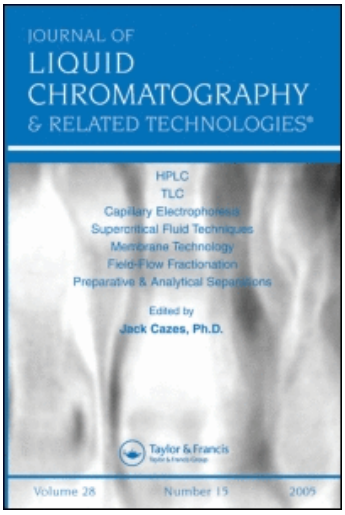

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SIMULTANEOUS DETERMINATION OF CORTISOL, CORTISONE, AND CORTICOSTERONE IN HUMAN PLASMA OF PARACHUTISTS IN VIEW OF PHARMACOKINETIC STUDIES

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SIMULTANEOUS DETERMINATION OF CORTISOL, CORTISONE, AND CORTICOSTERONE IN HUMAN PLASMA OF PARACHUTISTS IN VIEW OF PHARMACOKINETIC STUDIES

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□ *A rapid high-performance liquid chromatographic method with UV detection (HPLC-UV) for the simultaneous quantification of cortisol, cortisone, and corticosterone was developed and validated. The method involved solid-phase extraction (SPE) followed by chromatographic separation using a Nucleosil 100 C-18, 5 μm, 250 × 4 mm i.d. chromatographic column. The mobile phase was binary mixture of acetonitrile and water (30:70 v/v) at a flow rate of 1 mL/min. The proposed method was validated for specificity, linearity, limits of detection and quantitation, precision, and accuracy. Calibrations curves prepared in human plasma extracts were linear with $r > 0.9997$ for all analytes. The concentration of each steroid was determined in the range from 2 to 300 ng/mL and accuracy ranged from 95.10 to 103.88% for cortisol, from 101.40 to 102.47%, and from 94.00 to 102.21% for cortisol, cortisone, and corticosterone, respectively. Good repeatabilities (RSD < 9.74%) were found for all compounds in the biological matrix. The limit of quantitation (LOQ) was 2 ng/mL for cortisol, cortisone, and corticosterone, respectively. The described method was successfully applied for monitoring three endogenous steroid levels in human plasma. Studies were performed on 20 healthy volunteers of both sexes, comprising two sociably diversified groups of people, namely, 10 parachutists before and after effort and 10 patients with depression. The method proposed is simple, inexpensive, and reproducible with the capabilities of accurate quantification of steroids. The method is suitable for a wide applications in human and physiological studies, thus appearing as a fast and reliable alternative to be used for routine steroids analysis.*

Keywords corticosterone, cortisol, cortisone, high-performance liquid chromatography method, human plasma, SPE extraction

INTRODUCTION

Simultaneous determination of biologically active corticosteroids in blood, urine, saliva, or tissue samples is very important to elucidate their diverse physiological roles in biological system. Especially, it can be

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considered in relation to a diagnosis of adrenocortical insufficiency and adrenal hyperplasia. However, they also have been recently used as biomarkers in depression or endocrinological stress.[1]

Cortisol, cortisone, and corticosterone (Figure 1) are major natural glucocorticoids produced by the adrenal glands in humans regulating a myriad of biological functions and processes. Excess production of cortisol over a prolonged period of time can cause a disease known as Cushing's syndrome. Cortisol and corticosterone in plasma have been also used as a marker of adrenocortical function.[2] Furthermore, cortisol may serve as diagnostic tool for depressive disorders and chronic fatigue.[3] Moreover, cortisol may be used as biomarker of stress.[4–6]

It should be emphasized that the ratio between cortisol (the physiologically active steroid hormone) and its metabolite cortisone might be both together useful to study, because they are necessary in several metabolic processes (e.g., adaptation to stress). In fact cortisol and cortisone increase when nervous tension occurs, e.g., during sport training or competition.[7]

Recently, the cortisol/cortisone ratio has been proposed as a useful doping test in athletes because they illicitly use corticoids to improve their performance.[8]

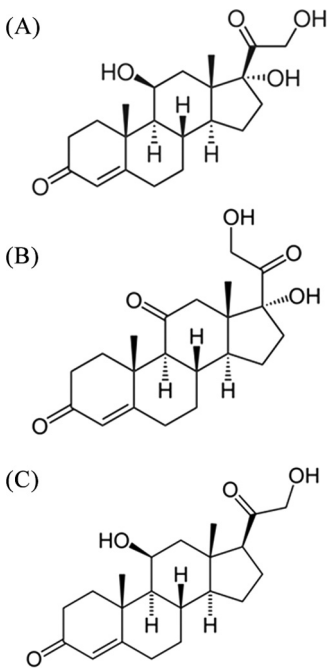

FIGURE 1 Chemical structures of three cortiocosteroids: cortisol (A), cortisone (B) and corticosterone (C).

