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Separation and detection of the isomeric equine conjugated estrogens, equilin sulfate and $\Delta^{8,9}$-dehydroestrone sulfate, by liquid chromatography–electrospray-mass spectrometry using carbon-coated zirconia and porous graphitic carbon stationary phases

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Abstract

Equilin-3-sulfate and $\Delta^{8,9}$-dehydroestrone-3-sulfate are two isomers found in equine conjugated estrogens that differ in structure only by the position of a double bond in the steroid B-ring. These geometric isomers were not resolved on a C_{18} column during the analysis of conjugated estrogen drug products by LC–MS using acetonitrile-ammonium acetate buffer as the mobile phase. While no separations of these two isomers were observed on C_{18} or other alkyl-bonded silica based phases using a variety of mobile phase conditions, partial separations were achieved on phenyl bonded silica phases with a resolution of 1.5 on a diphenyl phase, and baseline separations were readily achieved on two carbonaceous phases with resolutions routinely exceeding three on graphitic carbon-coated zirconia (Zr-CARB) and resolutions as high as 19 on porous graphitic carbon (Hypercarb). An examination of a selected few conjugated estrogens in the complex drug substance by LC–MS on Hypercarb is presented.
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Keywords: Conjugated estrogens; Equilin sulfate; Dehydroestrone sulfate; Porous graphitic carbon; Carbon coated zircona; Zr-CARB

1. Introduction

Conjugated estrogens, a drug derived from pregnant mare's urine and marketed for more than 50 years, is used primarily for estrogen replacement therapy to treat menopausal symptoms and to prevent post-menopausal osteoporosis. As a natural product, it contains numerous steroidal compounds in the form of covalently bonded sulfates and glucuronides. The United States Pharmacopeia (USP) [1] defines conjugated estrogens in terms of specific estrogenic compounds that must be present and the levels at which they should be found expressed as a percent of labeled content of conjugated estrogens; the percent is given as an upper limit or an acceptable range for each component. The labeled content of the drug in a dosage form (e.g., 0.625 mg or 1.25 mg per tablet) is not the sum of all estrogens, but rather the sum of the main three components in conjugated estrogens: estrone sulfate (ES), equilin sulfate (EqS), and 17α-dihydroequilin sulfate (17αEqS). The structures of these three compounds are shown in Fig. 1.

While the USP 27 recognizes 10 components in conjugated estrogens, an analysis in our laboratory by liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS) reveals that there are more than 100 steroid conjugates in the product, the actual number depending on the level of detection. An analysis of such a complex mixture by LC–MS is a daunting task particularly since the structures of many components are undefined, and there are few reference standards commercially available. In fact, the components exist as conjugates in their native state, while the USP reference standard materials used in their analysis are in the form

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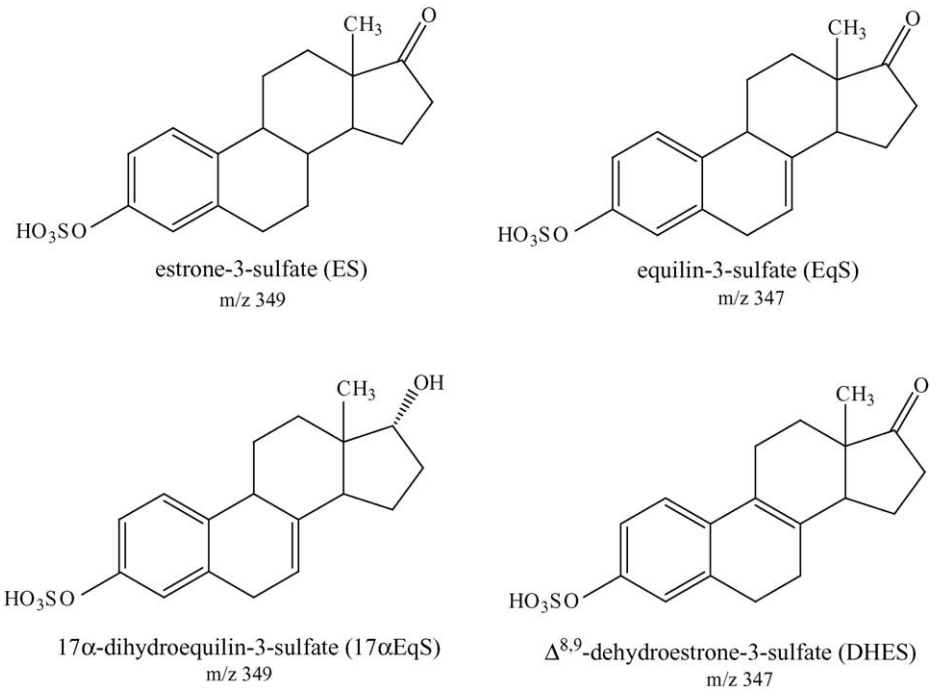

Fig. 1. Chemical structures of selected equine conjugated estrogens.

of the free (unconjugated) estrogens. In the USP assay, the conjugates are converted to the free steroids, the free steroids are converted to trimethylsilane (TMS) derivatives, and the TMS derivatives are analyzed by gas chromatography. One advantage to such an assay is the availability of selected free steroids for use as reference standards. One disadvantage is that the drug product is not evaluated in its native state, and there are cases where multiple conjugates are converted to the same free estrogen upon hydrolysis. For example, both estrone-3-sulfate and estrone-3-glucuronide hydrolyze to estrone. Similarly, β-estradiol-3-sulfate, -17-sufate, and -3, 17-disulfate and all β-estradiol glucuronides are hydrolyzed to β-estradiol. Thus, components such as these are not differentiated by an analysis requiring hydrolysis to free steroids. Nevertheless, the USP assay offers simplification because there are fewer individual components of the complex drug substance to analyze.

