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RESEARCH ARTICLE

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Detecting the misuse of 7-oxo-DHEA by means of carbon isotope ratio mass spectrometry in doping control analysis

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Rationale: The misuse of 7-oxo-DHEA (3β-hydroxyandrost-5-ene-7,17-dione) is prohibited according to the World Anti-Doping Agency (WADA) code. Nevertheless, it is easily available as a dietary supplement and from black market sources. In two recent doping control samples, significant amounts of its main metabolite 7β-OH-DHEA were identified, necessitating further investigations.

Methods: As both 7-oxo-DHEA and 7β-OH-DHEA are endogenously produced steroids and no concentration thresholds applicable to routine doping controls exist, the development and validation of a carbon isotope ratio (CIR) mass spectrometry method ha been desirable. Excretion studies encompassing 7-oxo-DHEA, 7-oxo-DHEA-acetate, and in-house deuterated 7-oxo-DHEA were conducted and evaluated with regard to urinary CIR and potential new metabolites of 7-oxo-DHEA.

Results: Numerous urinary metabolites were identified, some of which have not been reported before, while others corroborate earlier findings on the metabolism of 7-oxo-DHEA. The CIRs of both 7-oxo-DHEA and 7β-OH-DHEA were significantly influenced for more than 50 h after a single oral dose of 100 mg, and a novel metabolite (5α-androstane-3β,7β-diol-17-one) was found to prolong this detection time window by approximately 25 h. Applying the validated method to routine doping control specimens presenting atypically high urinary 7β-OH-DHEA levels clearly demonstrated the exogenous origin of 7-oxo-DHEA and 7β-OH-DHEA.

Conclusions: As established for other endogenously produced steroids such as testosterone, the CIR allows for a clear differentiation between endo- and exogenous sources of 7-oxo-DHEA and 7β-OH-DHEA. The novel metabolites detected after administration may help to improve the detection of 7-oxo-DHEA misuse and simplify its detection in doping control specimens.

1 | INTRODUCTION

According to the Prohibited List of the World Anti-Doping Agency (WADA), the administration of 7-oxo-dehydroepiandrosterone (7-oxo-DHEA) and its 7β- and 7α-OH-derivatives is forbidden.[1] Similar to e.g. testosterone (T), these steroids are considered as endogenous but, in contrast to T, no urinary concentration or concentration ratio thresholds have been defined. Both 7-oxo- and 7β-OH-DHEA are found in the low-ng/mL range in urine, and only for 7α-OH-DHEA could urinary concentration levels be described from population-based studies, as demonstrated by van Renterghem et al.[2]

The current approach of the Cologne doping control laboratory to detect the potential misuse of 7-oxo-DHEA is therefore based on monitoring appropriate ion transitions as established by steroid reference materials for all three compounds and to identify outliers by comparison of the area under the peak for each compound with that of negative quality control urine samples. In early 2019, one sample yielded substantially elevated areas under the peak for 7β-OH-DHEA and a slightly elevated area for 7-oxo-DHEA. To further investigate this sample, the development and validation of a method to determine the carbon isotope ratios (CIRs) of 7-oxo-DHEA and 7β-OH-DHEA conducted. Later in the same year, a sample with similarly elevated urinary concentrations was observed in the Romanian Doping Control Laboratory.

The existing isotope ratio mass spectrometry (IRMS) method for the determination of T and its metabolites was adopted in order to enable the purification of the additional target compounds (TCs).[3, 4] CIRs are expressed as $\delta^{13}C_{VPDB}$ values against the international standard Vienna Pee Dee Belemnite (VPDB) based on Equation (1)[5] and are given in ‰:

$$\delta^{13}C_{VPDB} = \frac{\left(^{13}C/^{12}C\right)_{SAMPLE}}{\left(^{13}C/^{12}C\right)_{VPDB}} - 1 \quad (1)$$

Pregnanediol (PD) and 3α-hydroxyandrost-16-ene (16EN) were used as endogenous reference compounds (ERCs). Differences between an ERC and a TC are expressed as Δ-values following Equation (2):

$$\Delta[‰] = \delta^{13}C_{ERC} - \delta^{13}C_{TC} \quad (2)$$

While for T and its metabolites thresholds for Δ-values have been defined by WADA, the basis for the interpretation of the results presented here will be the threshold as implemented for *in situ* formed steroids such as boldenone or norandrosterone.[6, 7]

In order to assess the applicability of the developed method, an excretion study involving one male volunteer was used and the samples thoroughly analyzed with regard to the CIR and Δ-values of all steroids considered relevant for the detection of 7-oxo-DHEA misuse. Exemplarily, samples from two additional excretion studies encompassing the administration of 7-oxo-DHEA-acetate were also investigated.

