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The simultaneous determination of serum cortisol and cortisone by high-performance liquid chromatography with fluorimetric detection

JUNICHI GOTO, FAZEL SHAMSA, NOBUHARU GOTO and TOSHIO NAMBARA*

Pharmaceutical Institute, Tohoku University, Aobayama, Sendai 980, Japan

Abstract: A new method for the simultaneous determination of cortisol and cortisone in serum by high-performance liquid chromatography with fluorimetric detection has been developed. The two steroids were derivatized by treatment with 9-anthroyl nitrile in triethylamine–acetonitrile to give the fluorescent esters through the 21-hydroxyl group. These derivatives were efficiently separated on a straight-phase Cosmosil 5SL column with hexane–ethyl acetate (6:5) as eluent. The detection limit was 20 fmol, using the 9-anthroyl derivative of prednisolone as an internal standard. The use of Sep-Pak C_{18} and Clin-Elut cartridges proved to be efficient for the clean-up of cortisol and cortisone in serum samples.

Keywords: *Cortisol; cortisone; 9-anthroyl nitrile; pre-column derivatization; fluorimetric detection; high-performance liquid chromatography.*

Introduction

The blood level of corticosteroids is widely used as a diagnostic index for pituitary and adrenal function. Several papers have reported the determination of corticosteroids in biological fluids by high-performance liquid chromatography (HPLC) [1–5]. The methods so far reported are, however, of doubtful sensitivity and reliability. In earlier reports the present authors proposed new types of fluorescent derivatization reagent for the alcoholic hydroxyl group, *viz.* 4-dimethylamino-1-naphthoyl nitrile [6], 1- and 9-anthroyl nitriles [7]. The present paper describes the derivatization of cortisol and cortisone with 9-anthroyl nitrile to generate the fluorescent esters through the primary hydroxyl group at C-21, followed by separation and determination by HPLC with fluorimetric detection.

Experimental

High-performance liquid chromatography

The apparatus used for this work was a Waters 6000A solvent delivery system (Waters Assoc., Milford, Ma., U.S.A.), a 650-10 LC fluorescence spectrophotometer with a

* To whom correspondence should be addressed.
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20 μl flow cell (Hitachi Ltd, Tokyo, Japan), used at excitation and emission wavelengths of 360 and 460 nm respectively. Samples were applied to the chromatograph by a U6K loop-valve injector (Waters Assoc.) with an effective volume of 2 ml. The Cosmosil 5SL column (150 × 4 mm i.d.) (Nakarai Kagaku Co., Kyoto, Japan) and LiChrosorb RP-18 column (125 × 4 mm i.d.) (E. Merck, Darmstadt, F.R.G.) were used at ambient temperature.

Materials

Cortisol, cortisone and prednisolone (Sigma Chemical Co., St. Louis, Mo., U.S.A.) were purified by recrystallization prior to use. The reagent 9-anthroyl nitrile was prepared as described previously [7]. All other chemicals employed were of analytical-reagent grade. Solvents were purified by distillation. Clin-Elut cartridges were obtained from Analytichem International (Harbor City, Ca., U.S.A.). Sep-Pak C_{18} cartridges (Waters Assoc.) were washed thoroughly with ethanol (10 ml) and water (10 ml) before use.

Sample preparation

A serum sample (100 μl) was mixed with prednisolone (12 ng) as internal standard, diluted with 0.5 M NaH_2PO_4 (1 ml), and then passed through a Sep-Pak C_{18} cartridge. After successive washings with water (2 ml) and 10% ethanol (3 ml), the desired fraction was obtained by elution with ethanol (3 ml). To the dried eluate was added 9-anthroyl nitrile (100 μg) in 10% v/v triethylamine–acetonitrile (100 μl). The solution was allowed to stand at room temperature for 30 min. After evaporation by a stream of nitrogen, the residue was redissolved in acetone (100 μl), diluted with hexane (2 ml) and heated at 70–80°C to remove the acetone. The solution was applied to a Clin-Elut cartridge which was impregnated with 0.1 M NaOH (300 μl). The cartridge was washed with hexane (4 ml) to remove excess reagent and the desired fraction was obtained by elution with dichloromethane (4 ml). After evaporation of the solvent, the residue obtained was redissolved in ethyl acetate (100 μl), and a 10 μl aliquot of the solution was injected onto the chromatograph.

Recovery test for cortisol and cortisone added to human serum

Spiked samples were prepared by adding 10 ng each of cortisol and cortisone to human serum (100 μl). A serum sample was diluted with 0.5 M NaH_2PO_4 (1 ml) and subjected successively to clean-up with a Sep-Pak C_{18} cartridge, derivatization with 9-anthroyl nitrile and HPLC as described above.

