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Note

Isocratic reversed-phase high-performance liquid chromatography of free and sulphoconjugated steroids

LEIF DIBBELT* and ERICH KUSS
Laboratorium für Klinische Chemie und Biochemie, I. Frauenklinik der Universität München, Maistrasse 11, D-8000 München 2 (F.R.G.)
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The isolation and identification of unconjugated steroids by high-performance liquid chromatography (HPLC) has become routine in the past few years. The HPLC analysis of conjugated steroids, however, has not been developed to the same extent. This is mainly due to the fact that special chromatographic techniques are needed to cope with the effects of the highly polar glucuronide or sulphate moiety[1]. One of such techniques, ion-pair reversed-phase HPLC, has recently been shown to resolve free and conjugated steroids simultaneously[1–3].

One of our interests has been the synthesis of substrates for placental steroid sulphatase[4,5]. In order to test the purity of the synthesized steroid sulphates, we developed a reversed-phase HPLC system based on a ion-pairing or solvent-induced anion-exchange mechanism. In the present communication we report on the efficiency of separation of various free and sulphoconjugated steroids using this simple isocratic system.

MATERIALS AND METHODS

^{3}H-labelled dehydroepiandrosterone and estrone were purchased from Amersham Buchler (Braunschweig, F.R.G.). The sodium salts of androsterone sulphate, 17β-estradiol-3-sulphate, estriol-3-sulphate and pregnenolone sulphate were from Sigma Chemie (Taufkirchen, F.R.G.), 16α- and 16β-hydroxydehydroepiandrosterone from Steraloids (Wilton, NH, U.S.A.). Dehydroepiandrosterone, estrone, 17β-estradiol and estriol were purchased from Merck (Darmstadt, F.R.G.), as were all other chemicals (p.a. grade) with the exception of triethylamine (puriss.) which was from Fluka Feinchemikalien (Neu-Ulm, F.R.G.).

Dehydroepiandrosterone sulphate, 16α-hydroxydehydroepiandrosterone-3-sulphate and estrone sulphate were prepared as described previously[4–6]. 16β-Hydroxydehydroepiandrosterone-3-sulphate was obtained as a by-product during the synthesis of its 16α-epimer from which it was separated by crystallization from methanol–water and methanol–diethyl ether. When both 16α- and 16β-hydroxydehydroepiandrosterone-3-sulphate were hydrolyzed by solvolysis[7], the products obtained were identical to commercially available 16α- and 16β-hydroxydehydroepiandrosterone, respectively, as indicated by the HPLC chromatogram.

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Liquid chromatography was performed using a Model 6000 A solvent-delivery system, an U6K injector, a radial compression separation system equipped with a 10-μm C_{18} RP Radial-Pak catridge (100 × 5 mm) and a Model 441 UV absorbance detector (214 nm), all from Waters (Eschborn, F.R.G.). Methanol–water (48:52) containing various concentrations of triethylammonium sulphate and adjusted to pH 4 with sulphuric acid was used as solvent. Chromatography was carried out at room temperature at a flow-rate of 1 ml/min. When ^{3}H-labelled steroid sulphates were chromatographed, aliquots of the corresponding eluate fractions were analyzed in a liquid scintillation counter using Picofluor-30 (Packard Instrument, Frankfurt, F.R.G.) as scintillation fluid.