As little is known about the metabolism of 7-oxo-DHEA, the samples collected during the excretion study were also analyzed by high resolution/high accuracy mass spectrometric methods in order to identify novel metabolites,[8–10] allowing us also to compare some of the results with those from an earlier study.[11] In order to further corroborate the conversion of 7-oxo-DHEA into metabolites present in the blank urine (i.e. endogenously occurring steroids) and to support structural elucidation, the excretion study was repeated with deuterium-labeled 7-oxo-DHEA. Finally, the developed and validated method was applied to both routine doping control samples.

2 | EXPERIMENTAL

2.1 | Chemicals and steroids

All solvents and reagents were of analytical grade. Chromabond® C-18 cartridges (500 mg, 6 mL) were obtained from Macherey & Nagel (Düren, Germany) and β-glucuronidase from *Escherichia coli* was from Roche Diagnostics GmbH (Mannheim, Germany).

Steroid reference materials, 3β-hydroxy-5α-androstane (RSTD), 5α-androstanediol (5a), 5β-androstanediol (5b), and 16EN were supplied by Steraloids (Newport, RI, USA) and T, epitestosterone (E), etiocholanolone (ETIO), androsterone (A), dehydroepiandrosterone (DHEA), PD, and 5α-androstane-3β,7β-diol-17-one (5aM), were supplied by Sigma Aldrich (Taufkirchen, Germany). Arimistane, 7-oxo-DHEA, 7β-OH-DHEA, and 7α-OH-DHEA were from Toronto Research Chemicals (North York, ON, Canada).

The CO_2 tank gas (Linde, Pullach, Germany) was calibrated against a secondary reference material USADA 33-1 provided by Cornell University (Ithaca, NY, USA).[12]

2.2 | Sample preparation for IRMS analysis

The approach used here represents an adaption of published protocols.[3, 4] Up to 20 mL of urine were applied to pre-conditioned (3 mL of MeOH followed by 3 mL of water) C-18 solid-phase extraction (SPE) cartridges, washed with 3 mL of water, and eluted with 3 mL of MeOH. After evaporation to dryness at 50°C under a gentle stream of compressed air, the residue was reconstituted in 2 mL of an aqueous sodium phosphate buffer (pH 7) and unconjugated steroids were extracted with 5 mL of *tert*-butyl methyl ether (TBME). Then, 100 µL of β-glucuronidase were added and the sample was incubated for 1 h at 50°C. The hydrolysis was stopped by adding 1 mL of an aqueous carbonate buffer (pH 10) and the formed glucuronidated steroids were extracted with 5 mL of TBME. The organic layer was transferred and evaporated to dryness.

As all aliquots were designated for further high-performance liquid chromatography (HPLC) clean-up, they were transferred into autosampler vials with 200 µL of MeOH, evaporated to dryness and reconstituted in 100 µL of acetonitrile/water (50/50, v/v).

2.3 | HPLC setup

The HPLC clean-up was performed on a model 1100 HPLC system (Agilent, Waldbronn, Germany) equipped with a XBridge™ Shield RP18 5 mm column (4.6 × 250 mm; Waters, Eschborn, Germany) and protected by a XBridge™ BEH Shield RP18 VanGuard cartridge (130 Å, 5 µm, 3.9 mm × 5 mm; Waters). The eluents were pure acetonitrile and water: increased from 20% acetonitrile to 70% in 15 min, then to 100% in 10 min, hold for 5 min followed by

re-equilibration for 5 min. The column flow rate was set to 1 mL/min, and the injection volume was 100 μL. Fractions were collected using an automated fraction collector (FOXY R1; Axel Semrau, Sprockhövel, Germany) and typical fraction collection times were from 8.30 to 9.80 min (F1 containing 7α- and 7β-OH-DHEA), from 9.81 to 10.80 min (F2, 7-oxo-DHEA), from 17.80 to 19.30 min (F3, PD) and from 24.80 to 25.80 min (F4, 16EN). Other TCs remained optional such as T (fraction from 13.80 to 14.60 min), 5b (15.00 to 15.90 min) or 5a (15.91 to 16.90 min).

