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Cortisol and cortisone ratio in urine: LC-MS/MS method validation and preliminary clinical application

Abstract

Background: The determination of urinary cortisol/cortisone ratio is of clinical utility in cases of Cushing's syndrome, apparent mineralocorticoid excess, and also provides information on 11β-hydroxysteroid dehydrogenase (11β-HSD) type 2 activity. It is therefore of utmost importance to ensure accurate cortisol and cortisone measurement and establish appropriate reference ranges.

Methods: After the isotopic dilution of urine, sample cleanups were obtained with on-line solid-phase extraction and cortisol and cortisone, separated using a Zorbax Eclipse XDB-C18 HPLC analytical column, were analyzed by tandem mass spectrometry with an electrospray ionization source in positive ion mode operation.

Results: The method was linear, with concentrations of up to 625 and 1125 nmol/L and lower limit of quantitation (LLOQ) of 5 and 6 nmol/L, for cortisol and cortisone, respectively. Within-run and between-run coefficients of variation were <5% and 6% for cortisol and 6% and 8% for cortisone, respectively. No ion suppression was observed. The non-parametric reference range for the cortisol/cortisone ratio was 0.14–1.09.

Conclusions: A simple and sensitive liquid chromatography tandem mass spectrometry method was developed and validated for the measurement of cortisol and cortisone in urine. Our findings indicate that the proposed analytical method is suitable for routine purposes and useful in many pathological conditions.

Keywords: cortisol; cortisone; liquid chromatography; ratio; reference range; tandem mass spectrometry.

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Introduction

Urinary free cortisol (UFC) analysis represents the first biochemical laboratory approach for the screening of endogenous Cushing's syndrome (CS). Endogenous CS is caused by prolonged exposure to elevated levels of endogenous cortisol that may occur from excess production by one or both adrenal glands, or from overproduction of the adrenocorticotropic hormone (ACTH), which normally regulates cortisol production. As symptoms are always non-specific, including hypertension, truncal obesity and mood disorders, specific biochemical tests are required for diagnosing CS. One of the first-line tests for diagnosis, the measurement of 24-h UFC, can also be useful in other clinical conditions characterized by a high serum cortisol level, such as in non-autonomous hypercortisolism (pseudo-CS), psychiatric disorders, morbid obesity, poorly controlled diabetes mellitus and alcoholism [1–3].

Altered cortisol metabolism is also responsible for a condition called apparent mineralocorticoid excess (AME) syndrome. Type 2 11β-hydroxysteroid dehydrogenase (11β-HSD) regulates the cortisol level by oxidizing it to its inactive form, cortisone. While cortisol is mainly essentially secreted by the adrenal gland, cortisone is mainly produced by 11β-HSD type 2, which interconverts bioactive cortisol to hormonally inactive cortisone to prevent activation of the mineralocorticoid receptor by cortisol. Hence the simultaneous determination of cortisol and cortisone can help in the diagnosis of AME syndrome, but also in congenital adrenal hyperplasia and adrenal insufficiency [4, 5].

Immunoassays are widely used for urinary cortisol measurement, but it is now accepted that these methods

suffer from various interferences due to antibody cross-reactions with endogenous steroid metabolites, including cortisone and synthetic glucocorticoids such as prednisolone [6–9]. It is now also well known that only chromatographic methods can accurately measure UFC: recently developed, more specific methods based on liquid chromatography with ultraviolet detection [10–13] allow the simultaneous determination of more than one analyte. Despite their advantages over immunoassay, liquid chromatography-ultraviolet methods are still susceptible to some interferences and poor specificity [14, 15]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been recognized as the best available method for the accurate analysis of endogenous steroid hormones in biological samples [15–17]. LC-MS/MS typically provides high analytical sensitivity and specificity, has the ability to simultaneously determine multiple analytes, has a wide dynamic range and usually requires fewer sample preparation steps than gas chromatography-mass spectrometry (GC-MS)-based methods. Because of these advantages, LC-MS/MS is widely accepted in clinical laboratories [17, 18].

Our aim was to develop and validate a suitable method for the routine measurement of cortisol/cortisone ratio in urine samples and to define the appropriate reference ranges.

