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HPLC method for determination of fluorescence derivatives of cortisol, cortisone and their tetrahydro- and allo-tetrahydro-metabolites in biological fluids

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ABSTRACT

11β-Hydroxysteroid dehydrogenase isoform 2 (11β-HSD2) is responsible for conversion of cortisol (F) to inactive cortisone (E). Disturbance of its activity can cause hypertension. To estimate 11β-HSD2 activity, besides F and E, their tetrahydro- (THF, THE) as well allo-tetrahydro- (allo-THF, allo-THE) metabolites should be determined. This study describes HPLC-FLD method for the quantitative determination of endogenous glucocorticoids (GCs) in plasma and urine (total and free) and their metabolites in urine. Following extraction at pH 7.4 using dichloromethane, GCs (F, E, THF, allo-THF, THE, allo-THE and internal standard – prednisolone) were derivatized with 9-anthroyl nitrile and purified by SPE using C_{18} cartridges. The enzymatic hydrolysis of conjugated steroids was provided using β-glucuronidase. The influence of organic bases on 9-AN derivatization of steroids was investigated. The best yield of the derivatization was obtained in presence of the mixture of 10.0% triethylamine (TEA) and 0.1% quinuclidine (Q). Chromatographic separation was accomplished in the Chromolith RP-18e monolithic column. The elaborated method was validated. Calibration curves were linear in the ranges: for F, E and THF 5.0–1000.0 ng mL^{-1}, for allo-THF and THE + allo-THE 10.0–1000.0 ng mL^{-1}. LOD (S/N = 3:1) for all analytes amounted 3.0 ng mL^{-1}. Recoveries of GCs exceeded 90%. The method was precise and accurate, intra- and inter-day precision were 3.0–12.1% and 9.2–14.0%, respectively. Accuracy ranged from 0.2 to 15.1%. The method was applied for estimating endogenous GCs in plasma and urine. Plasma levels of F and E were in the ranges: 133.0–174.5 ng mL^{-1} and 17.4–35.9 ng mL^{-1}, respectively. Free urinary steroids were in the ranges: 12.0–54.1 μg/24 h (UFF) and 37.8–76.2 μg/24 h (UFE). The ratio of (THF + allo-THF)/(THE + allo-THE) amounted from 1.01 to 1.23. The obtained results confirmed utility of the elaborated method in the assessment of 11β-HSD2 activity in man.

1. Introduction

11β-Hydroxysteroid dehydrogenase (11β-HSD) is an enzyme which catalyzes the interconversion of the active glucocorticoid (GC) – cortisol (F) to biologically inactive cortisone (E). Two isozymes of 11β-hydroxysteroid dehydrogenase have been characterized (11β-HSD1 and 11β-HSD2). The specific presence in tissues of those isoforms has a pivotal meaning in regulating activation of glucocorticoid and mineralocorticoid receptors (GR and MR, respectively) [1–5]. F and E are metabolized in the liver by a two-step reduction of steroid A-ring. The reaction is catalyzed by α-reductase followed by 3α-hydroxysteroid dehydrogenase (3α-HSD), obtaining allo-tetrahydrocortisol (allo-THF) and allo-tetrahydrocortisone (allo-THE). For β-reductase followed by 3α-HSD, the products are: tetrahydrocortisol (THF) and tetrahydrocortisone (THE) [1,6,7].

Type 1 of 11β-HSD is most abundant in liver and fat tissue. It modulates glucocorticoid action in various tissues, e.g. it regulates hepatic gluconeogenesis and body fat mass [1–4]. It has a very important contribution to metabolic syndrome [2,5,8]. 11β-HSD2 is most abundantly present in kidney, colon and salivary glands. The role of 11β-HSD2 is pivotal in corticosteroids physiology, because *in vitro* the affinity of MR for F and aldosterone is the same (E has no binding affinities with MR). *In vivo* in physiological conditions 11β-HSD2 protects MR from F and thus ensures the selectivity of aldosterone binding with MR. Defective conversion of F to E causes MR activation by F, whose circulating concentration is about 1000-fold higher than aldosterone [1,3,4]. This phenomenon is a base of apparent mineralocorticoid excess (AME), and it is suggested to be crucial in essential hypertension [1,3–6,9]. The equilibrium between 11β-HSD1 and 11β-HSD2 activity ensures the proper F availability and action in its target tissues. That balance can be demonstrated by measuring the ratio of reduced metabolites of F and E in urine [6,10–13] as well as by determining of urinary free

