Journal of Chromatography B, 877 (2009) 2321–2329

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Validation and performance comparison of two carbon isotope ratio methods to control the misuse of androgens in humans☆

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ARTICLE INFO

Article history:
Received 15 August 2008
Accepted 9 December 2008
Available online 24 December 2008

Keywords:
Method validation
Isotope ratio mass spectrometry (IRMS)
Testosterone misuse
Doping control

ABSTRACT

Carbon isotope ratio of androgens in urine specimens is routinely determined to exclude an abuse of testosterone or testosterone prohormones by athletes. Increasing application of gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) in the last years for target and systematic investigations on samples has resulted in the demand for rapid sample throughput as well as high selectivity in the extraction process particularly in the case of conspicuous samples. For that purpose, we present herein the complimentary use of an SPE-based assay and an HPLC fractionation method as a two-stage strategy for the isolation of testosterone metabolites and endogenous reference compounds prior to GC/C/IRMS analyses. Assays validation demonstrated acceptable performance in terms of intermediate precision (range: 0.1–0.4‰) and Bland–Altman analyses revealed no significant bias (0.2 ‰). For further validation of this two-stage analyses strategy, all the specimens (n = 124) collected during a major sport event were processed.

1. Introduction

The detection of testosterone or testosterone prohormones is of particular concern in drug testing, because of their potential misuse in sports and society [1,2]. Mainly two methodologies are currently available to determine the application of endogenous steroids [3]. The first one relies on the longitudinal monitoring of the steroid profile of the athlete and the interpretation of selected parameters sensitive to the application of xenobiotic testosterone. In this context, the ratio of testosterone glucuronide over the epitestosterone glucuronide levels (T/E) in urine specimen represents a valuable decision-marker when suitable statistical approaches are applied. Recently, it has been demonstrated that a Bayesian screening test whose T/E threshold progressively evolves from a population basis to a subject basis has a major sensitivity for the detection of testosterone abuse with respect to other existing approaches [4,5]. Besides the use of longitudinal steroid profiles, the guidelines described by the World Anti-Doping Agency (WADA) focus on the application of isotope ratio mass spectrometry (IRMS) for the determination of $^{13}C/^{12}C$ ratio of relevant steroids, expressed as $\delta^{13}C$-values (‰) versus a reference standard material. This alternative methodology allows to directly assess the origin of urinary testosterone metabolites, as xenobiotic testosterone or testosterone prohormone contains less ^{13}C than their endogenous homologs [6,7]. Precisely, comparisons are made between the $^{13}C/^{12}C$ ratio of testosterone metabolites with those of urinary reference steroids within the sample [8,9]. Reference endogenous steroids (ERC) reflect the diet composition of the athlete and are considered as such, if their carbon isotope values are not affected by xenobiotic compounds. Clearly, the administration of an endogenous steroid is demonstrated when a difference $\Delta^{13}C$ of 3.0‰ or more between the $^{13}C/^{12}C$ ratio of testosterone or a testosterone metabolite relative to an ERC is determined [10].

Selection of urine specimens for IRMS analyses is based on one of the several criteria stated in a WADA technical document [10]. These current cut-off limits for the detection of testosterone misuse are derived from population-based thresholds. Reason for applying this strategy is likely to restrict the number of urine samples for IRMS analyses; a technique that requires extensive clean-up procedures prior to analysis. However, it may be emphasized that exogenous steroid may be detected by means of carbon isotope ratio analysis in urine specimens characterized by steroid levels below the established thresholds [11–13].

☆ This paper is part of a special issue entitled "Method Validation, Comparison and Transfer", guest edited by Serge Rudaz and Philippe Hubert.

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doi:10.1016/j.jchromb.2008.12.020

For compound-specific isotope analysis (CSIA) by GC/C/IRMS, it is mandatory to have a full baseline separation of GC components to avoid partial peak integration that will alter the measured δ-value [14,15]. To enable this for isotopic analyses of steroids isolated from human urines, several assays have been reported in the literature. Most of these purification procedures based on multiple solid phase extraction (SPE) steps were developed for the isolation of urinary testosterone metabolites together with reference endogenous compounds [16,17,9,18]. Nevertheless, combining SPE steps to semi-preparative HPLC has undoubtedly demonstrated a higher separation efficiency of the analytes from matrix interferences [12,19].

In order to apply IRMS as a screening method for the detection of doping with androgens in replacement of T/E ratio and other parameters from the steroid profile [20], rapid extraction of the urine specimen should be achieved. For that purpose, Aguilera et al. reported a carbon isotope ratio method based on the extraction of urine specimen by means of SPE C18 cartridges capable to process up to 60 samples each week [6]. Nevertheless, in the case of an adverse analytical finding, it is necessary that the record must include GC/C/IRMS chromatograms of the analytes free of peak contamination. If the screening method could not reach this standard, a confirmation assay including labor-intensive purification by means HPLC fractionation should be applied. This paper presents the performance comparison of a screening assay and a confirmation procedure based on HPLC fractionation for the extraction of testosterone metabolites (androsterone, etiocholanolone, 5β-androstanediol) and endogenous reference compounds (16(5α)-androstenol, 5β-preganediol, 11-ketoetiocholanolone) in human urine specimens prior to GC/C/IRMS analysis. As depicted in Fig. 1, these endogenous reference compounds (ERC) are formed through different routes of the steroids metabolism.

2. Experimental

2.1. Chemicals

Methanol (>99.9%) and cyclohexane (>99.9%) were purchased from Merck (Darmstadt, Germany). Acetic anhydride (>99.0%, lot 1131689), 1-octadecanol (>99.5%, lot 15027/1) and pyridine (>99.8%) were obtained from Fluka (Buchs, Switzerland). Acetonitrile (>99.95%) was from Biosolve B.V. (Valkenswaard, Netherlands). Tert-butyl methyl ether (TBME) 99% pure was purchased from Acros Organics (Geel, Belgium). Water was produced by a Milli-Q Gradient A10 water purification system with a Q-Gard® 2 and a Quantum™ EX Ultrapure organex cartridge purchased by Millipore Corp. (Billerica, MA, USA). Bakerbond

