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Article

Qualification and Quantification of Seventeen Natural Steroids in Plasma by GC–Q-MS and GC-IT–MS/MS

A.F. Toribio-Delgado[1*], M. Maynar-Mariño[2], M.J. Caballero-Loscos[3], M.C. Robles-Gil[2], G.J. Olcina-Camacho[2] and J.I Maynar-Mariño[1]

[1]Department of Analytical Chemistry, University of Extremadura, Badajoz, Spain, [2]Department of Physiology, University of Extremadura, Cáceres, Spain, and [3]Department of Medical-Surgical Therapeutics, University of Extremadura, Badajoz, Spain

[*]Author to whom correspondence should be addressed. Email: afertd@unex.es

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Studying the plasma steroid profile offers information about the possible existence of endocrinological alterations. This study describes the development and validation of gas chromatographic–mass spectrometric and gas tandem mass spectrometric methods for the simultaneous identification of 17 steroid hormones in human plasma using five different solvents. The *n*-hexane/ethyl acetate solvent mixture, in a proportion of 70/30 (*v*/*v*) provided the best results. The extracts were derivatized with *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide. The obtained limits of detection were below 1 ng/mL in the majority of the studied steroids and the limits of quantification were below 5 ng/mL; the method obtained good linearity, reproducibility, repeatability, accuracy and recoveries above 95% in most cases.

Introduction

Sex hormones and corticosteroids are a group of hormones derived from cholesterol that are widely distributed in humans. They are involved in many physiological systems such as reproductive function, stress and metabolism. The analysis of sex hormones and corticosteroids in biological samples can be employed as a diagnostic tool in endocrine disorders, for pharmaceutical investigation and for the detection of possible misuse in athletes (1).

The need to determine steroids in different biological matrices has led to the development of different techniques for steroid determination in these matrices (2). Steroid hormones are normally measured by immunoassay (IA) in routine clinical laboratories because of its simplicity, speed and analytical sensitivity. Nevertheless, the use of IA is limited because of the cross-reactivity of the antibodies used with structurally related compounds. One alternative technique is chromatography coupled to mass spectrometry, which has been accepted for a long time as a potent technique for sieving and confirming the presence of substances prohibited in our biology (3).

The primary biological samples used for the determination of steroid hormones are serum and urine. In recent years, this study and others have reported several methods for their determination in urine samples involving gas chromatography–mass spectrometry (GC–MS) (4, 5, 6) or liquid chromatography–mass spectrometry (LC–MS) (7, 8, 9).

Besides urine, blood is one of the most commonly used fluids for the determination of endocrinological disorders (10) and for anti-doping analyses in sport; the illicit use of anabolites to improve the performance of sportsmen is prohibited by most sporting federations, including the International Olympic Committee (11) and the World Anti-Doping Agency (WADA).

Many efforts have been made to develop sensitive detection methods of steroids in blood samples. Blood offers certain advantages; for example, the matrix is relatively uniform, which permits the detection of drug traces, and another advantage is that the precursors of the metabolites can be found in the matrix (12).

Several GC–MS methods have been developed for the measurement of individual steroids or a profile of steroids and their metabolites in serum (13, 14, and 15); however, the number of steroids tested is lower than in the present work.

This work proposes a method to determine a total of 17 steroid hormones in plasma by gas chromatography- quadrupole- mass spectrometer (GC–Q-MS) and gas chromatography- ion trap- mass/mass spectrometer (GC-IT–MS-MS) in addition to five different extractor solvents. The derivation of the steroids is performed by using silynization or oxime/silynization reactions according to the properties of the steroids (8, 16). The selectivity, reproducibility, repeatability, accuracy, recovery, limits of detection (LOD), limits of quantification (LOQ) and linearity of the method were measured, and thus a reliable method was obtained for determining and quantifying this group of steroid hormones.

Experimental

Reagents and materials

Testosterone, 17β-estradiol, androstenedione, methyltestosterone, androsterone, 5β-androsterone, estrone, dihydrotestosterone (DHT), progesterone, cortisone, cortisol, tetrahydrocortisone (THE), nandrolone, dehydroepiandrosterone (DHEA), estriol, epitestosterone, epiandrosterone, *N*-methyl *N*-trimethylsilyl-trifluoroacetamide (MSTFA) and arylsulphatase were obtained from Sigma-Aldrich, tetrahydrocortisol (THF), androsterone glucuronide, 5β-androsterone glucuronide, androsterone sodium sulphate and 5β-androsterone sodium sulphate were supplied by Steraloids, β-glucoronidase (type *Escherichia coli* K 12) was obtained from Boheringer Mannheim, dithioerythritol was acquired from Serva, ammonium iodide (NH_4I) was acquired from Panreac and human albumin at 20% was from Grifols. All other reagents and solvents were of analytical grade and were mainly supplied by Scharlau, J.T. Baker and Panreac.

Equipment

The GC-Q–MS analysis of the samples was conducted on an Agilent Technologies 6890N chromatograph with MS 5973 Network quadrupole spectrometer. Separation was performed with a factor four capillary column VF-1ms 25 m × 0.25 mm i.d.

Table I
Selected Ions for the Identification of Steroids

GC-Q–MS

Compound	Retention time (min)	Relative retention time	Quantification ion	Qualification ion
Androsterone	20.398	0.882	419	434–329
5β-androsterone (etiocholanolone)	20.453	0.884	434	419–329
Dehydroepiandrosterone (DHEA)	21.090	0.911	432	417–327
Epiandrosterone	21.190	0.916	419	434–329
Epitestosterona	21.421	0.926	432	417
Nandrolone	21.539	0.931	418	403
Dihydrotestosterone (DHT)	21.598	0.933	434	405
Estrone	21.715	0.938	414	309
Androstanedione	21.743	0.940	415	430
17β -estradiol	21.955	0.949	285	416
Testosterone	21.949	0.949	432	417
Estriol	24.415	1.055	504	386–345
Progesterone	25.020	1.081	458	443
Tetrahyidrocortisone (THE)	26.419	1.142	635	530
Tetrahydrocortisol (THF)	27.092	1.171	637	532
Cortisone	28.373	1.226	616	630
Cortisol	29.897	1.292	632	559
Methyltestosterone	23.139	1.000	301	446

