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DETERMINATION OF 17-OXOSTEROIDS IN SERUM AND URINE BY FLUORESCENCE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING DANSYL HYDRAZINE AS A PRE-LABELING REAGENT

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SUMMARY

A fluorescence high-performance liquid chromatographic method is described for the determination of 17-oxosteroids in biological fluids. 17-Oxosteroids in urine samples are extracted with dichloromethane after enzymatic hydrolysis (β-glucuronidase—sulfatase), and dehydroepiandrosterone sulfate in serum samples is solvolysed with sulfuric acid in ethyl acetate. 17-Oxosteroids are labeled with dansyl hydrazine in trichloroacetic acid—benzene solution, and then chromatographed on the microparticulate silica gel column using dichloromethane—ethanol—water (400:1:2) as the mobile phase. The eluate is monitored by a fluorophotometer at 365 nm (excitation) and 505 nm (emission). Linearities of the fluorescence intensities (peak heights) with the amounts of various 17-oxosteroids were obtained between 60 and 1000 pg. The assay proved satisfactory with respect to sensitivity, precision and accuracy. The results obtained by a radioimmunoassay and this method were in good agreement ($r = 0.964$, $n = 81$) for serum dehydroepiandrosterone sulfate. This method is also useful for the simultaneous determination of individual 17-oxosteroids in serum and urine.

INTRODUCTION

The measurement of the so-called total 17-oxosteroids in serum and urine samples has been widely used in routine clinical analysis. However, in certain clinical conditions, such as adrenogenital syndrome, carcinoma of the adrenal cortex and gonadal disorders, it is desirable to obtain information about the urinary excretion of individual 17-oxosteroids. Many methods have been reported for the determination of individual 17-oxosteroids in biological fluids, including radioimmunoassay [1—3], gas chromatography [4, 5], and gas chromatography—mass spectrometry [6]. Recently, a high-performance liquid chromatographic (HPLC) method [7] was also applied. However, the sensitivity of

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HPLC methods was too low due to the use of a refractive index or UV detector. Especially 17-oxosteroids have no strong UV-absorbing groups in their structures. 2,4-Dinitrophenylhydrazine was used as a labeling reagent in order to increase the detection limit [8, 9]. Fluorescent labeling techniques [10] have been used for several years. In a previous paper [11] we used dansyl hydrazine as an fluorescent labeling reagent for Δ^4-3-oxosteroids, such as cortisol and 11-desoxycortisol.

In this paper, we describe a highly sensitive fluorescence HPLC method for the determination of 17-oxosteroids in biological samples.

EXPERIMENTAL

Materials

Dehydroepiandrosterone (DHEA) and androsterone (AN) were obtained from Tokyo Kasei Co. (Tokyo, Japan), and etiocholanolone (ETIO), androsta-3,5-diene-17-one, androstanedione, Δ^4-androstene-3,17-dione, 11-oxo-androsterone (11-oxo-AN), 11-oxo-etiocholanolone (11-oxo-ETIO), Δ^5-androstene-3β,16α-diol-17-one (16-OH-DHEA), 11β-hydroxyandrosterone (11-OH-AN), and 16α-hydroxyandrosterone (16-OH-AN) were purchased from Sigma (St. Louis, MO, U.S.A.). Potassium salt of dehydroepiandrosterone sulfate (DHEA-S) was prepared using DHEA and sulfamic acid according to the description of Joseph et al. [12]. (Anal. calc. for $C_{19}H_{27}O_5SK \cdot 1/2\ H_2O$: C, 53.57; H, 6.99. Found: C, 53.07; H, 6.88.) Dansyl hydrazine was of reagent grade (II) from Sigma. Sodium pyruvate, trichloroacetic acid, dichloromethane, ethyl acetate and other chemicals were obtained commercially. Helicase (β-D-glucuronidase—arylsulfatase) was purchased from Boehringer Mannheim—Yamanouchi Co. (Tokyo, Japan).

