*Journal of Chromatography*, 432 (1988) 47–56
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4340

# SIMULTANEOUS ASSAY FOR INDIVIDUAL SULPHATED 3$\alpha$- AND $\beta$-HYDROXYSTEROIDS IN SERUM USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COMBINED WITH 3$\alpha$- AND $\beta$-HYDROXYSTEROID DEHYDROGENASES IMMOBILIZED ON ONE COLUMN

KENJI TAKAGI*, KAZUTADA OKUMURA, NORIYUKI MORIKAWA and SUMIHIKO OKUYAMA

*Nagoya University College of Medical Technology, 1-20, Daikominami-1-chome, Higashi-ku, Nagoya 461 (Japan)*

and

MING-CHAO WU and OSAMU NARITA

*Department of Obstetrics and Gynaecology, Nagoya University Branch Hospital, 1-20, Daikominami-1-chome, Higashi-ku, Nagoya 461 (Japan)*

(First received February 22nd 1988; revised manuscript received June 7th 1988)

## SUMMARY

A high-performance liquid chromatographic method for the simultaneous determination of individual sulphated 3$\alpha$- and $\beta$-hydroxysteroids in serum using 3$\alpha$- and $\beta$-hydroxysteroid dehydrogenases (3$\alpha$-HSD and $\beta$-HSD, respectively) immobilized on one column and a fluorimeter to detect the resulting $NAD^+$ to NADH transformation is described. Individual sulphated 3$\alpha$- and $\beta$-hydroxysteroids in serum are extracted with ethanol, solvolysed with sulphuric acid in ethyl acetate and then separated by high-performance liquid chromatography. The hydroxysteroids thus separated are subsequently mixed with $NAD^+$ and then passed through the column in which the following catalytic reaction occurs:

$$3\alpha\text{- or }\beta\text{-hydroxysteroids} + NAD^+ \xrightarrow{3\alpha\text{-HSD or }\beta\text{-HSD}} 3\text{-oxosteroids} + NADH + H^+$$

The detection limits are as low as 0.5–1.0 $\mu$g/dl for sulphated 3$\alpha$- or $\beta$-hydroxysteroids in serum. The present assay method is highly specific, reliable and reproducible and is thus applicable to a clinical study on the metabolism of sulphated 3$\alpha$- and $\beta$-hydroxysteroids in patients with adrenal or gonadal diseases.

## INTRODUCTION

Sulphated $C_{19}$ steroids are secreted from the gonads and the adrenal cortex. The estimation of total $C_{19}$ steroids serves as a screening test for the diagnosis of

0378-4347/88/$03.50 © 1988 Elsevier Science Publishers B.V.

Step I Extraction of conjugated steroids from serum
Step II Deconjugation of conjugated steroids through solvolysis
Step III Separation of deconjugated steroids on Bilepak column in HPLC
Step IV Catalysis of $NAD^+$ mediated by HSD as shown in

$$3\alpha\text{- and }\beta\text{-hydroxysteroids} + NAD^+ \xrightarrow{3\alpha\text{- and }\beta\text{-HSD}} 3\text{-oxosteroids} + NADH + H^+$$

Step V Fluorimetry of reduced NAD

Fig. 1. Principle of the assay method.

gonadal or adrenal diseases. However, the determination of individual components of the steroids gives more meaningful information. The determination of individual sulphated $C_{19}$ steroids in various biological fluids has been carried out by gas chromatography (GS) [1–5] and also by gas chromatography–mass spectrometry (GC–MS) [6–10]. All the methods reported so far involve hydrolysis as well as derivatization, hence require large-scale equipment, so may not be suitable for an ordinary clinical laboratory.

Although high-performance liquid chromatography (HPLC) [11,12] has already applied to these problems, the sensitivity of the HPLC–UV method [11] is low and the HPLC pre-label method [12] requires a troublesome pre-labelling procedure.

