Journal of Chromatography B, 878 (2010) 845–852

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Quantification of endogenous steroids in human urine by gas chromatography mass spectrometry using a surrogate analyte approach

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ARTICLE INFO

Article history:
Received 29 July 2009
Accepted 24 January 2010
Available online 29 January 2010

Keywords:
Endogenous steroid
Surrogate analyte
Calibration curve
Urine
Gas chromatography mass spectrometry

ABSTRACT

Providing "real blank sample" is a problem in determination of endogenous steroids in complex matrices. A new quantification strategy is proposed in the present study, which is based on using isotope-labeled steroids instead of natural steroids for constructing calibration line. This approach is called surrogate analyte and it is shown that its accuracy is better than some of the previously described methods at low concentrations and comparable to standard addition method at medium and high concentration levels. The method was fully validated to satisfy the ICH criteria and it was applied for determination of endogenous steroids in several urine samples.

1. Introduction

A major part of many of quantitation experiments is constructing a calibration line, which is usually accomplished by making serial dilutions of the analyte in neat organic or aqueous solvents and obtaining the response for each of the dilutions followed by plotting the concentrations vs. the corresponding responses measured for the analyte in those dilutions. This method will only be applicable for quantification of the analyte in simple and rather neat unknown solutions. However, it would not be proper for samples with complex matrices, which need extraction. In these cases, extraction efficiency is a crucial part of the quantitation. Biological fluids such as plasma and urine are among these cases. This problem is surmounted by spiking a blank of the sample with sequentially increased amounts of the analyte and making a calibration equation based on these calibration samples. Using suitable internal standard accompanied with the analyte will eliminate many of the random errors and improve the accuracy and precision of quantification [1]. This approach is a routine method in most of the analytical experiments. In cases where the quantitation of an endogenous compound (e.g., a steroid) is concerned, unfortunately none of the above approaches can completely solve the problem. In these cases, because of unknown amounts of endogenous analyte that already exist in the samples, a true blank sample is not conceivable. Therefore, the samples cannot be simply spiked with the known amounts of analyte standard and used for constructing the calibration line. One solution to this problem might be pretreatment of the sample to remove any residue of the endogenous analyte. Stripping the sample off the analyte residue with active charcoal is an example of this approach [2]. Unfortunately, this process is costly, labor-intensive and is not universally applicable. Compounds, which are bound to plasma lipoproteins, are not removed and also for other compounds the depletion may not be complete [3]. Furthermore, pretreatment of the sample will eliminate many of natural components and dramatically change the nature of original matrix, which may result in other types of errors [4–7]. The second option might be the usage of background subtraction during data processing. This may also be impractical if the background level of the analyte is greater than the expected experimentally measured signal or change in signal [8,9]. The third approach is the method of standard addition, which relies on addition of certain aliquots of the sample to all the standard solutions, and constructing the calibration curve using these calibration samples. Then the analyte concentration can be determined by extrapolating the calibration line to the negative part of the concentration axis [10]. The major disadvantage of this method is its necessity for constructing separate calibration line for each sample and therefore, besides requiring a large amount of sample, which

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doi:10.1016/j.jchromb.2010.01.040

is not feasible in many cases, it is also time and labor-intensive [11–13]. Use of artificial matrices was proposed as a practical solution for the matrix problem [3,14,15]. Artificial matrices can vary widely in complexity. In its simplest form, an artificial matrix is pure water or a buffer, such as phosphate-buffered saline, which is frequently used for plasma and serum analyses because of its similar pH (7.4) and ionic strength (150 mM). If a closer correspondence to the actual biological matrix is desired (e.g., in clinical analysis), more complex synthetic solutions as artificial matrices can be prepared [3,16]. However, in some situations there are considerable variations among real matrices which could result in different analytical responses even at equal analyte concentration. In these cases artificial matrices may be not adequate [3,17].