GC-IT–MS

Compound	Retention time (min)	Relative retention time	Ion parent	Qualification ion	Quantification ion
Androsterone	21.942	0.776	420	420–329–239	329
5β-androsterone (etiocholanolone)	22.207	0.785	420	420–329–239	329
Dehydroepiandrosterone (DHEA)	23.530	0.832	418	418–327–237	327
Epiandrosterone	23.786	0.841	420	420–329–239	329
Epitestosterona	24.303	0.859	432	432–417–342–327	417
Nandrolone	24.491	0.866	418	418–403	403
Dihydrotestosterone (DHT)	24.491	0.866	434	434–419–405–377	419
Estrone	24.961	0.882	399	399–309–281–267–229	309
Androstanedione	24.934	0.881	430	430–415–325	415
17β -estradiol	25.364	0.897	416	416–326–285	326
Testosterone	25.311	0.895	432	432–417–327–301	417
Estriol	29.752	1.052	504	504–311–295–281–269	311
Progesterone	30.022	1.061	458	458–368–353–299	368
Tetrahyidrocortisone (THE)	33.035	1.168	635	635–619–530	619
Tetrahydrocortisol (THF)	34.232	1.210	636	636–531–430	531
Cortisone	36.009	1.273	617	617–525–435	525
Cortisol	38.155	1.349	632	632–543–437	543
Methyltestosterone	28.288	1.000	446	446–356–301	301

DF = 0.25. The analysis was started at 100°C for 1.5 min and was increased 10°C per min until 280°C, which was maintained for 5 min, and then increased 10° C per min until 300°C, which was maintained for 2 min. The analysis was performed in splitless mode, with He gas carrier at a flow rate of 0.6 mL/min. The injected volume was 3 μL and the temperature of the injector was 250°C.

The GC-IT–MS-MS analysis of the samples was conducted on a Varian 3800 gas chromatograph directly coupled to an MS–MS Saturn 2000 ion-trap mass spectrometer and equipped with a Varian 8200 autosampler with a capacity for 48 samples. Separation was performed using an HP-5MS (crosslinked 5%Ph Me silicone) capillary column with 30 m × 0.25 mm i.d.; film thickness, 0.25 μm. The analysis was performed in splitless mode. Helium was used as the carrier gas (1 mL/min) and the column oven temperature was programmed to maintain at 100°C for 1.5 min, increase to 25°C per min until reaching 190°C, increase 3°C per min until reaching 280°C, maintain for 5 min, increase 25°C per min until 325°C and maintained for 3 min. Analysis was performed in splitless mode. The injected volume was 3 μL and the temperature of the injector was 280°C.

The quantification and qualification ions of the studied compounds are shown in Table I.

Preparation of the standard solutions and calibration curves

Stock standard solutions (100 ng/mL) of each analyte were prepared using methanol as solvent. Successive dilutions with methanol of the stock solutions were made to prepare working solutions. All solutions were stored at −20°C in the dark and were removed every six months.

To have a matrix as close as possible to the true samples, human albumin at 20% was used to prepare triplicate calibration curves with the following concentrations: 1, 2, 4, 6, 8 and 10 ng/mL for sex steroids and 5, 10, 20, 30, 40 and 50 ng/mL for corticosteroids.

Sample storage

The plasma samples were conserved at −20°C. The plasma was very stable and did not require special precautions to be conserved, and storage at −20°C sufficed (17).

Sample preparation

Extraction procedure

Extraction was carried out with 2 mL of the following solvents: *n*-hexane, *n*-hexane–ethyl acetate (70/30, *v*/*v*, 50/50, *v*/*v*,

25/75, *v*/*v*), ethyl acetate, ethyl ether and tert-butyl methyl ether.

Free fraction extraction

To one mL of plasma sample, 2 ng of methyltestosterone as internal standard was added and the pH was adjusted to 9.5 using NaOH 1M and carbonate buffer 0.2M, pH 9.5. Extraction was carried out with 2 mL of the following solvents: ethyl ether, tert-butyl methyl ether, *n*-hexane–ethyl acetate (25/75, *v*/*v*, 50/50, *v*/*v* and 70/30, *v*/*v*), ethyl acetate and *n*-hexane. After 30 min of shaking, the mixture was centrifuged for 5 min and the organic phase was dried under a stream of nitrogen.

Glucuroconjugated fraction extraction

To the previous aqueous part, 2 ng of methyltestosterone as internal standard was added and the pH was adjusted to 7 using acetic acid 1M and phosphate buffer pH 7. After the addition of 50 μL of β-glucuronidase, enzymatic hydrolysis was performed in a thermoblock for 1 h at 50°C. To adjust the pH of the sample to 9.5 to optimize the extraction process of the steroids, the same procedure was followed as that described for the free fraction.

Sulphoconjugated fraction extraction

To the aqueous fraction of the previous stage, after the addition of 2 ng of methyltestosterone as internal standard, the pH was adjusted to 5 using the addition of acetic acid 1M and an acetate buffer pH 5, and 50 μL of arylsulfatase was added; it was left in the thermoblock for 15 h at 50°C, then left to cool at room temperature. To adjust the pH of the sample to 9.5 to optimize the extraction process of the steroids, the same procedure was followed as that described for the free fraction.

Derivatization procedure

To the dried plasma extracts 50 μL of a mixture of MSTFA–NH_4I–dithioerythritol (1000:2:4) (*v*/*w*/*w*) was added. The reaction mixture was heated in a thermoblock for 30 min at 60°C to perform the derivatization reaction, and was then encapsulated and injected into the chromatograph.

Derivatization test

To carry out the derivatization test, 1 mL of albumin (20%) androsterone and etiocholanolone were added in a known quantity (10 ng/mL), and the relationship was measured between the mono-trimethylsilil and the bi-trimethylsilil areas, after performing the derivatizacion as explained in the previous section.

The percentage of derivatization was checked in the following way:

$$\%derivatization = 100 - \left(\frac{Amonoderivatized}{Abiderivatized} \bullet 100\right)$$

Hydrolysis test

To perform the hydrolysis test, androsterone and etiocholanolone glucuroconjugates were used to control the glucuroconjugated fractions and androsterone and ethiocolanolone sulfoconjugates were used to control the sulfoconjugated fraction.

Ion 419 was monitored for the androsterone, 434 for the etiocholanolone and 301 for the internal pattern (methyltestosterone).

A known concentration (10 ng/mL) of androsterone and etiocholanolone in glucuroconjugate and sulfoconjugate form was added to 1 mL of 20% albumin; in parallel, the same known concentration of androsterone and etiocholanolone was added in free form to another milliliter of albumin, and the hydrolysis of the glucuroconjugate and sulfoconjugate compounds was performed as described in previous sections.

The calculations of the percentage of hydrolysis were conducted in the following way:

$$\%hydrolysis = \frac{\frac{ACdeconjugated}{API}}{\frac{ACfree}{API}} \bullet 100$$

where AC deconjugated is the area of the compound after hydrolysis, AC free is the area of the compound without added conjugation and API is the corresponding area of the internal standard added.

