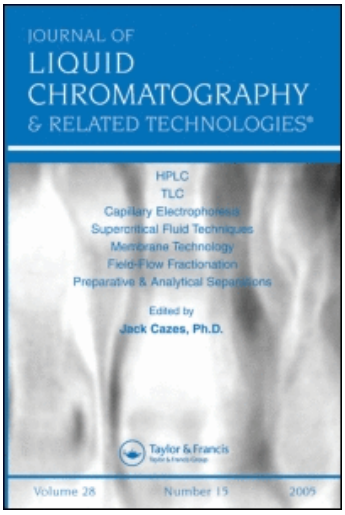

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STUDIES ON STEROIDS CLXXXII. DETERMINATION OF 6β-HYDROXYCORTISOL IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

A new sensitive method for the determination of 6β-hydroxycortisol in urine by high-performance liquid chromatography with fluorescence detection has been developed. 6β-Hydroxycortisol and its C-6 epimer (internal standard) were transformed quantitatively into the 21-(9-anthroyl) derivatives when treated with 9-anthroyl nitrile in the presence of triethylamine in acetonitrile. The resulting fluorescent esters were readily separated on a Cosmosil 5SL column using ethyl acetate/hexane (2:1) as a mobile phase with a detection limit of 25 pg. The efficient clean-up was achieved by the combined use of Bond Elut and Clin Elut cartridges. The present method is applicable to the quantification of 6β-hydroxycortiol in human urine with satisfactory accuracy and precision.

INTRODUCTION

6β-Hydroxycortisol is a polar unconjugated metabolite of cortisol in human urine (1). The urinary excretion is significantly elevated in the newborn, pregnancy and cancer as well as in humans who administered drugs such as diphenylhydantoin, phenobarbital and o,p'-DDD. Several methods which involve radioimmunoassay (2,3) and enzyme immunoassay (4), have already been

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developed for the determination of 6β-hydroxycortisol in biological fluids. For this purpose high-performance liquid chromatography (HPLC) has also been employed (5). The procedure, however, is still unsatisfactory with respects to the sensitivity and simplicity. The present paper deals with a new method for the determination of 6β-hydroxycortisol in urine by HPLC with fluorescence detection using 9-anthroyl nitrile as a pre-column labeling reagent.

MATERIALS AND METHODS

Apparatus

The apparatus used was a Waters 6000A solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) equipped with a Hitachi 650-10 LC fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan) (excitation wavelength 360 nm; emission wavelength 460 nm). The test samples were applied to the chromatograph by a U6K sample loop injector (Waters Assoc.) with an effective volume of 2 ml. The Cosmosil 5SL (5 μm) (150 mm x 4 mm i.d.) (Nakarai Kagaku Co., Kyoto, Japan) and LiChrosorb RP-18 (5 μm) (125 mm x 4 mm i.d.) (E. Merck AG, Darmstadt, F.R. Germany) columns were used under ambient conditions.

Chemicals and Reagents

6β-Hydroxycortisol and 6α-hydroxycortisol were prepared in these laboratories by the known methods and purified by repeated recrystallization. 9-Anthroyl nitrile was synthesized in the manner described in the previous paper (6). All other chemicals employed were of analytical-reagent grade. Solvents were purified by distillation prior to use. The Clin Elut CE1000M and Bond Elut C_{18} 607101 cartridges were purchased from Analytichem International, Inc. (Harbor City, CA, U.S.A.). A Bond Elut cartridge was washed successively with ethanol (10 ml) and water (10 ml) before use.

Derivatization with 9-Anthroyl Nitrile

To a test sample was added a solution of 9-anthronyl nitrile (100 μg) in 5% triethylamine/acetonitrile (100 μl), and the whole was kept at room temperature for 1 hr. After removal of the solvent with an aid of nitrogen gas stream, the residue was redissolved in acetone (100 μl), mixed with hexane (2 ml), and heated at 70-80°C for a few minutes to remove acetone. The solution was applied to a Clin Elut cartridge which was previously impregnated with 0.1 N NaOH (0.3 ml). The cartridge was washed with hexane (4 ml) to remove the excess reagent, and the desired fraction was eluted with dichloromethane (4 ml). The eluate obtained was dried in vacuo and redissolved in ethyl acetate (0.5 ml). A 10 μl aliquot of the solution was injected into the chromatograph.

Procedure for the Determination of 6β-Hydroxycortisol in Urine

A urine sample (0.5 ml) was pipetted into a test tube containing a known amount of 6α-hydroxycortisol (ca. 50 ng) in phosphate buffer (pH 7) (1 ml). The mixture was applied to a Bond Elut cartridge impregnated with phosphate buffer (pH 7) (1 ml). The cartridge was washed successively with water (3 ml) and 5% ethanol (3 ml), and the desired fraction was eluted with 70% ethanol (2 ml). The eluate obtained was dried in vacuo and redissolved in ethyl acetate (0.3 ml). The solution was applied to a Clin Elut cartridge impregnated with 15% ammonium carbonate (0.3 ml). The desired fraction was eluted with ethyl acetate (4 ml). The eluate obtained was subjected to pre-column derivatization followed by HPLC in the manner described above.

