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DETERMINATION OF SERUM Δ^5-3β-HYDROXYSTEROID SULPHATES BY COMBINED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND IMMOBILIZED 3β,17β-HYDROXYSTEROID DEHYDROGENASE IN COLUMN FORM

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SUMMARY

We studied the use of an immobilized enzyme, covalently bound to aminopropyl-CPG, in the analysis of individual Δ^5-3β-hydroxysteroid sulphates. A microcolumn with immobilized 3β,17β-hydroxysteroid dehydrogenase was prepared and used together with high-performance liquid chromatogaphy (HPLC). The reduced nicotinamide-adenine dinucleotide produced from Δ^5-3β-hydroxysteroids by this enzyme was fluorimetrically determined. The immobilized enzyme was sufficiently stable for at least one month or for 180 tests when used repeatedly. A clinical trial demonstrated that this HPLC—immobilized enzyme method is superior to the soluble enzyme method, giving reliable and reproducible results at a low cost.

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INTRODUCTION

Δ^5-3β-Hydroxysteroid sulphates [5-androstene-3β,17β-diol (Δ^5-androstene-diol), dehydroepiandrosterone (DHEA), pregnenolone and 17-hydroxypregnenolone] are secreted almost exclusively from the adrenal gland. DHEA sulphate is the major Δ^5-3β-hydroxysteroid sulphate and the most common adrenal steroid in the blood. Serum DHEA sulphate, therefore, has served as a valuable indication of adrenal activity [1].

Many methods have been reported for the determination of DHEA sulphate in biological fluids, including gas chromatography [2, 3], gas chromatography–mass spectrometry [4] and radioimmunoassay (RIA) [5—7].

Here, we describe a high-performance liquid chromatographic (HPLC) method for the determination of individual Δ^5-3β-hydroxysteroid sulphates in which specific on-line detection is performed using immobilized 3β,17β-hydroxysteroid dehydrogenase (β-HSD) and a fluorimeter to detect the resulting nicotinamide-adenine dinucleotide transformation from the oxidized to the reduced form ($NAD^+ \rightarrow NADH$). The principle of this method is as follows: after separation of Δ^5-3β-hydroxysteroids by HPLC, NADH is produced by use of the enzyme β-HSD, which catalyses the reaction:

$$3\beta,17\beta\text{-hydroxysteroids} + NAD^+ \xrightarrow{\beta\text{-HSD}} 3,17\text{-oxosteroids} + NADH$$

EXPERIMENTAL

Materials

5-Androstene-3β,17β-diol, testosterone, oestradiol, dehydroepiandrosterone, dehydroepiandrosterone sulphate, 5α-androstane-3β,17β-diol, 5α-androstane-3α,17β-diol, 17-hydroxypregnenolone, pregnenolone, oestriol, androsterone, etiocholanolone, cortisol, internal standard (5β-pregnan-3β,20α-diol), 3β,17β-dehydroxysteroid dehydrogenase (β-HSD) (Grade II from *Pseudomonas testosteroni* Lot No. 40F-6809), sulphatase (Type VIII, from Abalone Entralis Lot No. S-9754) and NAD were obtained from Sigma (St. Louis, MO, U.S.A.). Methanol and other chemicals were obtained commercially.

Apparatus

A JASCO (Tokyo, Japan) Trirotar-V high-performance liquid chromatograph was used throughout this work. The instrument was equipped with a JASCO FP-110C spectrofluorimeter and a gradient elution accessory G-A 40. A JASCO Bilepak column (25 cm × 4.6 mm I.D., particle size 5 μm) was used for the separation. Peak area and retention time were determined with a JASCO DP-L220 data processor.

Preparation of immobilized enzyme column

Fig. 1 outlines the immobilization procedure. An immobilized enzyme column (3.5 cm × 4.6 mm I.D.) was prepared by binding β-HSD to an aminopropyl-CPG glass with glutaraldehyde via Schiff base formation [8] and was placed into a continuous-flow system using HPLC, the diagram of which is shown in Fig. 2.

