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Simultaneous determination of cortisol and cortisone in urine by reversed-phase high-performance liquid chromatography Clinical and doping control applications

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Abstract

A reversed-phase high-performance liquid chromatography (HPLC) method for the simultaneous determination of cortisol and cortisone in human urine samples using methylprednisolone as the internal standard is described. The method involves the systematic use of isocratic mobile phases of water and methanol, acetonitrile or tetrahydrofuran and a reversed-phase Hypersil C_{18} column. A water–acetonitrile mixture used as the mobile phase proved to be the most adequate one for analyzing urine samples purified by solvent extraction. The proposed method is sensitive, reproducible and selective. It was applied to the determination of cortisol and cortisone in several human urine samples: healthy subjects, sportsmen before and/or after stress for doping control purposes, and patients with Cushing's syndrome.

1. Introduction

Cortisol (F) and cortisone (E) are steroids (structures in Fig. 1) needed in several metabolic processes and in adaptation to stress (an increase of F and E is involved when an emotion occurs, e.g. during sport training or competition) [1,2]. Only F is produced and secreted by the adrenal gland, adrenal production of E in vivo being negligibly low. The reversible conversion of F to E by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD] is a major step in the metabolism of F mainly in the liver, kidney and lung [2,3]. Moreover, they are involved in the inhibition of allergic and inflammatory reactions.

Urinary free F excretion has been shown to be probably the most reliable single index of adrenocortical secretion and is generally accepted as being a measure of the free fraction in plasma

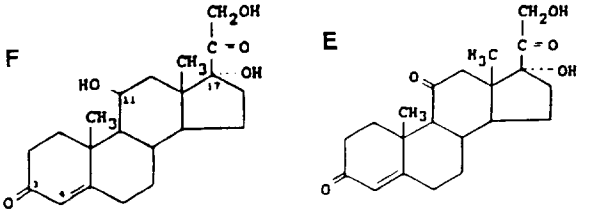

Fig. 1. Structures of cortisol (F) and cortisone (E).

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[4]. The most frequent cause of Cushing's syndrome is cortisol overproduction by an adrenal tumor. Other causes include an excess in ACTH production as a result of a hypophysary tumor (Cushing's disease). Diagnosis is carried out by urinary analysis of free cortisol and 17-hydroxycorticoids [5]. It is also known that athletes use corticoids to improve their performance. Since 1975, the International Olympic Committee Medical Commission has restricted the use of these compounds to legitimate medical purposes. For this reason, it could be interesting to assess the F/E ratio in the doping test to predict any abnormality of their use. Therefore, the accurate measurement of F and/or E in urine may be used for clinical or doping control purposes.

A literature survey revealed that nearly all the previous analyses have been directed towards the determination of F in different biological fluids, using radioimmunoassay (RIA) methods characterized by a high sensitivity but also by an important lack of selectivity owing to the cross-reactivity of related compounds [6]. HPLC procedures have been tried [4,7–11], but their specificity has not always been sufficient. Other procedures including different studies in biological fluids related to F, E and other steroids by HPLC [12–17], liquid chromatography–mass spectrometry (LC–MS) [17–18] and gas chromatography–mass spectrometry (GC–MS) [19]. However, there are only a few reports on the simultaneous determination of F and E by HPLC [20] and GC–MS [21], probably because clinical laboratories mostly use the cortisol assay. Prior to chromatographic analysis, either liquid–liquid or solid-phase extraction procedures have been used for the analysis of these steroids [22].

In this paper, an optimized method for the simultaneous determination of F and E by HPLC using methylprednisolone (MPL) as the internal standard (I.S.) is reported. After sample preparation, the optimized separation was applied to the simultaneous determination of F and E in urine samples obtained from healthy subjects, patients with Cushing's syndrome and sportsmen (before and/or after stress test).

2. Experimental

2.1. Chemicals and reagents

Cortisone (E) (17,21-dihydroxy-4-pregnene-3,11,20-trione), cortisol (F) (11β,17,21-trihydroxy-4-pregnene-3,20-dione), and methylprednisolone (MPL) (6-methyl-11β,17,21-trihydroxy-1,4- pregnadiene-3,20-dione) were from Sigma (St. Louis, MO, USA). Disodium hydrogenphosphate dihydrate and anhydrous sodium sulphate of analytical grade from Merck (Darmstadt, Germany). HPLC-grade methanol, acetonitrile, and tetrahydrofuran were from Promochem (Wesel, Germany). Water was purified with a Milli-Q system (Millipore, Molsheim, France). Millipore 0.45-μm nylon filters (Bedford, MA, USA) were also used. All other chemicals were of analytical reagent grade.

