Method Development for Cortisol and Cortisone by Micellar Liquid Chromatography Using Sodium Dodecyl Sulphate: Application to Urine Samples of Rugby Players

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Abstract

The chromatographic behavior of cortisol and cortisone using a micellar medium of sodium dodecyl sulphate (SDS) as surfactant, a Hypersil C_{18} (150- × 3.2-mm i.d., 5 µm) column, a flow rate of 0.5 mL/min, and UV absorbance detection at 245 nm is described. The effect of several organic modifiers and the surfactant concentration on the separation is studied. A mobile phase of 18mM SDS and 8.3% tetrahydrofuran allows for the separation of cortisol and cortisone up to baseline. These results are also achieved by applying a bivariant optimization method. The proposed method is sensitive, reproducible, and selective. In addition, it is less expensive than conventional high-performance liquid chromatography methods for cortisol and cortisone. The method is applied to the determination of cortisol and cortisone in urine samples of rugby players before and after stress for doping control purposes.

Introduction

Cortisol (F) and cortisone (E) are steroid hormones secreted by adrenal glands that regulate a myriad of biological functions and metabolic processes. They are used for therapeutic purposes to reduce pain and allergic and inflammatory reactions (1,2), and they are frequently abused in sports (3,4). Urinary free cortisol (UFF) excretion has been shown to be the single most reliable index of adrenocortical secretion, and it is generally accepted to be a measure of the free fraction in plasma (5). UFF is also used in the diagnosis of depressive disorders and as the most frequent marker for different kinds of stress-induced reactions (6). In addition, the International Olympic Committee Medical Commission has prohibited the use of corticoids via oral, rectal, intravenous, or intramuscular administration. For this reason, it could be interesting to assess the F–E ratio for the useful prediction of abnormality of their use in the doping test. Therefore, the accurate measurement of F or E (or both) in urine may be used for doping control purposes (7).

Radioimmunoassay and competitive protein-binding assay are routinely used to measure UFF. However, it is well known that these methods, though very sensitive, suffer from a lack of specificity, which may become crucial in complex biological matrices such as urine (8). Gas chromatography (GC)–mass spectrometry (MS) is also used for F and E analysis and provides good sensitivity and selectivity (9–11). However, the required derivatization of the analytes complicates sample preparation and reduces the quality of information (4). High-performance liquid chromatography (HPLC) has also been used for the analysis of some endogenous steroids (8,12–14). Other methods, such as liquid chromatography (LC)–MS, provide high sensitivity and selectivity (4,15,16). Unfortunately, this analytical technique is still very expensive for routine analysis, and it is not always available in laboratories. Recently, the importance of chromatographic techniques for the determination of natural steroids, especially in biological fluids, has been emphasized (17).

Micellar liquid chromatography (MLC) is an alternative to HPLC because of the large number of interactions of solutes with the mobile and stationary phases (enhanced selectivity). The most important drawback of the MLC is the decrease of chromatographic efficiency (poor wetting of the stationary phase and restricted mass transfer) as compared to that obtained in HPLC. Several methods have been proposed to improve chromatographic efficiency in MLC, such as: the use of columns with a smaller i.d. than those employed in HPLC, increase of the column temperature, addition of small amounts of organic modifiers, and, recently, use of wide-pore HPLC column packings (18–21).

In previous papers, the optimization of the separation of complex samples of corticoids (CC) and urinary steroids (URST), including F and E by HPLC (22,23) and MLC using sodium dodecyl sulphate (SDS) (19,24) and hexadecyltrimethylammonium bromide (25) for screening purposes, was reported. The optimal separations achieved in MLC for CC (19)

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and URST (24) involved the use of a Hypersil column (Phenomex, Torrance, CA) and mobile phases consisting of 1.9% BuOH and 36mM SDS [allowing the separation of 13 out of 16 CC in 27 min (19)], and 5% PrOH and 40mM SDS [allowing the separation of 13 out of 13 URST in 23 min (24)]. However, these separations were not adequate for quantitative purposes of F and E in urine samples because these compounds were not completely separated (the resolution for both separations was close to 0.75). MLC methods have also been proposed for cortisol, other individual CCs in different pharmaceuticals (26), and urinary free cortisol (27).