Apparatus

An Hitachi Model 634 high-speed liquid chromatograph, an Hitachi Model 204 spectrofluorophotometer equipped with a xenon lamp, and a Jasco FP-110 fluorescence spectrophotometer equipped with a mercury lamp were used.

Reagent solutions

Dansyl hydrazine solution (0.2%, w/v): a 0.2% (w/v) solution of dansyl hydrazine was prepared by dissolving 20 mg of dansyl hydrazine in 10 ml of ethanol, and stored at 4°C until used.

Sodium pyruvate suspension (0.5%, w/v): a 0.5% (w/v) suspension was prepared by suspending 50 mg of fine powder of sodium pyruvate in 10 ml of benzene; it should be vigorously mixed before use.

Trichloroacetic acid—benzene solution (0.5%, w/v).

Stock solution of DHEA-S: an aliquot of DHEA-S was dissolved in ethanol to make a stock solution (10 μg/ml), and stored at 4°C until used.

DHEA-S standard solution: aliquots of 0.75 ml, 1.0 ml and 1.5 ml of the DHEA-S stock solution were taken, evaporated under a stream of nitrogen gas, and each was then dissolved in redistilled water and made up to 100 ml before use.

Synthesis of dansyl hydrazone of DHEA. Dansyl hydrazine (159 mg) was

added to a solution of DHEA (150 mg) in 0.25% trichloroacetic acid—benzene solution and the mixture was heated at 60°C for 45 min on a water-bath. Sodium pyruvate (300 mg) was added to the reaction mixture and heated for 15 min on a water-bath. After cooling to room temperature, the solvent was removed by evaporating to dryness under reduced pressure. The residue was dissolved in dichloromethane and washed respectively with 0.25 *N* sodium hydroxide solution and water. After drying with sodium sulfate, evaporation of the solvent afforded a yellow solid, which was recrystallized from ethanol—diethyl ether to give colorless needle crystals (yield: 152 mg), m.p. 231—233°C. Anal. calc. for $C_{31}H_{41}N_3O_3S \cdot 1/2\ H_2O$: C, 68.38; H, 7.72; N, 7.72. Found: C, 68.30; H, 7.76; N, 7.54. IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 2800 (Ar—$N(CH_3)_2$), 1614 (—N=C—), 1590, 1579, 1500 (aromatic). NMR (C^2HCl_3) δ ppm: 2.85 (s, 6H, N—Me_2), 0.60 (s, 3H, C(19)—Me), 1.00 (s, 3H, C(18)—Me), 3.35 (d, 1H, C(3)—OH), 3.50 (m, 1H, C(3)—H), 5.30 (d, 1H, C(6)—H), 7.20 (t, 1H, arom.), 7.29 (s, 1H, —SO_2NH—N=), 7.52 (t, 1H, arom.), 8.25—8.64 (4H, arom.). Mass spectrometry *m/z*: 535 (M^+).

Chromatographic conditions

Stainless-steel columns 250 mm × 4 mm I.D. and 250 mm × 4.6 mm I.D. were packed with Hitachi gel No. 3042 (silica gel, particle size 5 μm) and Zorbax SIL (particle size 5—6 μm), respectively. The eluent was the organic layer separated from the mixture dichloromethane—ethanol—water (400:1:2, v/v) after shaking for 30 min. Flow-rate, column pressure, and column tempe ature were 1 ml/min, 35 kg/cm^2, and 35°C, respectively. The effluent was monitored at 505 nm emission against 350 nm or 365 nm excitation with an Hitachi Model 204 spectrofluorophotometer or a Jasco FP-110 fluorimeter.

Procedure

Serum sample. To 50—100 μl of serum in a centrifuge tube is added water to a volume of 1.5 ml. After the addition of 5.0 ml of dichloromethane, the contents of the tube are mixed with a Vortex-type mixer for 1 min and centrifuged at 1000 *g* for 2 min. Unconjugated steroids are extracted into dichloromethane. One milliliter of the supernatant aqueous layer is taken exactly, transferred to another tube and then mixed with a mixture of 10 ml of ethyl acetate and 0.1 ml of concentrated sulfuric acid for 1 min. After discarding the aqueous layer, the ethyl acetate layer is incubated for 3 h at 40°C. The ethyl acetate layer is washed successively with 1 ml of 1 *N* sodium hydroxide solution and 2 ml of water. After drying with anhydrous sodium sulfate, 7.0 ml of the ethyl acetate layer are transferred to another tube and evaporated to dryness under a stream of nitrogen gas. The resultant residue is assayed by the procedure described below.

