Determination of selected endogenous anabolic androgenic steroids and ratios in urine by ultra high performance liquid chromatography tandem mass spectrometry and isotope pattern deconvolution

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ABSTRACT

An isotope dilution mass spectrometry (IDMS) method for the determination of selected endogenous anabolic androgenic steroids (EAAS) in urine by UHPLC-MS/MS has been developed using the isotope pattern deconvolution (IPD) mathematical tool. The method has been successfully validated for testosterone, epitestosterone, androsterone and etiocholanolone, employing their respective deuterated analogs using two certified reference materials (CRM). Accuracy was evaluated as recovery of the certified values and ranged from 75% to 108%. Precision was assessed in intraday (n=5) and interday (n=4) experiments, with RSDs below 5% and 10% respectively. The method was also found suitable for real urine samples, with limits of detection (LOD) and quantification (LOQ) below the normal urinary levels. The developed method meets the requirements established by the World Anti-Doping Agency for the selected steroids for Athlete Biological Passport (ABP) measurements, except in the case of androsterone, which is currently under study.

INTRODUCTION

Misuse of steroids is nowadays a significant social issue. Apart from doping in sports, endogenous anabolic androgenic steroids (EAAS) use has become a problem of public health [1]. Regarding substances prohibited in sports, over the years consensus has been achieved about which steroidal markers must be controlled as an additional part of the World Anti-Doping Agency (WADA) Athlete Biological Passport (ABP), the steroidal module [2]. Testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5αAdiol), 5β-androstane-3α,17β-diol (5βAdiol) and the ratios T/E, A/T, A/Etio, 5αAdiol/5βAdiol, 5βAdiol/E are the parameters of choice. An abnormal steroidal or longitudinal profiles may constitute a suspicion of doping, thus, reliable analytical methods are needed to assess the concentration of those EAAS. Moreover, clear verification of exogenous administration of EAAS is still a challenge. The general workflow includes an initial screening method followed by a confirmation if adverse results are found. However, in spite of WADA efforts, a completely standardized methodology has not been established yet, neither by the mass detector (Q or QqQ), nor by the sample treatment method used in that initial screening step [3]. Although the accepted WADA quantification method for EAA determination in urine is GC/MS [2], among current instrumental techniques in drug testing, UHPLC-MS/MS is mainly used due to its high throughput, chromatographic performance and sensitivity [4-6].

On the other hand, ESI, the most employed ionisation source in LC-MS instrumental techniques can suffer severe matrix effect problems, mainly related with ion suppression or enhancement [7-9]. The use of isotope labelled internal standards (ILIS) is widely recognized as the best way to overcome matrix effect problems. Thus, quantification through isotope dilution mass spectrometry (IDMS) works out the issues related with signal alteration [10,11]. A recently developed IDMS method of quantification, isotope pattern deconvolution (IPD),

does not rely on the construction of any calibration graph. IPD involve the artificial alteration of the natural isotopomer abundances of a compound in a sample by the addition of a known amount of a labelled analogue. The isotopic composition of the blend is a linear combination of two isotope patterns: that of the natural abundance compound and the isotope pattern of the labelled analogue. The separate contribution of each 'isotope pattern' to the whole mass spectrum can be calculated by multiple linear regression and provides the molar fractions of both labelled and unlabelled compound in the sample. [12-14]. This method has been satisfactorily tested for rapid quantifications in different complex matrices, such as food and environmental samples [15-18]. IDMS together with IPD can be considered a reliable (precise and accurate) methodology, free of matrix effect and fast, providing one result per injection. However, except for a recent paper related with testosterone determination in urine [19], IPD has never been applied to steroid determination.

In this work, an UHPLC-MS/MS method, based in IDMS and IPD quantification approach, is developed and validated for the determination of selected EAAS in human urine. T, E, A and Etio were selected among the EAAS included in the ABP, excluding the diols due to the known ionization difficulties by ESI of hydroxyandrostane compounds [20]. Accuracy and precision has been checked for the selected compounds, as well as ratios, through the analysis of NMIA MX002 and MX005 freeze dried human urine CRMs.