An interesting and attractive case of endogenous compound analysis is determination of steroids in different biofluids. The isolation and quantification of endogenous steroids related to endocrine disorders has become an important field of investigation for clinical laboratories. High sensitivity and selectivity are mandatory in the analytical determination of steroids, which can be present at very low concentrations in relatively small and complex biological samples [18]. This is often required for veterinary growth promoter investigations [19], as well as for environmental studies [20], doping control [21–23], Alzheimer disease [24], many inherited human disease [25] and cancer research projects [26]. The methodology of human steroid profiling in urine was adapted and introduced in doping controls in 1983 by Donike et al. to allow for the determination of testosterone and other related endogenous steroids misuse in sport [27]. Because of tremendous impact of positive results of doping test on athletes' career, it is of utmost importance that the analytical methods of doping laboratory be as reliable as possible. This is guaranteed by comprehensive validations and proficiency tests, and the use of isotope-dilution mass spectrometry (IDMS) employing certified reference material allows the precise and accurate evaluation of steroid profile data [27]. For the determination of steroids in urine, a mixture of diisopropylamines (DIPAs) as artificial urine was successfully employed by doping analysts to make the external calibration samples [28,29]. In their routine analysis, doping laboratories usually use a single point calibration procedure [30]. Many years of experiences and a large number of scientific publications support the reliability and suitability of doping laboratories standard procedures [27,30–33]. In the present study, a new quantification strategy was developed for accurate determination of endogenous steroids in human urine. The main novelty of this method is its new approach for constructing the calibration line, which is based on the utilization of isotope-labeled steroids (surrogate analyte) instead of natural steroids as calibration standards. The concept of using surrogate analyte for determination of endogenous compounds was first introduced by Li and Cohen, in 2003 for measurement of α-ketoisocaproic acid in rat plasma [3,8]. In our previous studies, this approach was used for quantification of endogenous androgens in cattle's meat [34,35]. In the present study, the applicability of the "surrogate analyte" approach for quantification of endogenous steroids in human urine was investigated.

2. Experimental

2.1. Reagents and chemicals

Most of the reagents and solvents were of analytical grade quality and purchased from Merck (Darmstadt, Germany). Pure standards of both natural and synthetic hormones including testosterone (T, 4-androstene-17β-ol-3-one), epitestosterone (EpiT, 4-androstene-17α-ol-3-one), dihydrotestosterone (DHT, 5α-androstane-17β-ol-3-one), etiocholanolone (Etio, 5β-androstane-3α-ol-17-one), androsterone (A, 5α-androstane-3α-ol-17-one), methyltestosterone (MT), testosterone-d_3 (T-d_3), β-glucuronidase from *Helix pomatia* type H-2 and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) were obtained from Sigma (St. Louis, MO, USA). epitestosterone-d_3 (EpiT-d_3), dihydrotestosterone-d_3 (DHT-d_3), androsterone-d_4 (A-d_4) and eticholanolne-d_4 (Etio-d_4), were obtained from National Measurement Institute, Australia (NMIA) (Sydney, Australia). All standards were stored at −20 °C in airtight container. The derivatization vials and the liners of GC-MS were silanised with a solution of 5% dimethyldichlorosilane (Merck) in toluene overnight before use. To avoid contamination, all the glassware were rinsed with analytical grade methanol and baked for 4 h at 220 °C prior to use. Stock solutions were prepared for all standard substances at 100 μg mL^{-1} in methanol. The derivatization reagent was prepared by dissolving 30 mg ammonium iodide (NH_4I) and 45 μL ethanthiol (ET) in 5 mL MSTFA, after heating for 45 s at 60 °C another 10 mL of MSTFA was added to this solution. The reagent is stable for two weeks in a tightly sealed brown vial (silanised and filled with dried pure Argon) at 0 °C. Sep-Pak C_{18} cartridge (Vac 200 mg, 3 mL) was purchased from Waters Co. (Milford, MA, USA).

2.2. Instrumentation

GC-MS analysis was carried out according to the procedure described by Donike and co-workers with some modifications [28]. The instrument used for GC-MS analysis was an Agilent (Agilent Technologies, Palo Alto, CA, USA) 6890 plus gas chromatograph equipped with a 5973 mass selective detector quadrupole mass spectrometer. A 1 μL aliquot of the final derivatized extract was injected into the system operated in the split-less mode. The injector temperature was set at 280 °C. The column was an Ultra-1 cross-linked methylsilicone, 17 m × 0.2 mm i.d., film thickness 0.11 μm (Agilent Technologies, Palo Alto, CA, USA). The GC oven temperature was initially set at 180 °C and then programmed to 231 °C at a rate of 3 °C min^{-1} then to 310 °C at 30 °C min^{-1} and maintained for 2 min. The temperature of the transfer line was maintained at 310 °C. Helium (99.999%) was used as carrier gas at 1 mL min^{-1}. The source and quadrupole temperatures were kept at 230 and 150 °C, respectively. The electronic beam energy of the mass spectrometer was set at 70 eV. The mass selective detector was operated in electron impact (EI) mode using selected ion monitoring (SIM). The dwell time of each ion was set at 100 ms. The GC conditions were selected to minimize the time of analysis while allowing all the analytes to elute in acquisition groups containing suitable number of ions for monitoring (Table 1). For the identification purpose, full scan mass spectrum, the ratios of four characteristic ions and the ±0.5% relative retention time (RRT) tolerance criteria in comparison to the standard were used and for quantification purpose, the most intense ion of each analyte (M) was used. Representative chromatograms are shown in Fig. 1.

