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DETERMINATION OF 17-OXOSTEROID GLUCURONIDES AND SULFATES IN URINE AND SERUM 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 direct determination of conjugated 17-oxosteroids in biological fluids without hydrolysis. Conjugated 17-oxosteroids are extracted with Sep-Pak C_{18} cartridge, labeled with dansyl hydrazine in trichloroacetic acid—benzene solution and then separated by high-performance liquid chromatography on reversed-phase μBondapak C_{18} column using 0.01 *M* sodium acetate in methanol—water—acetic acid (65:35:1, v/v) as the mobile phase. The eluate is monitored by a fluorophotometer at 365 nm (excitation) and 520 nm (emission). Linearities of fluorescence intensities (peak heights) with the amounts of various conjugated 17-oxosteroids were obtained between 10 pmol and 100 pmol. This method is sensitive, reliable and useful for the simultaneous determination of conjugated 17-oxosteroids in urine and serum.

INTRODUCTION

The measurement of steroids in serum and urine samples has been used in clinical laboratories as a clinical indicator of adrenal function [1]. Many methods have been reported for analysis of metabolic profiles of steroids in urine by gas chromatography [2—6] and gas chromatography—mass spectrometry [7, 8]. As the steroids excreted in urine are mainly in the conjugated form, the analytical methods generally involve hydrolysis of the conjugates prior to chromatographic analysis. Therefore, important information about metabolic pathways such as subtle changes in type or site of conjugation may be overlooked by the use of such procedures.

Recently, high-performance liquid chromatography (HPLC) has been studied

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for the separation of conjugated steroids [9—13], mainly estrogen glucuronide and estrogen sulfate. Conjugated 17-oxosteroids were separated by reversed-phase HPLC [13], which showed poor efficiency, however.

In the previous paper [14], we reported a highly sensitive HPLC method using dansyl hydrazine as a fluorescent labeling reagent for the determination of urinary 17-oxosteroids after enzymatic hydrolysis and extraction of the liberated steroids.

In this paper, we describe a highly sensitive fluorescence HPLC method for the direct determination of conjugated 17-oxosteroids in urine and serum samples without hydrolysis.

EXPERIMENTAL

Materials

Androsterone glucuronide (AN-G)* and androsterone sulfate (AN-S) were obtained from Sigma (St. Louis, MO, U.S.A.). Etiocholanolone glucuronide (ETIO-G) and etiocholanolone sulfate (ETIO-S) were purchased from Makor (Jerusalem, Israel). Dehydroepiandrosterone sulfate (DHEA-S), androsterone (AN), etiocholanolone (ETIO) and dehydroepiandrosterone (DHEA) were those used in previous studies [14]. β-Glucuronidase and arylsulphatase were from Boehringer Mannheim-Yamanouchi (Tokyo, Japan). Sep-Pak C_{18} cartridges from Waters Assoc. (Milford, MA, U.S.A.) and Amberlite XAD-2 from Rohm and Haas Co. (Philadelphia, PA, U.S.A.) were used. All other reagents and solvents were of analytical reagent grade from commercial sources.

Instruments and chromatographic conditions

We used an Hitachi Model 635 high-performance liquid chromatograph equipped with a Kyowa Seimitsu KHP-UI-130 injection valve, a stainless-steel column, a Jasco Model FP-110 fluorescence spectrophotometer equipped with a mercury lamp and a micro flow cell. A reversed-phase μBondapak C_{18} column (Waters, 300 × 3.9 mm I.D., particle size 10 μm) was used for the separation of all conjugated 17-oxosteroids. The solvent system used was 0.01 *M* sodium acetate in methanol—water—acetic acid (65:35:1, v/v) at a flow-rate of 1 ml/min. A Zorbax SIL column (250 × 4.6 mm I.D., DuPont, Wilmington, DE, U.S.A.) was also used for free steroids liberated by enzymatic hydrolysis. The detector wavelength was set at 365 nm and 505 nm for excitation and emission, respectively.

Reagent solutions

Dansyl hydrazine solution. A 0.2% (w/v) solution was prepared by dissolving 20 mg of dansyl hydrazine in 10 ml of benzene, and stored in a refrigerator until use.