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cortisol (UFF) and cortisone (UFE) [10,12,13]. Plasma F/E ratio is another valuable parameter in estimation of the coordinated activity of two isoforms of 11β-hydroxysteroid dehydrogenase [13].

F and its metabolites were assayed in biological samples by various methods such as: RIA, competitive protein binding assay, gas chromatography, HPLC. Radioimmunoassay [14,15] or competitive protein binding assay [16] were used for determination of UFF. F and E were determined using RIA method in plasma after chromatographic separation [17]. Simultaneous determination of UFF and UFE and their metabolites by chromatographic methods is an undisputed advantage over competitive binding assays [11,16].

Methyloxime-trimethylsilyl derivatives of urinary corticosteroids were estimated using gas chromatography with flame ionization detection [18–20] as well as mass spectrometry [10,11,20–27]. However, the derivatization process was very complicated and time-consuming. Moreover, the methyloxime-trimethylsilyl derivatives were unstable in air [20] and did not permit sufficient separation of two stereoisomeric compounds, THF and allo-THF [28]. Two-step reaction to produce bismethylenedioxy-3,11-dipentafluoropropionyl derivatives was suggested to avoid the problem with stability and to ensure better resolution [28]. HPLC-UV with normal phase [29] and reversed phase [16,30–34] was applied to determine glucocorticoids in clinical samples. LC–MS–MS methods were presented for simultaneous determination of THF, allo-THF and THE in urine with the lowest concentration detected ranging from 0.1 ng mL^{-1} [12] to 0.5 ng mL^{-1} [35]. However, problems with the specificity of methods using tandem mass spectrometry were emphasized. Urine matrix is rich in steroidic compounds which cause most fragment ions to be common to different components. Moreover, selectivity is further reduced by the tendency of steroids to lose water molecules in the source [35]. HPLC-normal phase with fluorescence detection was applied for estimating 9-anthroyl nitrile (9-AN) derivatives of F and E in plasma and urine in presence of prednisolone and prednisone [36]. 9-AN derivatives of urinary metabolites of F and E (THF, allo-THF and THE) were determined in similar HPLC system [37]. RP-HPLC method with fluorescence detection was evaluated for determination of triamcinolone (TMC) in the presence of endogenous glucocorticoids [38]. Separation of E, F in plasma and their metabolites in urine has been received but the method has not been validated.

The paper presents a specific, sensitive HPLC method with complete validation for determination of fluorescence derivatives of F and E in plasma as well as UFF, UFE and conjugated steroids in urine. The method was applied for estimation of the analytes in biological samples to confirm its usefulness for the assessment of 11β-hydroxysteroid dehydrogenase activity.