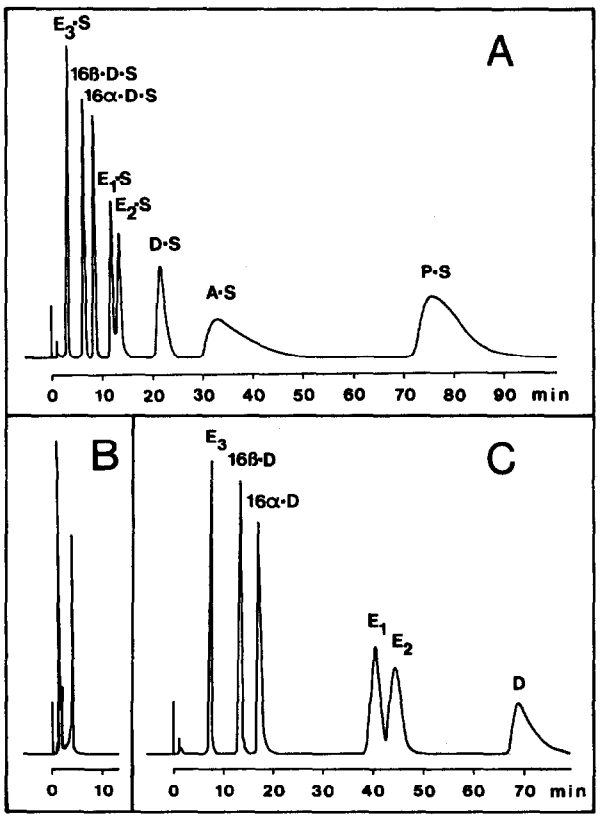

Fig. 1. Reversed-phase HPLC of steroid sulphates (A, B) and unconjugated steroids (C). Methanol–water (48:52) was used as solvent containing either no (B) or 25 m*M* triethylammonium sulphate (A, C). Steroid sulphates applied in (A, B) were: 2 μg estriol-3-sulphate (E_3-S); 28 μg 16β-hydroxydehydroepiandrosterone-3-sulphate (16β-D-S); 28 μg 16α-hydroxydehydroepiandrosterone-3-sulphate (16α-D-S); 2 μg estrone sulphate (E_1-S); 2 μg 17β-estradiol-3-sulphate (E_2-S); 28 μg dehydroepiandrosterone sulphate (D-S); 775 μg androsterone sulphate (A-S) and 85 μg pregnenolone sulphate (P-S). Unconjugated steroids applied in (C) were 3.5 μg estriol (E_3); 22 μg 16β-hydroxydehydroepiandrosterone (16β-D); 22 μg 16α-hydroxydehydroepiandrosterone (16α-D); 3.5 μg estrone (E_1); 3.5 μg 17β-estradiol (E_2) and 31 μg dehydroepiandrosterone (D). The flow-rate was 1 ml/min, and the steroids were detected by their absorbance at 214 nm.

RESULTS

The resolution by isocratic reversed-phase HPLC of a mixture of androsterone sulphate, dehydroepiandrosterone sulphate, the 16α- and 16β-hydroxy derivatives of dehydroepiandrosterone sulphate, estrone sulphate, 17β-estradiol-3-sulphate, estriol-3-sulphate and pregnenolone sulphate was studied using a methanol–water eluent with or without the addition of triethylammonium sulphate. In the presence of 25 m*M* triethylammonium sulphate, all of the eight steroid sulphates were resolved with the exception of the pair estrone sulphate/17β-estradiol-3-sulphate which could not be completely separated at the given methanol–water ratio (Fig. 1A). In the absence of the quaternary ammonium salt, the steroid sulphates were only poorly resolved (Fig. 1B): estriol-3-sulphate, 16α- and 16β-hydroxydehydroepiandrosterone-3-sulphate, estrone sulphate and 17β-estradiol-3-sulphate were eluted together (first peak), followed by dehydroepiandrosterone sulphate (shoulder) and by androsterone sulphate and pregnenolone sulphate (second peak). A mixture of unconjugated dehydroepiandrosterone, 16α- and 16β-hydroxydehydroepiandrosterone, estrone, 17β-estradiol and estriol was also chromatographed under identical conditions. The elution pattern of the free steroids (Fig. 1C) resembled that of the corresponding steroid sulphates in the presence of salt; the retention times, however, were significantly longer and did not change when triethylammonium sulphate was omitted from the eluent.

The effect of the salt concentration on the retention times of the steroid sulphates was tested in the range of 1–25 m*M* triethylammonium sulphate. Apart from the pair estrone sulphate/17β-estradiol-3-sulphate mentioned above, baseline separation of all other steroid sulphates was achieved at the lowest salt concentration tested. By increasing the triethylammonium sulphate concentration from 1 to 25 m*M*,

TABLE I

EFFECT OF SOLVENT (TRIETHYLAMMONIUM SULPHATE) CONCENTRATION ON THE RETENTION OF STEROID SULPHATES

Reversed-phase HPLC of sulphoconjugated steroids was performed as described in Materials and methods. Mean values of capacity factors determined from triplicate analyses are given. The "retention time" of a non-retained peak was 1.1 min. Capacity factors of free steroids were 5.2 (estriol), 11.0 (16β-hydroxydehydroepiandrosterone), 14.5 (16α-hydroxydehydroepiandrosterone), 35.4 (estrone), 39.4 (17β-estradiol) and 62.1 (dehydroepiandrosterone) independent of the triethylammonium sulphate concentration employed.

