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Article

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Bioanalytical LC–MS Method for the Quantification of Plasma Androgens and Androgen Glucuronides in Breast Cancer

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Abstract

The physiological and pathological development of the breast is strongly affected by the hormonal milieu consisting of steroid hormones. Mass spectrometry (MS) technologies of high sensitivity and specificity enable the quantification of androgens and consequently the characterization of the hormonal status. The aim of this study is the assessment of plasma androgens and androgen glucuronides, in the *par excellence* hormone-sensitive tissue of the breast, through the application of liquid chromatography–mass spectrometry (LC–MS). A simple and efficient fit-for-purpose method for the simultaneous identification and quantification of dehydroepiandrosterone sulfate (DHEAS), androstenedione (A4), androsterone glucuronide (ADTG) and androstane-3α, 17β-diol-17-glucuronide (3α-diol-17G) in human plasma was developed and validated. The presented method permits omission of derivatization, requires a single solid-phase extraction procedure and the chromatographic separation can be achieved on a single C18 analytical column, for all four analytes. The validated method was successfully applied for the analysis of 191 human plasma samples from postmenopausal women with benign breast disease (BBD), lobular neoplasia (LN), ductal carcinoma *in situ* and invasive ductal carcinoma (IDC). DHEAS plasma levels exhibited significant differences between LN, IDC and BBD patients ($P < 0.05$). Additionally, ADTG levels were significantly higher in patients with LN compared with those with BBD ($P < 0.05$).

Introduction

Steroid hormones are lipophilic, low-molecular weight compounds known to exert a profound biological effect on target organs (1, 2). They include estrogens, androgens, progestagens and corticosteroids (3). Androgens and estrogens can exert intracrine action in the cells where they are synthesized (4). Subsequently, they are inactivated in the presence of steroid-modifying enzymes and are released in the circulation (4). Androgens circulate mainly in the form of inactive glucuronide metabolites of androsterone (ADT) and androstane-3α, 17β-diol (3α-diol), namely androsterone glucuronide (ADTG), and androstane-3α, 17β-diol-glucuronide (3α-diol-G) (5). 3α-diol-G exists in two different forms, androstane-3α, 17β-diol-3-glucuronide (3α-diol-3G) and androstane-3α, 17β-diol-17-glucuronide (3α-diol-17G) (6). Metabolic inactivation takes place mainly in the liver but also in other target tissues, like the breast (7).

Breast is both a steroid target organ and a site of active steroid metabolism. Its physiological and pathological development is strongly affected by the hormonal environment (8). The stimulatory activity of estrogens in combination with the inhibitory activity of androgens results in the appropriate hormonal balance for maintaining organ integrity and physiology (8, 9). An imbalance in androgen–estrogen ratio can lead to the development of malignancy (9). Androgens are the predominant steroids in the mammary gland and are synthesized from dehydroepiandrosterone (DHEA) (8). DHEA sulfate conjugate (DHEAS) is the most abundant steroid and is present in a 500-fold higher concentration than its precursor DHEA in human plasma (10, 11). DHEAS is the precursor of 75–100% of the estrogens in women (8, 12). It has been proposed as a tumor marker of breast cancer and as a risk factor for disease progression in patients treated with tamoxifen or fulvestrant (12, 13). It is noteworthy that besides DHEAS, glucuronide metabolites including ADTG, 3α-diol-3G and 3α-diol-17G are also present in the blood in considerably high concentrations (14, 15).

Steroids, transported via the blood, induce their effect on target tissues by binding to nuclear or cytosolic receptors at nano- or picomolar levels (16, 17). The type and magnitude of steroid action is strongly dependent on their concentration levels (2). They are potent enough so as to exert major physiological effects even at low concentrations, while level variations can be used for diagnostic purposes (17). In postmenopausal women, all estrogens and the vast majority of androgens are made locally in the peripheral target tissues with minimal diffusion (5). Therefore, the measurement of intratissular steroid activity in tissue samples obtained surgically is the ideal approach for accurate steroid quantification (18). Nevertheless, steroid metabolites represent the obligatory exit route of all steroids and can be measured in the circulation (5). Consequently, quantification of their circulating levels can be a powerful tool for the investigation of steroids effect on target organs and of their role in the etiology of related disorders (2).

The identification and quantification of steroids from minimally invasively acquired biological samples, through the application of novel analytical techniques like liquid chromatography–mass spectrometry (LC–MS), is the ultimate trend in modern laboratory medicine. Minimally invasive sampling eliminates potential changes in hormone levels that are due to the collection procedure (19). LC–MS analysis enables the accurate simultaneous measurement of multiple steroids, even at low concentrations, with high analytical sensitivity and specificity (20). Additionally, derivatization can be circumvented and the assays can be performed at room temperature, allowing the analysis of thermally sensitive compounds, like glucuronide metabolites (21). Another advantage of this technique is the ability to distinguish between structurally similar metabolites (22). As an example, the differential detection of the two isoforms of 3α-diol-G has been applied for the study of androgen tissue-specific glucuronidation and its correlation with the unique effect of each metabolite (6).

In a previous review, we queried whether LC–MS analysis of androgen glucuronides could raise new perspectives in the diagnostic field of hormone-dependent malignancies (23). In the present work, we performed a study on selected androgens and androgen glucuronides by LC–MS on the typical hormone-dependent malignancy of breast cancer. The goal of this study was 2-fold: first, to develop and validate a simple and efficient LC–MS method for the quantification of DHEAS, ADTG, 3α-diol-17G and androstenedione (A4), and second, and equally important, to apply the method for the quantification of the four steroids in four distinct groups of patients with benign and malignant breast diseases. As presented subsequently, we developed an advantageous method of androgen analysis. In comparison to previous studies, the method enables the simultaneous measurement of the four analytes from a single human plasma sample and allows A4 quantification without prior derivatization (5, 18, 24–26). Moreover, the method involves a single, simple solid-phase extraction (SPE) procedure, and the chromatographic separation is performed on a single analytical column. To the best of our knowledge, this is the first study evaluating androgen level variations between distinct histological types of breast diseases.