Table 1
Selected ions and time widows used for detection of the analytes in SIM mode.

Ion groups	Analyte	Time window (min)	Selected ions (m/z)
1	Androsterone	3–11.5	419, 434
1	Androsterone-d_4	3–11.5	423, 438
1	Etiocholanolone	3–11.5	419, 434
1	Etiocholanolone-d_4	3–11.5	423, 438
2	Epitestosterone	11.5–12.8	417, 432
2	Epitestosterone-d_3	11.5–12.8	420, 435
2	Dihydrotestosterone	11.5–12.8	419, 434
2	Dihydrotestosterone-d_3	11.5–12.8	422, 437
2	Testosterone	11.5–12.8	417, 432
2	Testosterone-d_3	11.5–12.8	420, 435
3	Methyl testosterone	12.8–22	431, 446

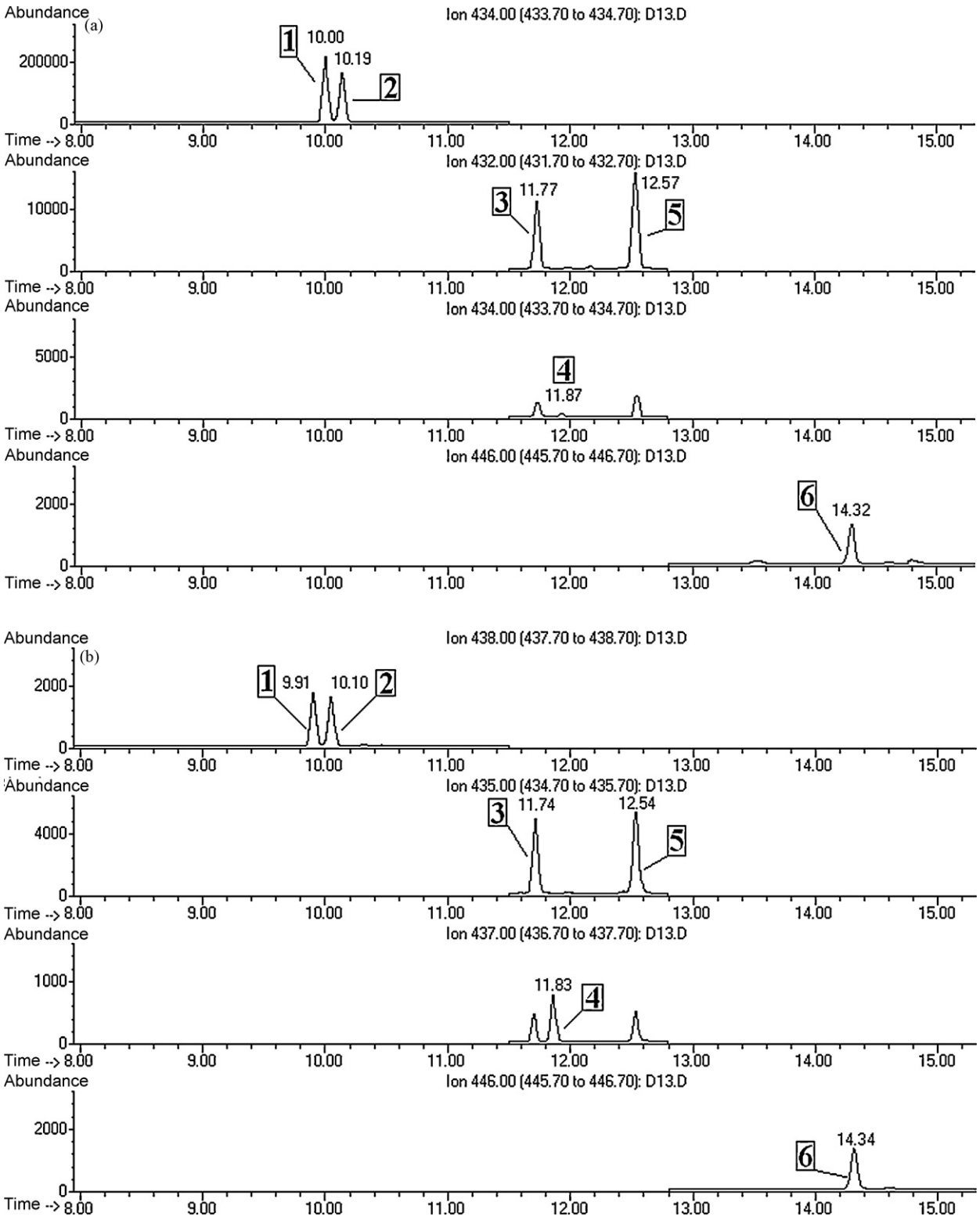

Fig. 1. Representative SIM GC-MS ion chromatograms of the final extract of a urine sample, (a) monitoring of the natural analytes (1) androsterone (2487 ng mL^{-1}), (2) etiocholanolone (1785 ng mL^{-1}), (3) epitestosterone (47 ng mL^{-1}), (4) dihydrotestosterone (5 ng mL^{-1}), (5) testosterone (64 ng mL^{-1}), (6) methyl testosterone (10 ng mL^{-1}). (b) Monitoring of the surrogate analytes in the same sample after fortification with the isotope-labeled analytes (1) androsterone-d_4 (20 ng mL^{-1}), (2) etiocholanolone-d_4 (20 ng mL^{-1}), (3) epitestosterone-d_3 (20 ng mL^{-1}), (4) dihydrotestosterone-d_3 (20 ng mL^{-1}), (5) testosterone-d_3 (20 ng mL^{-1}), (6) methyl testosterone (10 ng mL^{-1}).

2.3. Sample preparation

Samples were prepared according to the method described by Donike and co-workers for analysis of anabolic steroids [28] with some modifications as follow:

2 mL of urine sample spiked with 20 ng methyl testosterone as internal standard (I.S.) and applied over a Sep-Pak C_{18} cartridge pre-conditioned with 5 mL of methanol and 5 mL of water. The cartridge was then washed with 5 mL of water to eliminate most of the water-soluble urinary constituents, which were not adsorbed on the solid

support. The steroids (free and conjugate) were then eluted with 5 mL of methanol. The entire effluent was evaporated to dryness under a nitrogen stream at 45 °C and the residue was dissolved in 1 mL of 0.2 M acetate buffer (pH 5.2). Following addition of 100 μL of β-glucuronidase from *Helix pomatia* the mixture was incubated for 3 h at 55 °C. The hydrolysate was then cooled to room temperature and Ca. 100 mg of potassium carbonate was added. The mixture was gently vortexed for a few seconds and extracted with 5 mL diethyl ether. After centrifugation, the organic layer was transferred to a silanised vial and evaporated to dryness under nitrogen stream at 45 °C then kept in a desiccator containing P_2O_5 under vacuum for at least 60 min before derivatization. To derivatize the hydroxyl and active ketone groups, 50 μL of derivatizing mixture containing MSTFA/NH_4I/ET (1000:2:3, v/w/v) were added to the dried residue. The reaction vial was mixed thoroughly using vortex mixer followed by heating at 65 °C for 30 min. The resulting solution was analyzed by GC-MS.

