Journal of Chromatography B, 878 (2010) 283–289

Contents lists available at ScienceDirect

Journal of Chromatography B

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

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

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ARTICLE INFO

Article history:
Received 14 June 2009
Accepted 10 November 2009
Available online 26 November 2009

Keywords:
Urinary free cortisol
Urinary free cortisone
11β-Hydroxysteroid dehydrogenase
9-Anthroyl nitrile derivatization
Enzymatic hydrolysis

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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doi:10.1016/j.jchromb.2009.11.016

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.

The urine was purified from endogenous GCs by triple SPE procedure. Cartridges with C_{18} were activated with 6 mL of methanol and 6 mL of water. Then 3 mL of urine was transferred to a cartridge. The urinary steroids were retained on the stationary phase, the filtrate was collected. To remove all endogenous steroids the process was repeated three times. Finally, blank urine was obtained.

2.3.2. Extraction and derivatization of steroids

The samples with steroids, spiked with 1 mL of phosphate buffer pH 7.4, were extracted using 4 mL of dichloromethane. The mixture was shaken out for 10 min with the amplitude of 4 cm and the frequency of 300 cycles min^{-1}. Then it was refrigerated for 10 min in 4 °C and centrifuged with 1740 × *g*. Collected dichloromethane layer was evaporated to dryness at a gentle stream of nitrogen at 40 °C. The evaporated residue of extracts were dissolved in 100 μL of the 10.0% TEA and 0.1% Q solution, then 150 μL of derivatizing agent, 9-AN, was added. The mixture was left to react for 30 min at the room temperature and was protected from light, then solvent was evaporated to dryness at 30 °C. The evaporated residue was dissolved in 0.2 mL of acetonitrile and 0.8 mL of demineralised water. The mixture was carefully vortexed and transferred to cartridges with C_{18}, previously activated with 2 mL of acetonitrile and 2 mL of water. The adsorbed analytes were rinsed with 2 mL of water and allowed to dry by air flushing for 10 min. Subsequently, the 9-AN derivatives of the corticosteroids were purified using 2 mL of mixture of n-hexane and dichloromethane at the ratio of 1:1 (v/v) and eluted with 1 mL of acetone. The obtained eluates were dried in a gentle stream of nitrogen at 40 °C. The residue was dissolved in 50 μL of acetonitrile and 50 μL of mobile phase and 50 μL was injected into the analytical column.

2.4. Analysis of steroids in plasma and urine

The samples of plasma and urine – without enzymatic hydrolysis, were processed in similar manner as for calibration curve (described in Section 2.3). The aliquot of 0.5 mL of plasma or urine was spiked with 50 μL of only I.S. and vortexed. After extraction with 4 mL of dichloromethane, the derivatization and purification were processed as described in Section 2.3.2. In this way urinary free steroids and plasma levels of GCs were determined.

To assess total steroids in urine, hydrolysis of the conjugated steroids in 0.5 mL of urine in environment of phosphate buffer (1 mL); pH 7.4, using 25 μL of β-glucuronidase from *Helix pomatia* (5800 units for 1 mL of urine) was carried out. The mixture was incubated for 20 h in 37 °C. Then hydrolyzed mixture was transferred to the glass vial and 50 μL of I.S. was spiked. It was carefully vortexed and extracted using 4 mL of dichloromethane. Then derivatization and purification were processed as described in Section 2.3.2.

2.5. Optimization of the hydrolysis conjugated steroids and derivatization procedure

2.5.1. Hydrolysis of conjugated steroids

The influence of incubation time, temperature, and buffer on the yield of hydrolysis of conjugated steroids was estimated. For that purpose four series of the same pooled urine were prepared. Two series of urine consisting of 5 samples each contained phosphate buffer of pH 7.4, in remaining two series, acetate buffer of pH 5.1 was applied. Then samples were incubated in following ways: in 37 °C for 20 h series I with phosphate buffer of pH 7.4 and series II with acetate buffer. Series III with phosphate buffer and series IV with acetate buffer were incubated in 55 °C for 2 h. After incubation and adding 50 μL of the solution of I.S. (2 μg mL^{-1}) further proceedings were as described previously in Section 2.3.2.