Trichloroacetic acid—benzene solution. A 0.5% (w/v) solution was prepared by dissolving 50 mg of purified trichloroacetic acid in 10 ml of benzene.

Steroid stock solutions. AN-G, ETIO-G, DHEA-S, AN-S and ETIO-S were

*The following trivial names are used: androsterone (AN), 3α-hydroxy-5α-androstan-17-one; dehydroepiandrosterone (DHEA), 3β-hydroxy-androst-5-ene-17-one; etiocholanolone (ETIO), 3α-hydroxy-5β-androstan-17-one.

dissolved separately in methanol to make each stock solution (0.5 μmol/ml), and stored at −20°C until use.

Extraction method

Urine sample. A 0.5-ml aliquot of urine was diluted to 1.0 ml with redistilled water, applied onto a Sep-Pak C_{18} cartridge and washed successively with 5 ml of water and 3 ml of 20% ethanol. Conjugated 17-oxosteroids were eluted with 2 ml of methanol and the eluent was then evaporated to dryness at 40°C under a stream of nitrogen gas. The resultant residue was assayed as described below.

Serum sample. A 0.2-ml volume of serum was diluted to 1.0 ml with 0.025 *M* phosphate buffer (pH 7.0), applied onto a Sep-Pak C_{18} cartridge and washed with 4 ml of water. Conjugated 17-oxosteroids were eluted with 2 ml of methanol and the eluent was then evaporated to dryness under a stream of nitrogen gas at 40°C. The resultant residue was assayed by the following procedure.

Labeling reaction

The residue in the test tube was dissolved by adding 0.2 ml of 0.5% trichloroacetic acid—benzene solution, admixed with 50 μl of 0.2% dansyl hydrazine solution, left to stand for 20 min at 60°C, and then evaporated to dryness under a stream of nitrogen gas. The labelled residue was dissolved in 200 μl of methanol and an aliquot of the solution was injected into the chromatograph described above.

RESULTS

Effect of eluent composition

For a reversed-phase μBondapak C_{18} column, many solvent systems were examined to obtain the complete separation of 17-oxosteroid glucuronides and sulfates. The effect of the methanol concentration on the capacity factors was investigated. The mixture of methanol and water containing 0.075 *M* sodium acetate and 2% acetic acid was used as mobile phase because glucuronides and sulfates were separated completely from the peak of excess dansyl hydrazine by addition of acetic acid and sodium acetate to the mobile phase. As shown in Fig. 1, the most suitable k' value was obtained by the use of 65% methanol but AN-S and ETIO-G did not separate. As shown in Fig. 2, sodium acetate concentration is important for the separation of glucuronides and sulfates. At low sodium acetate concentration, the sulfates are eluted first and then the glucuronides. The addition of acetic acid to the mobile phase gave a distinct effect on the separation of glucuronides and excess dansyl hydrazine but the capacity factors did not change to a large extent. Therefore, 0.01 *M* sodium acetate in methanol—water—acetic acid (65:35:1) was used as eluent. The chromatogram presented in Fig. 3 shows good separation of standard conjugated 17-oxosteroid mixture including DHEA-S, ETIO-S, AN-S, ETIO-G and AN-G.

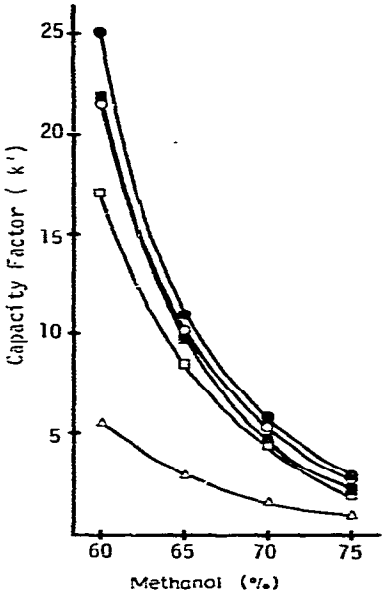

Fig. 1. Effect of methanol concentration on capacity factor. Eluent: methanol—water + 2% acetic acid + 0.075 *M* sodium acetate. (●) AN-G; (■) ETIO-G; (○) AN-S; (□) ETIO-S; (△) DHEA-S.