Participants and biospecimens

Participants

It has been well documented that higher plasma levels of endogenous steroid hormones and aging are strongly associated with an increased risk of breast cancer in postmenopausal women (27–29). In line with the above observations, our study included only postmenopausal women who underwent breast biopsy or surgery at the First Department of Propaedeutic Surgery, of Hippokratio Hospital of Athens. The study population consisted of 49 women with benign breast disease (BBD), 44 with lobular neoplasia (LN), 48 with ductal carcinoma *in situ* (DCIS) and 50 with invasive ductal carcinoma (IDC). BBD patients were diagnosed with fibroadenomas or fibrocystic breast disease while LN patients were diagnosed with atypical lobular hyperplasia (ALH) or lobular carcinoma *in situ* (LCIS). A detailed questionnaire with anthropometric data, gynecological anamnesis and family breast cancer anamnesis was obtained from all participants. Institutional Research Committee approved the protocol, and written informed consent was obtained from all the patients prior to study entry. Exclusion criteria included the presence of metabolic disorders, history of androgenic disorders and history of other cancer.

Biospecimens

Peripheral venous blood samples were collected in the morning between 8:00 a.m. and 10:00 a.m. after a period of overnight fasting, into separator vacutainers with anticoagulant ethylenediaminetetraacetic acid (EDTA). Subsequently the samples were centrifuged at 4,400 × *g* for 15 min at 8°C, divided into aliquots on ice and stored frozen (−80°C) until being assayed, at the Research Center of the Hellenic Anticancer Institute. Method development, validation and application in real samples were performed in the Laboratory of Forensic Medicine and Toxicology, School of Medicine, University of Athens, which is accredited with an EN/IEC ISO 17025.

Experimental

Materials and standards

Reference standards of ADTG, 3α-diol-17G, A4 and DHEAS were purchased from Steraloids (Newport, RI, USA). Internal standard (IS) androstenedione-d7 was obtained from Steraloids and was found to be suitable for all four analytes. All steroids and deuterated analog have reported chemical and isotopic purity greater than 99%. HPLC-grade acetonitrile, HPLC-grade methanol, HPLC-grade ammonia solution, HPLC-grade isopropanol, HPLC-grade formic acid and phosphate buffered saline (PBS) were purchased from Merck (Darmstadt, Germany). Water was deionized and purified on a Direct-Q water purification system (Millipore SA, Molsheim, France). All solutions were filtered through a 47-mm, 0.2-μm nylon membrane filter and degassed (Sartorius Stedim Biotech, Gnbh, Goettingen, Germany). HF Bond Elut LRC-C18 SPE cartridges (200 mg) were purchased from Agilent Technologies (Lake Forest, CA, USA).

Instrumentation and LC–MS conditions

A high-performance liquid chromatograph mass spectrometer LCMS-2010EV (HPLC system from Shimadzu, Kyoto, Japan) was employed for the study. The analyzer consists of a CBM-20Alite system controller, a CTO-20AC column oven, a LC 20AB pump equipped with a DGU $20A_5$ degasser, a SIL-20AC autosampler and a LCMS-2010EV single stage quadrupole mass analyzer coupled with an electrospray ionization source (ESI), operating in either negative or positive mode.

Sample solutions of ADTG, 3α-diol-17G, A4 and DHEAS were separated on a C18 Synergi 4u Hydro-RP, analytical column (150 mm × 4.6 mm, 4 μm; Phenomenex, USA) protected by a SecurityGuard C18 precolumn (4 mm × 3 mm, Phenomenex, USA) using a 20–80% gradient program of acetonitrile–0.05% formic acid buffer mobile phase at a flow rate of 250 μL/min. The chromatographic parameters were optimized to allow the selective separation of the four analytes. The autosampler temperature was set at 22°C. The injection volume of the reconstituted sample was 15 μL. A second confirmatory analysis was performed on an analytical column of different polarity (XTerra® MS C_8, 250 mm × 2.1 mm i.d., 5 μm; Waters Corporation, Milford, MA, USA) under the same analytical parameters, in order to collect the appropriate identification points and to meet the confirmation criteria as they are described in EC guidelines (30).

Nitrogen was used as drying and nebulizer gas. The nebulizer gas flow was set to 1.5 L/min. Curve desolvation line (CDL) and heat block temperatures were both optimized at 290°C. The appropriate Interface, CDL, Q-array DC and Q-array RF voltages were adjusted to 3, 5, −5 and 140 V, respectively. Detector voltage was 2.8 kV for ADTG, 3α-diol-17G, and A4 and 1.8 kV for DHEAS. Mass spectra of target compounds were obtained using scan mode (*m/z* 100–500). The instrument parameters were optimized by tuning the $[M-H]^-$, $[M+H]^+$ ions of the analytes, by direct infusion of their standard solutions. Quantitative analysis was performed by processing peak areas in selected ion monitoring chromatograms (SIM). SIM was used for detection of ionized molecules. MS coupling with ESI interface was used in negative ion mode and SIM at *m/z* 367, 465, 467 for DHEAS, ADTG, and 3α-diol-17G, respectively, and in positive ion mode and SIM at *m/z* 287 for A4. The mean retention times of the selected compounds were 7.22, 7.20, 8.59 and 26.76 min for DHEAS, 3α-diol-17G, ADTG and A4, respectively. The chromatographic data were acquired and processed using LC–MS solution software (version 3, Shimadzu).