Results and Discussion

Selectivity was verified by injecting five replicates of albumin 20% (control sample). No peaks were observed at the lower limit of quantification (LLOQ), which is defined as the lowest concentration of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy (18), and which corresponds to the analyte response at the LLOQ being at least five times the response compared to blank response:

$$LLOQ = \frac{Y_c - \bar{Y}_b}{b} = \frac{5s_b}{b}$$

where S_b is the deviation standard of the white, b is the calibration curve slope, y_c is the critical value of the brute signal and y_b is the average of the target signals.

Repeatability is the best match possible between the results obtained with the same method, test material and conditions (operator, apparatus and laboratory) after a short interval of time. To examine the repeatability of the current extraction method, the variation coefficient ($N = 5$) was calculated for all the compounds studied in each of the studied solvents with the GC-Q–MS and GC-IT–MS-MS. The results are shown in Table II. Some of these studied compounds were not detected in some of the solvents; these situations have been termed N.D. With the solvent that gave the best results, the repeatability was studied by calculating the coefficient of variation (CV) of the injection of five replicates of three different concentrations

Table II
Coefficient of Variation of the Compounds Studied in Different Solvents

Compound	*n*-Hexane	*n*-Hexane–ethyl acetate (70/30)	*n*-Hexane–ethyl acetate (50/50)	*n*-Hexano–ethyl acetate (25/75)	Ethyl acetate	diethyl ether	tert-butyl methyl ether
Androsterone	42.98	12.01	63.14	54.95	N.D.	24.33	N.D.
5β-androsterone	45.00	13.09	50.70	57.27	N.D.	24.47	N.D.
DHEA	N.D.	11.03	45.77	60.44	83.72	12.55	32.09
Epiandrosterone	N.D.	5.94	46.38	62.45	70.72	N.D.	36.30
Epitestosterone	N.D.	9.80	54.77	56.31	59.39	23.95	37.18
Nandrolone	46.57	11.42	40.24	71.42	71.13	N.D.	30.84
DHT	45.51	7.98	55.63	45.97	69.88	N.D.	N.D.
Estrone	N.D.	10.77	49.44	20.03	N.D.	N.D.	N.D.
Androstenodione	N.D.	13.86	42.55	52.42	N.D.	N.D.	N.D.
β-estradiol	N.D.	12.83	45.44	56.94	73.24	N.D.	N.D.
Testosterone	N.D.	12.22	38.00	64.76	65.95	37.41	N.D.
Estriol	N.D.	10.63	N.D.	N.D.	71.74	38.29	29.17
Progesterone	28.76	7.20	48.10	84.37	N.D.	N.D.	N.D.
THE	28.85	13.79	61.31	98.80	N.D.	56.77	24.81
THF	N.D	9.06	62.34	96.83	48.36	56.12	N.D.
Cortisone	N.D.	8.19	34.50	98.00	95.29	45.85	29.86
Cortisol	N.D.	13.84	72.41	84.21	53.94	53.54	29.89

Table III
Repeatability of the Compounds Studied at Low Concentration (ppb)

Compound	Quadrupole				Ion trap			
	Mean	S	CV (%)	15 % CV	Mean	S	CV (%)	15 % CV
Androsterone	0.96	0.11	10.89	1.63	0.99	0.01	1.36	0.20
5β-androsterone	0.99	0.02	2.07	0.31	0.99	0.07	6.70	1.00
DHEA	0.97	0.06	6.51	0.98	0.98	0.03	3.00	0.45
Epiandrosterone	0.97	0.05	5.00	0.75	0.97	0.05	5.32	0.80
Epitestosterone	0.98	0.02	1.86	0.28	0.98	0.04	3.90	0.59
Nandrolone	0.99	0.05	4.98	0.75	0.97	0.04	4.22	0.63
DHT	0.95	0.01	0.58	0.09	0.99	0.01	1.10	0.17
Estrone	0.99	0.03	3.30	0.49	0.99	0.04	3.63	0.54
Androstenodione	0.99	0.01	1.24	0.19	0.92	0.03	2.92	0.44
β-estradiol	0.98	0.02	1.55	0.23	0.93	0.01	1.18	0.18
Testosterone	0.97	0.03	2.77	0.42	0.94	0.05	5.22	0.78
Estriol	0.99	0.03	3.50	0.52	0.97	0.06	6.02	0.90
Progesterone	0.99	0.01	0.85	0.13	0.99	0.04	3.57	0.54
THE	4.99	0.01	0.26	0.04	4.99	0.08	1.51	0.23
THF	4.99	0.07	1.40	0.21	4.99	0.01	0.17	0.03
Cortisone	4.98	0.06	1.22	0.18	4.97	0.02	0.47	0.07
Cortisol	4.98	0.03	0.69	0.10	4.98	0.01	0.14	0.02

Table IV
Repeatability of the Compounds Studied at Medium Concentration (ppb)

Compound	Quadrupole				Ion trap			
	Mean	S	CV (%)	15 % CV	Mean	S	CV (%)	15 % CV
Androsterone	5.93	0.05	0.77	0.12	5.96	0.03	0.50	0.07
5β-androsterone	5.96	0.08	1.34	0.20	5.95	0.03	0.48	0.07
DHEA	5.97	0.11	1.87	0.28	5.97	0.02	0.32	0.05
Epiandrosterone	5.97	0.04	0.66	0.10	5.99	0.11	1.80	0.27
Epitestosterone	5.98	0.07	1.17	0.18	5.98	0.04	0.66	0.10
Nandrolone	5.98	0.06	0.95	0.14	5.95	0.11	1.89	0.28
DHT	5.95	0.04	0.73	0.11	5.95	0.07	1.14	0.17
Estrone	5.97	0.03	0.42	0.06	5.98	0.12	1.98	0.30
Androstenodione	5.97	0.07	1.22	0.18	5.95	0.03	0.42	0.06
β-estradiol	5.95	0.10	1.65	0.25	5.98	0.11	1.84	0.28
Testosterone	5.94	0.02	0.26	0.04	5.96	0.16	2.68	0.40
Estriol	5.93	0.07	1.26	0.19	5.93	0.11	1.86	0.28
Progesterone	5.97	0.07	1.17	0.17	5.99	0.22	3.66	0.55
THE	29.93	0.01	0.04	0.01	29.93	0.01	0.04	0.01
THF	29.98	0.01	0.04	0.01	29.97	0.01	0.04	0.01
Cortisone	29.88	0.01	0.04	0.01	29.87	0.01	0.04	0.01
Cortisol	29.98	0.01	0.04	0.01	29.98	0.01	0.04	0.01