Since conjugated estrogens are derived from a natural source, there will be a certain amount of sample variation depending on such factors as individual animal variation and time of collection during the mare's gestation period. Such variations are recognized by the USP, in which acceptable ranges or upper limits are given for selected estrogens. For example, an acceptable range for sodium estrone sulfate is given as 52.5–61.5%, and for sodium equilin sulfate, 22.5–30.5%.

Our laboratory has developed an LC–MS method for the direct analysis of steroid sulfates and glucuronides found in conjugated estrogens in their natural state [2]. Separations were performed on C_{18}-bonded silica gel using a mobile phase gradient with acetonitrile-ammonium acetate buffer as the mobile phase. Using this method, most components were differentiated through a combination of chromatographic separation and differentiation on the basis of mass by selected ion monitoring (SIM) ESI-MS. Several chromatograms were generated for a single analysis, one for each selected atomic mass unit. However, two isomers found in conjugated estrogens, equilin-3-sulfate (EqS) and $\Delta^{8,9}$-dehydroestrone-3-sulfate (DHES), defied separation on the C_{18}-bonded stationary phase, and because they generate pseudo molecular ions with the same mass (*m*/*z* 347), they were not differentiated by ESI-MS. Because DHES co-eluted with EqS, it contributed to the sum of the three main components used to determine the level of each component as a percent of the labeled content of drug. The presence of DHES in conjugated estrogens was recognized years ago [3] and is listed as a component by the USP. Furthermore, DHES is present in conjugated estrogens at reasonable levels and is itself reported to have estrogenic activity [4]. For these reasons, it is important to separate this compound from EqS.

As seen in Fig. 1, the structures of EqS and DHES differ only by the position of one double bond in the steroid B ring. EqS has an unconjugated double bond at the 7,8 position of the steroid ring system, while DHES has a double bond at the 8,9 position conjugated with the aromatic A ring. The purpose of this work was to evaluate various types of columns, mobile phases, and chromatographic conditions in order to provide a procedure suitable for the separation of these two isomeric compounds using a mobile phase that is compatible with LC–MS.

Normal phase silica gel [5–8] and silver ion chromatography [9–12] have been highly successful and usually

superior to reversed-phase partition chromatography for the separation of closely related olefinic isomers. Sadler, et al. [8] separated isomers of indenestrol, a metabolite of diethylstilbestrol, which differ only in the position of the indene double bond, by normal phase chromatography on silica gel. In a study on the separation of isomeric cholesterol-related C_{27} sterols, Ruan et al., [11,12] achieved remarkable separations with silver ion chromatography, but had limited success using normal-phase silica gel HPLC or reversed-phase C_{18} HPLC. Silica gel and silver ion chromatography are normally conducted under normal phase conditions. Conjugated estrogens possess a non-polar steroid ring covalently bonded to a polar sulfate or glucuronide moiety. These conjugates exist as anions at neutral or basic pH in water, making the steroids water soluble. Their water solubility makes them more suitable to RP HPLC.

C_{18} stationary phases have been used in the separation of geometric and positional olefinic isomers of fatty acids, carotenoids, retinoids, and tocopherols [11,13–19]. In the separation of these olefinic compounds that differ in the number and position of carbon-carbon double bonds, it has been generally recognized that polymeric bonded C_{18} silica based phases, in which the silane of an octadecylsilane group is covalently bonded to more than one position on the silica gel surface, are superior to the monomeric C_{18} phases [16,19,20]. Even better separations of unsaturated lipids and structurally related polyaromatic hydrocarbons have been achieved on polymeric C_{30} phases [16,20,21]. Superior resolution of C_{30} phases has been attributed to differentiation of isomers based in part on their molecular shape [20–22].

Carbonaceous columns show unique properties compared to conventional reversed-phase supports and have been used to separate isomers or other structurally related compounds. Zr-CARB [23–26], a zirconia based material coated with 1% graphitic carbon, and Hypercarb [27–35], a porous graphitic carbon (PGC) phase that is 100% carbon, have been used to separate diastereomers, *cis*/*trans* isomers, and other geometric isomers. Both of these carbon phases often show higher selectivity for closely related compounds than alkyl bonded silica phases, and they are used in reversed phase mode. Two stereoisomeric steroids, dexamethasone and betamethasone, differing in the orientation of a methyl group at the C-16 position of the steroid ring, were separated on PGC but not on a C_{18} stationary phase [33].

Since EqS and DHES differ only in the position of a double bond, one where the bond is conjugated to an adjacent aromatic ring and the other where it is not, it seems reasonable that a phenyl-bonded phase may be able to exploit some steric or electronic differences through π–π interactions. Phenyl, diphenyl, and pentafluorophenyl columns were thus included in this work to determine their ability to resolve these two isomers. This paper summarizes the various columns and conditions evaluated to identify a system that could separate EqS and DHES while maintaining good separation between other conjugated estrogens.

