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DETERMINATION OF 17-OXOSTEROID SULPHATES IN SERUM BY ION-PAIR EXTRACTION, PRELABELLING WITH DANSYLHYDRAZINE AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A new high-performance liquid chromatographic (HPLC) method with fluorescence detection is described for the direct determination of four serum 17-oxosteroid sulphates. Each serum sample was deproteinated with methanol, the methanol was evaporated and 17-oxosteroid sulphates in the residue were extracted with benzene as ion pairs in the presence of tetrapentylammonium ion. The ion pairs were labelled with dansylhydrazine and the hydrazones were separated by HPLC on a Capcell-Pak C_8 (silicone polymer-coated silica gel modified with octyl groups) reversed-phase column using methanol–0.5% (w/v) sodium acetate–50% (v/v) acetic acid (57:42:1, v/v/v) as the mobile phase. The eluent was monitored with a fluorometric detector at an excitation wavelength of 330 nm and an emission wavelength of 540 nm.

INTRODUCTION

The measurement of sulphates of 17-oxosteroids (17OS) in serum is of clinical significance for clarifying the situation of adrenal androgen. Sulphates of 17OS in serum include dehydroepiandrosterone (DHEA), androsterone (Ad), epiandrosterone (EA) and etiocholanolone (Ec) sulphates. Among these four steroids, DHEA sulphate is secreted abundantly from the adrenal. A low level of secretion is shown in adrenal cortex insufficiency, whereas a high level of secretion is indicated in cases of adrenal hyperplasia such as Cushing's syndrome. In contrast, cases of adenoma show a marked reduction in secretion, and this feature can be utilized in the differential diagnosis of adrenal lesions[1]. Thus, DHEA sulphate is the only adrenal androgen generally measured, and the clinical significance of the other 17OS sulphates has not yet been clarified.

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Measurement methods using gas chromatography (GC)[2–4] and GC combined with mass spectrometry[5–7] were reported. However, as the procedure necessary for such measurements is too complex, at present determinations are generally conducted by radioimmunoassay[8–11], but this method has a problem of cross-reaction.

For highly sensitive high-performance liquid chromatography (HPLC) with fluorescence detection, two methods have been reported[14,15]. In one, 17OS sulphates in serum are solvolysed after removal of free 17OS present in the serum[14]. In the other, serum is applied on to a Sep-Pak C_{18} cartridge[15].

DHEA sulphate and Ad sulphate have been separated by HPLC with fluorescence detection using dansylhydrazine as a prelabelling reagent, following these two pretreatment methods, but two other 17OS sulfates, EA and Ec sulphates, were not separated.

Independently, we have developed a method that involves the formation of 17OS sulphate ion pairs with tetrapentylammonium ion (TPA), extraction of the ion-paired 17OS sulphates with benzene, labelling of the ion pairs with dansylhydrazine, resulting in dansylhydrazones, and separate measurement by HPLC on a Capcell-Pak C_8 column followed by spectrofluorimetric detection. This method does not require hydrolysis. Compared with the usual methods of GC and radioimmunoassay, this new method requires a shorter measurement time and is highly accurate and reproducible. Furthermore it is able to measure DHEA, Ad, EA and Ec sulphates at the same time.

EXPERIMENTAL

Apparatus

The high-performance liquid chromatograph (Model 655A-11), equipped with a controller (Model L-5000LC), spectrofluorimeter (Model F-1000), recorder (Model D-2000 chromato-integrator) and column oven (Model 655A-52) were from Hitachi (Tokyo, Japan). A 250 mm × 4.6 mm I.D. stainless-steel column packed with Capcell-Pak C_8 (silicone polymer-coated silica gel modified with octyl groups; particle size 5 μm) was purchased from Shiseido (Tokyo, Japan).

Materials

DHEA, EA, Ec and Ad sulphates were obtained from Sigma (St. Louis, MO, U.S.A.) and tetrapentylammonium bromide from Aldrich (Milwaukee, WI, U.S.A.). All other reagents and solvents were of analytical-reagent grade or HPLC grade and were purchased from commercial sources.

Reagent solutions

TPA solution. A 0.1 *M* TPA solution was prepared by dissolving TPA bromide in water.

Dansylhydrazine solution. A 0.2% (w/v) solution was prepared by dissolving 20 mg of dansylhydrazine in 10 ml of acetonitrile; the solution was stored in a refrigerator until use.

