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DIRECT DETERMINATION OF STEROIDAL SULFATES*

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SUMMARY

The acylation of steroid sulfates by heating at 70°C for 0·5 h with heptafluorobutyric anhydride occurs in quantitative yield. The ester formed is the same as that obtained when the free steroid was so treated with heptafluorobutyric anhydride. Proof was provided by classical chemical analyses.

The steroid sulfates of cord blood plasma were extracted and separated on Sephadex LH-20. Acylation with heptafluorobutyric anhydride was followed by gas chromatography with detection by electron capture. Results of analyses of steroid sulfates in human cord blood show the presence of estriol and 16α-hydroxydehydroepiandrosterone.

INTRODUCTION

The present paper describes the methodology and results of acylation of steroidal sulfates by heating with heptafluorobutyric anhydride (HFBA) to give derivative formation without prior hydrolysis. Electron capture gas chromatography provided sensitive detection and quantitation. Estrogen sulfates [1, 2] and dehydroepiandrosterone sulfate† [3] are major metabolites in the peripheral circulation and the methods described herein may make it possible to quantitate them more rapidly.

The study of sulfates, particularly steroid sulfate esters in biological systems, has been hampered by tedious methodology. Most procedures involve hydrolysis by enzymes or solvolysis before quantitation can be carried out. Boiling with acid is often carried out but is not suitable in case of labile compounds.

In the method described here, the steroid sulfates were extracted from plasma by the method of Loriaux *et al.*[1]. The sulfates were then separated by chromatography on Sephadex LH-20, and this fraction was subjected to acylation with heptafluorobutyric anhydride. Results obtained with samples of cord blood plasma are given.

MATERIALS AND METHODS

The sulfate esters were purchased from Schwarz–Mann and Sigma Chemical Co. Routinely, all samples were evaluated for purity by chromatography on Sephadex LH-20 or thin-layer chromatography. Solutions were made in alcohol (0·1 μg/μl) and were kept at 4°C when not in use. The sulfates remained stable, except for estrone-3-sulfate sodium salt, which decomposed with time in ethanol solution. It was found that a few drops of NH_4OH added to the solution increased the stability.

[6,7-^{3}H(N)]-Estrone sulfate, ammonium salt and testosterone[7-^{3}H-(N)]-sulfate, ammonium salt were obtained from New England Nuclear, Boston, Mass. These were periodically evaluated by thin-layer and Sephadex LH-20 chromatography. [6,7-^{3}H]-Estriol-16-glucuronide was a generous gift of Dr. Mortimer Levitz.

Gas chromatograph. A Glowall 310 gas chromatograph (Willow Grove, Pa.) equipped for electron capture detection was used. This contained a Lovelock detector with a 22·5 μCi radium foil that is suitable for beta-ionization as well as electron capture work. The 6 ft coiled glass column (3·4 mm i.d.) was packed with Supelcoport (Supelco, Bellefonte, Pa.) coated with 5% OV-210, plus 2·5% OV-17. Operating conditions were: flash, 270°; column, 225°; and detector oven, 240°C. Argon inlet pressure was 30 psi, giving a flow rate of 60 ml/min.

Heptafluorobutyric anhydride was obtained from Pierce Chemical Co. and was distilled.

Extraction of the sulfates

The extraction procedure followed a modification of that described by Loriaux *et al.*[1]. Plasma (4 ml) was saturated with sodium chloride until crystals were seen on the bottom of the 50 ml glass stoppered tube. The plasma was extracted twice with 10 ml of tetrahydrofuran. Centrifugation was required to separate the phases. The upper phase (tetrahydrofuran) was siphoned off, and was evaporated *in vacuo*

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† Trivial—IUPAC Name Listings: estrone sulfate: 17-oxo-1,3,5(10)-estratrien-3-yl sulfate; estriol sulfate: 16α, 17β-dihydroxy-1,3,5(10)-estratrien-3-yl sulfate; dehydroepiandrosterone sulfate (DHEAS): 17-oxo-5α-androsten-3β-yl sulfate; dehydroepiandrosterone (DHEA): 3β-hydroxy-5-androsten-17-one; 16α-hydroxydehydroepiandrosterone (16α-hydroxy-DHEA); 3β,16α-dihydroxy-5-androsten-17-one; androsterone sulfate: 17-oxo-5α-androstan-3-yl sulfate.

at a water bath temperature not over 50°C. Isotopically labelled steroid sulfates were added before extraction to assess recovery.