Urine sample. To 1.0 ml urine in a centrifuge tube are added 500 μl of 2 *M* acetate buffer (pH 5.2) and 40 μl of Helicase. The contents of the tube are then mixed and incubated overnight at 37°C. After incubation, 6 ml of dichloromethane are added and mixed with a Vortex-type mixer for 1 min. The aqueous layer is discarded and 4 ml of the dichloromethane layer are transferred to another tube and evaporated to dryness under a stream of nitrogen gas. The resultant residue is assayed by the following procedure.

Labelling reaction. To the evaporated residue in a test tube are added 0.2 ml of 0.5% trichloroacetic acid—benzene solution and 0.1 ml of dansyl hydrazine solution. After incubation for 20 min at 60°C, 0.2 ml of sodium pyruvate suspension is added and warmed for 10 min at 60°C. The solvent is evaporated to dryness under a stream of nitrogen gas. To the residue in a test tube are added 3.0 ml of dichloromethane and 1.0 ml of 0.25 *N* sodium hydroxide solution; the tube contents are mixed with a Vortex-type mixer for 1 min, followed by washing with 2 ml of water. The aqueous layer is discarded and the dichloromethane layer is dried by addition of anhydrous sodium sulfate. In the assay of urine sample, an aliquot of this extract solution is injected into the chromatograph. Two milliliters of dichloromethane extract obtained from each serum sample are transferred to another tube and evaporated to dryness under a stream of nitrogen gas. The resultant residue is redissolved by the addition of 100 μl of chloroform, of which an aliquot is injected into the chromatograph.

RESULTS

Fluorescence spectrum

Dansyl hydrazone of DHEA was prepared by the reaction of DHEA with dansyl hydrazine in trichloroacetic acid—benzene solution. As shown in Fig. 1, dansyl hydrazone of DHEA has an excitation maximum at 350 nm and an emission maximum at 505 nm. It was stable in chloroform for at least 1 week at 4°C without any change of fluorescence. The fluorescence of the solution obtained from the assay procedure was also stable for 3 days at 4°C.

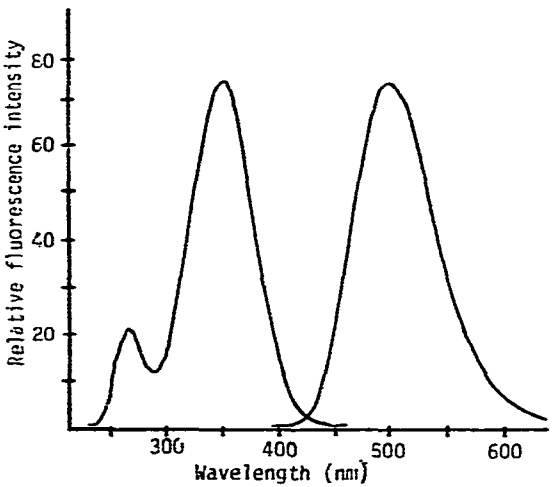

Fig. 1. Excitation and emission spectra of the dansyl hydrazone of dehydroepiandrosterone in chloroform. Excitation maximum = 350 nm; emission maximum = 505 nm.