In a previous paper, we reported a specific and reliable HPLC method using 3$\beta$,17$\beta$-hydroxysteroid dehydrogenase ($\beta$-HSD) immobilized on the column for the determination of serum $\Delta^5$-3$\beta$-hydroxysteroid sulphate [13]. The present paper describes an HPLC method to determine individual sulphated 3$\alpha$- and $\beta$-hydroxysteroids in serum. This method affords a specific on-line separation of these steroids using 3$\alpha$-hydroxysteroid dehydrogenase (3$\alpha$-HSD) and $\beta$-HSD immobilized together on one column and a fluorimeter to measure the reduced NAD.

The principle of the assay method is outlined in Fig. 1.

## EXPERIMENTAL

### *Steroids and other chemicals*

5-Androstene-3$\beta$,17$\beta$-diol ($\Delta^5$-diol), 3$\beta$-hydroxy-5-androstene-17-one (dehydroepiandrosterone, DHEA), 3$\alpha$-hydroxy-5$\alpha$-androstane-17-one (androsterone, AN), 3$\alpha$-hydroxy-5$\beta$-androstane-17-one (etiocholanolone, ETIO), 5$\beta$-pregnane-3$\beta$,20$\alpha$-diol (internal standard, I.S.), 3$\alpha$-HSD, $\beta$-HSD, sulphatase (Type VIII, from Abalone Entrails), dehydroepiandrosterone sulphate (DHEA-S), androsterone sulphate (AN-S), dehydroepiandrosterone glucuronide (DHEA-G), androsterone glucuronide (AN-G), etiocholanolone glucuronide (ETIO-G) and $\beta$-$NAD^+$ were all purchased from Sigma (St. Louis, MO, U.S.A.). Etiocholanolone sulphate (ETIO-S) and 5-androstene-3$\beta$,17$\beta$-diol sulphate ($\Delta^5$-diol-S) were obtained from Steraloid (Wilton, NH, U.S.A.). Methanol and other chemicals were obtained from commercial sources.

### *Apparatus and chromatographic conditions*

The apparatus and the chromatographic conditions are outlined in Fig. 2.

| | |
|---|---|
| Chromatograph | JASCO 801-SC |
| Solvent programmer | 880-02 |
| Fluorimeter | FP-210 |
| | excitation, 350 nm; emission, 470 nm |
| Data processor | DP-L220 |
| Pump | 880-PU |
| Column | JASCO Bilepak |
| | 250 mm × 4.6 mm I.D. |
| Column temperature | 25 °C |
| Mobile phase | A, methanol–water (70:30) |
| | B, methanol–water (30:70) |
| | A:B (40:60) to A:B (70:30), linear, in 64 min |
| Flow-rate | 0.6 ml/min |
| Immobilized enzyme column | 35 mm × 4.6 mm I.D. |
| Column temperature | 25 °C |
| Reagent | 0.6 m*M* $NAD^+$ + 10 m*M* potassium dihydrogenphosphate |
| | + 1 m*M* EDTA |
| | + 0.05% 2-mercaptoethanol (pH 7.8) |
| Reagent flow-rate | 0.5 ml/min |

Fig. 2. Apparatus and chromatographic conditions for assay of sulphated steroids using an immobilized enzymes column integrated into the HPLC system.

*Preparation of immobilized enzymes column*

3$\alpha$-HSD and $\beta$-HSD were respectively coupled to aminopropyl-CPG glass beads (Electro-Nucleonics, NJ, U.S.A.) with glutaraldehyde through Schiff base formation [13,14]. These immobilized enzymes were packed in one column (3.5 cm × 4.6 mm I.D.), which was placed in a continuous-flow HPLC system [14].