Stock solutions, calibration curves and quality control sample preparation

A stock solution of each standard and deuterated IS (1 mg/mL) was prepared by dissolving in methanol. Sub-stock of 100, 10 and 1 μg/mL solutions were also prepared in methanol and used to prepare spiking standards to achieve appropriate concentrations. The calibration curves of the standard solutions had a dynamic range from 1.0 to 100 ng/mL using seven calibration points (1.0, 2.0, 5.0, 25, 50, 75 and 100 ng/mL) for ADTG and A4, 1.0 to 75 ng/mL using six calibration points (1.0, 2.0, 5.0, 25, 50 and 75 ng/mL) for 3α-diol-17G and 0.05 to 10 μg/mL using five calibration points (0.05, 0.1, 1.0, 5.0 and 10 μg/mL) for DHEAS. Calibrators and quality control (QC) samples of appropriate concentration were prepared by spiking blank plasma with an appropriate analyte stock or sub-stock solution. "The authentic analyte in authentic matrix" is considered the most appropriate approach for reliable assay performance. For endogenous steroid compounds quantification specifically, analyte levels in authentic matrix can in some situations be essentially negligible due to gender, age, diurnal changes, etc. (31). Based on this information, blood samples collected from elderly healthy individuals (80–90 years) were used as blank plasma, after they were tested by established analytical techniques that verified the negligible levels of the steroids of interest in these samples.

The standard curves were calculated by plotting peak area ratio (*Y*) of each analyte and IS versus concentration (*X*, ng/mL or μg/mL) with $1/x^{0.5}$ linear regression. The lowest standard on the calibration curve was defined as the limit of quantification (LOQ). In the present work, LOQ was adapted to the aim of the study and to expected concentrations for the study population (32). QC samples at concentrations of low 1.5 μg/mL, medium 4 μg/mL and high 8 μg/mL were prepared in human plasma for DHEAS. A low QC of 3 ng/mL and a high of 8 ng/mL were prepared for ADTG, 3α-diol-17G and A4. All solutions, samples and spiked calibrators and QC samples were stored in a frozen state (−20°C) until use.

Human plasma extraction

For extraction from plasma, 1 mL of human plasma sample was transferred to a glass tube. PBS (1 mL) was added and the tubes were vortexed. 50 μL of methanolic solution containing the deuterated IS was added to each tube. The C18 solid-phase cartridges were conditioned using 2 mL methanol followed by 2 mL of deionized and purified water. Samples were transferred to the SPE columns. Each column was washed with a solution (3 mL) of methanol:water (10:90, v/v). The analytes of interest were eluted using a solution (3 mL) of methanol:5% ammonia. The eluates, were evaporated to dryness under nitrogen at 50°C at 15 psi. The dried residue was reconstituted with 100 μL methanol and the sample was transferred into a vial for analysis.

Method validation parameters and acceptance criteria

The analysis of endogenous compounds in biological samples using chromatographic techniques lacks of established protocols or gold standard methods. The complexity of steroids, arising from their structure and their way of action, perplexes both the analytical and validation procedures. Considering these limitations and as thoroughly described by van de Merbel, a fit-for-purpose method was developed and validated for the simultaneous determination of four androgens and androgen glucuronides, generally following the Commission Decision 2002/657/EC (30, 32, 33). Any modifications were adjusted to the properties of the biological samples and to the aims of the study. The primary purpose was to detect the molecular ions of the analytes and their applicability for the quantitative analysis of the compounds in spiked human plasma and samples from real cases. Thus, the testing of the validation parameters in human plasma was a prerequesite. The method has been validated for selectivity, linearity, precision, accuracy and trueness (as corrected percentage recovery). Retention time and *m/z* were set as acceptance criteria for the identification of the compounds. The retention time of the analytes with specific *m/z* corresponded to that of the calibrator at a tolerance of ±2.5%. Additionally, the identification and quantification of the analytes were evaluated according to the ratios of signal-to-noise (S/N), S/N ≥ 3 and S/N ≥ 10, respectively.

Selectivity

Selectivity of the method was evaluated by analyzing structurally similar compounds (DHEA, androstane-3α, 17β-diol-3-glucuronide, estradiol, estriol, estrone, estrone sulfate, 2-hydroxy estrone, 4-hydroxy estrone, 16-hydroxy estrone). DHEA and androstane-3α, 17β-diol-3-glucuronide were not detectable due to low sensitivity at

Table I. Calibration Curve Characteristics

Analyte	Concentration range	Equations	LOD	LOQ
ADTG (ng/mL)	1–100	$y = 0.0093x + 0.1500$	0.333	1
3α-diol-17G (ng/mL)	1–75	$y = 0.0035x + 0.0344$	0.333	1
A4 (ng/mL)	1–100	$y = 0.0173x + 0.0176$	0.333	1
DHEAS (μg/mL)	0.05–10	$y = 0.5000x + 0.6740$	0.016	0.05

Table II. Intra- and Inter-Day Precision and Accuracy of Spiked QC Samples

Analyte	QC low			QC med			QC high		
	Mean	RSD%	Accuracy%	Mean	RSD%	Accuracy%	Mean	RSD%	Accuracy%
Intra-day variation ($n = 6$)									
ADTG (ng/mL)	3.08	4.83	2.78				7.90	2.12	−1.31
3α-diol-17 G (ng/mL)	2.59	8.54	−13.62				8.70	3.93	8.78
A4 (ng/mL)	3.05	1.33	1.53				8.40	1.76	5.06
DHEAS (μg/mL)	1.69	4.83	12.92	4.32	1.51	8.12	9.06	2.28	13.20
Inter-day variation ($n = 12$)									
ADTG (ng/mL)	3.13	8.88	4.50				8.10	4.31	1.31
3α-diol-17 G (ng/mL)	2.73	9.64	−8.99				8.13	7.71	1.68
A4 (ng/mL)	3.00	2.85	0.06				8.38	3.96	4.70
DHEAS (μg/mL)	1.60	13.46	6.59	4.13	11.20	3.14	8.08	18.74	1.04

the MS conditions of the method. Selectivity was tested between the analytes by separate injection and analysis of individual standard solutions and of a mixed solution at the concentration of 100 ng/mL. The absence of interfering peaks at the retention time of the analytes and/or peak interferences with area 3.3 times lower than the limit of detection (LOD: 3.3 S/N) certified the selectivity of the method.