2. Experimental

2.1. Chemicals

Conjugated estrogens tablets (Premarin), 0.625 mg, were manufactured by Wyeth-Ayerst (Philadelphia, PA, USA). Equilin-3-sulfate piperazine salt and 8,9-dehydroestrone were obtained from Productos Quimicos Naturales, S.A. de C.V. (Proquina; Mexico). DHES as a pyridinium salt was synthesized from the free steroid using a reported sulfation procedure [36]. HPLC-grade Omni-Solve acetonitrile, methanol, tetrahydrofuran, isopropanol, and reagent grade glacial acetic acid and ammonia were purchased from EM Science (Gibbstown, NJ, USA). Ammonium fluoride, certified reagent, was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Water was purified using a Milli-Q Water System to 18 MΩ-cm (Millipore, Bedford, MA, USA). Triethylamine, minimum 99%, was obtained from Sigma (St. Louis, MO, USA). Ammonium acetate was obtained from Taylor Chemical (St. Louis, MO, USA).

2.2. Sample solutions and solid phase extraction

Aqueous solutions of DHES and EqS were prepared separately at 0.1 mg ml^{-1} and mixed in equal volumes. Individual and combined standards were injected (2 μl) and analyzed by LC–UV–MS.

The outer coatings of twenty 0.625 mg conjugated estrogens tablets were removed by washing with water leaving the inner cores protected with shellac coating. The twenty inner cores were dried, weighed and ground in a mortar. An amount of this composite equivalent to 0.250 mg conjugated estrogens was mixed with 2 ml water using a vortex mixer and an ultrasonic bath. The mixture was placed on a Waters Sep-Pak Cartridge, C18, 3cc (Part No. 20805) that had been prewashed with MeOH and water, and the cartridge was eluted with two 3-ml portions of acetonitrile-water (5:95), then two 3-ml portions of acetonitrile. The combined acetonitrile fractions, which contained the conjugated estrogens, were evaporated to dryness; the residue was dissolved in 2.0 ml water and analyzed by LC–UV–MS. For the examination of the more prominent conjugated estrogens by LC–MS, a portion of this solution was diluted 1:50 with water.

2.3. LC–UV–MS system

Experiments were carried out on an Agilent 1100 HPLC-single quadrapole mass spectrometer equipped with a binary pump, a vacuum degasser, a thermostatted autosampler, a thermostatted column compartment, a diode array detector, and an atmospheric pressure electrospray ionization source using ChemStation software, version A.08.03. Chromatographic columns and mobile solvents that were used in experiments for separation of EqS and DHES are given in Table 1. Flow rates were normally 0.3 ml min^{-1} and the injection volume was typically 10 μl. Column temperatures were ambient

Table 1
Resolution of equilin sulfate (EqS) and $\Delta^{8,9}$-dehydroestrone sulfate (DHES) by reversed-phase HPLC using various stationary phases and mobile phases