Borate buffer. A 0.4 *M* buffer solution (pH 9.0) was prepared.

Standard solution. A 1-mg amount each of DHEA, EA, Ec and Ad sulphates were dissolved separately in 10 ml of water (concentration 100 mg/l) and an equal

volume of each was combined (four volumes), then 16 volumes of water were added (resulting in a concentration of each steroid of 5 mg/l), and the solution was stored in a refrigerator. Working standard solutions (0.05–5 mg/l of each steroid), used to determine calibration graphs, were prepared immediately before use by further dilution with water.

Mobile phase. A methanol–0.5% (w/v) sodium acetate–50% (v/v) acetic acid (57:42:1, v/v/v) mixture was prepared.

Procedure

Deproteination of serum sample. A 0.6-ml volume of serum was mixed with 3 ml of methanol in a glass test-tube, ultrasonicated for 15 min and centrifuged for 10 min at 1100 *g*. Then, 2.4 ml of the supernatant (0.4 ml as serum) were transferred into a glass test-tube and evaporated to dryness under reduced pressure.

Extraction of 17OS sulphates. The above residue was dissolved in 1.0 ml of water, then 0.6 ml of borate buffer was added and the mixture was shaken with 4 ml of benzene for 30 s. The mixture was centrifuged for 5 min at 700 *g* and the benzene layer was removed. Free 17OS was extracted in the benzene layer. 17OS can be measured by the method of Kawasaki *et al.*[14]. To the aqueous layer, 0.4 ml of TPA solution was added and mixed well. The mixture was shaken with 4 ml of benzene for 30 s, and centrifuged for 10 min at 700 *g*. A 2-ml volume of the benzene solution was transferred into a glass-stoppered test-tube and evaporated to dryness under reduced pressure (ion pairs of 17OS sulphates with TPA).

Labelling reaction. Labelling was done by adding 200 μl of dansylhydrazine solution and 50 μl of acetic acid to the extracted ion pairs of 17OS sulphates with TPA. The mixture was evaporated under reduced pressure at 50°C and allowed to stand for 20 min. The labelled ion pairs were dissolved in 100 μl of acetonitrile and an aliquot (5 μl for the calibration graph and normal values, 1 μl for high values, and 10 μl for low values) was then chromatographed.

Chromatographic conditions. The separation of 17OS sulphates was carried out on a Capcell-Pak C_8 column at 30°C with a mobile phase flow-rate of 1 ml/min. The effluent was monitored with a spectrofluorimeter at an excitation wavelength of 330 nm and an emission wavelength of 540 nm.

The above procedure was also used for working standard solutions.

Evaluation of serum deproteination

Preparation of sample. For evaluation of the methanol method and the Sep-Pak C_{18} cartridge method by the recovery test, serum–17OS sulphate solutions and serum–water solutions (9:1, v/v) were prepared. 17OS sulphate solutions were prepared by dissolving 1 mg each of the 17OS sulphates in 10 ml of water.

Methanol method[16,17]. A 1.0-ml serum sample was mixed with 5 ml of methanol and ultrasonicated for 15 min, before being centrifuged for 10 min at 1100 *g*. Then 1.0 ml (for direct dansylation) or 2.0 ml (for dansylation of ion pairs of 17OS sulphates with TPA) of the supernatant were evaporated. The 17OS sulphates were either directly dansylated, or extracted as ion pairs and dansylated by the extraction and dansylation procedure used for the present method.

Sep-Pak C_{18} cartridge[15]. The cartridge was activated with 5 ml of methanol and washed with 20 ml of water before use. A 0.6-ml volume of serum was diluted to

3.0 ml with 0.025 *M* phosphate buffer (pH 7.0) and applied to a Sep-Pak C_{18} cartridge, which was then washed with 4 ml of water. The 17OS sulphates adsorbed by the cartridge were eluted with 3 ml of methanol. Then 1 ml (for direct dansylation) or 2 ml (for dansylation of ion pairs) of the effluent were evaporated under reduced pressure.

Procedure for determinating 17OS sulphates by solvolysis

Solvolysis of 17OS sulphates[12,13]. The sample was acidified to pH 1.0 with 50% sulphuric acid, adjusted to a final salt concentration of 20% with sodium chloride and extracted with 4 ml of ethyl acetate. The organic phase was dried with 0.1 g of anhydrous sodium sulphate. The subsequent filtrate was kept at 30°C for 20 h, washed with 2 ml of 10% (w/v) potassium hydroxide and then twice with 2 ml of water. The washed solvent was dried with sodium sulphate and evaporated under reduced pressure (17OS from 17OS sulphates).