Linearity

The linearity of the method was tested in the range of 1–100 ng/mL for ADTG and A4, 1–75 ng/mL for 3α-diol-17G and 0.05–10 μg/mL for DHEAS. The calibration curves were linear in the corresponding dynamic ranges and can be described by the equation $y = ax + b$, whereas the slopes were reproducible and the correlation coefficients (r) were greater than 0.998. Calibration characteristics of typical curves obtained by the analytical method are reported in Table I.

Accuracy and precision

Accuracy was defined as the percentage of the measured concentration of the spiked QC sample after substraction of the nominal concentration against the nominal concentration. Intra-assay accuracy and precision were determined by measuring six human spiked QC samples on the same day. Inter-assay accuracy and precision were evaluated by 12 replicate analyses of the QC samples by a three-day experiment. Over the range of the assays, the intra-day and the inter-day precision were <15%, except for the high QC of DHEAS. However, 18.4% inter-day precision is considered acceptable, since DHEAS is an endogenous compound and might present higher relative standard deviation (% RSD) values, provided that they are in accordance with the respective QC values in patient samples (34). The intra- and inter-day accuracy was acceptable at all concentrations. For all the compounds, the results were within the accepted range, confirming their validity. The results obtained are summarized in Table II.

LOQ and LOD

The LOQ was hereby calculated based on the criterion of S/N ≥ 10, as 1 ng/mL for ADTG, A4 and 3α-diol-17G and 0.05 μg/mL for DHEAS. Under the chromatographic and detection conditions used, the LOD was calculated based on the criterion of S/N ≥ 3 to be at the level of 0.333 ng/mL for ADTG, A4 and 3α-diol 17-G and 0.016 μg/mL for DHEAS (Table I).

Recovery

In this method, recovery (trueness) was calculated as a percentage of the measured concentration minus the endogenous concentration against the nominal concentration. The presence of the deuterated IS enabled the correction of the recovery for potential losses during sample preparation. Quantitative recovery was assessed from a plasma sample with low endogenous steroid levels, spiked with the analytes at the QC concentrations. More specifically, spiked plasma samples were prepared at the concentration of 3 and 8 ng/mL for ADTG, 3α-diol-17G and A4 and at 1.5, 4.0 and 8.0 μg/mL for DHEAS. The intra-assay recovery ranged from 86.38 to 113.20% and the inter-assay from 91.55 to 107.90%. Recovery was also calculated from the ratio of areas between spiked extracted sample and unextracted standard sample. Similar results were yielded in both cases.

Stability

Stability was validated by checking the storage conditions and by performing analysis of stock solutions, spiked and unspiked plasma samples. Stock solutions of all analytes were stable for at least two years at −20°C. Spiked plasma samples were stable in the instrument autosampler for at least 24 h. Long-term stability was assessed with standard methanolic solutions. The stability samples were run on Days 0, 3, 15, 45 and 150. Stability was tested by comparing each sample with a freshly prepared standard. ADTG, 3α-diol-17G, A4, DHEAS and androstenedione-d7 were stable at −20°C for 5 months (data not shown).

Quantitative analysis

The quantitative analysis of the analytes in the validation and patient samples was interpolated from calibration curves constructed by calculating the ratios of analyte peak area/IS peak area. Representative