Determination of steroid hormone level in parachutists is of interest as it is a unique group to research. It is difficult to find parachutists of both sexes.

Radioimmunoassay (RIA), immunoassay (EIA), and competitive protein-binding assay are often used as methods of choice for the determination of corticosteroids. However, it is also well known that these methods, although very sensitive, suffer from a lack of specificity, which may become crucial in complex biological matrix.[9–12] This led to the use of high-performance liquid chromatography (HPLC) separation methods before RIA quantitation,[13,14] which, in turn, increased analysis time and overall cost.

Separation of steroid hormones by HPLC method with fluorescence detection has also been used. However, some of these methods use fluorimetric derivatization before HPLC analysis, which is limited by the precisely controlled reaction conditions and the instability of the fluorescent analogue.[15,16]

Recent works in several laboratories have shown that HPLC with UV detection can be used to determine low concentrations of several steroids. UV detector is widely available for analysts and often represents enough sensitivity for investigations of steroids in biological fluids, such as urine,[17–21] serum,[22–25] and plasma.[20,26,27]

Other procedures including liquid chromatography-mass spectrometry (LC/MS)[28–32] or gas chromatography-mass spectrometry (GC-MS)[33] have also been developed for determination of steroids in biological samples; however, the methods based on these techniques still involve extensive effort for routine use. Moreover, LC-MS methods often require a complicated extraction or derivatization procedures, which are tedious and time-consuming.[34]

The aim of this work was to develop and validate an HPLC-UV method for the simultaneous quantification of cortisol, cortisone, and corticosterone in human plasma. An efficient and simple solid-phase extraction (SPE) procedure was developed and allowed a sensitive determination of glucocorticoids in small plasma samples. Measurement of these endogenous corticosteroid concentrations in human volunteers, parachutists, and patients with depression (females and males) were demonstrated.

MATERIALS AND METHODS

Reagents

Cortisol, cortisone, corticosterone, and dexamethasone (internal standard, I.S.) were purchased from Sigma-Aldrich (St. Louis, USA). Reagents used for the preparation of samples and mobile phases, methanol and acetonitrile, were obtained from Merck (Darmstadt, Germany). All chemicals

were of HPLC grade and used as received without further purification. Water was purified by triple distillation.

Instrumental Parameters and Conditions

HPLC Technique

The high-performance liquid chromatography system was purchased from Knauer (Berlin, Germany), and was equipped with a solvent pump (Mini-Star K-500), a K-2500 UV detector, and a computer system for data acquisition (Eurochrom 2000). A Nucleosil-100 C_{18}, analytical column (5 µm, 250 × 4 mm mm i.d.) from Knauer (Berlin, Germany) was used. The mobile phase consisted of acetonitrile and water (30:70, *v*/*v*) was used at a flow of 1 mL/min with an isocratic elution. The chromatographic separation was performed at room temperature. The analytes were monitored at 240 nm. Under these conditions, the retention times for cortisol, cortisone, corticosterone, and the internal standard (dexamethasone) were 8.98, 9.98, 20.08, and 15.65 min, respectively. The total run for each sample analysis was 25 min.

Experimental Design

The reported analytical method was applied to the monitoring of steroid concentrations in human plasma. The study was performed on three groups of both sexes (20 healthy volunteers and two diversified socially groups of people: 10 patients with depression and 6 parachutists). Volunteers were selected for this study on the basis of medical history, clinical examination, and laboratory investigation. All of them provided written consent. The study protocol was approved by The Ethical Committee of Medical University of Gdańsk, Gdańsk, Poland.

The plasma samples were collected between 7 and 9 a.m. from 20 healthy volunteers and 10 depression patients. In the case of 10 parachutists plasma samples were taken before and after jump between 7 and 9 a.m. All samples were immediately separated by centrifugation and frozen in 2 mL aliquots at −20°C until analysis.

Standard Solutions and Sample Preparation

Stock solutions of cortisol, cortisone, corticosterone, and internal standards were made by accurately weighing 10.0 mg of both analytes into 10 mL of acetonitrile. Further solutions were obtained by serial dilution of stock solution with acetonitrile to prepare the calibration solutions containing 1.0, 10.0, and 100.0 µg/mL of analytes, respectively. The I.S. stock solution was further diluted with acetonitrile to prepare the working standard solution contained 10 µg/mL of dexamethasone. All solutions were stored in the refrigerator at 4°C and protected from light.