2.4. Calibration curves

For each one of the endogenous steroids which were subject of this study (testosterone, T; epitestosterone, EpiT; androsterone, A; etiocholanolone, Etio and dihydrotestosterone, DHT) calibration curves were constructed using three different methods: (a) routine as described in Section 1, (b) standard addition and (c) surrogate analyte method.

2.4.1. Routine method

Calibration curves were constructed by adding the fixed amount of I.S. solution (20 ng MT, i.e., 20 μL; 1 μg mL^{-1} in methanol) and varying quantities of the unlabeled steroid standards to 2 mL of 2 years old child urine as blank matrix to make the final concentrations range of 2–500 ng mL^{-1}. The samples were then extracted, hydrolyzed and derivatized as described above.

2.4.2. Standard addition method

Calibration curves were constructed by adding the fixed amount of I.S. solution and varying quantities of the unlabeled steroid standards to 2 mL of some test (n = 12) and quality control (QC_s) samples. The test urine samples were obtained from 12 healthy male volunteers. The age range of the volunteers was 21–40 years and the weight range 62.7–90.5 kg. All the volunteers signed informed consent and the study was approved by the Ethic Medical Committee of Tehran University of Medical Sciences. The QC samples were prepared by fortification of child urine (which has already been stripped of endogenous steroids by active charcoal) with the steroid standards (three levels, six replicates). To compare the performance of the three methods these test and QC samples were analyzed by routine and surrogate analyte methods too. The samples were then extracted, hydrolyzed and derivatized as described above.

2.4.3. Surrogate analyte method

In this approach, calibration curves were constructed by adding the fixed amount of I.S. solution and varying quantities of the appropriate isotope-labeled steroids (T-d_3, EpiT-d_3, A-d_4, Etio-d_4 and DHT-d_3) standards to 2 mL of the child urine (without any pretreatment) to make the final concentration range of 2–500 ng mL^{-1}. The samples were then extracted, hydrolyzed and derivatized as described above.