In this paper, micellar mobile phases containing SDS and different organic modifiers are examined to evaluate F and E retention and separation characteristics using a reversed-phase Hypersil C_{18} 3.2-mm i.d. column with a flow rate of 0.5 mL/min. Based on this study, a separation method for the simultaneous determination of F and E using tetrahydrofuran (THF) as organic modifier, giving good resolution with not too long of an elution time, has been developed and applied to urine samples of rugby players before and after stress for doping control purposes.

Experimental

Chemicals and reagents

E (4-pregnene-17α,21-diol-3,11,20-trione), F (11β,17α,21-trihydroxypregnene-4-en-3,20-dione), and fluorocortisone acetate (FLA) (9α-fluoro-11β,17α,21-trihydroxy-4-pregnene-3,20-dione acetate) were from Sigma (St. Louis, MO). Stock solutions of these analytes (1000 µg/mL) were prepared in methanol. Working solutions (2–10 µg/mL) of a single corticoid or an appropriate mixture were also prepared in methanol from stock solutions. SDS, disodium hydrogen phosphate, and anhydrous sodium sulphate of analytical-reagent grade were from Merck (Darmstadt, Germany). HPLC-grade acetonitrile (ACN), 1-propanol (PrOH), and THF were purchased from Promochem (Wesel, Germany) and dichloromethane from Carlo Erba (Milan, Italy). Water was purified with a Milli-Q system (Millipore, Molsheim, France). Bond elut C18 cartridges (3 mL and 500 mg) from Varian (Harbor City, CA) and Millipore 0.45-µm nylon filters (Bedford, MA) were also used. Other chemicals used were of analytical-reagent grade.

Apparatus

The chromatographic system consisted of the following components, all purchased from TSP (Riviera Beach, FL): a Constametric 4100 solvent delivery system, a spectromonitor 5000 photodiode-array detector (DAD) covering the range 190–360 nm and interfaced to a computer for data acquisition, and a recorder model CI 4100 data module. A 6-port valve with a 20-µL sample loop injector (Rheodyne, Cotati, CA), a Jones Chromatography block heated series 7960 for thermostating columns in the range 30–70ºC (Seagate Technology, Scotts Valley, CA), a vacuum membrane degasser Model Gastor (SAS Corporation, Tokyo, Japan), and a bonded-silica Hypersil ODS (150- × 3.2-mm i.d., 5 µm) column from Phenomenex were used. A vortex mixer Mixo-Tub-30 from Crison (Barcelona, Spain) was also used.

Mobile phase and chromatographic analysis

Isocratic micellar mobile phases were prepared daily, mixing well-known volumes of THF, ACN, or PrOH with aqueous solutions of SDS (prepared with Milli-Q water), by programming the pump (e.g., 8.3% THF and 18mM SDS). Binary mobile phases consisted of PrOH (5–9.2%) and 18mM SDS, ACN (11.8–16.7%) and 18 mM SDS, or THF (5.0–8.3%) and 18mM SDS.

Other mobile phases consisted of 5% THF and SDS (18–118mM), and THF (2–10%) and SDS (20–100mM). All solvents and mobile phases were filtered under vacuum through 0.45-µm nylon filters and then degassed using a vacuum membrane degasser.