Materials and methods

Standards and reagents

Cortisol (F), cortisone (E), cortisol-9,11,12,12-d_4 (d_4F), cortisone-2,2,4,6,6,12,12-d_7 (d_7E), HPLC-MS grade methanol and formic acid (99%) were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA); Milli-Q water was used (Millipore SpA, Milano, Italy). Potassium hydrogen phosphate, sodium dihydrogen phosphate, sodium chloride, urea and creatinine were obtained from Carlo Erba (Limito, Italy).

Powdered F and E were dissolved in methanol to produce primary stock solutions with concentrations of 4.08 and 2.77 mmol/L, respectively, and stored at -80°C. Secondary stock solutions were obtained by diluting the primary stock solutions in methanol to give concentrations of 40.8 and 55.4 µmol/L F and E, respectively. These solutions were stored at -80°C for up to 4 months. Calibrators, containing both analytes, were obtained by diluting the secondary stock solutions in a water solution (assay diluent) containing 16 mmol/L potassium hydrogen phosphate, 5 mmol/L sodium dihydrogen phosphate, 60 mmol/L sodium chloride, 340 mmol/L urea and 9 mmol/L creatinine.

Powdered d_4F and d_7E were dissolved in methanol to produce internal standard primary stock solutions, at the concentration of 5 g/L for both standards, and stored at -80°C. An internal standard secondary mixed solution, obtained by diluting the internal standard primary stock solution in methanol to give 1 mg/L of d_4F and d_7E, was stored in 3-mL aliquots at -80°C for up to 2 months.

Sample preparation

A 10-mL aliquot was taken out from the 24-h collected urine and centrifuged at 3000×*g* for 10 min at room temperature. The urine surnatants were stored at -80°C until analysis. Samples were thawed at room temperature before sample preparation was performed. After adding 2 µL of formic acid and 50 µL of internal standard secondary mixed solution to 500 µL of urine surnatant or calibrators, the solution was vortexed for 30 s and then centrifuged at 16,000×*g* for 5 min at room temperature; 20 µL of the surnatant was added to 200 µL of 0.1% formic acid water solution and placed in the LC-MS/MS autosampler.

On-line SPE-LC-MS/MS

The instrumentation consisted of an Agilent HPLC series 1200 with a column oven, an autosampler, a binary LC pump and a degasser together with an additional isocratic pump with a switching valve for on-line solid-phase extraction (SPE) and a triple quadrupole mass spectrometer (Agilent 6430) equipped with an electrospray ionization source, operating in positive ion mode (Agilent Technologies, Palo Alto, CA, USA).

The on-line clean-up/enrichment was carried out by a Zorbax Extend-C18 cartridge (2.1×12.5 mm, 5 µm particle size) and the HPLC separation by a Zorbax Eclipse XDB-C18 analytical column (4.6×50 mm, 1.8 µm particle size) (Agilent Technologies).

Analyte enrichment and sample clean-up on the SPE column were carried out with 70:30 water/methanol (solvent C) solution. The subsequent HPLC separation was carried out (maintaining the column at 50°C) by a two-solvent gradient system, in which solvent A was methanol with 0.1% formic acid and solvent B was water with 0.1% formic acid. The SPE-HPLC conditions are summarized in Table 1.

Quantitative analysis was performed in multiple reaction monitoring (MRM) mode. Three positive ion selected transitions were monitored for F and E, and a single ion transition was monitored for d_4F and d_7E, using optimized collision energies (CEs) and a fragmentor voltage (FV) as shown in Table 2. Further operative parameters were as follows: drying gas (N_2) flow and temperature, 10 L/min and 300°C, respectively; nebulizer pressure, 35 psi; capillary voltage, 4000 V; and dwell time, 120 ms.

Method validation

The assay was validated against published acceptance criteria for linearity, imprecision, uncertainty and stability proposed by the Food and Drug Administration (http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf).

Ion suppression

Post-column infusion studies were made to evaluate ion suppression. Urine samples (n=10) with a low analyte concentration and water were injected, while a solution of cortisol and cortisone (1000 nmol/L) in 70:30 (v/v) mobile phase A:B was infused into a mass spectrometer at 16 µL/min. Ion suppression was observed by monitoring the ion counts for each *m/z* transition throughout the 7-min run time.