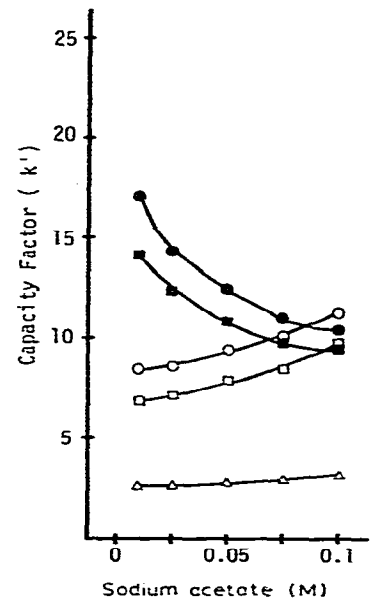

Fig. 2. Effect of sodium acetate concentration on capacity factor. Eluent: methanol—water—acetic acid (65:35:2) + sodium acetate. (●) AN-G; (■) ETIO-G; (○) AN-S; (□) ETIO-S; (△) DHEA-S.

Working curves and sensitivities

Typical working curves are shown in Fig. 4. Linearity of fluorescence intensity (peak height) with the injected amounts of conjugated 17-oxosteroids were obtained between 10 pmol and 100 pmol, and detection limits were about 1.0 pmol. When 0.2 ml of serum sample was used for the assay, the detection limit for DHEA-S was about 8 μg/dl from this working curve.

Recovery and reproducibility

Recovery tests were carried out by determining urine samples spiked with a mixture of known amounts of the five conjugated 17-oxosteroids and pooled serum sample with DHEA-S. As shown in Table I, conjugated 17-oxosteroids were recovered in the range 94.9—105.2% with C.V. (%) in the range 1.7—3.4%.

Typical chromatograms from urine and serum samples

Typical chromatograms obtained from normal human and patient urine samples and normal human serum are shown in Fig. 5; urinary conjugated 17-oxosteroids and serum DHEA-S were clearly separated and identified by comparison with authentic samples.

Comparison with results obtained by the deconjugation method

The reliability of the newly devised HPLC method for the direct determination of urinary and serum conjugated 17-oxosteroids was assessed by com-