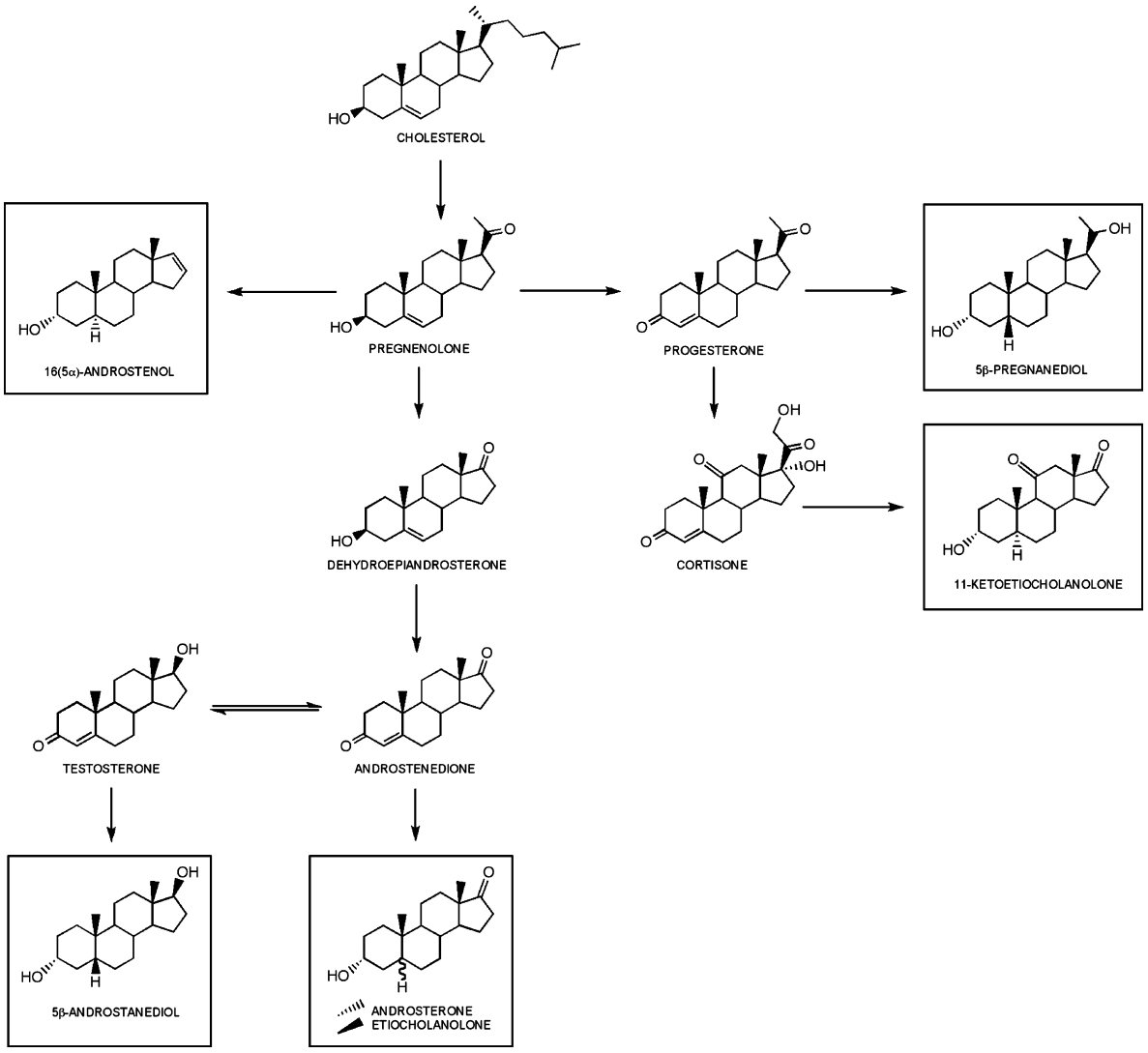

Fig. 1. Simplified metabolic pathways of the steroids targeted for IRMS analysis.