RESULTS AND DISCUSSION

In the previous papers we reported the reactivities of 9-anthroyl nitrile and 1-anthroyl nitrile toward various hydroxyl groups on the steroid nucleus (6,7). The former reagent is capa-

ble of reacting selectively with the primary hydroxyl groups at C-21 in cortisol and cortisone in triethylamine/acetonitrile. Therefore, 9-anthroyl nitrile appeared to be also promising for derivatization of 6β-hydroxycortisol. Initially, the suitable condition for acylation of 6β-hydroxycortisol with 9-anthroyl nitrile in triethylamine/acetonitrile was investigated (Figure 1). The excess of the reagent which would disturb the chromatogram, could be efficiently removed by passing the reaction mixture through a Clin Elut cartridge. The effects of concentration of triethylamine in acetonitrile and reaction time on the formation of the ester were examined. The reaction rate was significantly influenced by the concentration of triethylamine where the maximum yield was obtained with 5% triethylamine in acetonitrile. In addition, the formation of the ester increased with the reaction time up to 60 min and reached a plateau as illustrated in Figure 2.

The HPLC separation of derivatized 6β-hydroxycortisol and 6α-hydroxycortisol was more satisfactorily achieved on a normal phase column (Cosmosil 5SL) using ethyl acetate/hexane (2:1) as a mobile phase rather than on a reversed phase column (LiChrosorb RP-18).

A calibration curve was constructed by plotting the ratio of peak area of 6β-hydroxycortisol to that of 6α-hydroxycortisol (internal standard) against the amount of the former where a linear response was observed in the range of 50 to 400 ng of 6β-hydroxycortisol.

The combined use of Bond Elut and Clin Elut cartridges proved to be effective for the clean-up of urinary 6β-hydroxycortisol. As illustrated in Figure 3, the desired 6β-hydroxycortisol was recovered at the rate of 95% when eluted with 2 ml of 70% ethanol after washing with 5% ethanol. The typical chromatograms of 6β-hydroxycortisol together with 6α-hydroxycortisol are shown in Figure 4. A known amount of 6β-hydroxy-

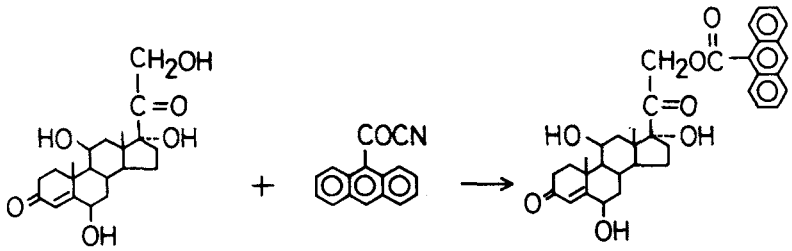

FIGURE 1 Reaction of 6β-Hydroxycortisol with 9-Anthroyl Nitrile

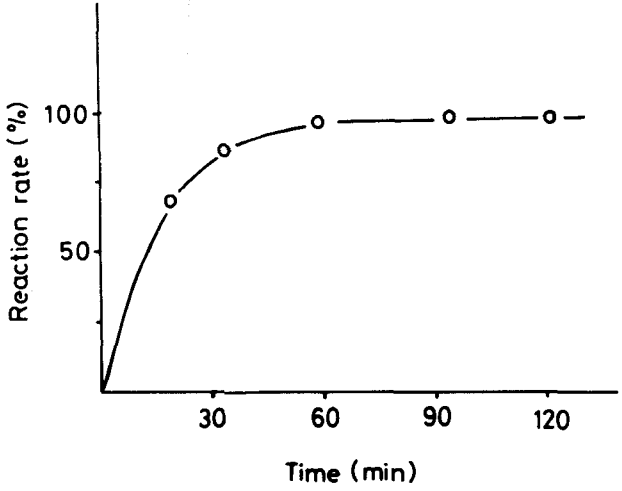

FIGURE 2 Time Course for Derivatization of 6β-Hydroxycortisol with 9-Anthroyl Nitrile

cortisol was added to human urine specimens at two levels and their recovery rates were determined by the standard procedure. It is evident from the data in Table I that the satisfactory results are obtainable by the proposed method.

The present method was then applied to the determination of 6β-hydroxycortisol in urine specimens collected from seven male healthy volunteers. As listed in Table II, the urine levels of 6β-hydroxycortisol were observed in the range previously estimated by the enzyme immunoassay method (4).