The plasma samples to be analyzed were removed from the freezer and thawed. Quality control samples (QC), calibration curve, and blank plasma samples were extracted using a solid-phase extraction technique. Standard samples of calibration curves and QC samples were prepared by spiking different samples of 1.0 mL plasma with known amounts of each steroid and internal standard solution (dexamethasone 200 ng/mL). QC samples were prepared by spiking each steroid to produce the concentration pools of 10, 50, and 200 ng/mL. Next, 4 mL of distilled water were added to each plasma sample. The resulting mixture was shaken mechanically for 10 min and centrifuged for 15 min at 3500 rpm/min. The plasma sample was rapidly passed through the SPE columns (Merch, LiChrolut RP-18, 100 mg), preconditioned with 3 ml of methanol and washed twice with 3 mL of deionized water using vacuum. After passing the respective spiked plasma samples, the SPE columns were washed with 2 mL of deionized water. The final elution of steroids was achieved using 0.5 mL methanol. The organic layer was transferred into a clean test tube and evaporated to dryness in a water bath at 45°C with the aid of a gentle stream of air. The residue was reconstituted with 70 µl of acetonitrile-water (90:10, *v*/*v*). After shaking for 0.5 min and centrifugation for 8 min at 10 000 rpm/min, 20 µl was injected into the HPLC system.

Validation of Analytical Method

Validation of analytical method was carried out according to the guidelines of the main regulatory agencies, such as those issued by the International Conference of Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), by the United States Pharmacopeia (USP) and by Food and Drug Administration (FDA). Quantification procedure for the HPLC method was based on the internal standard method. Concentration of analyte of interest in biological material was determined by plotting peak-height ratios (each steroid/internal standard) vs. each steroid concentration (ng/mL). This assay has been carried out on the basis of the replicate analysis of samples containing known amounts of analytes. Selectivity was determined by analyzing blank human plasma samples, which were tested for interference using the proposed methods, and these results were compared with those ones obtained from human plasma samples containing steroids and internal standard. Linearity of the method was tested for the range of steroid concentrations 2–300 ng/mL. The assay has been evaluated on the basis of the analysis of the calibration samples constructing the calibration curves consisted of the eight concentrations: 2, 5, 10, 20, 50, 100, 200, and 300 ng/mL for all analytes tested, namely cortisol, cortisone, and corticosterone. The limit

of detection (LOD) was determined as the lowest measurable sample concentration which was distinguished from zero, defined as the peak three times that of the baseline noise. The limit of quantification (LOQ) was defined as the lowest concentration at which the precision expressed by RSD% was lower than 15%, the accuracy expressed by a percent of the nominal concentrations was within 80–120% and the ratio of signal to noise was better than 10. Intra- and inter-day precision and accuracy were assessed by analyzing the quality control samples (10, 50, and 200 ng/mL), which were tested in the same day ($n = 6$) and on ten different days, respectively. Intra-day and inter-day precision and accuracy were evaluated using back calculated concentrations. Intra-day precision was determined by calculating the relative standard deviations (RSD%). Assay accuracy was assessed by calculating the estimated concentrations as a percent of the nominal concentrations.

The absolute recoveries of steroids and internal standard were determined by direct comparison of peak heights from extracted plasma samples vs. non-extracted ones.

As part of the validation, the freeze-thaw stability of steroids was evaluated by comparing the stability of samples containing analyzed substance that had been frozen and thawed three times with plasma samples that were thawed only once. This evaluation was based on back-calculated concentrations.

Selectivity

Selectivity of the method was confirmed on the basis of the analysis of different blank human plasma samples and plasma extracts containing of steroids and internal standard ($n = 6$). Typical chromatograms (HPLC) obtained with a blank plasma and a plasma sample containing 300 ng/mL of cortisol, cortisone, corticosterone, and internal standard are illustrated in Figures 2A and 2B, respectively. The absence of any signal at the same migration time as the selected steroids indicated that there were no matrix interferences. Additionally, the specificity of method has been confirmed by the identification of peaks of steroids and internal standard in term of the retention time and UV spectrum.