EXPERIMENTAL

Reagents and materials

Testosterone (T, purity 99%) and etiocholanolone (Etio, purity 98%) were provided by Sigma-Aldrich (Saint Louis, MO, USA), epitestosterone (E, purity 96.1%) was provided by LGC Standards (Luckenwalde, Germany) and androsterone VETRANAL® (A, purity 98.2%) by Sigma-Aldrich (Seelze, Germany). D3-Testosterone (d3-T, d3≈91%), d3-epitestosterone (d3-E, d3≈94%), d4-androsterone (d4-A, d4≈81%), d5-etiocholanolone (d5-Etio, d5≈92%) and certified reference materials (CRMs) NMIA MX002 and MX005 were all purchased to NMI Australia (North Ryde, NSW, Australia). Molecular structure of the selected EAAS are shown in Figure 1.

Methanol (HPLC quality), acetonitrile (HPLC quality) and methyl tert-butyl ether (MTBE, GC quality) were provided by Scharlau (Barcelona, Spain). For the sample hydrolysis, β-glucuronidase from *E. coli* K12 provided by Roche (Indianapolis, IN) was employed. A 1 M phosphate buffer was prepared by dissolving the proper amount of $(NH_4)_2HPO_4$ (Merck, Darmstadt, Germany) in Milli-Q water and adjusted to pH=7 with HCl 37% from Scharlau (Barcelona, Spain). Also, a $NaHCO_3/Na_2CO_3$ (1:2, w/w) (Sigma-Aldrich Co., Madrid, Spain) solid buffer was prepared. Formic acid (LC additive quality) and a 500 mM solution of NH_4COOH (Scharlau, Barcelona, Spain) in methanol HPLC were used as modifiers for mobile phases.

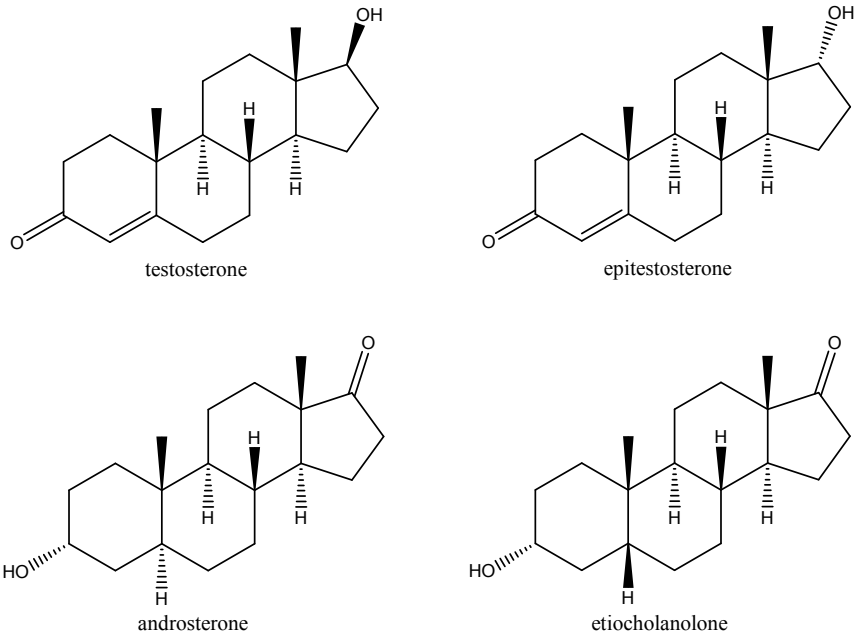

Figure 1. Molecular structure of the selected endogenous steroids. Location of D atoms are displayed for the labeled compounds.

