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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF STEROID 3-SULFATES AND THE CORRESPONDING UNCONJUGATED STEROIDS

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SUMMARY

Simultaneous separation of steroid 3-sulfates and the corresponding free steroids has been accomplished by reversed-phase high-performance liquid chromatography (RP-HPLC). Addition of ammonium sulfate to the aqueous component of the mobile phase results in retention of the steroid sulfates to octadecyl silica packings by an apparent ion-pairing mechanism without affecting the retention of the free steroids. The retention time of steroid sulfates was dependent on the ammonium sulfate concentration in the mobile phase. Biologically important C_{19} Δ^5,3β-hydroxysteroids and corresponding 3-sulfate conjugates or estrogen and estrogen 3-sulfates have been resolved with this RP-HPLC system.

INTRODUCTION

Sulfate conjugates of steroids occur in biological systems as end products for excretion[1] and as precursors in steroid biosynthesis[2]. An example of the latter role is the C_{19} Δ^5,3β-hydroxysteroid, dehydroepiandrosterone sulfate (DHAS), which is the major steroid secreted by the human fetal adrenal cortex[3] and serves as a precursor for estrogen synthesis by the placenta[4]. In addition, this tissue also produces the unconjugated steroid dehydroepiandrosterone (DHA) and its precursors pregnenolone and 17α-hydroxypregnenolone along with the 3-sulfate conjugates of the latter two steroids[5].

Previously, chromatographic separations of a mixture of free and sulfate-conjugated steroids from biological sources usually has required a differential extraction of the two classes of steroids, then solvolysis of the sulfate conjugate and analysis of the solvolyzed steroid product by gas chromatography[5], thin-layer chromatography (TLC)[6] or reversed-phase high-performance liquid chromatography (RP-HPLC)[7]. Steroid sulfates can be directly resolved by TLC without prior solvolysis after removal of the free steroids[8]. However, none of these methods can resolve the free and sulfate conjugate steroids simultaneously. The present study demonstrates the simultaneous separation of unconjugated steroids and steroid 3-sulfates by ion-pair RP-HPLC.

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MATERIALS AND METHODS

DHA, DHAS, pregnenolone, estriol, 17β-estradiol, estrone, estriol-3-sulfate, 17β-estradiol-3-sulfate and estrone-3-sulfate were purchased from Sigma (St. Louis, MO, U.S.A.) and 17α-hydroxypregnenolone and pregnenolone sulfate were purchased from Steraloids (Wilton, NH, U.S.A.). All purchased steroid sulfates were sodium salts. 17α-Hydroxypregnenolone 3-sulfate (triethylammonium salt) was a gift from the steroid reference collection of the Medical Research Council (London, U.K.). [7-^{3}H]DHAS, ammonium salt (25 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). Methanol (Omnisolve) and ammonium sulfate were from MCB (Cincinnati, OH, U.S.A.).

RP-HPLC was performed on 150 × 4.6 mm I.D. stainless-steel columns packed with octadecylsilyl, 5-μm particles of either Apex ODS or Spherisorb S5-ODS from Jones Chromatography (Columbus, OH, U.S.A.). Columns were slurry-packed in the laboratory with a Shandon column-packing instrument (Jones Chromatography). The efficiencies of the Apex ODS and of the Spherisorb S5-ODS columns were 50,300 and 45,500 plates/m, respectively, as determined with 20 μg DHA as the solute and a methanol–20 m*M* ammonium sulfate (59:41) solvent system at a flow-rate of 1 ml/min at room temperature. Isocratic chromatography was performed at room temperature with a Beckman 110A pump, Model 210 injector and a Model 155-40 variable wavelength detector (Beckman, Berkeley, CA, U.S.A.). Gradient chromatography was performed at 45°C with a Beckman 324 chromatograph, (two Model 100A pumps, Model 421 controller and Model 210 injector), with UV detection by a LDC Spectromonitor III variable-wavelength detector (Laboratory Data Control, Riviera Beach, FL, U.S.A.) and fluoresence detection by a Kratos Model FS 950 Fluoromat (Kratos, Westwood, NJ, U.S.A.). All steroids were detected by UV absorbance at 280 nm and estrogens were also detected by fluorescence at 214 nm excitation and 340 nm emission cut-off. The pH of the aqueous component of the mobile phase was unadjusted unless noted otherwise. All steroids were dissolved in absolute ethanol.