Separation of the sulfates on Sephadex LH-20

Sephadex LH-20 was swelled with chloroform–methanol (1:1 v/v) containing 0·01 M sodium chloride. A 1 × 20 cm. glass column was packed to a depth of 10 cm. using 2 g of Sephadex. The column was washed with 24 ml of the eluting solution prior to adding the extract to the column, in 0·5 ml of the eluting solution. The first 12 ml of eluting solvent was discarded. The second 12 ml was collected (sulfate fraction). Then the column was eluted with 12 ml of methanol (disulfates). These last two collections were combined. After concentration *in vacuo* a 1/10 aliquot was removed for scintillation counting to assess recovery.

The residue after evaporation of the eluting solvent was reacted with heptafluorobutyric anhydride as described below.

Acylation with heptafluorobutyric anhydride

The dried sample (100 μg) or extract containing steroid sulfate was placed in a 100 × 13 mm screw capped (Teflon lined) test tube. Pesticide grade benzene (redistilled daily) (200 μl) and heptafluorobutyric anhydride (100 μl) were added. The mixture was sealed in the test tube and heated in a block at 70°C for 0·5 h. The reaction mixture was evaporated to dryness under a stream of dry nitrogen in a warm water bath. Predetermined amounts of benzene were added for injection of aliquots into the gas chromatograph. The same procedure was followed for preparation of the heptafluorobutyrates of unconjugated steroids [5–7].

RESULTS AND DISCUSSION

Proof of acylation of the steroid sulfate was made by classical chemical characterization and comparison of the products of reaction of heptafluorobutyric anhydride with the pair DHEA and DHEAS. The results of these experiments follow.

Thin-layer chromatography

The reaction products of both DHEA and DHEAS were applied to a silica gel G thin-layer plate. After development with the solvent benzene–ethylacetate (90:10 v/v) the plates were dried in air. The plate was then sprayed with 5% phosphomolybdic acid in ethanol. Heating at 120°C for 10 min served to bring out the blue color of the reaction products. The R_F of the two substances was the same. No trace of unreacted or unknown substances was seen on the chromatograms. One major zone was seen.

Table 1 shows the mobilities of the product of heptafluorobutyrylation of several other steroid sulfates prepared in the same way.

TABLE 1. R_f Values for Steroidal Heptafluorobutyrates on Silica Gel G Thin Layers

Steroid	Free	Free + HFBA[a]	Sulfate + HFBA[b]
estrone	0.29	0.29	0.29
estriol	0.00	0.44	0.44
DHEA	0.14	0.51	0.51
androsterone	0.10	0.53	0.53

a - free steroid reacted with heptafluorobutyric anhydride

b - steroid sulfate reacted with heptafluorobutyric anhydride

Gas chromatography

Gas chromatography of the product of acylation of the steroid and steroid sulfate pair also showed the agreement of identity of the esters. The non-acylated steroid always had a longer retention time. On columns of 5% OV-1 on Supelcoport, the retention times of estrone-heptafluorobutyrate and androsterone-heptafluorobutyrate were the same. Subsequently it was found that gas chromatography with a mixed phase column of 5% OV-210 and 2·5% OV-17 gave a separation of these two steroids. Table 2 shows the retention times of several steroidal heptafluorobutyrates on this mixed phase packing. In each case the acylation product of the free steroid and its sulfate gave the same retention time. The chromatograms showed only trace amounts of unreacted or unknown substances.

Linear calibration curves were obtained from the heptafluorobutyrate derivatives at the picogram level. Linear curves were obtained after derivatization of estriol sulfate from 10 to 100 pg.

Melting point determination

Milligram quantities of the heptafluorobutyrate of DHEA and DHEAS were prepared for determination of melting point and mixed melting points and elemental analysis. After thin-layer chromatography the heptafluorobutyrates were subjected to vacuum sublimation at a temperature of 120°C with 2 Torr pressure. The ester readily sublimed.

The melting point of the sublimate of both products was 117°C. When the two substances were mixed the melting point was 116–117°C (uncorrected).