*Procedures to determine sulphated 3$\alpha$- and $\beta$-hydroxysteroids*

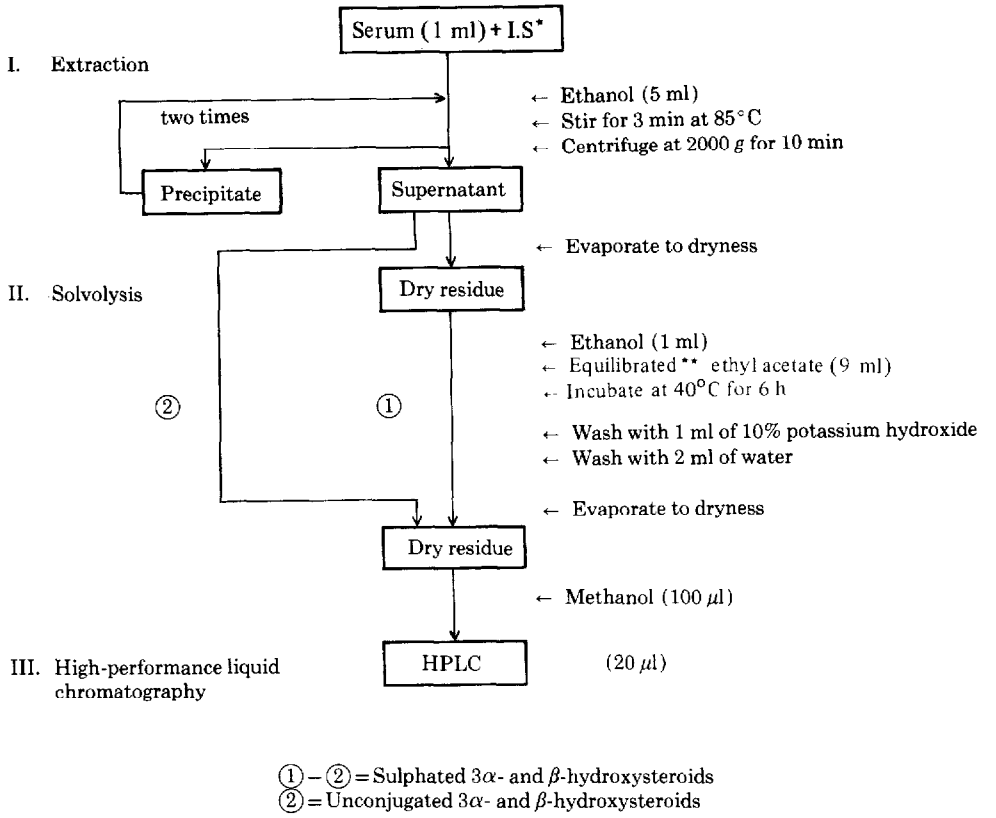
*Extraction of sulphated 3$\alpha$- and $\beta$-hydroxysteroids.* As shown in Fig. 3, individual sulphated 3$\alpha$- and $\beta$-hydroxysteroids in serum were extracted with ethanol, as described previously [13].

*Enzymic hydrolysis.* Enzymic hydrolysis of sulphated 3$\alpha$- and $\beta$-hydroxysteroids was carried out with sulphatase (Sigma, Type VIII, from Abalone Entrails) [13].

*Solvolytic procedure.* The procedures of Kawasaki et al. [12], Kornel [15] and DePaoli et al. [16] for solvolysis of conjugated steroids were modified so as to be applicable to sulphated 3$\alpha$- and $\beta$-hydroxysteroids. A flow-chart of this technique is shown in Fig. 3. To the dry residues obtained after evaporation in the test-tube were added 1 ml of absolute ethanol and 9 ml of equilibrated ethyl acetate. After incubation at 40 °C for 6 h, the organic layer was washed with 1 ml of 10% potassium hydroxide solution and 2 ml of water. The solvents were again evaporated to dryness.

*Identification and quantitation of 3$\alpha$- and $\beta$-hydroxysteroids.* Based on the peak retention times and areas recorded, individual 3$\alpha$- and $\beta$-hydroxysteroids and I.S. were automatically identified and quantified by the DP-L220 data processor (Japan Spectroscopic, Tokyo, Japan) with high accuracy and precision.