Table 1 HPLC pumps timetable.

Step	Binary pump				Isocratic pump		
	Time, min	Flow, mL/min	%A	%B	Time, min	Flow, mL/min	%C
1	0.00	0.4	30	70	0.00	0.1	100
2	0.50	0.4	30	70	0.09	0.1	100
3	0.51	0.4	50	50	0.10	1.0	100
4	1.00	0.4	50	50	0.49	1.0	100
5	2.99	0.4	70	30	0.50	0.1	100
6	4.99	0.4	70	30	3.99	0.1	100
7	5.00	0.8	100	0	4.00	0.5	100
8	5.99	0.8	100	0	4.99	0.5	100
9	6.00	0.8	30	70	5.00	0.1	100
10	6.99	0.8	30	70	7.00	0.1	100
11	7.00	0.4	30	70			

Table 2 MRM transitions and the related optimized parameters for analytes and internal standards.

Analyte		MRM transition	FV (V)	CE, eV
d_4F	Quantifier	367.1>121.1	125	24
d_7E	Quantifier	369.3>169.1	125	25
F	Quantifier	363.2>121.1	140	33
	Qualifier a	363.2>327.2	140	13
	Qualifier b	363.2>145.1	140	29
E	Quantifier	361.2>163.1	125	21
	Qualifier a	361.2>121.1	125	33
	Qualifier b	361.2>145.1	125	37

Linearity

The linearity was assessed by the correlation coefficients (r^2) on the calibrators at five different concentrations ranging from 5 to 625 nmol/L for F and from 6 to 1125 nmol/L for E. Calibration curves were obtained by plotting the ratios between the analyte peak area and the internal standard peak area against concentration in nanomoles per liter.

Lower limit of quantification and limit of detection

The lower limit of quantification (LLOQ), also defined as functional sensitivity, was determined as the lowest concentration at which accuracy and imprecision were within ±20% and represents the first point of the calibration curve. The limit of detection (LOD) was defined as the lowest concentration at which signals were three times greater than background noise for both primary and secondary transitions.

Imprecision

Intra-day imprecision was evaluated by 10 repeated measurements of three urine samples in a single analytical run, at 9.9, 20 and 164 nmol/L for F and at 36, 94 and 186 nmol/L for E. Inter-day imprecision was determined by 10 repeated measurements of three urine samples in 10 different analytical runs, at 20, 179 and 292 nmol/L for F and at 34, 78 and 213 nmol/L for E. Imprecisions were expressed as coefficients of variation (CV%).

Uncertainty: dilution and recovery tests

Dilution tests

Three different urine samples, each diluted with the assay diluent to a ratio of 1:2, 1:4, 1:8 and 1:16, were treated as described in the Sample Preparation section and then analyzed.

Recovery tests

Three different urine samples were spiked with three different levels of the standards: 5.4 and 14.7 nmol/L (first level), 13.6 and 36.9 nmol/L (second level) and 34.0 and 92.3 nmol/L (third level) for F and E, respectively. The recovery percentage was expressed as a ratio of [(measured concentration-endogenous concentration)/(spiked concentration)]×100.

Sample stability

Urine stability was determined by keeping the aliquots of four urine samples (obtained after the first centrifugation) at room temperature (RT) for 0 h (basal sample – immediately stored at -80°C), 2 h, 4 h, 7 h and 24 h, and at +4°C for 0 h (basal sample – immediately stored at -80°C), 2 h, 4 h, 7 h, 24 h, 48 h, 72 h, 96 h and 120 h before storage at -80°C; successively, all these samples were analyzed simultaneously, in the same analytical run. The results were expressed as percentages with respect to their basal measured values (time 0 h).

Reference values and patient samples

In order to calculate the reference range, 24-h urine samples were collected from 98 apparently healthy adult volunteers (43 males, 55 females; age range, 8–94 years). Pathological samples were obtained from 26 patients (7 males, 19 females; age range, 8–84 years) with a diagnosis of CS according to a recent guideline [19] and recruited from the endocrinology operative unit. After collection, samples were quickly stored at -80°C until analysis. The study

was approved by the local ethics board, and the participants gave informed written consent.