Individual stock solutions were prepared with 500 µg/mL of T, 200 µg/mL of E, 500 µg/mL of A, 500 µg/mL of Etio and 100 µg/mL of each deuterated analog (d3-T, d3-E, d4-A and d5-Etio) by dissolving the proper amounts of solid standards in methanol. Then, 10 µg/mL working solutions of each compounds were prepared by dilution of stock solutions with methanol. A mix of labelled compounds was prepared in MeOH containing 1 µg/mL of d3-T and d3-E and 25 µg/mL of d4-A and d5-Etio. All standard solutions were stored in amber glass bottles at -20ºC. CRMs were reconstituted following the procedure indicated by the manufacturer and stored in a refrigerator until use.

Ultrapure water was obtained from a Milli-Q gradient A10 from Millipore (Bedford, MA, USA).

Instrumentation

Characterization and determination of analytes were performed on an Acquity UPLC system equipped with binary solvent and sample managers from Waters Corp. (Milford, MA, USA), coupled to a TQD quadrupole-hexapole-quadrupole tandem mass spectrometer and a Z-spray-electrospray interface (Waters Corp.). Chromatographic separation was achieved at 55ºC on an Acquity UPLC BEH C18 column (1.7 µm, 2.1 mm x 100 mm, Waters Corp.) at 0.3 mL/min flow rate and 10 µL injection volume. Mobile phases consisted in H_2O/ACN (95/5, v/v) as phase A and H_2O/ACN (5/95, v/v) as phase B, both containing 0.01% of formic acid and 0.1 mM of NH_4COOH as modifiers. The gradient applied was: 10% B (0-1 min), linear increase to 50% B in 4.3 min, 50% B (5.3-9 min), 95% B (9.5-10.5 min), 10% B (11-13 min).

Ionization was performed at 120ºC desolvation temperature and 350ºC source temperature, while cone gas and desolvation flows were set at 80 and 800 L/h respectively. 3.5 kV capillary voltage was applied in positive mode. Multiple reaction monitoring (MRM) conditions and retention times are listed in Table 1.

Drying and nebulizing gas was N_2 from a nitrogen generator N_2 LC-MS adapted for LC-MS analyzers (Claind, Teknokroma, Barcelona, Spain). Collision cell was kept at approximately 5 x 10^{-3} mbar of argon 99.995% provided by Praxair (Madrid, Spain). Dwell time was set to 0.1 s

per scan for all quantification measurements. Analytical data was processed using Masslynx v4.1 (Waters) and homemade Excel spreadsheets (Microsoft Office).

Table 1. Experimental conditions of the LC-(ESI)-MS/MS for natural and labeled steroids

Compound	Ret. Time (min)	Precursor ion	Cone voltage (V)	Collision voltage (V)	MRM transitions*
T	6.43	$[M+H]^+$	35	25	**289.2 > 96.9** **290.2 > 96.9** 289.2 > 109.1
E	7.06	$[M+H]^+$	35	25	**289.2 > 96.9** **290.2 > 96.9** 289.2 > 109.1
A	8.29	$[M+NH_4]^+$	35	10	**308.3 > 273.1** **309.3 > 274.1** 308.3 > 291.1
Etio	8.05	$[M+NH_4]^+$	35	10	**308.3 > 291.1** **309.3 > 292.1** 308.3 > 273.1
d3-T	6.40	$[M+H]^+$	35	25	**292.2 > 96.9** **293.2 > 96.9** 292.2 > 109.1
d3-E	7.03	$[M+H]^+$	35	25	**292.2 > 96.9** **293.2 > 96.9** 292.2 > 109.1
d4-A	8.24	$[M+NH_4]^+$	35	10	**312.3 > 277.1** **313.3 > 278.1** 312.3 > 295.1
d5-Etio	8.00	$[M+NH_4]^+$	35	10	**313.3 > 296.1** **314.3 > 297.1** 313.3 > 278.1

*In bold: MRM transitions employed for IPD quantification.

Sample treatment

A previously developed and widely used sample treatment method based on WADA guidelines [2] has been directly applied. 25 µL of labelled mix was added into 2.5 mL of sample in clean 15 mL-glass tubes followed by 1 mL of 1 M phosphate buffer (pH=7). Hydrolysis was performed by adding 30 µL of β-glucuronidase solution and incubated in a water bath at 55 ± 2 ºC for 1 h.