*Estimation of sulphated 3$\alpha$- and $\beta$-hydroxysteroids.* The present method does not directly measure sulphated 3$\alpha$- and $\beta$-hydroxysteroids. Therefore, the differences between the concentrations of 3$\alpha$- and $\beta$-hydroxysteroids before and after




*Internal standard (5β-pregnane-3β,20α-diol)
**The equilibration is done by shaking 50 ml of ethyl acetate in a separatory funnel with 10 ml of 2 *M* sulphuric acid

Fig. 3. Flow-chart of the assay method.

solvolysis were presumed to be the amounts of sulphated 3α- and β-hydroxysteroids (1 and 2 in Fig. 3).

## RESULTS

### *Selection of eluent*

We examined many organic solvent systems as eluents for the separation of 3α- and β-hydroxysteroids and found the methanol–water gradient elution system to be the most suitable when used with a JASCO Bilepak column (Fig. 2).

### *Optimal conditions of reagent for immobilized enzymes*

*pH value.* Measurements were carried out with 10 m*M* phosphate buffers between pH 6.0 and 8.5 at 25°C with a large excess of $NAD^+$ (1 m*M*), in order that equilibrium should be rapidly reached in the HPLC system with immobilized enzymes. The equilibrium was practically sufficient in favour of the forward reaction at pH 7.8.

*$NAD^+$ concentration.* Measurements were carried out at various $NAD^+$ concentrations in 10 m*M* phosphate buffers (pH 7.8) at 25°C. The fluorescence intensity reached a plateau at 0.6 m*M* $NAD^+$. Therefore, the reagent was made from 0.6 m*M* $NAD^+$ in 10 m*M* potassium phosphate buffer (pH 7.8) with 1 m*M* EDTA and 0.05% 2-mercaptoethanol.

*Optimal conditions for solvolysis*

*Influence of incubation time.* The incubations were carried out at 40°C with sulphated 3$\alpha$-hydroxysteroids (ETIO-S and AN-S) and $\beta$-hydroxysteroids ($\Delta^5$-diol-S and DHEA-S), and glucuronidated 3$\alpha$-hydroxysteroids (ETIO-G and AN-G) and $\beta$-hydroxysteroids (DHEA-G) as substrates. As shown in Fig. 4, maximum solvolysis of sulphated $\beta$-hydroxysteroids was achieved within 3 h, and that of sulphated 3$\alpha$-hydroxysteroids within 6 h. However, glucuronidated 3$\alpha$- and $\beta$-hydroxysteroids were not hydrolysed at all.

*Rate of solvolysis or enzymatic hydrolysis.* As shown in Table I, rates of solvolysis were from 76.2 to 97.2%. Enzymatic hydrolysis with Abalone Entrails, how-

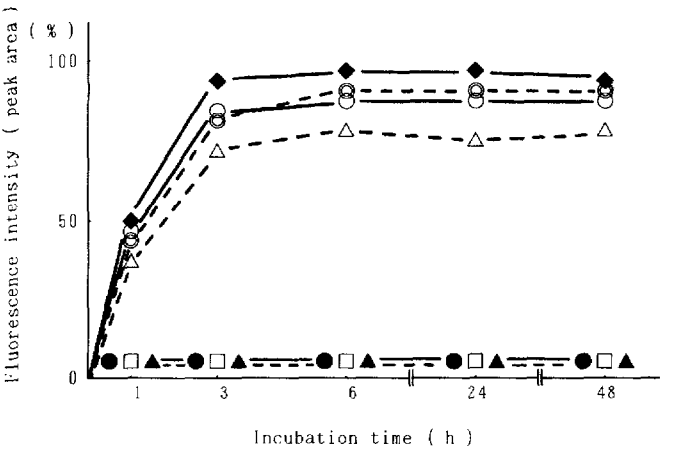



Fig. 4. Influence of incubation time on solvolytic yield: (◆) 5-androstene-3$\beta$,17$\beta$-diol-S; (○) DHEA-S; (◎) etiocholanolone-S; (△) androsterone-S; (●) DHEA-G; (□) etiocholanolone-G; (▲) androsterone-G.