Optimal conditions of dansylation

Though a 0.02% dansyl hydrazine solution was used in the previous paper, 17-oxosteroids could not be quantitatively labeled under the same conditions. Therefore, various factors were examined with DHEA. A 0.2% dansyl hydra-

zine solution was used in this method because the excess reagent could be removed by extraction after the reaction with pyruvate. Fig. 2 shows the effects of reaction time and the concentration of trichloroacetic acid solution on the fluorescence intensity (assessed by peak height in the chromatogram). The reaction time required to reach a maximum and constant peak height decreased with increased trichloroacetic acid concentration in benzene up to 1.0%. Using 0.2% dansyl hydrazine solution, the peak height reached a maximum at 20 min with 0.5% trichloroacetic acid solution. Though the peak height showed a slight decrease after 20 min, 0.5% trichloroacetic acid solution was used and a reaction time of 20 min was decided upon. The effect of temperature on the reaction was examined. It was decided to employ a reaction temperature of 60°C because the maximum peak height was obtained by 60°C, and the unknown minor peaks appeared at 80°C.

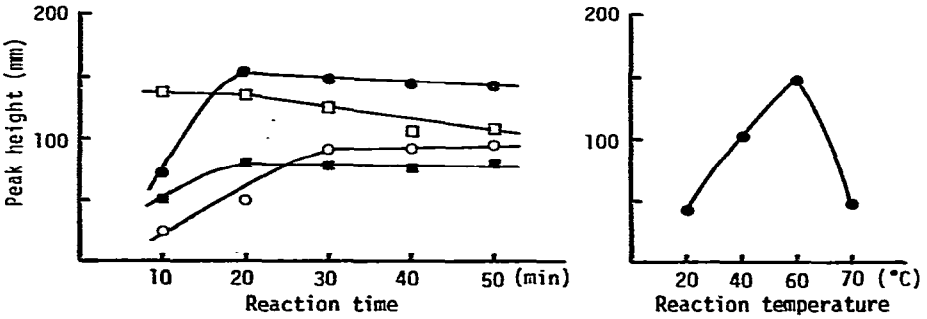

Fig. 2. Effects of trichloroacetic acid concentration, reaction time and reaction temperature on fluorescence intensity (peak height). The concentrations of trichloroacetic acid—benzene solution were: 2.0% (■), 1.0% (□), 0.5% (●) and 0.25% (○).

Selection of eluent

Many solvent systems were examined in order to obtain the complete separation of 17-oxosteroids. The organic layer of dichloromethane—ethanol—water (400:1:2) was found to be suitable when used with an Hitachi gel No. 3042 column and a Zorbax SIL column. The chromatograms presented in Fig. 3A and B show a good separation of standard 17-oxosteroids including androsta-3,5-diene-17-one, androstanedione, androstadienedione, Δ^4-androstene-3,17-dione, AN, DHEA, ETIO, 11-oxo-AN, 11-oxo-ETIO, 16-OH-DHEA, 11-OH-AN, and 16-OH-AN.

Working curves and sensitivities

Typical working curves are shown in Fig. 4. When the Jasco FP-110 fluorimeter was used as detector, standard working curves of AN, DHEA, and ETIO showed linearities in the range of 60 pg to 1 ng, corresponding to 0.2—3.4 pmol. When 0.1 ml of serum sample was used for the assay, the detection limit of DHEA sulfate was about 0.6 μg/dl from this working curve.

