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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.

1. 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

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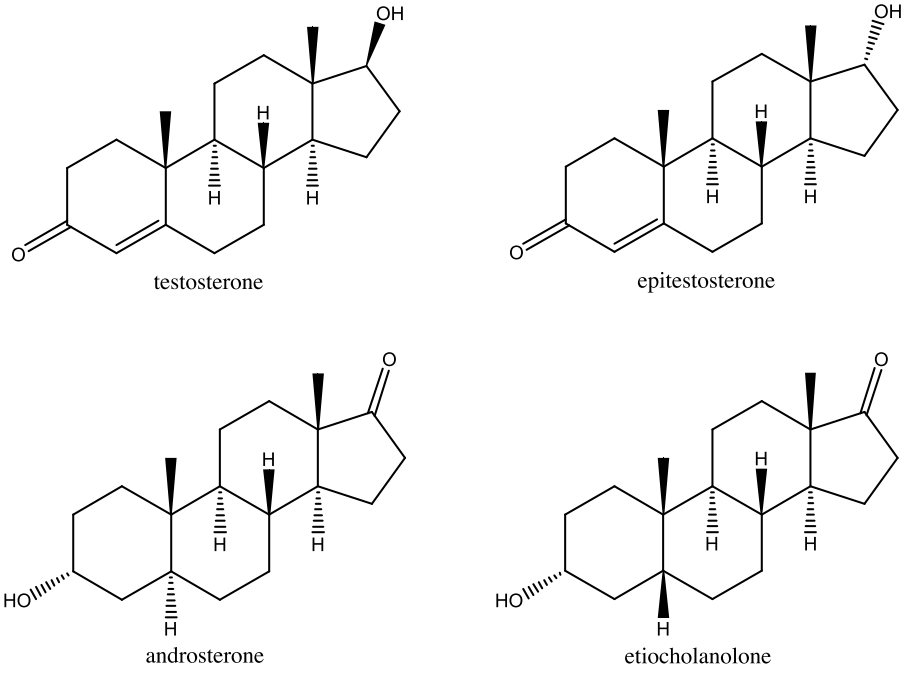

Fig. 1. Molecular structure of the selected endogenous steroids. Location of D atoms are displayed for the labeled compounds.

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.

2. Experimental

2.1. 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 Fig. 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.

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 −20 °C. 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).

2.2. 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 55 °C on an Acquity UPLC BEH C18 column (1.7 μm, 2.1 mm × 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 120 °C desolvation temperature and 350 °C source temperature, while cone gas and desolvation