TABLE I

PERCENTAGE CLEAVAGE OF SULPHATED STEROIDS BY SOLVOLYSIS OR ENZYMATIC HYDROLYSIS

| Sulphated steroid | Solvolysis (mean ± S.D.) | Enzymatic hydrolysis (mean ± S.D.) |
|---|---|---|
| 5-Androstene-3$\beta$,17$\beta$-diol-S | 97.2 ± 3.8 | Not detected |
| Dehydroepiandrosterone-S | 87.1 ± 4.3 | 88.8 ± 4.0 |
| Androsterone-S | 76.2 ± 2.3 | Not detected |
| Etiocholanolone-S | 88.4 ± 4.5 | 9.5 ± 0.5 |

ever, hydrolysed none or only a little of the 3$\alpha$-sulphated groups and 3$\beta$,17$\beta$-disulphated groups.

*Stability of immobilized enzymes*

The activity of the immobilized 3$\alpha$-HSD and $\beta$-HSD assessed from assays of individual 3$\alpha$- and $\beta$-hydroxysteroid standards remained at 80% or more for at least one month while five serum samples were assayed every day.

*Calibration curves and sensitivities*

Calibration curves for individual 3$\alpha$-hydroxysteroids (ETIO and AN) and $\beta$-hydroxysteroids ($\Delta^5$-diol and DHEA) [13] were all linear in the range 1–200 ng. Calculated from these calibration curves, a minimum detectable concentration of individual sulphated 3$\alpha$- and $\beta$-hydroxysteroids was 0.5 $\mu$g/dl, when 1.0 ml of serum sample was used for the assay.

*Chromatogram of standard sample and serum sample*

Fig. 5 shows an example of a chromatogram demonstrating a good separation of individual 3$\alpha$- and $\beta$-hydroxysteroid standards. Fig. 6 shows a typicl chromatogram of a normal human serum obtained from a 20-year-old female subject. The peaks for $\Delta^5$-diol, DHEA, ETIO and AN were clearly separated.

*Recovery*

The overall recovery tests were carried out with human serum samples obtained from healthy male or female subjects, spiked with known amounts of sul-

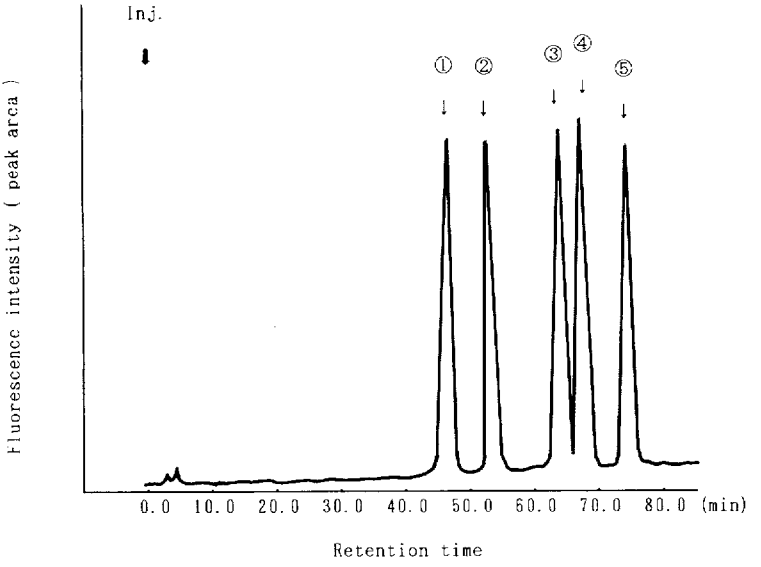



Fig. 5. Chromatogram of NADH produced by individual steroids. Peaks: 1 = 5-androstene-3$\beta$,17$\beta$-diol (100 ng); 2 = DHEA (300 ng); 3 = etiocholanolone (50 ng); 4 = androsterone (50 ng); 5 = internal standard (5$\beta$-pregnane-3$\beta$,20$\alpha$-diol) (400 ng).