3. Results and discussion

As mentioned in Section 1, few methods have been proposed to solve the problem of "lack of a true blank matrix" in the quantification of endogenous steroids. The developed method in Cologne doping laboratory based on isotope dilution technique, artificial matrix and single point calibration is well validated and has been shown to be completely reliable [27,28]. In the present study, a new quantification strategy was developed, which is named "surrogate analyte approach" and is based on using isotope-labeled steroids instead of their natural forms to generate the desired calibration lines. Then the concentrations of endogenous steroids in each real sample can be calculated based on the regression equations of these calibration lines. Unlike isotope dilution technique that exploits the use of stable isotope-labeled internal standards, in surrogate analyte approach the stable isotope-labeled analyte is not added to every calibration and test sample, but instead is added and quantified only in the calibration samples [36,37]. There are few reports about application of surrogate analyte in chemical analysis [1,3,8,34,35]. The key point in this approach is that for small molecules, the occurrence of isotope-labeled form of the analyte with more than 2-Da mass difference is naturally negligible and therefore, all the samples would be blank with respect to this form of the analyte (Fig. 1). Therefore, calibration samples could be prepared simply by enrichment of the real samples with the sequentially increasing amounts of the isotope-labeled standard. In this way, the stable isotope-labeled analyte functions not as an internal standard but as a surrogate analyte. Since the endogenous analyte and its isotope-labeled form are chemically identical, they will show similar GC retention time but their masses are different and therefore, by using mass detector, separate ion chromatogram will be obtained for each one of them. However, the prerequisite of this approach is the identical behavior of analyte and its isotope-labeled form in all aspects of analysis from sample preparation to instrumental analysis. If these requirements are met, the calibration curve, which is constructed by using the labeled standard as analyte, could be utilized for quantitation of natural analyte without being concerned about the interference of endogenous analyte.

Development of the surrogate analyte approach involved the following steps [8]:

(a) Derivatization and measurement of the peak areas of the natural and deuterium-labeled analyte using the neat solutions, followed by determination of the response factor (RF) of the natural analyte to the surrogate analyte to take into account any isotope effect or difference in ionization efficiency using the following equation (Eq. (1)):

$$\mathrm{RF} = \frac{\mathrm{area}_{\text{deuterium-labeled analyte}}}{\mathrm{area}_{\text{natural analyte}}} (\text{at equivalent concentrations}) \quad (1)$$

For calculating the RF value, the most intense ion of each analyte (i.e., M for natural and $M+n$ in the case of deuterium labeled standard, n = No. of deuterium) was selected.

(b) In another approach for determining the differences between the analytical responses of labeled and unlabeled steroids, calibration curves were constructed for both surrogate and natural analyte standard solutions over the concentration range of 2–500 ng mL^{-1}. The slopes and intercepts of the equations were compared (t-test, α = 0.05). No significant difference was observed, which indicates similar responses for surrogate and natural analytes over the studied concentration range.

(c) Preparation of deuterium-labeled calibration standards over a desired concentration range in urine matrix.

In order to compensate for possible interference due to the natural existence of $M+3$ ion along with the endogenous testosterone and epitestosterone, the raw areas measured for testosterone-d_3 and epitestosterone-d_3 were corrected using the equations suggested by Nolteernsting et al. [38] (Eqs. (2)

Table 2
Determined response factor (RF) of the steroids at different concentrations.

Analyte	Concentration (ng mL^{-1})	RF[a] (Mean)	No. of replicate	S.D.[b]	CV%[c]
Testosterone-d_3	2	1.01	6	0.066	6.53
	5	1.03	6	0.072	6.99
	25	0.98	6	0.024	2.45
	100	1.03	6	0.062	6.02
	250	0.99	6	0.012	1.21
	500	1.00	6	0.020	2.00
Epitestosterone-d_3	2	0.96	6	0.016	1.67
	5	0.98	6	0.026	2.65
	25	0.96	6	0.046	4.79
	100	0.95	6	0.019	2.00
	250	0.94	6	0.036	3.83
	500	0.97	6	0.025	2.58
Dihydrotestosterone-d_3	2	0.96	6	0.012	1.25
	5	0.94	6	0.042	4.47
	25	0.95	6	0.034	3.58
	100	1.03	6	0.047	4.55
	250	0.96	6	0.033	3.44
	500	1.04	6	0.047	4.52
Androsterone-d_4	2	0.97	6	0.018	1.86
	5	0.97	6	0.029	2.99
	25	0.97	6	0.019	1.96
	100	0.97	6	0.014	1.44
	250	0.98	6	0.025	2.55
	500	0.98	6	0.038	3.88
Eticholanolone-d_4	2	0.99	6	0.047	4.75
	5	0.98	6	0.053	5.41
	25	1.01	6	0.043	4.26
	100	0.97	6	0.035	3.61
	250	1.01	6	0.037	3.65
	500	0.98	6	0.040	4.07