Linearity

The linearity of the method was tested for the range of concentrations 2–300 ng/mL using a 1.0 mL plasma sample. Proposed method exhibited a reliable linear response for the validated range of concentrations of steroids. The mean equations of the calibration curves including eight points for HPLC with correlation coefficients $r > 0.9997$ for analyzed steroids were shown in Table 1, where H/H_{IS} represents the ratio of steroids peak height

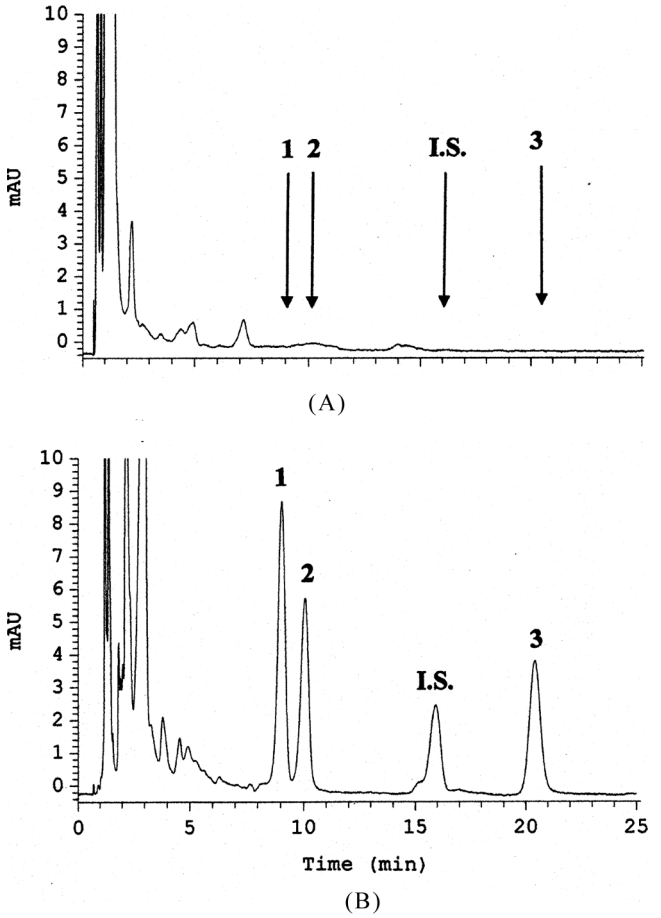

FIGURE 2 Typical chromatograms of blank human plasma extract (A) and plasma spiked with three steroids: (1) cortisol (300 ng/mL); (2) cortisone (300 ng/mL); and (3) corticosterone (300 ng/mL) and dexamethasone (I.S.) (200 ng/mL) (B).

and internal standard one and C represents the steroid concentration in ng/mL. The low value of intercept and its relatively large standard errors confirms the specificity of the presented method.

TABLE 1 Summary of Validation Data in the Range of 2–300 ng/mL for Steroids in Human Plasma Obtained with HPLC Calibrations (n = 6)

	Equation Parameter		Standard Error	Correlation Coefficient		
Steroids	Slope	Intercept	s	r	LOD [ng/mL]	LOD [ng/mL]
Cortisol	0.0111 ± 0.0001	0.0473 ± 0.0159	0.034	0.9997		
Cortisone	0.0073 ± 0.00005	0.0764 ± 0.0071	0.015	0.9998	0.5	2
Corticosterone	0.0048 ± 0.00003	-0.0003 ± 0.0046	0.010	0.9998		

Limit of Detection and Quantitation

The limits of detection (LOD) for steroids were calculated from six independently made replications and were determined at the level of 0.5 ng/mL for each steroid. The lowest standard concentration in the calibration curves were considered as the lower limit of quantitation. The limit of quantitation for steroid was proved to be 2 ng/mL (Table 1). The method was sufficiently sensitive, with a quantification limit comparable[23,27] or lower than for the earlier published HPLC methods with spectrophotometric detection.[7,18,20]

Precision and Accuracy

The intra-day and inter-day precision and accuracy results for presented method are shown in Table 2. The intra-day precisions of HPLC method for chosen steroids, expressed as RSD%, ranged from 1.75 to 7.19, from 2.26 to 8.55, and from 1.49 to 6.33% for cortisol, cortisone, and corticosterone, respectively. The inter-day precision assay was found to be better than 9.74% for analyzed steroids. The intra- and inter-day accuracies for analytes were better than 94.00% and 97.20%, respectively.

Recovery

The absolute recovery of each steroid in plasma was determined at two different concentration levels (low and high) with six replicates for each concentration. Mean recoveries were 99.15 ± 8.45 and $99.67 \pm 2.04\%$ for cortisol, 98.72 ± 5.02 and $98.44 \pm 4.16\%$ for cortisone, and 93.24 ± 8.50 and $93.37 \pm 4.27\%$ for corticosterone at the 10 and 100 ng/mL

TABLE 2 Assay Variability Obtained from Intra- and Inter-day Experiments for Analysed Steroids