Column (manufacturer)	Mobile phase[a]	R_s[b]
YMC ODS-AM S3, 3.0 mm × 150 mm, 3 μm, 17% carbon load (Waters, Milford, MA, USA)	(1) NH_4OAc–water–MeCN, pH 4 to pH 6	0
	(2) NH_4OAc–water–MeOH	0
	(3) 1–5% HOAc–water–MeCN	0
Luna ODS(2), 4.6 mm × 150 mm, 3 μm, 17.5% carbon load (Phenomenex, Torrance, CA, USA)	NH_4OAc–water–MeCN, pH 6	0
Xterra RP-18, 4.6 mm × 250 mm, 5 μm, 15% carbon load (Waters, Milford, MA, USA)	(1) NH_4OAc–water–MeCN, pH 6	0
	(2) NH_4OAc–water–MeOH, pH 6	0
	(3) HOAc–water–MeCN	0
Vydac diphenyl, 4.6 mm × 150 mm, 5 μm, 5% carbon load (W.R. Grace, Columbia, MD, USA)	10 mM NH_4OAc–water–MeCN, pH 6; 12–20.2% MeCN over 0–34 min; flow rate 0.35 ml min^{-1}; column temperature 30 °C	1.5
Vydac diphenyl, 2.1 mm × 250mm, 5 μm, 5% carbon load (W.R. Grace, Columbia, MD, USA)	10 mM NH_4OAc–water–MeCN, pH 6; 12–16.8% MeCN over 0–40 min; flow rate 0.2 ml min^{-1}	1.3
Inertsil phenyl, 4.6 mm × 250 mm, 5 μm, 10% carbon load (G.L. Sciences, Distributed by Alltech, Deerfield, IL, USA)	(1) NH_4OAc–water–MeCN, pH 6	0
	(2) NH_4OAc–water–MeOH, pH 6	0
	(3) HOAc–water–MeCN	0
Inertsil phenyl, 3.0 mm × 150 mm, 5 μm, 10% carbon load (G.L. Sciences, Distributed by Alltech, Deerfield, IL, USA)	(1) 10 mM NH_4OAc,19% MeCN in water, pH 6; 0.4 ml min^{-1}	0.6
	(2) NH_4OAc–water–MeOH, pH 6	0
Zorbax phenyl, 4.6 mm × 250 mm, 5 μm, 5% carbon load (Agilent Technologies, Wilmington, DE, USA)	(1) 10 mM NH_4OAc–water–MeCN, pH 6; 19–31% MeCN 0–40 min; 0.5 ml min^{-1}	0.8
	(2) NH_4OAc–water–MeOH, pH 6	0
	(3) HOAc–water–MeCN	0
Chromegabond PFP (Pentafluorophenyl) 250 mm × 4 mm, 5 μm (E.S. Industries, West Berlin, NJ, USA)	(1) 1–5% HOAc–MeCN–water	0
	(2) NH_4OAc–water–MeCN	0
Luna phenyl-hexyl, 2.1 mm × 250 mm, 3.5 μm, 17.5% carbon load (Phenomenex, Torrance, CA, USA)	(1) NH_4OAc–water–MeCN	0
	(2) HOAc–water–MeOH	0
Supelcosil-DP (diphenyl), 4.6 mm × 250 mm, 5 μm, 6% carbon load (Supelco, Bellefonte, PA, USA)	10 mM NH_4OAc–water–MeCN, pH 6; 22–31% MeCN 0–40 min; flow rate 0.5 ml min^{-1}	0.7
Xterra phenyl, 3.0 mm × 150 mm, 3.5 μm, 12% carbon load (Waters, Milford, MA, USA)	(1) 10 mM NH_4OAc–water–MeCN, pH 6; 18.5% MeCN 0–1 min, 18.5–27.5% MeCN 1–41 min; Flow rate 0.3 ml min^{-1}	0.6
	(2) NH_4OAc–water–MeCN–THF	0
	(3) NH_4OAc–water–MeCN–MeOH	0
Zorbax SB-phenyl, 4.6 mm × 250 mm, 5 μm, 5.5% carbon load (Agilent Technologies, Wilmington, DE, USA)	NH_4OAc–water–MeCN, pH 6	0
Spherisorb phenyl, 4.6 mm × 250 mm, 5 μm, 3% carbon load (Waters, Milford, MA, USA)	10 mM NH_4OAc–water–MeCN, pH 6; 5–12% MeCN 0–28 min; flow rate 0.35 ml min^{-1}	1.1
apHera C_{18} polyvinyl alcohol, 4.6 mm × 250 mm, 5 μm, 17% carbon load (Advanced Separation Technologies, Whippany, NJ, USA)	NH_4OAc–water–MeCN, pH 6	0
Prontosil C_{30}, 4.6 mm × 150 mm, 3 μm, 200 Å (Mac-Mod Analytical, Chadds Ford, PA, USA)	NH_4OAc–water–MeCN, pH 6	0
Zr-CARB, 2.1 mm × 150 mm, 3 μm, operated at 80 °C (ZirChrom Separations, Anoka, MN, USA)	(1) MeCN–5% THF–25 mM NH_4F–75 mM NH_3–H_2O; 10–50% MeCN 0–20 min; flow rate 0.4 ml min^{-1}; column temperature 80 °C	4.1
	(2) 50 mM NH_3–water–MeCN; 19–38% MeCN 0–30 min; flow rate 0.2 ml min^{-1}; column temperature 80 °C	2.9

Table 1 (*Continued*)

Column (manufacturer)	Mobile phase[a]	R_s[b]
Hypercarb, 3.0 mm × 150 mm, 5 μm (Thermo-Hypersil-Keystone, Bellefonte, Pa, USA)	(1) A = 50 mM NH_3–water, B = 50 mM NH3–MeCN–IPA (1:1); 30–85% B 0–35 min; flow rate 0.3 ml min^{-1}; column temperature 30 °C	8.0
	(2) A = 20 mM Et_3N–water, B = 20 mM Et3N–MeCN–IPA (1:1); 20–35% B 0–30 min, 35–100% B 30–95 min; flow rate 0.3 mL min^{-1}; temperature 30 °C	10.1
Hypercarb, 4.6 mm × 250 mm, 5 μm (Thermo-Hypersil-Keystone, Bellefonte, Pa, USA)	(1) A = 10 mM Et_3N–water, B = 10 mM Et_3N–MeOH–IPA (1:4); 35–45% B 0–25 min, 45–100% B 25–67 min; flow rate 0.3 ml min^{-1}; temperature 30 °C	12.6
	(2) A = 10 mM Et_3N–water, B = 10 mM Et_3N–acetone–MeCN (1:9); 35–50% B 0–37 min, 50–100% B 37–70 min; flow rate 0.3 ml min^{-1}; temperature 30 °C	19.3

[a] NH_4OAc = ammonium acetate, MeCN = acetonitrile, MeOH = methanol, HOAc = acetic acid, THF = tetrahydrofuran, IPA = 2-propanol, Et3N = triethylamine. The column temperature was ambient when not otherwise specified. For columns which afforded no resolution, the mobile phase % compositions and gradient conditions were varied and are too numerous to list in detail here.
[b] Resolution (R_s) was calculated using the method of the United States Pharmacopeia [1].

or 30 °C, except for the Zr-CARB column, which was normally operated at 80 °C. When the diode array detector was used, the signal was monitored at 215 or 275 nm, or both, depending upon the UV transparency of the selected mobile phase. The ESI-MS was operated in negative ion mode with the fragmentor set to 100 V, drying gas flow at 10 l min^{-1}, nebulizer pressure at 310.3 kPa (45 psi), drying gas temperature at 350 °C, and capillary voltage at 3.5 kV. MS data were collected in the SIM mode at *m/z* 347, $[M-H]^-$, for detection of EqS and DHES, although numerous other ions were monitored during the analysis of conjugated estrogens.