Once the analytical column had been conditioned with the micellar mobile phase (30 min), chromatograms were obtained at 30ºC. For optimization purposes, based on the use of different isocratic micellar mobile phases, a methanolic solution containing F, E, and FLA (IS), or an appropriate mixture of the compounds (2–10 µg/mL), was injected (20 µL). UV detection and a flow rate of 0.5 mL/min were used. Peak identification and purity was performed by comparing the retention time and UV spectra of the chromatographic peaks with those of reference compounds previously registered by injection of each one individually. In addition, single steroid standards (3 µg/mL) were spiked to the steroids mixture, and the increase of the corresponding peak area in the chromatogram was checked. Detection was performed at 245 nm (wavelength of absorption maximum).

Urine collection

Urine samples were collected from rugby players (20–22 years old) before (PRE) (11 samples) and after stress (POST) (11 samples) 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 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 4ºC for further analysis.

Sample preparation

Blank urine samples

Steroid-free urine samples were prepared by percolating urine samples through Bond elut C_{18} cartridges. By doing this, urinary steroids and other potential interfering compounds were retained in the cartridges. The unretained fraction was then collected and checked for endogenous steroids [with negative result following the solvent extraction procedurereported in the literature (28)] and used as a matrix for F and E spikes.

Solvent extraction

Urine samples (3 mL) were placed in a stoppered centrifuge tube, spiked with FLA (IS)(444 ng/mL), and processed according to a similar previously described liquid–liquid extraction (LLE) procedure, in which recoveries were found to be 98.5%, 108%, and 91.0% for F, E, and FLA, respectively (14). Briefly, 0.35 g NaCl was added to the samples in order to avoid

emulsions, and pH close to 9 was obtained using 0.5 g of Na_2HPO_4. Next, 4 mL of dichloromethane was added. The mixture was shaken and centrifuged. The organic phase (the lower layer in the extraction flask) was removed and dried over anhydrous Na_2SO_4. A 3-mL aliquot was evaporated to dryness. The dry residue was reconstituted with 200 µL of MeOH and 20 µL was injected into the HPLC system. The absolute preconcentration factor was close to 11.

Results and Discussion

Column, surfactant, and organic modifier choice

In previous works, the separation of complex samples of CC and urinary steroids in MLC has been studied (19,24). However, when these separations were applied to urine samples for quantitative analysis of F and E, the results obtained were not satisfactory because the resolution was close to 0.75. On these grounds, to obtain a separation for F and E with better performance than that previously obtained, a Hypersil column (30°C) 3.2 mm i.d. was initially selected [to improve column efficiency in MLC, smaller flow rates and column inner diameters than those typically employed in HPLC have been recommended (18)]. An 18mM SDS (larger than the critical micelle concentration = 8.1mM) (29) and several common organic modifiers (PrOH, THF, and ACN) were also selected.

Effect of organic modifiers on the separation

The effect of PrOH, THF, and ACN on the retention and separation characteristics of F and E using 18mM SDS was studied. The solvent concentration ranges (Φ, %) were: (5–9.2) for PrOH, (5.0–8.3) for THF, and (11.6–16.7) for ACN. The retention factors (k) were obtained from the retention times of F and E and from the retention time of a solution of KNO_3. ln k were plotted versus Φ (18) and the linear least squares analysis data for PrOH, THF, and ACN was obtained. The slopes with negative sign (solvent strength parameter) and intercepts were 0.125, 0.134, and 0.095, and 2.56, 2.81, and 3.58 for E, and 0.135, 0.182, and 0.100, and 2.50, 2.97, and 3.53 for F, respectively. The correlation coefficients (r) were always higher than 0.997. The slope values indicate that THF interacts more strongly with micelles and, consequently, solvates them more effectively.

Selectivity between consecutive peaks ($\alpha = k_F/k_E$) has been examined quantitatively by analysis of ln k versus Φ plots. Selectivity increases slightly as Φ increases (lines tend to diverge) for PrOH and THF. However, parallel lines were obtained for ACN. A similar behavior can be observed by plotting the selectivity factor α versus Φ for the three solvents. In HPLC using MeOH or THF, the elution order was the same as that obtained in MLC using THF, PrOH, or ACN. In contrast, in HPLC using ACN, the elution order was the opposite (14).

