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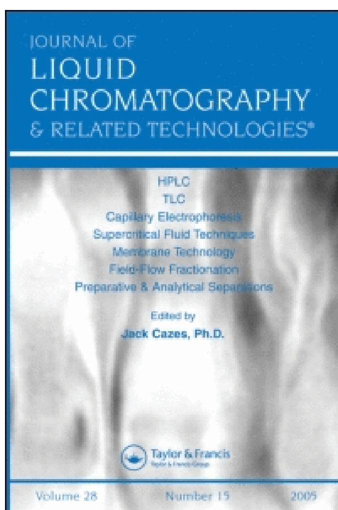

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Simultaneous Determination of 18-Oxygenated Corticosteroids by High-Performance Liquid Chromatography with Fluorescence Detection

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SIMULTANEOUS DETERMINATION OF 18-OXYGENATED CORTICOSTEROIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

A method for differentially measuring the 18-oxygenated corticosteroids, 18-hydroxycortisol, 18-hydroxycortisone and 18-oxocortisol, in human urine from the patients with primary aldosteronism has been developed by high-performance liquid chromatography with fluorescence detection. The method involves the derivatization of 18-oxygenated corticosteroids into the fluorescent 21-anthroyl esters by the action with 1-anthroyl nitrile. Urine was first extracted with a mixture of ether-dichloromethane, and solid-phase

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extraction of the anthroyl derivatives on a Bond Elut CN cartridge column was used as a clean up step before final separation by high-performance liquid chromatography with fluorescence detection. 11,18-Epoxypredonisolone was used as an internal standard. The linearity of the calibration curve for each steroid was from 0.5 pmol to 25 pmol per injection, and the detection limit was 0.1 pmol (SN = 5).

INTRODUCTION

18-Oxygenated corticosteroids, such as 18-hydroxycortisol, 18-oxocortisol and 18-hydroxycortisone (Fig. 1) are often called hybrid steroids since they have the characteristic structure of glucocorticoids and mineralcorticoids (1). Such cortisol derivatives were first reported by Chu and Urick (1) in the urine of patients with primary aldosteronism, and a number of studies have shown that these compounds are effective discriminators for primary aldosteronism (2-5). Although gas chromatography - mass spectrometry (1), radioimmunoassay (4, 6), enzyme immunoassay (7) and high-performance liquid chromatography (5) have been reported for the measurement of 18-hydroxycortisol, a method for the simultaneous determination of the above three 18-oxygenated corticosteroids in human biological fluids has not been established. To clarify the excretion of these steroids in urine, a highly sensitive quantitation method is required because of the low concentration of such steroids in biological fluids.

We wish to report here the method developed for the quantitation of 18-oxygenated corticosteroids, and its application to the quantitative determination of them in human urine.

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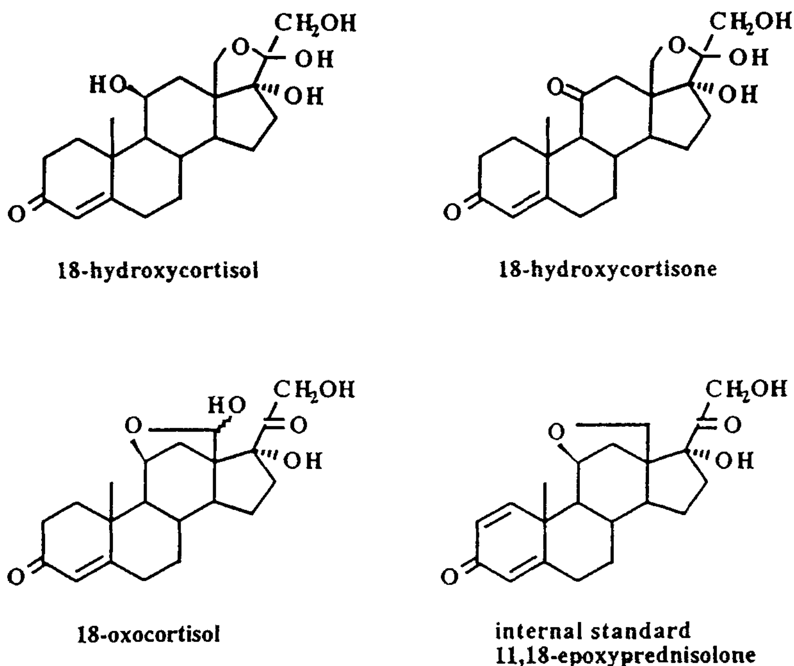