Table V
Repeatability of the Compounds Studied at High Concentration (ppb)

Compound	Quadrupole				Ion trap			
	Mean	S	CV (%)	15 % CV	Mean	S	CV (%)	15 % CV
Androsterone	9.96	0.03	0.33	0.05	9.96	0.04	0.40	0.06
5β-androsterone	9.96	0.02	0.23	0.03	9.94	0.07	0.75	0.11
DHEA	9.95	0.09	0.89	0.13	9.99	0.05	0.55	0.08
Epiandrosterone	9.98	0.02	0.23	0.03	9.92	0.04	0.39	0.06
Epitestosterone	9.96	0.06	0.60	0.09	9.96	0.04	0.37	0.06
Nandrolone	9.97	0.02	0.15	0.02	9.98	0.08	0.83	0.13
DHT	9.96	0.04	0.36	0.05	9.93	0.07	0.75	0.11
Estrone	9.96	0.02	0.18	0.03	9.97	0.02	0.18	0.03
Androstenodione	9.98	0.01	0.13	0.02	9.97	0.01	0.09	0.01
β-estradiol	9.97	0.07	0.72	0.11	9.96	0.02	0.18	0.03
Testosterone	9.95	0.04	0.43	0.07	9.95	0.03	0.29	0.04
Estriol	9.96	0.02	0.24	0.04	9.44	0.11	1.21	0.18
Progesterone	9.97	0.02	0.15	0.02	9.93	0.05	0.47	0.07
THE	49.99	0.01	0.03	0.01	49.98	0.01	0.02	0.01
THF	49.97	0.01	0.02	0.01	49.97	0.01	0.02	0.01
Cortisone	49.99	0.01	0.02	0.01	49.99	0.01	0.02	0.01
Cortisol	49.95	0.01	0.02	0.01	49.96	0.01	0.02	0.01

within the range of expected concentrations: low concentration, medium concentration and high concentration (Tables III, IV and V). The precision measure for each of the three concentrations should not exceed 15% CV of these measures (18) in all cases for both GC-Q–MS and GC-IT–MS-MS was carried out this premise.

Reproducibility is the degree of approximation between the results obtained with the same method on identical test material but under different conditions (different operators, different apparatus, different laboratories and after different time intervals). To determine the reproducibility, the CV was determined when five replicates of three different concentrations were injected within the range of expected concentrations (low concentration, medium concentration and high concentration) using ethyl *n*-hexane–ethyl acetate mixture (70/30, *v*/*v*) as solvent extractor by the GC-Q–MS and the GC-IT–MS-MS on different days. The condition to be fulfilled in all cases is that the precision measure for each of the three concentrations

Table VI
Reproducibility of the Compounds Studied at Low Concentration (ppb)

Compound	Concentration set	Mean	S	CV (%)	15% CV
Androsterone	1	0.98	0.07	7.34	1.10
5β-androsterone	1	0.99	0.05	4.70	0.70
DHEA	1	0.98	0.05	4.82	0.72
Epiandrosterone	1	0.97	0.05	4.87	0.73
Epitestosterone	1	0.98	0.03	2.91	0.44
Nandrolone	1	0.98	0.04	4.49	0.67
DHT	1	0.97	0.03	2.64	0.40
Estrone	1	0.99	0.03	3.28	0.49
Androstenodione	1	0.95	0.04	4.48	0.67
β-estradiol	1	0.95	0.03	2.96	0.44
Testosterone	1	0.96	0.04	4.24	0.64
Estriol	1	0.98	0.05	4.79	0.72
Progesterone	1	0.99	0.02	2.45	0.37
THE	5	4.99	0.05	1.02	0.15
THF	5	4.99	0.05	0.94	0.14
Cortisone	5	4.98	0.04	0.88	0.13
Cortisol	5	4.98	0.02	0.47	0.07

Table VII
Reproducibility of the Compounds Studied at Medium Concentration (ppb)

Compound	Concentration set	Mean	S	CV (%)	15% CV
Androsterone	6	5.94	0.04	0.66	0.10
5β-androsterone	6	5.96	0.06	0.95	0.14
DHEA	6	5.97	0.08	1.26	0.19
Epiandrosterone	6	5.98	0.08	1.30	0.19
Epitestosterone	6	5.98	0.05	0.90	0.13
Nandrolone	6	5.96	0.09	1.44	0.22
DHT	6	5.95	0.05	0.90	0.14
Estrone	6	5.98	0.08	1.35	0.20
Androstenodione	6	5.96	0.05	0.88	0.13
β-estradiol	6	5.96	0.10	1.68	0.25
Testosterone	6	5.95	0.11	1.80	0.27
Estriol	6	5.93	0.09	1.50	0.22
Progesterone	6	5.98	0.15	2.57	0.39
THE	30	29.93	0.01	0.04	0.01
THF	30	29.98	0.01	0.04	0.01
Cortisone	30	29.88	0.01	0.04	0.01
Cortisol	30	29.98	0.01	0.04	0.01

Table VIII
Reproducibility of the Compounds Studied at High Concentration (ppb)

Compound	Concentration set	Mean	S	CV (%)	15% CV
Androsterone	10	9.96	0.03	0.34	0.05
5β-androsterone	10	9.95	0.05	0.53	0.08
DHEA	10	9.97	0.07	0.74	0.11
Epiandrosterone	10	9.95	0.04	0.44	0.07
Epitestosterone	10	9.96	0.05	0.47	0.07
Nandrolone	10	9.98	0.06	0.57	0.08
DHT	10	9.95	0.06	0.58	0.09
Estrone	10	9.97	0.02	0.17	0.03
Androstenodione	10	9.97	0.01	0.14	0.02
β-estradiol	10	9.96	0.05	0.50	0.07
Testosterone	10	9.95	0.03	0.35	0.05
Estriol	10	9.70	0.28	2.94	0.44
Progesterone	10	9.95	0.04	0.40	0.06
THE	50	49.99	0.01	0.01	0.01
THF	50	49.97	0.01	0.01	0.01
Cortisone	50	49.99	0.01	0.01	0.01
Cortisol	50	49.96	0.01	0.01	0.01

does not exceed 15% CV of these measures (18) (Tables VI, VII and VIII).

The accuracy of the method was determined by measuring three concentrations in the range of expected concentrations (low concentration, medium concentration and high concentration) and five replicates per concentration. The average value should be within 15% of actual value (18). This is true for all concentrations studied, as shown in Tables IX, X and XI.