All fractions were evaporated to dryness and acetylated by adding 75 μL of acetic anhydride and 75 μL of pyridine followed by incubation at 70°C for 1 h. After evaporation, all fractions were dissolved in 200 μL of MeOH each, transferred to autosampler vials, and evaporated again.

Fractions 1 and 2 underwent a second HPLC clean-up to achieve sufficient peak purity. This second HPLC run started at 60% acetonitrile, which was increased to 84% in 10 min, then to 98% in 0.1 min and hold for 5 min followed by re-equilibration for 5 min at the starting conditions. From F1 the acetylated derivatives of 7α- and 7β-OH-DHEA were collected from 6.80 to 7.20 min, and from F2 acetylated 7-oxo-DHEA was collected from 5.30 to 6.20 min. Again, the collected fractions were evaporated, transferred, and subjected to IRMS analysis in the same manner as F3 and F4.

2.4 | GC/C/IRMS system setup

All CIR measurements were performed on a Delta V Plus isotope ratio mass spectrometer coupled to a Trace GC 1310 gas chromatograph equipped with a TriPlus RSG autosampler (all from Thermo Fisher, Bremen, Germany). The systems were coupled via a GC IsoLink CNH operated at 950°C and a ConFlo IV interface (both from Thermo Fisher). The GC column was a J&W Scientific DB-17MS (length 30 m, i.d. 0.25 mm, film thickness 0.25 μm; Agilent). The initial oven temperature was 100°C, held for 2 min and increased at 40°C/min to 273°C, at 2°C/min to 301°C, at 40°C/min to 320°C and finally held for 2 min. Carrier gas was helium (purity grade 5.0) with a programmable flow starting with 2.5 mL/min during injection and then 1.4 mL/min during the analytical run. Splitless injections were performed as a sandwich with 1 μL of a cyclohexane solution containing 40 μg/mL RSTD and 4 μL of sample reconstituted in cyclohexane at a temperature of 280°C. Isodat 3.0 software (Thermo Fisher) was used for data acquisition and evaluation.

To ensure specificity and peak purity, an ISQ single quadrupole mass spectrometer (Thermo Fisher) was hyphenated by a micro channel device (SGE, Sydney, Australia) and a restriction capillary (length 5 m, i.d. 0.15 mm, SGE) to the GC column effluent. The mass spectrometer was operated in electron ionization (EI) mode and total ion chromatograms were recorded from *m/z* 50 to 500 using Xcalibur software (version 2.2; Thermo Fisher).

2.5 | Method validation

The method has been validated taking into account the requirements for IRMS as defined by WADA.[7]

2.5.1 | Quality control urines

For the blank urine (QCN), four morning urines of a male volunteer producing only small amounts of the TCs 7-oxo-DHEA, 7α- and 7β-OH-DHEA were pooled and stored refrigerated until use. The positive quality control urine (QCP) was a pooled urine of the first six samples collected after the administration study employing product **1** (*vide infra*, *experiment a*), further diluted by urine sampled pre-administration from the same volunteer.

2.5.2 | Isotopic fractionation of the method and intra-day precision

The absence of isotopic fractionation during the sample preparation process was ensured by fortifying the QCN with 40 ng/mL of 7β-OH- and 7-oxo-DHEA and comparison of the results with those obtained for direct measurements of standards. The obtained standard deviations (SDs) were calculated and used to evaluate the method's repeatability.

2.5.3 | Inter-day precision

For inter-day precision the QCP was prepared six times on different days and again SDs were used to evaluate the method performance. In addition, these measurements were used to build up Shewhart charts for 7β-OH- and 7-oxo-DHEA to allow for a long-term monitoring of the established method's performance.