Next, approximately 200 mg of $NaHCO_3/Na_2CO_3$ (1:2, w/w) solid buffer were added to the tube and stirred until total dissolution of the solid. Liquid-liquid extraction was carried out by adding 6 mL of MTBE, stirring in a vortex for 1 min and centrifuging at 3500 rpm for 5 min. Then, the upper organic phase was carefully transferred to clean glass tubes using disposable Pasteur pipettes, avoiding transferring any aqueous phase. MTBE was eliminated by evaporation in a MiVac at 40ºC for 20 min, the residue was redissolved in 300 µL of $MeOH/H_2O$ (1:1, v/v) and transferred to LC vials.

Method Validation

Accuracy

Accuracy was validated by recovery experiments applying the method to two CRMs with different steroid concentrations and ratios, NMIA MX002 and NMIA MX005 freeze dried human urine. The method was regarded as accurate if the recovery was between 70% and 110%.

Precision

Using the same CRMs, intraday and interday precisions were validated. Intraday repeatability was assessed performing the analysis of five replicates. Interday reproducibility was obtained by the application of the method to four replicates in four consecutive weeks.

In order to assess precision in terms of WADA guidelines [2], total combined uncertainty, u_c, was also calculated according to WADA technical document TD2014DL [21] and Nordtest Guide [22]. A detailed explanation of the measurement uncertainty determination, using T as model compound in urine, is explained elsewhere [19]. The combined uncertainty for the ratios was assessed taking into account also the general propagation equation [23] to calculate the uncertainty associated to the reference material. A detailed explanation is shown in the supplementary information.

LOD and LOQ

A rough estimation of detection and quantification limits were conducted using the signal to noise ratio obtained in a real life sample. To this end, 9 urine samples from healthy female volunteers were collected and analyzed in order to get samples with low concentration of EAAS. Limits of quantification (LOQ) and detection (LOD) of the method were estimated as S/N equal to 10 and 3, respectively, in the lowest concentrated sample.

Quantification by isotope pattern deconvolution (IPD)

Isotope pattern deconvolution is a mathematical tool based on multiple linear regressions that provides the molar fractions of natural and labelled analytes in the spiked sample. The addition of the labelled analog alters the natural isotopic distribution of abundances $A_{nat}^{SRM_i}$ due to the overlap of the labelled isotopic distribution $A_{lab}^{SRM_i}$. Hence, the deconvolution of the measured distribution in the mix $A_{mix}^{SRM_i}$ is performed by solving the multiple linear regression in matrix notation:

$$\begin{bmatrix} A_{mix}^{SRM1} \\ A_{mix}^{SRM2} \\ \vdots \\ A_{mix}^{SRMn} \end{bmatrix} = \begin{bmatrix} A_{nat}^{SRM1} & A_{lab}^{SRM1} \\ A_{nat}^{SRM2} & A_{lab}^{SRM2} \\ \vdots & \vdots \\ A_{nat}^{SRMn} & A_{lab}^{SRMn} \end{bmatrix} \begin{bmatrix} X_{nat} \\ X_{lab} \end{bmatrix} + \begin{bmatrix} e^{SRM1} \\ e^{SRM2} \\ \vdots \\ e^{SRMn} \end{bmatrix}$$

Where the error vector e^{SRMi} is the minimized parameter in the regression to solve the system and to obtain the molar fractions of natural and labelled analytes (x_{nat} and x_{lab} respectively). This can be easily achieved with the LINEST function in Microsoft Excel or any spreadsheet software. Then, since the amount of labelled compound N_{lab} is known, the amount of natural compound in the sample N_{nat} is readily calculated as follows:

$$N_{nat} = N_{lab} \frac{x_{nat}}{x_{lab}}$$

In contrast with commonly used analytical methodologies, IPD does not need methodological calibration and a concentration value is obtained from a single injection of the spiked sample. However, an extensive characterization of natural and labelled compounds is required to construct the calculation matrix, including isotopomer abundance distributions and exact

concentration of labelled standard solutions (determined by reverse isotope dilution). If the individual isotopomer distribution is theoretically calculated, extent of labeling and spectral purity must be also characterized. Description of the general IPD methodology as well as examples of characterization of standards can be consulted in the literature [12,24]. In the present work, isotopomer abundances corresponding to the selected transitions for natural and labeled compounds have been experimentally obtained according to the method reported in previous works [19].