	Intra-day (n = 6)			Inter-day (n = 10)		
Nominal Concentration (ng/mL)	Concentration Measured ± SD (ng/mL)	Precision RSD (%)	Accuracy (%)	Concentration Measured ± SD (ng/mL)	Precision RSD (%)	Accuracy (%)
Cortisol						
10	9.51 ± 0.68	7.19	95.10	10.17 ± 0.95	9.38	101.70
50	51.94 ± 2.11	4.05	103.88	50.24 ± 2.27	4.52	100.51
200	195.88 ± 3.42	1.75	97.94	199.39 ± 4.20	2.11	99.69
Cortisone						
10	10.14 ± 0.87	8.55	101.40	9.72 ± 0.93	9.59	97.20
50	51.23 ± 2.45	4.78	102.47	49.21 ± 1.98	4.03	98.42
200	203.29 ± 4.58	2.26	101.64	201.81 ± 5.88	2.91	100.90
Corticosterone						
10	9.40 ± 0.59	6.33	94.00	10.15 ± 0.99	9.74	101.50
50	51.10 ± 2.18	4.28	102.21	50.55 ± 3.28	6.48	101.09
200	197.98 ± 2.95	1.49	98.99	199.17 ± 5.33	2.68	99.59

TABLE 3 The Results of the Recovery for Steroids and Internal Standard (I.S.) from Human Plasma (n = 6)

	Recovery (%)	
Nominal Concentration (ng/mL)	Mean ± SD	RSD (%)
Cortisol		
10	99.15 ± 8.45	8.53
100	99.67 ± 2.04	2.05
Cortisone		
10	98.72 ± 5.02	5.08
100	98.44 ± 4.16	4.23
Corticosterone		
10	93.24 ± 8.50	9.12
100	93.37 ± 4.27	4.57
Dexamethasone (I.S.)		
200	96.46 ± 6.16	6.38

concentration, respectively (Table 3). The absolute recovery of internal standard (dexamethasone) was 96.46 ± 6.16%. These data confirmed that the extraction provided adequate sensitivity.

Freeze-thaw Stability

The freeze-thaw stability was evaluated using plasma samples containing 10.0, 50.0, and 200 ng/mL of each steroid. These tests were performed by measuring three replicates at each concentration during two months and obtained data were given in Table 4. The stability results confirm that the

TABLE 4 The Results of the Stability Studies for Processed Plasma Sample Freeze-thaw Cycles During 2 Months (n = 3)

	Plasma Concentration (ng/mL)			
Nominal Concentration of Steroids	Initial	After I Freeze-thaw Cycle	After II Freeze-thaw Cycle	After III Freeze-thaw Cycle
Cortisol				
10	10.03 ± 0.62	9.77 ± 0.49	10.13 ± 0.51	9.87 ± 0.72
50	49.53 ± 1.06	50.53 ± 1.16	49.47 ± 1.65	50.20 ± 1.83
200	199.53 ± 5.48	203.53 ± 5.37	197.47 ± 4.98	200.21 ± 5.79
Cortisone				
10	9.64 ± 0.50	10.21 ± 0.66	9.77 ± 0.52	10.48 ± 0.64
50	50.43 ± 0.75	49.83 ± 1.30	50.23 ± 1.27	50.57 ± 1.00
200	194.86 ± 5.09	198.21 ± 2.34	202.67 ± 5.77	194.62 ± 5.41
Corticosterone				
10	9.98 ± 0.39	10.3 ± 0.54	10.55 ± 0.49	9.94 ± 0.61
50	49.80 ± 1.40	50.36 ± 1.20	48.93 ± 1.53	50.67 ± 1.44
200	201.57 ± 6.46	200.72 ± 6.62	202.71 ± 4.46	196.94 ± 5.73

analytes were stable in human plasma for three cycles of freeze and thaw when stored at −20°C and thawed to room temperature and could be handled under normal laboratory conditions without any significant loss.

RESULTS AND DISCUSSION

There are a number of techniques that have been reported for sample preparation of complex biological fluids including liquid-liquid extraction, solid-phase extraction, and ultrasonic extraction. Specifically SPE method was optimized for purposes of the presented study. The SPE procedure implemented for the sample pre-treatment, based on C-18 cartridges, provided good extraction yields (>93.24%) and satisfactory precision (RSD < 9.74%). The method was also selective. The selectivity test demonstrated that there was no interference with the analyzed steroid peaks and internal standard peak in human plasma samples extracted using the described procedure (Figure 2A, B). The retention times for cortisol, cortisone, corticosterone, and dexamethasone were 8.98, 9.98, 20.05, and 15.65 min, respectively. The proposed method possesses also high accuracy and a wide response function range, which allows determinations of analytes not only at the therapeutic doses of steroids but also considering natural endogenous rate of glococorticoids in healthy volunteers and human beings with depression or even found in extreme situations (parachutists). The aforementioned procedure provides a simple, sensitive, and specific determination of plasma glucocorticoids. Thus, very low concentrations can be determined (2 ng/mL), which competes with most RIA procedures[9–12] and allows the use of small plasma volumes for the assay. Other advantages over RIA procedures include the lack of cross-reactivity and the suppression of costs associated with the elimination of radioactive material.

