Journal of Pharmaceutical and Biomedical Analysis 140 (2017) 174–181

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Detailed analysis of cortisol, cortisone and their tetrahydro- and allo-tetrahydrometabolites in human urine by LC–MS/MS

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ARTICLE INFO

Article history:
Received 10 January 2017
Received in revised form 17 March 2017
Accepted 18 March 2017
Available online 21 March 2017

Keywords:
11β-hydroxysteroid dehydrogenase
5α-reductase
Glucocorticoids
Allo-tetrahydrocortisone
Bioanalysis

ABSTRACT

Cortisol (F) and cortisone (E) are metabolized to A-ring reduced metabolites in the reactions catalyzed by 5α- and 5β-reductase. 5α-tetrahydrocortisol (alloTHF) and 5β-tetrahydrocortisol (THF) are produced from F. The metabolism of E takes place in analogy to form alloTHE and THE. Up to now, the analysis of endogenous glucocorticoids did not consider alloTHE, limiting the metabolism of E to THE only. Nevertheless, such simplification can generate inaccuracy in the assessment of the function of enzymes crucial for glucocorticoids metabolism: 11β-hydroxysteroid dehydrogenase type 1 and type 2 (11β-HSD1 and 11β-HSD2), as well as 5α- and 5β-reductase.

The paper presents the new LC–MS/MS method for the simultaneous analysis of F and E with their tetrahydro- (THF and THE) and allo-tetrahydrometabolites (alloTHF and alloTHE) in urine. The method was fully validated and allows determining both the unconjugated and total concentrations of urinary glucocorticoids.

The method meets the EMA's recommendations and was proved to be useful in the analysis of clinical samples. The LLOQ of 1 ng/mL allows the determination of free urinary F, E, THF and THE, but not alloTHF and alloTHE, in samples obtained from pregnant women. The range of concentrations is wide enough for the analysis of total levels of F, E, THF, alloTHF, THE and alloTHE. The undisputed advantage of the method, distinguishing it among others, is the ability to determine F and E and their both 5α- and 5β-metabolites. Taking alloTHE into consideration enables the thorough analysis of the glucocorticoid equilibrium in human.

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1. Introduction

Cortisol (F), the main glucocorticoid (GC) in human, is produced in adrenal cortex and plays a crucial role in many physiological processes. Disturbances in F secretion lead to life-threatening conditions, such as Addison's syndrome (primary adrenal insufficiency resulting in F deficiency) and Cushing's disease (excess in F production) [1,2]. Moreover, the alterations in levels of GCs in urine and/or in plasma were observed in many other clinical conditions, which are not directly caused by the dysfunction of adrenal glands, eg. in apparent mineralocorticoid excess (AME) syndrome [3,4], polycystic ovary syndrome (PCOS) [5,6], obesity and insulin resistance [7,8], cholestasis [9], hypertension [10], pre-eclampsia (PE) [11], and mental disorders: depression [12], bipolar disorder and schizophrenia [13]. Those observations led the researchers to the conclusion of the potentially disturbed metabolism of F in the above mentioned diseases. This, in turn, may be reflected in abnormal values of specific parameters: plasma or serum cortisol to cortisone

Abbreviations: ACN, acetonitrile; allo-THE, 5α-tetrahydrocortisone, allo-tetrahydrocortisone; alloTHF, 5α-tetrahydrocortisol, allo-tetrahydrocortisol; AME, apparent mineralocorticoid excess; DCM, dichloromethane; E, cortisone; EMA, European Medicines Agency; F, cortisol; GC, glucocorticoid; IS, internal standard; LLOQ, lower limit of quantitation; ME, matrix effect; MF, matrix factor; MRM, multiple reaction monitoring; PCOS, polycystic ovary syndrome; PE, pre-eclampsia; RE, recovery; THE, 5β-tetrahydrocortisone, tetrahydrocortisone; THF, 5β-tetrahydrocortisol, tetrahydrocortisol; UFE, urinary free cortisone; UFF, urinary free cortisol; ULOQ, upper limit of quantitation; 11β-HSD, 11β-hydroxysteroid dehydrogenase.

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http://dx.doi.org/10.1016/j.jpba.2017.03.039
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ratio (F/E), urinary free cortisol to cortisone (UFF/UFE), or the sum of A-ring reduced metabolites of cortisol (tetrahydrocortisol, THF and allo-tetrahydrocortisol, alloTHF) to A-ring reduced metabolite of cortisone (tetrahydrocortisone, THE) expressed as the ratio: (THF + alloTHF)/THE. The parameters are impacted by the function of 11β-hydroxysteroid dehydrogenases (11β-HSD) – type 1 and type 2–that are responsible for the interconversion of F and E. Moreover, the ratio (THF + alloTHF)/THE is influenced by the activities of 5α- and 5β-reductases, which catalyze the production of A-ring reduced metabolites of both F and E [2–12,14].

The analysis of urinary F, and sometimes E, was done with the use of various methods starting from radioimmunoassay [15,16], competitive protein binding assay [17], and ending with chromatography, both gas and liquid [1,9,17–20]. Chromatographic methods allow also the determination of A-ring reduced metabolites of F and E. Among the chromatographic methods with different types of detection, those with MS/MS are considered the most specific and selective ones and were proved to be the most sensitive ones. There are LC–MS/MS methods focusing on urinary F and E [21,22], or only on their metabolites: THF, alloTHF and THE [23–25].