RESULTS AND DISCUSSION

Optimization of LC conditions

In a first approach, gradient conditions using methanol and water both containing 0.01% HCOOH/1mM NH_4COOH as mobile phases were tested, as employed for testosterone determination in previous works [19]. However, due to the similarity between A and Etio (5α/5β-position isomers of one H), separation could not be accomplished even with long run times. Therefore, acetonitrile (ACN) was tested as mobile phase on the basis of the chromatographic conditions used by Hauser *et al.* [25], which consisted in water/ACN (95/5, v/v) (Eluent A) and water/ACN (5/95, v/v) (Eluent B), both containing HCOOH and NH_4COOH. Different modifier concentrations were tested and 0.01% HCOOH plus 0.1 mM NH_4COOH provided the optimal sensitivity and peak shape.

Optimization of A/Etio separation was performed starting from isocratic conditions at different %B (10, 20 and 30%) to ensure that separation was possible with a C_{18} column. Once separation was observed using 30% Eluent B, peak shape and time analysis were tried to be improved by performing a gradient prior to an isocratic step. Thus, gradients of the same slope were tested starting from 0% or 10% Eluent B and arriving up to 30, 40 and 50% Eluent B, followed by the isocratic step. Separation of the A/Etio pair was achieved in all six experiments, but lower isocratic and initial % of Eluent B produced longer run times and decreased sensitivity due to peak broadening. Thus, starting conditions were set at 10 % eluent B, followed by a gradient up to 50% Eluent B in 4.3min and an isocratic step until 9 min. (Figure 2)

Characterization of analytes

Characterization of natural (T, E, A, Etio) and labelled compounds (d3-T, d3-E, d4-A, d5-Etio) consisted in the determination of the experimental isotopic distribution of abundances and exact concentration of labelled standard solutions.

Experimental abundances were measured by injecting (n=5) individual 500 ng/mL of T, E, d3-T and d3-E, and 5 µg/mL of A, Etio, d4-A and d5-Etio in MeOH/H2O (1:1, v/v). MRM transitions were selected on the basis of theoretical fragmentation calculations by IsoPatrn software [26], selecting the 10-12 most abundant transitions.