2.2. Apparatus

The chromatographic system consisted of the following components, all from LDC Analytical (Riviera Beach, FL, USA): a ConstaMetric 4100 solvent delivery system, a Spectromonitor 5000 photodiode-array detector (DAD) covering the wavelength range 190–360 nm and interfaced to a computer for data acquisition, recorder Model CI 4100 data module. A Rheodyne 20 μl loop injector (Cotati, CA, USA) and a Jones-chromatography block heated series 7960 for thermostating columns in the range 30–60°C (Seagate Technology, Scotts Valley, CA, USA) were used. The reversed-phase bonded-silica Hypersil 5-ODS column (250 × 4.6 mm I.D., 5-μm average particle size) from Phenomenex (Torrance, CA, USA) was used. A vortex-mixer Mixo-Tub-30 from Crison (Barcelona, Spain) and a Visiprep vacuum manifold system from Supelco (Bellefonte, PA, USA) were used.

2.3. Mobile phase

The mobile phase was prepared by mixing Milli-Q water with methanol, acetonitrile, or tetrahydrofuran in a required volume ratio by

programming the pump. All solvents and mobile phases were previously filtered under vacuum through 0.45-μm Nylon filters and degassed using helium.

2.4. Chromatographic analysis

Once the column had been conditioned with the mobile phase, chromatograms were obtained at 30°C. A methanolic solution containing F, E and MPL (I.S.) or an appropriate mixture of them (4 μg/ml) was injected (20 μl). The flow-rate was 1.0 ml/min, and UV–DAD detection was in the range 190–360 nm. F and E were identified by comparison of the UV spectra of the chromatographic peaks with those of the reference compounds. Subsequent confirmation was performed by co-injection with the reference compounds. Detection was performed at 245 nm (wavelength of absorption maximum).

2.5. Urine collection

(i) Urine samples were collected from healthy subjects (25–30 years old) between 9 and 12 a.m.

(ii) Urine samples (24 h, mean value of urinary excretion = 1.865 l) from subjects with potential Cushing's syndrome were collected in Puerta de Hierro Hospital (Madrid, Spain).

(iii) Urine samples were collected from basketball players (17–20 years old) before (9 samples) and after (9 samples) stress in the Sportive Medical Center (Institute of Physical Education and Sport Sciences, Madrid, Spain). In order to induce stress, a maximal aerobic exercise test protocol was followed. The treadmill incremental test was applied to all of them under the following conditions: warm-up for 2 min at 6 km/h; initial speed 8 km/h; increases of 2 km/h for 2 min; and a 3% slope. After collection, samples were stored at 4°C for further analysis.

2.6. Solvent extraction

Human urine samples (3 ml) spiked with I.S. (MPL) 0.033 μg/ml were processed according to a previously described solvent extraction procedure in which recoveries for F, E, and MPL were evaluated [22]. In brief, NaCl was added to samples to avoid emulsions, and pH was adjusted with Na_2HPO_4. Dichloromethane was added. The mixture was shaken (1 min) and centrifuged for 3 min at 3700 g. The organic phase was removed and dried over anhydrous Na_2SO_4. A 3-ml aliquot was evaporated to dryness, and the residue was reconstituted with 200 μl MeOH, and 20 μl were injected into the HPLC system.

3. Results and discussion

3.1. Separation of cortisol and cortisone

The calculated retention factors, k, and the resolution for cortisol and cortisone are shown in Table 1. As can be observed, the mobile phase containing water–methanol gives the best results, and the elution order for F and E changed when water–acetonitrile was used.