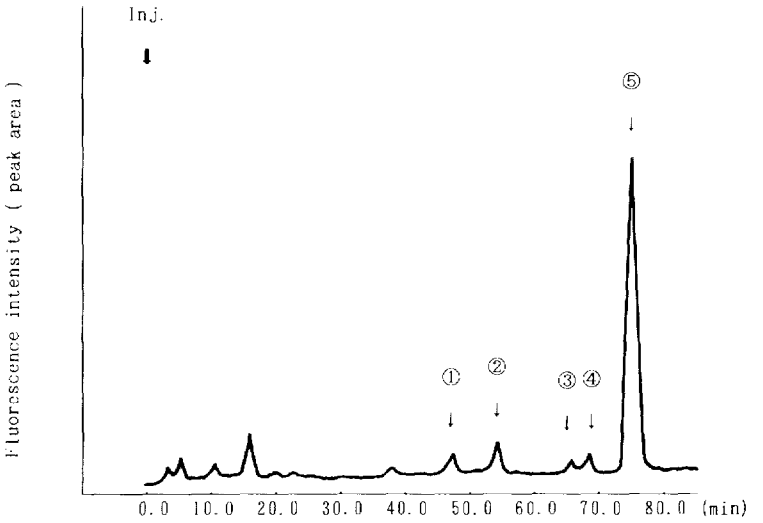



Fig. 6. Typical chromatogram of normal human serum (female, aged 20 years). Peaks: 1 = 5-androstene-3$\beta$,17$\beta$-diol; 2 = DHEA; 3 = etiocholanolone; 4 = androsterone; 5 = internal standard (5$\beta$-pregnane-3$\beta$,20$\alpha$-diol).

TABLE II

RECOVERY OF SULPHATED STEROIDS ADDED TO HUMAN SERUM ($n$ = 5)

| Sulphated steroid | Added ($\mu$g/dl) | Expected ($\mu$g/dl) | Found ($\mu$g/dl) | Recovery (mean ± S.D.) (%) |
|---|---|---|---|---|
| 5-Androstene-3$\beta$,17$\beta$-diol-S | 5.0 | 11.9 | 10.2 | 85.6 ± 4.4 |
| Dehydroepiandrosterone-S | 100.0 | 251.6 | 203.5 | 80.9 ± 6.4 |
| Androsterone-S | 30.0 | 73.9 | 44.6 | 60.3 ± 5.3 |
| Etiocholanolone-S | 5.0 | 5.0 | 4.3 | 85.2 ± 5.0 |

phated 3$\alpha$- and $\beta$-hydroxysteroids. As shown in Table II, recoveries ranged from 60.3 to 85.6% with a coefficient of variation (C.V.) of 4.4–6.4%.

*Comparison with radioimmunoassay*

The reliability of the present HPLC–immobilized enzymes method for the simultaneous determination of serum sulphated DHEA was assessed by comparing the results with those obtained by radioimmunoassay (RIA). The coefficient of correlation between these two measurements was 0.938 ($n$ = 15) and the regression line was $y = 0.80x + 8.74$, in which $x$ is the value determined by the present method. A good correlation was observed between the two methods.