Table IX
Accuracy of the Compounds Studied at Low Concentration (ppb)

Compound	True value	Ion trap			Quadrupole		
		Mean	+15%	−15%	Mean	+15%	−15%
Androsterone	1.00	0.99	1.13	0.84	0.96	1.11	0.82
5β-androsterone	1.00	0.99	1.14	0.84	0.99	1.14	0.84
DHEA	1.00	0.98	1.13	0.83	0.97	1.11	0.82
Epiandrosterone	1.00	0.97	1.12	0.83	0.97	1.12	0.82
Epitestosterone	1.00	0.98	1.13	0.83	0.98	1.12	0.83
Nandrolone	1.00	0.97	1.11	0.82	0.99	1.14	0.84
DHT	1.00	0.99	1.14	0.84	0.95	1.09	0.80
Estrone	1.00	0.99	1.13	0.84	0.99	1.14	0.84
Androstenodione	1.00	0.92	1.06	0.78	0.99	1.14	0.84
β-estradiol	1.00	0.93	1.07	0.79	0.98	1.12	0.83
Testosterone	1.00	0.94	1.09	0.80	0.97	1.12	0.83
Estriol	1.00	0.97	1.11	0.82	0.99	1.14	0.84
Progesterone	1.00	0.99	1.14	0.84	0.99	1.14	0.84
THE	5.00	4.99	5.74	4.24	4.99	5.74	4.24
THF	5.00	4.99	5.74	4.24	4.99	5.74	4.24
Cortisone	5.00	4.97	5.72	4.22	4.98	5.73	4.24
Cortisol	5.00	4.98	5.73	4.23	4.98	5.72	4.23

Table X
Accuracy of the Compounds Studied at Medium Concentration (ppb)

Compound	True value	Ion trap			Quadrupole		
		Mean	+15%	−15%	Mean	+15%	−15%
Androsterone	6.00	5.96	6.85	5.06	5.95	6.84	5.05
5β-androsterone	6.00	5.95	6.85	5.06	5.97	6.87	5.08
DHEA	6.00	5.97	6.87	5.08	5.99	6.88	5.09
Epiandrosterone	6.00	5.99	6.89	5.09	5.98	6.87	5.08
Epitestosterone	6.00	5.98	6.88	5.08	5.98	6.88	5.08
Nandrolone	6.00	5.95	6.84	5.05	5.98	6.87	5.08
DHT	6.00	5.95	6.84	5.06	5.95	6.84	5.06
Estrone	6.00	5.98	6.87	5.08	5.97	6.87	5.08
Androstenodione	6.00	5.95	6.85	5.06	5.97	6.87	5.08
β-estradiol	6.00	5.98	6.88	5.08	5.95	6.84	5.05
Testosterone	6.00	5.96	6.86	5.07	5.94	6.84	5.05
Estriol	6.00	5.93	6.81	5.04	5.93	6.82	5.04
Progesterone	6.00	5.99	6.89	5.09	5.97	6.87	5.07
THE	30.00	29.93	34.42	25.44	29.93	34.42	25.44
THF	30.00	29.97	34.47	25.48	30.00	34.50	25.50
Cortisone	30.00	29.87	34.36	25.39	29.88	34.36	25.39
Cortisol	30.00	29.98	34.47	25.48	29.98	34.48	25.49

The recovery of the method was determined by calculating the recovery rate when following the previously described procedure for five replicates of three concentrations within the range of expected concentrations. Valid recoveries above 90% were found in the three concentrations in study. This is true for all studied concentrations, as shown in Table XII.

Standard curves were prepared in triplicate for each steroid. Peak area ratios (analyte/internal standard) were plotted against concentration, and the slope, interception and correlation coefficient were determined by linear regression analysis without including the zero point (Table XIII). These curves were linear within the concentration ranges studied, with correlation coefficients $R^2 > 0.9$. To assess the linearity of the calibration curve, a method was applied to test two hypotheses: the null hypothesis (H0), where there is no significant correlation between concentration and signal; and the alternative

Table XI
Accuracy of the Compounds Studied at High Concentration (ppb)

Compound	True value	Ion trap			Quadrupole		
		Mean	+15%	−15%	Mean	+15%	−15%
Androsterone	10.00	9.96	11.45	8.46	9.96	11.45	8.46
5β-androsterone	10.00	9.94	11.43	8.45	9.96	11.45	5.08
DHEA	10.00	9.99	11.49	8.49	9.95	11.44	5.09
Epiandrosterone	10.00	9.92	11.41	8.43	9.98	11.48	5.08
Epitestosterone	10.00	9.96	11.45	8.46	9.96	11.46	5.08
Nandrolone	10.00	9.98	11.47	8.48	9.97	11.47	5.08
DHT	10.00	9.93	11.42	8.44	9.96	11.46	5.06
Estrone	10.00	9.97	11.46	8.47	9.96	11.46	5.08
Androstenodione	10.00	9.97	11.46	8.47	9.98	11.48	5.08
β-estradiol	10.00	9.96	11.45	8.46	9.97	11.46	5.05
Testosterone	10.00	9.95	11.45	8.46	9.95	11.44	5.05
Estriol	10.00	9.44	10.86	8.02	9.96	11.45	5.04
Progesterone	10.00	9.93	11.42	8.44	9.97	11.47	5.07
THE	50.00	49.98	57.48	42.48	49.99	57.49	25.44
THF	50.00	49.97	57.47	42.48	49.97	57.47	25.50
Cortisone	50.00	49.99	57.49	42.49	49.99	57.49	25.39
Cortisol	50.00	49.96	57.45	42.46	49.95	57.44	25.49

Table XII
Recovery of the Compounds Studied at Low, Medium and High Concentrations

Compound	Low concentration		Medium concentration		High concentration	
	Quadrupole	Ion trap	Quadrupole	Ion trap	Quadrupole	Ion Trap
Androsterone	96.40	98.60	99.10	99.30	99.56	99.58
5β-androsterone	98.80	99.40	99.57	99.23	99.56	99.40
DHEA	96.80	98.20	99.77	99.53	99.46	99.90
Epiandrosterone	97.00	97.20	99.63	99.83	99.82	99.22
Epitestosterone	97.60	98.20	99.67	99.67	99.64	99.58
Nandrolone	98.80	96.80	99.60	99.10	99.74	99.76
DHT	94.60	99.20	99.20	99.17	99.62	99.28
Estrone	99.20	98.60	99.57	99.63	99.64	99.66
Androstenodione	99.00	91.80	99.57	99.23	99.82	99.66
β-estradiol	97.60	92.80	99.10	99.70	99.66	99.58
Testosterone	97.40	94.40	99.07	99.37	99.48	99.54
Estriol	99.00	96.60	98.80	98.77	99.60	94.40
Progesterone	98.80	99.00	99.50	99.80	99.74	99.32
THE	99.76	99.80	99.78	99.77	99.98	99.96
THF	99.80	99.76	99.99	99.91	99.94	99.94
Cortisone	99.68	99.40	99.59	99.58	99.98	99.98
Cortisol	99.52	99.60	99.95	99.92	99.90	99.92