3.2. Calibration

Standards containing mixtures of F, E, and MPL (I.S.) were prepared at five different concentrations in the range 2.0–10.0 μg/ml using 5.0 μg/ml MPL. These solutions were separated with a mobile phase composed of water–acetonitrile (70:30, v/v), a flow-rate of 1.0 ml/min, a Hypersil column (30°C), and UV–DAD detection at 245 nm. The results were analyzed by

Table 1
Retention factors, k, for F, E, and MPL (I.S.) and resolution values, R_s, for F and E

Mobile phase	Retention factors, k			R_s
	E	F	MPL	
A	2.11	2.78	5.04	2.0
B	4.11	3.63	6.66	1.4
C	3.35	4.08	6.61	1.8

Mobile phases: A, water–methanol (42:58, v/v); B, water–acetonitrile (70:30, v/v) and C, water–tetrahydrofuran (75:25, v/v).

linear regression analysis. By plotting the ratio of corticoid peak area to MPL (I.S.) (*PAR*) against the concentration (x) of each corticosteroid, the calibration equations, $PAR = A + Bx$ (μg/ml), were obtained. In Table 2 parameters A (intercept), B (slope) and r (regression coefficient) are shown. In all cases the intercepts were not significantly different from zero.

3.3. Precision, limits of detection (L.D.) and selectivity

The precision of the chromatographic method was examined by analyzing ten different standard solutions of F and E containing 5 μg/ml each using the calibration graphs. The R.S.D. for F and E and the limits of detection (L.D.) obtained for a signal/noise ($S/N = 3$) are shown in Table 2. Potential interference with the following corticoids was studied: prednisone, prednisolone, corticosterone, deoxycorticosterone, 11α-hydroxyprogesterone, fluorocortisone (FL), fluorocortisone acetate, methylprednisolone, triamcinoloneacetonide, deflazacort (11β,16β)-21-acetyloxy-11-hydroxy-2′-methyl-5′H-pregna-1,4-diene-[17,16-d]oxazole-3,20-dione and 21-OH-deflazacort (11β,16β)-21-hydroxy-11-hydroxy-2′-methyl-5′H-pregna-1,4-diene-[17,16-d]oxazole-3,20-dione. Only FL was found to interfere because it co-eluted with F.

3.4. Urine sample analyses

To determine urinary cortisol and cortisone levels, sample preparation was required due to the occurrence of interfering compounds or to the low levels of the analytes in the samples. In previous papers [22,23] solvent and solid-phase extraction procedures for urine samples containing corticoids by exhaustive optimization of the main extraction variables have been described. These procedures include F, E, and MPL over a wide range of concentrations. Solvent extraction recoveries, (%E) ± R.S.D., found for F, E, and MPL added to urine samples were 98.7 ± 0.5, 108 ± 5.6 and 94.4 ± 5.2, respectively. Thus, the optimized mobile phases were used with these samples under solvent extraction conditions. Typical chromatograms are shown in Fig. 2. A mobile phase containing water–acetonitrile (70:30, v/v), was finally chosen because it provided the best selectivity and was adequate for quantitative analysis. Detection and identification of F and E based on retention times and UV spectra were carried out [24]. The UV spectrum of each peak in the chromatogram was stored and subsequently compared with that of the corresponding standard. The instrument can provide a contour plot, showing the relationship between absorbance, wavelength, and time. This can often be used for the detection and identification of otherwise unsuspected impurities in the sample. Impurities were investigated further by displaying the spectra obtained at different points across the peak. The spectra were normalized and overlaid. If the peak is not chromatographically pure then the spectra will not match properly. As urinary endogenous compounds can present similar spectra to those of F and E, for investigating peak purity the second derivatives of the spectra and absorbance ratios (A_{275}/A_{245}) across the peak were also obtained. When interferences occur, a change in the mobile phase composition is recommended.

Table 2
Linear regression equations ($PAR = A + Bx$) for F and E

Compound	A	B	r	D.L. (ng)	R.S.D. (%)
F	−0.030 ± 0.044	0.211 ± 0.008	0.999	0.051	3.1
E	−0.006 ± 0.004	0.193 ± 0.008	0.998	0.055	2.4

PAR is the peak area ratio to MPL (I.S.) 5 μg/ml, x = μg/ml F or E; r = correlation coefficient; D.L. = detection limit.