FIGURE 1. Molecular structures of steroids.

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MATERIALS AND METHODS

Materials

18-Hydroxycortisol, 18-hydroxycortisone and 11,18-epoxyprednisolone (11β,18-epoxy-17α,21-dihydroxypregna-1,4-diene-3,20-dione) were chemically synthesized as reported previously(8). 1-Anthroyl nitrile was prepared as reported by Goto *et al.*(9) Bond Elut CN cartriges were obtained from Analytichem International (Haber City, CA, U.S.A). All other reagents were of analytical grade. 18-Oxocortisol

was synthesized as follows: The mixture of 18-hydroxycortisol-17α, 21α-acetonide (480 mg), lead tetraacetate (450 mg) and iodine (300 mg) in dry 1, 2-dichloroethane (20 ml) and cyclohexane (20 ml) was irradiated with a 500 W tungsten lamp for 30 min at room temperature. The reaction mixture was filtered and the filtrate was washed with 5 % sodium thiosulfate, then water, and then dried over sodium sulfate. The crude product was subjected to silica-gel column chromatography eluted with chloroform - methanol (30 : 1, v/v) to give 18-oxocortisol actonide (25 %) as a hemiacetal form, mp 215 - 218 °C. The acetonide was hydrolyzed in the usual manner to give 18-oxocortisol. mp 198 - 200 °C. Analysis Calculated for $C_{21}H_{28}O_6$: C, 67.00; H, 7.50. Found : C, 67.16; H, 7.35.

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Urine samples

Urine samples (24 h) were collected without preservatives from patients with primary aldosteronism before or after adrenalectomy, and from healthy subjects as a control were collected. All specimens were stored at -25°C until analyzed.

Apparatus and chromatographic conditions

The HPLC apparatus consisted of a LC-6A system equipped with RF-535 spectrofluorometer (Shimadzu, Kyoto, Japan). The chromatographic separation was performed on a μBondasphere phenyl (150 x 3.9 mm i.d., 5 μm, Waters, Milford, MA, USA) column using gradient elution mode at a flow rate of 1.0 ml/min. The column temperature was ambient. The mobile phases

were 10 mM ammonium acetate - methanol (50 : 50, v/v; mobile phase A) and acetonitrile (mobile phase B). The gradient program was as follows; an isocratic elution with 15 % of mobile phase B for 10 min, then a linear gradient to 30 % of mobile phase B over a period of 30 min. The fluorescence intensity of the eluent was monitored with an emission wavelength at 470 nm and an excitation wavelength at 370 nm.

Sample extraction

To a urine sample (100 - 500 µl), an appropriate amount of 11,18-epoxyprednisolone was added as an internal standard, and the whole was extracted twice by shaking for 3 min with 5 ml of diethyl ether - dichloromethane (3 : 2, v/v). After being centrifuged at 800 x g for 10 min, the organic layer (ca. 8 ml) was evaporated to dryness below 40°C under reduced pressure. The residue was subjected to derivatization with 1-anthroyl nitrile.