[a] RF = $\text{area}_{\text{deuterium-labeled analyte}}/\text{area}_{\text{natural analyte}}$.
[b] Standard deviation (S.D.).
[c] Coefficient of variation (CV%).

and (3)):

$$a\_\text{T-d}_{3(\text{corr})} = a\_\text{T-d}_{3(\text{raw})} - a\_\text{T}_{(\text{raw})} \times 0.027/0.999649 \quad (2)$$

$$a\_\text{EpiT-d}_{3(\text{corr})} = a\_\text{EpiT-d}_{3(\text{raw})} - a\_\text{EpiT}_{(\text{raw})} \times 0.027/0.999649 \quad (3)$$

$a\_\text{T}_{(\text{raw})}$: Area of testosterone detected, $a\_\text{EpiT}_{(\text{raw})}$: Area of epitestosterone detected, $a\_\text{T-d}_{3(\text{raw})}$: Area of testosterone-$d_3$ detected, $a\_\text{EpiT-d}_{3(\text{raw})}$: Area of epitestosterone-d_3 detected, $a\_\text{T-d}_{3(\text{corr})}$: Corrected area of testosterone-d_3, $a\_\text{EpiT-d}_{3(\text{corr})}$: Corrected area of epitestosterone-d_3.

Since previous studies clearly indicated the negligible influences of M on $M+4$ and vice versa (below 0.0971%) there is no need for this correction in the cases of d_4-labeled analytes [38,39].

(d) Extraction, hydrolysis and derivatization of the prepared calibration, quality control (QC) and real samples using the procedures described above.
(e) Analysis of the tests, calibrations and QCs by GC-MS and measurement of the peak area responses for the endogenous analyte, deuterium-labeled analyte and internal standard. The calibration curve was constructed based on the peak area ratio of the deuterium-labeled analyte to that of internal standard (methyl testosterone).
(f) Calculation of the concentration of endogenous analyte in urine based on the regression equation of the calibration line and the peak area ratio of endogenous analyte to internal standard.

Table 3
Estimated recoveries for isotope-labeled and natural[a] forms of steroid standards ($n = 6$) at different concentrations from a urine sample.

Biofluids: Urine	Spiked level (10 ng mL^{-1})		Spiked level (200 ng mL^{-1})		Spiked level (400 ng mL^{-1})	
Compound	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
Methyl testosterone	93.7	9.5	94.6	7.1	95.1	6.5
Testosterone-d_3	92.3	9.2	92.5	6.6	93.4	6.2
Testosterone	89.5	9.5	90.1	8.7	91.5	7.2
Epitestosterone-d_3	93.3	9.8	93.8	7.3	93.5	7.8
Epitestosterone	89.2	10.7	90.4	8.1	90.2	7.2
Androsterone-d_4	92.5	10.6	92.8	9.4	94.1	8.5
Androsterone	89.3	9.8	89.8	8.6	91.5	7.1
Dihydrotestosterone-d_3	93.1	8.2	93.9	7.6	94.5	6.1
Dihydrotestosterone	88.4	10.8	91.6	8.5	92.5	8.5
Etiocholanolone-d_4	92.1	10.8	93.2	8.5	93.5	7.3
Etiocholanolone	90.4	11.5	90.7	8.9	90.5	7.8

[a] Recoveries for natural steroids were calculated after background subtraction.

Table 4
Quantification of testosterone in some real and QC samples to compare different methods.

Sample	Replicate no.	Surrogate method			Routine method			Standard addition method		
		Measured conc. ($ng\,mL^{-1}$)	CV%	RE%	Measured conc. ($ng\,mL^{-1}$)	CV%	RE%	Measured conc. ($ng\,mL^{-1}$)	CV%	RE%
1	3	102	4.8		80	6.3		99	10.6	
2	3	87	5.5		65	5.6		79	11.8	
3	3	56	6.3		34	5.3		49	13.7	
4	3	64	6.2		42	6.4		58	12.3	
5	3	108	4.5		86	6.3		98	13.2	
6	3	121	5.2		99	4.7		115	10.1	
7	3	78	5.8		56	5.4		69	11.6	
8	3	43	7.2		21	7.7		37	14.1	
9	3	157	6.4		135	7.8		149	12.6	
10	3	68	6.5		46	4.6		58	11.6	
11	3	79	5.8		57	5.8		72	10.2	
12	3	83	5.9		61	5.8		76	12.2	
QC conc.										
LQC 10 $ng\,mL^{-1}$	6	9	9.4	−10	−13	10.3	−230	7	14.3	−30
MQC 200 $ng\,mL^{-1}$	6	196	7.8	−2	174	8.2	−13	191	12.4	−4.5
HQC 400 $ng\,mL^{-1}$	6	396	6.5	−1	374	8.7	−6.5	389	12.2	−2.7

Abbreviations: LQC, low quality control; MQC, medium quality control; HQC, high quality control; CV%, percent coefficient of variation; RE%, percent relative error; conc., concentration.