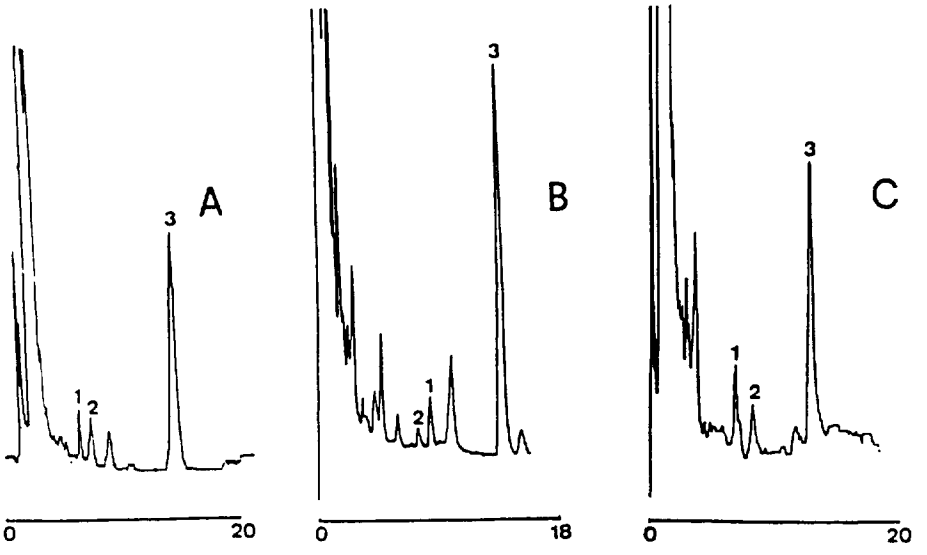

Fig. 2. Chromatograms obtained from human urine samples after solvent extraction. Mobile phase: water–methanol (42:58, v/v) for A, water–acetonitrile (70:30, v/v) for B, and water–tetrahydrofuran (75:25, v/v) for C. Peaks: 1 = cortisone, 2 = cortisol and 3 = methylprednisolone (I.S.).

3.5. Applications

The sportsmen samples were studied in three different ways by taking into account:

(i) The total available samples both before and after stress (BAS). From these results useful information can be gained because the doping test can be performed during training time or after competition.

(ii) The pre- and post-stress samples (PRE and POST) were also considered separately to obtain data on the effect of stress on basketball players.

(iii) Samples from three basketball players were selected to obtain individual information about their urinary excretion.

In Table 3 the urinary F, E, and F/E data found for CUS, HS and BAS samples are summarized, along with some statistical parameters of interest. In Table 4 are shown the F, E, and F/E data (pre- and post-stress) of 3 basketball players as a representative example of individual excretion, and the mean values of pre- and post-stress samples ($n = 9$). Fig. 3 shows the mean values of F, E, and F/E ratios of the following samples: BAS (along with separate pre- and post-stress data), HS and CUS.

In order to test the normality of F, E, and F/E data, a Kolmogorov–Smirnov test was carried out using the Statgraphics software. The Kolmogorov–Smirnov statistics (KSS) are summarized in Tables 3 and 4. The KSS values at a significance level in the range 0.01–0.1 allow us to conclude that there is no evidence to reject a normal distribution in each data set.

A comparative study using the t-test at a 0.05 significance level for F, E, and F/E mean values obtained from Tables 3 and 4 has been carried out. Significant differences were found in the following cases: between PRE, POST, BAS or CUS and HS samples for cortisol, but significant differences for PRE and CUS samples were not found; between BAS, PRE or POST and HS or CUS for cortisone, but significant differences for HS and CUS, and POST and PRE were not

Table 3
Urinary cortisol and cortisone levels (μg/ml) in different samples (n = sample size)

	HS (n = 27)			CUS (n = 20)			BAS (n = 18)		
	F	E	F/E	F	E	F/E	F	E	F/E
KSS	0.102	0.149	0.113	0.282	0.172	0.142	0.217	0.141	0.144
Mean	0.036	0.094	0.373	0.111	0.095	1.016	0.079	0.166	0.451
S.D.	0.016	0.036	0.139	0.066	0.085	0.528	0.062	0.077	0.188
C.V. (%)	45.26	38.7	36.8	59.39	89.39	51.97	78.94	46.44	41.56
Minimal value	0.008	0.024	0.200	0.049	0.006	0.291	0.020	0.066	0.115
Maximal value	0.066	0.158	0.730	0.267	0.354	2.000	0.242	0.361	0.748
Median	0.035	0.101	0.380	0.079	0.071	1.105	0.054	0.146	0.426

found. The F/E ratio also reveals significant differences for CUS and BAS, PRE, POST or HS, but not for PRE and HS (Fig. 3).