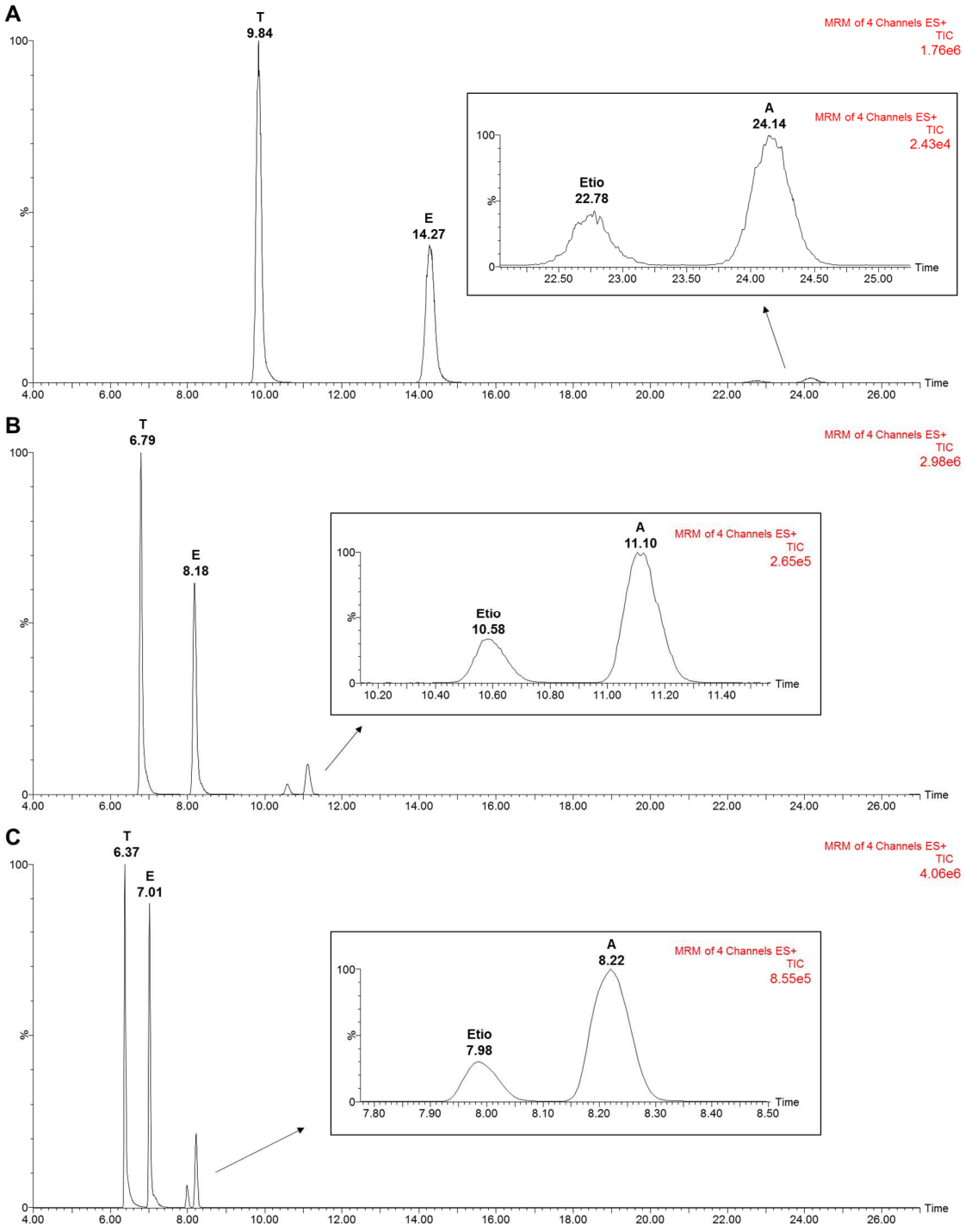

Figure 2. Chromatographic optimization using gradients of the same slope from 10% Eluent B at 1 min to A) 30% at 3.2 min, B) 40% at 4.25 min and C) 50% at 5.3 min, followed by an isocratic step (see text). All three injections correspond to a standard with 500 ng/mL T and E and 1.0 µg/mL A and Etio in MeOH/H_2O (1:1, v/v).

Finally, concentrations of labelled 100 µg/mL standard solutions were checked by reverse isotope dilution (RID). That is, quantification of labelled compound spiking the standard solution with an accurately prepared natural standard solution using IPD [27]. Results obtained by RID were: (100 ± 4) µg/mL for d3-T, (105.8 ± 1.1) µg/mL for d3-E, (98.2 ± 1.1) µg/mL for d4-A and (112.7 ± 1.9) µg/mL for d5-Etio.

Method validation

Accuracy assessment was carried out by applying the developed IPD methodology to 2 certified reference materials (CRM) from NMI Australia: NMIA MX002 and NMIA MX005. Recovery, calculated as the percentage ratio between the found and the certified concentration value, is shown in tables 2 and 3. It is worth noting the high accuracy of the method for T, E and Etio, with recovery values between 95% and 108% in all experiments. However, results for A were lower than expected, between 75% and 79%, which may be caused by insufficient hydrolysis time and/or temperature. Though, interference of matrix components with d4-A mass spectrum is being also considered since an abnormally high response in labelled transition measurements produces lower quantifications by IPD. All these possibilities are currently under study.

