 12 minutes for elution. All solutes were partially separated in four peaks. It can be concluded that the bonded phase prepared with Speier's catalyst is different than the bonded phase prepared with the free radical catalyst.

Figure 40 shows another separation on a C18 bonded column. This column was shorter (75 mm) in length compared to other columns, and it was packed by a commercial vendor. The column showed successful baseline separation for all the solute molecules in

26 minutes. The elution order for this column was corticosterone < adrenosterone < 4 androstene-3,17-dione < 11 α -acetoxyprogesterone < estradiol < estrone. It suggests that a shorter column with a smaller sample size results in better resolution.

The other column used for the comparative study was a C18 column designed to fit into the MS instrument. The column was directly attached to the APCI probe inlet. This column is shorter in length and has a smaller inner diameter in comparison to the other columns; 20 mm in length and 21 mm internal diameter. When a 10 μ L injection volume was used on this column, it became overloaded and two peaks appeared for each solute. To avoid this, a smaller injection volume was used which solved the overloading problem. Also, as this column was not evaluated earlier with HPLC (because it was only for an LC-MS format) the separation was carried out using the following methanol:water mobile phases containing 0.5 % formic acid: 70:30, 60:40, 50:50 and 40:60. The best separation was achieved with a methanol:water ratio 40:60, which is shown in Figure 41. A partial separation with five peaks was achieved on this column in 7 minutes. Compared to the other bonded columns this column took shortest time for elution. The resolution can be further improved by a smaller injection volume which was not achieved with the current HPLC instrument.

The same separation was performed on all four estradiol bonded columns. Both the 17 α -ethynylestradiol bonded and ethynylestradiol 3-methylether bonded columns with free radical catalyst did not show any separation and gave only a single peak at 2 minutes for the mixture. As discussed above, these columns behave like a bare silica column. One of the possible explanations is that reaction of estradiol with t-butyl

peroxide might be giving a new compound which is polar, and makes these columns different than estradiol columns prepared with a Pt catalyst. Both bonded phases prepared with Speier's catalyst showed partial separation. Figure 42 shows the separation obtained on the 17 α -ethynylestradiol column with Pt catalyst. Elution was achieved in 25 minutes with the elution order as corticosterone < estradiol + adrenosterone < 4 androstene-3,17-dione < estrone + 11 α -acetoxyprogesterone. Figure 43 shows the separation on the ethynylestradiol 3-methylether column with Pt catalyst. Total elution took 8 minutes; three partially separated peaks were observed for the six steroid solutes. The first peak was from adrenosterone + corticosterone, the second peak was from 4 androstene-3,17-dione + estradiol and the third peak was due to 11 α -acetoxyprogesterone + estrone. The 17 α -ethynylestradiol (Pt) column showed better resolution than ethynylestradiol 3-methylether (Pt) column. This can be explained by the fact that a greater amount of organic group was bonded to silica hydride surface when 17 α -ethynylestradiol was used for hydrosilation compared to ethynylestradiol 3-methylether hydrosilation.

Figure 44 shows the separation of the same mixture on the UDC cholesterol column which was packed by a commercial vendor. Previous studies have shown that cholesterol columns are very powerful due to their liquid crystal-like properties. UDC cholesterol column showed baseline separation for the steroid mixture. A complete baseline separation was achieved on this column in 50 minutes. In addition there was a small peak, which may be due to some impurity.

Figure 31. APCI (+ve) mass spectra of adrenosterone (A), corticosterone (B), 4 androstene-3,17-dione (C), and 11 α -acetoxypregesterone (D).

Figure 32. Total ion chromatogram of estrone on endcapped silica hydride column.

Figure 33. APCI (+ve) mass spectra of estrone for peak 1 (A) and peak 2 (B).

Figure 34. Total ion chromatogram of estradiol on endcapped silica hydride column.

Figure 35. APCI (+ve) mass spectra of estradiol for peak 1 (A) and peak 2 (B).

Table 21. Molecular weight of steroids and m/z values for their corresponding ions.