Infrared spectrometry

The acylation products of DHEA and DHEAS after sublimation were subjected to infrared spectro-

TABLE 2. Retention Times of Steroidal Heptafluorobutyrates (as mm from injection peak)

Steroid	Free	Free + HFBA[a]	Sulfate + HFBA[b]
estrone	110	105	105
estriol	260	116	116
DHEA	110	104	104
16α-hydroxy-DHEA	360	142	---[c]

a - free steroid reacted with heptafluorobutyric anhydride (HFBA)

b - steroid sulfate reacted with heptafluorobutyric anhydride

c - steroid sulfate standard not available

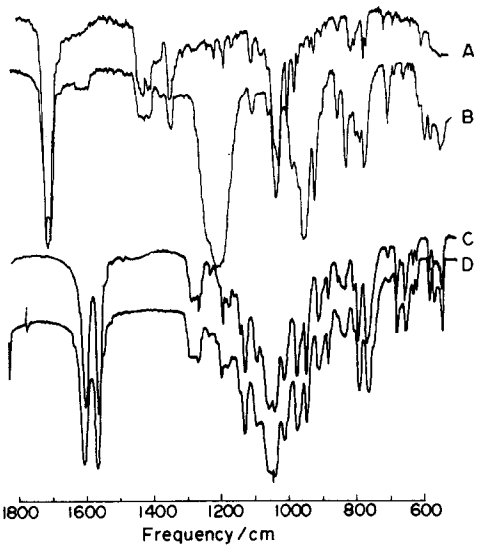

Fig. 1. Infrared spectra of: a. dehydroepiandrosterone; b. dehydroepiandrosterone-3-sulfate; c. dehydroepiandrosterone-heptafluorobutyrate; d. product of reaction of (b) with heptafluorobutyric anhydride.

metry. Each was prepared in KBr pellets. Infrared spectrometry (Perkin–Elmer 421 Spectrometer) showed that the two products were the same. The characteristic broad bands in the 1240–1125 and 1070–1060 cm. regions of the spectrum were no longer present in the spectrum of the sulfate after reaction with the anhydride. The bands common to carbonyl stretching of the ester linkage were present in both cases. This provided further evidence for the identity of the reaction products. Figure 1 shows the spectra of the free, and sulfurylated DHEA and the products of their reaction with heptafluorobutyric anhydride. The fingerprint regions were identical.

Mass spectrometry

Mass spectrometry of the heptafluorobutyrates of DHEA and its sulfate showed that they were the same. The mass spectrometry was performed by William Comstock, Heart and Lung Institute of the National Institutes of Health, through courtesy of Dr. Henry Fales. Samples of dehydroepiandrosterone (100 μg) and dehydroepiandrosterone sulfate (100 μg) were reacted with heptafluorobutyric anhydride as described. These were purified by thin-layer chromatography and the zones were scraped and eluted. Silica gel particles were removed by filtration through Millipore filters (40 μm). The spectra of the two derivatives, after mass spectrometry using a direct probe, were the same. The spectra, after gas chromatography–mass spectrometry, showed a small peak at *m/e* 484 corresponding to DHEA monoheptafluorobutyrate. A large peak at *m/e* 270 and corresponding to $(M\text{-}214)^+$, the loss of a heptafluorobutyryloxy residue (mass = 213) and resultant lack of hydrogen of the –O– at C_3 of the parent molecule was present with equal magnitude in each of the spectra. These results agree with those observed by Challis and Heap[5]. Segments of the mass spectra including the ion at *m/e* 270 are shown in Fig. 2.

Elemental analysis

After thin-layer chromatography and vacuum sublimation, the product of acylation of DHEA sulfate was subjected to elemental analysis for $C_{23}H_{26}F_7O_3$:

	C	H	F
Theoretical, %	57·12	5·38	27·54
Found	57·82	5·93	27·52

Recovery and reproducibility

Assessment of the accuracy of the procedure during the extraction and chromatography was made from recovery of the radioactivity added to the plasma. Recovery of radioactivity averaged 81·31% with standard deviation of 15·0%. The standard error was 3·75%. These results were obtained using testosterone sulfate or estrone sulfate added to the plasma, with counting after separation on Sephadex LH-20.

Presentation of evidence for the specificity of the procedure involving amounts of sulfates available in biological samples is difficult. The separation of the conjugates on the Sephadex LH-20 column has been well documented [8]. Steroids and their glucuronides when subjected to LH-20 chromatography always eluted in the first 12 ml fraction. No glucuronide or free steroid were found in the "sulfate" fraction.