Statistical method

The results are expressed as average±SD. A one-way analysis of variance test was used to analyze stability test results. The Mann-Whitney test was used to compare groups. All statistical analyses were performed using the Analyse-it software (Analyse-it Software, Ltd., Leeds, UK).

Results

On-line SPE-LC-MS/MS

The developed method allowed the separation of F and E to produce clean, discrete peaks with E eluting first at 4.4 min and F at 4.7 min with co-elution of their respective internal standards (Figure 1).

Method validation

Ion suppression

No ion suppression was observed at the time of elution of either F or E (Figure 2). Ion counts were stable between 4.0 and 5.0 min for each *m/z* count. This effect was reproducible following the injection of different patient samples (n=10) and water. At the corresponding elution times of analytes, no significant difference was observed between matrix and water for ion suppression.

Linearity

For F and E, the calibration curves demonstrated linearity up to a concentration of 625 and 1125 nmol/L, respectively (r^2>0.998).

Lower limit of quantification and limit of detection

The LLOQ was 5 nmol/L for F and 6 nmol/L for cortisone. The LOD was 4 and 5 nmol/L for F and E, respectively.

Imprecision

Intra-assay CVs for the three urine samples were 3%, 4% and 6% for F and 5%, 5% and 2% for E. Inter-assay CVs for the urine samples (n=3) were 5%, 8% and 6% for F and 5%, 6% and 4% for E.

Uncertainty: dilution and recovery tests

Dilution tests

The correlations between expected and measured concentrations were y=0.982x+6.388 (r^2=0.998, p<0.0001) and y=0.982x+5.552 (r^2=0.999, p<0.0001) for F and E, respectively.

Recovery tests

The mean recoveries of the three urine samples were 106% for cortisol and 104% for cortisone, as shown in Table 3.

Sample stability

As shown in Figure 3, no significant differences were observed in F and E values measured in four urine samples kept at RT for 0 h (basal value – immediately stored at -80°C), 2 h, 4 h, 7 h and 24 h, and at +4°C for 0 h (basal value – immediately stored at -80°C), 2 h, 4 h, 7 h, 24 h, 48 h, 72 h, 96 h and 120 h before storage at -80°C. The basal values were as follows: urine A, 40 nmol/L for F and 133 nmol/L for E; urine B, 254 nmol/L for F and 409 nmol/L for E; urine C, 168 nmol/L for F and 225 nmol/L for E; and urine D, 20 nmol/L for F and 67 nmol/L for E.

Reference values and patient samples

Nonparametric reference intervals for apparently healthy adults were 0.14–1.09 for the F/E ratio (median 0.39), determined as central 95%; 16–170 nmol/24 h for F (median 52 nmol/24 h); and 41–364 nmol/24 h for E (median 135 nmol/24 h). The F/E ratio interval obtained for CS patient samples was 0.45–4.69, the values being 27–6219 nmol/24 h for F and 58–1606 nmol/24 h for E. Urine F/E ratio, as well as F and E concentrations measured with the proposed method, was significantly higher in patients than in healthy subjects (p<0.001).

Discussion

Cortisol and cortisone assay in a complex matrix such as urine is a challenge for the laboratory since many

