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Title: A comprehensive study for the validation of a LC-MS/MS method for the determination of free and total forms of urinary cortisol and its metabolites

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Abstract: Several pathological conditions can be related to the alteration of the urinary levels of cortisol (F) and its metabolites. The determination of each of them in the free and free plus conjugated form can provide a deeper insight into the impaired activity of the cortisol metabolism enzymes, thus improving the diagnosis protocol currently based only on the determination of total amount of urinary cortisol metabolites.
In that view, an LC-MS/MS method for the determination of the free and total amount of urinary F, cortisone (E), tetrahydrocortisol (THF), allo-tetrahydrocortisol (A-THF) and tetrahydrocortisone (THE) was thus developed and validated. Deconjugation of glucocorticoids was carried out by enzymatic hydrolysis. Analytes were extracted by solid phase extraction, separated by liquid chromatography and analyzed via electro-spray ionization (negative ion mode) triple-quadrupole mass spectrometry in the selected reaction monitoring mode using a stable isotope-labeled internal standard.
Baseline separation for all compounds, in particular the two stereoisomers A-THF and THF, was obtained. Matrix effects, not reported so far, were observed and minimized for the determination of urinary free E and THE. Validated range was 0.5-1000 ng/mL for A-THF and THF, 5-800 ng/mL for E and THE and 1-1000 ng/mL for F, with R2 values greater than 0.9981. The LOD and LOQ of the described method ranged from 0.1 to 3.0 ng/mL, while the extraction recoveries resulted close to 100% for all the glucocorticoids determined. Precision and accuracy were well within ±10%. As suggested by the results obtained in the preliminary study on polycystic ovary syndrome (PCOS) urine samples, the method can be used to support clinical diagnosis of pathologies related to cortisol metabolism. In fact, levels of free and total glucocorticoids in control subjects were in agreement with previously reported data, as well as free and total A-THF/THF ratio in PCOS patients. Conversely, in the latter free F/E and A-THF + THF/THE ratios were lower than in control subjects ($P<0.01$), suggesting a possible alteration of 11b-HSD1 and 11b-HSD2 activity, to be further investigated.

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Dear Editor

Please find below the details regarding the revisions made to the manuscript:

Ms. Ref. No.: JPBA-D-13-01131
Submitted Title: "Development and validation of a liquid chromatography-tandem mass spectrometric method to profile urinary cortisol metabolism"
New title :" A comprehensive study for the validation of a LC-MS/MS method for the determination of free and total forms of urinary cortisol and its metabolites"
Journal of Pharmaceutical and Biomedical Analysis

addressed by the referees.

Changes and corrections in the manuscript are highlighted in yellow.
Below we reported the comments to each point raised by the referees

- 1. In the title, the concept of "evaluation of cortisol metabolites with LC-MS/MS" is not good to explain what it has been done. Please reconsider the title to emphasize your technical achievement.
Authors answer : We changed the title into "A comprehensive study for the validation of a LC-MS/MS method for the determination of free and total forms of urinary cortisol metabolism"

- 2. In the abstract, the extraction recoveries should be mentioned along with results from free and total fractions.

Authors answer : Extraction recoveries and preliminary results on PCOS patients were mentioned in the abstract

- 3. In the experimental, the glucuronide conjugates should be also contained in the sample preparation for free fraction. Besides, concentrations of free and total fractions should be compared.

Authors answer: The referees observation is not clear: urine samples for free fraction determination contain glucuronide conjugates. The latter are washed away (NH_4OH and H_2O-MeOH washings) during the SPE purification of the urine for free fraction determination. The determination of free and total analytes are then made in two separated steps and samples.
Glucuronides are about 99% of the total content of urinary glucocorticoids, thus the concentrations range are quite different. This difference is well know, in particular regarding the tetrahydro form, and it is quite evident from the results reported in the tables. However, we added a sentence at the end of section 3.6 to stress this aspect.

- 4. Although the authors evaluated the dilution effects, the analyte-free urine could be conducted to establish the comprehensive analytical protocol. The urinary cortisol and its metabolites can be easily removed by charcoal-stripping and Oasis HLB SPE purifications.

Authors answer : As described in section 3.4, we carried out experiments also using analyte-free

urine obtained by charcoal treatment, among the others. We used Oasisi HLB purification too, with and without a previous charcoal treatment. Using all those matrices to build calibration curves for the analytes under investigations provided beautiful curves, very close to the solvent calibration curves, but they proved to be not suitable in reproducing the urine matrix and the related effects on some compounds. That's why we came to dilution evaluation. All of the above is described in details in section 3.4.

- 5. Did you synthesize a deuterated cortisol in this study? If not, a sentence "Deuterated cortisol was easily prepared ~" is not necessary.

Authors answer : Deuterated cortisol was prepared in our lab, following a reported procedure. That is claimed at line 108 in the Experimental. We stressed this aspect adding "in our lab" at line 116 and 281 (in yellow in the revised manuscript text)

- 6. A representative chromatogram obtained from the used blank urine should be also presented in Figure 2.

Authors answer : The blank urine we used for the calibration curves was a pool of urines sample, thus not so blank, as indicated in the text. We don't see which improvement to the manuscript can provide the chromatogram of that urine, as it resembles to a typical urine sample. Anyway, if the editor considers it essential, there is no problem to provide the chromatogram.

- 7. The efficiency of hydrolysis was mentioned in the results section. How did you evaluate the efficiency? Did you use the reference standards of conjugates of all analytes? Please explain in details in the experimental section.

Authors answer : The efficiency of conjugates enzymatic hydrolysis was evaluated following the approach reported in ref 26 and 27. Samples coming from the same pool of urines were hydrolyzed using different amount of enzyme and for different incubation periods, as reported in the Results section. When the concentration of the free forms reached a plateau, efficiency was assumed to be 100%.
We added a sentence explaining how we evaluated the efficiency in section 2.3.2 in the Experimental.

- 8. Figure captions should be revised to explain in brief.
Authors answer: captions of figures where changed as follows (yellow in body text):

Figure 1 : Structures of cortisol and its metabolites. The enzymes involved in conversion of cortisol into cortisone and the corresponding tetrahydrometabolites are reported

Figure 2 : Typical LC-MS/MS chromatographic profiles for the determination of cortisol and its metabolites in an urine sample. The chromatographic traces for the MS/MS transition of each compound (see Table 1 for conditions) are superimposed. It is worth notice the separation of THF from A-THF peak, as the two compounds share the same transition.