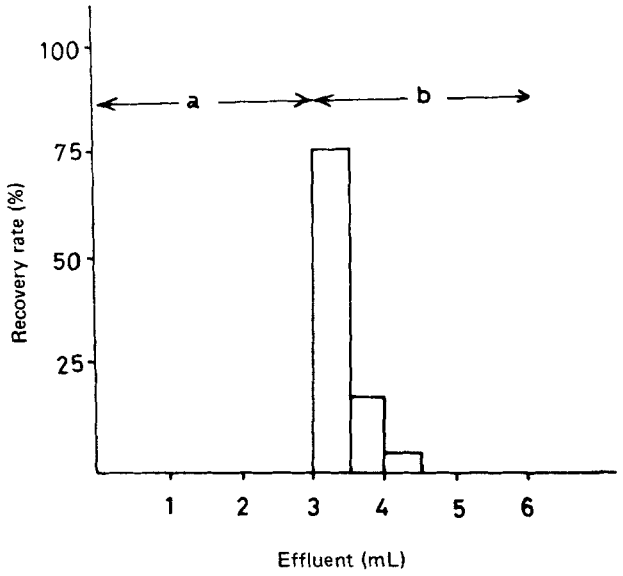

FIGURE 3 Elution Pattern of 6β-Hydroxycortisol on a Bond Elut Cartridge
Eluent: a) 5% ethanol; b) 70% ethanol.

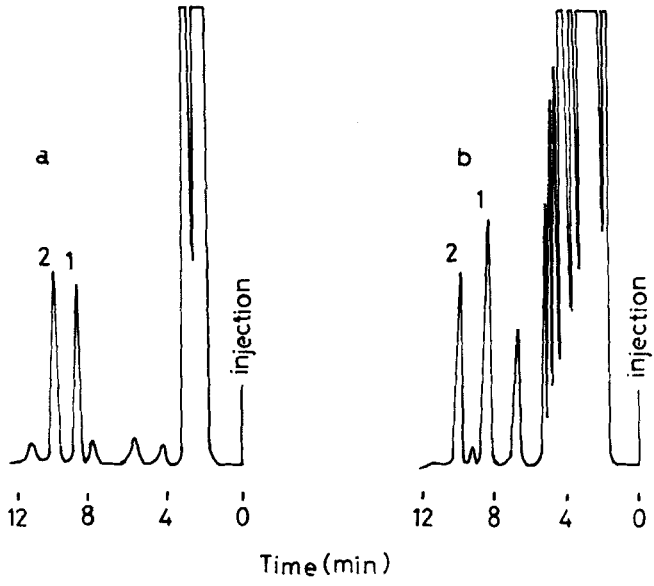

FIGURE 4 Chromatograms of 6β-Hydroxycortisol and 6α-Hydroxycortisol Derivatives formed with 9-Anthroyl Nitrile
a) Standard sample; b) urine sample. 1: 6β-hydroxycortisol, 2: 6α-hydroxycortisol (internal standard).

TABLE I
Recovery of 6β-Hydroxycortisol added to Human Urine

Urine	Added (ng/0.5 ml)	Expected	Found	Recovery (% ± S.D.*)
86	50	136	137	101 ± 1.6
193	100	293	270	92 ± 2.1

* n=8

TABLE II
Urine Levels of 6β-Hydroxycortisol in Healthy Male Volunteers (μg/24 hr)

Subject	A	B	C	D	E	F	G	Mean
	407	254	503	212	278	219	246	303 ± 45

TABLE III
The k' Values and Peak Area Ratios of 6β-Hydroxycortisol in Human Urine to Internal Standard

	A		B		C	
	S	U	S	U	S	U
6β-Hydroxycortisol	0.77	0.77	0.79	0.79	0.84	0.84
6α-Hydroxycortisol	0.80	0.80	0.83	0.83	0.86	0.86
Peak area ratio (6β/6α)	1.32	0.82	-	-	1.32	0.80

S: standard sample, U: urine sample.
Conditions: A) Cosmosil 5SL, ethyl acetate/hexane (2:1), 1 ml/min; B) Cosmosil 5SL, ethyl acetate/chloroform (3:4), 1.5 ml/min; C) LiChrosorb RP-18, methanol/water (3:1), 0.6 ml/min.

The disadvantage of HPLC in structural elucidation, because the information provided is insufficient, has already been pointed out. In order to solve these problems, several methods have already been devised. Previously, we demonstrated an attempt for unequivocal characterization of bile acid sulfates in bile by HPLC using mobile phases of varying pH (8). In this study, a similar approach was done for structural characterization of 6β-hydroxycortisol in urine. The eluate corresponding to the peak on the chromatogram was collected and, after the addition of the derivatized 6α-hydroxycortisol, was subjected to HPLC under three different conditions. As listed in Table III, k' values of 6β-hydroxycortisol in human urine were completely identical with those of the authentic sample. Moreover, the peak area ratio of the two epimers showed the same value under two different HPLC conditions. These results imply that the present method undergoes no interferences with coexisting substances and is favorable for the determination of 6β-hydroxycortisol in urine.

The newly developed method for the determination of 6β-hydroxycortisol in biological fluids is highly sensitive, convenient and may be useful for monitoring the effects of hormones and drugs acting as inducers of microsomal enzymes.

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