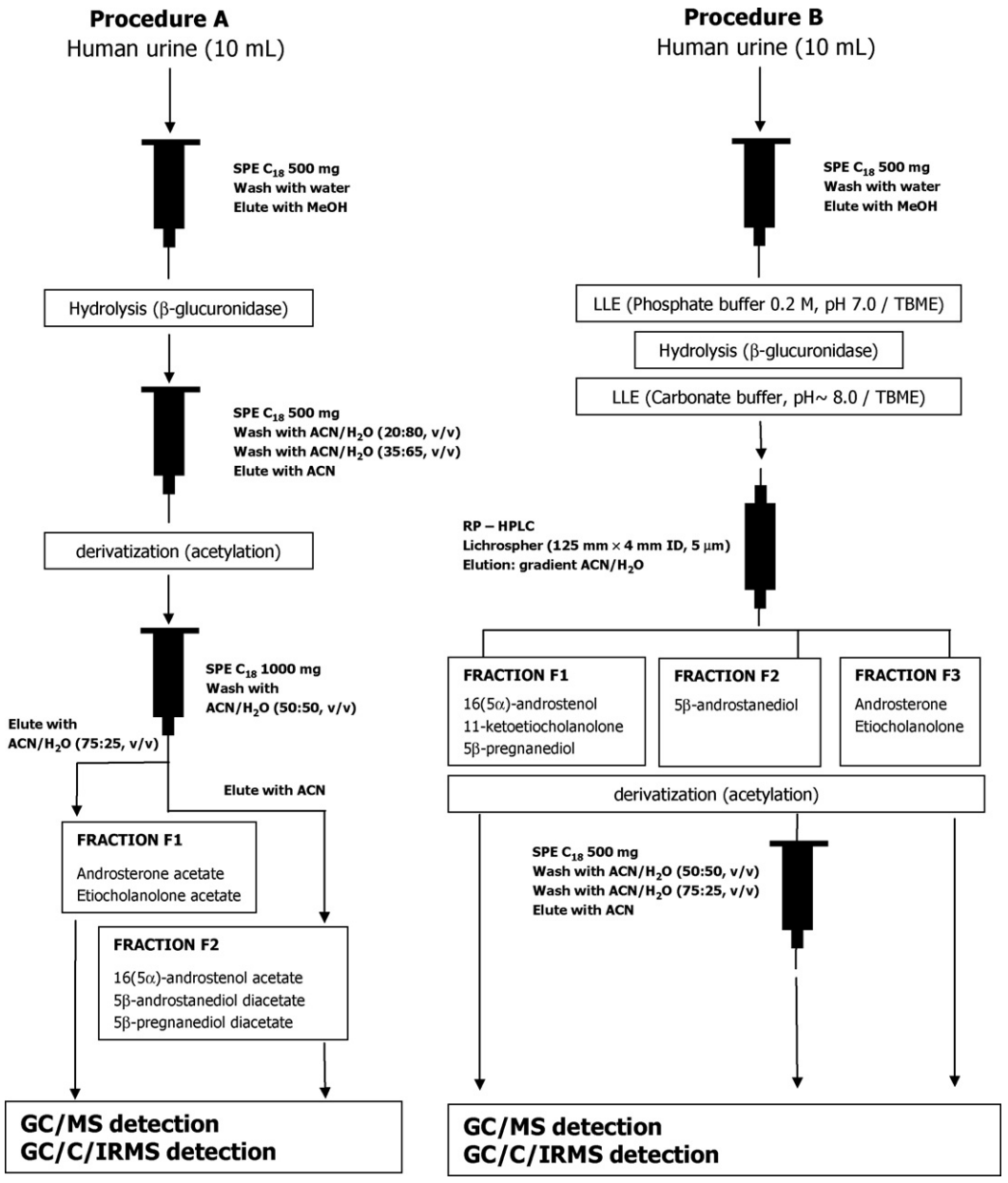

Fig. 2. Flow chart of a screening assay (procedure A) and a confirmation procedure (procedure B) for the determination of carbon isotope ratio of target steroids in human urine specimens.

spe™ 500 mg or 1000 mg octadecyl C_{18} disposable extraction columns were obtained from JT Baker (Phillipsburg, NJ, USA). β-Glucuronidase from *Escherichia coli* in a 50% glycerol solution (pH 6.5, 140 U/mL at 37 °C) was supplied by Roche Diagnostics GmbH (Manheim, Germany). Helium (Quality 60, >99.9999%) and carbon dioxide gas (quality 40, >99.99%) were purchased from Carbagas (Domdidier, Switzerland). The mixture of three alkanes (0.15 mg of each in 1 mL cyclohexane), C_{15} (n-pentadecane), C_{20} (n-eicosane) and C_{25} (n-pentacosane) for the calibration of $^{13}C/^{12}C$ ratio of the reference CO_2 was supplied by Chiron AS (Trondheim, Norway) and further diluted with 14 mL cyclohexane (purity 99.9%). 5α-androstan-3α-ol-17-one (androsterone, Lot TH868), 5β-androstan-3α-ol-17-one (etiocholanolone, Lot C305), 5α-androstan-3β-ol acetate (SI, Lot DO125), 5β-pregnane-3α,20α-diol (5β-pregnanediol, Lot 1934), 5β-androstan-3α,17β-diol (5β-androstanediol, Lot TP257), 16(5α)-androsten-3α-ol (16(5α)-androstenol, Lot TH877) and 5β-androstan-3α-ol-11,17-dione (11-ketoetiocholanolone) were obtained from Steraloids Inc. (Newport, RI, USA).

2.2. Sample extraction

The whole two-stage extraction procedures of androsterone (A), etiocholanolone (E), 16(5α)-androstenol (16EN), 5β-androstanediol (5βA) and 5β-pregnanediol (5βP) from human urine specimens is described in Fig. 2. The HPLC based extraction procedure encompassed 11-ketoetiocholanolone (11-ketoE) as a supplementary ERC.

Procedure A. The urine sample (10 mL) was centrifugated in glass tubes at 2500 rpm for 5 min and was applied onto a C_{18} column (500 mg) previously conditioned by successive addition of 5 mL methanol and 5 mL water. The column was washed with 5 mL of water and the steroid fraction was eluted with 8 mL methanol. After evaporation of the eluate to dryness, the residue was dis-

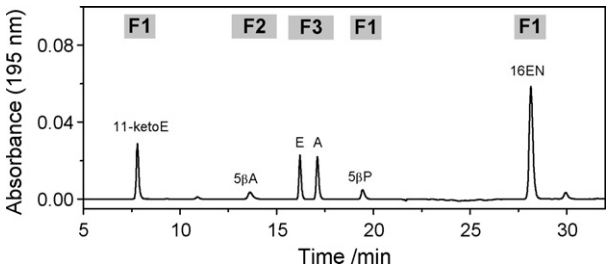

Fig. 3. HPLC chromatogram of a standard solution containing androsterone (A), etiocholanolone (E), 5β-androstanediol (5βA), 16(5α)-androstenol (16EN), 11-ketoetiocholanolone (11-ketoE) and 5β-pregnanediol (5βP) at a concentration level of 20 μg/mL each. Three fractions (F1, F2 and F3) were collected for the isolation of these compounds after injection of 55 μL.

solved in 1 mL of 0.2 M phosphate buffer (pH 7.0) and 50 μL of β-glucuronidase prior to incubate the mixture at 50 °C during 1 h (or at 37 °C overnight) in a thermostated water bath. Then, the supernatant was applied on C_{18} column (500 mg) and the deconjugated steroids were purified by washing with 6 ml acetonitrile:H_2O (20:80, v/v) and 6 ml acetonitrile:H_2O (35:65, v/v) prior to elution with 12 mL acetonitrile. The eluate was evaporated to dryness and the residue was lyophilized for 20 min. The target steroids were subsequently acetylated for 1 h at 60 °C in a reaction medium made of pyridine (50 μL) and acetic anhydride (50 μL). The reaction reactor used for this step was a 15-mL sealed glass tube. After evaporation, the residue was dissolved in 3 mL of acetonitrile:H_2O (50:50, v/v) and applied onto a C_{18} column (1000 mg) previously conditioned by successive addition of 5 mL methanol and 5 mL water. The cartridge was washed with 12 mL of acetonitrile:H_2O (50:50, v/v). Then, the first fraction (F1) containing androsterone and etiocholanolone acetates was collected after elution with 15 mL acetonitrile:H_2O (75:25, v/v). Finally, 5β-androstanediol diacetate, 5β-pregnanediol diacetate and 16(5α)-androstenol acetate were subsequently isolated after elution with 15 mL of acetonitrile (fraction F2). Both fractions F1 and F2 were evaporated to dryness and dissolved in 300–500 and 50–120 μL of cyclohexane, respectively. Each fraction contained 5α-androstan-3β-ol acetate (SI) at a level of 25 μg/mL.