Compound	*Triethylammonium sulphate concentration (mM)*				
	1	*2.5*	*5*	*10*	*25*
Estriol-3-sulphate	0.1	0.3	0.7	1.2	1.5
16β-Hydroxydehydroepiandrosterone-3-sulphate	0.5	0.9	2.4	3.5	4.5
16α-Hydroxydehydroepiandrosterone-3-sulphate	1.0	1.8	3.5	4.9	6.2
Estrone sulphate	1.5	3.3	5.4	7.5	9.9
17β-Estradiol-3-sulphate	1.6	3.8	6.1	8.6	11.1
Dehydroepiandrosterone sulphate	2.7	6.1	10.0	13.8	18.3
Androsterone sulphate	5.0	9.5	15.6	21.1	27.5
Pregnenolone sulphate	10.7	22.1	36.0	49.1	68.8

the retention times of all sulphoconjugated steroids were increased at least six-fold (Table I).

The lowest amount of dehydroepiandrosterone sulphate detectable in the eluate by absorbance at 214 nm was 200 ng. A linear relationship between the peak area and the amount of dehydroepiandrosterone sulphate injected was observed from 200 ng up to 1 mg. When ^{3}H-labelled dehydroepiandrosterone sulphate or estrone sulphate was added to the mixture of sulphoconjugated steroids, 97.4 ± 1.3% (eight experiments) and 95.9 ± 1.6% (ten experiments) of the expected radioactivity, respectively, were eluted together with the corresponding unlabelled steroid sulphates.

DISCUSSION

HPLC on non-polar adsorbents with organic solvent–water mixtures as eluent is a powerful tool in the analysis of unconjugated steroids. It is of little value, however, in the analysis of conjugated steroids; since the negative charge of the conjugating moiety hampers the interaction between steroid sulphates or glucuronides and the stationary phase, these compounds are hardly retained and thus poorly resolved. This drawback can be overcome by addition of suitable counter ions to the eluent. Ammonium ions have been used as counter ions to resolve steroid conjugates and free steroids by reversed-phase HPLC with a methanol gradient in water[2,3]. The increase in retention of steroid sulphates resulting from the addition of ammonium sulphate was explained by ion pairing in the solvent of the ionized sulphate group and the ammonium cation[3].

In the present investigation, triethylammonium ions have been used as counter ions. Due to their considerable hydrophobicity, the ions may interact with the stationary phase giving rise to a solvent-induced anion-exchange mechanism of steroid sulphate retention[1] in addition to the ion-pairing mechanism mentioned above. At least at the concentrations tested, triethylammonium sulphate seems to be more effective in retaining sulphoconjugated steroids than ammonium sulphate. Comparison of the data of Simonian and Capp[3] with our results supports this suggestion: in the presence of 5, 10 and 20 m*M* ammonium sulphate the capacity factors of dehydroepiandrosterone sulphate were 6.1, 12.2 and 17.3% of that of free dehydroepiandrosterone, whereas in the presence of 5, 10 and 25 m*M* triethylammonium sulphate the respective values were 16.2, 22.4 and 29.7%.

As shown in Fig. 1, the simple isocratic reversed-phase HPLC system with methanol–water–triethylammonium sulphate as eluent efficiently resolves a number of sulpohoconjugated steroids including strongly related ones like 16α- and 16β-hydroxydehydroepiandrosterone-3-sulphate. The system can easily be modified to fit individual separation problems by varying the triethylammonium sulphate concentration and/or the methanol–water ratio. Simultaneous separation of both free and sulphoconjugated steroids, for instance, can be achieved at low triethylammonium sulphate concentrations as is evident from the capacity factors given in Table I. Since the recovery of steroid sulphates is nearly quantitative, the HPLC system can be used not only for purification but also for quantification of sulphoconjugated steroids, either by direct evaluation of the detector output or by subsequent analysis of the separated fractions, for instance by radioimmunoassay.

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