Derivatization procedure

The sample extract was mixed with 100 µl of 0.4 % 1-anthroyl nitrile in acetonitrile and 100 µl of 0.4 % quinuclidine in acetonitrile (10). The mixture was allowed to stand at room temperature for 10 min, and then the excess reagent was decomposed by the addition of 25 µl of water. After standing at room temperature for 5 min, the mixture was neutralized with 25 µl of 1 % acetic acid, and the solvent was evaporated to dryness below 40°C under reduced pressure. The residue was dissolved in a small amount of benzene and loaded on a Bond Elut CN

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cartridge equilibrated with hexane for elimination of interfering peaks on the chromatogram. The cartridge was washed with 6 ml of ethyl acetate - hexane (1 : 15, v/v), and the resultant fluorescent derivatives were eluted with 6 ml of ethyl acetate - hexane (3 : 2, v/v). After evaporation of the solvent under reduced pressure, the residue was dissolved in methanol, and applied to HPLC analysis.

RESULTS AND DISCUSSION

Derivatization of 18-oxygenated corticosteroids with 1-anthroyl nitrile

A highly sensitive derivatization of hydroxysteroids with 1- or 9-anthroyl nitrile for HPLC with fluorescence detection, has been previously developed by Goto *et al.*(9). It was noted that, in the derivatization of cortisol with both reagents, the primary hydroxyl group at C-21 was quantitatively converted to the corresponding esters, but the 11β-secondary and 17α-tertiary hydroxyl groups underwent no reaction. In order to employ these reagents for fluorescence labeling for the precolumn derivatization of 18-hydroxycortisol, 18-hydroxycortisone and 18-oxocortisol, the reactivities of both reagents for 18-oxygenated corticosteroids were initially examined according to the method described by Goto *et al*. A marked difference in the reactivity between the two reagents was observed; the reactivity of 9-anthroyl nitrile for the three 18-oxygenated steroids was less than 10 % of that of 1-anthroyl nitrile, despite the modification of

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conditions such as reaction time, reaction temperature and kinds of base catalysts. These results may be due to steric interaction between the hydrogens at the 1- and 8- positions of the 9-anthroyl nitrile and the intramolecular hemiacetal system of the 18-oxygenated steroids. Therefore, 1-anthroyl nitrile was selected as the derivatization reagent for the development of a sensitive HPLC method.

The quantitative formation of the anthroyl derivatives of all the steroids with 1-anthroyl nitrile was easily achieved at room temperature within 10 min in the presence of quinuclidine as a catalyst. Unfavorable side reactions and the dehydration of the hydroxyl groups through the derivatization procedure were not observed. All the fluorescent derivatives, the detector responses of which were of the same degree, showed a single peak on the chromatogram, showing that derivatization with 1-anthroyl nitrile occured in the C-21 primary hydroxyl group.

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Separation of 1-anthroyl derivatives

Various combinations of column, pH of buffer solution and organic solvent were examined in order to obtain a complete separation of the anthroyl derivatives of the three 18-oxygenated corticostroids and 11,18-epoxyprednisolone. 11,18-Epoxyprednisolone, which is not present in human biological fluids and gaves the fluorescent derivative in the same yield, was used as an internal standard (5). When the C18 columns were tested with mixtures of ammonium acetate and organic solvents such as methanol and

acetonitrile as mobile phase, the peaks due to 18-oxocortisol and 18-hydroxycortisol could not to be resolved successfully. The use of the C8 and phenyl columns gave a good separation of the anthroyl derivatives of the four 18-oxygenated steroids, however, the peak widths obtained on the C8 columns were broader than those obtained on the phenyl columns. Therefore, the phenyl columns were selected for the method. The elution with mixtures containing both methanol and acetonitrile provided more sufficient separation, and sharp and symmetrical peaks, compared with that with methanol or acetonitrile alone as a organic solvent, when combinations of ammonium acetate and organic solvents were tested on phenyl columns. The pH and the concentration of ammonium acetate of mobile phase had little effect on the separation and the peak shape of each steroid. From the results, the best separation of the anthroyl derivatives was achieved on a μBondasphere phenyl column by gradient elution with mixtures of 10 mM ammonium acetate - methanol and acetonitrile within 35 min. A typical chromatogram obtained with authentic specimens of 18-oxygenated corticosteroids and the internal standard is shown in Fig. 2. These derivatives were completely separated from each other without noticeable peak asymmetry. The retention time of the 18-oxygenated corticosteroids increased in the following order : 18-oxocortisol < 18-hydroxycortisol < 18-hydroxycortisone < 11,18-epoxyprednisolone. Reproducible retention times were obtained for all the 18-oxygenated corticosteroids under the present HPLC conditions.