2.5.4 | Limit of quantification

The limit of quantification (LOQ) was set to 10 ng/mL for 7β-OH-DHEA and to 20 ng/mL for 7-oxo-DHEA and verified by six-fold preparations of 20 mL aliquots of the QCN spiked at these concentrations.

2.5.5 | Linear range of the IRMS setup

Pure standards were injected into the IRMS setup at approximate amounts ranging from 5 to 200 ng of steroid on column, resulting in peak signal heights between 100 and 9000 mV. As long as the mean value of replicate measurements at the same concentration showed SDs <0.5‰ and did not differ by more than 0.5‰ from the mean

value estimated for the standard, data were considered to fall into the linear range of the instrument.

2.5.6 | Population samples

Due to the very low urinary amounts of 7α-OH-, 7β-OH- and 7-oxo-DHEA it was not possible to investigate a reference population as commonly recommended for endogenously produced steroids.[7, 13, 14] As an alternative, approximately 1000 routine doping control samples were re-evaluated regarding their amount of 7β-OH- and 7-oxo-DHEA as both compounds are included with diagnostic precursor/product ion pairs in routine initial testing procedures (ITPs). Of the 1000 samples, 11 were found to fulfil the criteria of 'no adverse analytical finding', ' available for research' as permitted by the tested athlete, and showing slightly elevated concentrations of the target analytes compared with the NQC urine samples included in the same batch of the ITP. These samples were analyzed in order to probe for the endogenous CIR of 7β-OH- and 7-oxo-DHEA in presumably negative urine samples. However, as the collection of these samples was the subject of strict regulations, they can (by definition) not serve as a reference population, even if the number of samples were higher.

2.5.7 | Specificity

Due to the hyphenation of the ISQ to the GC/C/IRMS system, it was possible to evaluate any co-elutions that appear in samples under investigation by visual inspection of each full scan mass spectrum of interest. In addition to detection of possible co-elutions, the identification of analytes was also feasible.

2.5.8 | Proof-of-concept samples

As proof-of-concept, samples from three different excretion studies employing three different individuals and two different commercially available preparations were analysed.

2.6 | Correction for the acetate moieties

In order to enhance analyte clean-up and the chromatographic behaviour, all analytes were acetylated. During acetylation, carbon with a different CIR is added to the steroidal backbone necessitating acetate correction to enable the comparison of mono- and di-acetylated compounds. Acetate correction was performed following published protocols and all values given in this article refer to the underivatized steroid.[13, 15]

2.7 | Urinary steroid concentrations

The steroid profile of the excretion study samples was determined using the normal routine ITP employing TMS-derivatives and deuterated internal standards.[16, 17]

2.8 | Elimination study samples

Overall, four different excretion studies were conducted and evaluated:

a. One healthy male volunteer (44 years, 180 cm, 83 kg) administered orally one capsule of 7-keto-DHEA (100 mg) sold as dietary supplement (referred to in this study as product **1**) via the Internet. The capsule was investigated regarding its content and the CIR of 7-oxo-DHEA was determined. One blank urine was sampled prior to the administration and then 21 urines within 93 h were collected and stored frozen at −20°C until analysis.
b. Four months later, the same volunteer administered orally 10 mg of two-fold deuterated 7-oxo-DHEA (preparation described below) dissolved in 10 mL ethanol/water (20/80 v/v). In addition to one blank urine collected prior to the administration, all urines for the next 24 h were collected and stored frozen at −20°C.
c. One healthy male volunteer (28 years, 178 cm, 78 kg) administered orally one capsule of a different dietary supplement (product **2**) also obtained via Internet-based suppliers. The capsule was investigated and found to contain 7-oxo-DHEA-3-acetate (amount not specified) and caffeine. The CIR of 7-oxo-DHEA-3-acetate was determined. Samples were collected for up to 120 h after administration and stored as described.
d. One healthy male volunteer (48 years, 195 cm, 130 kg) administered orally four capsules of the same dietary supplement (product **2**) and collected samples for 120 h.

All studies were approved by the Ethical Committee of the National Institute of Sports of Romania (Bucharest, Romania, approval #2283/2016) and written informed consent was provided by the volunteers.