Compound name	Molecular weight	Parent ion in the spectrum, m/z	Other ions in the spectrum, m/z
Adrenosterone	300.39	301	283 $[M+H - H_2O]^+$ 333 $[M+H+O_2]$
Corticosterone	346	347	329 $[M+H - H_2O]^+$ 311 $[M+H - 2H_2O]^+$ 379 $[M+H+O_2]$
4-Androstene-3,17-dione	286.41	287	319 $[M+H+O_2]$
11 α acetoxyprogesterone	372.51	373	313 $[M+H-H_3COOH]^+$
Estrone	270.37	271	253 $[M+H - H_2O]^+$ (1 st peak) 295 (1 st &/or 2 nd peak) 205 (2 nd peak) 181 (2 nd peak) 163 (2 nd peak)
Estradiol	272.38	271	255 $[M+H - H_2O]^+$ (1 st peak) 295 (1 st &/or 2 nd peak) 205 (2 nd peak) 181 (2 nd peak) 163 (2 nd peak)

Figure 36. LC-MS separation of steroids on AstroSil hydride column (A), and endcapped silica hydride column (B), Mobile phase methanol:water 50:50 + 0.5% formic acid, specification of individual ions: 1 = adrenosterone, m/z 300, 2 = corticosterone m/z 346, 3 = 4 androstene-3,17-dione, m/z 286, 4 = 11 α -acetoxyprogesterone, m/z 373, 5 = estradiol, m/z 272, 5' = impurity, m/z = 295, 6 = estrone, m/z 270, 6' = impurity, m/z = 295.

Figure 37. LC-MS separation of hormones on C8 hydride column (A), and C8 MicroSolv column (B). Conditions same as Figure 36.

Figure 38. LC-MS separation of hormones on C18 (Pt) column. Conditions same as Figure 36.

Figure 39. LC-MS separation of hormones on C18 (free radical) column.
Conditions same as Figure 36.

Figure 40. LC-MS separation of hormones on C18 (Higgins) short column, 75×4.6 mm. Conditions same as Figure 36.

Figure 41. LC-MS separation of hormones on C18 (Sprite) LC-MS format column, 20×2.1 mm, mobile phase methanol:water 40:60, LC pump flow 0.3 mL/min, small injection. Conditions same as Figure 36.

Figure 42. LC-MS separation of hormones on 17 α -ethynylestradiol (Pt) column.
Conditions same as Figure 36.

Figure 43. LC-MS separation of hormones on ethynylestradiol 3-methylether (Pt) column. Conditions same as Figure 36.

Figure 44. LC-MS separation of hormones on UDC cholesterol column.
Conditions same as Figure 36.

4. LC-MS analysis of catecholamines

For catecholamine studies, dopamine hydrochloride, (-)-3-(3,4-dihydroxyphenyl)-2-methyl-L-alanine sesquihydrate [L- α -methyl-dopa], (-)-norepinephrine, (-)-epinephrine, and a mixture of all of the above compounds were used as the test solutes. Figure 45 shows the structures of the solutes cited above.

In this study all LC-MS separations were performed isocratically in the reverse-phase mode using 25 mM ammonium formate:acetonitrile (95:5) as the mobile phase. Selection of this mobile phase was based on the results of the commercial column. Figure 46 shows the separation of the same catecholamines used in this study on a BioBasic SCX ion exchange commercial column (Thermo Electron corporation), with detection in the APCI+ mode. The mobile phase used was 20 mM ammonium formate:acetonitrile (95:5). All of the parameters used for this separation are listed in Figure 46. These conditions were used as reference for the current study. Ionization and MS acquisition conditions are listed in Chapter 2 (B.5). The LC pump flow rate was set at 0.5 mL/min, at ambient column temperature and with an injection volume of 10 μ l. First the retention times for all the catecholamines were determined individually and finally a mixture was run isocratically. The test samples were prepared by dissolving 1mg of sample into 10 mL of mobile phase. All the samples were light sensitive and decomposed in very short time. Therefore all injections were done using fresh samples and the samples were constantly kept in a dark cool place to avoid decomposition. All the columns showed reversed phase behavior with an elution order as follows: L- α -methyl-dopa < (-)-norepinephrine < (-)-epinephrine < dopamine hydrochloride.

Compound name	Structure
Dopamine hydrochloride
L- α -methyl-dopa
(-)-Norepinephrine
(-)-Epinephrine

Figure 45.

Structures of catecholamine solutes.

Figure 46. LC-MS separation of catecholamines on BioBasic SCX cation exchange column.

As mentioned above, catecholamines show very poor UV absorbance. Coupling of a mass spectrometer to LC makes it easier to identify solutes in the mixture based on the molecular weight of the solute. All the catecholamines used in this study were able to form ions in the presence of acetonitrile and ammonium formate. As the MS was equipped with an APCI probe in the positive mode it was expected to show an $[M+H]^+$ ion as the most intense peak in the spectrum but most of the solutes did not show a parent ion peak. Mass spectra for all the solutes are discussed below.