Figure 3 : Dilution plot of E. Plateau level for the concentration of E was obtained using a 0.1 or less dilution factor, indicating that a ten-fold or more dilution is necessary to eliminate the matrix effects for E.

- 9. In line 408, "epatic" must be corrected.
Authors answer: corrected

- 10. In conclusion, the quantitative results from the method validation should be omitted.
Authors answer: corrected

- 11. In the references, there are many typo including abbreviation of journal name (ref. 6, 8, 12 and 18) and authors' name (ref. 16).
Authors answer: References were checked and corrected when necessary.

- 12. In table 1, "transition" should be changed to "transition ion (m/z)".
Authors answer: corrected.

13. Table 3 and 4 should be merged.
14. Table 6 and 7 should be merged as well.

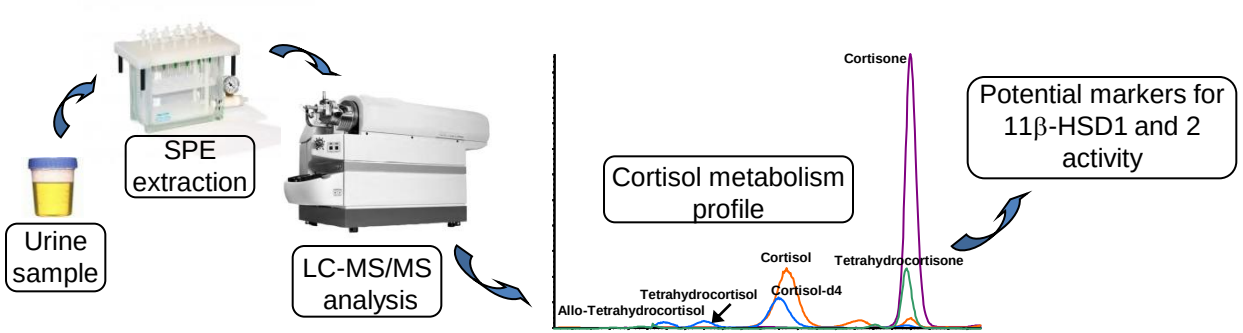
Authors answer: Tables 3 and 4 were merged into the new table 3, and table 6 and 7 were merged in the new table 5.

15. In Graphical Abstract, the analytical scheme is too much general. It would be better to describe cortisol metabolism associate with the results obtained from the PCOS.
Authors answer: we changed the graphical abstract to stress more the potential of a complete cortisol metabolism profile to assess 11β-HSD1 and 2 activity. We did not stress the association with the results obtained from PCOS samples, as those results are still preliminary and more samples should be examined to confirm them.

We hope that the comments are clear and these revisions meet the Journal of Pharmaceutical and Biomedical Analysis requirements for publication.

Yours sincerely
Dr. Angela Cuzzola

***Graphical Abstract**



Highlights

- An SPE LC–MS/MS method was validated to profile cortisol metabolism for clinical diagnosis
- Free and total cortisol and its metabolites (E, A-THF, THF, THE) were determined in human urine
- Interferents were separated and matrix effects thoroughly evaluated
- The method is sensitive, precise, accurate and adequate for clinical analysis
- The method was successfully applied in a preliminary study on PCOS urine samples

A comprehensive study for the validation of a LC-MS/MS method for the determination of free and total forms of urinary cortisol and its metabolites

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Abstract

Several pathological conditions can be related to the alteration of the urinary levels of cortisol (F) and its metabolites. The determination of each of them in the free and free plus conjugated form can provide a deeper insight into the impaired activity of the

cortisol metabolism enzymes, thus improving the diagnosis protocol currently based only on the determination of total amount of urinary cortisol metabolites.

In that view, an LC-MS/MS method for the determination of the free and total amount of urinary F, cortisone (E), tetrahydrocortisol (THF), allo-tetrahydrocortisol (A-THF) and tetrahydrocortisone (THE) was thus developed and validated. Deconjugation of glucocorticoids was carried out by enzymatic hydrolysis. Analytes were extracted by solid phase extraction, separated by liquid chromatography and analyzed via electro-spray ionization (negative ion mode) triple-quadrupole mass spectrometry in the selected reaction monitoring mode using a stable isotope-labeled internal standard.

Baseline separation for all compounds, in particular the two stereoisomers A-THF and THF, was obtained. Matrix effects, not reported so far, were observed and minimized for the determination of urinary free E and THE. Validated range was 0.5–1000 ng/mL for A-THF and THF, 5–800 ng/mL for E and THE and 1–1000 ng/mL for F, with R^2 values greater than 0.9981. The LOD and LOQ of the described method ranged from 0.1 to 3.0 ng/mL, while the extraction recoveries resulted close to 100% for all the glucocorticoids determined. Precision and accuracy were well within ±10%. As suggested by the results obtained in the preliminary study on polycystic ovary syndrome (PCOS) urine samples, the method can be used to support clinical diagnosis of pathologies related to cortisol metabolism. In fact, levels of free and total glucocorticoids in control subjects were in agreement with previously reported data, as well as free and total A-THF/THF ratio in PCOS patients. Conversely, in the latter free F/E and A-THF + THF/THE ratios were lower than in control subjects ($P<0.01$), suggesting a possible alteration of 11β-HSD1 and 11β-HSD2 activity, to be further investigated.

Keywords Cortisol Metabolites Urine LC-MS PCOS

1. Introduction

Among glucocorticoids, cortisol (F) and its metabolites (Fig. 1) play different roles in human physiology and several pathological conditions [1,2]. Cortisol deficiency has been reported in patients with Addison's syndrome leading to hypotension, while excess cortisol is observed in Cushing's syndrome and determined by the onset of hypertension [3]. Altered metabolism of cortisol is also responsible for the apparent mineralocorticoid excess (AME) syndrome, congenital adrenal hyperplasia and adrenal insufficiency [4]. Recent evidences suggest that obesity, insulin resistance, hypertension, polycystic ovary syndrome [5] and hyperthyroidism [6] may also be related to this alteration. These and other disorders can be detected by monitoring the interconversion of cortisol and cortisone (E) and the related metabolites [7,8], in which several enzymes are involved. In the kidney and colon, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) inactivates cortisol to cortisone, while in the liver and adipose tissue, cortisol is regenerated from cortisone by 11β-hydroxy steroid dehydrogenase type 1 (11β-HSD1) [9]. Moreover, in the liver, the A-ring reductases (5α, 5β and 3α reductase) convert cortisol and cortisone to their tetrahydrometabolites, allo-tetrahydrocortisol (A-THF), tetrahydrocortisol (THF) and tetrahydrocortisone (THE) [10].