Procedure B. An aliquot of 10 mL urine specimen was first centrifugated in glass tubes at 2500 rpm for 5 min. Then, the supernatant was applied onto a C_{18} column (500 mg) previously conditioned by successive addition of 5 mL methanol and 5 mL water. The column was washed with 2 mL of water and the steroid fraction was eluted with 5 mL methanol. After evaporation to dryness of the eluate, the residue was dissolved in 1 mL of 0.2 M phosphate buffer (pH 7.0) and a liquid–liquid extraction was performed with 5 mL TBME during 10 min. The aqueous layer was isolated and 50 μL of β-glucuronidase was added prior to incubation at 50 °C during 1 h (or at 37 °C overnight) in a thermostated water bath. The hydrolysis reaction was stopped under fresh water and by adding 150 μL of a saturated solution of bicarbonate (pH 8–9). A liquid–liquid extraction was performed with 5 mL TBME during 10 min. After centrifugation at 2500 rpm for 5 min, the organic layer was transferred in a glass tube and evaporated to dryness under an air stream. The dried residue was dissolved in 60 μL methanol and then submitted to a semi-preparative HPLC procedure for further purification of the target compounds (Fig. 3). The HPLC system used was composed of the Agilent 1100 Series modules (degasser, binary pump, standard autosampler, thermostated column compartment, diode-array detector) coupled to the 1200 Series fraction collector AS (Agilent Technologies, Waldbronn, Germany). A Lichrospher™ RP-18 column (250 mm × 4 mm, 5 μm) from Merck (Darmstadt, Germany) was employed for the isolation of the steroids in different fractions. Water and acetonitrile were used as mobile phase solvents. The gradient percentage of organic solvent started with 40%, changed linearly to 50% during a period of 3 min, followed by an isocratic elution with 50% for 12 min. Then, the proportion of acetonitrile was changed to 70% and maintained as such for 5 min. Finally, the mobile phase was modified at 18 min to reach a proportion of 95% acetonitrile at 19 min, followed by an isocratic elution with 95% until 28 min. Subsequently, the column was re-equilibrated for 10 min. The flow rate was set to 1.0 mL/min in constant flow mode and the injected volume was 55 μL. A standard solution (20 μg/mL) with all target steroids was injected initially and at the end of each batch to monitor the fraction collection and also detect the possible drifts in the retention time of the steroids. For that purpose, UV detection at 195 nm was employed. After collection of fractions F1 (16(5α)-androstenol, 5β-pregnanediol and 11-ketoetiocholanolone), F2 (5β-androstanediol) and F3 (androsterone and etiocholanolone), the solvent of each fraction was evaporated to dryness under an air stream and the residue was lyophilized during 20 min. The steroids were subsequently acetylated for 1 h at 60 °C in a reaction medium made of pyridine (50 μL) and acetic anhydride (50 μL). The reaction reactor used for this step was a 15-mL sealed glass tube. Then, the solvent of all fractions was evaporated to dryness under an air stream. The residue of fraction F2 was dissolved in 3 mL of acetonitrile:H_2O (50:50, v/v) for further purification. The solution containing 5β-androstanediol diacetate was applied onto a C_{18} column (500 mg) previously conditioned by successive addition of 5 mL methanol and 5 mL water. Then, the cartridge was washed with 6 mL of acetonitrile:H_2O (50:50, v/v) and 6 mL of acetonitrile:H_2O (75:25, v/v). 5β-androstanediol diacetate was isolated after elution with 6 mL of acetonitrile and the solvent evaporated to dryness. Following the estimated concentrations of the analytes by means of GC/MS analyses, the residues in fractions F1, F2 and F3 were dissolved in 50–120 μL, 80–300 μL and 300–1000 μL of cyclohexane, respectively. Each fraction contained 5α-androstan-3β-ol acetate (SI) at a level of 25 μg/mL.

2.3. GC/MS analysis

Prior to GC/C/IRMS analysis, identification of the substance was ensured by GC chromatographic retention time agreement within 1% of the retention time of reference material analyzed in the same batch and by measurement of full EI MS spectrum between *m*/*z* 40 and 450 with an acceptable maximum tolerance edited in a WADA technical document [21]. The three diagnostic ions selected for identification of each compound in their acetylated form were the following: androsterone (*m*/*z* 218, 257 and 272), etiocholanolone (*m*/*z* 228, 257 and 272), 16(5α)-androstenol (*m*/*z* 148, 241 and 316), 5β-androstanediol (*m*/*z* 241, 256 and 316), 5β-pregnanediol (*m*/*z* 269, 284 and 344), 11-ketoetiocholanolone (*m*/*z* 191, 271 and 286).

The GC/MS analysis were performed on a Hewlett-Packard 5890 Serie II Plus chromatograph (HP Analytical Division, Waldbronn, Germany) equipped with a HP 7673 auto-sampler and coupled with a HP 5971 mass selective detector (MSD). GC separation was achieved on a DB-17MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) from J&W Scientific (Folsom, CA, USA). Helium was used as carrier gas with a constant flow of 0.8 mL/min and at the initial column head pressure of 10 psi. For the identification of the target compounds, the oven temperature was increased from 80 °C (1 min) to 270 °C at 30 °C/min, then to 280 °C (3 min) at 30 °C/min and finally to 300 °C (5 min) at 5 °C/min. Injections of samples (1 μL) were made at 280 °C in the splitless mode. EI mass spectra were recorded with the instrument autotuned by continuous scanning in the 40–450 amu range at an ionization potential of 70 eV.

2.4. GC/C/IRMS analysis

The carbon isotope measurements were performed on a Delta V Plus IRMS system (ThermoFisher Scientific, Bremen, Germany) coupled to a Trace GC Ultra Gas Chromatograph) via a GC-C/TC III interface (ThermoFisher Scientific, Bremen, Germany. The sample was injected via a TriPlus™ autosampler (ThermoFisher Scientific, Bremen, Germany). The mass spectrometer consisted of an electron impact source held at 3.0 kV acceleration voltage for CO_2 gas, a magnet and three Faraday collectors for measurement of the ions at m/z 44, 45 and 46. Chromatographic separations were achieved on a DB-17MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) from J&W Scientific (Folsom, CA, USA). The GC injection port, combustion oven and reduction oven temperatures were set to 250 °C, 940 °C and 600 °C, respectively. Reference carbon dioxide gas pulses (20 s durations) were introduced at four different times during the course of the chromatographic separation. Regarding the procedures for the determination of δ^{13}C-value of the acetyl group and the calibration of the reference gas with the alkane mixture, the oven temperature was increased from 80 °C (1 min) to 220 °C (6.0 min) at 15 °C/min, then to 250 °C at 15 °C/min, and maintained at the final temperature for 3 min. For the analysis of the fractions containing the purified steroids, the oven temperature was increased from 80 °C (1 min) to 270 °C (8.3 min) at 15 °C/min, then to 300 °C at 35 °C/min, and maintained at the final temperature for 3 min. The volume of injection was 1 μL and the extracts were injected in the splitless mode (1:30 min).