2.9 | Sample preparation for metabolite identification

In accordance with the above-mentioned sample preparation procedure, 5 mL of urine were passed through a pre-conditioned SPE cartridge, washed, eluted and evaporated to dryness. After reconstitution in 2 mL of buffer, the fraction of free steroids was extracted with 5 mL of TBME, transferred to a new test tube and evaporated to dryness prior to derivatization employing MSTFA/NH_4I/ethanethiol 1000:2:3 (v/w/v).[18] The aqueous residue was fortified with β-glucuronidase and incubated, and the formed glucurono-conjugated steroids were extracted with TBME, dried and

derivatized. In order to account for the sulfo-conjugated steroids, the retained aqueous residue was acidified with glacial acetic acid and applied onto another SPE cartridge, washed, eluted and evaporated to dryness. After reconstitution in ethyl acetate, MeOH and sulfuric acid, the samples were incubated at 50°C for 1 h.[14] After adding a 1 M solution of sodium hydroxide in MeOH, the samples were dried, reconstituted in water, and extracted with TBME to yield the formed sulfo-conjugated steroids, which were also subjected to high-resolution mass spectrometric analysis after trimethylsilyl (TMS)-derivatization.

2.10 | Gas chromatography/high-resolution mass spectrometry

All studies on samples prepared as described for metabolite identification were conducted on a Q Exactive GC Orbitrap GC/MS/MS system (Thermo Fisher). The gas chromatograph was equipped with a HP-Ultra 1 analytical column (length 17 m, 0.2 mm i.d., film thickness 0.11 μm; Agilent) and the temperature program started at 180°C, increased at 3°C/min to 240°C and then at 40°C/min to 320°C and held for 2 min. Injections were performed in split mode at 300°C with a split ratio of 1/5 and a purge flow rate of 5 mL/min using helium (purity grade 4.6). Constant flow was set to 1 mL/min and the transfer line temperatures were 280°C for the first transfer line and at 300°C for the second. Data was acquired either in Full MS mode covering a scan range of *m/z* 50 to 700 or in Parallel Reaction Monitoring using an isolation window of 1.3 *m/z* units. The resolution was set to 60,000 and data was collected and evaluated using Xcalibur software (version 4.0). Daily mass calibration of the instrument yielded mass accuracy in the range of ±2 ppm.

Samples analyzed for structural elucidations, which may have been derivatized differently (acetylation or MSTFA/ethyl acetate, 20/80 v/v, 45 min at 70°C), were injected onto an model 7200 Accurate-Mass Q-TOF system coupled to a model 7890A gas chromatograph (both Agilent, Santa Clara, CA, USA). The GC column and parameters were equivalent to those described for the IRMS system, the injection volume was reduced to 2 μL and the Pulsed Splitless option was chosen with an initial pressure of 40 psi. Data covering a scan range from *m/z* 50 to 500 with a scan speed of 333 ms/spectrum was acquired and evaluated using MassHunter software (version B.06, Agilent). The instrument was mass calibrated prior to each sequence in order to achieve mass accuracy in the range of ±5 ppm.

2.11 | Deuteration of 7-oxo-DHEA

As the common procedure to introduce deuterium atoms into the steroid backbone employing MeOD, D_2O and NaOD was not applicable to 7-oxo-DHEA, the reaction conditions were adjusted as follows: 20 mg of 7-oxo-DHEA were dissolved in 5 mL of MeOD in a round-bottomed flask. Then, 500 μL of D_2O and finally 100 μL of NH_4 in H_2O (25%) were added and the mixture was stirred for 16 h at 50°C. Afterwards the sample was dried under a stream of air, reconstituted in 15 mL of water and extracted twice with 15 mL TBME. The organic layers were combined, dried and purified using the above-mentioned HPLC setup. The clean deuterated 7-oxo-DHEA (12 mg) was analyzed to determine its deuterium content and used for the above-mentioned excretion study.

3 | RESULTS AND DISCUSSION

3.1 | Two-fold deuterated 7-oxo-DHEA

Applying the common procedure to introduce deuterium atoms to 7-oxo-DHEA resulted in six-fold deuterated arimistane. This disadvantageous conversion of 7-oxo-DHEA has been described for the derivatization process before, and the labile character of this steroid towards basic conditions was corroborated here.[11] Adopting the reaction conditions as described employing a weaker base resulted in a two-fold deuteration exclusively within the steroidal D-ring. In Figure 1, the corresponding mass spectra of the deuterated and the native steroid are presented for both the acetylated and the trimethylsilylated compound.