Labelling reaction of free-type 17OS. The 17OS in each residue was labelled by the dansylhydrazine method of Kawasaki *et al.*[14].

HPLC conditions. 17OS was separated by using a Zorbax SIL column (250 mm × 4.6 mm I.D.) at 25°C using the organic layer separated from the mixture of dichloromethane, ethanol and water (400:7:7, v/v/v). The effluent from the column was monitored with the spectrofluorimetric detector at an excitation wavelength of 350 nm and an emission wavelength of 505 nm[14].

RESULTS AND DISCUSSION

Optimization of assay conditions

Serum deproteination methods. Experiments were performed to find an adequate deproteination method for the determination of 17OS sulphates in serum.

Serum was deproteinated with either methanol or a Sep-Pak C_{18} cartridge. The 17OS sulphates in the deproteinated samples were directly dansylated either without using ion-pair extraction or after ion-pair extraction. The recoveries (± S.D., $n = 5$) of DHEA, EA, Ec and Ad sulphates obtained by deproteination with methanol, ion-pair extraction and dansylation were 96.8 ± 2.5%, 101.6 ± 3.3%, 101.1 ± 3.2% and 97.5 ± 2.8%, respectively, these values being better than those obtained with other methods. The recoveries (± S.D., $n = 5$) of 17OS sulphates obtained by deproteination with methanol and dansylation were 59.5 ± 2.3 to 78.7 ± 5.7% those obtained by treatment with the Sep-Pak C_{18} cartridge, ion-pair extraction and dansylation were 44.1 ± 3.3 to 52.2 ± 10.8% and those obtained by treatment with the Sep-Pak C_{18} Cartridge and dansylation were 32.1 ± 3.3 to 52.2 ± 10.8%.

Optimum pH extraction of 17OS sulphates. To determine the optimum pH for extraction of 17OS sulphates, we adjusted the pH of the mixture for five volumes of sample (4 mg/l of each DHEA sulphate) and one volume of TPA solution (pH 6, 7, 8, 9, 10, 11 or 11.5) with phosphate buffer (pH 6, 7 and 8) and borate buffer (pH 9, 10, 11 and 11.5) at a concentration of 0.2 *M*. For extraction with benzene, twice the volume of aqueous solution was used. The extraction (± S.D., $n = 5$) of DHEA sulphates in the pH range 6–11.5 was 98.8 ± 1.5%.

TPA extraction of 17OS sulphates. To determine the concentration of TPA at which extraction of 17OS sulphates with benzene would be maximized, 17OS sul-

phates were extracted with five different concentrations of TPA solutions. Maximum extraction was obtained at a molar ratio of TPA to DHEA sulphate of 50:1. When the molar ratios of TPA to DHEA sulphate were 10:1, 25:1, 50:1, 75:1 and 100:1, the extraction (± S.D., n = 5) was 78.0 ± 4.0, 91.5 ± 2.7, 101.1 ± 2.0, 99.6 ± 2.3 and 100.3 ± 2.5%, respectively.

Labelling. Dansylhydrazine has been used as a labelling agent for the chromatographic determination of free 17OS and 17OS sulphates. The dansylation has been conducted in the presence of a low concentration of strong acid in an organic solvent, including (1) concentrated hydrochloric acid in ethanol (final concentration 0.325%, v/v)[18]; (2) trichloroacetic acid in benzene (final concentration 0.33%, v/v[14], or 0.4%, v/v[15]) or (3) trichloroacetic acid in a mixture of benzene and ethanol (final concentration 0.33%, w/v)[19]. We tested various concentrations for dansylating ion pairs of the 17OS sulphates with TPA to determine directly the individual serum 17OS sulphates by HPLC. We found that acetic acid was as effective for dansylation as a low concentration of trichloroacetic acid. The optimum final concentrations of acetic acid and trichloroacetic acid were 20% (v/v) and 0.25% (w/v), respectively.