Lately, the methods were presented that allow the simultaneous determination of F, E and the reduced metabolites [26–32]. Their lower limit of quantitation (LLOQ) ranges from 0.1 [28,29,32] up to 3 ng/mL [27] for F and E, and from 0.1 [29] up to 5 ng/mL [32] for the A-ring reduced metabolites. Interestingly, none of the LC–MS/MS methods considers allo-tetrahydrocortisone (alloTHE) in the analysis, simplifying the metabolism of E to THE only, even though it is known that the proportion of 5α- and 5β-reduction can differ in various clinical conditions [5,13,33] and even depends on gender [34]. The mentioned methods allow the analysis of unconjugated [27,30] or total GCs in human urine [29,31,32], as well as both free and total levels [26].

The paper presents the LC–MS/MS method for the simultaneous quantitative analysis of F and E with their tetrahydro- (THF and THE) and allo-tetrahydro metabolites (alloTHF and alloTHE) in urine. The elaborated method was fully validated and confirmed to be useful in the analysis of urine samples obtained from volunteers and pregnant women. The described method allows determining the unconjugated as well as total concentrations of urinary GCs. Importantly, it focuses not only at THE as the main metabolite of E, but it takes into account also alloTHE, bringing the new quality in the estimation of 11β-HSD and 5α- and 5β-reductase function.

2. Materials and methods

2.1. Standards and reagents

Standards of F and E, hydrocortisone-9,11,12,12-D_4 (F-D_4; used as internal standard – IS) as well as formic acid (≥95%) were purchased from Sigma-Aldrich (St. Louis, USA). Metabolites of F and E: THF, alloTHF, THE and alloTHE were obtained from Steraloids (Newport, USA). Organic solvents (at least LC gradient grade): acetonitrile (ACN), methanol and dichloromethane (DCM), as well as β-glucuronidase from *Helix pomatia* were purchased from Merck (Darmstadt, Germany). Di-sodium hydrogen phosphate anhydrous (Fluka Chemie, Buchs, Switzerland) and potassium phosphate monobasic (Xenon, Łódź, Poland) were used to prepare buffer of pH 7.5. Standard samples, QC samples and buffer were prepared using demineralized water (Simplicity UV, Merck-Millipore, Darmstadt, Germany), while mobile phase for HPLC included water (LC–MS grade) that was purchased from J.T.Baker (Deventer, The Netherlands).

Stock and working solutions of six analyzed GCs were prepared in anhydrous ACN, while those of the IS (F-D_4) were prepared in methanol. Working solutions of analytes covered the concentration ranges: 0.02–20.0 μg/mL for F, E, alloTHF and alloTHE, and 0.02–100.0 μg/mL for THF and THE. Working solution of the IS was prepared at 7.5 μg/mL. All stock and working solutions of analyzed GCs were stored at −25 °C and 4 °C, respectively, until required. Stock and working solutions of IS were both stored at −25 °C.

2.2. HPLC–MS/MS conditions

Determination of F, E, THF, alloTHF, THE and alloTHE was carried out in the Shimadzu (Kyoto, Japan) system comprising Nexera chromatograph interfaced to a triple quadrupole mass spectrometer LCMS-8030. Mass spectrometer was equipped with ESI. The HPLC system consisted of a solvent degasser (DGU-20A_5), a binary pump (LC-20AD) set at a total flow rate of 0.30 mL/min, a thermostated (10 °C) autosampler (SIL-30AC), a thermostated (30 °C) column compartment (CTO-20AC) and a flow-line selection valve (FCV-20AH_2) used to divert the eluent to waste from 0 to 4.5 min. The separation was accomplished in the Core-Shell Kinetex XB-C18 column (2.1 × 100 mm; 2.6 μm particle size) guarded by a pre-column SecurityGuard ULTRA C18, both from Phenomenex (Torrance, USA). The isocratic mobile phase consisted of 0.1% formic acid in water and 0.1% formic acid in ACN (762:238, v/v). Before application to the HPLC system, the mobile phase was de-aerated using an ultrasonic bath (Polsonic, Warszawa, Poland). To minimize the carry-over effect, the autosampler needle was rinsed before and after aspiration of the sample using 500 μL of the de-aerated solution: methanol-water (70:30, v/v).The desolvation line and the heat block temperature was maintained at 250 °C and 400 °C, respectively. Nitrogen was used as the both nebulising gas (flow rate of 2 L/min) and the drying gas (flow rate of 15 L/min), while argon was used as the collision gas for collision-induced dissociation CID (with the pressure set at 230 kPa). The electrospray needle voltage was 4.5 kV. MS/MS detection processed in multiple reaction monitoring (MRM) mode (Table 1). The LabSolutions software (Shimadzu, Kyoto, Japan) was used for the instrument control and data acquisition.

2.3. Preparation of calibration standards and QC samples

Two calibration curves were prepared for the quantification of endogenous GCs in urine, using water as a matrix. They comprised low and high concentrations: calibration curve A covered 1.0–75.0 ng/mL in matrix for all analytes; calibration curve B cov-

Table 1
LC–MS/MS parameters for quantitative analysis of glucocorticoids studied.

Compound (trivial name)	Retention time [min]	ESI mode	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Collision energy [eV]
Cortisol-D_4 (F-D_4)	5.23	+	366.90	121.10	−28
Cortisol (F)	5.32	+	362.90	121.10	−27
Cortisone (E)	5.73	+	361.00	163.15	−29
Allo-tetrahydrocortisol (alloTHF)	8.05	−	411.10[a]	335.25	18
Tetrahydrocortisol (THF)	8.49	−	411.10[a]	335.25	20
Allo-tetrahydrocortisone (alloTHE)	10.63	−	409.20[a]	333.25	16
Tetrahydrocortisone (THE)	11.61	−	409.20[a]	333.25	19

[a] Precursor ions are adducts of particular GCs with $HCOO^-$.

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