3. Results and discussion

3.1. UV spectral properties of EqS and DHES

The UV spectra for EqS and DHES are shown in Fig. 2. DHES (Fig. 1) has a double bond in the B ring conjugated with the aromatic A ring, which renders a chromophore with a λ_{max} at 275 nm, while EqS, where the double bond is unconjugated, exhibits only weak absorbance at 275 nm. Ordinarily, with such a marked difference in spectra, it would be possible to determine the ratio of these two compounds by UV spectral comparison during HPLC analysis even if the compounds were not resolved. However, other components in the complex mixture of natural source conjugated estrogens (with different masses) also co-elute with EqS and DHES and contribute to the UV absorbance, thus, complicating any effort to distinguish the isomers by UV analysis.

3.2. Separation of EqS and DHES

Conjugated estrogens have an anionic polar sulfate or glucuronide group, polar ring substituents, and a nonpolar steroid ring system. EqS and DHES have the same polar groups: a sulfate group at position 3 and a carbonyl group at position 17 of the steroid ring. They differ only by the position of a single olefinic bond in the nonpolar steroid ring system. Therefore, chromatographic differentiation depends upon differences in the hydrophobic region of the molecule.

Various columns and mobile phases used in trial separations of EqS and DHES and the results for each are given in Table 1. Three C_{18} silica-based columns, one C_{18} polymer

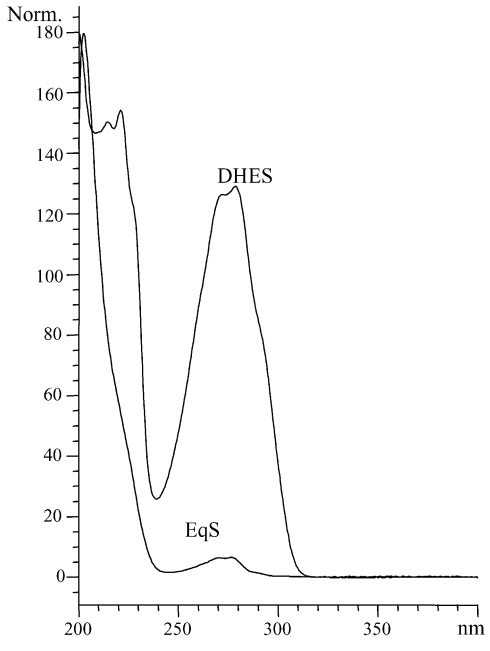

Fig. 2. UV spectra of equilin-3-sulfate (EqS) and $\Delta^{8,9}$-dehydroestrone-3-sulfate (DHES). The conjugated double bond of DHES promotes absorbance at 275 nm.

column, and one C_{30} silica-based column gave no detectable separation under a variety of mobile phase conditions. When extracted samples of conjugated estrogens tablets were analyzed on a C_{18} silica based column using mobile phases containing ammonium acetate at pH 4.0, 4.5, 5.0, 5.5, and 6.0, chromatographic efficiency was poor at pH 4.0 and improved as the pH was increased. Therefore, the ammonium acetate mobile solvent was routinely buffered at pH 6.0.

In the analysis of estrone sulfate by LC–MS, electrospray ionization (ESI) is more sensitive than atmospheric pressure chemical ionization (APCI), and negative ion ESI is more sensitive than positive ion ESI [37]. Analysis of a steroid sulfate or steroid glucuronide by negative ion LC–ESI-MS, in most cases, generates its pseudo molecular ion $[M-H]^-$. For example, full MS scans of estrone-3-sulfate (M_r 350), 17α- or 17β-estradiol-3-sulfate (M_r 352), and estradiol-17β-glucuronide (M_r 448), give base peaks corresponding to molecular anions at *m*/*z* 349, 351, and 447, respectively [37–40]. Similarly, from LC negative ion ESI-MS experiments conducted in our laboratory, EqS and DHES generate pseudo molecular ions at *m*/*z* 347 due to $[M-H]^-$, while ES and 17αEqS generate pseudo molecular ions at *m*/*z* 349. Extracted ion chromatograms (EIC) at *m*/*z* 347 and 349 for the analysis on a C_{18} bonded phase of conjugated estrogens extracted from tablets, are shown in Fig. 3(a). The top plot shows that EqS and DHES elute as a single peak. The bottom plot shows that ES and 17αEqS, two other major components in conjugated estrogens, are well resolved. The plots show the presence of other components at these mass units as well.

Since the double bond of DHES is conjugated with the steroid aromatic A ring, the aromatic π cloud and ring planarity will differ from that of EqS. Phenyl bonded stationary phases may take advantage of these differences by forming aromatic π–π interactions with the analytes. As shown in Table 1, partial resolution was achieved on selected phenyl and diphenyl columns. The degree of separation by phenyl stationary phases varied with different columns and different mobile phases. Generally, separations are more successful on diphenyl phases than phenyl phases, more successful on columns with low carbon load, and more successful with mobile phases containing MeCN than MeOH (Table 1). The separation of EqS and DHES in a conjugated estrogen sample on a Vydac diphenyl column is shown in Fig. 3(b). No separations are seen with phenyl-hexyl or pentafluorophenyl phases.

As shown in Table 1, DHES and EqS were easily separated on Zr-CARB, a graphitic carbon coated zirconia stationary phase, and Hypercarb, a porous graphitic carbon stationary phase. Fig. 3(c) shows the separation of these two isomers on Zr-CARB and well resolved components at *m*/*z* 349 as well. Fig. 3(d) shows the two isomers separated by 8 min on Hypercarb during an analysis of a conjugated estrogen tablet by LC–MS using a mobile phase gradient, with adequate but not quite as wide a separation for the components at *m*/*z* 349. These two carbon phases show a remarkable selectivity toward these two steroid olefinic isomers.