The above results show the usefulness of the simultaneous determination of F and E in urine samples. In this way it is possible to distinguish CUS from HS, POST, PRE or BAS samples by their F/E ratio, because cortisol only increases for Cushing's syndrome samples without increasing cortisone.

On the other hand, the t-test applied to F, E, and F/E mean values of PRE and POST stress samples (Table 4) does not reveal significant differences. An increase of F, E, and F/E (13.5%, 12.9%, and 1.2%, respectively) under stress conditions was obtained. Attention should be paid to the unusual urinary excretion of F and E for basketball player number 2 (Table 4).

The results obtained under stress conditions are in agreement with those obtained by Park et al. [17] for sportsmen after competition (Olympic Games of Seoul): the mean values ± S.D. for F and E (male) were 0.13 ± 0.19 μg/ml and 0.23 ± 0.14 μg/ml, respectively. These results show that the stress test applied to basketball players was adequate. Also, the results obtained for healthy subjects and patients with Cushing's syndrome are in agreement with those reported by Nakamura and Yakata [25,26]. They found F levels of 0.019 ± 0.010 μg/ml for apparently healthy individuals and 0.188 μg/ml for two patients with Cushing's syndrome.

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Table 4
Urinary cortisol and cortisone levels for basketball players before and after a maximal aerobic exercise test

Sportsmen	F (μg/ml)		E (μg/ml)		F/E	
	PRE	POST	PRE	POST	PRE	POST
1	0.059	0.077	0.105	0.147	0.560	0.524
2	0.033	0.242	0.142	0.361	0.232	0.670
3	0.046	0.049	0.109	0.183	0.422	0.268
Mean (n = 3)	0.046	0.123	0.119	0.230	0.405	0.487
KSS	0.237	0.271	0.197	0.221	0.139	0.215
Mean (n = 9)	0.073	0.084	0.158	0.172	0.456	0.446

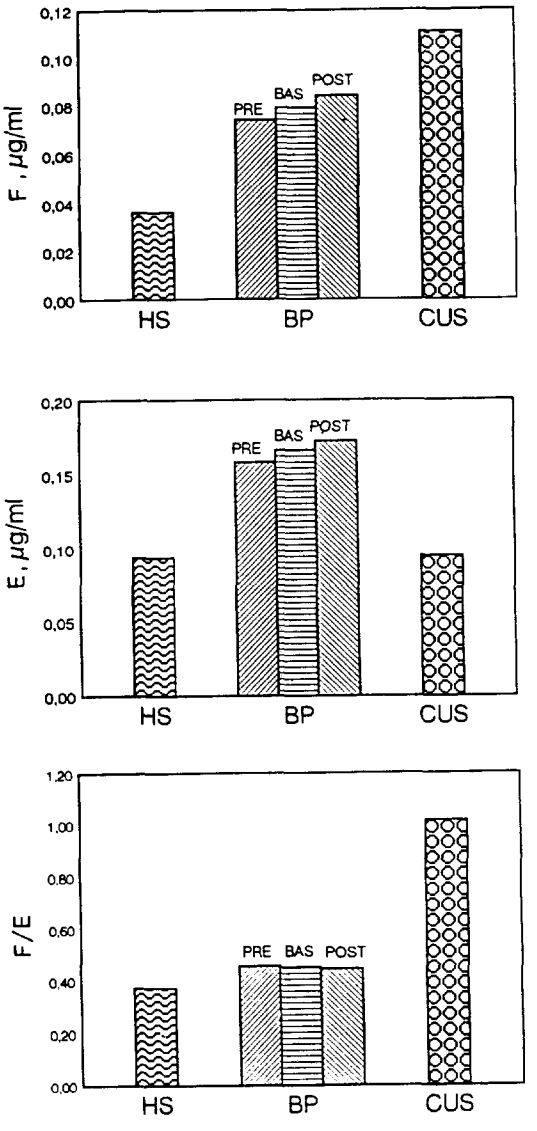

Fig. 3. Bar diagram showing the mean values of urinary cortisol, cortisone and cortisol/cortisone ratios obtained from Tables 3 and 4.

plying urine samples of sportsmen and patients with Cushing's syndrome, respectively.

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