2.5.2. Influence of organic bases on derivatization

The results of the derivatization process in the absence of one or both of organic bases were analyzed. For that purpose the dry residues of steroids extracts were dissolved in 100 μL of 10.0% TEA (the absence of Q), 100 μL of 0.10% Q (the absence of TEA) or 100 μL of anhydrous acetonitrile (absence of both TEA and Q) before adding the derivatizing agent, 9-AN. The residual conditions and procedures were as described in Section 2.3.2.

The influence of Q concentration on yield of derivatization was studied. Following solutions Q in acetonitrile were prepared: 0.10%; 0.15%; 0.20% and 0.30%. The dry residues after dichloromethane extraction were dissolved only in 100 μL of the above Q solutions.

In another experiment, the influence of the amount of 9-AN on the yield of the derivatization was estimated. Therefore, after dissolving the dry residue of extracts in the mixture of TEA and Q, increasing volumes 100, 150, 200, 300, 400 and 500 μL of 0.2 mg mL^{-1} 9-AN were added. The rest of derivatization and purification conditions were the same as described in Section 2.3.2.

The stability of steroids' derivatives was also investigated in higher temperature (40 °C) of evaporation after derivatization.

2.6. Validation parameters

2.6.1. Linearity

Linearity of the calibration curve was estimated for the peak area of each corticosteroid after derivatization with 9-AN to I.S., as a function of concentration of the analytes in matrices covering the range of 5.0–1000.0 ng mL^{-1} for E, F and THF and the range of 10.0–1000.0 ng mL^{-1} for allo-THE + THE and allo-THF. The equations of calibration curves were used to calculate unknown endogenous GC concentration in human plasma and urine. Mandel's fitting test was applied for the evaluation of the linearity of a straight line regression model. The correlation coefficients *r* were also calculated.

2.6.2. Limit of detection (LOD), limit of quantitation (LOQ) and control samples

LOD of the corticosteroids was determined as an S:N baseline ratio of 3:1. LOQ was defined as the lowest concentration of E, THE, allo-THE, F, THF and allo-THF determined by the method within the relative standard deviation (%RSD) not exceeding 20% of its nominal value. The control samples with concentration 5.0 ng mL^{-1} of F, THF, E and 10.0 ng mL^{-1} of allo-THE, THE, allo-THF in water and blank urine were independently prepared and determined.

2.6.3. Precision and accuracy

The intra-day precision was examined by determination of endogenous GCs in plasma and urine samples. Six samples of each series were assayed on the same day. Urine samples were examined without enzymatic hydrolysis and after 20 h incubation with β-glucuronidase. Precision was expressed as percent relative standard deviation. The inter-day precision was estimated by determination of endogenous GCs in plasma and urine (without and after enzymatic hydrolysis) samples. Three series consisting of nine samples each (3 samples of plasma, 3 samples of urine without hydrolysis and 3 of urine after enzymatic hydrolysis) were prepared and assayed within 3 days (each series on another day). Accuracy was calculated for the same ranges of steroids as for evaluation of precision of the method and expressed as the percent difference between the mean determined concentration and the nominal concentration.

2.6.4. Recovery

The total recovery of GCs from the biological matrices (plasma and urine) was estimated. First, the initial concentrations of endogenous GCs in human samples of plasma and urine were

determined. Later, known amount of GCs was added to the same plasma and urine prior to extraction and the final concentrations of GCs were determined. Each series consisted of six samples urine or plasma. Recovery was estimated as a ratio of found and expected (initial + added) concentration of GCs in plasma and urine, expressed in % [34]:

$$\text{Recovery [\%]} = \left(\frac{\text{found concentration}}{\text{expected concentration}}\right) \times 100\%$$

The recoveries of GCs from urine were analyzed both without enzymatic hydrolysis as well as after incubation with β-glucuronidase.