The solvolysis of [^{3}H]DHAS (0.2 μCi) was performed by one of two methods[9]. First, the [^{3}H]DHAS and 50 μg of unlabeled DHAS were added to 2 ml tetrahydrofuran which was made to 0.01 *M* perchloric acid and incubated at 37°C for 2 h. The solution was then neutralized with potassium hydroxide, evaporated under nitrogen, 2 ml of water was then added to the residue and extracted with three volumes of dichloromethane. The second method used 2 ml sodium chloride–saturated ethyl acetate that was acidified to 0.01 *M* sulfuric acid and incubated at 37°C for 2 h. This solution was neutralized with sodium bicarbonate, evaporated under nitrogen, 2 ml of water was then added to the residue and extracted with three volumes of dichloromethane. The dichloromethane extracts from each solvolysis method were evaporated under nitrogen and the residue dissolved in 0.1 ml of methanol–20 m*M* ammonium sulfate (30:70). As a control, unsolvolyzed DHAS was added directly to 2 ml of water-saturated *n*-butanol, evaporated under nitrogen and the residue dissolved in 0.1 ml of methanol–20 m*M* ammonium sulfate (30:70). A 50-μl aliquot of each sample was analyzed with the gradient chromatograph and 1.0-ml fractions were collected, scintillation fluid containing 33% Triton X-100[10] was added to each collected fraction and the radioactivity was determined in a liquid scintillation counter.

The identity of the radioactive peaks was determined by comparison to the retention times of authentic unlabelled standards.

RESULTS

The Δ^5,3β-hydroxysteroids, DHA, 17α-hydroxypregnenolone and pregnenolone, and the corresponding 3-sulfate ester conjugates were separated by RP-HPLC using a linear gradient of methanol (30–100%) in 20 m*M* ammonium sulfate (Fig. 1A and B). Each of the three sulfate conjugates could be resolved from one another

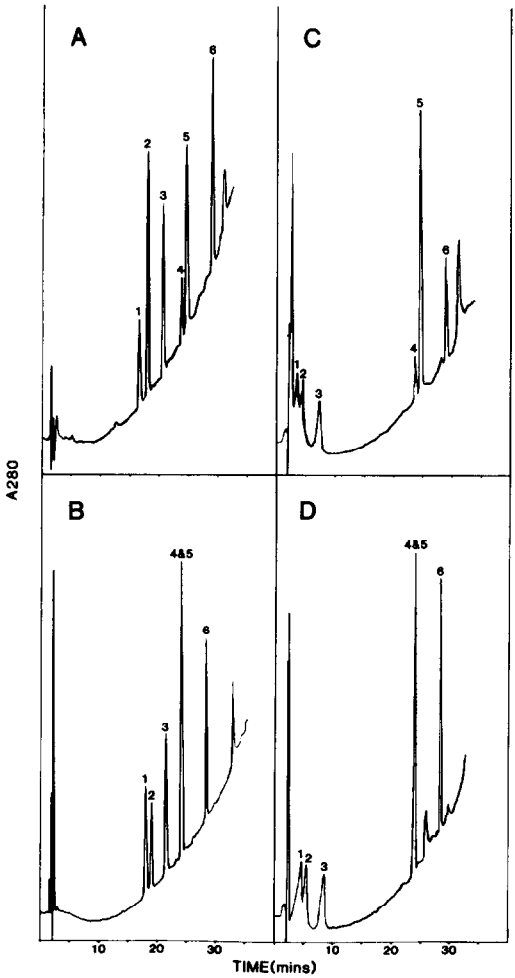

Fig. 1. RP-HPLC of Δ^5,3β-hydroxysteroids and their sulfate conjugates. The separations were performed at a flow-rate of 1 ml/min with linear gradient of methanol (2%/min) from 30 to 100% in either 20 m*M* ammonium sulfate (A and B) or water (C and D) and on columns packed with Spherisorb ODS (A and C) or Apex ODS (B and D). The steroids injected were 10 μg each of DHAS (1), 17α-hydroxypregnenolone sulfate (2), 17α-hydroxypregnenolone (4), DHA (5) and 20 μg each of pregnenolone sulfate (3) and pregnenolone (6). Detection is by UV absorbance at 280 nm and attenuation is 0.02 a.u.f.s.

and from each of the corresponding unconjugated steroids on a C_{18} Spherisorb-packed column (Fig. 1A) and on a C_{18} Apex-packed column (Fig. 1B). With this gradient system, the Spherisorb-packed column resulted in slightly shorter retention times of all six steroids as compared to the Apex-packed column, and in addition the Spherisorb column resolved 17α-hydroxypregnenolone and DHA into two peaks whereas these two steroids have the same retention time on the Apex column. If