In accordance with acetylated DHEA, the molecular ion of the acetylated 7-oxo-DHEA is not visible in the EI spectra but the de-acetylated fragment ion (loss of 60 Da) is the base peak. Here, a mass shift from *m/z* 284 to *m/z* 286 (elemental composition of $C_{19}H_{24}O_2$ and $C_{19}H_{22}{}^2H_2O_2$, respectively) is observed. In order to verify the location of the introduced deuterium atoms, the TMS-derivative of the substance was analyzed. Once again here, the ion corresponding to the three-fold trimethylsilylated 7-oxo-DHEA is not present in the spectra and the base peak at *m/z* 428 is attributed to the two-fold trimethylsilylated steroid remaining after the loss of TMSOH (90 Da) and H_2 (2 Da). This loss can be explained by the above-mentioned labile structure of the analyte and may occur during derivatization or as a result of thermal degradation during the transfer from the injector on the GC column.[11] Nevertheless, applying this derivatization technique, only the mono-deuterated signal at *m/z* 429 is visible (Figure 1), which points towards a deuteration vicinal to an oxo-function. Taking into consideration that the D-ring fragment ion at *m/z* 169 shifts to *m/z* 170 enables us to conclude that 16,16-$^2H_2$-7-oxo-DHEA was synthesized.[19]

3.2 | Method validation

Several aspects usually considered during method validation for an IRMS method have been addressed and are summarized here. In addition, due to the labile nature of the target analytes, another issue was investigated. The TCs under consideration here (7-oxo-DHEA, 7α- and 7β-OH-DHEA) showed the tendency to significantly de-acetylate, probably during the transfer from the injection port liner onto the GC column. Optimizing the chromatographic conditions and