Moreover, this procedure allows the simultaneous determination of cortisol, cortisone, and corticosterone, which makes it suitable for a variety of clinical applications like the assessment of adrenocortical status in hypercorticism,[1–3] depression,[2,3,35] and other psychiatric disorders,[7] or the diagnosis of the apparent mineralocorticoid excess syndrome.[1,36] For these applications, it compares favorably with GC-MS procedures,[33] offering a simpler and less expensive alternative.

The present method was applied to the determination of the ratio of cortisone/cortisol in the plasma of 20 human volunteers (n = 20), patients with depression (n = 10) and parachutists (n = 10) by measuring the individual concentrations of corisol, cortisone, and corticosterone and using a small volume (1 mL) of plasma. The results are shown in Table 5. Representative chromatograms of plasma extracted from healthy volunteers (A), patients with depression (B), and parachutists before (C) and after

TABLE 5 Steroid Concentrations Results of Plasma Measured by HPLC-UV Method in Human with Depression vs. Human Healthy Volunteers and in Athletes Before and After Extreme Sport

Subjects	Cortisol (ng/mL)	Cortisone (ng/mL)	Cortisone/ Cortisol ratio	Corticosterone (ng/mL)
Healthy volunteers n = 20	116.93	72.94	0.62	50.51
Female n = 10	108.39	75.22	0.69	46.23
Male n = 10	125.48	70.67	0.56	54.79
Depression patients n = 10	162.96	54.56	0.33	26.42
Female n = 5	182.38	50.86	0.28	29.68
Male n = 5	143.53	58.26	0.40	23.15
Athletes before extreme exercise n = 10	139.63	68.31	0.49	28.14
Female n = 5	135.15	61.19	0.45	25.40
Male n = 5	144.12	75.43	0.52	30.85
Athletes after extreme exercise n = 10	233.02	77.93	0.33	27.42
Female n = 5	225.20	97.26	0.43	30.24
Male n = 5	240.84	58.60	0.24	24.59

jump (D) are shown in Figures 3A–D, respectively. The mean cortisol concentration level measured by this method for healthy volunteers was 116.93 ng/mL (n = 20), and this result was in agreement with those ones obtained during the previous observations.[22–26] Additionally, concentrations of steroids for females (n = 10) and males (n = 10) were also determined. The mean concentrations for females were found to be 108.39 and 75.22 and 46.23 ng/mL, and for males 125.48, 70.67, and 54.79 ng/mL for cortisol, cortisone, and corticosterone, respectively. Higher mean plasma cortisol level and lower mean concentrations of cortisone in 10 patients with depression (182.38 ng/mL and 50.86 ng/mL) than control group (116.93 and 72.94 ng/mL) were observed. These results were in accordance with previously published articles.[35,37] The same results were received, comparing female and male groups separately. It should be also emphasized that the investigation was performed on a group of patients with depression disease. They were no hospitalized but the patients' received antidepressant drugs and were receiving psychiatric care. Thus, although there was an absolute steroid plasma concentration difference between healthy volunteers and patients with depression disease, it requires statistical confirmation. Analysis of steroids in plasma by the developed method enabled the estimation of the rate of stress in 10 parachutists before and after a jump. Plasma steroid concentrations for parachutists was higher after extreme effort than before a jump. However, the stressful exercise did not provide any statistically significant difference in the cortisol/cortisone ratio. That result is also in accordance with a previous article concerning athletes (cyclists), before and after effort.[7]

Statistical analysis was performed using the statistical software package *Statistica*, version 8.0 (StatSoft, Kraków, Poland). The plasma steroids were