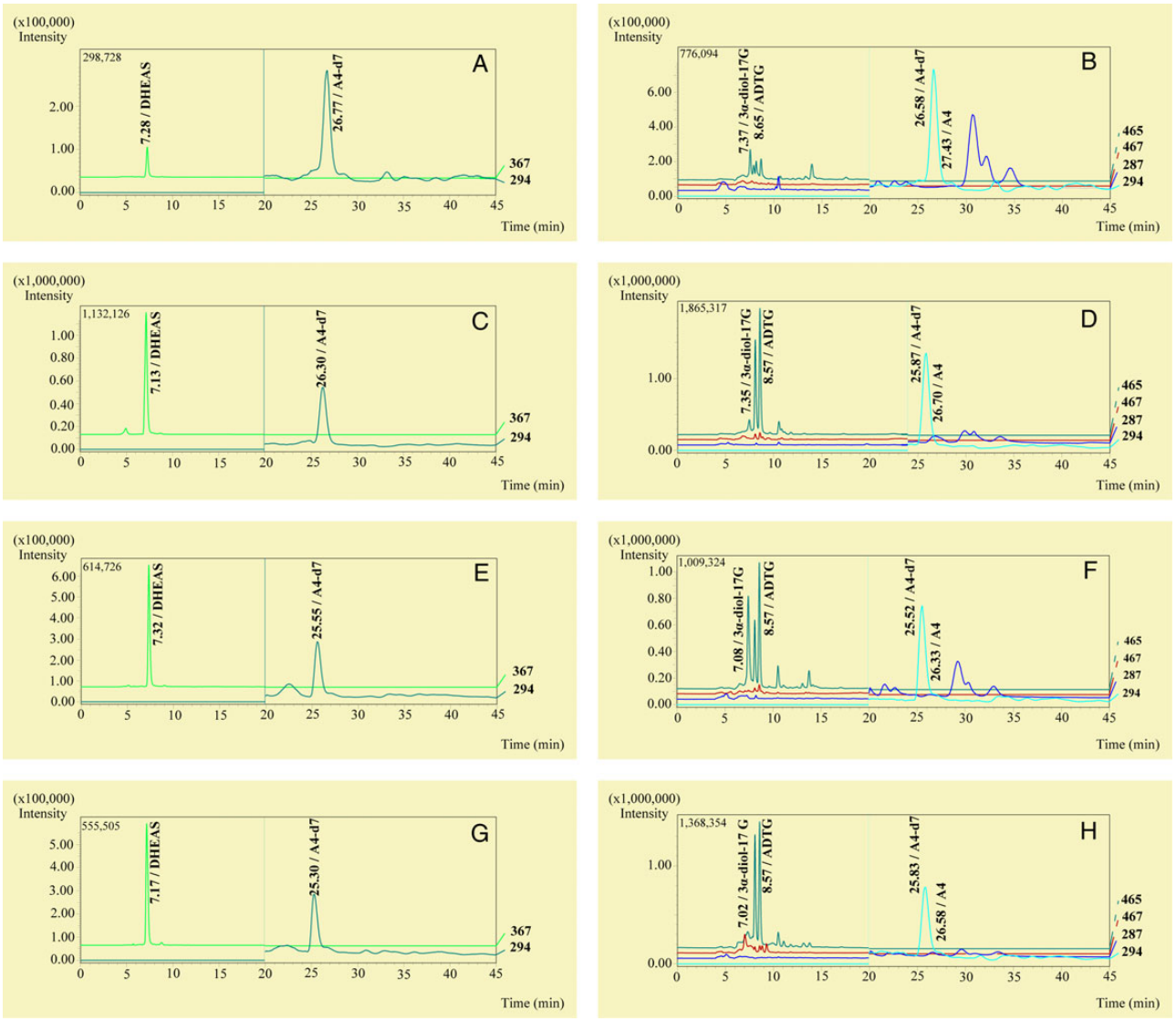

Figure 1. Representative LC–MS chromatograms of DHEAS and IS (A, C, E, G), and ADTG, 3α-diol-17G, A4 and IS (B, D, F, H) in plasma samples of patients with BBD (A, B), LN (C, D), DCIS (E, F) and IDC (G, H).

SIM chromatograms obtained from patient plasma samples are shown in Figure 1.

Statistical analysis

The statistical analysis of the validated method was performed using the SPSS 10.00 software (SPSS Inc, Chicago, IL, USA), while the analyses of plasma hormone levels were conducted using the SAS software version 9.3 (SAS Institute Inc., Cary, NC, USA). All statistical tests were two-tailed. The level of statistical significance was set at 0.05.

We assessed differences in the quantitative characteristics among the four groups using the analysis of variance (ANOVA) test or the non-parametric Kruskall–Wallis test, as appropriate. We compared qualitative characteristics with the χ^2-test of independence. Characteristics are presented as mean ± SD for continuous variables with normal distributions, as median [Q1, Q3] for non-normally distributed variables and as frequency (%) for categorical variables. Plasma hormone values below the LOQ were set equal to 1/2 of its value. Spearman's correlation coefficients were used to examine the interrelationships among plasma hormone levels. Comparisons of hormonal values between LN vs. BBD and LN vs. IDC were performed using the non-parametric Wilcoxon rank-sum test. As DHEAS, A4, ADTG and 3α-diol-17G were not normally distributed, their values were logarithmically transformed before further statistical analysis. Univariate linear regression models were used to evaluate the association of selected characteristics of the participants, including body mass index (BMI), age at menarche, age at menopause, parity, age at first full-term childbirth, time between age at menarche and first full-term childbirth, hormone therapy and family history of breast cancer, with the mean value of the log transformed hormonal levels.

Application and results

Characteristics of participants

Descriptive statistics of the study population are presented in Table III. Participants had a mean age of 62 ± 9 years. The four groups were similar with regard to mean BMI, parity, hormone therapy and mean age at first full-term childbirth. LN patients were younger,

Table III. Selected Demographic Characteristics of Patients Presented as Mean ± SD for Continuous Variables and as percentage (%) for Categorical Variables

	ALL ($N = 191$)	BBD ($N = 49$)	LN ($N = 44$)	DCIS ($N = 48$)	IDC ($N = 50$)	*P*-value
Age (years)	61.75 ± 9.44	60.77 ± 8.77	56.48 ± 8.12	63.15 ± 8.31	66.06 ± 9.98	**<0.001**
BMI (kg/m^2)	27.56 ± 4.98	27.55 ± 5.29	27.08 ± 4.11	27.75 ± 4.89	27.64 ± 5.30	0.960
Age at menarche (years)	12.77 ± 1.43	13.34 ± 1.50	12.04 ± 1.47	12.67 ± 1.22	12.66 ± 1.36	**0.002**
Age at menopause (years)	49.24 ± 4.09	49.06 ± 4.39	50.54 ± 2.55	50.04 ± 3.75	47.80 ± 4.52	**0.015**
Age at first full-term childbirth (years)	24.89 ± 5.43	23.74 ± 5.50	26.00 ± 5.98	26.34 ± 4.33	24.12 ± 5.71	0.094
Time between menarche and first full-term childbirth (years)	12.09 ± 5.67	10.39 ± 5.77	14.09 ± 6.39	13.64 ± 4.47	11.27 ± 5.65	**0.016**
Parity						
No	12.7%	10.4%	8.3%	15.6%	14.6%	0.772
Yes	87.3%	89.6%	91.7%	84.4%	85.4%	
Hormone therapy						
No	81.4%	85.4%	82.6%	81.0%	77.1%	0.770
Yes	18.6%	14.6%	17.4%	19.0%	22.9%	
Family history of breast cancer						
No	68.3%	83.3%	54.2%	67.4%	61.2%	**0.039**
Yes	31.7%	16.7%	45.8%	32.6%	38.8%	

P-values depicted in bold denote statistical significance.

