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Evaluation of uncertainty sources in the determination of testosterone in urine by calibration-based and isotope dilution quantification using ultra high performance liquid chromatography tandem mass spectrometry☆

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ABSTRACT

Three quantification methodologies, namely calibration with internal standard (Cal-IS, non-weighted), weighted calibration with internal standard (wCal-IS) and isotope pattern deconvolution (IPD) have been used for the determination of testosterone in urine by LC–MS/MS. Uncertainty has been calculated and compared for the three methodologies through intra- and inter-laboratory reproducibility assays. IPD showed the best performance for the intra-laboratory reproducibility, with RSD and combined uncertainty values below 4% and 9% respectively. wCal-IS showed similar performance, while Cal-IS where not constant and clearly worse at the lowest concentration assayed (2 ng/mL) reaching RSD values up to 16%. The inter-laboratory assay indicated similar results although wCal-IS RSD (20%) was higher than IPD (10%) and Cal-IS get worse with RSD higher than 40% for the lowest concentration level. Uncertainty budgets calculated for the three procedures revealed that intercept and slope were the most important factors contributing to uncertainty for Cal-IS. The main factors for wCal-IS and IPD were the volumes of sample and/or standard measured.

1. Introduction

The use of drugs to enhance performance in sports is a well-known and documented issue. Despite the continuous introduction of new compounds, endogenous androgenic anabolic steroids (EAAS) are among the most popular doping agents [1–3]. EAAS determination still represents an important challenge due to the complexity to differentiate exogenous administration of endogenous substances. The goal requires collaborative efforts as well as advanced methodologies [1–7]. Longitudinal fluctuations measurement for a given athlete is nowadays regarded as the most effective approach to suspect the EAAS misuse. In this way, the steroidal profile of the Athlete Biological Passport (ABP) represents a powerful tool to reveal doping with endogenous compounds [1,3,6].

For most drugs, urine is the matrix generally used since it involves a non-invasive sampling procedure, large volumes are easily obtained, shows wide time windows and concentrations are high enough [1,6,7]. However, sample preparation is mandatory to ensure matrix effect attenuation and good sensitivity and selectivity. Usual treatment techniques such as solid phase extraction (SPE), liquid–liquid extraction (LLE) and simple matrix dilution are normally used. Due to its simplicity, efficiency and low cost, LLE at basic pH is still widely used in EAAS determination in urine samples [5,6].

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Concerning identification and quantification, LC–MS based techniques – equipped with Electrospray Ionization source (ESI)- tend to replace GC–MS(/MS) – considered as the gold World Anti-doping Agency (WADA) standard for quantifications [8]- since the former shows suitable sensitivity and faster instrumental run time. Specially UHPLC–MS/MS with its demonstrated separation efficiency is considered the method of choice in doping analysis [1,5,6,9].

A relevant problem with the use of ESI source is the signal alteration due to matrix effect [10–12]. Matrix effect can affect drastically to sensitivity, precision and accuracy of the analytical results. The most robust approach to minimize matrix effect rely on the use of Stable Isotope Labeled-Internal Standard (SIL-IS) [11,12]. Thus, matrix-effects associated to complex matrices can be properly overcome using a quantification methodology based on isotope dilution mass spectrometry (IDMS). Classical IDMS is based on the preparation of methodological calibration curves with the associated time consumption. An alternative method of quantification, based on the measurement of isotopic abundances in the spiked sample by multiple linear regression, can also be used. This method, known as isotope pattern deconvolution (IPD), do not requires the construction of any calibration graph and has been tested satisfactorily for rapid quantifications in different complex matrices [13–16]. IDMS together with IPD is a fast and reliable methodology, which provides one result per injection with high accuracy and free of matrix effect.

In the field of doping analysis, improvements of reliability and robustness of analytical results is continuously and still required [1,2,5,6]. WADA highlights the need of good inter-laboratory precision, particularly relevant in ABP profiling [5]. Analytical results for ABP are obtained from different laboratories for the same athlete, thus, improving inter-laboratory precision seems of maximum concern to allow universal application of any developed methodology. In this way, the need of calculating and minimizing measurement uncertainty deserves to be treated thoroughly [2,17,18].