The RF of the endogenous analyte to that of the stable isotope-labeled standard should be incorporated in the calculation, if the value does not equal 1 [8] (Eq. (4)).

$$\text{Conc.}_{\text{Analyte}} = \frac{(\text{Area}_{\text{analyte}}/\text{Area}_{\text{I.S.}}) \times RF - b}{a} \quad (4)$$

$\text{Conc.}_{\text{Analyte}}$: Concentration of endogenous analyte; $\text{Area}_{\text{analyte}}$: Area under the peak of analyte; $\text{Area}_{\text{I.S.}}$: Area under the peak of internal standard; RF: Response factor of the natural analyte to the surrogate analyte; b: Intercept of the calibration line; a: Slope of the calibration line.

(g) Measurement of the concentration of analyte in some real test and QC samples using the standard addition method and comparing the results with those obtained by the routine and surrogate analyte methods.

Peak area responses of the five endogenous steroids in neat solutions at different concentrations (six levels, six replicates) and their deuterium-labeled forms were measured and the response factor (RF, $\text{Area}_{\text{d-Steroid}}/\text{Area}_{\text{Steroid}}$) for each one of them was calculated (Table 2). No significant difference was determined between the mean RF values of each concentration level (*t*-test, $\alpha = 0.05$). These results indicate that the RF values were concentration independent in the experimental concentration range. On the other hand, all the RF values are near one, which means equal amounts of the deuterium-labeled and natural analytes would produce similar MS responses. This means that there is no isotope effect or difference in ionization efficiency between the analytes and their labeled forms. However, these response factors, which are determined, by comparison of signals from neat solutions of steroids and their deuterated forms do not account for the urine matrix effect. To check this matrix effect and any possible isotope effect in extraction, the recovery of the natural steroids and their labeled forms were compared at three levels ($n = 6$) (Table 3). The results show equal recoveries for the steroids and their deuterated forms. Little differences between recoveries of the natural steroids and their deuterated forms can be due to the different ways of calculation, since the recovery values for natural steroids were calculated after background subtraction. In conclusion, by considering the results of neat standard solutions (Table 2), and recovery studies (Table 3) it seems that there are no significant isotopic effect and isotopic exchange in the whole procedure. Theoretically, the C–D bond is not susceptible to isotope exchange unless other side reactions cause C–D breaking and forming during multiple steps [40]. Since by considering the chemistry of sample preparation and instrumental analysis steps it is hard to conceive that such reactions occur, the exchange loss of deuterium will be improbable during the analysis. This is in agreement with the results of previous studies which showed identical chromatographic and mass spectrometric properties of natural and isotope-labeled forms of testosterone and other steroids, which are labeled at suitable positions [41,42]. In order to investigate the applicability and efficacy of surrogate analyte approach for the analysis of steroids in urine samples and make a comparison between different methods, concentration of the analytes in twelve real urine samples was determined by the three methods. Since the results for all the analytes showed similar pattern, only the results for testosterone are presented (Table 4) in order to avoid lengthening the article. As the results indicate, the surrogate analyte and standard addition methods show comparable efficiency in terms of accuracy (RE%) at medium and high concentration levels. However, at low concentration the surrogate analyte approach shows better accuracy than standard addition method (*t*-test, $\alpha = 0.05$). Both surrogate and standard addition methods show better accuracy than routine method especially at low and medium concentrations which could be due to the existence of analytes residuals in calibrator samples. In addition, the concentrations of steroids in real samples calculated by surrogate approach are closer to those which are calculated by standard addition in comparison to those obtained by routine method. In conclusion, the surrogate approach shows better accuracy than standard addition and routine methods at low concentration and comparable accuracy with standard addition at medium and high concentrations. On the other hand, the routine method shows considerable inaccuracy at low and medium concentration levels, which makes its results uncertain. The precision (CV%) of both surrogate and routine methods were better than standard addition at all concentration levels. However, this difference is not important since most of CV% values over the calibration concentration range are bellow 15% which comply with the ICH criteria for a valid bioanalytical method [43,44].

The method was also found to be valid according to the FDA guideline on validation of bioanalytical methods (Table 5) [43]. The mean accuracy of QC samples was found to be within ±10%. Precision as measured by the coefficient of variation was within ±15% for the lower limit of quantification (LLQ) and <±15% for all other concentrations. The limit of detections and quantifications for all the

Table 5
Accuracy and precision values for quantification of three deuterium-labeled steroids in QC samples using surrogate analyte method.