2.7. Application for clinical studies

Usefulness of the elaborated method has been confirmed by quantitative determination of F and E in plasma; UFF and UFE (in urine without enzymatic hydrolysis) and all remaining corticosteroids (F, E and their A-ring reduced metabolites) in urine after 20 h incubation with β-glucuronidase. Human plasma (Regional Centre of Blood Donation in Poznań) and urine were obtained from 3 healthy volunteers. The urine samples were collected for 24 h. The volumes were noted and the aliquots of 7 mL were taken. All the samples were stored frozen in plastic vials until analyzed.

3. Results and discussion

3.1. Matrix

Since F, E and their reduced A-ring metabolites are endogenous compounds, then urine and plasma cannot be used directly as matrix for preparing calibration curves of the analytes. Stripping the plasma and urine of endogenous GCs by SPE was presented to avoid mentioned problem, but no details were stated [36]. Other proposed solution was preparing calibration curves using urine diluted with water in the ratio of 1:50 [32] or pure water [30,31] as matrix. We also used the cartridges with C_{18} phase to purify the plasma and urine from endogenous GCs. After double extraction urine was successfully stripped of steroids (Fig. 1A) but not plasma. In plasma some amounts of F were found even after fourfold SPE extraction. Finally, pure water and urine purified by SPE were used to prepare calibration curves. Actually, we received identical calibration parameters for GCs in pure water and blank urine used as matrix (Table 1). The results correspond with published data where no difference was found using pure water and diluted urine for determination of UFF and 6β-hydroxycortisol [32].

3.2. HPLC conditions

Separation of F, E, THF, allo-THF, THE, allo-THE and I.S. was provided using monolithic column Chromolith RP-18e. Application of the monolithic column with comparison to commonly used column with C_{18} phase LiChrospher RP-18e resulted in better resolution of THF, allo-THF and E in shorter time of the analytical run (Fig. 1B and C). However, using Chromolith RP-18e better separation of THE and allo-THE has not been received, similarly when LiChrospher RP-18e has been used. The time of a single assay was reduced from about 50 min for LiChrospher column to 30 min for Chromolith column (Fig. 1). The assay following the elaborated method is the least time-consuming with comparison to other HPLC-FLD methods [36,37]. The retention times presented in the literature [37] were for A-ring metabolites of F and E about 45–50 min but the separation of F and E was not provided. Similarly, shorter retention times were obtained for F and E if compared to method presented by Shibata et al. [36]. Resolution of the steroidic compounds was received using simple mobile phase consisted of acetonitrile and acidified water (pH 4.6)