Solvolysis conditions of sulfates

Most 17-oxosteroids are excreted as sulfate or glucuronide conjugates. To

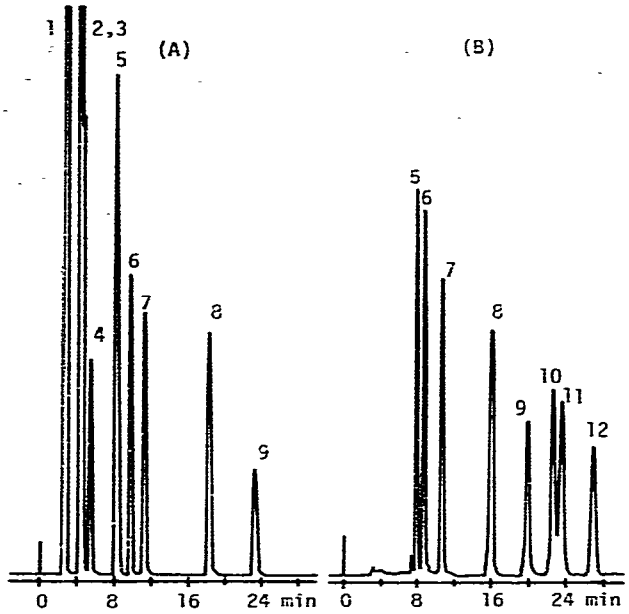

Fig. 3. Chromatograms of dansyl hydrazone derivatives of 17-oxosteroid standard mixture. Peaks: 1 = androsta-3,5-diene-17-one, 2 = androstanedione, 3 = androstadienedione, 4 = androst-4-ene-3,17-dione, 5 = androsterone, 6 = dehydroepiandrosterone, 7 = etiocholanolone, 8 = 11-oxoandrosterone, 9 = 11-oxoetiocholanolone, 10 = 16α-hydroxydehydroepiandrosterone, 11 = 11β-hydroxyandrosterone, 12 = 16α-hydroxyandrosterone. (A) Hitachi gel No. 3042 (250 × 4 mm I.D.) column; mobile phase, dichloromethane—ethanol—water (400:1:2), 1 ml/min; Hitachi 204 fluorescence detector (excitation 350 nm; emission 505 nm). (B) Zorbax SIL (250 × 4.6 mm I.D.) column; JASCO FP-110 fluorescence detector (excitation 365 nm; emission 505 nm); other conditions as in (A).

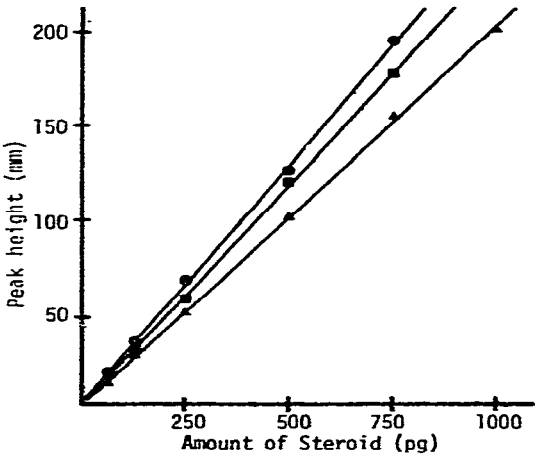

Fig. 4. Standard curves for 17-oxosteroids: androsterone (●), dehydroepiandrosterone (■), etiocholanolone (▲).

assay DHEA sulfate in serum or urine, various hydrolysis procedures were examined. In the case of acid hydrolysis, the duration of hydrolysis is very critical; less than 10 min caused incomplete hydrolysis and more than 10 min lead to a gradual destruction of steroids and an increased number of unknown peaks. The amount of free DHEA obtained from the sulfate conjugate was only 50.2% of the calculated amount by acid hydrolysis and unknown peaks other than that of the dansyl hydrazone of DHEA appeared in the chromatogram. Therefore, hydrolysis of DHEA sulfate was done by solvolysis with ethyl acetate—sulfuric acid as reported by Burstein and Lieberman [13]. DHEA sulfate was almost quantitatively hydrolyzed to yield 94.5% DHEA after incubation for 3 h at 40°C. This value agreed closely with the yield reported by Kulpmann and Breuer [14].

Enzymatic hydrolysis

Urine samples containing both sulfate and glucuronide conjugates were hydrolyzed with Helicase according to the literature [5, 15].

Recovery and reproducibility

The recovery test was carried out by determining pooled serum samples spiked with known amounts of DHEA sulfate. As illustrated in Table I, the recoveries of added DHEA sulfate varied from 97% to 106.5% with the coefficient of variation (C.V.) ranging from 1.1 to 5.8%. Interassay variations were also measured with three serum samples containing 43.4, 62.5, and 97.9 μg/dl DHEA sulfate, respectively. The respective C.V. values were 2.1, 0.7 and 2.6% (five replicate determinations).