The molecular weight of hydrated L- α -methyl-dopa is 238.24. The mass spectrum for L- α -methyl-dopa (Figure 47-A) had four peaks with equal intensity; the first one at m/z 212 which is obtained by the loss of 1.5 water molecules $[M+H-1.5H_2O]^+$, the second peak observed at m/z 195 which is due to the loss of one more water molecule $[M+H-2.5H_2O]^+$ and two more peaks were at m/z 166 m/z and m/z 139. These observations suggest that before studying mixture separation, it is important to first find the ion peaks from individual solute molecules. This interpretation has been inferred in the case of L- α -methyl-dopa, being a hydrated molecule, which lost water molecules when the sample was injected in the ion source at a temperature of 600⁰C and hence did not show a parent ion peak.

The mass spectrum for (-)-norepinephrine is shown in Figure 47-B. Two intense peaks were obtained by loss of water molecules, the first peak for ion 152 m/z and the second peak at 135 m/z . The mass spectrum for (-)-epinephrine is shown in Figure 48-A. The spectrum gave a parent ion peak at m/z 184 with very low relative intensity. The most intense peak was observed for ion at m/z 166 due to loss of one water molecule, and

a second peak for ion 148 m/z with lower intensity obtained by loss of a second water molecule. The mass spectrum of dopamine hydrochloride is shown in Figure 48-B. The most intense peak was observed for ion 154 m/z due to loss of an HCl molecule. Two more peaks were observed for ions 136 m/z and 119 m/z by loss of water molecules. All the ions obtained for the catecholamine solutes are listed in Table 22.

The mass spectrometric study of the catecholamine solutes was helpful to identify each solute in the mixture based on their fragmentation with the ammonium formate/acetonitrile mobile phase. The LC-MS separation of catecholamines on each column is discussed below.

The LC-MS separations on 17 α -ethynylestradiol columns are shown in Figure 49 and separations on ethynylestradiol 3-methylether bonded columns are shown in Figure 50. None of the columns showed complete baseline separation for all four compounds. The best separation was achieved with the 17 α -ethynylestradiol (Pt) column. The elution order on this column was L- α -methyl-dopa < (-)-norepinephrine < (-)-epinephrine + dopamine hydrochloride. Total elution time was 5 minutes. When compared to the commercial ion exchange column (Figure 46), the 17 α -ethynylestradiol (Pt) column showed better isolation. The 17 α -ethynylestradiol (free radical catalyst) column showed partial separation in 4 minutes and the elution order was L- α -methyl-dopa < single peak from (-)-norepinephrine + (-)-epinephrine < dopamine hydrochloride. None of the peaks showed baseline separation. The ethynylestradiol 3-methylether (Pt) column showed partial resolution with two peaks, the first being L- α -methyl-dopa and the second peak the remaining three solutes, (-)-norepinephrine, (-)-epinephrine, and dopamine

hydrochloride. The ethynylestradiol 3-methylether (free radical) column produced partial separation in 3 minutes; a single peak was partly split into three peaks, with the first one being L- α -methyl-dopa, the second (-)-norepinephrine and the third (-)-epinephrine and dopamine hydrochloride. The separation of the catecholamines was very similar on all the four estradiol bonded columns. Although elemental analysis and CP-MAS NMR results showed that the bonded phase prepared with a Pt catalyst and a free radical catalyst are completely different although they showed the same chromatographic behavior for the catecholamine solutes. A comparison of the steroid hormones separation and the catecholamine separation on these columns suggest that the estradiol bonded columns are very selective for steroid separations.

Four C18 bonded columns were used for the comparative study and all of them showed different separation behavior. The C18 column prepared with the Pt catalyst (Figure 51) gave complete baseline resolution for all the four solutes in 10 minutes, as well as one small peak, most probably from impurity. The elution order was L- α -methyl-dopa < (-)-Norepinephrine < (-)-epinephrine < dopamine hydrochloride. The C18 column prepared with a free radical catalyst (Figure 52-A) did not show baseline separation and the partial separation took 5 minutes with same elution as above. The C18 columns (LC-MS format) did not show complete baseline separation for all four solutes (Figure 52-B). The same elution order was observed as for the C18 (free radical) column with the only difference being that the separation took 2 minutes; half the time because it was a short column with a smaller diameter. The second C18 commercial column (short

length, 75mm) showed complete baseline separation but it took 22 minutes for elution (Figure 53). The elution order was the same as the other columns.

