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GROUP FRACTIONATION OF FREE AND CONJUGATED STEROIDS BY MEANS OF DISPOSABLE SILICA-BASED ANION-EXCHANGE COLUMNS

ERIC VANLUCHENE* and DIRK VANDEKERCKHOVE
University Hospital, Department of Gynaecology, State University of Ghent, De Pintelaan 185, B-9000 Ghent (Belgium)

SUMMARY

Adsorption and desorption modes have been elaborated which allow the separation of complex steroid mixtures of biological fluids into an unconjugated, a glucuronic acid conjugated and a sulphuric acid and mixed conjugated fraction. Capillary gas chromatographic analysis of the fractions obtained provides "total steroid profiles" of urine, blood, ovarian follicle fluid and amniotic fluid.

INTRODUCTION

In studies dealing with complex gas chromatographic steroid profiles an important gain in sensitivity and specificity of detection can be obtained by applying fractionation of the steroid extracts according to the conjugate forms. The Sephadex LH-20® column chromatography method[1] using chloroform–methanol containing 0.01 *M* sodium chloride is still frequently used. Apart from a sulphate and a disulphate fraction, a mixed unconjugated and glucuronic acid-conjugated fraction are obtained. Alternative methods use hydrophilic or lipophilic Sephadex-based anion exchangers. Separate glucuronide, monosulphate and disulphate conjugated steroids can be obtained with the anion exchanger diethylaminohydroxypropyl (DEAP)-Sephadex LH-20[2]. It was also possible to separate simultaneously a phenolic and a neutral steroid fraction if a strong anion exchanger triethylaminohydroxypropyl-Sephadex LH-20 was used[3]. Recently strong anion exchangers based on silica have been made available as disposable sample preparation columns. We have determined the best working conditions to allow them to be used for the fractionation of steroids and their conjugated forms.

MATERIALS AND METHODS

Bonded octadecylsilica and quaternary amine strong anion-exchange disposable columns (500 mg dry material), respectively Bond Elut® C_{18} (BE C_{18}) and Bond Elut® SAX (BE SAX), were from Analytichem International (Harbor City, CA, U.S.A.). Also from Analytichem was a ten-place vacuum manifold (Vac Elut) allowing simultaneous elution of up to ten disposable columns. Methanol was of residue-

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free quality. Triethylammonium sulphate (TEAS, 0.5 *M*, pH 5.0) was prepared by titrating triethylamine with 0.5 *M* aqueous sulphuric acid. This solution was used to prepare the 0.25 *M* triethylammonium sulphate in methanol–water (1:1). Lipidex 5000® was obtained from United Technologies Packard. The *Helix pomatia* juice (Industrie Biochimique Francaise, Clichy, France) contains 100 000 F.U. β-glucuronidase and 1 000 000 R.U. sulphatase per ml [one Fishman Unit (F.U.) is the amount of β-glucuronidase liberating 1 μg of phenolphtalein in 1 h at 37°C; one Roy Unit (R.U.) is the amount of sulphatase that hydrolyses 1 μmol *p*-nitrocatechol sulphate per h at pH 5 and 37°C].

Sample processing

Anion exchange. To the biological fluid (serum, urine, amniotic fluid) was added water, 1.5 *M* potassium acetate buffer and 0.5 *M* triethylammonium sulphate solution in water (both pH 5.0) so that final concentrations of respectively 0.15 and 0.125 *M* were obtained[4]. This mixture was applied to the BE C_{18} column (1.5 cm × 0.9 cm I.D.) (primed with 5 ml methanol and 10 ml water) and sucked through at two drops per second. After rinsing with 7.5 ml 0.15 *M* potassium acetate buffer (pH 5.0), the column was sucked dry and removed from the Vac Elut. The BE SAX column (1.5 cm × 0.9 cm I.D.) was washed successively with 4 ml methanol, 5 ml water, 25 ml 0.5 *M* acetic acid in water and again 25 ml water in order to replace the original chloride counter ion by the acetate ion. Then 4 ml methanol were applied and sucked through, except for 1 ml which was left in the anion-exchanger column. The BE C_{18} column upon which the steroids were adsorbed was attached to the BE SAX column. A 4-ml volume of methanol was applied to desorb the free and conjugated steroids from the C_{18} column and simultaneously load them on the SAX column (rate: two drops per second). The non-retained unconjugated steroids were collected using Rotavapor-adapted 40-ml ground-glass tubes. After removing the C_{18} column an additional 1 ml methanol was sucked through the SAX column, which completed the elution of the unconjugated steroids. The glucuronic acid conjugates were eluted with 8.4 ml 0.2 *M* formic acid in methanol–water (1:1). The steroid sulphates were desorbed with 8.4 ml of the 0.25 *M* TEAS solution.