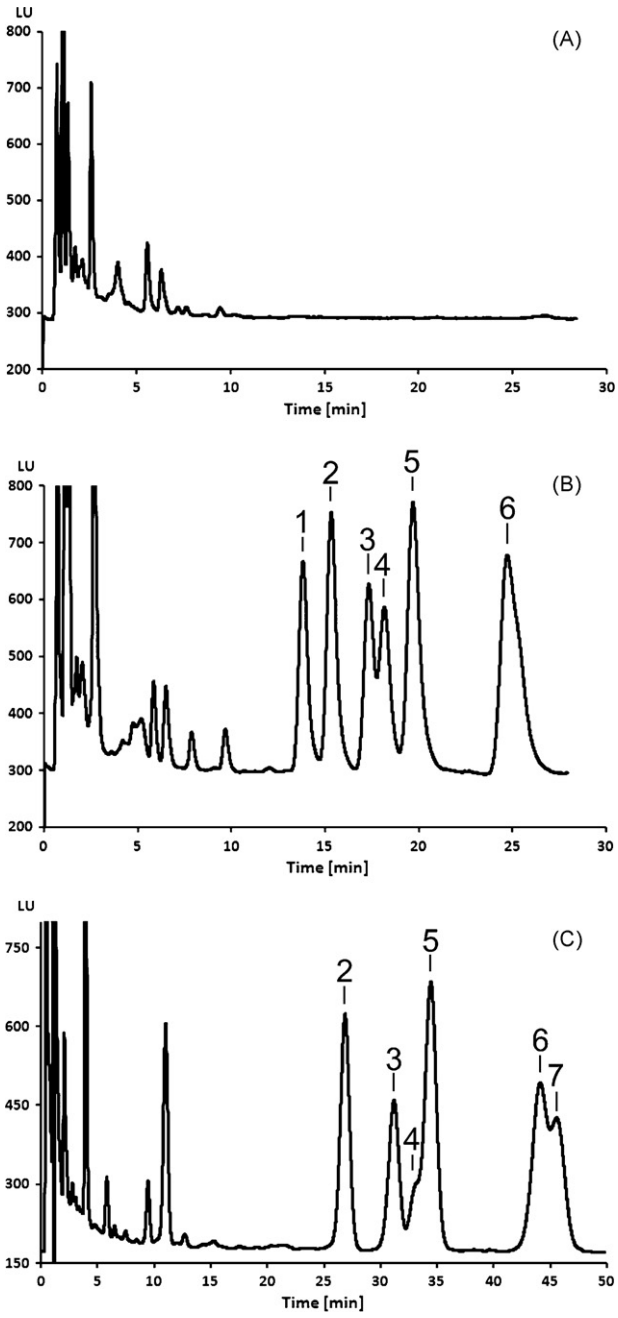

Fig. 1. Chromatograms A and B were obtained after extraction of healthy volunteer's urine previously stripped of endogenous GCs by SPE; separation of GCs using monolithic Chromolith column: A—blank urine; B—urine spiked with the standard solution of I.S. (1), F (2), THF (3), allo-THF (4), E (5) and THE + allo-THE (6); (final concentrations in urine were: 250 ng mL^{-1} for analytes and 200 ng mL^{-1} for I.S.). Chromatogram C was obtained after extraction of water as a matrix spiked with the standard solution of F (2), THF (3), allo-THF (4), E (5), THE (6), allo-THE (7); (final concentrations of GCs in matrix were 500 ng mL^{-1}), separation received using LiChrospher column.

which is also much more friendly to the environment than applied earlier [36,37] organic normal phases.

3.3. Enzymatic hydrolysis of conjugated steroids

Free F and E represent about 20–40% of their total urinary amount, remaining part is excreted as glucuronides and sulfates [39]. Tetrahydro-metabolites of F and E are mostly excreted into

Table 1
Equations of calibration curves[a] of F, E and their tetrahydro- and allo-tetrahydro-metabolites.

Compound	Calibration curve range (ng mL^{-1})	Calibration curve equations	Correlation coefficient, r
F	5.0–1000.0	$P_F/P_P = (0.00509 \pm 0.00010)C_F + 0.02968$	0.9999
THF	5.0–1000.0	$P_{THF}/P_P = (0.00363 \pm 0.00011)C_{THF} - 0.00442$	0.9999
allo-THF	10.0–1000.0	$P_{allo\text{-}THF}/P_P = (0.00364 \pm 0.00014)C_{allo\text{-}THF} - 0.03745$	0.9996
E	5.0–1000.0	$P_E/P_P = (0.00730 \pm 0.00117)C_E - 0.02636$	0.9997
THE + allo-THE	10.0–1000.0	$P_{THE+allo\text{-}THE}/P_P = (0.00889 \pm 0.00146)C_{THE+allo\text{-}THE} - 0.04670$	0.9995

[a] Six calibration curves were prepared in a period of 4 weeks.