Table XIII
Calibration Curves of Quantification of the Compounds Studied

Compound	Calibration curves		R2	
	MS–MS	MS	MS–MS	MS
Androsterone	y = 9.80x + 4.65	y = 0.82 + 1.02x	0.98	0.98
5β-androsterone	y = 0.27x + 0.11	y =1.76 + 0.60x	0.92	0.93
DHEA	y = 5.73x − 0.83	y =1.16 + 0.31x	0.94	0.99
Epiandrosterone	y = 0.01x + 0.03	y = 5.80 + 4.50x	0.99	0.92
Epitestosterone	y = 0.05x + 0.03	y = 0.08 + 0.44x	0.92	0.98
Nandrolone	y = 0.42x + 1.84	y = 0.65 + 0.32x	0.99	0.96
Estrone	y = 0.09x − 0.01	y = 4.03 + 2.16x	0.97	0.95
DHT	y = 0.15x + 0.01	y = 0.30 + 0.32x	0.95	0.94
Androstenodione	y = 3.84x + 2.66	y = 0.55 + 1.12x	0.96	0.95
17β-estradiol	y = 0.01x + 0.00	y = 1.26 + 2.26x	0.98	0.98
Testosterone	y = 0.38x − 0.04	y = 1.03 + 1.36x	0.95	0.96
Estriol	y = 6.93x + 32.27	y = 0.18 + 0.44x	0.98	0.92
Progesterone	y = 0.14x − 0.01	y = 0.38 + 0.19x	0.97	0.98
THE	y = 0.29x + 0.04	y = 0.04 + 0.04x	0.98	0.97
THF	y = 21.36x + 1.28	y = 0.03 + 0.30x	0.94	0.93
Cortisone	y = 5.59x + 0.68	y = 0.05 + 0.03x	0.98	0.94
Cortisol	y = 3.62x + 4.27	y = 0.01 + 0.01x	0.99	0.98

Table XIV
Linearity of the Calibration Curves

Compound	t Calculated (Ion trap)	t Calculated (Quadrupole)	t Tabulated
Androsterone	15.65	15.65	2.57
5β-androsterone	7.58	8.15	2.57
DHEA	8.85	22.25	2.57
Epiandrosterone	22.25	7.58	2.57
Epitestosterone	7.58	15.65	2.57
Nandrolone	22.25	10.95	2.57
DHT	12.71	9.75	2.57
Estrone	9.75	8.85	2.57
Androstenodione	10.95	9.75	2.57
β-estradiol	15.65	15.65	2.57
Testosterone	9.75	10.95	2.57
Estriol	15.65	7.58	2.57
Progesterone	12.71	15.65	2.57
THE	15.65	12.71	2.57
THF	8.85	8.15	2.57
Cortisone	15.65	8.85	2.57
Cortisol	22.25	15.65	2.57

hypothesis (H1), where there is significant correlation between concentration and signal. This defines two "t" values, the tabulated t corresponding to the value found in the table of student's t n-2 degrees of freedom, a 2-tailed $\alpha = 0.05$, and t calculated that corresponds to calculation using the following formula:

$$t_{calculated} = \frac{r \bullet \sqrt{(n-2)}}{\sqrt{1-r^2}}$$

If t calculated > t tabulated, the alternative hypothesis is true that there is significant correlation between concentration and signal. The results of the method are shown in Table XIV. In this case, both calibration lines drawn by the quadrupole analyzer to the calibration curve drawn by the ion trap team shows that t calculated > t tabulated; thus satisfying the alternative hypothesis (H1) and ensuring a significant correlation between concentration and signal.

The derivatization must be complete to ensure that the measuring of the analytes being quantified is correct. The current measurement of derivatization was performed with androsterone and etiocolanolone, both of which are isomeric in the derivatization test, thus ensuring that this derivatization was correct for both the alpha disposition and the beta disposition of the analytes. In this case, this derivatization was 100% for the androsterone and etiocholanolone.

To undertake the quantification of analytes, they must first be separated from their links to the glucuronide acid and sulphate group; two specific enzymes were used to do this: arylsulphatase and β-glucuronidase, which work in specific conditions. If these links are not broken, the steroid will not be freed and, thus, its later derivatization and analysis will not be possible.

It is very important to ensure that the enzymes hydrolyze the links well in the working conditions of the current method. Measurement of the percentage of hydrolysis was performed with androsterone and etiocolanolone in both sulfoconjugate and glucuroconjugate form in the hydrolysis test. In this case, the percentage of hydrolysis of the androsterone glucuro-conjugate was 100%, and the etiocholanolone glucuroconjugate 98%. The percentages of hydrolysis of the

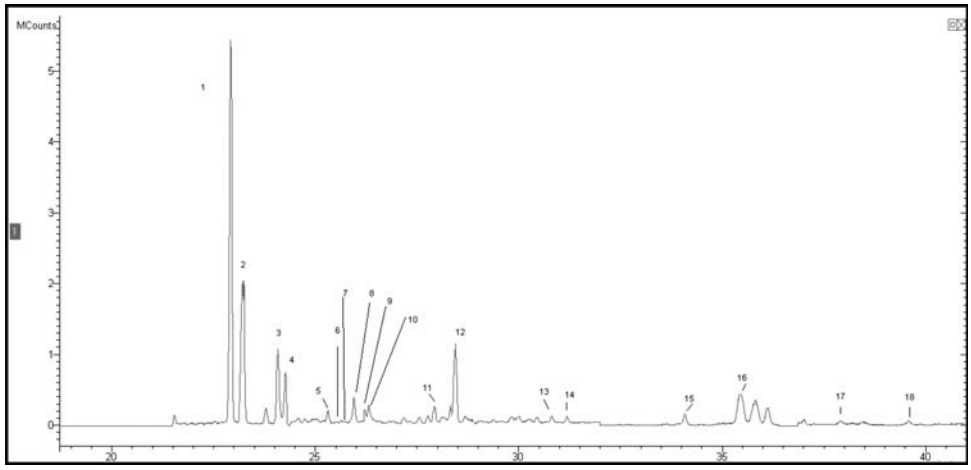

Figure 1. Free steroids in human plasma extracted with *n*-hexane-ethyl acetate 70/30 (*v*/*v*): 1 = androsterone; 2 = etiocolanolone; 3 = DHEA; 4 = epiandrosterone; 5 = epitestosterone; 6 = nandrolone; 7 = DHT; 8 = androstenodione; 9 = testosterone; 10 = 17β-estradiol; 11 = estrone; 12 = metiltestosterone; 13 = estriol; 14 = progesterone; 15 = THE; 16 = THF; 17 = cortisone; 18 = cortisol.