Figure 1 shows the chromatograms obtained under optimal conditions. From these results, 8.3% THF was finally selected for further experiments, taking into account the analysis time, resolution (R_s = 2.17), and matrix interferences (the major impediment to reduce the analysis time).

Effect of SDS concentration

A study of the effect of SDS (range 18–118mM) on the separation of F and E using 5% THF was carried out. The concentration of THF was decreased with respect to that considered as optimum because F and E coeluted when SDS concentration was increased. The k values and selectivity decreased as SDS increased (α values ranged from 1.18–1.04). The absence of an intersection between F and E lines when 1/k versus SDS are plotted (18) indicates that there is no variation in the elution order.

Bivariant optimization method for the SDS–THF system

A bivariant method has been applied for the optimization of an adequate composition of the micellar mobile phase SDS–THF. SDS concentration was decreased but that of THF was increased. The ranges of THF, SDS, and SDS–THF concentration ratio were 2–10%, 20–100mM, and 2–50, respectively. α has also been examined from F and E retention data and SDS–THF concentration ratio in the range 2–50. As can be observed in Figure 2, larger α values were obtained when using lower values of the SDS–THF ratio. From this study, an

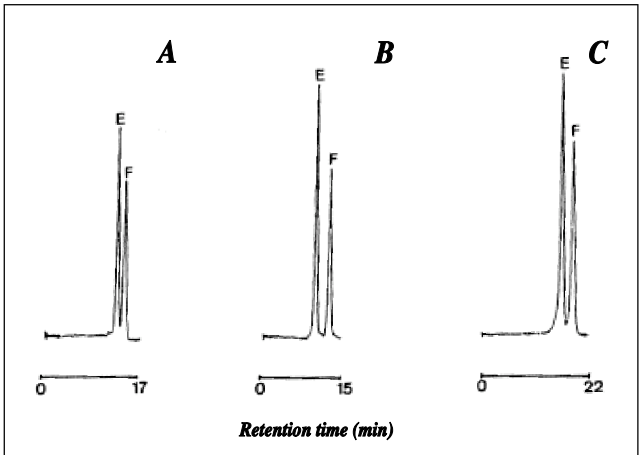

Figure 1. Chromatograms obtained from a standard mixture of F (10 µg/mL) and E (10 µg/mL) using micellar mobile phases 18mM SDS and different organic modifiers: A (5.0% PrOH), B (8.3% THF), and C (15% ACN).

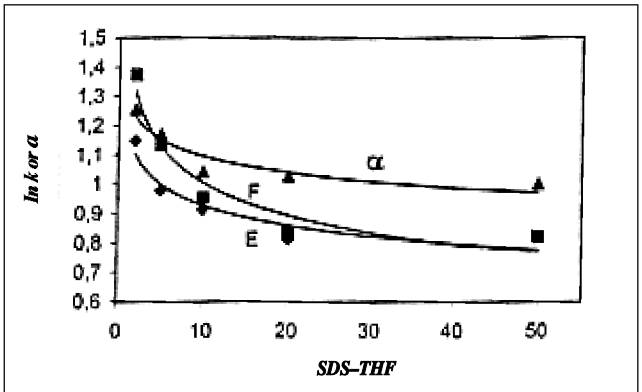

Figure 2. Influence of the SDS–THF concentration ratio on retention and selectivity for cortisol (F) and cortisone (E). $\alpha = k_F/k_E$.

optimum separation is achieved using a mobile phase 20mM SDS and 10% THF. However, a refined study leads to a 18mM SDS and 8.3% THF mobile phase.