urine as conjugated forms [25,39]. Therefore, enzymatic hydrolysis has to be applied to determine total amounts of urinary glucocorticoids. The incubation with β-glucuronidase from *Helix pomatia* was provided at 37 °C but the time differed in published methods from overnight incubation [18] to 48 h [19,25]. It was suggested that the same yield of hydrolysis can be achieved in time of 2–3 h raising the temperature of incubation to 55 °C [39]. Therefore, we investigated the influence of buffer, time and temperature on the extent of hydrolysis. The extent of the hydrolysis in different conditions (mean values) is presented in Fig. 2. The best yield of hydrolysis of conjugated forms of F, E and THF was observed when phosphate buffer (pH 7.4) was used and incubation in 37 °C remained for 20 h. For THE + allo-THE the extent of hydrolysis was practically the same when samples were incubated with acetate buffer in 55 °C for 2 h and with phosphate buffer in 37 °C for 20 h (100.0% and 98.3%, respectively). The greatest amount of allo-THF was liberated after incubation with acetic buffer (pH 5.1) in 37 °C for 20 h. However, hydrolysis provided with phosphate buffer instead of acetic buffer ensured for allo-THF the yield exceeding 80%. Finally, incubation with phosphate buffer at 37 °C for 20 h has been chosen.

3.4. Fluorescence derivatives

The reaction of steroids with 9-anthroyl nitrile to receive fluorescent derivatives has been reported [36–38]. The temperature of the reaction was found as very important in reaction selectivity, especially considering tetrahydro-steroids as THF, allo-THF and THE. The products of the reaction at 60 °C were 21-monoderivative but also 3,21-diderivative. The same reaction at lower temperature gave only one type of products, 21-monoderivatives [37]. Therefore, it may be assumed that at the room temperature the acylation of the hydroxyl group occurs selectively at the 21st position in the carbon chain of GCs.

The presence of catalysts was required to ensure the high yield of derivatization process [36]. However, it was reported that the reaction rate of anthracene-9-carbonyl chloride with hydroxyl compounds was slowed in presence of tertiary organoamine bases such as triethylamine [40,41]. It might be supposed that 9-AN would react in similar way. Our experimental data did not corroborate that formation of derivatives in the uncatalyzed reaction is more rapid as it was observed in case of steroids derivatized with 9-anthroyl chloride [41]. On the contrary, the reaction of GCs with 9-AN in absence of TEA and Q practically did not occur. It was found that using the mixture of 10.0% TEA and 0.1% Q provided the best results in the yield of the reaction of all analyzed GCs (Fig. 3). The reaction of steroids with 9-AN in presence of only 10.0% TEA resulted in lower extent of derivatization of all analytes. The derivatization process with only 0.1% Q as a catalyst was also investigated. We stated that yields of the reactions of F, E and allo-THF were comparable to the situation when the mixture of TEA and Q was used. However, for THF and THE + allo-THE the yields of the reaction catalyzed by 0.1% Q only were significantly lower, comparing to mixture of the bases. Therefore, the mixture of 10.0% TEA and 0.1% Q was chosen as optimal environment for the reaction of GCs with 9-AN.

The influence of the amount of derivatizing agent (0.2 mg mL^{-1}) on the yield of the reaction was investigated as well. Maximum extent of the derivatization was received after adding 200 μL of 9-AN solution. However, application of 150 μL of the same solution resulted in the yield of the reaction exceeding 90% comparing to the usage of 200 μL of 9-AN. Considering the yield of the reaction, economy and environmental protection, it was concluded that the amount of 150 μL of 9-AN would be sufficient for the derivatization process.

It was noted that it is important to maintain anhydrous conditions during derivatization [36,37]. Keeping the samples in the dark is also advantageous to derivatives stability [37]. In such conditions,

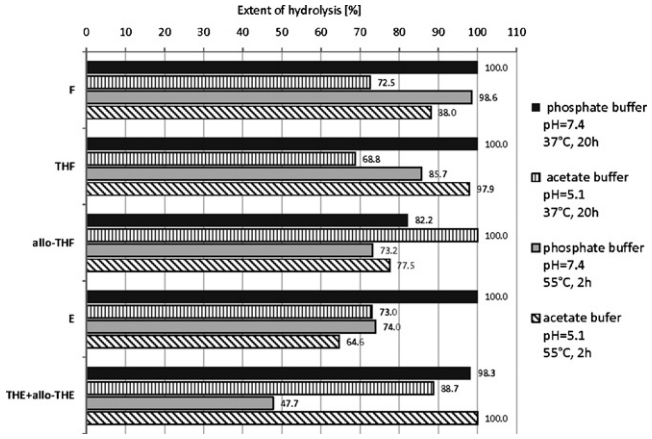

Fig. 2. Effects of buffer, temperature and time of incubation on the extent of enzymatic hydrolysis of conjugated urinary GCs. The highest yield of hydrolysis for each steroid is set as 100% and all other values are given as a % of this yield.