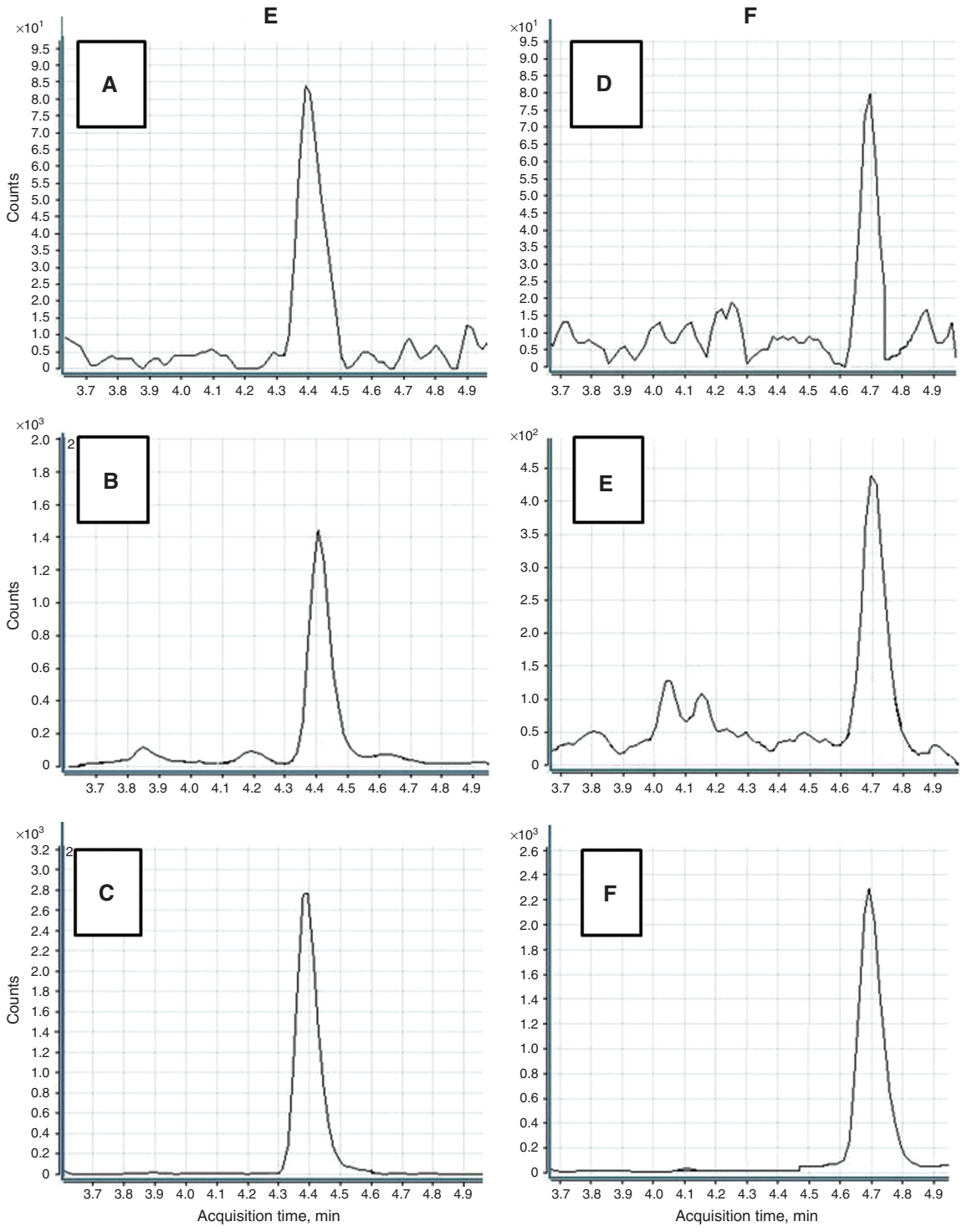

Figure 1 MRM chromatograms of cortisol (F) and cortisone (E) and their respective internal standards.
(A) E elution at 4.4 min (*m/z* 361.2>163.1) for 6 nmol/L of E standard; (B) E elution at 4.4 min (*m/z* 361.2>163.1) for a urine sample at 20 nmol/L of E; (C) d_7E elution at 4.4 min (*m/z* 369.3>169.1); (D) F elution at 4.7 min (*m/z* 363.2>121.1) for 5 nmol/L of F standard; (E) F elution at 4.7 min (*m/z* 363.2>121.1) for a urine sample at 10 nmol/L of F; (F) d_4F elution at 4.7 min (*m/z* 367.1>121.1).

structurally similar steroids and metabolites are present. Immunoassay-based methods lead to falsely elevated cortisol values consequent to poor antibody specificity [7–9]. In clinical conditions calling for F/E ratio determination, it is of utmost importance to employ a highly specific method allowing the simultaneous measurement of both