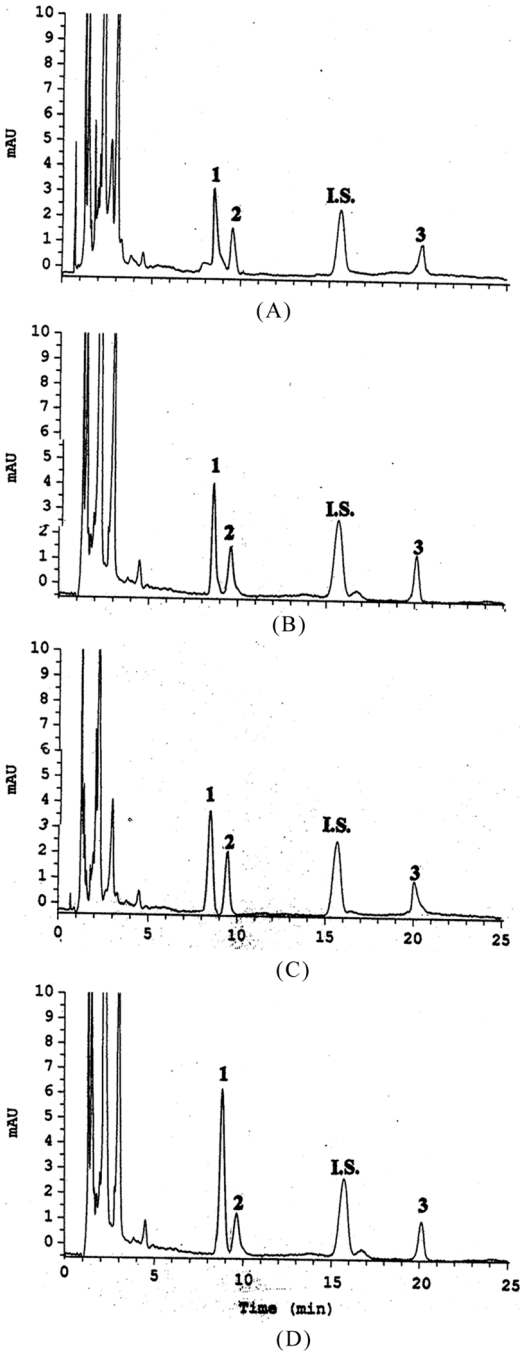

FIGURE 3 Representative HPLC chromatograms of human plasma extract obtained from healthy volunteer (A), patient with depression (B), parachutist before the jump (C), and parachutist after the jump. Concentration levels of cortisol (1); cortisone (2); corticosterone (3), respectively: A – 125.97; 75.66; 72.04; B – 146.21; 82.78; 94.36; C – 132.56; 110.87; 87.05 ng/mL; D – 222.56; 58.67; 30.85**,** respectively and dexamethasone (I.S.) at a concentration of 200 ng/mL.

analyzed by parametric tests. All experimental data concentrations of steroids in plasma samples corresponding to volunteers were divided into four groups (healthy volunteers, parachutists before jump, parachutists after jump, and people with depression disease) and compared using analysis of variance (ANOVA). ANOVA enabled the separation of the variation between each group mean from the overall mean for all the groups (the inter-group variability) and the variation between each study participant and the participants' group mean (the intra-group variability). If the inter-group variability is much greater than the intra-group variability, there are likely to be differences between the group means, which should be tested further by post hoc tests. The first step of parametric analysis was investigation of normal distribution for variables using the Shapiro-Vilk test and the Kolmogorov-Smirnov test for analyzed groups, performed separately. Next, the homogeneity of variance for variables was checked. Restrictions for the aforementioned variables enabled the use of analysis of variance. For this reason, a number was attributed, in turn, to each analyzed group and then calculated by the ANOVA tool, post-hoc test. The data were reported as average ± standard deviations. The statistical analysis of the steroid concentration data leads to the conclusion that the examined groups show significant differences between them. Results received after statistical calculations are shown in Table 6.

Especially, the measurement carried out in parachutists and sedentary subjects demonstrated that the plasma cortisol and cortisone levels in parachutists significantly increased after a stressful situation, such as fear before jump. The difference was annulled in the case of corticosterone; there were not statistically significant differences in corticosterone plasma levels in all analyzed groups. In the case of cortisol, statistically significant differences were observed between healthy volunteers and parachutists after jump as between healthy people and people with depression disease. However, there were no significant differences between cortisol and cortisone plasma levels between sedentary healthy volunteers and parachutists before jump, in spite of exciting situations in parachutists. It should be emphasized that significant differences exists in cortisol and cortisone in parachutists after jump and depression disease. For this reason, it may be concluded that there exists a stronger influence of physical effort than the influence of depression on cortisol and cortisone levels in plasma.

The scope of the extended application of the method proposed to the metabolic profiles of the tested corticoid concentrations in relation to the diagnosis of adrenocortical function and metabolic syndrome are currently under further investigation.