Results and Discussion

Derivatization of cortisol and cortisone with 9-anthroyl nitrile

In previous work the reactivities of 9-anthroyl nitrile toward the hydroxyl groups of several steroids in triethylamine–acetonitrile were investigated and it was shown that cortisol underwent facile acylation through the 21-hydroxyl group [7]. Initially, suitable conditions for quantitative derivatization of corticosteroids with this reagent were examined. Cortisol or cortisone (50 ng) and 9-anthroyl nitrile (100 μg) were dissolved in triethylamine–acetonitrile (100 μl) at various concentrations and allowed to stand either at room temperature or at 60°C. To the resulting solution was added the 9-anthroyl derivative of prednisolone (50 ng) as an internal standard and an aliquot was applied to HPLC. As illustrated in Fig. 1, the extent of reaction was significantly influenced by the

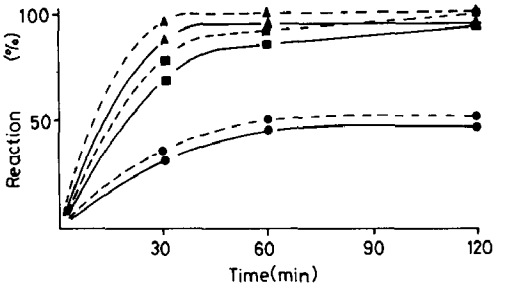

Figure 1
Time course of reaction for derivatization of cortisol with 9-anthroyl nitrile. Concentration of triethylamine in acetonitrile: ●0.1% v/v; ■4% v/v; ▲10% v/v; —— 60°C, - - - room temperature.

concentration of triethylamine. When cortisol was treated with the reagent in 10% v/v triethylamine–acetonitrile at room temperature, the yield of the ester increased to reach a plateau at 30 min. Quantitative formation of the ester was attained with 10% v/v triethylamine, whereas a lower yield was observed with 0.1% and 4% v/v triethylamine. When derivatization was carried out at 60°C, the yield was again reduced. Corticosteroids were therefore treated with 9-anthroyl nitrile (100 μg) in 10% v/v triethylamine–acetonitrile (100 μl) at room temperature to form the esters. Under these conditions acylation proceeded selectively toward the 21-hydroxyl group and did not favour the 11β- and 17α-hydroxyl groups.

HPLC of 9-anthroyl derivatives of cortisol and cortisone

HPLC separation of the 9-anthroyl derivatives of cortisol, cortisone and prednisolone was performed using the straight-phase Cosmosil 5SL column and the LiChrosorb RP-18 reversed-phase column. Cortisol and cortisone were resolved on the reversed-phase column with acetonitrile–water or methanol–water. Under these conditions, however, prednisolone displayed a very similar retention time to that of cortisol. The Cosmosil 5SL column with a solvent system of hexane–ethyl acetate (6:5) was therefore chosen as being more suitable for the separation of the three 9-anthroyl derivatives by HPLC. A typical chromatogram is illustrated in Fig. 2.

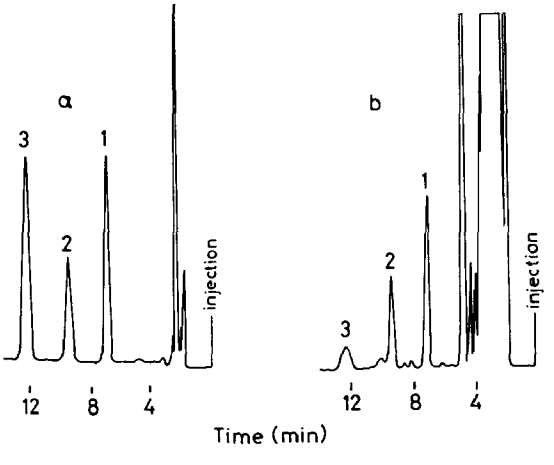

Figure 2
Chromatograms of cortisol and cortisone derivatives formed with 9-anthroyl nitrile. (a) Mixed standards; (b) serum sample. 1, Cortisol; 2, prednisolone (internal standard); 3, cortisone.

The 9-anthroyl derivatives were monitored fluorimetrically: a detection limit of 20 fmol was observed (signal-to-noise ratio = 5). A calibration graph of the peak area ratio of cortisol and cortisone to that of the internal standard prednisolone was linear in the range 1–50 ng, the regression equations being $y = 0.0992\,x + 0.0908$ for cortisol ($n = 10$) and $y = 0.0984\,x - 0.0072$ for cortisone ($n = 10$).

Clean-up of cortisol and cortisone in serum

The recovery of cortisol and cortisone in blood is significantly influenced by the clean-up procedure employed. For the separation of corticosteroids from biological fluids, extraction with an organic solvent such as dichloromethane is widely used. This procedure, however, proved not to be applicable to cortisol and cortisone in serum because the recovery and reproducibility were unsatisfactory and interfering peaks appeared on the chromatogram. In previous work the utility of a Sep-Pak C_{18} cartridge for the clean-up of drugs [8, 9] and bile acids [10] in biological fluids was demonstrated. This cartridge was therefore examined for the purification of serum cortisol and cortisone by dissolving 50 ng of each in 0.5 M NaH_2PO_4 and applying the mixture to the cartridge. After successive washing with water and 10% m/v ethanol, the cortisol and cortisone were obtained by elution with ethanol. Each 0.5 ml fraction of the eluting solvent was collected, prednisolone (10 ng) added and then subjected to derivatization with 9-anthroyl nitrile, followed by separation and determination by HPLC. It was found that both cortisol and cortisone were quantitatively eluted in the first 2 ml of the eluting solvent. This procedure was effective for the removal of endogenous substances in serum, which could potentially interfere with the derivatization reaction and the chromatogram.