Enzymatic hydrolysis and solvolysis. The steroid glucuronide fraction was taken nearly to dryness using a rotary evaporator. The residue was dissolved in 4.5 ml water, brought to pH 4.8 with 0.5 *M* sodium hydroxide (three drops) and incubated with 40 μl *Helix pomatia* extract at 37°C for 24 h. The incubate was saturated with solid sodium bicarbonate and extracted with 20 ml ethyl acetate. Finally, the extract was washed with 5 ml water.

The steroid sulphate fraction was taken to dryness and redissolved in water (4.5 ml). A 1-g amount of sodium chloride was added and the pH adjusted to $\leqslant 1$ using one drop of concentrated sulphuric acid. A 30-min extraction with 20 ml ethyl acetate (automatic shaking apparatus) was then performed. After centrifugation at 1500 *g*, the water layer was removed and the upper layer incubated during 16 h at 37°C. Finally the ethyl acetate was washed with saturated bicarbonate and water.

Derivatization and gas chromatography. Methoxime-trimethylsilyl ethers were formed, purified using Lipidex 5000® columns and injected into a 25 × 0.32 mm I.D. polydimethylsiloxane capillary column (CP Sil 5 from Chrompack, The Netherlands) using a falling needle injector, as described previously[4,5]. A Varian 3500 in-

strument was used. The hydrogen carrier gas flow-rate was 2 ml/min. The oven temperature was first programmed from 160°C at 20°C/min to 220°C and then at 4°C/min to 285°C. Detection was done by a flame thermionic detector except for compounds without a methoxime function. These were detected using flame ionization.

Recovery estimation. Recovery of steroid glucuronides was assessed by analysing urine samples from adults (containing merely glucuronide-conjugated steroids) and comparing the results with those obtained with a similar procedure not including the BE SAX fractionation[5]. Likewise, steroid sulphate recovery was estimated in serum samples from adults as in this fluid the vast majority of steroids occur as sulphate conjugates.

Measurement of elution curves and recoveries with radioactive steroids and steroid conjugates. Twenty thousand counts of respectively [2,4,6,7-$^3H_4$]oestradiol, [2,4,6,7-$^3H_4$]oestradiol-17β-glucuronide and [7-^{3}H]dehydroepiandrosterone-3β-sulphate were mixed with 1 ml of an urine sample and the conjugates separated as described, except that fractions of 1.4 or 1 ml were collected. Solvents were evaporated with nitrogen, the residues dissolved in scintillation fluid and counted.

Thin-layer chromatographic confirmation of steroid conjugation in different fractions. This was done on high-performance silica gel plates from Merck using propan-2-ol–chloroform–methanol–water (10:10:5:2) as the eluent. Detection of the steroids was made possible by heating at 120°C after spraying with an ethanolic solution of sodium *p*-phenolsulphonate (0.2 g per 100 ml) and phosphoric acid (8.5%).

Quantitation. Appropriate amounts of different internal standards were added: for unconjugated steroids 1.25 μg tetrahydrocortisol were added before the adsorption on BE C_{18}. Testosterone was added after the BE SAX column to both sulphate and glucuronide fractions (for 1 ml urine, 2.5 and 10 μg respectively). Relative response coefficients for each particular steroid were determined in relation to the internal standard and used in the calculation of concentrations.

RESULTS*

As shown in Fig. 1, radioactive steroids and steroid conjugates are completely separated from each other. Quantitative elution is obtained with only small amounts of the indicated eluents. The recovery for unconjugated radioactive oestradiol was 98%. Less than 2% of the total radioactivity was eluted in the formic acid-containing eluate. Of oestradiol glucuronide, less than 1% eluted in both the unconjugated and sulphate fraction. Up to 99% dehydroepiandrosterone (DHA) sulphate was found in the 0.25 *M* TEAS fraction.