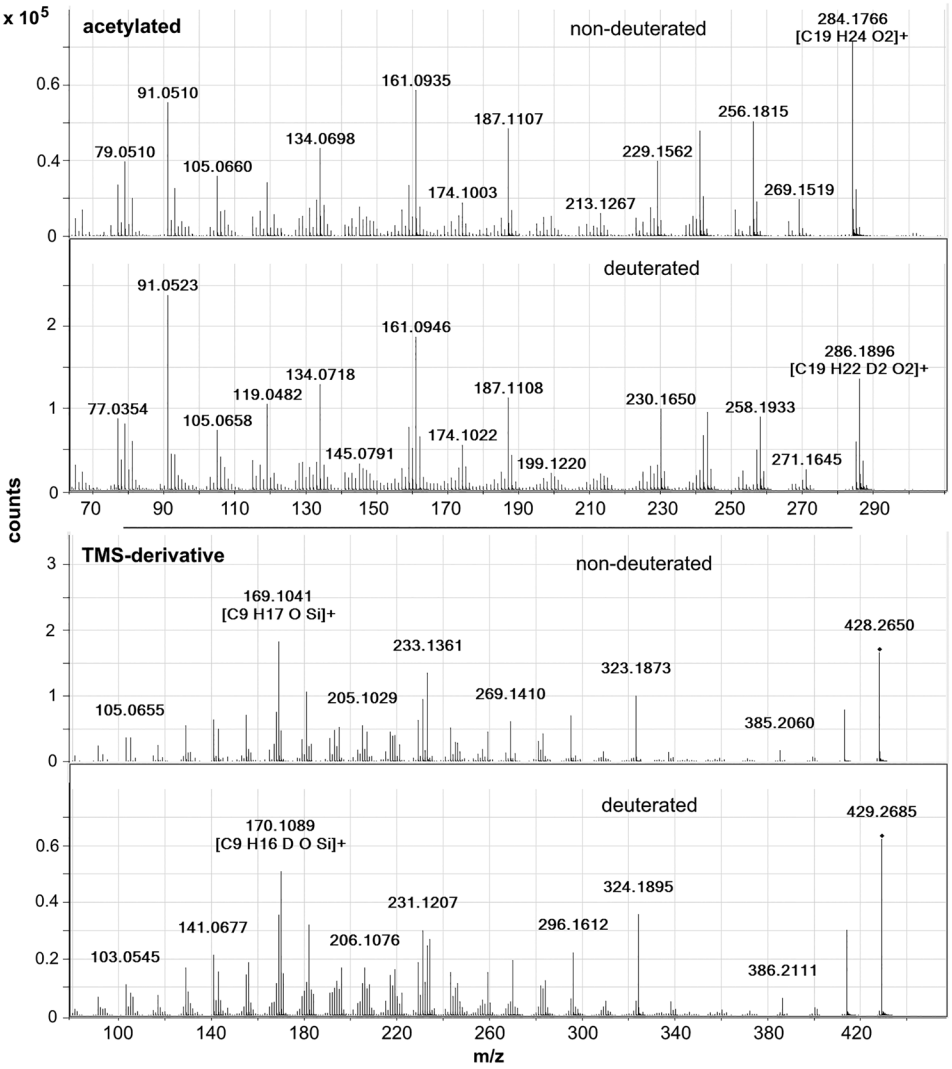

FIGURE 1 GC/Q-TOF high-resolution mass spectra obtained for the acetylated (upper part, TOF spectrum) and the degradation product of the trimethylsilylated (lower part, Q-TOF spectrum) 7-oxo-DHEA. Shown are the spectra for the native and the two-fold deuterated compound. Further information can be found in the text

lowering the temperature of the liner to 280°C enabled us to ameliorate the problem for 7-oxo-DHEA, with more than 90% of analyte remaining acetylated. In the case of 7α- and 7β-OH-DHEA, the acetylated steroids still accounted for less than 60%. To ensure the absence of isotopic fractionation or at least the stability of a possible isotopic fraction, if present, for the thermal de-acetylation process, standards of each compound were injected and the acetylated and the de-acetylated signal were compared to ensure that both show the same isotopic ratio within the analytical error.

As demonstrated by the obtained results (Table 1), the de-acetylation was not accompanied by isotopic fractionation. Especially for 7β-OH-DHEA the results for the acetylated steroid were less repeatable than they were for the de-acetylated steroid (probably due to suboptimal chromatography), so the decision was taken to pursue analyzing de-acetylated 7β-OH-DHEA and acetylated 7-oxo-DHEA in this study. 7α-OH-DHEA was not further considered during method validation as in both the negative QC urines and the urine samples collected after the administration of 7-oxo-DHEA the amount of 7α-OH-DHEA was found to be negligible. This contradiction to the cited literature[2] may be explained by recent findings demonstrating that the quantification of 7α-OH-DHEA may be affected by a co-elution of androst-4,6-diene-3,17-dione.[11] Further investigations may be necessary to clarify this point.

3.2.1 | Isotopic fractionation of the method and intra-day precision

After ensuring that the measurement itself is not affected by isotopic fractionation the sample preparation as a whole was carefully inspected. These measurements were combined with the intra-day precision and the results are listed in Table 2. For both 7β-OH- and 7-oxo-DHEA, a slight increase in the CIR is visible, which was found to be non-significant for 7-oxo-DHEA but significant for 7β-OH-DHEA (Student's t-test, p <0.05). This was probably not due to isotopic fractionation as discussed below in the context of the limit of quantification. The standard deviation of repeated measurements was found to be below 0.4‰ and therefore as expected for an IRMS method.

TABLE 1 Carbon isotope ratios obtained for repeated injections of acetylated standards in order to investigate possible isotopic fractionation during the thermal deacetylation in the GC injection port. All values are given in $\delta^{13}C_{VPDB}$ [‰]. Further information can be found in the text

#	7α-OH-DHEA_deAc	7α-OH-DHEA_Ac	7β-OH-DHEA-deAC	7β-OH-DHEA_Ac	7-oxo-DHEA_deAC	7-oxo-DHEA_Ac
1	−33.3	−35.5	−32.4	−35.2	−31.6	−32.9
2	−33.3	−35.5	−32.4	−34.2	−31.1	−33.3
3	−33.3	−35.5	−32.5	−34.2	−31.0	−33.4
4	−33.3	−35.4	−32.5	−34.0	−31.0	−33.4
5	−33.4	−35.4	−32.5	−35.8	−30.6	−33.3
6	−33.3	−35.4	−32.9	−34.5	−30.9	−33.3
Mean	**−33.3**	−35.5	**−32.5**	−34.6	**−31.0**	−33.3
SD	0.03	0.04	0.17	0.66	0.28	0.15
Corrected	**Mean**	**−33.4**		**−32.5**		**−31.0**
	SD	0.04		0.73		0.17