In order to select an adequate solvent for dansylation and for injection of the dansylated 17OS sulphates into the HPLC apparatus, the sample was dansylated by using ethanol, acetonitrile, 1,2-dichloroethane or benzene as the reaction solvent. The 17OS sulphate values obtained with benzene as reaction solvent were lower than those with acetonitrile and ethanol, and the values obtained with 1,2-dichloromethane were lower than those obtained with benzene. The relations of DHEA, EA, Ec and Ad sulphate values obtained with benzene or 1,2-dichloroethane to those obtained with acetonitrile were 0.20, 0.21, 0.23 and 0.23 (with benzene) and 0.11, 0.11, 0.12 and 0.11 (with 1,2-dichloroethane), respectively.

There was no difference between 17OS sulphate concentrations obtained with acetonitrile and those obtained with ethanol. However, with ethanol, some non-steroidal peaks were present on the chromatograms, and these peaks increased with time at room temperature. These peaks were not observed with acetonitrile (Fig. 1). We found that evaporation of the solvent in the reaction solution accelerated the labelling.

To bring the dansylation to an end after evaporation of the solvent in the reaction solution at the temperatures selected for the acceleration of the reaction, the optimum times for maintaining the various temperatures were determined, and were found to be 25 min at 40°C, 15–20 min at 50°C and 10 min at 60°C.

Mobile phase. In order to separate the four 17OS sulphates, the proportions of methanol, sodium acetate and acetic acid in the mobile phase on the Capcell-Park C_8 column were varied. The separation of 17OS sulphates was improved by decreasing the methanol concentration, but the capacity factor (k') increased and the retention time was prolonged. However the k' values decreased with increase in column temperature. The k' value of 17OS sulphates was slightly decreased with a reduction in the pH of the mobile phase (Fig. 2). The optimum mobile phase obtained from this experiment was methanol–5% (w/v) sodium acetate–50% (v/v) acetic acid (57:42:1, v/v/v).

Fluorescence spectrum. To obtain the fluorescence spectrum of DHEA sulphate extracted with benzene as ion pairs with TPA and then dansylated, the fractions of dansylated ion pairs separated by HPLC were collected, and monitored with a spec-

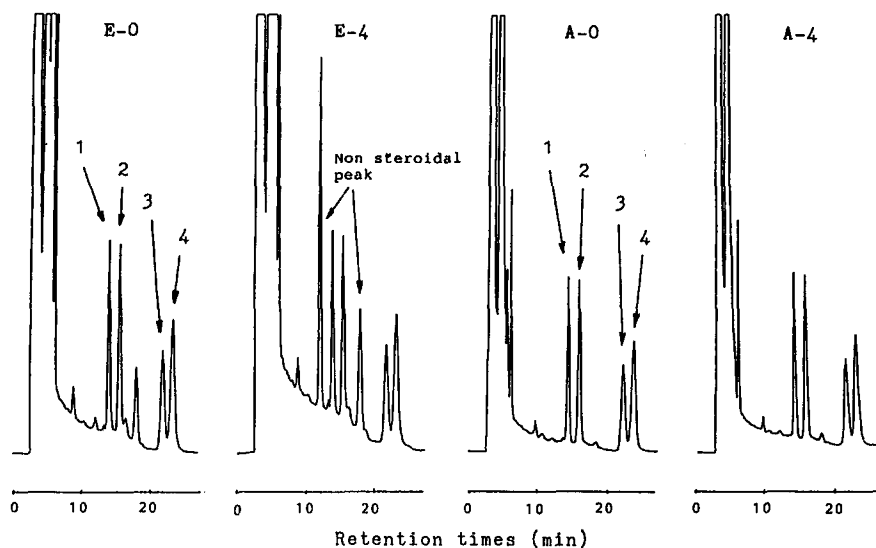

Fig. 1. Chromatograms obtained using ethanol (E-0, E-4) or acetonitrile (A-0, A-4) as solvents, for dansylated steroids E-0 and A-0 were obtained immediately after dansylation and E-4 and A-4 were obtained after E-0 and A-0 had remained for several hours at room temperature. Peaks: 1 = DHEA sulphate, 2 = EA sulphate; 3 = Ec sulphate; 4 = Ad sulphate.

trofluorimeter (Hitachi Model 4010). Dansylated ion pairs of DHEA sulphates with TPA had an extraction maximum at 332 nm and an emission maximum at 538 nm in the mobile phase.