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* Abbreviations used in the figures and tables: Andr = androsterone; Etio = etiocholanolone; DHA = dehydroepiandrosterone; KA = 11-oxo-androsterone; T = testosterone (internal standard); HA = 11β-hydroxyandrosterone; HE = 11β-hydroxyetiocholanolone; THE = tetrahydrocortisone; THA = tetrahydro-11-dehydrocorticosterone; THB = tetrahydrocorticosterone; aTHB = 5α-tetrahydrocorticosterone; THF = tetrahydrocortisol; aTHF = 5α-tetrahydrocortisol; E = cortisone; F = cortisol; eAndr = epiandrosterone; 16 DHA = 16α-hydroxydehydroepiandrosterone; 'Pon = pregnenolone; 16β-DHA = 16β-hydroxydehydroepiandrosterone; $\alpha\alpha$Pon = 3α-hydroxy-5α-pregnan-20-one; Pg = progesterone; 16'Pon = 16α-hydroxypregnenolone; B = corticosterone; IS = 20α-dihydroprogesterone (used as the internal standard); androstenediol = 3β,17β-dihydroxy-5-androstene; pregnenediol = 3β,20α-dihydroxy-5-pregnene; pregnenetriol = 3β,17α,20α-trihydroxy-5-pregnene; MeOH = methanol; TEAS = triethylammonium sulphate.

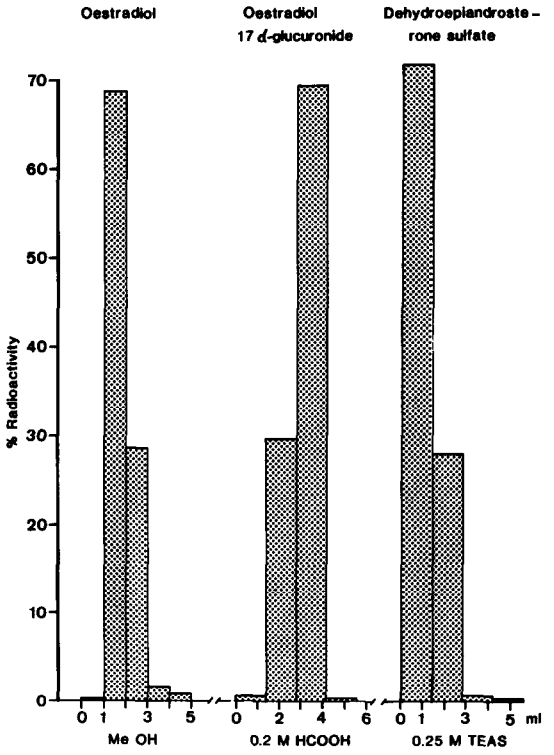

Fig. 1. Separation of radioactive steroid(conjugate)s oestradiol, oestradiol-17α-glucuronide and dehydroepiandrosterone sulphate using a disposable strong anion-exchange column.

TABLE I

RELATIVE RECOVERY OF THE BE SAX METHOD COMPARED WITH A REFERENCE METHOD FOR STEROID GLUCURONIDES (G) IN URINE AND FOR STEROID SULPHATES (S) IN SERUM

Steroid conjugate	*Recovery (%)*	*Coefficient of variation (%)*
Urine		
Androsterone G	106.3	6.4
Etiocholanolone G	99.2	3.1
Tetrahydrocortisone G	107.4	5.0
Tetrahydrocortisol G	104.7	3.7
5α-Tetrahydrocortisol G	106.6	4.0
Serum		
Androsterone S	116.0	5.1
Dehydroepiandrosterone S	95.7	4.3
Epiandrosterone S	100.5	4.7
Pregnenediol S and diS	105.2	6.3
16α-Hydroxydehydroepiandrosterone S	113.3	3.6

TABLE II

REPRODUCIBILITY DATA FOR FIVE ANALYSES OF AN URINE AND OF A SERUM POOL

Steroid conjugate	*Coefficient of variation (%)*
Urine	
Androsterone G	6.5
Etiocholanolone G	7.9
Tetrahydrocortisone G	5.4
Tetrahydrocortisol G	2.8
5α-Tetrahydrocortisol G	6.5
Serum	
Androsterone S	9.0
Dehydroepiandrosterone S	4.3
Androstenediol S and diS	5.0
Pregnenediol S and diS	4.2
Pregnenetriol S and diS	6.3

As we could not obtain a radioactive steroid disulphate conjugate, the elution of disulphates was tested with thin-layer chromatography (TLC). The disulphate of 5-androstene-3β,17β-diol exhibited an R_F value of 0.43 and its monosulphate one of 0.66. The R_F values for glucuronides were below 0.1. All conjugates tested were eluted quantitatively in their appropriate fraction. As expected, glucurosulphate double conjugates are eluted in the sulphate fraction.

Relative recoveries, calculated for urinary steroid glucuronides and steroid sulphates in serum, are given in Table I. As stated above, a method not including anion exchange is used as a reference here. Five different urines and six different serum samples have been analysed.

The reproducibility for determinations in urine and serum is given in Table II. In the urine samples represented in the chromatograms of Fig. 2 the concentrations were: 1.4 mg/l for androsterone glucuronide and 2.3 mg/l for tetrahydrocortisone. The androsterone sulphate concentration (Fig. 2B) was 0.54 mg/l and we calculated the dehydroepiandrosterone sulphate concentration to be 0.70 mg/l.