The symbol δ is the standard notation for expressing carbon isotope ratios. It is defined as parts per thousand deviation of isotopic compositions from that of Vienna Pee Dee Belemnite (VPDB) and is calculated according to:

$$\delta^{13}\mathrm{C}\,(‰) = \frac{(^{13}\mathrm{C}/^{12}\mathrm{C})_{\mathrm{sample}} - (^{13}\mathrm{C}/^{12}\mathrm{C})_{\mathrm{standard}}}{(^{13}\mathrm{C}/^{12}\mathrm{C})_{\mathrm{standard}}} \times 1000 \quad (1)$$

Data acquisition and evaluation of the GC/C/IRMS data were performed with the ISODAT 2.5 software (ThermoFisher Scientific, Bremen, Germany).

2.5. Data evaluation and analysis

All statistical analyses were performed using S-PLUS® 7.0 for Windows. For distribution testing, we employed a Kolmogorov–Smirnov test of normality. Testing of statistical differences among normally distributed groups used the two-sample t-test, with $p < 0.05$ was considered statistically significant. The Bland–Altman approach was used to further assess the differences between both methods by plotting the relative difference between the two assays versus the determined δ^{13}C-value [22,23]. The mean relative difference and the 95% limits of agreement were calculated.

3. Results and discussion

Assays based on off-line SPE approach prior to GC/C/IRMS significantly increase sample throughput with respect to HPLC purification procedures. Consequently, this ensures timely release of the IRMS data to National Sport Authorities and Sport Federations. For that purpose, our laboratory developed a fast IRMS method based on the works of Aguilera et al. [6,17]. As depicted in Fig. 2, this method (procedure A) allows to determine the δ^{13}C-values of two ERC and three testosterone metabolites in their acetylated forms. Using that procedure, about 8 h are required by a laboratory technician for the extraction of 10 urine specimens and 2 quality controls. Thereafter, the batch of samples to be analyzed by GC/C/IRMS may be injected overnight. Unfortunately, the relative rapid sample processing may contrast with extraction specificity. If the presence of interferents is demonstrated after a GC/MS analysis of the extract, the sample has to be purified using a more extensive procedure. For that purpose, a method including a semi-preparative HPLC fractionation (procedure B, Fig. 2), may potentially provide a higher specificity.

The forthcoming section will present the tests used for system suitability and run acceptance criteria. These tests are of major concern to enhance the quality of bioanalytical works [24]. The measurement of the δ^{13}C-value for the target steroids was considered acceptable when the criteria were in the established tolerance range. Acceptance criteria relative to chromatography and mass spectrometry were also applied to exclude that interferents were not co-eluted with any of the target steroids.

3.1. System suitability and run acceptance criteria

The potential application of GC/C/IRMS analysis to any multitude of research areas necessitates system suitability checks to ensure that the system is operating properly at the time of analysis [25]. Specifically, the repeatability and the linearity domain of the δ^{13}C-values are assessed in our laboratory by flushing 10 pulses (20 s duration) of the reference CO_2 gas over 10 min of time at the same intensity (2 V at m/z 44) and at various intensity (typically from 0.3 to 10 V), respectively. Then, it is expected to obtain a standard deviation less than 0.1‰ for both tests prior to proceed with GC/C/IRMS analyses. The reference CO_2 gas, which was calibrated by a mixture of three n-alkanes having certified carbon isotopic compositions: C_{15} (−30.22‰), C_{20} (−33.06‰) and C_{25} (−28.21‰), was used for the determination of the δ^{13}C-value of an internal standard added. Multiple injections ($n = 36$) of 5α-androstan-3β-ol acetate (SI) yielded a mean δ^{13}C-value of −30.60‰ and a 95% confidence interval ranging from −30.0 to −31.1‰. Based on the δ^{13}C-values of 5α-androstan-3β-ol acetate spiked in the extract, this confidence interval was subsequently considered as one of the criteria for run acceptance. Another acceptance criteria was derived from the δ^{13}C-values of the isotopic calibrators back-calculated from a selected reference CO_2 pulse. Repeated injections ($n = 40$) of the calibration mixture over a one month period allowed to determine 95 % confidence intervals: C_{15} (−30.30 to −29.70‰), C_{20} (−33.42 to −32.70‰) and C_{25} (−28.90 to −27.96‰) for guarantying that the accuracy of the measurement was acceptable.

3.2. Derivatization of the steroids: effect on the δ^{13}C-values

The steroids of interest were acetylated to efficiently isolate them into two different fractions following differential elution on a SPE cartridge (procedure A) and to improve the chromatographic resolution as well [6]. However, this step leads to a dilution of the native δ^{13}C-value of the steroid as each acetyl group introduces two carbon atoms into the molecule. In addition, the acetylation derivatization reaction is supposed to induce a kinetic isotope effect [26]. To account for the shift of the δ^{13}C-value due to the formation of acetyl derivative, the following mass-balance equation may be applied [12,27]:

$$n_{\mathrm{cd}}\delta^{13}\mathrm{C}_{\mathrm{cd}} = n_{\mathrm{c}}\delta^{13}\mathrm{C}_{\mathrm{c}} + n_{\mathrm{d}}\delta^{13}\mathrm{C}_{\mathrm{dcorr}} \quad (2)$$

where n is the number of moles of carbon, C_c the compound of interest, C_{cd} the acetylated compound and C_{dcorr} the correction factor for the molecule of interest. The term C_{dcorr} was determined indirectly by measuring the δ^{13}C-value of free and acetylated 1-octadecanol [28]. Accordingly, we determined from replicate analyses ($n = 5$) and the subsequent use of Eq. (2) that $\delta^{13}C_{dcorr}$ was of −57.54‰. Then, the original δ^{13}C-values of the steroids of interest were calculated for the GC/C/IRMS data of the acetylated species and the value obtained for $\delta^{13}C_{dcorr}$. Table 1 lists the δ^{13}C-values of