Congenital or acquired defects in the enzymes involved in the metabolism of cortisol induce alterations of the level of cortisol and its derivatives, leading to the consequent diseases cited above. Glucocorticoids are largely present in urine as conjugates derivatives [11]. The diagnosis is generally based on the determination of the total amount of urinary glucocorticoids, expressed as F/E, (A-THF + THF/THE) or A-THF/THF ratios. However, the evaluation of the amount of the free forms can provide further information [2,9,12] and a deeper insight into the activity of the enzymes

involved in the metabolism of cortisol (for instance 11β-HSD2 [13] or 5α-reductase [2]), thus affording a more accurate diagnosis of the related pathologies.

Several analytical methods have been developed for the determination of cortisol and its metabolites by GC–MS based techniques [14,15], but they involve time-consuming derivatization steps due to the low volatilities of corticosteroids. In recent, LC-MS methods have been applied to the analysis of corticosteroids in biological fluids, but very few of them address the determination of F, E, THE, THF and A-THF all at once. In particular, to the best of our knowledge, only two papers [16,17], reported the separation of the two isomers THF and A-THF without chemical derivatization [18], that otherwise are determined together as THF + A-THF [12], thus without providing any information regarding the 5α and β-reductase activity.

In that view, in our previous paper [16] we compared the application of hyphenated MS techniques for the simultaneous measurement of free glucocorticoids, LC/MS resulting the most suitable for the task. Starting from there, here we report a comprehensive development study, with particular regard to matrix effects, and the validation of a method for the determination of free and total urinary F, E, THE, THF and A-THF. The method, that comprises solid phase extraction (SPE) and LC-MS/MS quantification, was developed within our project on the possible correlation between the levels of those compounds (free and conjugated form) and the clinical diagnosis of related pathologies. In particular, here we also report a preliminary application of the developed protocol to urines coming from polycystic ovary syndrome (PCOS) patients.

*** Figure 1 here ***

Fig. 1. Structures of cortisol and its metabolites. The enzymes involved in the conversion of cortisol into cortisone and the corresponding tetrahydrometabolites are reported

2. Experimental

2.1 Reagents and chemicals

All chemicals used (methanol, water and formic acid) were HPLC grade from Carlo Erba Reagenti S.p.A. (Rodano, MI, Italy). 5β-Pregnan-3α,11β,17,21-tetrol-20-one (tetrahydrocortisol, THF, MW=366), 5α-Pregnan-3α,11β,17,21-tetrol-20-one (allo-tetrahydrocortisol, A-THF, MW=366) were purchased from Steraloids, Inc. (Newport, RI, USA); 5β-Pregnan-3α,17α,21-triol-11,20-dione (tetrahydrocortisone, THE, MW=364), Pregn-4-en-11β,17α,21-triol-3,20-dione (cortisol, F, MW=362), Pregn-4-en-17α,21-diol-3,11,20-trione (cortisone, E, MW=360), β-glucuronidase by *Helix Pomatia* type H-1 (arylsulfatase ≥10000 U/g solid) were from Sigma–Aldrich Corporation (St. Louis, MO, USA). Pregn-4-en-9,11,19,19-^{2}H-11β,17α,21-triol-3,20-dione (cortisol-d_4, Fd_4, MW=366) was prepared in our lab according to a reported procedure [19]. SPE was carried on Oasis HLB SPE cartdriges (20 mL, 30 mg; Waters) with a Supelco extraction vacuum manifold.

The activity of *Helix Pomatia* sample was evaluated following the rate of hydrolysis of 4-nitrophenyl-β-D-glucuronide by UV, according to the procedure reported by Cichna [20]. The β-glucuronidase activity resulted 570 U/g.

2.2 Calibrators and quality control samples

Original stock solutions of each compound (1 mg/mL in MeOH) were stored at -20°C up to 6 months without any changes and brought to room temperature prior to use. Standard solutions of the analytes were prepared by diluting original stock solutions with MeOH. Cortisol-d_4 (Fd_4) were used as internal standard (IS). Solvent based standard calibration curves were constructed by using neat solutions obtained by

dilution with H_2O (50:50) of the standard methanolic solutions, in the way of obtaining a range of concentration 0.5-1000 ng/mL containing the IS at 50 ng/mL.

A pool of 24h urine collections, provided by 25 healthy volunteers, was used as reference matrix for matrix based calibration curves. The latter were constructed by spiking aliquots of the pool of reference urine (2 mL) with standard solutions (100 μL), in the way of obtaining a range of concentration 0.5-1000 ng/mL in the sample, before injection, containing the IS at 50 ng/mL. A further urine aliquot spiked only with 50 μL of standard solution of IS (1 μg/mL in MeOH) was used as blank.

Quality control (QC) working solutions in MeOH at concentration of 150, 1500, 4500 ng/mL of each analyte, containing 500 ng/mL of IS, were prepared by dilution of stock solutions. All matrix based calibration standards and QC samples were extracted and analyzed in the same manner as the unknown samples.

For the evaluation of recovery and matrix effect three sets of samples were analyzed:

1) QC standard solutions in neat solvent obtained from QC working solution by dilution 1 to 10 with MeOH-H_2O (50:50, v/v).

2) Pre-spiked samples: QC working solutions (100 μL) were added to three aliquots (2 mL) of the urine pool and then extracted by SPE as described above. 100 μL of the eluate was concentrated under flow nitrogen at 35°C and the residue was reconstituted in 200 μL of MeOH-H_2O (50:50, v/v). An aliquot of urine spiked with 50 μL of the methanolic standard solution of IS (1 μg/mL) was used as blank.