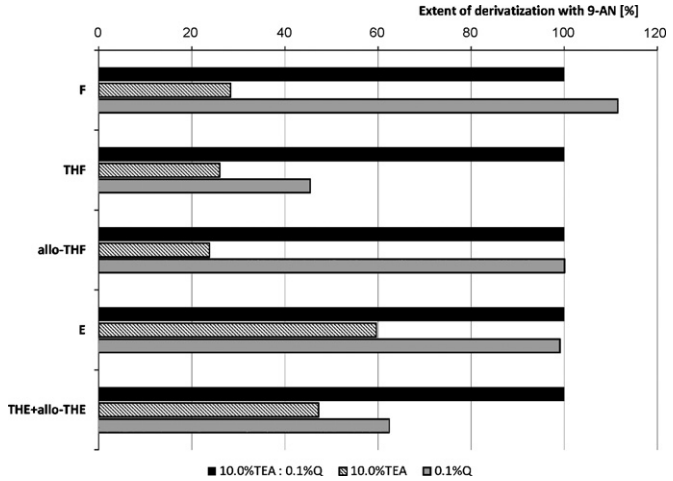

Fig. 3. The effects of organic bases on the extent of GCs derivatization using 9-AN. The yield of derivatization in presence of the mixture of 10.0% TEA and 0.1% Q in acetonitrile is set as 100% and all other values are given as a % of this yield.

extracts of GC derivatives were found to be very stable at room temperature, even for a week [36]. However, we investigated the influence of the temperature of the evaporation under the gentle stream of nitrogen and found it as another important parameter for derivatives stability. The temperature of evaporation was assumed as 40 °C (under the reduced pressure) [36], or it was not stated [37]. We have found that reducing the temperature of evaporation from 40 °C to 30 °C increased the derivatization yield of about 20%.

3.5. Validation parameters

3.5.1. Specificity

The peaks of the derivatives of endogenous GCs and P after extraction from plasma and urine are presented on the chromatogram (Fig. 4). The peaks of reduced metabolites of F, THF and allo-THF, are not completely separated but the resolution (Rs = 1.43) is acceptable. The peaks of THE and allo-THE are not separated and these steroids are determined in clinical samples as a sum. The separation of THE and its α-isomer is an actual problem. They can be partially separated but in time-consuming assay (Fig. 1C). However, in the literature most often only THE is considered as metabolite of E. The level of allo-THE is passed over [6,10,12,19–28,39]. The reason of such simplification may be much lower level of allo-THE in comparison to THE in urine [42] or difficulties in effective separation of those isomers. However, solving that problem was not necessary, because the ratio of total cortisol metabolites (THF + allo-THF) to total cortisone metabolites (THE + allo-THE) is estimated for the assessment of 11β-HSD activity.

3.5.2. Linearity, LOD and LOQ

Standard curves for endogenous GCs were linear in following ranges of concentrations: 5.0–1000.0 ng mL^{-1} for F, THF, E and 10.0–1000.0 ng mL^{-1} for allo-THF and THE + allo-THE. Statistical analysis using Mandel's fitting test confirmed linearity of the calibration curves. The correlation coefficients r were also calculated and they ranged from 0.9995 to 0.9999. The equations of mean standard curves were calculated using MSExcel and are presented in Table 1. The significance of intercept values was determined using t-Student test ($\alpha = 0.05$). The value of intercept was significant only for calibration curve for F. The rest of intercept values was not significant. Therefore, they were not considered in further calculations of GCs levels in biological samples.