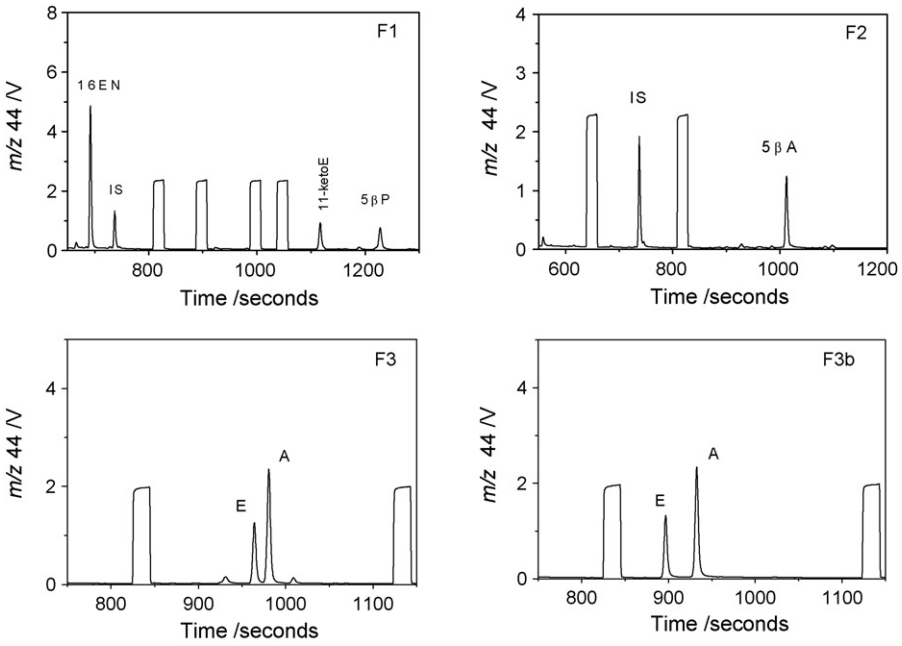

Fig. 4. GC/C/IRMS chromatograms (m/z 44) of the acetylated steroids isolated in fractions F1, F2 and F3 after purification of a urine specimen using the HPLC-based assay. The last panel (F3b) corresponds to the GC/C/IRMS chromatograms (m/z 44) of A and E extracted from the same specimen by the HPLC-based assay and analyzed without conversion to their acetate derivatives. The square-topped peaks represent 20 s pulses of carbon dioxide reference gas.

the target steroids measured underivatized, acetylated and after correction for the derivative carbon introduced during derivatization. Using an appropriate equation for the propagation of errors [29], the standard deviation associated with the calculated δ^{13}C-values of the native steroids could be assessed. It took into account the standard deviations associated with δ^{13}C measurements of free 1-octadecanol (S.D. = 0.12‰, n = 5), acetylated 1-octadecanol (S.D. = 0.11‰, n = 5) and acetylated steroids (S.D. in Table 1). The differences between the delta values of underivatized monohydroxy steroids and the corrected values for acetylation of the corresponding compounds were found to be comparable ($\Delta^{13}C_{max}$ = 0.5‰), hence indicating that the ^{13}C kinetic isotope effect associated with the acetylation reaction is likely to be similar. According to these data, A, E, 16-EN and 11-ketoE might be potentially analyzed underivatized using the HPLC purification assay (Procedure B). Actually, the GC/C/IRMS signals of A and E show an higher resolution in their free form compared to acetylated forms under the GC conditions used (Fig. 4). In contrast, the dihydroxy steroids 5βP and 5βA in their free form reveal a bias to more depleted ^{13}C isotopic composition with respect to the corrected delta values (Table 1).

The introduction of different quantities of analytes served to define the linear response of the IRMS. Typically, the signal intensities between 0.25 and 5 V on the m/z 44 channel resulted in stable δ^{13}C-values (from −0.07 to 0.07‰/V). This test was performed periodically to ensure that these intervals of signal intensities still provided these performances. Indeed, it might be that accuracy of GC/C/IRMS for a steroid compound is significantly affected when signal intensity is outside the linearity range [6].

3.3. Characteristics and comparison of the methods

The methods presented in this work display basically two different purification strategies for isolating steroids from human urine specimens (Fig. 2). With respect to multiple SPE extraction steps (procedure A), the HPLC based fractionation (procedure B) could potentially offer a better separation of analytes from interferences [29]. However, it may be emphasized that ^{13}C isotopic fractionation across an HPLC peak may be considerable (up to 18‰) [30]. Consequently, a significant isotopic fractionation may be observed in the case of an incomplete peak collection by semi-preparative HPLC. To minimize these effects which could seriously compromise accuracy of IRMS analyses, we monitored the absorbance signal of the target compounds at appropriate wavelengths in a standard mixture injected at the first and last position of a sequence analysis (Fig. 3). If the HPLC peaks of all standards are entirely included in the collection windows, the batch may be accepted and the specimens

Table 1
δ^{13}C-values (n = 5) of the steroid standards measured underivatized and acetylated. The δ^{13}C-values of the steroids measured in their acetyl forms were further corrected according to Eq. (2).

Steroids	Underivatized	Acetylated	Corrected values
	δ^{13}C-values [‰]	δ^{13}C-values [‰]	δ^{13}C-values [‰]
A	−26.90 ± 0.10	−29.63 ± 0.10	−26.67 ± 0.18
E	−29.36 ± 0.12	−31.80 ± 0.16	−29.06 ± 0.26
5βA	−29.55 ± 0.31	−33.64 ± 0.10	−28.57 ± 0.18
5βP	−18.63 ± 0.15	−23.63 ± 0.14	−17.13 ± 0.25
16EN	−26.86 ± 0.17	−29.32 ± 0.10	−26.34 ± 0.20
11(ketoE	−17.32 ± 0.13	−21.28 ± 0.13	−17.41 ± 0.23

Data are mean ± S.D.

Table 2
δ^{13}C-values (n = 5) of the steroid standards spiked in 10 mL of a 3-year-old boy urine sample (n = 5) and further extracted using the screening assay (method A) and the HPLC-based purification procedure (method B). The steroids of interest were measured in their acetyl forms and then the δ^{13}C-values were corrected according to Eq. (2).

Steroids	Method A	Method B
	δ^{13}C-values [‰]	δ^{13}C-values [‰]
A	−26.74 ± 0.21	−26.68 ± 0.19
E	−29.07 ± 0.18	−29.05 ± 0.23
5βA	−28.52 ± 0.18	−28.62 ± 0.17
5βP	−17.08 ± 0.17	−17.01 ± 0.17
16EN	−26.48 ± 0.22	−26.36 ± 0.21
11-ketoE	N.D.	−17.58 ± 0.34

Data are mean ± S.D. N.D. not determined.

Table 3
Intermediate precision of the δ^{13}C-values of a blank urine extracted by methods A and B. All values are in $\delta^{13}C_{VPDB}$ (‰).