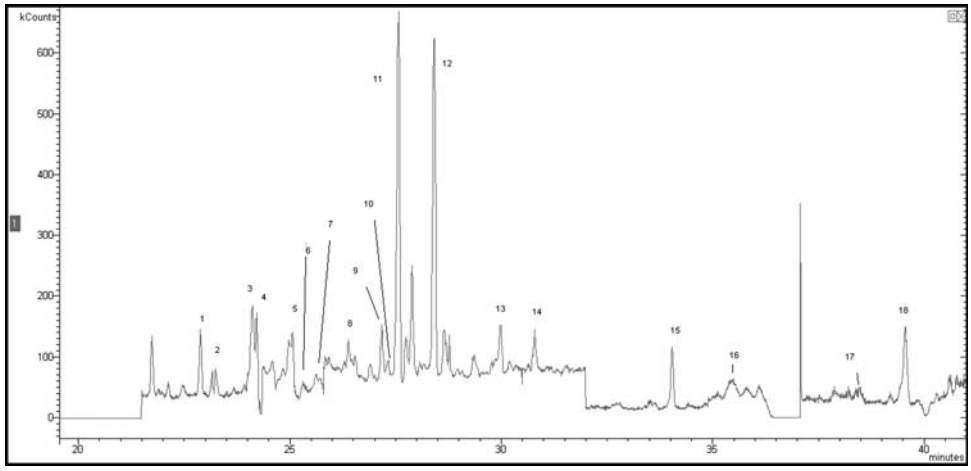

Figure 2. Glucuroconjugated steroids in human plasma extracted with *n*-hexane-ethyl acetate 70/30 (*v*/*v*): 1 = androsterone; 2 = etiocolanolone; 3 = DHEA; 4 = epiandrosterone; 5 = epitestosterone; 6 = nandrolone; 7 = DHT; 8 = androstenodione; 9 = testosterone; 10 = 17β-estradiol; 11 = estrone; 12 = metiltestosterone; 13 = estriol; 14 = progesterone; 15 = THE; 16 = THF; 17 = cortisone; 18 = cortisol

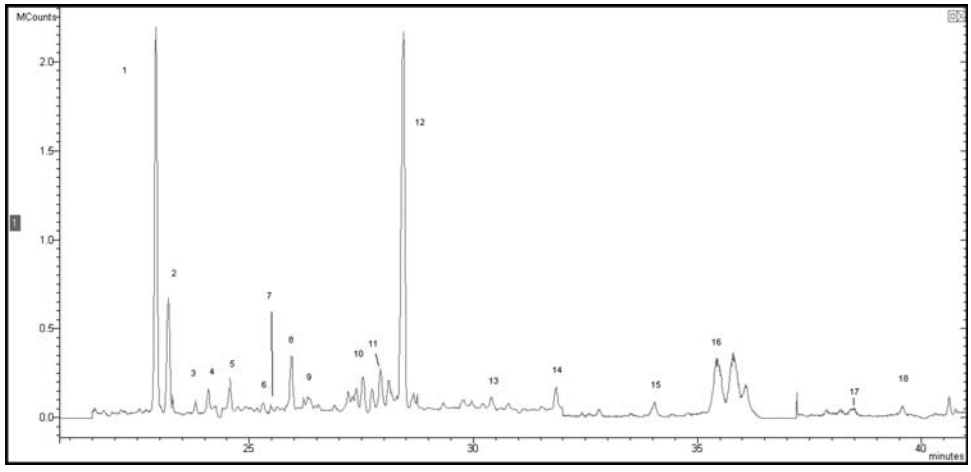

Figure 3. Sulphoconjugated steroids in human plasma extracted with *n*-hexane-ethyl acetate 70/30 (*v*/*v*): 1 = androsterone; 2 = etiocolanolone; 3 = DHEA; 4 = epiandrosterone; 5 = nandrolone; 6 = epitestosterone; 7 = DHT; 8 = androstenodione; 9 = estrone; 10 = testosterone; 11 = 17β-estradiol; 12 = metiltestosterone; 13 = estriol; 14 = progesterone; 15 = THE; 16 = THF; 17 = cortisone; 18 = cortisol.

Table XV
LOD, LOQ and LLOQ of the Compounds Studied

	LOD (ng/mL)		LOQ (ng/mL)		LLOQ (ng/mL)	
Compound	Ion trap	Quadrupole	Ion trap	Quadrupole	Ion trap	Quadrupole
Androsterone	0.20	0.35	0.33	0.33	0.33	0.58
5β-androsterone	0.20	0.52	0.33	0.33	0.33	0.87
DHEA	0.05	0.50	0.08	0.08	0.08	0.83
Epiandrosterone	0.36	0.02	0.60	0.60	0.60	0.03
Epitestosterone	0.05	0.01	0.08	0.08	0.08	0.02
Nandrolone	0.33	0.16	0.55	0.55	0.55	0.27
Estrone	0.11	0.02	0.18	0.18	0.18	0.03
DHT	0.02	0.39	0.03	0.03	0.03	0.65
Androstenodione	0.05	0.07	0.08	0.08	0.08	0.12
17β-estradiol	0.17	0.19	0.28	0.28	0.28	0.32
Testosterone	0.48	0.09	0.80	0.80	0.80	0.15
Estriol	0.06	0.74	0.10	0.10	0.10	1.23
Progesterone	0.13	0.18	0.22	0.22	0.22	0.30
THE	0.18	0.26	0.30	0.30	0.30	0.43
THF	0.04	1.60	0.07	0.07	0.07	2.67
Cortisone	0.29	0.05	0.48	0.48	0.48	0.08
Cortisol	0.02	0.14	0.03	0.03	0.03	0.23

androsterone and etiocholanolone sulfoconjugates were 95 and 94%, respectively.

Figures 1, 2 and 3 show the chromatograms obtained in unconjugated, glucoconjugated and sulfoconjugated serum fractions, respectively.

The detection limit can be defined according to the criteria 3s (19), which states that the LOD is the analyte concentration that provides the net signal that equals 3 times the standard deviation of the target signal:

$$LOD = \frac{Y_c - \bar{Y}_b}{b} = \frac{3s_b}{b}$$

where S_b is the deviation standard of the white, b is the calibration curve slope, y_c is the critical value of the brute signal and y_b is the average of the white signals.