3) Post-spiked samples: three aliquots of 2 mL of urine pool were extracted by SPE as described above. 100 μL of the eluates were concentrated under flow nitrogen at 35°C and the residues were reconstituted in 20 μL of the 150, 1500 and 4500 ng/mL QC working solutions, 80 μL of MeOH and 100 μL of H_2O to provide the three QC samples with a 15, 150, 450 ng/mL final concentration after spiking, respectively. The residue

from a fourth aliquot was reconstituted in 10 μL of the methanolic standard solution of IS (1 μg/mL), 90 μL of MeOH and 100 μL of H_2O, and used as blank.

2.3 Sample preparation

2.3.1 Determination of unconjungated glucocorticoids

50 μL of methanolic standard solution of IS (1 μg/mL) was added to urine sample (2 mL) previously centrifuged at 2000 g for 30 min. Each sample was loaded onto an Oasis HLB SPE cartdrige preconditioned with 1 mL of MeOH and followed by 1 mL of H_2O. The loaded cartdrige was subsequently washed with 1 mL of 10% MeOH and 0.5 mL of 2% NH_4OH in 40% MeOH, and then glucocorticoids were eluted with 0.5 mL of MeOH. For LC-MS analysis, 100 μL of eluate was diluted to 200 μL with H_2O.

2.3.2 Determination of total (free plus conjugated) glucocorticoids

Enzymatic hydrolysis conditions were optimized carrying out various experiments using different concentration of the enzyme and incubation periods, following the release of the unconjugated form by LC-MS/MS determination. When the concentration of the free forms reached a plateau, efficiency was assumed to be 100%.

Deconjugation of glucocorticoids was carried out by enzymatic hydrolysis on 125 μL of urine sample with 50 μL of *Helix Pomatia* solution (β-glucuronidase activity 40.5 U/mL in H_2O) in 200 μL of sodium acetate buffer (pH 5.1;0.5 M) for 4h at 55°C. The reaction mixture was then diluted to 1 mL with H_2O and spiked with 25 μL of standard solution of IS (20 μg/mL). The resulting solution was extracted by SPE as described above. For LC-MS analysis, 100 μL of eluate (0.5 mL) was diluted 1 to 20 with MeOH-H_2O (50:50 v/v).

2.4 Instrumentation and chromatographic conditions

An Applied Biosystems-MDS Sciex API 4000 triple-quadrupole mass spectrometer (Concord, Ont., Canada), equipped with Turbo-V IonSpray (TIS) source and interfaced to Perkin-Elmer Series 200 Micro High pressure mixing pump and a Series 200 Autosampler (Perkin-Elmer, Boston, MA, USA), was used for LC/MS/MS analysis.

The analytes were separated on a Restek Allure Biphenyl column (50 × 2.1 mm, 3 μm) (Bellefonte, PA, USA) using as mobile phase MeOH and H_2O with 0.1% of formic acid and flow-rate at 0.3 mL/min. Separation was achieved under the following chromatographic conditions: 48% MeOH for 13.5 min then increased to 52% MeOH in 0.1 min and maintained for 4.4 min. Column equilibration time was 1.5 min. The temperature of the chromatographic column was maintained at 30°C. Injection volume was 20 μL. We verified that injection of samples in pure methanol altered the chromatographic performances. Thus all the samples were injected as MeOH:H_2O 50:50 mixture, that substantially are the starting chromatographic conditions.

Quantitation was performed using Selected Reaction Monitoring (SRM) in negative ion mode with a dwell time of 200 ms. The Applied Biosystems Analyst software "Quantitative Optimization" wizard was used to discern the optimal parameters by flow injection analysis. The turboionspray temperature was 550°C, other optimized parameters were: ionspray voltage (IS), -4.2 kV; gas source 1 (GS1), 55 psi; gas source 2 (GS2), 55 psi; curtain gas (CUR), 35 psi. MS–MS product ions were produced by collisional induced dissociation (CID) of selected precursor ions in the LINAC collision cell (Q2) and mass-analyzed in the second mass filter (Q3). Nitrogen was used as collision gas (CAD) at 8 mPa pressure. Additional experimental conditions for MS–MS product ions spectra are reported in Table 1.

*** Table 1 here ***

2.5 Method validation

Calibration curves were obtained by plotting the analytes to IS peak areas ratios versus analytes to IS nominal concentrations ratios. The ratios of the peak area of the analytes to IS were corrected for the presence of endogenous compounds in the matrix, by subtracting the corresponding ratios of the blank. The concentrations of the analytes in unknown and QC samples were obtained multiplying the known IS concentration by the ratio of analyte to IS concentrations provided by the calibration curve. All calculations were performed in EXCEL® software (Microsoft Corporation, USA) using the function formulas for mean ("average") and standard deviation ("stdev"). The limit of detection (LOD) and limit of quantitation (LOQ) were determined from the slope (S) and the standard deviation of the intercept (σ_y) of matrix based calibration curve, according to the formulas $LOD = 3 \times (\sigma_y/S)$; $LOQ = 10 \times (\sigma_y/S)$ [21].

Intra-day accuracy and precision were evaluated from replicate analyses (five runs, five replicates per run) of QC samples at three different final concentrations (15, 150, 450 ng/mL) carried out in the same day. QC working solutions were pre-spiked to aliquots of the urine pool used as reference matrix. Inter-day accuracy and precision were assessed from the same sets of QC samples in five different days.

Precision was expressed as coefficient of variation (CV%) and calculated using the formula: CV% = (standard deviation/mean) × 100%.

Accuracy is the degree of closeness of the determined values to the nominal, and it was expressed as percentage of the measured versus the nominal concentration.

Absolute and extraction recoveries were expressed as percentage calculated comparing the peak areas of the QC pre-spiked samples ($A_{qc\text{-}pre}$) with the peak areas of the QC standard solutions (A_{std}) for absolute recovery (RE_{abs} (%) = 100 × $A_{qc\text{-}pre}$ / A_{std}), and with the peak area of QC post-spiked samples ($A_{qc\text{-}post}$) for extraction recovery (RE_{ext} (%) = 100 × $A_{qc\text{-}pre}$ / $A_{qc\text{-}post}$), respectively.