3.3. Bonding mechanisms

Silica gel, alkyl bonded silica gel, phenyl bonded silica gel, and carbonaceous stationary phases have different bonding mechanisms, and therefore, separate different types of analytes. The nonpolar hydrocarbon chains of C_{18} and C_{30} bonded stationary phases bind to analytes primarily through weak Van der Waal interactions and separations depend primarily on hydrophobic differences between the analyte molecules. Since EqS and DHES have a cyclopentylperhydrophenanthrene ring structure with only one olefinic bond in a different location within that ring structure, the compounds have similar hydrophobic properties, so separation on alkyl bonded silica phases is not successful. It has been pointed out that C_{30} phases can also separate analytes on the basis of molecular shape [21,26], but this pertains to the shape of long chain compounds containing olefinic bonds, such as carotenoids or retinoids, which fit onto the C_{30} phase. π-Bonding does not occur with these phases.

Phenylalkyl bonded stationary phases may form π–π interactions with aromatic rings, olefinic bonds or other compounds with π orbitals, which may exploit differences in molecular shape or molecular electronic properties. EqS and DHES are at least partially resolved by phenyl and diphenyl phases, whereas C_{18} phases fail to resolve these compounds.

On silica gel itself, analytes can be adsorbed onto the silica surface, and differences in analyte molecular shape plays a role in separation, often making silica more successful than C_{18} bonded phases in separating isomers or structurally related compounds. We discounted the use of silica gel because the conjugated estrogens are soluble in water and insoluble in nonpolar organic solvents that are used in normal phase conditions for silica gel chromatography. In addition, one would expect the polar anionic groups on the conjugated estrogens to bind strongly to the polar silica surface.

The two carbonaceous columns discussed here can be used with aqueous, aqueous–organic, or organic mobile solvents. Thus, they can be used in RP HPLC and provide an alternative to RP HPLC on C_{18} or other alkyl bonded silica phases. Like silica gel and unlike alkyl bonded silica gel, the carbonaceous phases can separate analytes based on molecular shape. Zr-CARB consists of a zirconia base coated with 1% porous graphic carbon (PGC), while Hypercarb is 100% PGC. PGC exists as layers of large planar sheets of a network of hexagonally arranged carbon atoms with sp^2 hybridization. Thus, compounds can be differentiated based on their ability to fit onto the flat surface of graphite, and this is the key to the high stereo-selectivity of PGC over conventional stationary phases. EqS elutes after DHES on the Zr-CARB and Hypercarb phases, which means that EqS has a stronger bonding affinity toward the graphite surface. The 3-dimensional structures of EqS and DHES are shown in Fig. 4. As expected, the main difference between these two structures is in the conformation of the B ring. All of the carbon atoms in the A and B rings of EqS are in the same plane, or close to it, providing a