The LOQ is the lowest concentration at which an analyte can be quantified correctly and the concentration by which an $S/N = 10$ is obtained, and the method used to calculate it is: $LOQ = (10/3)\ LOD = 10\ s_b/b$.

The results obtained by the current method for each of these analytes are reflected in Table XV. With the GC-IT–MS-MS and GC-Q–MS method, the greatest sensibility was found for dihydrotestosterone (0.08 ng/mL) and cortisol (0.08 ng/mL) and the lowest was found for epiandrosterone (1.59 ng/mL), whereas with GC–MS the greatest and lowest sensibility were found for epiatestosterone (0.05 ng/mL), epiandrosterone (0.02 ng/mL), estrone (0.02 ng/mL) and cortisone (0.05 ng/mL).

If LOD and LOQ of the two analyzers are compared, both the quadrupole analyzers and the ion trap analyzer are clearly valid for detecting and quantifying the steroid hormones being studied. It is worth observing that the advantage of the ion trap analyzer over the quadrupole analyzer is the possibility of making MS^2.

Conclusions

The aim of this study was to develop a sensitive method for the simultaneous determination of 17 natural steroids in plasma. The primary advantage of this method is the large number of steroids tested in a single run.

Regarding all the tested solutions, the only one that was able to determineall of the studied analytes, with a good variation coefficient was *n*-hexane–ethyl acetate (70/30, *v*/*v*).

The LOD of this method allowed a screening of the studied analytes to be absolutely sure of their presence or absence in human plasma, and the LOQ allowed a high level of certainty about the concentration of the studied steroids.

After performing the hydrolysis and derivatization tests with this method, the critical stages of the pretreatment procedure of the sample checked to be correct.

References

1. García, M., Carda, S.; Direct injection of physiological fluids in micellar liquid chromatography; *Journal of Chromatography B*, (1999); 736: 1–18.
2. Kuronen, P., Volin, P., Laitalainen, T.; Reversed-phase high-performance liquid chromatographic screening method for serum steroids using retention index and diode-array detection; *Journal of Chromatography B*, (1998); 718: 211–224.
3. Ceglarek, U., Kortz, L., Leichtle, A., Fiedler, G.M., Kratzsch, J., Thiery, J.; Rapid quantification of steroid patterns in human serum by on-line solid phase extraction combined with liquid chromatography–triple quadrupole linear ion trap mass spectrometry; *Clinica Chimica Acta*, (2008); 401: 114–118.
4. Galán-Martín, A., Maynar-Mariño, J., García, M., Rivero-Marabé, J., Caballero-Loscos, M., Maynar-Mariño, M.; Determination of nandrolone and metabolites in urine samples from sedentary persons and sportsmen; *Journal of Chromatography B*, (2001); 761: 229–236.
5. Bayle, M., Enea, C., Goetinck, P., Lafay, F., Boisseau, N., Dugué, B., *et al.*; Quantitative analysis of DHEA and androsterone in female urine: Investigating the effects of menstrual cycle, oral contraception and training on exercise-induced changes in young women; *Analytical and Bioanalytical Chemistry*, (2009); 393(4): 1315–1325.
6. Meklat, N., Tabet, J.C., de Ceaurriz, J.; Urine ratio of tetrahydrocortisol to tetrahydrodeoxycortisol to screen for the systemic administration of cortisone and hydrocortisone; *Forensic Science International*, (2009); 185: 13–17.
7. Leung, G., Chung, E., Ho, E., Kwok, W., Leung, D., Tang, F., *et al.*; High-throughput screening of corticosteroids and basic drugs in horse urine by liquid chromatography-tandem mass spectrometry; *Journal of Chromatography B*, (2005); 825: 47–56.
8. Strahm, E., Kohler, I., Rudaz, S., Martel, S., Carrupt, P., Veuthey, J., *et al.*; Isolation and quantification by high-performance liquid chromatography–ion-trap mass spectrometry of androgen sulfoconjugates in human urine; *Journal of Chromatography A*, (2008); 1196–1197: 153–160.
9. Pozo, O., van Eenoo, P., van Thuyne, W., Deventer, K., Delbeke, F.; Direct quantification of steroid glucuronides in human urine by liquid chromatography-electrospray tandem mass spectrometry; *Journal of Chromatography A*, (2008); 1183(1–2): 108–18.
10. Rauh, M.; Steroid measurement with LC–MS/MS in pediatric endocrinology; *Molecular and Cellular Endocrinology*, (2008); 301: 272–281.
11. Zieraua, O., Lehmann, S., Vollmer, G., Schänzer, W., Diel, P.; Detection of anabolic steroid abuse using a yeasttransactivation system; *Steroids*, (2008); 73: 1143–1147.
12. Yu, N., Ho, E., Tang, F., Wan, T., Wong, A.; Comprehensive screening of acidic and neutral drugs in equine plasma by liquid chromatography–tandem mass spectrometry; *Journal of Chromatography A*, (2008); 1189: 426–434.
13. Santen, R., Demers, L., Ohorodnik, S., Settlage, J., Langecker, P., Blanchett, D., *et al.*; Superiority of gas chromatography/tandem mass spectrometry assay (GC/MS/MS) for estradiol for monitoring of aromatase inhibitor therapy; *Steroids*, (2007); 72: 666–671.

14. Lee, J., Ettinger, B., Stanczyk, F., Vittinghoff, E., Hanes, V., Cauley, J., *et al.*; Comparison of methods to measure low serum estradiol levels in postmenopausal women; *Journal of Clinical Endocrinology and Metabolism*, (2006); 91: 3791–3797.
15. Shibasaki, H., Nakayama, H., Furuta, T., Kasuya, Y., Tsuchiya, M., Soejima, A., *et al.*; Simultaneous determination of prednisolone, prednisone, cortisol, and cortisone in plasma by GC-MS: Estimating unbound prednisolone concentration in patients with nephrotic syndrome during oral prednisolone therapy; *Journal of Chromatography B*, (2008); 870: 164–169.
16. Buiarelli, F., Coccioli, F., Merolle, M., Neri, B., Terracciano, A.; Development of a liquid chromatography–tandem mass spectrometry method for the identification of natural androgen steroids and their conjugates in urine samples; *Analytica Chimica Acta*, (2004); 526: 113–120.
17. Appelblad, P., Irgum, K.; Separation and detection of neuroactive steroids from biological matrices; *Journal of Chromatography A*, (2002); 955: 151–182.
18. Guidance for Industry Bioanalytical Method Validation. U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine (CVM), (2001) BP.
19. IUPAC; Analytical Chemistry Division: Nomenclature, symbol, units and their usage in spectrochemical analysis. II. Data interpretation. Spectrochim. *Acta*, (1978); 33 B: 241–246.