Nominal conc. ($ng\,mL^{-1}$)		Testosterone-d_3			Dihydrotestosterone-d_3			Androsterone-d_4		
		Measured conc. ($ng\,mL^{-1}$)	CV%	RE%	Measured conc. ($ng\,mL^{-1}$)	CV%	RE%	Measured conc. ($ng\,mL^{-1}$)	CV%	RE%
2 (LLQ)	Interday	1.6	12.6	−13.0	1.6	12.4	−14.0	1.7	12.5	−9.5
		1.7	12.3	−10.0	1.8	12.5	−12.0	1.8	12.1	−12.0
		1.8	12.8	−11.5	1.6	12.7	−13.5	1.6	12.6	−14.0
	Intraday	1.7	12.5	−10.0	1.7	12.3	−12.5	1.8	12.2	−11.5
10 (LQC)	Interday	9.0	9.3	−8.9	9.0	10.1	−8.6	9.0	9.6	−9.6
		9.1	8.4	−8.5	9.1	10.3	−9.4	9.0	9.5	−8.8
		9.0	9.6	−9.6	9.0	9.5	−9.7	9.2	9.3	−8.1
	Intraday	9.1	9.2	−9.4	9.0	9.6	−8.8	9.0	9.6	−9.8
200(MQC)	Interday	197.6	6.5	−1.2	190.4	7.5	−4.7	191.2	6.7	−4.4
		193.3	7.6	−3.3	191.3	6.1	−4.3	190.6	6.4	−4.6
		195.7	7.4	−2.1	193.2	7.3	−3.4	193.6	7.6	−3.1
	Intraday	194.2	7.5	−2.8	192.3	6.4	−3.8	192.7	6.2	−3.6
400(HQC)	Interday	395.4	5.7	−1.1	392.6	6.6	−1.7	391.1	6.5	−2.2
		391.5	5.8	−2.1	394.4	5.1	−1.4	389.5	6.9	−2.6
		390.6	6.4	−2.4	391.4	6.2	−2.2	384.6	6.8	−3.9
	Intraday	396.4	6.3	−0.9	394.1	6.1	−1.4	390.7	5.6	−2.3

Abbreviations: LLQ, lower limit of quantification; LQC, low quality control; MQC, medium quality control; HQC, high quality control; CV%, percent coefficient of variation; RE%, percent relative error; conc., concentration.

analytes were determined as 1 and 2 $ng\,mL^{-1}$ respectively, with a linear range of 2–500 $ng\,mL^{-1}$ and a correlation coefficient (r^2) of 0.997.

In addition to its better performance, surrogate analyte method could be a potential approach in situations where constructing separate calibration line for each sample is desirable. Each complex matrix such as urine has its own characteristics, which make it unique. In other words, each urine sample differs from all other urine samples with respect to quality and quantity of its components. These differences could result in different analytical responses which are clearly shown by previous studies [28,29]. Therefore, there are cases that the calibration line which has been constructed for a certain urine sample is not applicable for other urine samples. In these cases, the surrogate analyte approach could be a suitable solution. To this aim, a certain isotopic labeled forms of the analyte can be added to the sample and after sample preparation and instrumental analysis, a specific calibration line for accurate quantification of that sample could be constructed. Although in this way surrogate analyte is used similar to standard addition method, but as was indicated by the results of the present study, the superior performance of surrogate analyte approach makes it more reliable and feasible over the conventional standard addition method, which is based on extrapolation [10].

4. Conclusion

In this study, a new approach for determination of endogenous steroids in human urine was established. By using the "surrogate analyte" approach, the problem of endogenous steroids interferences is circumvented; the method development and validation are simplified and the method performance is improved over conventional approaches especially at low concentrations. The surrogate analyte approach shows better accuracy than standard addition and routine methods at low concentrations and comparable accuracy with standard addition at medium and high concentrations. The precision of surrogate analyte method is almost the same as routine method and better than standard addition method over the full calibration range. From the viewpoints of ICH performance criteria for bioanalytical methods, all the validation parameters of surrogate method were fully satisfactory for the analyte concentrations of interest. Application of this technique for quantitation of endogenous steroids in urine was demonstrated in a small-scale comparative study between the three methods. The developed method could be an alternative to the standard procedures in cases where quantification based on spiked calibration line is desired or there are considerable differences between the analytical responses of real and artificial matrices. By this approach, the true blank matrix, which is not accessible in the case of endogenous analytes and is a necessity for accurate quantification especially at trace level would be provided. The general procedure can be applied to any GC-MS quantitative analysis when the true blank matrix is unavailable.

Acknowledgment

This work was partially supported by INSF (Iran National Science Foundation) and TWAS-IRAN CHAPTER.

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