Reduction of matrix effect by dilution was evaluated and calculated according to the method described by Kruve et al. [22]. An aliquot of the pool of urine was extracted by SPE and then spiked with a standards solution to provide a final concentration of 450 ng/mL for all the analytes. The eluate and the reference standards solution (450 ng/mL) were analyzed at their initial concentration and after dilution from 1 to 2 to 1 to 100 with a MeOH-H_2O (50:50, v/v) mixture. A dilution factor of 0.5 (see section 3 for details) for the eluate coming from the SPE was then used to evaluate the matrix effect (ME) as percentage comparing the peak areas of the QC post-spiked samples with the peak areas of the QC samples prepared in neat solution (ME (%) = 100 × $A_{qc\text{-}post}$ / A_{std})

2.6 Preliminary study on PCOS samples

24 h urine samples coming from healthy volunteers (n = 25 female, age 19-49) and from PCOS patients (n = 12 female, age 18-37) were analyzed using the validated method here described for the determination of the level of free and total E, F, THE, THF and A-THF.

3. Results and Discussion

3.1 LC-MS/MS analytical method development

As already reported in our previous paper [16] on the determination of the free fraction of glucocorticoids, negative ion mode Electrospray Ionization (ESI) provides very high sensitivity and specificity for the determination of cortisol and its metabolites [23]. Under our experimental conditions, fragmentation of $[M+HCOO]^-$ adduct provided $[M\text{-}CH_2O\text{-}H]^-$ as the most abundant ion for all the compounds examined [16]. This transition was selected for quantification of the five glucocorticoids and internal standard, Fd_4, in SRM mode.

In order to further increase the ESI sensitivity, we moved from the 50 × 4.6 mm [16] to the 50 × 2.1 mm Restek Allure Biphenyl column. Isobaric endogenous compounds were detected in the SRM transition profile of A-THF and F coming from urine samples analysis, not present in standards mixtures and co-eluting with the two analytes using the 4.6 mm i.d. column. Thus, elution conditions were carefully tuned in terms of mobile phase composition and ramp, flow rate, injection volume and column temperature (see Experimental Section for details), leading to the baseline separation of the two stereoisomers THF and A-THF, besides the other compounds under study. Comparing the data coming from the previous and new developed method on a set of control urines, we found that the final improved chromatographic conditions avoided an overestimation of the amount of A-THF and F by 10-15% on average, in some cases reaching 30-40% for F.

LC-MS/MS ionization parameters were then optimized for strongest product ion signal intensities, following the transitions reported in Table 1.

We used Fd_4 in place of methyl-prednisolone as internal standard as it provided an accuracy increase by 10-15% in the determination of F and its metabolites, without substantially affecting the determination accuracy for E and THE. Deuterated cortisol was easily prepared in our lab at low cost in few simple steps starting form cortisone [19]. In Fig. 2, a representative chromatogram of the separation of the five components of an urine sample is reported.

*** Figure 2 here ***

Fig. 2. Typical LC-MS/MS chromatographic profiles for the determination of cortisol and its metabolites in an urine sample. The chromatographic traces for the MS/MS transition of each compound (see Table 1 for conditions) are superimposed. It is worth

notice the separation of THF from A-THF peak, as the two compounds share the same transition.

3.2 Enzymatic hydrolysis

We wanted to determine not only the five urinary free glucocorticoids but also their total amount, to have a complete overview of the analytes profile. As glucocorticoids are present in urine largely as conjugates derivatives (about 95% glucuronides and 3-4% sulfates), their total amount in urine is generally determined after enzymatic deconjugation. In that regard, one of the most used enzymes mixture is the *Helix pomatia* type H-1 juice, which is characterized by glucuronidase and arylsulfatase activities. As different deconjugation conditions were described in literature [24,25], we carried out a series of experiments to find the best procedure for our purposes.

We set the temperature of the enzymatic hydrolysis at 55°C, as lower temperatures (37-40°C) were reported to require very long incubation time (24–48 h) [26]. Hydrolysis experiments of urine samples were carried out varying the concentration of the enzyme and the incubation time. Differently from previously reported data [27], we observed that an enzyme concentration of 1.4 U/mL was not adequate to achieve complete hydrolysis of the analytes conjugates after 4 h of incubation time at 55°C, in particular as regard A-THF. Plateau levels of the five glucocorticoids were reached increasing the *Helix Pomatia* concentration up to 5.4 U/mL over 4 h of incubation. Thus, those conditions were employed to determine the total amount of E, F, THE, THF and A-THF in urine samples.

3.3 Sample preparation

We used Oasis HLB cartridges for the SPE extraction of the five analytes, as they proved to be very efficient in terms of recovery and reproducibility in the extraction of cortisol and its metabolites from urine [16,17]. The amount of urine sample required for the analysis of the free and total glucocorticoids was investigated. For the determination of free glucocorticoids, urine volumes below two milliliters afforded the accurate determination of E and F and THE, but not of THF and A-THF. Thus, washing and elution conditions were optimized on a two milliliters urine loading. For the determination of total glucocorticoids, the volumes of 500, 250 and 125 μL of urine were investigated. The latter resulted adequate for the detection of the studied analytes after the enzymatic hydrolysis and the appropriate dilution to avoid signal saturation.

Samples were always analyzed as MeOH-H_2O (50:50, v/v) solvent mixture, that practically are the starting elution conditions of the analytical method, as alterations of the chromatographic performances were observed for some compounds when injecting samples in pure methanol.

3.4 Calibration curves, matrix effect and recoveries

Calibration curves should be prepared in the same blank matrix as the samples. For this purpose, different blank urine samples have been reported in literature. Steroid free urine matrices were generally obtained by removing the endogenous steroids by treatment with sorbents (i.e. activated carbon [28]). In some cases, a mixture of water and salts and other components contained in urine (like NaCl, KCl, $CaCl_2$, urea and phosphates) was used as synthetic urine free of steroids [29]. We explored all those options and compared them with the calibration curve constructed using MeOH-H_2O as solvent. The steroid free matrices were extracted according to the developed SPE protocol and then spiked with specified amounts of standard solution to cover the 0.5-1000 ng/mL concentration range. The slope of the calibration curve built with neat