The LC-MS separation of the mixture of four catecholamines on the two C8 bonded column are shown in Figure 54. The C8 hydride column showed the same elution order as other columns and gave partial separation in 4 minutes. The C8 commercial column showed a different elution order than all other column. The elution order was (-)-norepinephrine < L- α -methyl-dopa < (-)-epinephrine + dopamine hydrochloride. The total elution time was 5 minutes. The only possible explanation for this behavior is that the synthesis of bonded phase via a hydride intermediate might be affecting catecholamine separation as the C8 commercial column was not prepared via a hydride intermediate but was prepared via organosilanization.

The LC-MS separation of the catecholamine mixture on the UDC cholesterol column is shown in Figure 55. The cholesterol column gave complete baseline separation for all four solutes in 16 minutes. The chromatogram also showed an impurity, which was not observed on other columns. The elution order was L- α -methyl-dopa < (-)-norepinephrine < (-)-epinephrine < dopamine hydrochloride. The cholesterol column showed complete baseline separation for steroids as well as for catecholamines.

Most surprisingly, the LC-MS separation on a bare silica column was as good as some of the bonded columns. The elution took 4 minutes and had baseline separation for L- α -methyl-dopa and (-)-Norepinephrine followed by a single peak for (-)-epinephrine + dopamine hydrochloride. The endcapped silica hydride column had a similar elution pattern but did not show baseline separation for any of the peaks. The AstroSil silica

hydride column gave partial separation in 4 minutes for three non-resolved peaks with L- α -methyl-dopa followed by (-)-norepinephrine and a single peak for (-)-epinephrine and dopamine hydrochloride. The chromatograms for the AstroSil silica column, the silica hydride column and the endcapped silica hydride columns are shown in Figure 56.

Table 22. Molecular weight of catecholamines and m/z values for their corresponding ions.

Compound name	Molecular weight	Parent ion in the spectrum (m/z)	Other ions in the spectrum (m/z)
L- α -Methyl-Dopa	238.24		212 [M+H-1.5H ₂ O] ⁺ 195 [M+H-2.5H ₂ O] ⁺ 166 139
(-)-Norepinephrine	169.18		152 [M+H-H ₂ O] ⁺ 135 [M+H-2H ₂ O] ⁺
(-)-Epinephrine	183.20	184 [M+H] ⁺	166 [M+H-H ₂ O] ⁺ 148 [M+H-2H ₂ O] ⁺
Dopamine hydrochloride	189.64		154 [M+H-HCl] ⁺ 136 [M+H-HCl-H ₂ O] ⁺ 119 [M+H-HCl-2H ₂ O] ⁺

Figure 47. APCI (+ve) mass spectra of L- α -methyl-dopa (A) and (-)-norepinephrine (B).

Figure 48. APCI (+ve) mass spectra of (-)-epinephrine (A) and dopamine hydrochloride (B)

Figure 49. LC-MS separation of catecholamines on 17α -ethynylestradiol (Pt) column (A), and 17α -ethynylestradiol (free radical) column (B), mobile phase 25 mM ammonium formate:acetonitrile 95:5, specification of individual peak : 1 = L- α -methyl-dopa, 2 = (-)-norepinephrine, 3 = (-)-epinephrine, 4 = dopamine hydrochloride.

Figure 50. LC-MS separation of catecholamines on ethynylestradiol 3-methylether (Pt) column (A), and ethynylestradiol 3-methylether (free radical) column. Conditions same as Figure 49.

Figure 51. LC-MS separation of catecholamines on C18 (Pt) column. Conditions same as Figure 49.

Figure 52. LC-MS separation of catecholamines on C18 (free radical) column (A), and 18 (LC-MS format) column (B). Conditions same as Figure 49.

Figure 53. LC-MS separation of catecholamines on C18 short length column (Higgins). Conditions same as Figure 49.

Figure 54. LC-MS separation of catecholamines on C8 hydride column (A) and C8 MicroSolv column (B), conditions same as Figure 49.

Figure 55. LC-MS separation of catecholamines on UDC cholesterol column.
Conditions same as Figure 49.

Figure 56. LC-MS separation of catecholamines on AstroSil silica hydride column (A), endcapped silica hydride column (B) and AstroSil silica column (C). Conditions same as Figure 49.

IV. CONCLUSION

The main goal of this study was to synthesize bonded stationary phases via the silanization/hydrosilation process. The hydrosilation method was performed using a free radical catalyst and Speier's catalyst (Pt catalyst). Bonded phases were prepared with estradiol steroid compounds and 1-octadecyne. These bonded stationary phases were characterized by carbon analysis, diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy, cross-polarization magic-angle spinning (CP/MAS) nuclear magnetic resonance spectroscopy (NMR), HPLC, and LC-MS.