2. Experimental

2.1. Materials

Cortisone, tetrahydrocortisone, allo-tetrahydrocortisone, tetrahydrocortisol and allo-tetrahydrocortisol were obtained from Research Plus Inc. (USA). Cortisol (hydrocortisone), 99% triethylamine (TEA) and quinuclidine (Q) were purchased from Sigma–Aldrich Chemie (Germany). Solutions of 12.5% TEA, 0.5% Q were prepared in anhydrous acetonitrile (J.T. Baker, The Netherlands). Then solutions of 12.5% TEA and 0.5% Q were mixed at the ratio of 4:1 to receive a final mixture containing 10.0% TEA and 0.1% Q. Prednisolone (P), the internal standard (I.S.), was obtained from Polfa (Pabianice, Poland). Di-sodium hydrogen phosphate anhydrous (Fluka Chemie, Switzerland), potassium phosphate monobasic (Xenon Łódź, Poland) and 85% ortho-phosphoric acid (Fluka Chemie, Switzerland) were used for preparing buffer of pH 5.0 and 7.4. Sodium acetate anhydrous (Sigma–Aldrich Chemie, Germany) and 99.5% acetic acid (P.O.Ch. Gliwice, Poland) were used for preparing acetate buffer of pH 5.1. Solution of β-glucuronidase from *Helix pomatia* (>100,000 U mL^{-1} of glucuronidase activity and ≤7500 U mL^{-1} of sulfatase activity), used for enzymatic hydrolysis, was purchased from Sigma–Aldrich Chemie (Germany). Derivatizing agent was 9-anthroyl nitrile (SynChem Laborgemeinschaft OHG, Germany), the concentration of 9-AN solution in anhydrous acetonitrile was 0.2 mg mL^{-1}. Acetone (P.O.Ch. Gliwice, Poland) used during the analysis was of analytical reagent grade. Acetonitrile, n-hexane (Merck, Germany), methanol (J.T. Baker, The Netherlands) and dichloromethane (Labscan, Ireland) were of HPLC grade. Demineralised water was always used (Simplicity UV, Millipore, USA).

2.2. Apparatus and HPLC conditions

F, E and their metabolites, after extraction from human plasma and urine and after pre-column derivatization, were determined in HPLC apparatus HP 1100 (Hewlett Packard, Vienna, Austria). Chromatographic separation of the endogenous GCs and I.S., all previously converted to 9-AN derivatives, was accomplished in the Chromolith Performance RP-18e monolithic column (100 mm × 4.6 mm) which was protected by a guard column (both from Merck, Germany). The column temperature was fixed at 25 °C. The mobile phase consisted of acetonitrile and 0.3 mM orthophosphoric acid at the ratio of 470:530 (v/v). The final pH of the mobile phase was 4.6. Water used for analysis was always filtered through a 0.45 μm cellulose membrane filter (Sartorius, Germany). Before application to the HPLC, the mobile phase was de-aerated using an ultrasonic bath (UM-4 Unitra, Poland) and a degasser (model G1322A) and then pumped by a quaternary pump (model G1322A) at the flow rate of 2 mL min^{-1}.

Extracted, derivatized and purified samples of analytes were injected into the analytical column using an autosampler (model 1314A). Fluorescence of steroids derivatives was measured at 460 nm emission wavelength with the excitation at 360 nm wavelength [36,38]. The system was controlled by ChemStation software.

The cartridges for solid phase extraction (SPE), of 1 mL capacity with 100 mg of octadecyl phase chemically bound to silica gel (Bakerbond SPE™, J.T. Baker, The Netherlands), were applied for isolation of analytes from plasma and urine after derivatization procedure. Urine to be used as a matrix for calibration curve was purified of endogenous GCs using cartridges for SPE of 3 mL capacity and packed with 200 mg of octadecylsilane (C_{18}) (Bakerbond SPE, J.T. Baker, The Netherlands).

2.3. Calibration curves

2.3.1. Standard solutions for calibration curve of GCs

Stock solutions of P (I.S.) and GCs were prepared by dissolving the appropriate amount of a compound in anhydrous acetonitrile. The stock solutions were of followed concentrations: 100.0 μg mL^{-1} for allo-THE and allo-THF; 400.0 μg mL^{-1} for P; 500.0 μg mL^{-1} for E, THE, F and THF. The standard solutions were prepared from stock solutions by diluting the appropriate volume of the stock solution with anhydrous acetonitrile in 10 mL glass flasks. The standard solutions were of 10.00; 5.00; 2.50; 1.00; 0.50; 0.25; 0.10; 0.05 μg mL^{-1} each GC and 2.00 μg mL^{-1} I.S. The 50 μL volume of the standard solutions were transferred to glass screw vials containing 0.5 mL of demineralised water or blank urine (stripped of endogenous GCs). The resulting concentrations of corticosteroids were 1000.0; 500.0; 250.0; 100.0; 50.0; 25.0; 10.0; 5.0 ng mL^{-1} and 200.0 ng mL^{-1} of I.S.