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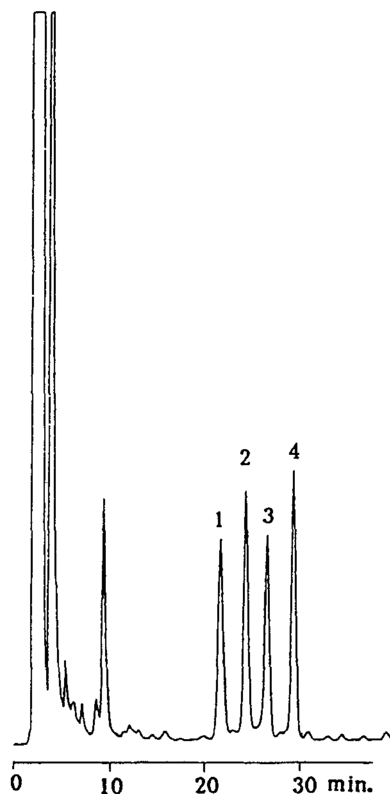

FIGURE 2. Chromatogram of standard 18-oxygenated steroids. 1: 18-oxocortisol 2: 18-hydroxycortisol 3: 18-hydroxycortisone 4: 11,18-epoxyprednisolone (internal standard).

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The calibration curves were constructed by plotting the relative peak area of each 18-oxygenated corticosteroid to the internal standard against the amounts of the corresponding 18-oxygenated corticosteroid. A good linear relationship to each 18-oxygenated steroid was obtained over the range of

0.5 - 25 pmol with the linear correlation coefficients of more than 0.999 for all the 18-oxygenated corticosteroids. The coefficients of variation for the measurement of 10 pmol of each standard 18-oxygenated steroid was less than 3 %. The detection limits of all the 18-oxygenated corticosteroids was estimated to be 0.1 pmol (signal-to-noise ratio = 5). The proposed HPLC method was at least 100 times more sensitive than the reported HPLC method using ultraviolet detection (5).

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Determination of 18-oxygenated corticosteroids in human urine

Extraction of 18-oxygenated corticosteroids from urine samples was performed with a mixture of diethyl ether - dichloromethane (3 : 2, v/v) in the usual manner. Maximum and consistent recoveries of all the 18-oxygenated corticosteroids were achieved by extracting twice with 10 ml of the organic solvent. Furthermore, to eliminate interfering peaks appearing on the chromatogram, extraction of the 1-anthroyl derivatives before HPLC analysis was tested by several cartridge columns such as Bond Elut and Sep-Pak series with mixtures of organic solvents. The interferences could be removed by a Bond Elut CN with mixture of ethyl acetate - hexane as an eluent.

The recovery rates through the extraction and derivatization as described in "MATERIALS AND METHODS" were tested by adding known amounts of 18-oxygenated corticosteroids (20 ng and 100 ng) to steroid-free urine (0.5 ml). As listed in Table I, all of the 18-oxygenated corticosteroids were

TABLE 1

Recoveries of 18-Oxygenated Corticosteroids

	added amounts* (ng)	recoveries (%)
18-Oxocortisol	22.3	87.6 ± 1.8**
	111.6	86.3 ± 1.2
18-Hydroxycortisol	20.5	89.1 ± 1.8
	102.7	91.1 ± 1.5
18-Hydroxycortisone	22.2	89.6 ± 1.8
	111.2	91.6 ± 1.9

* added amounts of each standard sample into 0.5 ml of steroid free urine, internal standard (19.5 ng).

** Mean ± S.E.M. (n = 6)

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recovered at a rate of more than 86 %, and the within-day and day-to-day coefficients of variation were less than 5 % (n=6) and 7 % (n=5), respectively.