TABLE I

RECOVERIES OF DEHYDROEPIANDROSTERONE SULFATE FROM SERUM

Sample	Added (μg/dl)	Found (μg/dl)	Recovery (%)	*n*	C.V. (%)
Serum A	0	55.1	—	10	1.10
	75	130.5	100.5	10	3.62
	150	214.9	106.5	10	5.75
Serum B	0	109.0	—	5	2.75
	200	303.0	97.0	5	4.48

Typical chromatograms of serum and urine samples

Fig. 5A shows a chromatogram of a normal serum sample containing AN and DHEA. Fig. 5B–D are typical chromatograms of urine samples obtained from patients with ovarian cystoma, adrenogenital syndrome, and hypertension, respectively. 11- and 16-oxygenated 17-oxosteroids were measurable simultaneously by increasing the sensitivity of the detector.

Comparisons with radioimmunoassay and a colorimetric method

To assess the reliability of the HPLC method for determination of DHEA sulfate in serum, DHEA sulfate levels in serum samples from 81 patients were

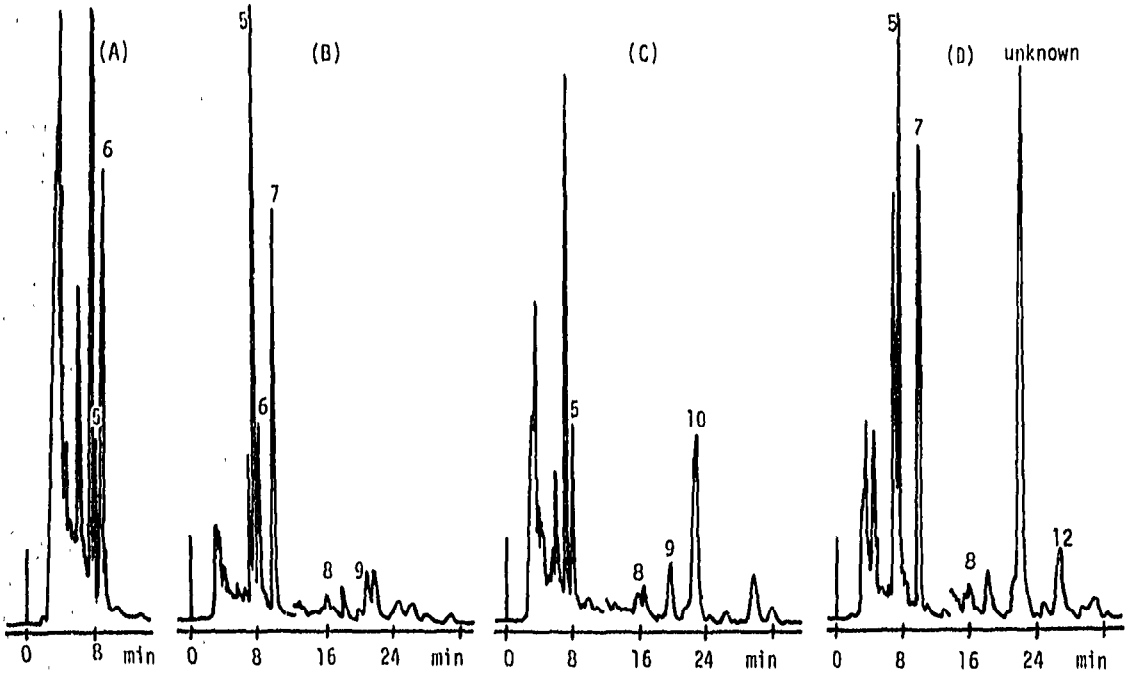

Fig. 5. Typical chromatograms of human serum and urine samples. (A) Normal human serum (male; age, 22): 5 = AN sulfate (32.8 μg/dl), 6 = DHEA sulfate (106.0 μg/dl). (B) Ovarian cystoma patient's urine (female, 21): 5 = AN (1.94 mg/day), 6 = DHEA (0.5 mg/day), 7 = ETIO (1.78 mg/day), 8 = 11-oxo-AN (38 μg/day), 9 = 11-oxo-ETIO (28 μg/day). (C) Adrenogenital syndrome patient's urine (male, 2): 5 = AN (22 μg/day), 8 = 11-oxo-AN (1.6 μg/day), 9 = 11-oxo-ETIO (5.24 μg/day), 10 = 16α-OH-DHEA (30 μg/day). (D) Hypertension patient's urine (male, 70): 5 = AN (4.32 mg/day), 7 = ETIO (4.64 mg/day), 8 = 11-oxo-AN (0.26 mg/day).