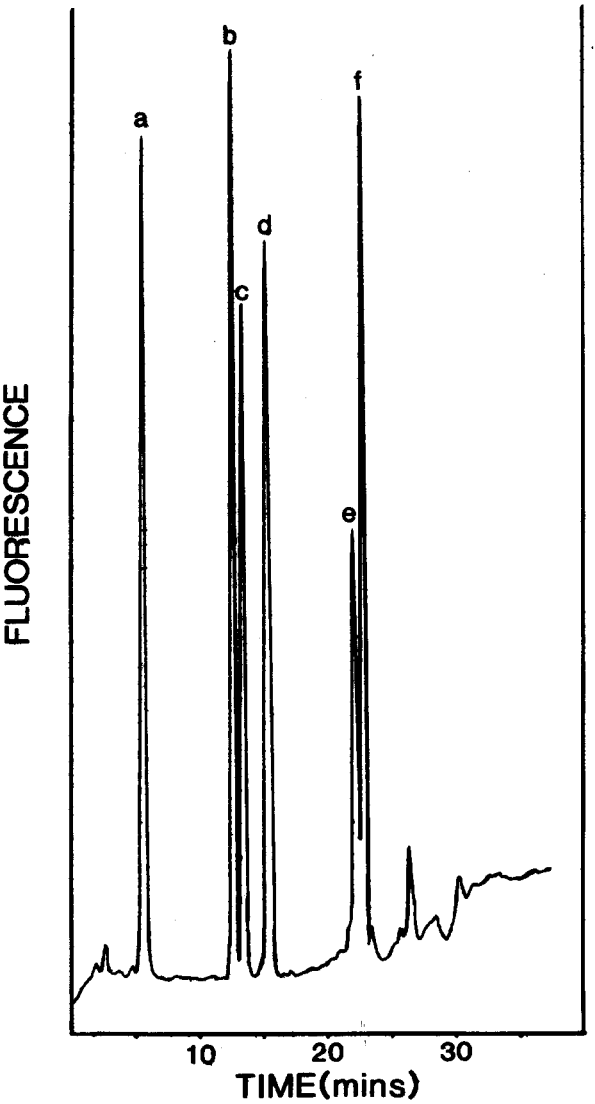

Fig. 2. RP-HPLC of estrogens and their 3-sulfate conjugates. The separations were performed as described in Fig. 1 with a linear gradient of methanol in 20 m*M* ammonium sulfate on a column packed with Spherisorb ODS. The steroids injected were 25 μg each of estriol-3-sulfate (a), estrone-3-sulfate (b), 17β-estradiol-3-sulfate (c), and 2.5 μg each of estriol (d), estrone (e) and 17β-estradiol (f). Detection is by fluorescence at 214 nm excitation and 340 nm emission cut-off and 0.5 range.

water is substituted for ammonium sulfate in the mobile phase, the sulfated Δ^5,3β-hydroxysteroids elute with poor resolution close to the unretained solvent peak whereas the unconjugated steroids elute with the same respective retention times with the methanol gradient in either water or ammonium sulfate on both types of packings (Fig. 1C and D).

The applicability of this gradient system to resolving other steroid 3-sulfates was determined with estrogen sulfates and their unconjugated pairs, estriol, 17β-estradiol and estrone. Each of the estrogen 3-sulfates were separated from one another and from the unconjugated estrogens by this RP-HPLC system (Fig. 2).

The effect of various ammonium sulfate concentrations on the chromatography of the steroid sulfates was analyzed isocratically with a mobile phase of methanol–ammonium sulfate solution (59:41). The range of ammonium sulfate concentration studied was 2.5–250 m*M*, the latter being the highest salt concentration miscible with 59% methanol. Capacity ratio (k'), selectivity (α) and resolution (R_s) for DHAS and pregnenolone sulfate were calculated for each ammonium sulfate concentration (Table I)[11]. With increasing salt concentrations in the mobile phase, the k' value for each steroid increased to a maximum at 100 m*M* ammonium sulfate. However, even at 250 m*M* ammonium sulfate in the mobile phase, the k' values for DHAS and pregnenolone sulfate were less than that for the unconjugated DHA (k' = 6.72), whose retention time was unaffected by the entire range of mobile phase salt concentration. The α value at the lowest salt concentration of 2.5 m*M* was quite large and reflects the very close proximity of the DHAS peak to the unretained

TABLE I

EFFECT OF SOLVENT SALT CONCENTRATION ON RP-HPLC OF STEROID SULFATES

The capacity ratio (k') and selectivity (α) were determined for DHAS and pregnenolone sulfate (PS) after RP-HPLC analysis. The steroids (10 μg each) were analyzed isocratically on an Apex ODS column in methanol–ammonium sulfate (59:41), with the salt concentration varied. (A) The pH of the aqueous component was not adjusted before mixing with methanol. (B) The pH of the aqueous component was adjusted to 7.5 with triethylamine prior to mixing with methanol.