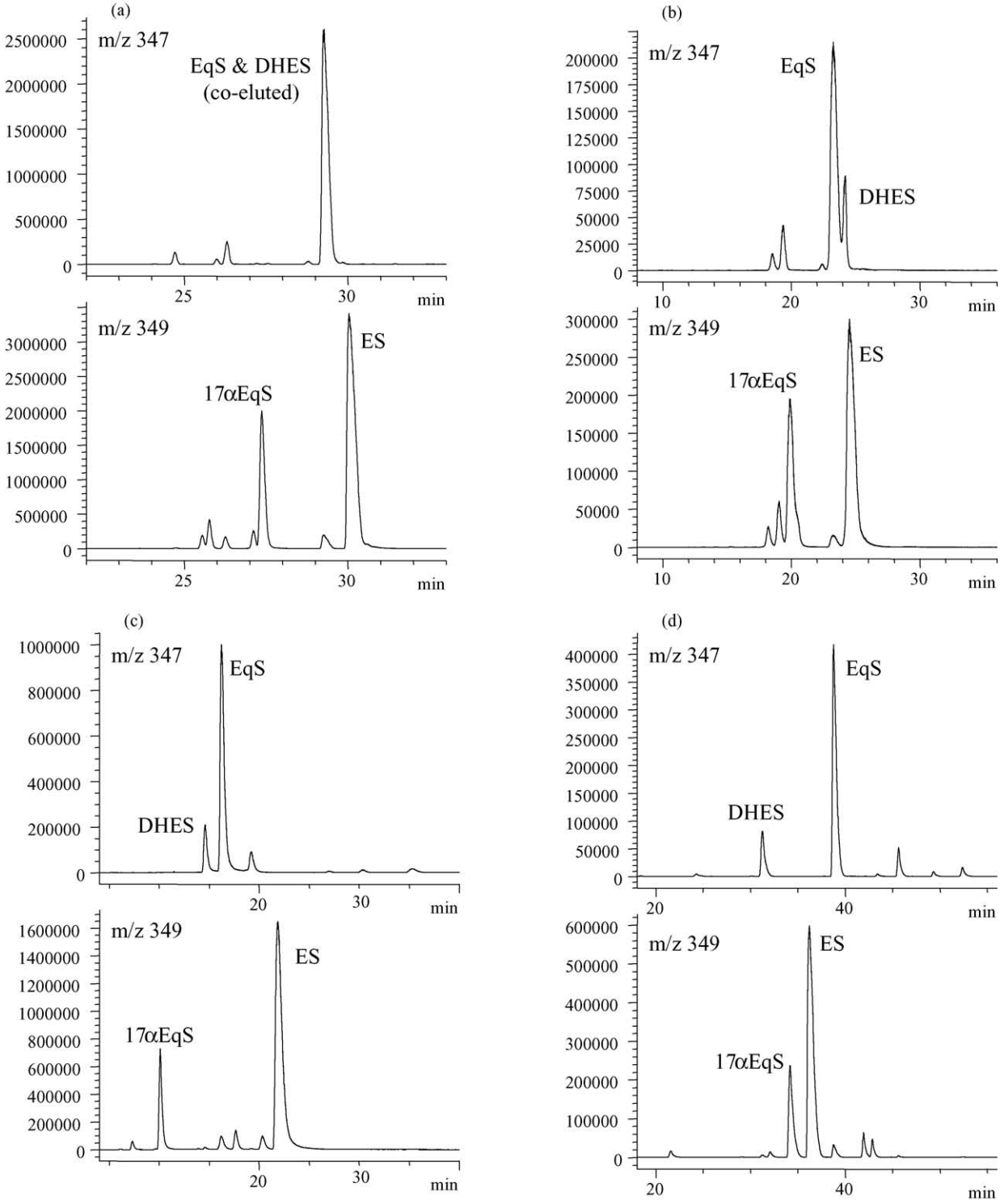

Fig. 3. Analysis of equine conjugated estrogens by LC–ESI-MS showing extracted ion chromatograms at *m/z* 347 and 349 for the following systems: (a) YMC ODS-AM S3 column, 3.0 × 150 mm, 3 μm, using a mobile phase gradient of 12–50% MeCN–10 mM ammonium acetate pH 6 in water over 47 min (b) Vydac diphenyl column, 4.6 mm × 150 mm, 5 μm, using a mobile phase gradient of 12–18% MeCN–10 mM ammonium acetate pH 6 in water over 28 min, (c) Zr-CARB column, 3.0 mm × 150 mm, 5 μm, using a mobile phase gradient of 19–38% MeCN–50mM NH_3 in water over 30 min, (d) Hypercarb column, 3.0 mm × 150 mm, 5 μm, mobile phase A: 20 mM triethylamine in water, mobile phase B: 20 mM triethylamine in isopropanol-MeCN (1:4), gradient: 30–40% B 0–20 min, 40–50% B 20–30 min, 50–100% B 30–55 min. Compound structures and abbreviations are defined in Fig. 1.

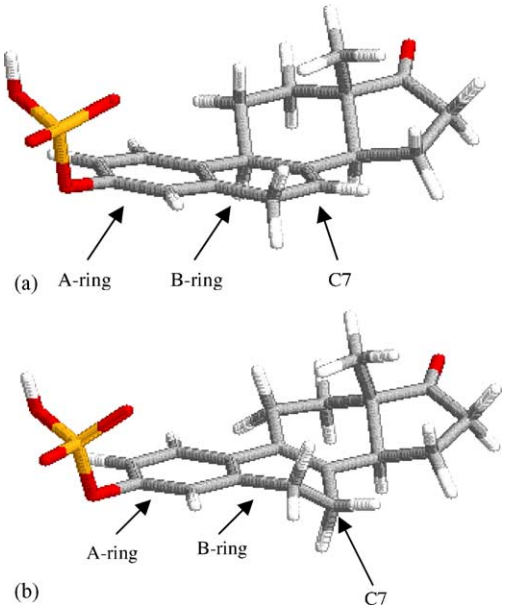

Fig. 4. 3-Dimensional structures of (a) EqS and (b) DHES.

flatter surface for binding to graphite. On the other hand, the B ring of DHES is puckered with the C7 atom prominently out of the plane and positioned beneath the ring. We attribute the separation of the two isomers on PGC to this difference in molecular shape.

3.4. Unique properties of Zr-CARB and Hypercarb

Because the chemistry of Zr-CARB and Hypercarb materials differs strikingly from that of C_{18} bonded silica, the elutotropic solvent series for C_{18} material does not apply to the carbonaceous columns. Unlike bonded silica materials, Zr-CARB and Hypercarb packing materials are stable to pH and temperature extremes.

Zr-CARB is stable at pH 0–14 and at temperatures up to 200 °C. It is not uncommon to operate these columns at 60–80 °C. Increasing the column temperature causes a drop in pressure, and therefore, it is possible to operate at higher flow rates with shorter analysis times. Higher temperatures promote mass transfer, sharper chromatographic peaks and improved resolution. Normally, the limiting factor in temperature control is sample stability, not column stability. Zirconium oxide is a strong Lewis acid, which may bind strongly to some compounds, and therefore, it is usually necessary to add ammonium fluoride or some other Lewis base to the mobile solvent to control the interaction of the analyte with the zirconia surface. During analyses by LC–ESI-MS with ammonium fluoride and ammonium acetate buffer in the mobile solvent (it is necessary to keep the mobile phase neutral or basic when using ammonium fluoride to prevent HF formation), the spray chamber became contaminated with a deposit of ammonium fluoride; ammonium hydroxide was equally effective for elution of the conjugated estrogens and did not cause contamination of the electrospray chamber.