Figures 3a and 3b show typical chromatograms obtained from the urine of a patient with primary aldosteronism and from the same patient after adrenalectomy, respectively. The peaks due to 18-hydroxycortisol, 18-hydroxycortisone and 18-oxo-cortisol in Fig. 2a were identified, respectively, on the basis of their retention times. The predominant 18-oxygenated corticosteroid was 18-hydroxycortisol as reported previously, accounting for more than 70 % of the total amounts of the three 18-oxygenated corticosteroids. Of particular interest was the

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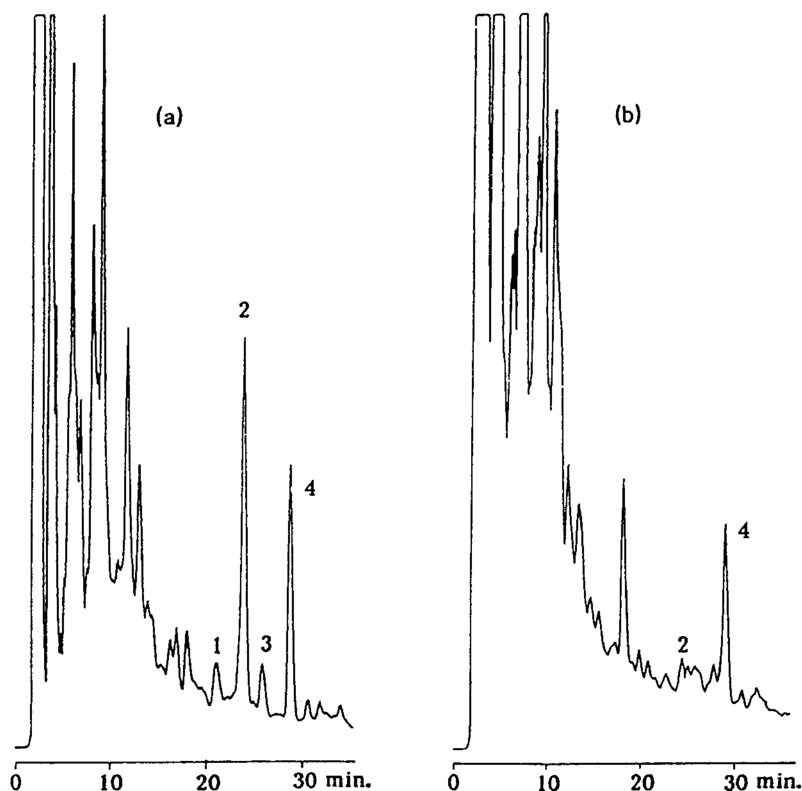

FIGURE 3. Chromatograms of urine samples from a patient with primary aldosteronism, before (a) and after adrenalectomy (b). 1:18-oxocortisol 2:18-hydroxycortisol 3:18-hydroxycortisone 4:internal standard.

identification of 18-hydroxycortisone in the urine of patients with primary aldosteronism in addition to 18-hydroxy and 18-oxocortisol. The concentrations of 18-hydroxycortisol, 18-hydroxycortisone and 18-oxo-cortisol in the urine of the patients with primary aldosteronism were estimated to be 350-2300, 45-100,

30-75 nmol / l, n=5), respectively. On the other hand, all the 18-oxygenated corticosteroid levels after adrenalectomy showed significant decreases to the level of the control in comparison with that before adrenalectomy, accounting for less than 100 nmol/l for 18-hydroxycortisol,and 4 nmol/l for 18-oxocortisol and 18-hydroxycortisone (Fig. 3b).

This study has provided an HPLC method with fluorescence detection for the simultaneous determination of 18-hydroxycortisol, 18-hydroxy-cortisone and 18-oxocortisol in human urine. Further application of this method is under way to clarify the physiological and pathophysiological roles of these 18-oxygenated corticosteroids in relation to primary aldosteronism.

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