In the present work, a previously developed method has been applied to assess the uncertainty in the testosterone concentration determined in several synthetic urine samples. Testosterone concentration has been calculated using three different methodologies, weighted and non-weighted calibration with IS (wCal-IS and Cal-IS, respectively) and IPD. In order to evaluate more in depth the associated uncertainty, an inter-laboratory comparison among five laboratories has been performed. For all three methodologies, intra- and inter-laboratory measurements have been conducted, combined uncertainties (u_c) and full uncertainty budgets have been obtained and compared.

2. Experimental

2.1. Reagents and materials

Testosterone (T, purity 99%) was provided by Sigma-Aldrich Co. (Madrid, Spain) and $^{13}C_2$-testosterone ($^{13}C_2$-T, purity 98% and $^{13}C_2$-enrichment 98%) by Cambridge Isotope Laboratories (Andover, MA, USA).

Methanol (MeOH, 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, USA) 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_4HCOO (Scharlau, Barcelona, Spain) in methanol HPLC were used for the mobile phase preparation.

A 250 μg/mL stock solution of T was prepared by dissolving 25 mg of solid standard, accurately weighed, in 100 mL of methanol. The stock solution of $^{13}C_2$-T was prepared by dissolving 10 mg of the purchased material in 50 mL of methanol. This provided a concentration by reverse isotope dilution against the natural compound of 237 μg/mL.

Individual 10 μg/mL and 1 μg/mL working solutions of the natural and labelled compounds were prepared by dilution of the stock solutions with methanol. All of the standard solutions were stored in amber glass bottles in a freezer.

The water purification system used was a Milli-Q gradient A10 from Millipore (Bedford, MA, USA).

2.2. Instrumentation

All participants in the inter-laboratory comparison have determined testosterone by LC–MS/MS. Additionally some laboratories have used other methodologies (see inter-laboratory comparison section).

This section describes the instrumentation used at Research Institute for Pesticides and Water (IUPA) laboratory, where the intra-laboratory measurements and all calculations have been done.

An Acquity UPLC system coupled to a TQD triple quadrupole mass spectrometer from Waters Corp. (Milford, MA, USA) was employed for sample analysis. Chromatographic separation was performed with an Acquity UPLC BEH C18 column (1.7 μm, 2.1 mm × 100 mm), also from Waters Corp., at a 0.3 mL/min flow rate and an injection volume of 10 μL. The column oven was kept at 55 °C and the sample manager at 10 °C. Mobile phase A was purified water and mobile phase B was MeOH HPLC, both containing 0.01% of formic acid and 1 mM of NH_4HCOO as modifiers. The gradient applied was: 45% B (0–1 min), linear increase to 77.5% B in 6.5 min, 95% B (7.51–8 min), 45% B (8.5–11.5 min). Chromatograms of blank and a selected sample can be seen in Figure S.8 in supplementary material.

Electrospray ionization in the mass spectrometer was performed at 120 °C and 350 °C source and desolvation temperatures, 80 and 800 L/h cone gas and desolvation flow, respectively, and 3.5 kV capillary voltage, operating in positive ion mode. MS/MS experimental conditions for T and $^{13}C_2$-T are listed in Table 1.

Nitrogen was employed as both drying and nebulizing gas, obtained from a nitrogen generator N_2 LC–MS adapted for LC–MS analyzers (Claind, Teknokroma, Barcelona, Spain). Collision cell was operated under a pressure of approximately 5.6×10^{-3} mbar of argon 99.995% (Praxair, Madrid, Spain). Dwell times of 0.1 s per SRM transition were chosen. MassLynx v4.1 (Waters) and homemade Excel spreadsheets were used to process the data obtained. Relative abundances of individual 100 ng/mL standards were determined (n = 5) under this conditions with RSD values under 1.5%.

2.3. Sampling and sample preparation

The aim of the study was explained to 15 healthy volunteers (8 men and 7 women with ages comprised between 16 and 59 years) and consent was obtained after confirmation that they fully understood the experiment. Urine samples were collected and stored at −20 °C until use. Testosterone concentration was approximately determined by IPD for all samples. 12 samples were selected and mixed in pairs in approximate 1:1 (v/v) ratios to obtain 6 synthetic urine samples, A to F, with increasing concentrations along the 2 ng/mL to 75 ng/mL testosterone range.

2.5 mL of the synthetic samples were transferred to individual glass tubes, together with 25 μL of 1 μg/mL $^{13}C_2$-T, and they were neutralized with 1 mL of 1 M phosphate buffer (pH 7.0). Then, 30 μL of β-glucuronidase solution were added. Samples were incubated at 55 ± 2 °C in a water bath for 1 h.