In the worked out conditions, LOD at an S/N baseline ratio = 3:1 for endogenous GCs in samples of plasma and urine amounted to 3.0 ng mL^{-1}. The LOQ for F, THF and E was determined to be 5.0 ng mL^{-1}, for allo-THF and THE + allo-THE it was 10.0 ng mL^{-1} (Table 2).

3.5.3. Precision, accuracy and recovery

The designed HPLC method was characterized by high intra-day and inter-day precision in the ranges of 3.0–12.1% (Table 3) and 9.2–14.0% (Table 2), respectively. Inter-day precision of F and E are given as a highest results estimated for three possible matrices (plasma, urine without hydrolysis and urine after enzymatic

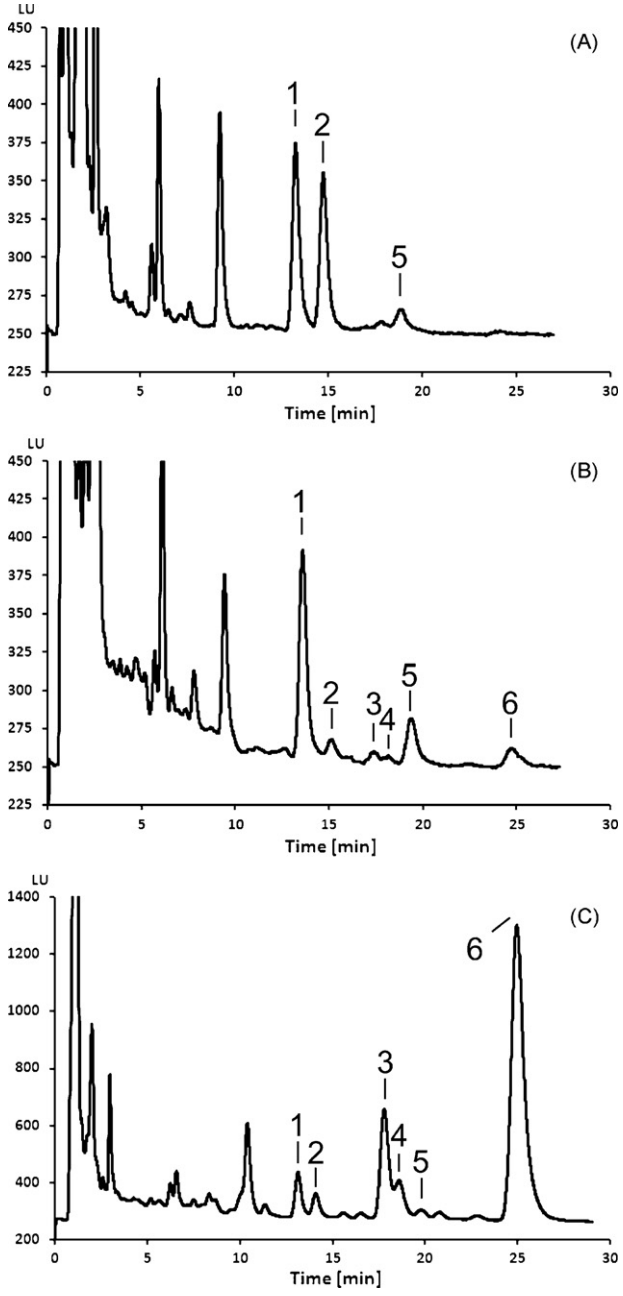

Fig. 4. Chromatograms obtained after extraction of biological samples: A—plasma; concentrations: 174.51 and 17.59 ng mL^{-1} for F and E, respectively; B—urine without enzymatic hydrolysis; determined concentrations amounted to 17.99 and 40.38 ng mL^{-1} for F and E, respectively. Chromatogram C—the same urine sample as B but after incubation with β-glucuronidase; concentrations: 38.67, 1595.51*, 460.74, 72.56, 2741.60* ng mL^{-1} for F, THF, allo-THF, E, THE + allo-THE, respectively. Peaks denoted on chromatograms: I.S. (1), F (2), THF (3), allo-THF (4), E (5), THE + allo-THE (6). *Samples were diluted before analysis to receive concentration of THF and THE + allo-THE in the ranges of calibration curves.