The serum sample (Fig. 3) contained 151 ng cortisol per ml. The dehydroepiandrosterone sulphate (Fig. 3C) concentration was 1287 ng/ml, whereas tetrahydrocortisone glucuronide (Fig. 3B) amounted to only 182 ng/ml.

DISCUSSION

The preliminary adsorption on octadecylsilica may seem at first glance unnecessary. We however were not able to bind quantitatively the steroid glucuronides when starting with an aqueous biological matrix as diluted urine or serum. Positive ions present in the biological fluids may from ion pairs with the steroid glucuronides, so avoiding sorption to the anion exchanger. An octadecylsilica purification step was found earlier[5] to retain quantitatively free steroids as well as conjugated steroids, if triethylammonium sulphate was used as an ion-pair-forming reagent. Moreover, by including adsorption on BE C_{18}, samples with different biological matrices, *e.g.*, tissue extracts, amniotic fluids with high protein concentrations, are made similar so

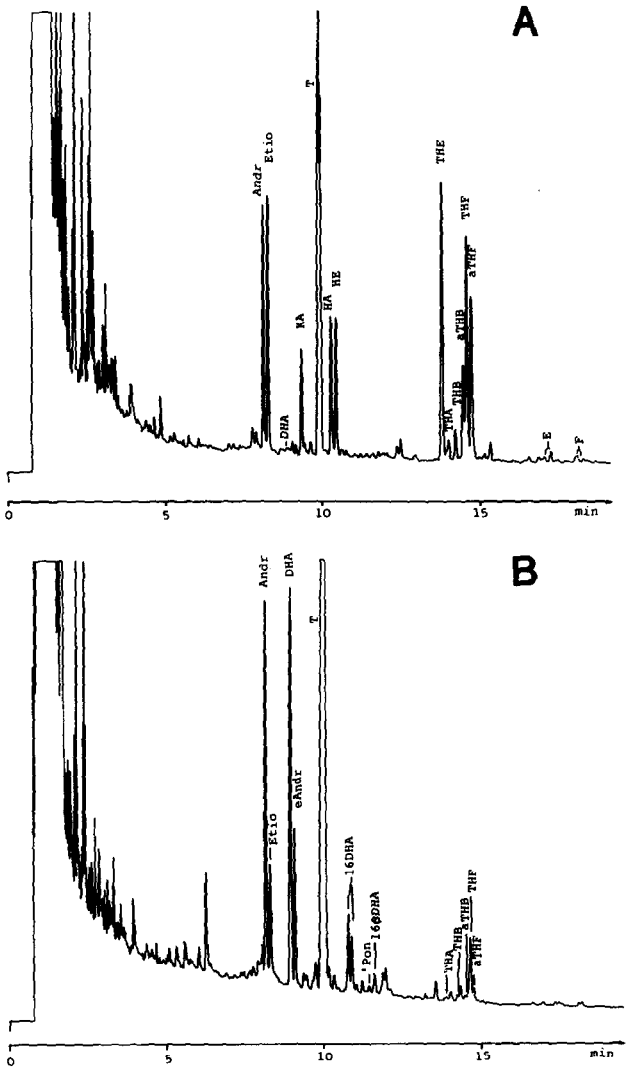

Fig. 2. Gas chromatograms of steroids in urine. (A) Steroid glucuronides; (B) steroid sulphates. For chromatographic conditions, see Materials and methods. Flame thermionic detection was used.

that the BE SAX fractionation is of general applicability. Conversion of the BE SAX into its acetate form is necessary because the results with the original chloride-ion form are strongly influenced by varying residual water concentrations in the methanol eluate from the BE C_{18} column.

The possibility to retain the conjugates on the BE SAX column from a methanol solution allowed direct elution of the C_{18} eluate into the coupled BE SAX column. Time-demanding transfers and the introduction of impurities from glassware are so avoided.