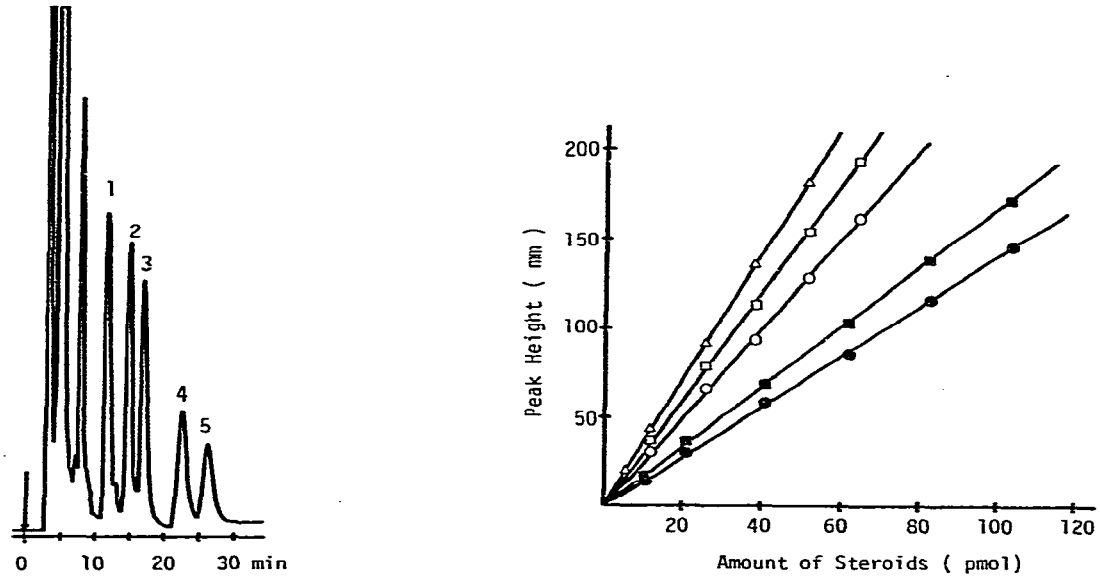

Fig. 3. Chromatogram of dansyl hydrazone derivatives of conjugated 17-oxosteroid standard mixture. Peaks: 1 = dehydroepiandrosterone sulfate, 2 = etiocholanolone sulfate, 3 = androsterone sulfate, 4 = etiocholanolone glucuronide, 5 = androsterone glucuronide. μBondapak C_{18} (300 × 3.9 mm I.D.) column; mobile phase, 0.01 *M* sodium acetate in methanol—water—acetic acid (65:35:1), 1 ml/min; JASCO FP-110 fluorescence detector (excitation 365 nm, emission 520 nm).

Fig. 4. Standard curves for conjugated 17-oxosteroids. (•) AN-G; (▪) ETIO-G; (○) AN-S; (□) ETIO-S; (△) DHEA-S.

TABLE I

RECOVERIES OF CONJUGATED STEROIDS ADDED TO HUMAN URINE AND SERUM

Urine (0.5 ml) with 0.75 nmol of each of the five conjugated 17-oxosteroids added was used. Serum (0.2 ml) with 0.5 nmol of added DHEA-S was used.

	Steroid	*n*	Recovery (%)	C.V. (%)
Urine	Androsterone glucuronide	4	95.3	2.2
	Etiocholanolone glucuronide	4	101.2	3.4
	Androsterone sulfate	4	101.0	1.7
	Etiocholanolone sulfate	4	105.2	3.2
	Dehydroepiandrosterone sulfate	4	96.8	3.4
Serum	Dehydroepiandrosterone sulfate	4	94.9	2.0

paring the results with those obtained by the previous method, involving enzymatic hydrolysis of urine samples with β-glucuronidase or arylsulphatase. The results obtained by both methods are illustrated in Table II.

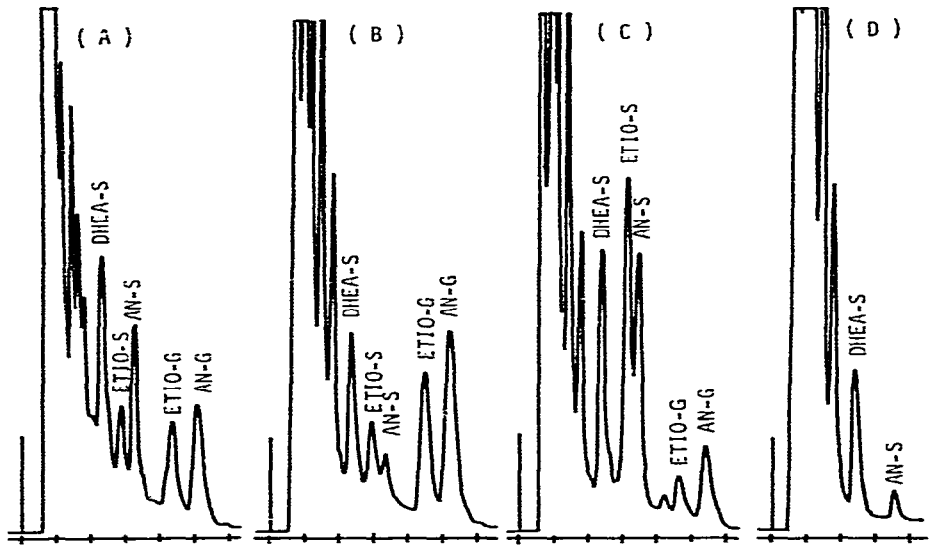

Fig. 5. Typical chromatograms of human urine and serum samples. (A) Normal human urine (male, aged 21). (B) Ovarian tumor patient's urine (female, aged 21). (C) Thyroiditis patient's urine (female, aged 50). (D) Normal human serum (male, aged 21).