TABLE III

LEVELS OF INDIVIDUAL SULPHATED STEROIDS

Levels of individual sulphated steroids in sera of normal subjects aged from 19 to 24 years old.

| Sulphated steroid | Concentration (mean ± S.D.) (μg/dl) | |
|---|---|---|
| | Female ($n=10$) | Male ($n=5$) |
| 5-Androstene-3β,17β-diol-S | 20.0 ± 6.7 | 65.2 ± 22.6 |
| Dehydroepiandrosterone-S | 99.2 ± 25.7 | 137.2 ± 23.8 |
| Etiocholanolone-S | 22.7 ± 5.8 | 46.2 ± 9.4 |
| Androsterone-S | 45.1 ± 17.4 | 73.9 ± 57.8 |

*Levels of sulphated 3α- and β-hydroxysteroids in normal subjects*

The levels of individual sulphated 3α- and β-hydroxysteroids in serum of normal subjects aged from 19 to 24 years old determined by the present HPLC–immobilized enzymes method are shown in Table III.

## DISCUSSION

Baulieu et al. [17] reported that the predominant $C_{19}$ steroids in human adrenal glands are $\Delta^5$-3β-hydroxysteroids, which are mainly composed of DHEA-S. There exists an equilibrium between $\Delta^5$-3β-hydroxysteroids and their sulphated derivatives, and this equilibrium is believed to control the transformation from DHEA-S via DHEA and $\Delta^4$-androstenedione to testosterone. Sulphated steroids are also linked to the production of other cortical steroid hormones and all $\Delta^4$-3-ketosteroids are biosynthesized from $\Delta^5$-3β-hydroxysteroids, depending on whether $\Delta^5$-3β-hydroxysteroids are sulphated or free. Accordingly, much attention has been paid to the determination of sulphated steroids. The serum level of DHEA-S, together with particular changes in pituitary–gonadal hormones, has been considered as an endocrine index of ageing [18].

At present, determination of sulphated adrenal steroids as a major component of serum steroids can be performed by RIA [19,20], GC or GC–MS. Although RIA has the advantage in direct measurements of high sensitivity without hydrolysis, it cannot be used to make simultaneous determinations of individual sulphated steroids and it is plagued by problems of disposal of radioactive reagents. The GC or GC–MS method gives much better resolution and higher sensitivity for the steroids. However, in order to separate sulphated steroids from the original specimen, isolation and purification is necessary after extraction.

The HPLC method [11–13] has been studied in many laboratories. The only prerequisite for this method is that the components to be analysed must be soluble in a mobile phase solvent. It was previously reported that a method using β-HSD immobilized in the detection system resulted in a high specificity, increased reliability and low cost of the determination of sulphated 3β-hydroxysteroids [13]. In the present study, we described a method for the simultaneous determination

of both sulphated 3$\alpha$- and $\beta$-hydroxysteroids by simply adding 3$\alpha$-HSD to the immobilized enzyme column.

Since sulphated steroids in serum have sulphate groups at the 3-position connected by ester bond, they have to be hydrolysed first so as to be detected with 3$\alpha$-HSD and $\beta$-HSD. Enzymatic hydrolysis with aryl sulphatase has no effect on AN-S of 3$\alpha$-sulphated group in 5$\alpha$-steroids and $\Delta^5$-diol-S of 3$\beta$,17$\beta$-disulphated groups, and only a little on ETIO-S of 3$\alpha$-sulphated group in 5$\beta$-steroids, while it is quite effective in DHEA-S of 3$\beta$-sulphated group. Thus, enzymatic hydrolysis can be used for steroids of 3$\beta$-sulphated groups [13] but not for steroids of 3$\alpha$-sulphated groups and $\Delta^5$-diol-S of 3$\beta$,17$\beta$-disulphated groups [21,22], because of their ambiguous substrate specificities.