Ammonium sulfate concentration (mM)	k'_{DHAS}	k'_{PS}	α
(A) Unadjusted pH			
2.5	0.023	0.70	31.0
5	0.41	1.39	3.39
10	0.82	2.52	3.08
20	1.16	3.34	2.88
50	1.61	4.41	2.73
100	2.07	5.77	2.79
250	2.07	5.59	2.70
(B) pH 7.5			
2.5	0.31	1.19	3.84
5	0.67	2.02	3.01
10	0.95	2.76	2.90
20	1.50	4.12	2.75
50	1.86	4.98	2.68
100	2.38	6.14	2.58
250	2.38	6.24	2.62

solvent peak. By doubling the ammonium sulfate concentration to 5 m*M*, the α value decreased nine-fold to 3.39 and then decreased slightly with increasing salt concentrations to a minimum of 2.70 at 250 m*M* ammonium sulfate. The calculated R_s values increased 2.9-fold with increasing salt concentration in the mobile phase until 100 m*M* ammonium sulfate, after which the R_s increased only 1% at 250 m*M* (Fig. 3). At all ammonium sulfate concentrations tested, the DHAS and pregnenolone sulfate peaks were symmetrical (0.8–0.9 half-width ratios) and baseline resolution was obtained throughout this range of salt concentrations.

The pH of the various concentrations of ammonium sulfate used ranged from 6.05 at 2.5 m*M* to 5.29 at 250 m*M* before mixing with the methanol component of the mobile phase. To determine the effect of pH on the chromatography of the steroid sulfates, DHAS and pregnenolone sulfate were again analyzed isocratically as described above except that the ammonium sulfate pH was adjusted to 7.5 with triethylamine prior to mixing with methanol. This adjusted pH was significantly higher than that for 2.5 m*M* ammonium sulfate mobile phase which had the highest pH and gave the lowest resolution value for the unadjusted pH solutions. The α values for DHAS and pregnenolone sulfate were very similar at each salt concentration independent of the pH except at 2.5 m*M* ammonium sulfate, which at pH 7.5 had an α value eight times smaller than that for the same salt concentration without pH adjustment (Table I). Adjusting the pH to 7.5 resulted in slightly greater k' values at each salt concentration. The R_s values for the pH 7.5 ammonium sulfate solutions are shown in Fig. 3 and again increased with increasing salt concentration in the mobile phase. At 5 m*M* or less ammonium sulfate the pH 7.5 solutions had higher R_s values than those for the corresponding unadjusted pH aqueous component,

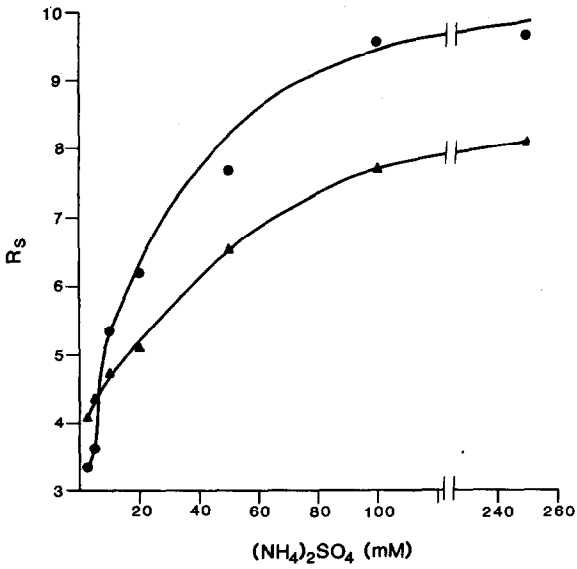

Fig. 3. Effects of ammonium sulfate concentration on R_s values. DHAS and pregnenolone sulfate were chromatographed as described in Table I. The calculated R_s values for unadjusted ammonium sulfate solutions (●) and for ammonium solutions adjusted to pH 7.5 with triethylamine (▲) are shown.

whereas the R_s values were greater for the unadjusted pH ammonium sulfate solutions $\geqslant$ 10 m*M*.