Table 2. Validation parameters, accuracy and precision, obtained for NMIA MX002 CRM.

NMIA MX002					
Compound	Certified value[1]	Intra-day repeatability (n=5)		Inter-day reproducibility (n=4)	
		Concentration[1]	Accuracy[2]	Concentration[1]	Accuracy[2]
T	16.6 ± 0.65	16.3 ± 0.3 (1.8%)	98%	15.9 ± 0.4 (2.4%)	96%
E	18.3 ± 1.3	19.7 ± 0.3 (1.8%)	108%	19.0 ± 0.5 (3%)	104%
A	1262 ± 39	963 ± 14 (1.4%)	76%	993 ± 81 (8%)	79%
Etio	814 ± 36	840 ± 15 (1.8%)	103%	804 ± 41 (5%)	99%

[1] Expressed as Mean ± SD (%RSD) ng/mL

[2] As % recovery respect to the certified value

Table 3. Validation parameters, accuracy and precision, obtained for NMIA MX005 CRM.

NMIA MX005					
Compound	Certified value[1,3]	Intra-day repeatability (n=5)		Inter-day reproducibility (n=4)	
		Concentration[1]	Accuracy[2]	Concentration[1]	Accuracy[2]
T	40.2 ± 1.8	37.3 ± 0.8 (2.0%)	93%	38.0 ± 0.7 (1.8%)	95%
E	10.74 ± 0.59	11.3 ± 0.3 (3%)	105%	11.0 ± 0.3 (3%)	102%
A	1184 ± 35	886 ± 40 (5%)	75%	890 ± 80 (9%)	75%
Etio	1290 ± 41	1229 ± 30 (2.4%)	95%	1246 ± 55 (4%)	97%

[1] Expressed as Mean ± SD (%RSD) ng/mL

[2] As % recovery respect to the certified value

[3] Corrected as indicated in the manufacturer's instructions (correction factor=0.9977), since the weighted water after reconstitution was 20.0461g

Intra-day repeatability (n=5) and inter-day reproducibility (n=4) were assessed for both CRMs. Results in terms of repeatability and reproducibility showed RSD values below 5% and 10%, respectively, in all cases (Tables 2 and 3). A shows the highest %RSD that should be related with the poorer recovery. Despite that, the developed method is characterised by a high precision.

In order to assess the method combined uncertainty, Nordtest calculations [22] were applied using the available data to obtain combined uncertainty for the determination of the four analytes (Table 4), plus the uncertainty of T/E, A/T and A/Etio ratios (Table 5). Total combined uncertainty, u_c, allows the comparison with the WADA requirements for a quantification method [2].

Since the obtained A concentrations differed from the certified values, its uncertainty derived from the bias (u(bias) around 23%) was found to be higher than the rest (u(bias) between 3.2% and 5.5%). Therefore, combined uncertainties of A, A/T and A/Etio determinations were worse than the rest of analytes and ratios. Lower uncertainty values for A and A ratios are expected once the aforementioned recovery problems are solved.

Regarding T, E and Etio, u_c was about 6% or lower in all cases. Taking into account that certified concentrations in the CRMs (Tables 2 and 3) are >5 ng/mL for T and E, and above five times the method LOQ for A and Etio (Table 6), the concentration uncertainty of the method was far lower than the limit of 20% set by the WADA for those three analytes [2].

Regarding T/E, the method u_c was 9%, also below the WADA requirements of 15%, and far lower than the observed longitudinal individual variation in male urine. In a recent paper [28] coefficients of variation of 30% and 46% for longitudinal T/E values where found when one or various laboratories were involved respectively. As might be expected, those values contain not only the individual variation but the method precision itself. Thus, a method with lower measurement uncertainty would help in assessing the actual variability in longitudinal steroidal profile for a given individual.

Table 4. Combined uncertainty, u_c, for the four selected EAAS.