Many reports about solvolysis have appeared. Kornel [15] reported that he successfully hydrolysed even more polar sulphated steroids by adding ethanol to the ethyl acetate–sulphuric acid method of Burstein and Lieberman [23]. We further modified the method of Kornel [15], referring to the methods of DePaoli et al. [16] and Kawasaki et al. [12]. The present method for solvolysis is simpler and thus easier, as well as more time-saving, than the methods reported so far [15,16,23], e.g. complete hydrolysis takes only 6 h. Another advantage of this solvolysis is its inability to hydrolyse glucuronide, in contrast to the ethyl acetate–perchloric acid method of DePaoli et al. [16], because the present study was aimed at developing a method to measure only sulphated steroids.

In the present study, gradient reproducibility was improved using 5$\beta$-pregnane-3$\beta$,20$\alpha$-diol as the internal standard and by introducing a data processor into the detection system. Thus, the serum levels of sulphated 3$\alpha$- and $\beta$-hydroxysteroids in normal subjects observed in the present study are in good agreement with these reported by Sekihara and Ohsawa [19].

The present HPLC method combined with an immobilized 3$\alpha$- and $\beta$-HSD column may be useful for studying the metabolism of sulphated steroids, and for analysing pituitary–adrenal–gonadal diseases as well as ageing.

REFERENCES


1 J. Sjövall and R. Vihko, Steroids, 6 (1965) 597.
2 H.H. Wotiz and S.J. Clark, Gas Chromatography in the Analysis of Steroid Hormones, Plenum Press, New York, 1966, p. 165.
3 C.H.L. Shackleton and J.W. Honour, Clin. Chim. Acta, 69 (1976) 267.
4 W.J.J. Leunissen and J.H.H. Thijssen, J. Chromatogr., 146 (1978) 365.
5 C.H.L. Shackleton, Clin. Chem., 27 (1981) 509.
6 J. Sjövall and R. Vihko, Steroids, 7 (1966) 447.
7 C.D. Pfaffenberger and E.C. Horning, J. Chromatogr., 112 (1975) 581.
8 H.Ch. Curtius, J. Völlmin, M.J. Zagalak and M. Zachmann, J. Steroid Biochem., 6 (1975) 677.
9 H. Breuer and L. Siekmann, J. Steroid Biochem., 6 (1975) 685.
10 M. Axelson, B.L. Sahlberg and J. Sjövall, J. Chromatogr., 224 (1981) 355.
11 I.R. Hunter, M.K. Walden and E. Heftmann, J. Chromatogr., 176 (1979) 485.
12 T. Kawasaki, M. Maeda and A. Tsuji, J. Chromatogr., 226 (1981) 1.
13 M.C. Wu, K. Takagi, S. Okuyama, M. Ohsawa, T. Masahashi, O. Narita and Y. Tomoda, J. Chromatogr., 377 (1986) 121.
14 S. Okuyama, N. Kokubun, S. Higashidate, D. Uemura and Y. Hirata, Chem. Lett., (1979) 1443.

15 L. Kornel, Biochemistry, 4 (1965) 444.
16 J.C. DePaoli, E. Nishizawa and K.B. Eik-Nes, J. Clin. Endocrinol., 23 (1963) 81.
17 E.E. Baulieu, C. Corpechot, F. Dray, R. Emiliozzi, M.C. Lebeau, P. Mauvais Jarvis and P. Robel, Rec. Progr. Hormone Res., 21 (1965) 411.
18 H. Ibayashi and K. Kato, in F. Labrie (Editor), Proceedings of the 7th International Congress of Endocrinology, Quebec, July 1–7, 1984, Elsevier, Amsterdam, 1984, p. 195.
19 H. Sekihara and N. Ohsawa, Steroids, 23 (1974) 245.
20 T. Aso, A.R. Aedo and S.Z. Cekan, J. Steroid Biochem., 8 (1977) 1105.
21 Y.A. Leon, R.D. Bulbrook and E.D.S. Corner, J. Biochem., 75 (1960) 612.
22 C.H.L. Shackleton, J. Sjövall and O. Wisen, Clin. Chim. Acta, 27 (1970) 354.
23 S. Burstein and S. Lieberman, J. Biol. Chem., 233 (1958) 331.