determined by both HPLC and radioimmunoassay (RIA). The RIA method used here is a direct assay of DHEA sulfate without hydrolysis after removing free steroids by dichloromethane extraction [16]. As shown in Fig. 6, the

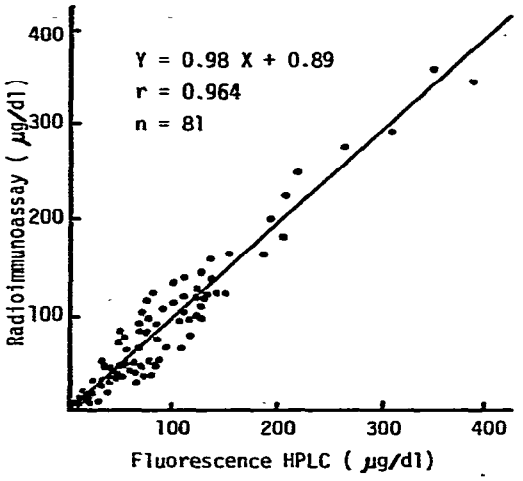

Fig. 6. Correlation between fluorescence HPLC and RIA values of dehydroepiandrosterone sulfate in serum.

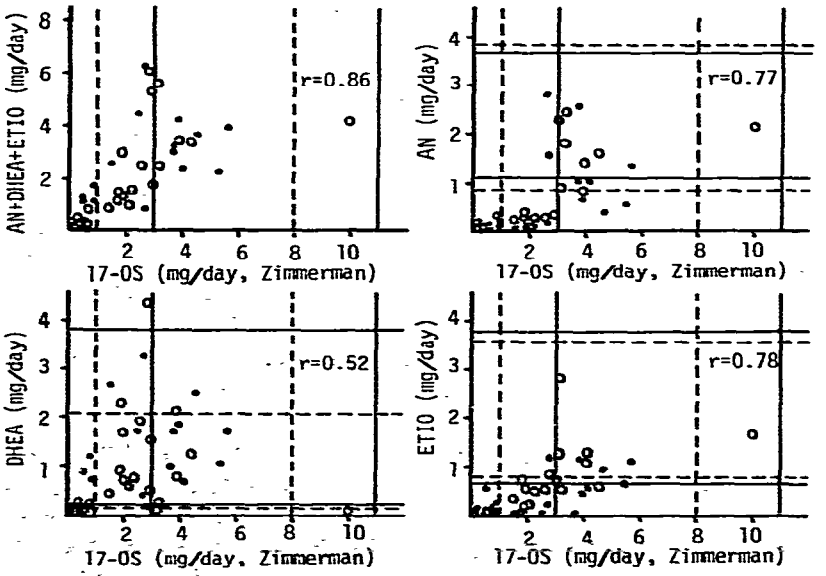

Fig. 7. Comparisons of results obtained with fluorescence HPLC and Zimmerman method for the determination of 17-oxosteroids in urine. (•) male, (○) female; normal value of male (straight line); normal value of female (broken line).

correlation coefficient was 0.964 and the regression line $Y = 0.98\ X + 0.89$, where X equals the values determined by the HPLC method.

Furthermore, the individual 17-oxosteroid levels in urine samples were determined by the HPLC method and the results were compared with the total values of 17-oxosteroids obtained by an ordinary colorimetric method used in routine assay. The results are shown in Fig. 7A—D.