Elimination of excess reagent in the reaction mixture was achieved using a Clin-Elut cartridge (a newly developed liquid–liquid partition type), since 9-anthroyl nitrile is less polar than the derivatized corticosteroids. The reaction mixture was applied to the cartridge, which was then impregnated with 0.1 M NaOH (300 μl). The excess reagent was easily removed by washing with hexane, the derivatized corticosteroids being quantitatively recovered by subsequent elution with dichloromethane. The use of this efficient clean-up procedure for HPLC of serum cortisol and cortisone yielded an excellent chromatogram without any interfering peaks (Fig. 2).

Determination of cortisol and cortisone in serum

The applicability of the present method to the determination of serum corticosteroids was examined by recovery experiments on human serum spiked with known amounts of cortisol and cortisone. As shown in Table 1, the recovery of cortisol and cortisone was

Table 1
Recovery of cortisol and cortisone added to human serum

	Serum (ng/ml)	Added (ng/ml)	Expected (ng/ml)	Found (ng/ml)	Recovery (% ± S.D.; $n = 8$)
Cortisol	129	100	229	225	98.1 ± 1.7
Cortisone	30	100	130	117	90.0 ± 3.3

90–98% with a standard deviation of 1.7–3.3%. The cortisol and cortisone levels of blood samples taken from ten healthy male volunteers were measured (Table 2). The ratio of serum cortisol to cortisone in the adult determined by this method was found to be less than that reported in the literature [11].

Table 2
Serum levels of cortisol and cortisone in healthy male volunteers

Subject	Cortisol (ng/ml)	Cortisone (ng/ml)	Cortisol/cortisone
A	96	45	2.1
B	109	58	1.9
C	85	41	2.1
D	101	31	3.3
E	75	45	1.7
F	78	38	2.1
G	73	27	2.7
H	91	28	3.3
I	146	52	2.8
J	99	49	2.0
Mean (± S.D.)	95 ± 21	41 ± 10	2.4 ± 0.6

Characterization of cortisol and cortisone in serum

It has been pointed out that HPLC is of limited value for structural elucidation, since the qualitative information provided is limited to the retention value. In a previous study the bile acid 3-sulphates in human bile were unequivocally characterized by HPLC, based upon their chromatographic behaviour at different pH values [12]. A similar approach was employed to characterize the peaks assignable to cortisol and cortisone. The eluate corresponding to each of the two peaks on the chromatogram was collected, combined with an internal standard and subjected to HPLC using the straight-phase and reversed-phase columns. It is evident from the data in Table 3 that the relative phase

Table 3
k' values and peak area ratios of cortisol and cortisone in human serum relative to internal standards

	k' value			Peak area ratio		
	A	B	C	A	B	C
Cortisol[a]	0.49	0.85	0.85			
Serum sample	0.49	0.85	0.85	0.58	0.59	0.59
Cortisone[b]	2.00	1.18	1.17			
Serum sample	2.00	1.18	1.17	0.59	0.57	0.60

Conditions: (A) Cosmosil 5SL, hexane–ethyl acetate (6:5), 1 ml/min; (B) LiChrosorb RP-18, methanol–water (3:1), 1 ml/min; (C) LiChrosorb RP-18, acetonitrile–water (24:15), 1 ml/min. Internal standard: a, cortisone; b, cortisol.

capacity ratios (k') of cortisol and cortisone in serum were identical with those of the respective authentic samples. Moreover, the peak area ratios of cortisol and cortisone to the internal standard yielded almost identical values for the three different systems. These results strongly suggest that the present method is not subject to interference by endogenous substances and is suited to the characterization and determination of cortisol and cortisone in biological fluids.

Although in the adult the amount of cortisone in serum is much less than that of cortisol, the situation is reversed in the human placenta, foetal plasma and amniotic fluid, where the amount of cortisone exceeds that of cortisol. Rapid interconversion of these steroids by 11β-hydroxysteroid dehydrogenase is of great importance in the foetus [11]. The HPLC methods previously reported for the determination of corticosteroids in biological fluids are not sufficiently sensitive, since they rely on u.v.-detection. Recently a new method for quantitation of cortisol by HPLC with fluorimetric detection using pre-column labelling with dansylhydrazine has been developed [3]. This procedure is, however, still insufficiently sensitive for the determination of cortisone in serum. The availability of the proposed method for the simultaneous quantitation of cortisol and cortisone in serum, with satisfactory reliability and sensitivity, may permit more precise information on the important roles of corticosteroids in endocrine regulation to be acquired. In addition, the use of fluorescent acyl nitriles for the HPLC of steroids and other physiologically active substances in biological fluids should be a fertile field for further investigation.

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