Carbon analysis showed that 17 α -ethynylestradiol and ethynylestradiol 3-methyl bonded phases prepared with a Pt catalyst had higher surface coverage compared to bonded phases prepared with a free radical catalyst. Similar results were observed with DRIFT spectroscopy. Solid state NMR spectra of estradiol bonded phases gave molecular confirmation regarding the presence of estradiol when prepared with a Pt catalyst. This however couldn't be seen when the bonded phases were prepared with a free radical catalyst. Success of carbon analysis, DRIFT spectra and NMR spectra confirmed that silanization/hydrosilation provides a suitable method for the preparation of silica-based stationary phases.

HPLC and LC-MS separation of a standard reference mixture and biomolecules suggests that estradiol columns prepared with a free radical catalyst has a polar surface and hence gave separations like a bare silica column. The estradiol columns prepared with a Pt catalyst showed partial separations for steroids and catecholamine solutes. It

can also be inferred that estradiol bonded columns showed selectivity for steroid solutes but not for catecholamine solutes.

The study suggests that steroids and catecholamines can be successfully detected with an APCI ion source in the positive mode. The LC-MS analysis of steroids and catecholamines are summarized in Table 23 and 24. Estrone and estradiol parent ion peaks were confirmed using MS which otherwise would have been difficult to identify based on retention time alone. However, the exact explanation for the multiple peaks observed on some columns is not completely understood. Future work should focus on improving the synthetic process in order to obtain a high surface coverage with estradiol bonded phases. Also, additional research should include analysis of the various peaks from estrone and estradiol.

Table 23. Comparative study on LC-MS separation of steroids

Column	Adreno-sterone	Cortico-sterone	4-androstene-3,17-dione	11α-acetoxy progesterone	Estradiol	Estrone	Time (Min)
AstroSil silica	2.0	2.0	2.0	2.0	2.0	2.0	2.0
17 α - ethynylestradiol (free radical)	2.5	2.5	2.5	2.5	2.5	2.5	3.0
Ethynylestradiol 3-methylether (free radical)	2.6	2.6	2.6	2.6	2.6	2.6	3.0
Silica hydride	3.2	2.7	3.8	3.8	2.6/4.4	2/4.4	5.0
Endcapped silica hydride	4.0	4.0	6.7	8.3	5.7/8.3	5.7/8.3	9.0
C8 hydride	6.0	7.6	13.1	16.8	13.8	13.7/16.6	18
C8 (MicroSolv)	10.6	21.3	31.6	42.5	33.8	36.3	44
C18 (Pt)	3.8	5.0	7.6	7.6	6.8/8.8	10.3/21.2	22
C18 (free radical)	5.6	6.6	9.9	11.5	9.3	9.3/11	13
C18 (short, Higgins)	6.4	8.5	14.1	16.9	19.6	25.4	26
C18 (sprite, Higgins)	1.4	1.9	3.1	4.2	4.2	5.7	7.0
17ethynylestradiol (Pt)	10.6	12	17.3	21.6	12	21.6	25
Ethynylestradiol 3-methylether (Pt)	4.9	4.9	6.0	7.0	6.0	7.0	8.0
UDC cholesterol	13.3	17	27.5	32	38	49	51

Table 24. Comparative study on LC-MS separation of catecholamines

Column	L-α-methyl-dopa	Norepinephrine	Epinephrine	Dopamine hydrochloride	Elution Time (Min)
AstroSil silica	2.1	3.0	3.6	3.6	4.0
Silica hydride	2.1	2.7	3.1	3.1	4.0
Endcapped silica hydride	2.1	2.1	2.2	2.6	4.0
C8 hydride	2.3	2.3	3.4	3.4	5.0
C8 (MicroSolv)	3.2	2.8	4.0	4.0	5.0
C18 (Pt)	2.4	4.9	7.3	9.3	12
C18 (free radical)	3.0	3.4	4.2	4.2	6.0
C18 (short, Higgins)	2.3	11.0	15.8	21.8	24
C18 (sprite, Higgins)	0.35	0.8	1.3	1.3	2.0
17 α -ethynylestradiol (Pt)	2.8	4.4	5.6	5.6	8.0
17 α -ethynylestradiol (free radical)	2.7	3.1	3.1	3.9	5.0
Ethynylestradiol 3-methylether (Pt)	2.9	3.5	3.8	3.8	6.0
Ethynylestradiol 3-methylether (free radical)	2.6	2.8	3.1	3.1	5.0
UDC cholesterol	4.2	8.9	12.3	16.1	18

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