Generally, efficiency was higher on C_{18} columns than Zr-CARB or Hypercarb columns. Although the Hypercarb column is stable to extremes in pH, for our system it was susceptible to contamination, which resulted in peak broadening, peak tailing, and sometimes retention time drift. It is important to wash these columns on a regular basis to prevent the build up of column contaminants in the graphite pores. For our application, the Hypercarb analytical and guard columns were regenerated daily using one procedure, and weekly using another. Since strongly bonded contaminants will most likely collect in the guard column and at the top of the analytical column, these columns were inverted and backward washed so that the flowing solvent would first contact the back end of the analytical column. The columns were backward washed after each day with acetone at a flow rate of 0.2 ml min^{-1} overnight, and after each week with 1 M ammonia in isopropanol at 0.2 ml min^{-1} for 900 min, followed by 95% MeOH at 0.4 ml min^{-1} for 30 min and acetone at 0.4 ml min^{-1} for 60 min. Susceptibility to column contamination is recognized by the manufacturer, who provides several different procedures for column regeneration.

3.5. Base additives in mobile phases

Conjugated estrogens exist as sulfate or glucuronide ions in solution, which chromatograph as paired ions, well suited for electrospray MS detection. This was particularly advantageous for the analysis of the conjugated estrogens because it permitted the use of selected amine additives in the mobile phase, which can interact with steroid conjugates to assist in their separation by paired ion reversed-phase chromatography. While amine additives are too strongly basic for most bonded silica phases, they are well suited for Zr-CARB and Hypercarb. As an added advantage, electrospray signal intensities were consistently several times higher with 10-mM ammonia or other amine additives in the mobile phase than with 10-mM ammonium acetate in the mobile phase. Ammonia, triethylamine, piperidine, and pyrrolidine were used successfully as amine additives in the mobile solvent, usually at 10 mM concentration.

Jacquet, et al. [31], used a PGC phase to separate three isomeric sulfobutyl ether β-cyclodextrins, each of which possesses one sulfonate group. Due to the polar retention effect of graphite (PREG) [31,34,35], these sulfonate compounds bind strongly to the PGC surface, and it was necessary to add ammonium acetate to the mobile solvent to serve as an electronic competitor in order to allow the sulfonate compounds to elute from the column. The sulfonate group on those compounds and the sulfate group on the estrogen sulfates are polar and ionic, and probably behave similarly on the PGC surface. We found that ammonia or other amine additives were more effective than ammonium acetate in overcoming the PREG, and therefore, the estrogen sulfates had significantly shorter retention times using ammonia than ammonium acetate in the mobile solvent.

Table 2
Area percent of the labeled content of conjugated estrogens tablets for selected estrogen sulfates determined by LC–MS using a Hypercarb column[a]

Lot	ES	EqS	17αEqS	DHES	ES + EqS
1	58.6	26.0	15.4	4.3	84.6
2	57.3	27.5	15.2	4.3	84.8
3	57.7	27.1	15.1	4.4	84.9
4	56.3	28.2	15.5	4.6	84.5
5	56.2	28.3	15.5	4.6	84.5
USP range:	52.5–61.5	22.5–30.5	13.5–19.5	≤6.25	79.5–88.0

[a] The labeled content of a conjugated estrogens tablet (e.g., 0.625 mg) is equivalent to the sum of the amounts of the three main components: ES, EqS, and 17αEqS.

3.6. Examination of selected components in conjugated estrogens tablets by LC–MS

Samples of conjugated estrogens were prepared from five lots of tablets and purified by solid phase extraction as described in the experimental section. Using a PGC stationary phase, each sample was analyzed by LC–MS for ES, EqS, 17αEqS (the three most prominent components), and DHES. Each component was determined as a percentage of the labeled content of conjugated estrogens and compared to the tolerance range permitted by the USP (Table 2). Each of the four compounds in each of the five lots fell within the specified range and showed good reproducibility from lot to lot.

While these principle components in equine conjugated estrogens can be separated easily by either the PGC or the carbon-coated zirconia columns, the PGC column was preferred because it provided better overall separation of the more than 100 conjugated steroids found in equine conjugates estrogens (data not shown). Ultimately, in the LC–MS analysis of the natural source conjugated estrogens drug product, it would be desirable to separate all components in each selected ion chromatogram containing multiple peaks attributed to steroid conjugates.

4. Conclusions

Two physiologically active steroid sulfate geometric isomers, DHES and EqS, which differ structurally only in the location of an olefinic bond in a steroid ring, appeared as a single peak during analysis by reversed-phase HPLC on C_{18}- or other alky-bonded silica gel stationary phases. It is difficult to separate geometric isomers by reversed-phase HPLC on C_{18} or other stationary phases that depend primarily on analyte hydrophobic differences. The two isomers were partially resolved on phenyl bonded silica stationary phases, with near baseline resolution on a Vydac diphenyl column. This separation was attributed to differences in aromatic π–π interactions.

Porous graphitic carbon (Hypercarb) and graphitic carbon coated zirconia (Zr-CARB) depend upon surface adsorption for separation, and therefore, can differentiate compounds based on molecular shape. These two phases can be used in reversed phase mode, and therefore, offer an alternative to separations by RP HPLC on alkyl bonded silica phases. The remarkable stereoselectivity exhibited by Zr-CARB and Hypercarb toward two conjugated estrogen isomers, differing only in the location of one double bond in the steroid ring system, implies that HPLC on PGC stationary phases may be generally applicable for the resolution of isomers or structurally related compounds.

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