339 solvent, synthetic urine and urine treated with activated carbon resulted almost the
340 same, suggesting the substantial absence of matrix effects. However, to verify how
341 close those matrices mimed real samples, we also constructed a calibration curve by
342 spiking urine aliquots, coming from a pool of healthy volunteers 24 h urines, with
343 standard solutions in the same range of the concentrations used before. The slope of the
344 latter curves for E and, in a lesser extent, for THE, proved to be different from those
345 obtained using steroid free matrices, indicating the presence of a matrix effect for these
346 two compounds. Thus, in our hands, the steroid free matrices proved to be not adequate
347 for the accurate determination of E and THE, differently from what reported elsewhere
348 [17,29].
349 We then investigated if sample dilution could reduce or eliminate the matrix effects,
350 following the approach employed recently by Kruve et al. [22]. According to this
351 approach, reduction or elimination of matrix effects upon dilution are evaluated through
352 the comparison of the calculated concentrations of undiluted and diluted samples.
353 In our case, we spiked a sample of the pool of urine extracted by SPE and investigated
354 dilution factors from 1 to 0.01. As expected, A-THF, THF and F were slightly affected
355 by dilution, showing the substantial elimination of the matrix effect just with a 0.5
356 dilution factor. Dilution 1 to 5 was necessary for THE to achieve the same result, while
357 E concentration reached the plateau using a dilution factor of 0.1 (see Fig. 3**)**. However,
358 the latter could not be used for our purposes, as free THF and A-THF are present in
359 urine in low amounts. Hence, matrix based calibration curves had to be used to
360 minimize inaccuracy in the determination of THE and E. From a set of experiments
361 carried out on several urine matrices, we eventually chose to employ a 0.5 dilution
362 factor for the eluate coming from SPE, in order to further avoid possible inaccuracy in
363 the determination of free forms. In fact, in that way we verified the complete

elimination of the small fluctuations in quantification observed using some matrices, maintaining the required level of sensitivity.

For the determination of the total amount of the five glucocorticoids, the calibration curve built in MeOH-H_2O was used for calculations, as the eluates coming from the SPE of the hydrolyzed samples were diluted 1 to 20 to fit their concentrations into the dynamic range of the calibration curve. In that case, the matrix effect is eliminated for all the components under study using such dilution, as described above.

*** Figure 3 here ***

Fig. 3. Dilution plot of E. Plateau level for the concentration of E was obtained using a 0.1 or less dilution factor, indicating that a ten-fold or more dilution is necessary to eliminate the matrix effects for E.

On the basis of the obtained results, the post-extraction spiked method [30] was employed to assess the matrix effect as percentage using a dilution 1 to 2 of the eluate coming from the SPE. Matrix effect values (ME) are reported in Table 2. A value equal to 100% indicates the absence of matrix effect, while values over or less than 100% indicates an ionization enhancement or suppression, respectively.

*** Table 2 here ***

The extraction recoveries resulted close to 100% (Table 2) for all the glucocorticoids examined at the three QC concentrations tested (15, 150, 450 ng/mL), in a reproducible way, indicating that the SPE protocol developed was very efficient. As regard the absolute recoveries (Table 2), almost the same results were obtained for F, A-THF and THF, while E and THE provided values lower than 100%, 71 and 85% in the worst case respectively, because of matrix effect resulting in signal suppression.

3.5 Linearity, limit of detection, precision and accuracy

The linearity of the method was evaluated by analyzing calibration standards of the five analytes in the range 0.5-1000 ng/mL, both in neat solution and in the presence of matrix. The range of linearity for each analyte for solvent and matrix based calibration curves are reported in Table 3. Solvent based curves were used for the determination of the amount of unconjugated plus conjugated glucocorticoids, as described above. The resulting correlation coefficients were very good, ranging from 0.9981 to 0.9995. Regarding E and THE, the linear range of solvent and matrix based calibration curves reflected the matrix effect observed in the determination of these two compounds. In fact, signal saturation for THE and E was obtained over 600 ng/mL for the solvent based calibration curve, while over 900 ng/mL for the matrix based calibration curve, indicating the suppression of the response due to the matrix effect. The effect influenced also the lower limit of detection and quantitation, that were higher for the matrix based curve compared to the standards solution curve, as expected. Limit of detection (LOD) and limit of quantitation (LOQ), reported in Table 3, were determined according to ICH (International Conference on Harmonization) guideline [31] from the slope (S) and the standard deviation of the intercept (σy) of the calibration curve.

*** New Table 3 here ***

Precision and accuracy were evaluated using QC samples pre-spiked in urine aliquots at three different concentrations (15, 150, 450 ng/mL). As reported in Table 4, intra and inter-day precision were 0.4–7.7% and 1.1–10% respectively, thus more than acceptable for the validation of the method. In that regard, also the accuracy data were pretty good, ranging from 90 to 109%.

*** Table 4 here ***

3.6. Preliminary study on PCOS urine samples

The validated reported protocol was employed in a preliminary study within our project regarding the possible relationship of urinary glucocorticoids to PCOS condition and other pathologies related to cortisol metabolism. Since conjugation is mainly a hepatic process, the ratio of total urinary tetrahydrometabolites of cortisol and cortisone (A-THF + THF/THE ratio) gives information on the activity of hepatic 11β-HSD1, while the ratio of urinary free F and E provides an index of activity of 11β-HSD2 [2]. In addition, the urinary A-THF/THF ratio can give information about the global activity of 5α-reductase [10]. Thus, the amount of the five analytes and the ratios related to the activity of 5α-reductase, 11β-HSD1 and 2 were determined both for the free and total forms in 24 h urine samples coming from healthy subjects and PCOS patients.

In agreement with previously published data [5,32], both free and total A-THF/THF ratio were higher than in control subjects, while A-THF + THF/THE ratio of total forms showed no significant differences between PCOS patients and controls (Table 5). As expected [11], free forms are present in very low amount compared to the total fractions, in particular for the tetrahydro metabolites (i.e. free A-THF is present about two thousands-fold lower than total A-THF form).

Conversely, a possible alteration of 11β-HSD1 and 11β-HSD2 activity was observed, as the free F/E and A-THF + THF/THE ratios were lower than in control subjects ($P<0.01$). That finding is particularly interesting and will be further investigated on a larger number of patients in the next future, as for the time being, the alteration of 11βHSD1 activity could be detected only using total A-THF + THF + αcortol/THE + cortolones ratio as marker [5,32].