Table IV. Plasma Hormone Levels in the Four Study Groups

Plasma hormones	BBD ($N = 49$)	LN ($N = 44$)	DCIS ($N = 48$)	IDC ($N = 50$)	*P*-value[a]	*P*-value for LN vs. BBD[b]	*P*-value for LN vs. IDC[b]
ADTG (ng/mL)							
Median	12.22	15.44	14.84	12.67	0.115	**0.019**	0.066
[Q1, Q3]	[7.23, 19.37]	[11.23, 29.85]	[7.84, 22.59]	[6.31, 23.80]			
3α-diol-17G (ng/mL)							
Median	1.92	2.11	1.86	2.12	0.796	0.457	0.876
[Q1, Q3]	[1.00, 3.02]	[1.39, 2.72]	[1.35, 2.63]	[1.37, 3.19]			
ADTG + 3α-diol-17G (ng/mL)							
Median	12.72	17.97	16.31	15.38	0.106	**0.011**	0.092
[Q1, Q3]	[8.71, 22.60]	[13.53, 33.83]	[9.69, 25.85]	[7.81, 26.60]			
A4 (ng/mL)							
Median	2.09	1.89	1.36	1.80	0.799	0.990	0.839
[Q1, Q3]	[0.50, 2.67]	[0.50, 2.44]	[0.50, 2.59]	[0.50, 2.53]			
DHEAS (μg/mL)							
Median	0.50	0.85	0.57	0.51	**0.019**	**0.015**	**0.002**
[Q1, Q3]	[0.28, 0.92]	[0.58, 1.41]	[0.29, 0.96]	[0.32, 0.77]			

Values are expressed as median [interquartile range]. *P*-values depicted in bold denote statistical significance.
[a]Derived from Kruskal–Wallis test.
[b]Derived from Wilcoxon test for independent samples.

with slightly lower mean age at menarche and slightly higher mean age at menopause. A higher proportion of these patients had a family history of breast cancer and a longer mean time interval between menarche and first full-term childbirth than DCIS, IDC and BBD patients.

Plasma hormone levels in patients with benign and malignant breast diseases

Median plasma concentrations of DHEAS, A4, ADTG, 3α-diol-17G and the sum of (ADTG + 3α-diol-17G) are shown in Table IV. We considered that the sum of ADTG and 3α-diol-17G is a fairly accurate estimate of total androgens, because ADTG accounts for 93% of the total androgen glucuronide derivatives while 3α-diol-17G is the predominant circulating isomer (≈5.6%) (8, 35).

According to our results, plasma levels of DHEAS, ADTG and the sum of (ADTG + 3α-diol-17G) were significantly higher in patients with LN compared with those with BBD ($P < 0.05$). The LN group also exhibited significantly higher levels of DHEAS compared with the IDC group ($P = 0.002$), while the difference with the DCIS group was marginally statistically significant ($P = 0.057$). Regarding plasma levels of 3α-diol-17G and A4, no statistically significant differences were observed between the four patient groups. Boxplots of plasma hormone levels in patients with BBD, LN, DCIS and IDC are presented in Figure 2.

Correlations between hormones

Table V presents the Spearman correlation coefficients between all four hormones. Considering that the studied hormones are metabolites of one another, we expected relatively strong correlations between them. Indeed, there was a strong correlation of DHEAS with ADTG and the sum of (ADTG + 3α-diol-17G). Moderate, albeit statistically