DISCUSSION

Several reports on the use of HPLC to determine steroids in biological fluids have been published [7, 17], but the sensitivity of HPLC was too low due to the use of a UV detector. Especially, 17-oxosteroids have no strong UV-absorbing group in their molecules so that their detection limits were more than 1 μg using a refractive index detector. In order to increase the sensitivity in the assay of 17-oxosteroids, 2,4-dinitrophenylhydrazine has been used by several workers [8, 9, 18]. The 2,4-dinitrophenylhydrazone derivatives of 17-oxosteroids were easily separated by thin-layer chromatography or HPLC, and could be detected in quantities as low as 1 ng. Though these methods have been applied to the assay of 17-oxosteroids and their conjugates in urine samples, they could not be applied to the assay of 17-oxosteroids in serum samples. In this report a fluorophotometric HPLC method for the determination of 17-oxosteroids in serum and urine samples has been developed. Dansyl hydrazine was used as a fluorescent pre-labeling reagent. In the previous paper [11] cortisol in serum or urine was determined sensitively by fluorescence HPLC using dansyl hydrazine as an pre-labeling reagent. Though 17-oxosteroids react with dansyl hydrazine in the presence of acid at room temperature as described in the previous paper [11], the yield of hydrazone derivatives was low and the peak of excess hydrazine interfered with the separation of some 17-oxosteroids and other unknown peaks appeared in chromatogram. Therefore, the reaction conditions of labeling were examined and the optimal conditions were selected as described in Procedure.

The chromatographic conditions were also selected to give a complete separation between dansyl hydrazones of 17-oxosteroids and the fluorescent coexisting substances in serum or urine samples in the shortest possible analysis time. As shown in Fig. 3, good separation of dansyl hydrazone derivatives of 17-oxosteroids can be achieved with an Hitachi gel No. 3042 column or a Zorbax SIL column using the organic layer of dichloromethane—ethanol—water (400:1:2, v/v) as mobile phase. The detection limit of the 17-oxosteroids was about 60 pg from the working curves, as shown in Fig. 4. Then, using 0.1 ml of serum or 1.0 ml of urine as sample in routine assay, the detection limits are 0.5 or 0.7 μg/dl, respectively. The detection limit depends on the efficiency of the fluorescence detector and the final injection volume. When the residue in the assay tube is dissolved in 100 μl of solvent at the final step, the detection limit of DHEA in urine is about 35 ng/dl. The sensitivity of this method is superior to those of other HPLC methods using a UV detector. Moreover, the peaks of 17-oxosteroids were overlapped by a strong band of UV-absorbing substances in serum or urine extracts and could not be detected. However,

they appeared in the chromatogram measured by a fluorophotometer and could be determined quantitatively.

Good correlation (r = 0.964) was obtained between the values of DHEA sulfate in serum samples determined by the method proposed here and by radioimmunoassay.

The estimation of total urinary neutral 17-oxosteroids serves as a screening test for the diagnosis of adrenal or gonadal disease. However, in order to derive meaningful information, the determination of individual components of this group of steroids is very important. For example, androsterone and etiocholanolone are primary metabolites of testosterone. The increased excretion of these compounds in a male, without proportionate changes of DHEA and 11-oxygenated 17-oxosteroids, is a positive indication of testicular dysfunction. When total 17-oxosteroids are estimated, such specific changes will go unobserved. Therefore, we examined the determination of individual 17-oxosteroids in urinary samples by the method proposed here and compared the results with the total 17-oxosteroid values obtained by a spectrophotometric method after enzymatic hydrolysis of urine samples. As shown in Fig. 5, typical chromatograms obtained from patients are different from each other. Fig. 7A shows the correlation between the total values of 17-oxosteroids obtained by the spectrophotometric method and the sum values of AN, DHEA, and ETIO determined by the HPLC method. The sum values of AN, DHEA, and ETIO correlate well with the total values of 17-oxosteroids. On the other hand, the individual values of AN, DHEA, and ETIO do not correlate with the total values of 17-oxosteroids, as shown in Fig. 7B—D. These results mean that the determination of individual components of 17-oxosteroids in urine samples is very important and the method described in this paper may have clinical potential in the routine assay of 17-oxosteroids in serum and urine samples.

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