200 mg of aminopropyl-CPG (Electro-Nucleonics; pore diameter 544 Å; particle size 120–200 mesh)

|

|—2 ml of 2.5% glutaraldehyde

|

degassing for 30 min at room temperature

|

reaction for 60 min at 1 bar

|

washing with distilled water

|

|—5 mg of β-HSD in 1 ml of 10 mmol/l KH_2PO_4 (pH 7.80)

|

degassing for 30 min in ice bath

|

reaction for 30 min at 1 bar

|

washing with distilled water

|

|—10 mmol/l KH_2PO_4 + 1 mmol/l EDTA + 0.05% 2-mercaptoethanol (pH 7.80)

|

packing

Fig. 1. Preparation of immobilized enzyme column.

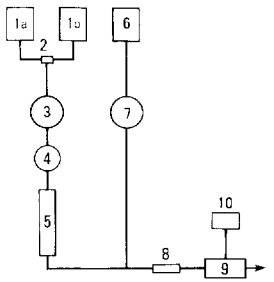


Fig. 2. Flow diagram for automated analysis of Δ^5-3β-hydroxysteroid sulphates using an immobilized enzyme column integrated into the HPLC system. 1a, 1b = Solvent reservoir; 2 = gradient elution accessory; 3, 7 = pump; 4 = sample injector; 5 = column; 6 = reservoir of NAD solution; 8 = column packed with immobilized 3β-17β-hydroxysteroid dehydrogenase; 9 = fluorimeter; 10 = recorder.

Chromatographic conditions

For the separation of individual Δ^5-3β-hydroxysteroids, the gradient elution technique was used, with methanol–water as eluent. The chromatographic conditions were as follows: solvent A (methanol–water, 70:30) and solvent B (methanol–water, 30:70) were mixed in the ratio of 40:60 and changed linearly to 70:30 by a gradient over a 64-min period. The flow-rate, column pressure and column temperature were 0.6 ml/min, 70 kg/cm^2 and 25°C, respectively. The β-NAD^+ solution introduced in this method was prepared as follows: 0.6 m*M* β-NAD^+ was dissolved in 10 m*M* potassium phosphate buffer (pH 8.5) containing ethylenediaminetetraacetic acid (EDTA) solution (1 m*M*) and 2-mercaptoethanol (0.05%). The flow-rate of the β-NAD^+ solution was 0.5 ml/min. NADH produced was monitored at 465-nm emission against 365-nm excitation with a JASCO FP-110C spectrofluorimeter.

Preparation of serum samples

Extraction procedure. A 1-ml volume of serum was added to a 12-ml glass-stoppered test tube. Then, 5 ml of ethanol were added. The mixture was stirred well and kept at 85°C for 1 min. After centrifuging for 5 min at 3500 *g*, the supernatant was transferred to another test tube and 5 ml of ethanol were added. This procedure was repeated three times and the final supernatant was evaporatored to dryness.

Enzymatic hydrolysis. To the evaporated residue in the test tube were added 0.1 ml of sulphatase (0.2% sodium chloride, 32 U/ml), 0.5 ml of 0.2 *M* acetate buffer (pH 5.0) and 0.4 ml of $25 \cdot 10^{-7}$ *M* D-saccharic acid 1,4-lactone. After incubation for 48 h at 37°C, the solvent was evaporated to dryness. The resultant residue was dissolved by the addition of 100 μl of methanol, of which 10 μl were injected into the chromatograph.

RESULTS

Fluorescence spectrum

NADH was prepared by the reaction of DHEA (1 μg per tube) with 2 ml of enzyme solution (in 10 ml of 0.1 *M* dipotassium hydrogen phosphate, pH 8.5, containing 0.3 U/ml β-HSD and 6 μM β-NAD^+) at 37°C for 20 min. As shown in Fig. 3, the NADH produced exhibited an excitation maximum at 365 nm and an emission maximum at 465 nm.

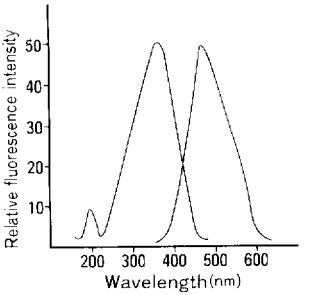


Fig. 3. Excitation and emission spectra of the NADH produced from DHEA. Excitation maximum = 365 nm; emission maximum = 465 nm.

Specificity of immobilized β-HSD

We tested the specificity of the immobilized enzyme, β-HSD, by the HPLC—immobilized enzyme method with a series of steroids. Our data (Table I) showed that the enzyme acted specifically on the 3β- and 17β-hydroxy groups. However, the concentration of 17β-hydroxysteroids in the serum did not significantly influence individual Δ^5-3β-hydroxysteroid sulphate measurements in this method.