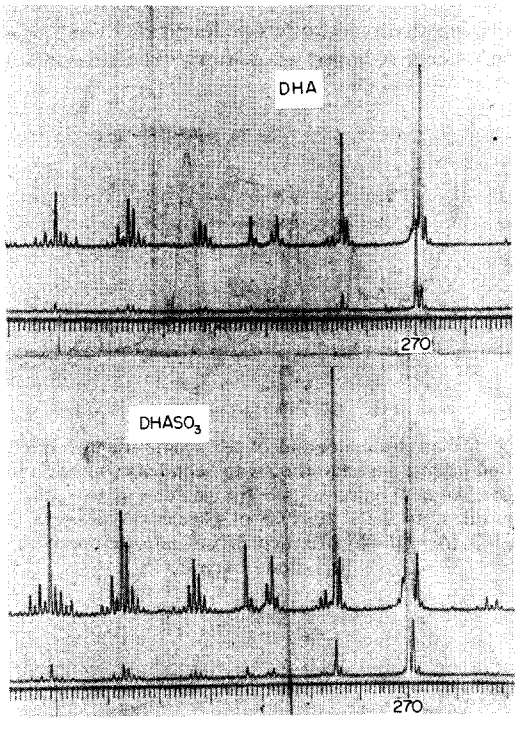

Fig. 2. Mass spectra of: DHA = dehydroepiandroterone heptafluorobutyrate; $DHASO_3$ = product of reaction of dehydroepiandrosterone sulfate with heptafluorobutyric anhydride.

Labelled estriol-6,7-H^3-16-glucuronide was found in the first fraction of the column.

The reaction of heptafluorobutyric anhydride with steroid sulfates has been verified with other steroids as shown in Tables 1 and 2. The reaction does not proceed with the glucosiduronates which were investigated. The reaction of the anhydride with the free steroid has been thoroughly investigated [5–7].

The use of the mixed phase (OV-17 + OV-210) packing in the gas chromatograph also increased the selectivity of the separation. The esters of estrone and DHEA could not be separated on OV-1 while they did separate on the mixed phase.

Gas chromatography of the heptafluorobutyrate obtained with biological samples which had been spiked with a known sulfate gave further evidence for the nature of the peaks which separated. An increase only in the peak in question was seen.

The peaks seen in the extract of cord blood were well separated and triangulation for quantitation was readily accomplished.

Results obtained with cord blood

Figure 3 shows a typical gas chromatogram of the steroid sulfates of cord blood plasma. It is known that the major steroids of this media are estriol and 16α-hydroxy-DHEA. The levels found in this study agree well with those reported by Magendantz and Ryan[9]. The nature of the other substances in the sulfate fraction indicated in the chromatograms is not at present known.

Table 3 shows the amounts of estriol and 16α-hydroxy dehydroepiandrosterone found in 15 samples of cord blood obtained at delivery. The amounts injected into the gas chromatograph represent $2{\cdot}5 \times 10^{-4}$ ml of blood. Since the proportion of steroids present in these samples is relatively high, well separated peaks with high signal to background ratios were obtained. Levels of 50 pg/ml of blood are readily detected.

TABLE 3. Steroid Sulfates in Cord Blood

pg/100 ml

Subject	Estriol[3]	16α-Hydroxy DHEA[3]
61	47	135
63	58	259
66	12	223
66-1	28	158
68	50	259
69	54	146
69-1	18	87
74	22	135
74-1	37	222
83	38	210
83-1	45	250
86	66	1
89	35	200
92	22	1

The method described gives a rapid determination of organic sulfates without hydrolysis of the sulfate moiety. Acid or enzyme hydrolyses are avoided and the procedure may be more reproducible. The labile substances can then be more readily detected.

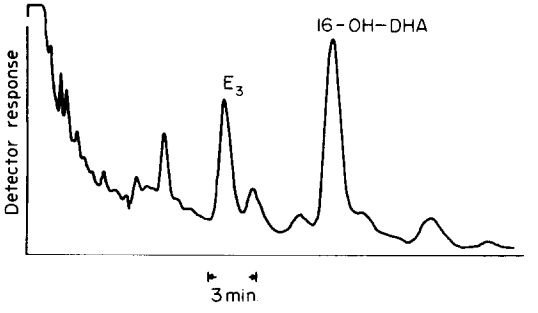

Fig. 3. Gas chromatogram of the sulfate fraction of cord blood plasma after acylation with heptafluorobutyric anhydride. Amount injected into the gas chromatograph was equivalent to $2{\cdot}5 \times 10^{-4}$ ml of plasma. E_3 = estriol 16-OH-DHA = 16α-hydroxydehydroepiandrosterone both as heptafluorobutyrate.

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