*** Table 5 here ***

4. Conclusions

A solid phase extraction LC–MS/MS bio-analytical method was developed and validated with a thorough study for the quantification of free and free plus conjugated cortisol, cortisone and their metabolites in human urine. The chromatographic method allowed the baseline separation of all the compounds under study, in particular the separation of the two stereoisomers A-THF and THF from the isobaric endogenous unknown compounds interfering with their determination, thus avoiding overestimation. Validated range for all the compounds, LOD, LOQ, intra and inter-day precision and accuracy meet the suggested industry and regulatory acceptance criteria. The resulting extraction and absolute recoveries proved to be very satisfactory and reproducible, showing the ruggedness of the procedure.

The study of matrix effects showed that free steroids urines used as matrix in several methods are not adequate for accurate determination of E and THE, that hence requires matrix based calibration curves.

The validated procedure was successfully applied in a preliminary investigation carried out to profile the five analytes in urine samples of PCOS patients. The data obtained support the hypothesis that the determination of both urinary free and total glucocorticoids can provide precious information and new perspectives in the diagnosis of pathological conditions related to the alteration of cortisol metabolism.

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Table 1. Experimental MS conditions for analytes determination.

	Transition Ion (*m/z*)	DP[a]	EP[a]	CE[a]	CXP[a]
F	407-331	-50	-8	-25	-8
E	405-329	-50	-10	-20	-8
THF-ATHF	411-335	-50	-8	-25	-8
THE	409-333	-45	-6	-25	-4
Fd_4	411-335	-50	-10	-25	-10

[a]DP = Declustering Potential; EP = Entrance Potencial; CE = Collision Energy; CXP = Cell Exit Potential

Table 2. Recoveries and matrix effect of each analyte (n. of replicates = 5)

	QC Conc. (ng/mL)	RE_{ext} (%, ±SD)	RE_{abs} (%, ±SD)	ME (%, ±SD)
ATHF	15	109±5	101±5	96±3
	150	105±6	99±6	102±2
	450	99±6	99±6	103±6
E	15	99±2	71±5	65±7
	150	102±2	74±4	70±6
	450	102±3	77±5	72±5
F	15	101±3	97±3	93±7
	150	103±3	102±3	96±9
	450	101±2	98±2	97±1
THE	15	104±1	88±4	90±7
	150	103±2	90±5	87±4
	450	100±3	85±3	84±6
THF	15	112±3	106±3	94±4
	150	105±2	97±4	95±7
	450	100±4	100±4	102±7

Table 3. Solvent and matrix based calibration curves parameters.

Solvent based calibration curves						
	Slope	Intercept	R^2	Calibration range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
A-THF	0.13	0.002	0.9994	0.5-1000	0.05	0.15
E	3.01	0.047	0.9981	0.5-500	0.2	0.6
F	1.75	0.241	0.9990	1-1000	0.1	0.3
THE	0.89	0.051	0.9960	0.5-500	0.1	0.3
THF	0.31	-0.014	0.9995	0.5-1000	0.05	0.15

Matrix based calibration curves						
	Slope	Intercept	R^2	Calibration range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
A-THF	0.12	-0.006	0.9994	0.5-1000	0.1	0.3
E	1.22	0.969	0.9940	5-800	1	3
F	1.73	0.229	0.9990	1-1000	0.2	0.6
THE	0.58	0.237	0.9890	5-800	0.2	0.6
THF	0.29	-0.007	0.9993	0.5-1000	0.1	0.3

Table 4. Intra-day and inter-day precision and accuracy of QC analytes determination (five runs, five replicates per run).

			Precision	
	QC Conc. (ng/mL)	Accuracy (%)	Intra-day (CV%)	Inter-day (CV%)
ATHF	15	100	0.8	2.4
	150	90	2	2.2
	450	90	2.5	3.5
E	15	105	0.8	1.8
	150	107	3.2	4.7
	450	97	2.8	9.6
F	15	101	0.8	2.8
	150	105	0.9	1.8
	450	95	0.4	1.8
THE	15	102	0.8	1.1
	150	109	0.9	1.3
	450	101	0.5	7.3
THF	15	109	1	1.3
	150	101	0.8	1.5
	450	94	1.2	1.9

Table 5. Concentration and relative ratios of free and total urinary corticoids in control subjects and patients with PCOS.

	Free Glucocorticoids (μg/day urine)		Total Glucocorticoids (mg/day urine)	
	Controls	PCOS	Controls	PCOS
	n = 25 (19-49 years)	n =12 (18-37 years)	n = 25 (19-49 years)	n =12 (18-37 years)
F	24.00 ± 1.10	6.52 ± 0.24	0.11 ± 0.01	0.11 ± 0.01
E	42.20 ± 2.05	23.14 ± 0.11	0.22 ± 0.01	0.23 ± 0.01
THF	8.99 ± 0.35	3.74 ± 0.21	2.70 ± 0.12	1.80 ± 0.06
A-THF	0.77 ± 0.02	0.52 ± 0.02	1.93 ± 0.12	1.57 ± 0.05
THE	10.04 ± 0.61	11.71 ± 0.72	4.07 ± 0.22	4.60 ± 0.18
THF+A-THF/THE	0.97 ± 0.10	0.61 ± 0.04	1.10 ± 0.12	0.83 ± 0.05
A-THF/THF	0.08 ± 0.01	0.17 ± 0.01	0.65 ± 0.07	0.90 ± 0.06
F/E	0.57 ± 0.05	0.30 ± 0.01	0.54 ± 0.06	0.51 ± 0.05