Table I. Linear Regression Equations (PAR = A + B*x*), Limits of Detection (LOD) and Quantitation (LOQ), and Within-Run and Between-Run Precision for F and E*

CC	A	B x 10^3	*r*	LOD (ng/mL)	LOQ (ng/mL)	CV_{Rep}	CV_{Repr}
F	–0.006	2.93	0.9999	3.7	12.3	3.2	5.8
E	–0.009	3.27	0.9999	3.3	11	2.6	6.4

* PAR is the peak area ratio of F or E to FLA (IS); *x* = ng/mL of F and E; *r* = correlation coefficient.

Table II. Urinary F and E Levels (µg/mL) in Different Samples (*n* = sample size)

	HS (14) (*n* = 27)			RUG (*n* = 22)		
	F	E	F–E	F	E	F–E
KSS	0.102	0.149	0.113	0.185	0.208	0.185
Mean	0.036	0.094	0.373	0.115	0.246	0.498
SD	0.016	0.036	0.139	0.036	0.119	0.242
CV, %	45.26	38.7	36.8	31.37	48.40	48.48
Minimal Value	0.008	0.024	0.200	0.065	0.104	0.182
Maximal Value	0.066	0.158	0.730	0.218	0.480	1.096
Median	0.035	0.101	0.380	0.114	0.218	0.452

Table III. Urinary F and E Levels for Rugby Players before or after a Maximal Aerobic Exercise Test

	F (µg/mL)		E (µg/mL)		F–E	
Rugby players	PRE	POST	PRE	POST	PRE	POST
KSS	0.207	0.242	0.214	0.207	0.176	0.255
Mean (*n* = 11)	0.110	0.120	0.223	0.270	0.554	0.451
SD	0.040	0.031	0.104	0.132	0.298	0.164
CV, %	35.9	25.8	46.7	49.7	54.8	36.4
Minimal value	0.065	0.080	0.104	0.123	0.182	0.264
Maximal value	0.218	0.175	0.461	0.480	1.096	0.765
Median	0.114	0.114	0.218	0.218	0.524	0.365

Analytical characteristics

Calibration graphs

Several corticoids were tested because of their suitability as internal standard (IS) under the optimal separation conditions. FLA was judged to be the best for quantitation purposes. This compound presents a higher retention than F and was separated up to baseline. Calibration graphs were obtained by adding to steroid-free urine sample standards of F, E, and FLA (IS) at nine concentrations in the range 44–622 ng/mL using 444 ng/mL FLA(IS) under solvent extraction conditions as described previously (Solvent extraction section). These solutions were separated using a mobile phase of 18mM SDS and THF 8.3%, flow rate of 0.5 mL/min, a Hypersil column, and UV absorbance–DAD detection at 245 nm. The results were analyzed by linear regression. Plotting each corticoid peak area to IS ratio (PAR) versus the concentration (*x*) of each one, the calibration equations, PAR = A + B*x* (ng/mL), were obtained. The parameters A (intercept), B (slope) and *r* (regression coefficient) are summarized in Table I. The slope, LOD, and LOQ values in Table I include the preconcentration factor previously assessed. The calibration equations (Table I) allow the calculation of F and E concentration levels in urine samples.

Precision, LODs, and selectivity

The repeatability (REP) (within run precision) was examined by analyzing 10 different mixtures of F and E within a day using an individual concentration of 5 µg/mL and by running each mixture once (*n* = 10), whereas reproducibility (REPR) (between-run precision) was evaluated for three different days (*n* = 30) using the calibration graphs. The CV values (CV_{rep} and CV_{repr}) for F and E and the LODs and LOQs for a signal-to-noise (s/n) of 3 and 10 (*n* = 10), respectively, are shown in Table I. Other corticoids such as triamcinolone, triamcinolone-acetonide, 11α-hydroxyprogesterone, prednisone, prednisolone, beta- and dexamethasone, fluorocortisone acetate, deoxycorticosterone, methylprednisolone, deflazacort, and21-OH-deflazacort were separated efficiently from F and E. Only fluorocortisone coeluted with F.