Using the 20 m*M* ammonium sulfate–methanol gradient RP-HPLC system, the recovery of [^{3}H]DHAS was compared before and after solvolysis. For the unsolvolyzed sample only a single peak of radioactivity was detected and corresponded to the retention time for DHAS. The observed radioactivity in this peak was 90% of that expected. Solvolysis of DHAS in either acidified tetrahydrofuran or acidified ethyl acetate resulted in a single major peak of radioactivity which corresponded to DHA. However, after the solvolysis procedures in tetrahydrofuran or ethyl acetate only 66% or 33%, respectively, of the radioactivity observed in the unsolvolyzed DHAS peak was recovered.

DISCUSSION

The addition of ammonium sulfate to the mobile phase has allowed the resolution by RP-HPLC of various steroid 3-sulfate esters from one another and from the unconjugated pairs. The absence of salt from the mobile phase does not effect the chromatography of the free steroids whereas decreased retention and poor resolution were observed for the steroid conjugates which probably results from a repulsion of the anionic steroid conjugates and the accessible silanol groups of the stationary phase. Although no reversals of retention order occurred when the Δ^5,3β-hydroxysteroids and their 3-sulfate conjugates were chromatographed by gradient elution on C_{18} Spherisorb packing and on C_{18} Apex packing, these two packings did exhibit different selective effects as evidenced by the separation of 17α-hydroxypregnenolone and DHA on the Spherisorb packing and the co-elution of these two steroids on the Apex packing. Others have shown that different selective effects of RP packings on steroid HPLC correlate to differences in accessible silanol groups on the packing materials[12]. Measurement of accessible silanols by methyl red adsorption[12] indicates a greater number of free silanol groups on the C_{18} Spherisorb packing than on the C_{18} Apex packing[13].

The increase in retention of the steroid sulfate esters with ammonium sulfate in the mobile phase is presumably due to ion-pairing of the ionized steroid sulfate and the cation of the mobile phase. With increasing counterion concentration, there appears to be a greater neutralization of the steroid sulfate charge which increases the interaction of the hydrophobic portion of the steroid conjugate with the bonded phase and this results in greater k' values. However, the ion-pairing even at high salt concentrations does not result in identical retention times for a conjugated and unconjugated steroid pair. Therefore, the steroid sulfates and the corresponding free steroids can be resolved simultaneously by RP-HPLC with the addition of a simple counterion to the aqueous portion of the mobile phase. The use of gradient elution enables a more complex mixture of free and conjugated steroids to be resolved simultaneously. Others have shown that isocratic RP-HPLC separation of a mixture of steroid sulfates and glucuronides can be accomplished by increasing the proportion of water in a water–methanol mobile phase above that required for separation of the free steroids[14,15]. Alkylammonium counterions have been used for isocratic RP-HPLC separation of a mixture of estrogen conjugates[16] and cortisol from cortisol phosphate[17]. The mechanism of RP-ion-pairing with counterions having a hydrophobic moiety has been suggested to occur between the solute and ion-pairing agent in the mobile phase and/or by the alkyl portion of the counterion interacting with the bonded hydrophobic groups of the stationary phase to result in a solvent-induced ion-exchange[16,18]. The mechanism of ion-pairing with ammonium sulfate in the pres-

ent study is most likely occurring by the former mechanism. However, when triethylamine is also present in the mobile phase, the second mechanism may also be occurring which may account for the higher R_s values at ammonium sulfate concentrations less than 10 mM as compared to the R_s values obtained without triethylamine present. Ion-pair formation by non-hydrophobic salts (sodium phosphate buffer or sodium sulfate) has been suggested to occur for RP-HPLC of carboxylic acids at a solvent pH where the solutes are fully ionized[19].

The RP-HPLC system described here can be used for rapid separation of a mixture of biologically important steroid 3-sulfates and their free steroids. Because the recovery of the steroid sulfates is 90% by this method, there is an additional advantage over solvolysis of the conjugate which varies in recoveries from 30 to 60%. The simultaneous separation of steroid sulfates and the free steroids by a single method has application in the study of steroid biosynthesis in tissues such as the adrenal cortex which in humans produces large quantities of DHA and DHAS[20]. Using the RP-HPLC system, this laboratory has investigated conversion of pregnenolone to pregnenolone sulfate, 17α-hydroxypregnenolone sulfate and DHAS in monolayer cultures of human adrenal cells[21].

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