	Method A					Method B					
	E	A	5βA	5βP	16EN	E	A	5βA	5βP	16EN	11-ketoE
1	−23.9	−23.3	−22.9	−22.9	−22.9	−23.5	−23.2	−22.8	−23.5	−22.7	−23.4
2	−23.6	−22.8	−22.3	−23.2	−22.3	−23.3	−23.5	−23.3	−22.9	−22.8	−23.0
3	−23.6	−23.0	−22.4	−23.5	−22.8	−24.2	−23.1	−23.0	−23.1	−23.2	−23.4
4	−23.8	−23.1	−22.4	−23.4	−22.8	−23.5	−22.6	−23.5	−23.0	−22.7	−23.5
5	−23.2	−23.4	−22.0	−23.5	−22.9	−23.2	−22.8	−23.2	−23.3	−22.9	−23.7
Mean	−23.6	−23.1	−23.3	−22.4	−22.7	−23.5	−23.0	−23.2	−23.2	−22.9	−23.4
S.D.	0.25	0.20	0.24	0.30	0.23	0.37	0.32	0.25	0.22	0.21	0.22

further processed. To determine potential isotopic fractionation of the steroids during extraction by each method, the pool of urine samples from a 3-year-old boy containing low concentration levels of the target endogenous steroids (<20 ng/mL) was enriched with surrogate standards A, E, 5βA, 5βP, 11-ketoE and 16EN at concentration levels of 1000, 1000, 200, 200, 200 and 1000 ng/mL. Except for 16EN, all steroids displayed recoveries higher than 70% using each procedure. Clearly, the yield of 16EN was variable (25–55%) owing to the relative volatility of the compound and its loss during the evaporation steps. Despite of non-quantitative recovery, the δ^{13}C-values of the analytes were found to be comparable to those of the methanolic standards (Tables 1 and 2), based on the outcome of two-sample Kolmogorov–Smirnov tests ($p > 0.05$). Thus, those findings indicated that no significant isotope fractionation was induced by both procedures for the isolation of the target compounds.

The intermediate precision was assayed by extractions ($n = 5$) of a blank urine over a 3-month period of time. As illustrated in Table 3, the standard deviations on the mean δ^{13}C-values of each compound extracted by either method A or method B were found to be similar. In view of previous validation data reported in the literature [12,13], it is likely that the instrument time-instability and sample preparation were the main contributors to the overall variability reflected by the magnitude of the standard deviations.

For the evaluation of method studies, we utilized negative and incurred urine specimens collected in clinical trials. Each sample was consecutively extracted by both methods and the data obtained for each compound were subsequently analyzed according to Bland–Altman representation [22,23,31]. The difference in the δ^{13}C-values of the analytes extracted by the two assays showed a bias of −0.16‰ (Fig. 5). Considering a 95% limit of agreement for the difference, only 1 out of the 100 values were out of the defined range; this is a non-significant result. These findings demonstrate that there was no systematic isotopic fractionation and hence that both purification assays may be indifferently used for the confirmation analyses of conspicuous urine samples, provided that a full baseline separation of the GC components is obtained.

3.4. Selectivity of the methods

As reported in a tutorial describing how to obtain valid δ^{13}C-values determination of urinary steroids [25], the fractions containing the compound of interests were also analyzed by full-scan GC/MS to assess identity of the species. The application of strict identification criteria for chromatography and mass spectrometry ensured that interferents were not co-eluted with any of the target steroids [21]. The example provided in Fig. 6 shows that the presence of a co-eluting compound can result in spurious δ^{13}C-values. Using the screening sample preparation, we determined δ^{13}C-values of −26.4‰ and −36.4‰ for IRMS signals corresponding to the retention times of etiocholanolone and androstesterone acetates, respectively. Indeed, the retention time of the interferent differed by 4 s with that of androsterone in the GC/C/IRMS chromatogram and therefore yielded very similar relative retention times (RRT = 1.329 versus 1.323 for androsterone acetate). A

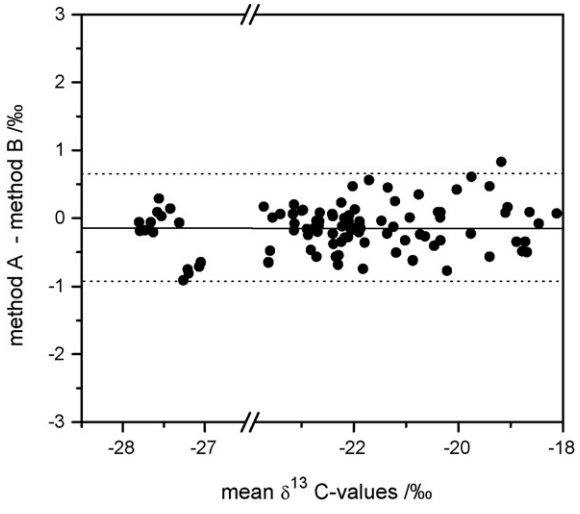

Fig. 5. Bland–Altman plot for etiocholanolone (E), androsterone (A), 16(5α)-androstenol (16-EN), 5β-androstanediol (5βA) and 5β-pregnanediol (5βP). The differences in δ^{13}C-values are plotted against the mean δ^{13}C-values determined by procedure A and procedure B. The solid line represents the mean difference between both tests and the dotted line the corresponding 95% confidence limits.

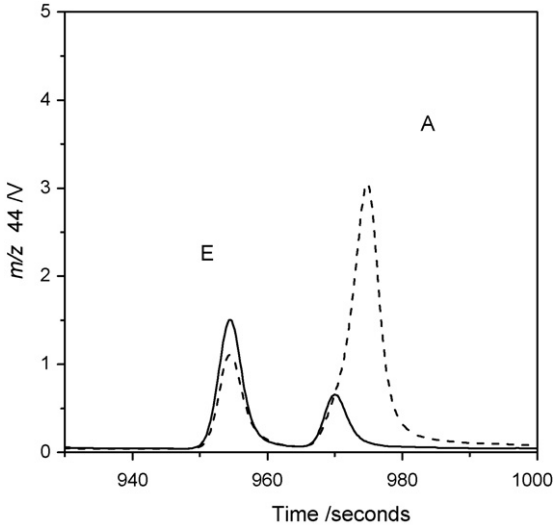

Fig. 6. GC/C/IRMS chromatograms (m/z 44) of the fraction containing androsterone and etiocholanolone in their monoacetylated form after extraction of a urine specimen using the screening assay (procedure A, dashed line) or the HPLC-based purification method (procedure B, solid line).