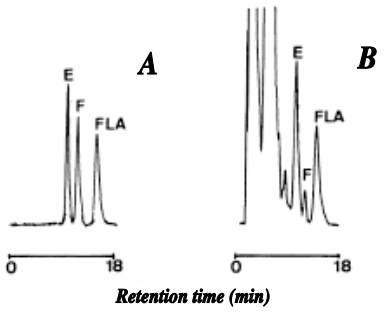

Figure 3. Chromatograms obtained at 245 nm using a mobile phase 18mM SDS and 8.3% THF: for a standard mixture of F, E, and FLA (IS) (A) and for a urine sample of a rugby player (POST) after solvent extraction (B). Peaks: cortisol (F), cortisone (E), and fluorocortisone acetate (FLA).

Urine sample analysis

The optimized mobile phase (18mM SDS and 8.3% THF) was

used with urine samples of rugby players under solvent extraction conditions. The comparison of Figures 3A and 3B indicates that this method is adequate for analysis of F and E in urine samples without matrix interferences [the CV (n = 6) of the retention factors for F and E was lower than 1% for each one]. A detection and identification process of F and E based on retention times and a diode array detector (DAD) was carried out (30). The UV absorbance 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. Because urinary endogenous compounds can present similar spectra to those of F and E, further investigation of the peak purity was carried out by obtaining the second derivatives of the spectra and absorbance ratios (A_{275}/A_{245}) across the peak. In this way, interferences were not found. When interferences occur, a change in the mobile phase composition is recommended.

Applications

The rugby players (RUG) samples were studied in two different ways, first by taking into account the total available samples (RUG) both before (PRE) and after stress (POST) [RUG = (PRE + POST)]. From these results, useful information can be gained because the doping test can be performed during training time or after competition. Second, the pre- and post-stress samples (PRE and POST) were also considered separately to obtain data on the effect of stress on rugby players.

Urinary F, E, and F–E data found for healthy subjects (HS) (14) and RUG and PRE and POST samples, along with some statistical parameters of interest, are summarized in Tables II and III, respectively. In order to test the normality of F, E, and F–E data, a Kolmogorov–Smirnov test was carried out using the Statgraphics software (Madrid, Spain). The Kolmogorov–Smirnov statistics (KSS) are in Tables II and III. The KSS values at a significance level in the range 0.01–0.1, allowing for the conclusion 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 II and III has been carried out. Significant differences were found for cortisol and cortisone between HS and PRE and POST and RUG samples. The F–E ratios also reveals significant differences for HS and RUG or PRE but not for HS and POST.

On the other hand, the t-test applied to F, E, and F–E mean values of PRE and POST stress samples (Table III) does not reveal significant differences. However, an increase of F and E, and a decrease of F–E (9.1%, 21.1%, and 17.1%, respectively) under stress conditions were obtained.

The results obtained under stress conditions are in agreement with those obtained by Park et al. (31) for athletes after competition in the Seoul Olympic Games using HPLC–DAD and thermospray LC–MS [the mean values ± SD for F and E (male) were 0.13 ± 0.19 µg/mL and 0.23 ± 0.14 µg/mL, respectively]. The results are also in agreement with those obtained by Santos et al. (14) for basketball players (the mean values ± SD for F and E were 0.084 ± 0.065 and 0.172 ± 0.079 µg/mL, respectively). These results show that the stress test applied to rugby players was adequate.

Conclusion

Mobile phases containing SDS and THF, ACN, or PrOH, as well as a Hypersil C_{18} column (3.2-mm i.d.) were tested for the separation of F and E. Different separations with different selectivities were obtained depending on the nature of organic modifier and SDS concentration. Satisfactory results were obtained using THF, which was adequate for urine sample analysis. MLC constitutes an alternative to conventional HPLC and presents several advantages, such as the decrease of adverse effects caused by endogenous compounds and the use of less toxic and cheaper mobile phases.

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