Complete desorption of steroid disulphates could in our hands be obtained only with a triethylammonium sulphate-containing eluent. More selective anions, *e.g.*,

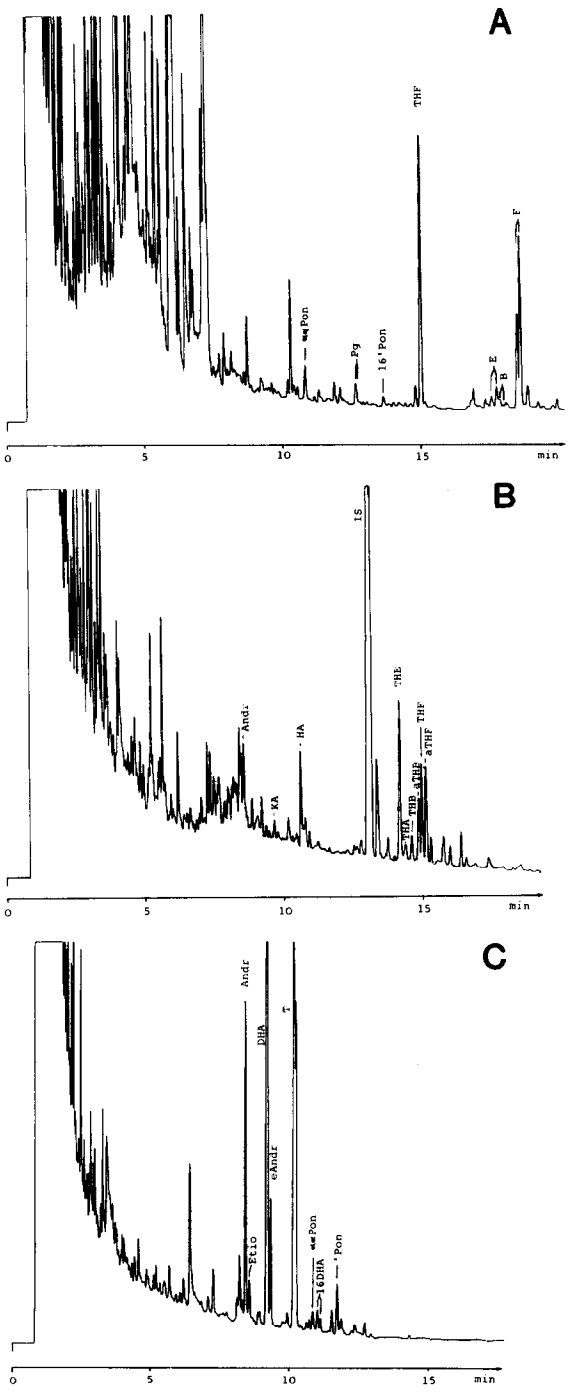

Fig. 3. Gas chromatograms of steroids in serum. (A) Unconjugated steroids; (B) steroid glucuronides; (C) steroid sulphates. For chromatographic conditions, see Materials and methods. Flame thermionic detection was used.

citrate or higher molarity (1 *M*) and higher pH (6.0) acetate buffers in 50% methanol–water did not result in quantitative recovery of, *e.g.*, 5-androstene-3β,17β-disulphate. Perhaps an adsorptive interaction mechanism with residual free silanol groups on the silica material (although the manufacturers claim complete end-capping) plays a role here. Amines are known to block specifically these silanol functions.

Initially, we included a second BE C_{18} adsorption step to remove triethylammonium sulphate prior to the solvolysis or enzymatic hydrolysis (a kind of desalting step). Experiments proved this to be unnecessary.

Steroid concentrations are generally higher (see Table I) after application of the BE SAX group fractionation. This is probably due to the more favourable hydrolysis conditions obtained after purification of the samples. Most urines contain androsterone and also tetrahydrocortisol as a sulphate. The reference method without group fractionation should thus provide higher steroid concentrations. This is not the case, indicating good relative recovery with the BE SAX method.

The solvolysis procedure described is necessary for the quantitative hydrolysis of especially androsterone and epiandrosterone sulphate (both 3α-sulphates of a 5α-steroid) in serum or urine. In urines of newborn infants, enzymatic hydrolysis resulted in only 20% of what was obtained using solvolysis for 16α-hydroxypregnenolone and 3β,16α-dihydroxy-5-androstene-17-one. These urines contain large quantities of mixed conjugates (especially of oestriol and 3β,16α,18-trihydroxy-5-androstene-17-one) for which both solvolysis and enzymatic hydrolysis has to be applied.

CONCLUSIONS

A less time-consuming and less labour-intensive method has been developed to separate steroids according to their conjugate forms. The total steroid profiles obtained after capillary gas chromatography of the different fractions allow quantitations in the presence of much larger amounts of other conjugate forms. Especially the recognition of steroid-synthesizing defects in neonates is made easier: here the high concentrations of sulphates of 5-ene-steroids interfere with the quantitation of gluco- and mineralo-corticosteroid glucuronides. The method has been applied to many different body fluids, *e.g.*, amniotic fluid containing high amounts of proteins and tissue extracts. It is predicted that, also for the analysis of conjugates of pharmaceutical products, the procedure would be of great value.

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