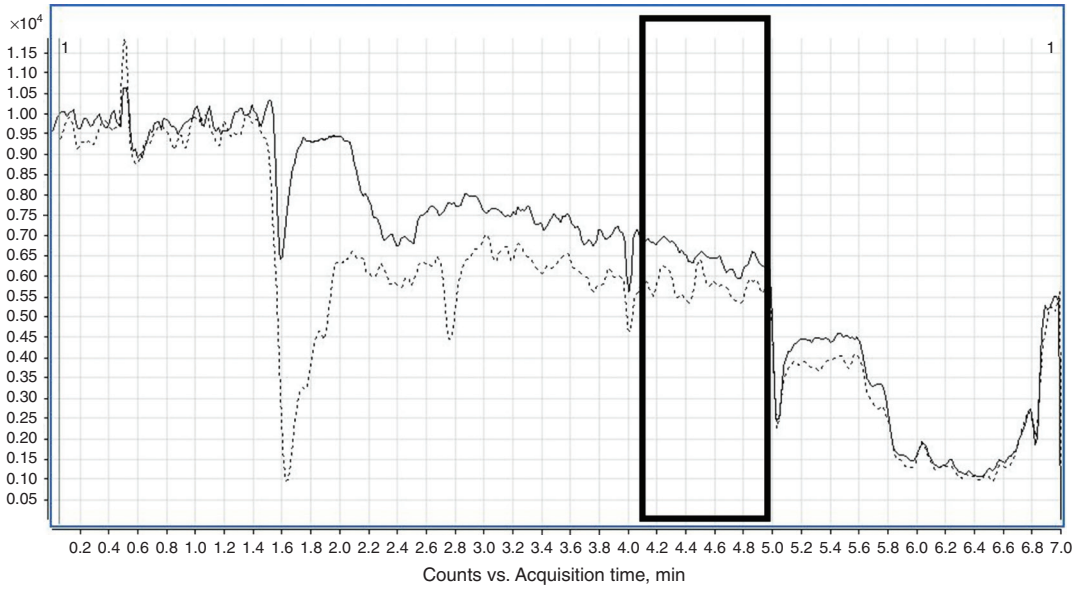

Figure 2 Ion suppression.
The total ion counts following post-column infusion of cortisol (F) and cortisone (E) solution for a urine sample (dotted line) and for water (solid line); the window of E and F separation (4.4 and 4.7 min) is highlighted.

Table 3 Recovery test results in urine samples for cortisol (F) and cortisone (E) method.

	F				E			
	Endogenous, nmol/L	Added, nmol/L	Found, nmol/L	Recovery, %	Endogenous, nmol/L	Added, nmol/L	Found, nmol/L	Recovery, %
Urine A	21	5.4	27	110	67	14.8	82	102
		13.6	36	110		36.9	106	106
		34.0	51	88		92.3	155	95
Urine B	34	5.4	39	92	80	14.8	97	115
		13.6	47	96		36.9	118	103
		34.0	74	118		92.3	174	102
Urine C	114	5.4	120	110	183	14.8	199	108
		13.6	127	96		36.9	225	114
		34.0	146	94		92.3	267	91

analytes, because some factors that affect method performances are nullified as they influence both molecules. Suitable for clinical purposes due to its characteristics, the tandem mass spectrometry-based method allows the simultaneous measurement of more than one analyte, guaranteeing highly analytical performances [15–18].

The developed method demonstrated satisfactory analytical performances. We obtained a functional sensitivity of 5 and 6 nmol/L for F and E, respectively, suitable imprecision (CV <10%) and uncertainty considering the dilution and the recovery tests. Nor did we find presence of ion suppression-enhancement since LC-MS/MS methods could also be affected by matrix effects. However, the use of specific deuterated internal standards for both F and E obviates unexpected matrix interferences and ionization problems [20].

Another potential source of variability is linked to the stability of steroids in biological samples [21, 22]. On evaluating pre-analytical variability in relation to temperature and time of storage before analysis, we found no difference up to 24 h at RT and 120 h at +4°C before storage at -80°C for either F or E.