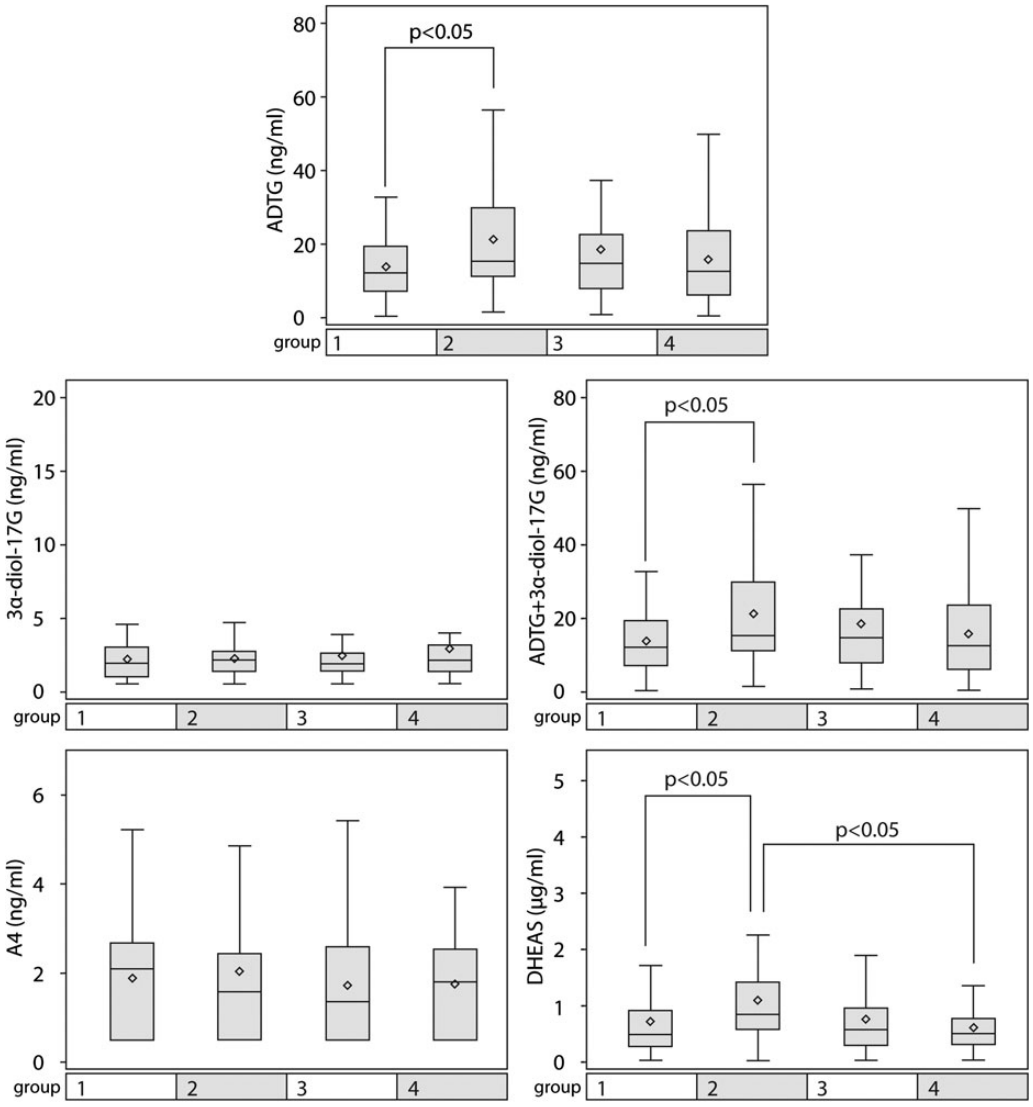

Figure 2. Plasma levels of ADTG, 3α-diol-17G, ADTG + 3α-diol-17G, A4 and DHEAS depicted as boxplots. Boxes represent the interquartile range; lines inside boxes represent the median value; whiskers represent the 5th and 95th percentiles. The 1–4 scale represents breast disease lesions as follows: 1: benign breast disease ($n = 49$), 2: lobular neoplasia ($n = 44$), 3: ductal carcinoma *in situ* ($n = 48$) and 4: invasive ductal carcinoma ($n = 50$).

Table V. Spearman's Rank Correlation Coefficient and Associated *P*-values Showing the Interrelationships Among Hormones

	ADTG	3α-diol-17G	ADTG + 3α-diol-17G	A4	DHEAS
ADTG					
3α-diol-17G	+0.31 (<0.0001)				
A4	+0.05 (0.4747)	+0.04 (0.5511)	+0.04 (0.5811)		
DHEAS	+0.71 (<0.0001)	+0.36 (<0.0001)	+0.70 (<0.0001)	+0.19 (0.009)	

Shaded cells indicate autocorrelations.

significant correlations were observed for 3α-diol-17G with ADTG. Finally, a statistically significant correlation, even though weak, was found between A4 and DHEAS.

Associations between hormones and patient characteristics

The crude effect of participant characteristics on the log transformed values of hormone plasma concentrations was investigated in each of the four groups using simple linear regression analysis. In the BBD group, a statistically significant association was observed between 3α-diol-17G and the age at menopause ($P = 0.02628$, $\beta = -0.05824$). In the LN group, statistically significant associations were found between DHEAS and the time interval between menarche and first full-term childbirth ($P = 0.02144$, $\beta = -0.0663$), as well as between A4 and the age at menopause ($P = 0.04704$, $\beta = -0.13073$). In the IDC group, the age at menarche was significantly related to ADTG ($P = 0.00148$, $\beta = -0.33361$) and to the sum of (ADTG + 3α-diol-17G) ($P = 0.00234$, $\beta = -0.27408$).

In the DCIS group, several significant associations were identified between hormone levels and patients characteristics. More specifically, ADTG was significantly associated with the age at first full-term childbirth ($P = 0.01720$, $\beta = -0.08060$), and the time between menarche and first full-term childbirth ($P = 0.02153$, $\beta = -0.07543$). The sum of (ADTG + 3α-diol-17G) was also significantly associated with the age at first full-term childbirth ($P = 0.01301$, $\beta = -0.06879$) and the time interval between menarche and first full-term childbirth ($P = 0.01457$, $\beta = -0.06555$). The age at first full-term childbirth ($P = 0.00285$, $\beta = -0.11537$) and the time between menarche and first full-term childbirth ($P = 0.00220$, $\beta = -0.11415$) were also associated with DHEAS. A4 was significantly associated with BMI ($P = 0.00047$, $\beta = -0.08845$). Finally, statistically significant associations were found between parity and ADTG ($P = 0.01239$, $\beta = -0.94932$), 3α-diol-17G ($P = 0.01914$, $\beta = -0.64437$) and the sum of (ADTG + 3α-diol-17G) ($P = 0.00307$, $\beta = -0.94277$).

Discussion

The application of MS as a diagnostic tool and as a biomarker discovery platform is emerging as one of the most promising new approaches in cancer diagnostics (36). MS offers high specificity, accuracy, precision and sensitivity, and when coupled with liquid chromatography (LC–MS) it is additionally qualified with a simplified sample preparation procedure (37). LC–MS has been recognized as a primary analytical technique for the accurate quantification of endogenous steroids in biological specimens (38). The biological material used for steroid analysis includes serum, plasma, urine and saliva (20). Human plasma is an extremely precious biofluid for biomarker discovery, as plasma proteome contains almost all proteins as a result of "tissue leakage" throughout the entire body (39). DHEAS and A4 are two of the most abundant circulating steroids and are thus an intriguing field of study (40). Since all androgens are inactivated in the glucuronide derivatives of ADT and 3α-diol, the blood concentrations of the latter two represent the sum of all the androgen metabolites released from all tissues (5, 8, 18, 24). Hence, the quantitative measurement of ADTG and 3α-diol-G permits an accurate assessment of total androgenic activity (8). Analysis of androgen glucuronides is considered more advantageous compared with that of bioactive androgens, because they have less day-to-day variability and thus provide a more accurate overall index of exposure to androgens (41). DHEAS may also be a valuable indicator of androgenic activity, considering that its half-life period is long (10–20 h) and that its concentration over a day varies little (40, 42).