Effect of organic solvent on fluorescence intensity (peak area)

The activity of immobilized enzyme was affected by the organic solvent. The organic solvent effects on the activity of the immobilized enzyme were therefore examined. Fig. 4 shows that the fluorescence intensity, assessed by the peak area in the chromatogram, decreased with an increase in methanol

TABLE I

SPECIFICITY OF 3β,17β-HYDROXYSTEROID DEHYDROGENASE

Each steroid at 0.7 nmol per 10 μl of methanol was determined with the HPLC—immobilized enzyme method and the intensity of reaction was expressed as a percentage relative to 5-androstene-3β,17β-diol.

Compound	Specificity (%)
5-Androstene-3β,17β-diol	100
Testosterone	42
Oestradiol	41
Dehydroepiandrosterone	54
5α-Androstane-3β,17β-diol	70
5α-Androstane-3α,17β-diol	7
17-Hydroxypregnenolone	25
Pregnenolone	29
Oestriol	0
Androsterone	0
Etiocholanolone	0
Cortisol	0

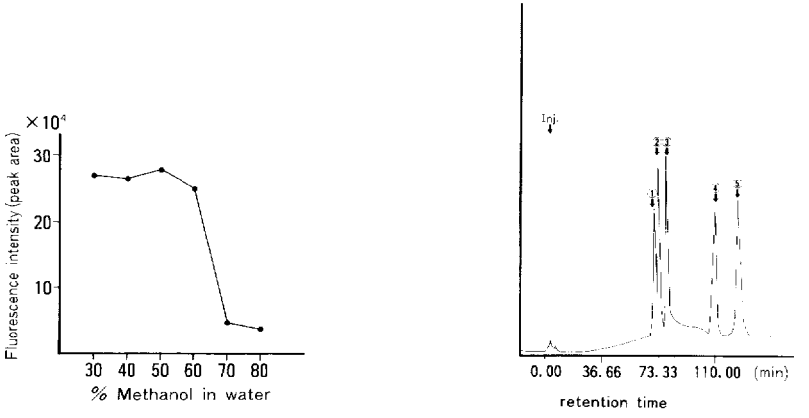


Fig. 4. Effect of methanol concentration on fluorescence intensity (peak area). The methanol concentrations in water as the mobile phase were: 30, 40, 50, 60, 70 and 80%. Injected volume: 10 μl of methanol containing 200 ng of DHEA.

Fig. 5. Chromatogram of NADH produced by individual Δ^5-3β-hydroxysteroids. Chromatographic conditions are as follows: mobile phase, A: methanol—water (70:30), B: methanol—water (30:70); gradient B/A ratio, 40:60 at zero time, and 70:30 at 64 min; slope, linear; flow-rate, 0.6 ml/min; JASCO FP-110C fluorescence detector (excitation 365 nm, emission 465 nm). Peaks: 1 = 5-androstene-3β,17β-diol; 2 = 17-hydroxypregnenolone; 3 = dehydroepiandrosterone; 4 = internal standard (5β-pregnan-3β,20α-diol); 5 = pregnenolone.

concentration in water up to 70%. If the methanol is used as a polar component of the gradient mixture in this method, the maximum gradient of methanol should be kept below 60%.

Chromatogram of standard samples

The solvent system was selected not only to separate the standard Δ^5-3β-

hydroxysteroids completely, but also to maintain the activity of immobilized enzyme. The chromatogram illustrated in Fig. 5 shows good separation of standard Δ^5-3β-hydroxysteroids, including 5-androstene-3β,17β-diol (80 ng), 17-hydroxypregnenolone (250 ng), DHEA (200 ng), internal standard (5β-pregnan-3β,20α-diol, 300 ng) and pregnenolone (300 ng).

Precision of peak area and retention time

A 10-μl volume of methanol, including individual Δ^5-3β-hydroxysteroids (200 ng), was injected into the HPLC system five successive times. As shown in Table II, the coefficient of variation (C.V.) of peak area and retention time was $< 0.3\%$ and $< 0.9\%$, respectively.