Also, sex-related differences have been determined by the ANOVA test. Significant differences exist between the sexes in all groups (healthy volunteers, parachutists before jump, parachutists after jump, and in the

TABLE 6 Results of ANOVA Calculations (Statistically Significant Differences are Indicated in Bold)

CORTISOL

	1	2	3	4	5	6	7	8
1		0.9704	0.9492	0.8069	**0.0005**	**0.0002**	0.0633	0.8195
2	0.9704		0.9999	0.9934	**0.0036**	**0.0006**	0.2788	0.9946
3	0.9492	0.9999		0.9999	**0.0112**	**0.0017**	0.5112	1.0000
4	0.8069	0.9934	0.9999		**0.0304**	**0.0051**	0.7485	1.0000
5	**0.0005**	**0.0036**	**0.0112**	**0.0304**		0.9978	0.6308	**0.0285**
6	**0.0002**	**0.0006**	**0.0017**	**0.0051**	0.9978		0.2487	**0.0047**
7	0.0633	0.2788	0.5112	0.7485	0.6308	0.2487		0.7340
8	0.8195	0.9946	1.0000	1.0000	**0.0285**	**0.0047**	0.7340	

CORTISONE

	1	2	3	4	5	6	7	8
1		0.9704	0.9492	0.8069	**0.0005**	**0.0002**	0.0512	0.8195
2	0.9504		0.9999	0.9934	**0.0036**	**0.0005**	0.2587	0.9946
3	0.9191	0.9999		0.9999	**0.0111**	**0.0016**	0.5112	1.0000
4	0.7068	0.9934	0.9999		**0.0203**	**0.0041**	0.7485	1.0000
5	**0.0004**	**0.0024**	**0.0101**	**0.0211**		0.9868	0.6308	**0.0193**
6	**0.0001**	**0.0005**	**0.0015**	**0.0042**	0.9978		0.2487	**0.0035**
7	0.0532	0.2788	0.5112	0.7485	0.6308	0.2487		0.7340
8	0.7093	0.8346	1.0000	1.0000	**0.0174**	**0.0037**	0.7340	

CORTICOSTERONE

	1	2	3	4	5	6	7	8
1		0.9704	0.9492	0.8069	0.0005	0.0002	0.0633	0.8195
2	0.9704		0.9999	0.9934	0.0036	0.0006	0.2788	0.9946
3	0.9492	0.8996		0.9999	0.0112	0.0017	0.5112	1.0000
4	0.8069	0.9934	0.9999		0.0304	0.0051	0.7485	1.0000
5	0.1225	0.0436	0.1212	0.0304		0.9978	0.6308	0.0285
6	0.0942	0.0316	0.2217	0.0752	0.9978		0.2487	0.0047
7	0.0833	0.2788	0.5112	0.7485	0.6308	0.2487		0.7340
8	0.8195	0.9946	1.0000	1.0000	0.2285	0.3247	0.7340	

1 – Healthy volunteers, Female; 2 – Healthy volunteers, Male; 3 – Depression patients, Female; 4 – Depression patients, Male; 5 – Athletes before extreme exercise, Female; 6 – Athletes before extreme exercise, Male; 7 – Athletes after extreme exercise, Female; 8 – Athletes before extreme exercise, Male.

depressive group). Because women are more sensitive to cortisol suppression, they may also be more sensitive to the effects on basophils and helper T lymphocytes;[38] and this provided an interesting observation of the sex differences in both pharmacokinetics and pharmacodynamics for cortisol and cortisone.

CONCLUSIONS

Cortisol, cortisone, and corticosterone were simultaneously analyzed by HPLC-UV method in a 25 min run. A SPE method for the extraction, purification, and concentration of a range of steroids from human plasma was also optimized. The SPE procedure was simple to adopt in the clinical

laboratory and can improve the accuracy of steroids quantitation by HPLC method. The developed method showed appropriate recovery and repeatability. Cortisol, cortisone, and corticosterone detection and quantification limits were lower or comparable to the minimum reported contents of these compounds in plasma. Accuracy and precision results verified that the method provided analytical results with high reliability and reproducibility. The lack of derivatization would be very useful as a versatile method for the sensitive assay of a wide variety of endogenous steroids by HPLC-UV method.

When compared to existing HPLC-UV methods,[22–24] it has the fundamental advantage of simultaneously analyzing three steroids with the same HPLC system and extraction procedure. The procedure here presented requires lower volumes of plasma (1 mL instead 2 mL),[23] shorter analysis time (25 min instead of 40 min), and is less laborious than the two-step liquid-liquid extraction.[24–26] Another method, based on HPLC with fluorescence detection, had been published previously.[16] However, it is only suitable for determination of steroids in urine, not in plasma, and does not allow the simultaneous analysis of three steroids, as of yet. The proposed method can be considered a useful tool for determination of cortisol, cortisone, and corticosterone in plasma samples and for the evaluation of stress before and after physical effort in athletes. Additionally, the HPLC-UV method proposed could be used for the follow-up of patients with depression.

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