Table 2
Validation parameters.

Concentration ranges of calibration curves (ng mL^{-1})		Inter-day precision[a] (%RSD)	Accuracy[b] (%error)	LOD (ng mL^{-1}) S:N = 3:1	LOQ[b] (ng mL^{-1}) (bias; %RSD)
F	5.0–1000.0	9.2	0.2–15.1	3.0	5.0 (−16.6; 8.3)
THF	5.0–1000.0	9.6	0.4–7.1	3.0	5.0 (15.1; 11.7)
allo-THF	10.0–1000.0	14.0	0.3–13.1	3.0	10.0 (−1.5; 7.4)
E	5.0–1000.0	10.9	0.3–9.9	3.0	5.0 (6.0; 12.4)
THE + allo-THE	10.0–1000.0	12.4	1.2–10.4	3.0	10.0 (7.9; 15.8)

[a] Inter-day precision was estimated in a period of 8 days ($n = 9$).
[b] $n = 6$.

Table 3
Recovery and intra-day precision of F, E and their metabolites.

Corticosteroid and matrix	Initial (ng mL^{-1})	Added (ng mL^{-1})	Expected (ng mL^{-1})	Found (ng mL^{-1})	Recovery %	RSD %
Plasma						
F	109.70	100.00	209.70	216.91 ± 4.36	103.4	3.0
E	13.71	100.00	113.71	112.21 ± 3.76	98.7	10.0
Urine without enzymatic hydrolysis						
F	<LOQ	50.00	50.00	51.59 ± 3.66	103.2	12.1
E	8.32	50.00	58.32	62.86 ± 7.50	107.8	10.2
Urine after 20 h incubation with β-glucuronidase						
F	36.08	50.00	86.08	85.31 ± 2.45	99.1	4.4
THF	207.97	50.00	257.97	253.40 ± 16.69	98.2	6.6
allo-THF	211.03	50.00	261.03	235.05 ± 22.32	90.0	8.7
E	14.17	50.00	64.17	73.22 ± 2.73	114.1	6.2
THE + allo-THE	185.05	50.00	235.05	237.72 ± 10.08	101.1	4.5

Each series consisted of six samples.

hydrolysis). THF, allo-THF, THE and allo-THE can be determined only in urine after hydrolysis with β-glucuronidase. Accuracy of the estimations has fitted the range required for endogenous compounds testing in body fluids. It has ranged from 0.2 to 15.1% (Table 2). Recovery of GCs from plasma and urine exceeded 90% (Table 3) and it was comparable to the literature [34,36].

3.6. Application for clinical studies

The elaborated method was applied for determination of endogenous F and E in plasma as well as UFF and UFE in urine and F, E, THF, allo-THF, THE + allo-THE in hydrolyzed samples of urine of three healthy volunteers. The obtained results correspond with those presented in the literature [6,12,13,15,21,25,35]. Plasma levels of F and E were in the range of 133.0–174.5 ng mL^{-1} and 17.4–35.9 ng mL^{-1}, respectively. UFF amounted to 12.0–54.1 μg/24 h, UFE was in the range of 37.8–76.2 μg/24 h. The ratio of THF + allo-THF to THE + allo-THE amounted from 1.01 to 1.23 and it covered the reference range 0.89–1.34 [6]. The data confirmed applicability of the elaborated HPLC-FLD method for estimation of 11β-HSD activity in man.

4. Conclusions

The elaborated HPLC method with fluorescence detection fulfills the validation requirements. It is precise, accurate and sensitive and undoubtedly can be used for studies on endogenous F, E and their metabolites levels both in plasma and urine. The presented data give an indication of the utility of the method for the determination of clinically important parameters in the assessment of 11β-hydroxysteroid dehydrogenase activity in man. Moreover, the obtained results agree with the data available in literature, which further confirms the usefulness of the approach.

Acknowledgement

The studies have been supported by Polish Ministry of Science and Higher Education, grant number N-405-01632/1498.

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