TABLE II

PRECISION OF RETENTION TIME AND PEAK AREA

Δ^5-3β-Hydroxysteroid	Retention time (mean ± S.D.) (min)	C.V. ($n = 5$) (%)	Peak area (mean ± S.D.) ($\times 10^4$)	C.V. ($n = 5$) (%)
Dehydroepiandrosterone	79.39 ± 0.24	0.30	69.20 ± 0.50	0.70
5-Androstene-3β,17β-diol	71.08 ± 0.14	0.20	129.40 ± 1.20	0.90
17-Hydroxypregnenolone	74.07 ± 0.07	0.10	32.20 ± 0.30	0.90
Pregnenolone	126.03 ± 0.25	0.20	37.10 ± 0.20	0.50

Calibration curves

When the JASCO FP-110C spectrofluorimeter was used, the standard calibration curve of 5-androstene-3β,17β-diol, dehydroepiandrosterone, 17-hydroxypregnenolone and pregnenolone showed a linearity in the range 2—200 ng (Fig. 6). Since 5-androstene-3β,17β-diol contains both 3β- and 17β-hydroxy groups, its fluorescence intensity (peak area) was higher than that of other Δ^5-3β-hydroxysteroids with the same injected amount.

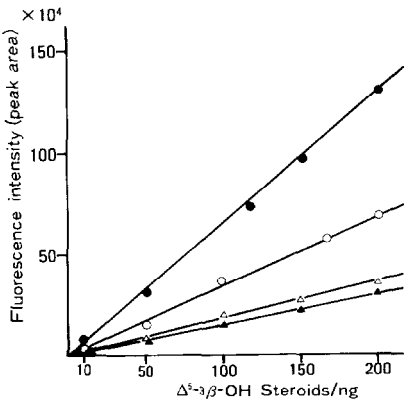


Fig. 6. Calibration curves for individual Δ^5-3β-hydroxysteroids: (●) 5-androstene-3β,17β-diol; (○) dehydroepiandrosterone; (△) pregnenolone; (▲) 17-hydroxypregnenolone.

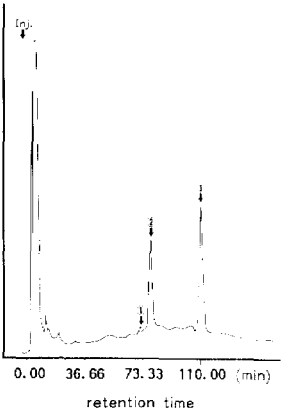


Fig. 7. Typical chromatogram of normal human serum (female; age 19). Chromatographic conditions as in Fig. 5. Peaks: 1 = 5-androstene-3β,17β-diol sulphate (32.2 ng/ml); 2 = dehydroepiandrosterone sulphate (1443 ng/ml); 3 = internal standard (5β-pregnan-3β,20α-diol, 300 ng).

TABLE III

RECOVERIES OF DEHYDROEPIANDROSTERONE SULPHATE ADDED TO HUMAN SERUM

Number of serum samples	Added (μg/dl)	Expected (μg/dl)	Found (μg/dl)	Recovery (%)	C.V. (%)
5	0	—	78.0	—	1.4
5	50	128	119.7	93.5	4.1
5	100	178	156.2	87.7	2.7
5	150	228	194.5	85.3	6.8

Deconjugation and recovery

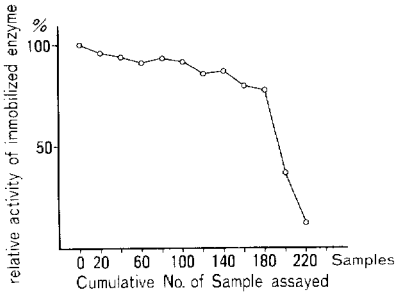
DHEA sulphate was hydrolysed with sulphatase to yield 86.6 ± 7.9% (mean ± S.D.) DHEA with a C.V. of 9.1% (five assays) after incubation for 48 h at 37°C. The recovery test was carried out by determining pooled serum samples spiked with known amounts of DHEA sulphate. As illustrated in Table III, the recoveries of added DHEA sulphate varied from 85.3 to 93.5%, with the C.V. ranging from 1.4 to 6.8%.

Chromatogram of serum sample

Fig. 7 shows a typical chromatogram of a normal serum sample obtained from a 19-year-old female. The peaks for 5-androstene-3β,17β-diol and dehydroepiandrosterone were clearly separated and identified by comparison with the authentic samples. The peaks that appeared in the chromatogram within 30 min are made up of unknown fluorescence compounds in serum.