The proposed method is validated for both F and E, since the F/E ratio may be helpful to physicians in diagnosing and monitoring patients with CS, but also in other endocrinological diseases in which there is an involvement

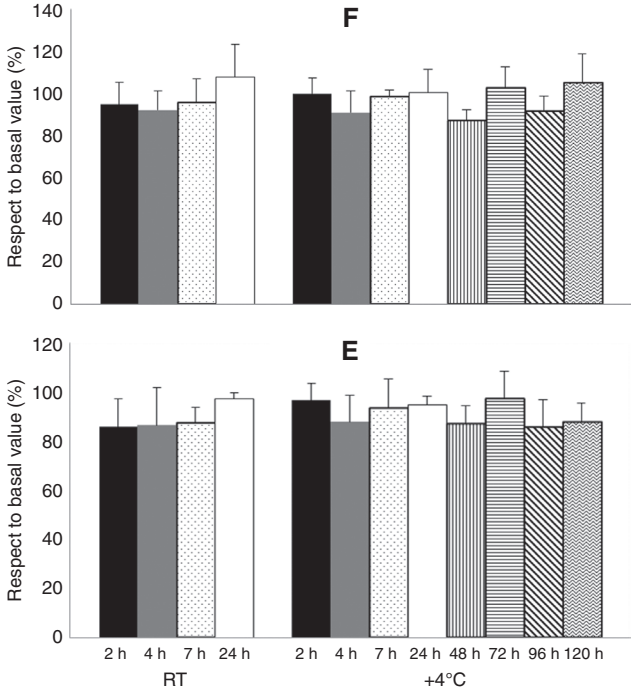

Figure 3 Sample stability: percentages of cortisol (F) and cortisone (E) concentrations measured in urine samples according to different temperatures and times in comparison to basal values (urine sample immediately stored at -80°C).

of the 11β-hydroxysteroid dehydrogenase (HSD) type 2, such as the AME syndrome [2]. Moreover, some authors [23] reported that urine E determination may be a useful complementary analyte to urine F for a more meaningful assessment of functional glucocorticoid activity. Furthermore, the simultaneous measurements of urine E and F compared to F alone are reported to be clinically useful in the identification of hypercortisolism and type 1 diabetes mellitus [12, 24]. As expected, in the present study, significantly higher levels for F, E and F/E ratio have been observed in CS patients than in healthy individuals [1, 5].

The reference range for urinary F calculated in our study is comparable to others obtained using LC-MS/MS methods [22, 25, 26], although different sample preparations have been proposed. Instead, in the current literature, few data have been published concerning the reference ranges for urine E [22, 26]; as with the F/E ratio, studied subjects on this issue are scarce [26–28]. Taylor et al. [22] reported the reference ranges for F and E in urine from 125 males and 140 females between the ages of 3 and 82 years without calculating the reference range for urine F/E ratio. McWhinney et al. [27] reported the urinary F/E ratio range (0.07–1.19, median 0.27) in 23 patients for whom hypercortisolism was ruled out on the basis of routine clinical and laboratory test results. Furthermore, the reported urine F/E ratio levels in 20 volunteers were 0.39±0.03 [28]. Nevertheless, the reference ranges obtained in our protocol (n=98 subjects) for both the E (41–364 nmol/24 h) and the F/E ratio (0.14–1.09) are comparable with those proposed in the studies made by these authors. Considering that the population involved in our protocol was more numerous than those in the literature, the obtained F/E ratio reference range suggested by us should be considered a useful tool in making a more reliable definition of physiological and pathological conditions.

Conclusions

A sensitive liquid chromatography tandem mass spectrometry method was developed and validated for the measurement of F and E in urine. The analytes and their stable isotope internal standards eluted within 5 min using a gradient elution. On-line SPE was employed for simple sample preparation, and the method was optimized to control for unpredictable interfering substances using two qualifier ions for both cortisol and cortisone. Our findings indicate that the analytical method developed and proposed by us is suitable for routine purposes since it is straightforward, relatively simple to perform, sensitive and has a satisfactory turnaround time. It thus provides clinicians with the opportunity to investigate into Cushing's syndrome and other endocrinological diseases in daily practice, with the F/E ratio adding information useful to the physician, since the reference range for the F/E ratio has now been established. Further studies are in progress to better evaluate the diagnostic accuracy of this method and for harmonizing all steps of the urinary measurement of cortisol and cortisone [29].

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.
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