Recent reports have focused on the study of the four analytes of interest in serum through the application of LC–MS-MS and GC–MS (5, 18, 24–26). These studies report different sample preparation processes for each analyte and the use of different analytical columns for the chromatographic separation. Specifically, the sample preparation for A4 requires derivatization while those for ADTG, 3α-diol-17G and DHEAS require different SPE procedures. In this study, we present the development and validation of an LC–MS method for the simultaneous multi-analyte quantification of ADTG, 3α-diol-17G, DHEAS and A4 from a single plasma sample. The multi-analyte capability gives the opportunity to explore entire metabolic pathways, revealing hormone profiles within steroid-producing and steroid-dependent tissues, like the breast (43). This method involves a single, simple and low-cost SPE procedure for all four analytes, eliminating the derivatization process prior to the assay. The assay can be performed at room temperature allowing the analysis of thermally sensitive compounds, including ADTG and 3α-diol-17G (21). An advantage of this method is that the chromatographic separation is achieved on a single analytical column. The analysis of plasma biospecimens demonstrates good linearity, precision and accuracy, allowing accurate measurement of all four analytes. Furthermore, our results are in accordance with recent studies reporting the application of mass spectrometric techniques as a cancer biomarker discovery tool (36, 38).

Breast cancer encompasses a genetically and clinically heterogeneous group of neoplasms. The diversity observed among breast cancer patients as well as between and within tumors determines the risk of disease progression and the choice of therapeutic intervention (44). Patients are stratified into subgroups exhibiting different risk factors, histological features, clinical behavior and response to treatment (36, 45). Quantification of circulating biomarkers is a valuable tool, which in combination with medical imaging, potentiates the optimization of clinical management (36).

Breast cancer is broadly categorized into *in situ* and invasive carcinoma and is further sub-classified as either ductal or lobular (46). LN is a distinct subtype of breast tumors, encompassing ALH and LCIS (47). LN is recognized as a non-obligate precursor of invasive breast cancer and as a marker of increased risk for subsequent breast carcinoma development (47). Although LN predominantly occurs in premenopausal women, epidemiological data report a great absolute increase in the incidence only in postmenopausal individuals, representing the majority of breast cancer patients (48).

Postmenopausal women normally have low circulating levels of androgens, but those who exhibit elevated levels are at a higher risk for developing breast cancer (28, 29). In line with the above observations, only postmenopausal women were included in our study. Interestingly, patients with LN exhibited significantly increased levels of DHEAS, ADTG and the sum of (ADTG + 3α-diol-17G) compared with BBD patients. Moreover, significant differences in plasma levels of DHEAS were observed between LN and IDC patients. Furthermore, the intriguing comparison of DHEAS plasma levels between LN and DCIS patients merits a comment although the result reached marginal statistical significance. The differential diagnosis of LCIS from low-grade DCIS is rather challenging, even in biopsy specimens (48). Thus, it will be of interest to perform further studies including a larger sample size which could eventually verify the above observation.

The quantitative analysis of plasma DHEAS and ADTG with the developed method enabled the discrimination between LN and BBD patients. Additionally, DHEAS can distinguish between LN and IDC patients. Distinguishing LN from other breast tumors is of essential importance, particularly for the patient's follow-up and treatment as LN patients are usually submitted to close follow-up and occasionally chemoprevention with tamoxifen (49). The effect of androgens on the mammary gland is influenced by the absence or presence of estrogens. In the first case, androgens are converted into estrogens in the presence of aromatase enzymes and they subsequently bind to estrogen receptors (ER) mediating ER stimulation (50). The administration of an anti-estrogen can be an effective treatment, blocking their effect. In the second case, androgens act as anti-estrogens by binding to the androgen receptor (AR) and inhibit the estrogenic stimulation of growth. In the latter case, anti-androgen treatment can obstruct their effect (50–53). Notably, studies report that androgens and their metabolites can bind directly to ER exerting estrogenic effects (54, 55). It has been suggested that DHEAS stimulatory effect on the proliferation of the MCF-7 human breast cancer cell line is mediated by the ER (55). Additionally, DHEAS can be converted in androstenediol, which is able to cause mitogenesis via ER binding (56). The study of patients with

elevated plasma levels of ADTG and DHEAS could provide a further insight of whether LN can be regarded as a marker of increased risk for breast carcinoma development, while their application as biomarkers could guide the therapeutic management of these patients.

ER is expressed as ERα or ERβ (57). The two ERs differ in the NH_2-terminal A/B domain, in the hinge region and also in their tissue distribution (57, 58). ERα is more abundant than ERβ in the breast and has a prominent role in the study and management of the hormone-dependent breast cancer (57, 59). ERα expression varies according to the developmental stage of the mammary gland, whereas ERβ is expressed in ~70% of breast epithelial cells at all stages of breast development (57, 60). It is of considerable interest that ERα is the primary target for endocrine therapies (57). Studies report that A4 and 3α-diol have a higher affinity for ERβ than for ERα (61). A recent study on the differential expression of ERs reports that lobular carcinoma expresses both ERα and ERβ whereas ductal carcinoma only the ERα (62). In our study, although A4 exhibited higher levels in the LN group and 3α-diol-17G in the IDC group the results were not statistically significant. Nevertheless, the affinity of A4 and 3α-diol-17G for ERβ has to be further elucidated as ERβ could provide an effective alternative for the management of hormone-resistant ER positive breast cancer.

Conclusion

A simple and efficient LC–MS method was developed and validated for the simultaneous determination of DHEAS, ADTG, 3α-diol-17G and A4 in human plasma and was efficiently applied in the study of breast cancer. To the best of our knowledge, this is the first multianalyte study of the four compounds on four well-defined groups of patients with benign and malignant breast diseases, based on the histological model of human breast cancer evolution (63). Our results support the potential usefulness of ADTG and DHEAS quantitative analysis by LC–MS in the differential diagnosis of different histological types of breast disease, particularly in postmenopausal women.

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