Stability. The fluorescence of the dansylhydrazones obtained by dansylation of ion pairs of the 17OS sulphates with TPA following evaporation of the solvent was stable for 30 days, and the fluorescence of the dansylated sample in the solvent dissolved in acetonitrile was stable for 1 week (both when stored in a refrigerator).

Calibration graphs. Calibration graphs were prepared by using 0.05–5 mg/l working standard solutions. The equations for the calibration graphs were $y = 3.9x$ for DHEA sulphate, $y = 3.4x$ for EA sulphate, $y = 3.8x$ for Ec sulphate and $y = 3.8x$ for Ad sulphate (y = amount of steroid, ng/injection volume; x = peak area, mV min). Coefficients of variation (C.V.) of the values obtained with the working standard solutions containing 0.05, 0.1, 0.5, 1.0 and 5.0 mg/l each of the 17OS sulphates were 4.3–6.1, 2.7–3.2, 2.2–2.8, 1.5–2.6 and 1.7–2.5% ($n = 5$), respectively.

Analytical Recovery. The serum sample recovery was determined by adding 0.1 ml of solution containing either 5 or 10 mg of all four 17OS sulphates at a ratio of 1 1 of water to 0.9 ml of serum sample (final concentration of each, 500 and 1000 μg/l). The recoveries obtained on addition of the solution of 5 or 10 mg of 17OS sulphates were in the ranges 95.8–104.0% ($n = 5$) and 96.3–102.2% ($n = 10$), respectively, as shown in Table I.

Within- and between-run precision. The within-day C.V. values, determined from ten repeated measurements of 17OS sulphates in serum and in serum with 5 or 10 mg of added 17OS sulphates as in the recoverey test, were in the range 2.56–7.40% and 2.78-3.34% (Table I). The between-day C.V. values, determined once a day on

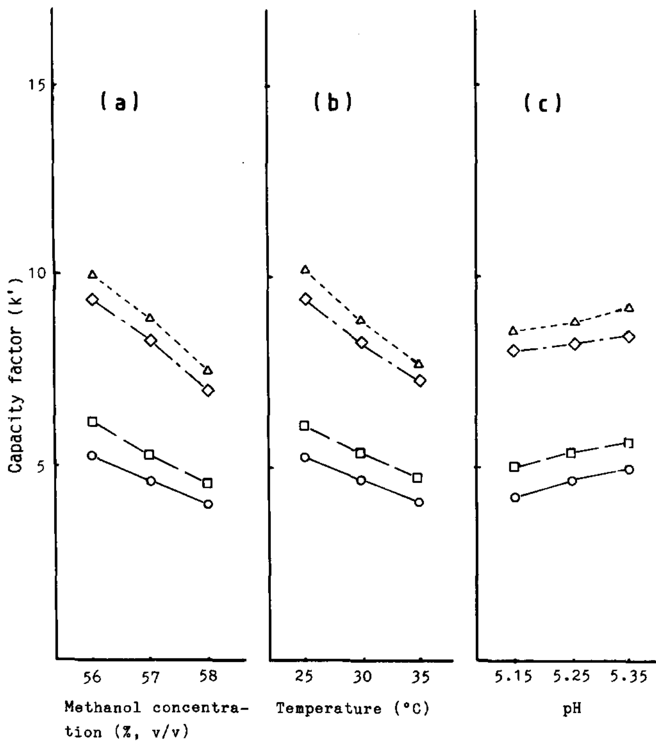

Fig. 2. Effect of methanol concentration and pH in mobile phase and column temperature. (a) The methanol concentration in the mobile phase [methanol–0.5% (w/v) sodium acetate–50% (v/v) acetic acid (57:42:1, v/v/v)] was changed from 56 to 58% at a column temperature of 30°C. (b) The column temperature was changed from 25 to 35°C using the proposed mobile phase. (c) The pH was changed from 5.15 to 5.35 by variation of the sodium acetate concentration in the mobile phase at a column temperature of 30°C. (○) DHEA sulphate; (□) EA sulphate; (◇) Ec sulphate; (△) Ad sulphate.

ten successive days from measurement of 17OS sulphates in the serum sample with added 17OS sulphates as in the recovery test, were in the range 4.11–5.98%.

Comparison with results obtained by deconjugation of 17OS sulphates in serum. The 17OS sulphate concentrations obtained with the present method (converted into free-type values) were compared with those obtained from solvolysis. Good correlations were observed for 17OS sulphates correlation coefficients of DHEA, EA, Ec and Ad were 0.927, 0.905, 0.929 and 0.948, respectively (n = 20)].