Stability of the immobilized enzyme

The stability of the immobilized enzyme was assessed by duplicate assays of a standard solution of DHEA (1 μg per 10 μl methanol). When the immobilized enzyme column was washed with β-NAD^+ solution for 20 min before storing at 4°C, the immobilized enzyme column retained 80% or more of its initial activity for 36 days when five serum samples were assayed every day (Fig. 8).



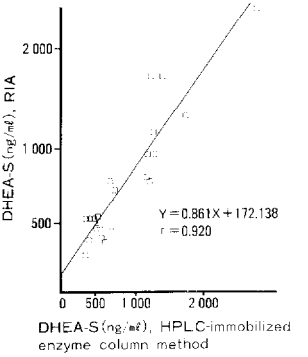


Fig. 8. The stability of immobilized enzyme column assessed in duplicate using DHEA (1 μg per 10 μl methanol). The initial activity is regarded as 100%.

Fig. 9. Correlation between HPLC—immobilized enzyme method proposed in this paper and RIA for serum DHEA sulphate value.

Comparison with radioimmunoassay and HPLC–immobilized enzyme column method

To assess the reliability of the present method for determination of DHEA sulphate in serum, DHEA sulphate levels in serum samples from 22 patients were simultaneously determined by both HPLC and RIA. As shown in Fig. 9, a good correlation was observed between the two methods.

Concentration of serum DHEA sulphate in control subjects and euprolactinaemic anovulatory patients

Serum concentrations of DHEA sulphate were measured in the 10 control subjects (0.77 ± 0.29 μg/ml) and in the 25 euprolactinaemic anovulatory patients (1.30 ± 0.79 μg/ml). The concentration of serum DHEA sulphate in anovulatory patients was significantly higher than in the control subjects ($P < 0.01$) (Fig. 10).

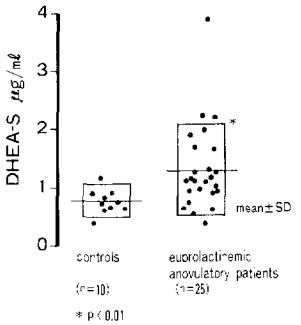


Fig. 10. Concentration of serum DHEA sulphate in control subjects and euprolactinaemic anovulatory patients.

DISCUSSION

Several HPLC methods were also applied to determine DHEA sulphate in biological fluids. However, these methods may be considered lacking in either sensitivity, specificity, reproducibility or convenience of analysis due to the use of a UV detector [9, 10] or fluorescence labelling technique [11, 12]. Enzymes have been used as spray reagents on thin-layer chromatographic plates for the determination of 3β-hydroxysteroids [13, 14], and have proved to be simple and specific. However, these methods consume considerable amounts of soluble enzymes, which makes the assay very expensive. Recent developments in immobilized enzyme technology offer new possibilities for the use of enzymes in clinical analysis; the immobilized enzyme reagents can give better stability and reusability than enzyme used in the form of solution [15–18].

Okuyama et al. [19] described an analytical method for individual bile acids using HPLC combined with a column of immobilized 3α-hydroxysteroid dehydrogenase. In this paper, we examined the applicability of immobilized β-HSD for the HPLC analysis of individual Δ^5-3β-hydroxysteroid sulphates.

The results presented indicate that an immobilized enzyme column used in a HPLC system showed excellent stability and sensitivity for at least one month or for 180 tests when used repeatedly. When 1 ml of serum sample was used for

the assay, the detection limit of Δ^5-3β-hydroxysteroid sulphates was ca. 2–10 μg/dl from the calibration curves. Therefore, dehydroepiandrosterone sulphate and 5-androstene-3β,17β-diol sulphate could be measured simultaneously.

A good correlation (r = 0.920) was observed between the values of DHEA sulphate in serum samples determined by the proposed method and by RIA.

Recently, several investigators have reported that the estimation of DHEA sulphate could serve as a screening test for diagnosis and treatment of anovulatory patients [20–23]. Our studies on the potential clinical use of serum DHEA sulphate assay for anovulatory women also suggest that adrenal androgen excess is a cause of anovulation.

In conclusion, the method presented here is inexpensive, simple, rapid, specific and reliable for analysing Δ^5-3β-hydroxysteroid sulphates.

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