TABLE II

COMPARISON BETWEEN THE DIRECT METHOD* AND THE METHOD WITH HYDROLYSIS** FOR CONJUGATED 17-OXOSTEROIDS IN URINE

Steroid	Regression line***	r	n
Androsterone glucuronide	$Y = 1.35\,X - 0.15$	0.894	20
Etiocholanolone glucuronide	$Y = 0.83\,X - 0.01$	0.948	20
Androsterone sulfate	$Y = 1.03\,X + 0.04$	0.972	16
Etiocholanolone sulfate	$Y = 1.01\,X - 0.06$	0.987	16
Dehydroepiandrosterone sulfate	$Y = 1.12\,X + 0.30$	0.914	18

*The method proposed in this paper.
**The previous method in the previous paper [14].
***X = the values obtained by the proposed method; Y = the values obtained by the previous method.

DISCUSSION

Urinary steroids, metabolized and excreted by conjugation with glucuronic acid or sulfuric acid, provide information on the physical condition of the organism because the state of conjugation depends on the structure and metabolic origin of the steroid metabolites. Several reports have been published for the determination of metabolic profiles of urinary steroids [2—8]. In most cases [2—6], hydrolysis of conjugated steroids is the first step in the assay method so that subtle changes in type or site of conjugation will be lost. Therefore, group separation of conjugates prior to hydrolysis based on ion-exchange triethylaminohydroxypropyl Sephadex LH-20 (TEAP-LH-20) [8]

and DEAE-Sephadex [15] has been developed. However, this method is time-consuming and less suitable for routine assay. A method that permits the direct determination of conjugated steroids without hydrolysis would probably give additional information on the pathological state.

Recently, HPLC has been studied for the separation of conjugated steroids [9—13] because they are not sufficiently volatile to allow their direct gas chromatographic separation. Lafosse et al. [13] reported the separation of conjugated 17-oxosteroids; however, they could not separate different types of conjugates, glucuronide and sulfate, and their determination range was at the microgram level. In the previous paper [14], we reported a highly sensitive fluorescence HPLC method for the determination of 17-oxosteroids, the liberated free steroids by hydrolysis, in serum and urine.

In this paper, we have developed a fluorescence HPLC method for the direct determination of conjugated 17-oxosteroids in urine or serum without hydrolysis.

Amberlite XAD-2 has been commonly used to extract conjugated steroids from urine [8, 16], and recently the extraction method with Sep-Pak C_{18} cartridge was reported by Shackleton and Whitney [17] and Axelson and Sahlberg [18]. In this assay, when Amberlite XAD-2 was used, unknown peaks appeared occasionally and interfered with the separation of conjugated steroids. Urinary conjugated steroids could be extracted with no chromatographic interferences by using Sep-Pak C_{18} cartridge. This extraction method was superior with respect to speed, simplicity and recovery for conjugated 17-oxosteroids.

The derivatization conditions of conjugated 17-oxosteroids with dansyl hydrazine were examined and the optimal conditions were similar to those of free 17-oxosteroids as described in the previous paper [14]. We chose chromatographic conditions which, in the shortest possible assay time, gave acceptable resolution between the dansyl hydrazones of conjugated steroids and the fluorescent coexisting substances in urine or serum samples. As shown in Fig. 3, good separation could be achieved on μBondapak C_{18} column using 0.01 *M* sodium acetate in methanol—water—acetic acid (65:35:1) as the eluent. The sensitivity of this method was superior to other HPLC methods using a UV detector.

As shown in Table II, the correlation between the values of urinary conjugated 17-oxosteroids obtained by the present method and by the previous method [14] involving enzymatic hydrolysis prior to chromatographic separation is not good for three of them (AN-G, ETIO-G and DHEA-S) as indicated by the regression line and *r* value. Since there are many kinds of conjugated oxosteroids in urine, it is preferable that the peaks should be isolated and analyzed by an independent method. In this study, the peaks in the chromatogram of the sample were identified by their retention times as compared with the chromatogram of the standard solution measured at the same time. The differences between the present and previous methods may be due to interfering substances or efficiency of enzymatic hydrolysis.

In conclusion, the present method gives, with a very short assay time, direct information on the different concentrations of conjugated 17-oxosteroids in urine and serum and on the rate of conjugation with glucuronic acid or sulfuric

acid. The extraction with Sep-Pak C_{18} cartridge represents a simple procedure with almost complete recovery of conjugated 17-oxosteroids. The newly developed fluorescence HPLC method may be clinically useful in the routine assay of urine and serum conjugated 17-oxosteroids.

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