Results using samples from both patients and healthy males

Typical chromatograms from serum samples. Typical chromatograms of 17OS sulphates in serum from a patient with Cushing's syndrome and from a healthy male are shown in Fig. 3.

Concentration of 17OS sulphates in serum. The determination of 17OS sulphates in serum is generally only performed for DHEA sulphate by radioimmunoassay. However, radioimmunoassay of steroids has the problem of steroid cross-reactivity. The direct determination of 17OS sulphates in serum by HPLC shows excellent speci-

TABLE I

ANALYTICAL RECOVERY OF 17OS SULPHATES FROM AND PRECISION OF ASSAY

Sulphate	*Amount added (μg/l)*	*Final amount (μg/l)*	*Recovery (%)*	*n*	*C.V. (%)*
DHEA	0	2014		5	2.89
	500	2493	95.8	5	3.75
	0	1263		10	2.56
	1000	2226	96.3	10	3.13
EA	0	174		5	5.16
	500	694	104.0	5	4.04
	0	76		10	7.40
	1000	1094	102.2	10	2.86
Ec	0	227		5	5.32
	500	733	101.2	5	3.87
	0	110		10	7.07
	1000	1095	98.5	10	3.34
Ad	0	669		5	3.60
	500	1154	97.0	5	3.62
	0	475		10	3.16
	1000	1461	98.6	10	2.78

ficity, but so far has been reported only for DHEA and Ad sulphates by Kawasaki *et al.*[15]. We applied our method for the direct determination of DHEA, Ad and two other 17OS sulphates to serum from 50 healthy men and a patient with Cushing's syndrome.

Table II shows that the levels of DHEA and Ec sulphates in the serum of the patient were higher than those in healthy men. Yamaji and Ibrayashi[1] reported the levels of DHEA sulphate for healthy men and women to be 1650 ± 471 μg/l (21–30-year-old men, n = 25) and 1090 ± 298 μg/l (21–30-year-old women, n = 20), Seki-

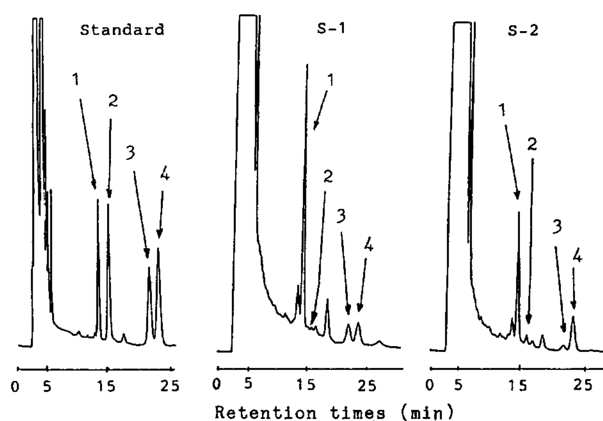

Fig. 3. Chromatograms of Serum 17OS sulphates. Standard: 2 mg/l of each steroid. S-1 = serum from 29-year-old man with Cushing's syndrome. S-2 = serum from a 25-year-old healthy man. Mobile phase: methanol–0.5% (w/v) sodium acetate–50% (v/v) (57:42:1, v/v/v).

TABLE II

17OS SULPHATE CONCENTRATIONS IN SERUM

Sulphate	*Concentration (μg/l)* 29-year-old man with Cushing's syndrome	*Healthy* 31–55-year-old men
DHEA	2595	1156 (± 572)
EA	162	97 (± 80)
Ec	298	91 (± 39)
Ad	273	454 (± 287)

hara *et al.*[20] 1780 ± 630 μg/l (20–29-year-old men, n = 15) and 980 ± 410 μg/l (20–29-year-old women, n = 14) and Kokubo *et al.*[11] 1290 ± 590 μg/l (20–50-year-old men, n = 10) and 830 ± 620 μg/l (20–50-year-old women, n = 5). The value obtained by the present method was 1156 ± 574 μg/l (31–55-year-old men, n = 50). Any difference between the previously reported values, determined by radioimmunoassay, and the values obtained by the present method are likely to have been due to steroid cross-reactivity (Kawasaki *et al.*[15] reported a method for the separation of DHEA and Ad sulphates by HPLC, but no values were given).

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