Compound	u(bias)	R_w	u_c[1]
T	5,5%	1,8%	5,8%
E	4,3%	1,8%	4,7%
A	23,2%	10,3%	25,4%
Etio	3,2%	5,2%	6,1%

[1] u_c calculated as the square root of the sum of the squares of interday reproducibility (R_w, random uncertainty) and u(bias), the uncertainty associated to any source of bias including that associated to CRMs [19,22]

Table 5. Combined uncertainty, u_c, for the selected ratios.

Ratio	u(bias)	R_w	u_c[1]
T/E	8,6%	2,7%	9,0%
A/T	19,2%	8,8%	21,1%
A/Etio	21,5%	5,6%	22,2%

[1] u_c calculated as the square root of the sum of the squares of interday reproducibility (R_w, random uncertainty) and u(bias), the uncertainty associated to any source of bias including that associated to CRMs [22,23]

Finally, limits of quantification (LOQ) and detection (LOD) of the method were roughly estimated as S/N equal to 10 and 3, respectively. To this end, the lowest concentrated sample among 9 healthy female volunteers were selected (see experimental section). Lowest values found within the samples for each analyte are shown in Table 6 along with their corresponding LOQ and LOD.

Table 6. Concentration of steroids in the selected urine sample and calculated LOD and LOQ.

Compound	Sample ID[1]	Mean ± SD (%RSD) ng/mL	LOD (ng/mL) [2]	LOQ (ng/mL) [3]
T	4	0.520 ± 0.024 (5%)	0.2	0.7
E	8	1.48 ± 0.09 (6%)	0.5	1.7
A	8	301 ± 5 (1.8%)	7.3	24.5
Etio	8	587 ± 15 (3%)	28.6	95.4

[1] Results for all 9 samples can be consulted in the Supplementary Information (Table S.4)

[2] S/N = 3

[3] S/N = 10

A brief summary of figures of merit of the here developed method reveals that accuracy (75-108% recovery) compares well with already published results, while precision shows equal CV values or better, specifically for T and E and inter-day precision study. On the other hand, as IPD methodology does not require the use of calibration curves and derivatization steps are ommitted in LC, the application of IPD quantification makes the method fast and reliable. Table S.5 in supplementary information shows validation results from some selected methods including those of the present work.

CONCLUSIONS

In this work, an IDMS method for UHPLC-MS/MS has been proved suitable for EAAS determination in human urine. Isotope pattern deconvolution (IPD) was employed as mathematical tool to perform the quantification of testosterone, epitestosterone, androsterone and etiocholanolone, using deuterium-labelled analogs for that purpose.

The high similarity of molecular structure between A and Etio required of an extensive optimization of the chromatographic separation using an acetonitrile gradient.

The method was successfully validated with its application to two certified reference materials in terms of intraday repeatability and interday reproducibility with low relative standard deviations (%RSD < 10%) in both experiments, as well as in terms of trueness or recovery respect the certified concentration values (between 75% and 110%).

In addition, LODs and LOQs of the method were estimated in real life, low concentrated, female urine samples. All limits were found suitable for the determination of EAAS since they fell below the normal range of concentration in adults.

Combined standard uncertainty for T, E, Etio and T/E were below the WADA required limits for a method to be useful in doping suspicion. Moreover, u_c(T/E) is well below the observed coefficients of variation for individual longitudinal profiles, thus allowing to improve future variability assessment studies. Regarding the lower performance of A and their ratios current studies are being conducted to improve the associated uncertainty.

Therefore, the present IPD method by LC-MS/MS is highlighted as a robust, exact and precise, and constitutes a potential alternative approach for endogenous steroid analysis and a capable alternative to traditional GC- and calibration-based quantifications. Since the ionization source

used in this work, ESI, is not suited for the determination of 5αAdiol and 5βAdiol, future works will be focused on their inclusion in the method as alternate forms. In this sense, the direct determination of glucuronide conjugates seems to be a good alternative to continue developing IPD as a reliable approach in EAAS determination.

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