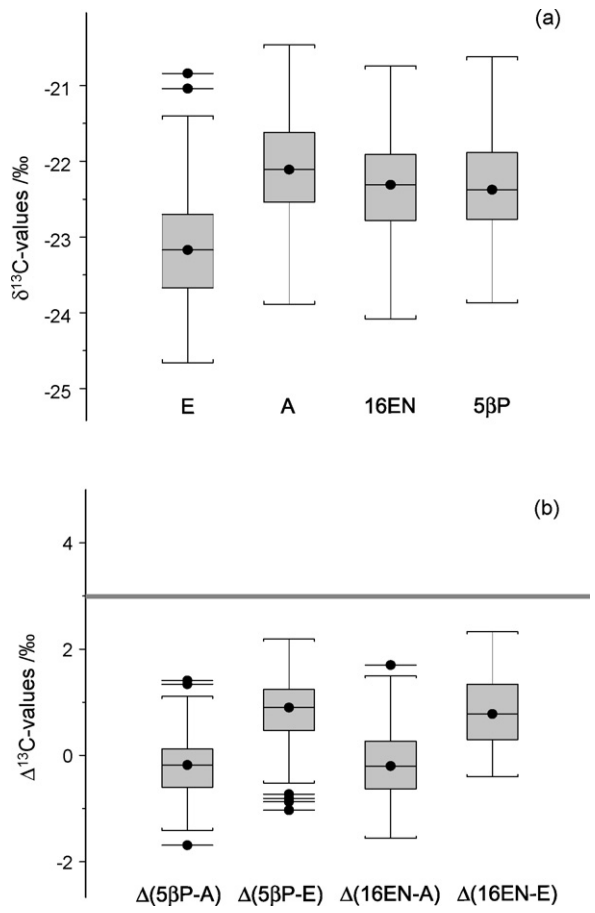

Fig. 7. Panel (a) represents the distribution of δ^{13}C-values determined for etiocholanolone (E), androsterone (A), 16(5α)-androstenol (16-EN), and 5β-pregnanediol (5βP) whereas panel (b) depicts the corresponding calculated Δ^{13}C-values for the 124 in-competition tests.

subsequent GC/MS analysis of the fraction clearly revealed the presence of an interference in the neighborhood of androsterone acetate retention time. In that case, the identification criteria for qualitative assay as described in the experimental part were not fulfilled. The re-extraction of the sample by the confirmation method allowed to discard the interference compound, and subsequently the GC/C/IRMS analysis yielded δ^{13}C-values of −26.7‰ and −25.4‰ for etiocholanolone and androstesterone acetates, respectively.

3.5. Application to specimens collected during competition events

IRMS may evidence a doping with testosterone and related prohormones, while the levels of endogenous steroids in the specimens appear to be in a normal range. For that reason, all samples collected during a major football competition in 2008 were submitted to GC/C/IRMS. Regarding the in-tournament tests, two-players per team were randomly drawn to undergo the doping control and, in addition, some players were target tested. Then, results were produced within 24 h after specimen reception using the screening procedure. Except for one specimen which was subsequently re-extracted with procedure B to remove an interfering compound eluting with androsterone acetate in the GC/C/IRMS run, the δ-values of androsterone, etiocholanolone, 16(5α)-androstenol and 5β-pregnanediol were determined following the extraction of all samples by the screening method (procedure A). It is noteworthy that the GC/C/IRMS signals of 16(5α)-androstenol were found to be at least two times more intense than those of 5β-pregnanediol in 77% of the samples analyzed. Despite of a lower extraction recovery, these findings confirmed the conclusion that this compound is generally excreted in male's urine in large quantities compared to the progesterone metabolite [16].

The δ^{13}C-values (n = 286) of the IS were comprised in the range of acceptance defined above, while negative quality controls (a blank urine) processed with each batch (n = 19) resulted in Δ^{13}C(ERC-A) and Δ^{13}C(ERC-E) values lower than 0.8 and 1.6‰). The δ^{13}C-values obtained for both testosterone metabolites and the endogenous reference compounds in the 124 in-competition testing are depicted in Fig. 7A. The values for each steroid were found to be normally distributed for a population of European subjects, with mean values of −23.13, −22.10, −22.31 and −22.32‰ for androsterone, etiocholanolone, 16(5α)-androstenol and 5β-pregnanediol, respectively. In addition to the analytical uncertainty, the variability in the dietary composition and exercise workload of the athletes may account for the distribution of the δ-values. Although it may not be excluded that endogenous steroids were administered, the range of values for Δ^{13}C(5βP-A) and Δ^{13}C(5βP-E) are similar to those obtained for a reference population of healthy Caucasian males in a European country [12]. As illustrated in Fig. 7B, these criteria used in anti-doping testing to evidence an administration of testosterone or related prohormones are lower than the threshold established by WADA at 3‰. Nevertheless, it is interesting to note that the differences of the δ^{13}C-values of etiocholanolone with respect to the ERC compounds were more pronounced than those for androsterone. A difference of –1‰ between the mean δ^{13}C-values of both androsterone and etiocholanolone was found to be statistically significant ($p < 0.05$). This isotopic fractionation between both 5α- and 5β-metabolites is supposed to originate from a kinetic isotope effect during the reduction step of the steroid A-ring [19].

4. Conclusions

We presented herein a two-stage IRMS analyses strategy to efficiently determine, in terms of rapidity and specificity, the δ^{13}C-values of the commonly investigated androgens in doping control. While the screening assay based on successive SPE steps was used to rapidly extract urine specimens for the identification of suspicious samples with elevated Δ^{13}C-values, an HPLC purification assay was implemented to complement this assay, mainly for confirmation purposes.

For the determination of the δ^{13}C-values of the relevant androgens in urine specimens, the use of the routine assay demonstrated a high throughput and acceptable specificity for most of the cases. Despite its time and expense, we showed that the confirmation procedure based on HPLC fractionation significantly increased specificity, particularly when interfering compounds co-eluted with testosterone metabolites, following application of the screening assay. Comparison of the extraction protocols on blank and incurred urine specimens demonstrated no significant bias and, independently, both methods proved to be reproducible. Basically, the extraction of a sample by different assays might further validate the data obtained for each steroid, and thus occurring isotopic fractionation in particular issues such as conversion of the steroids in their acetate derivatives and collection of the HPLC fractions might be excluded.

Acknowledgements

This study was entirely supported by the Fédération Internationale de Football Association (FIFA) Medical Assessment and Research Center (Prof. J. Dvorak, FIFA/F-MARC, Zürich, Switzerland), the Union European Football Association (UEFA) and the Swiss Federal Office of Sport (FOSPO). We thank P.H. Beyrière for technical assistance and Dr. S. Rudaz for scientific support.

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