Figure 1

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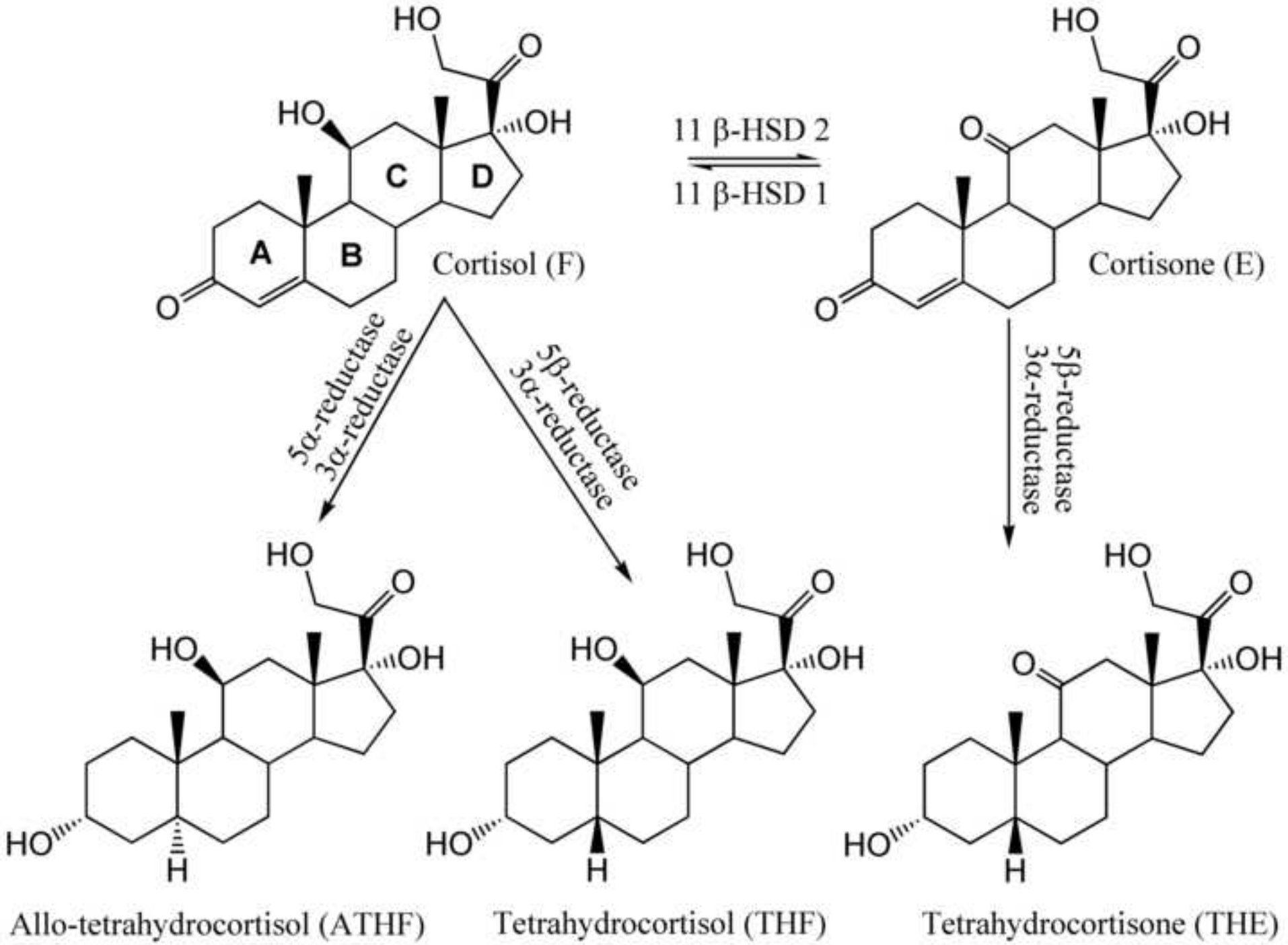

Figure. 2.

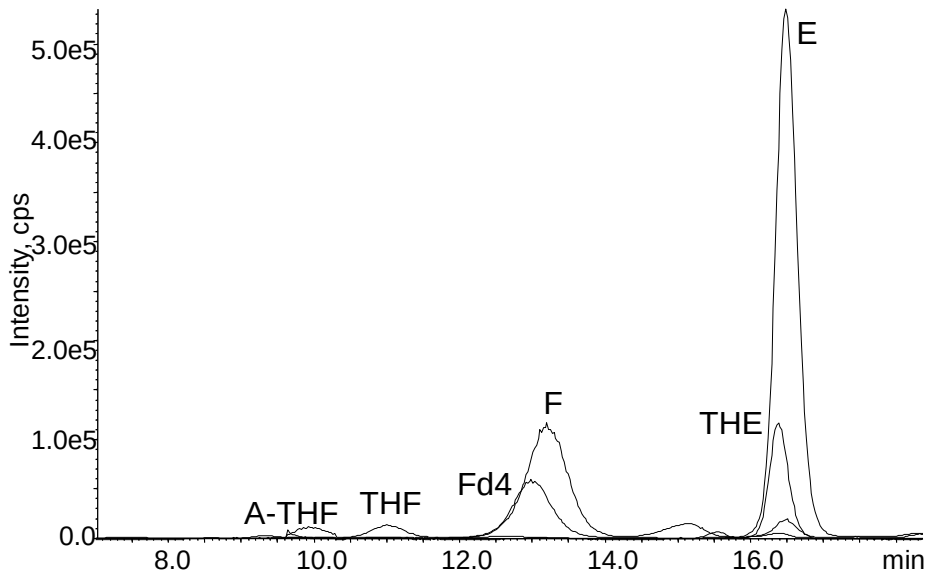
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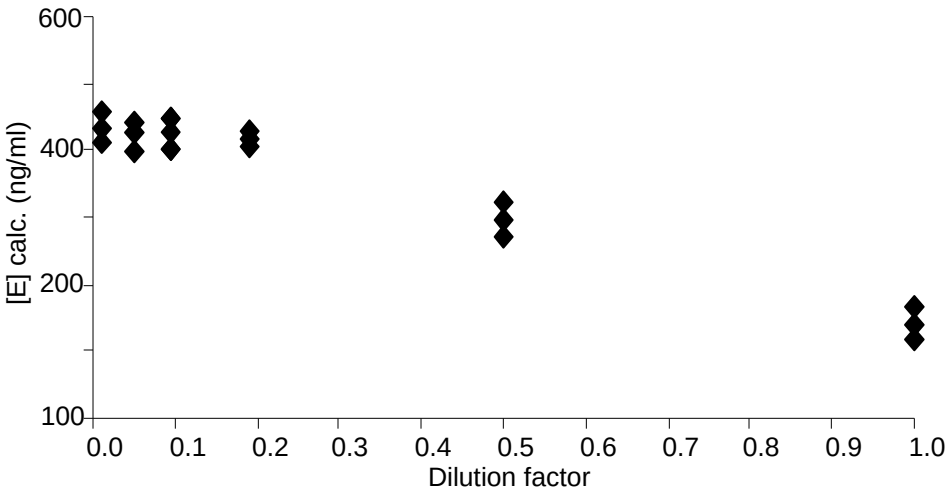
Figure 3.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