TABLE 2 Carbon isotope ratios obtained for the different method validation experiments. All values are given in $\delta^{13}C_{VPDB}$ [‰]. Further information can be found in the text

	Intra-day		LOQ		Inter-day	
Amount added [ng/mL]	**40**	**40**	**10**	**20**	**Excretion study sample**	
#	**7β-OH-DHEA**	**7-oxo-DHEA**	**7β-OH-DHEA**	**7-oxo-DHEA**	**7β-OH-DHEA**	**7-oxo-DHEA**
1	−31.6	−30.7	−28.1	−30.2	−31.7	−30.0
2	−31.3	−30.2	−28.1	−30.5	−31.9	−30.3
3	−31.8	−30.8	−28.1	−29.9	−31.4	−30.3
4	−31.9	−30.5	−28.4	−30.3	−32.0	−30.3
5	−31.9	−31.2	−28.8	−31.3	−31.7	−30.3
6	−31.9	−30.2	−29.0	−29.7	−31.7	−30.3
Mean	**−31.7**	**−30.6**	**−28.4**	**−30.3**	**−31.7**	**−30.3**
SD	0.22	0.36	0.38	0.51	0.18	0.10

3.2.2 | Inter-day precision

The results for the inter-day precision were found to be even better than for the intra-day precision (Table 2), which may be attributable to the elevated concentrations found in the post-administration samples pooled here.

3.2.3 | Limit of quantification

The results for the LOQ experiment are also shown in Table 2 and, again, an increase compared with the pure standards (Table 1) is visible and significant for both 7β-OH- and 7-oxo-DHEA (Student's t-test, p <0.05). Especially for 7β-OH-DHEA, a shift from −32.5 to −28.4‰ was found. This is not due to isotopic fractionation accompanying the method but can be explained by the endogenous presence of both analytes. An endogenous concentration of approximately 5 ng/mL of 7β-OH-DHEA, with a signature around −22‰, would perfectly explain both increased values found for the samples spiked with 40 ng/mL and 10 ng/mL. Calculating the expected CIR using a mixing model approach results in values of −31.4‰ for the 40 ng/mL sample (measured as −31.7‰) and −29.1‰ for the lower concentration (measured as −28.4‰). The same applies to 7-oxo-DHEA if the endogenous concentration were 2 ng/mL, matching plausibly the measured values. If the endogenous concentration of the analyte of interest is sufficiently high, linear mixing models (LMMs) are the method of choice to validate an IRMS method.[20] As the concentrations here were found too low in the QCN to use a LMM, a standard addition approach was chosen but in the end the results obviously reflect a mixture of both approaches.

3.2.4 | Linear range of the IRMS instrument

The linear range approved for 7β-OH-DHEA was from 400 to 5000 mV and for 7-oxo-DHEA from 400 to 6000 mV. Signals with

TABLE 3 Carbon isotope ratios obtained for 11 samples chosen from routine doping control samples due to slightly elevated concentrations of 7β-OH- and 7-oxo-DHEA. Deviations for Δ-values are due to rounding. All values are given in $\delta^{13}C_{VPDB}$ [‰]

Sample	PD	7β-OH-DHEA	7-oxo-DHEA	PD - 7β-OH-DHEA	PD - 7-oxo-DHEA
A	−20.3	−20.6	−21.6	0.3	1.3
B	−20.7	−21.2	−21.0	0.5	0.3
C	−21.0	−20.9	−24.5	−0.1	3.5
D	−21.6	−21.4	−21.9	−0.3	0.2
E	−22.8	−22.0	−22.0	−0.9	−0.8
F	−19.1	−19.0	−19.1	0.0	0.0
G	−19.2	Nd	−21.5	Nd	2.3
H	−20.8	−23.0	−22.7	2.2	1.8
I	−19.3	−20.9	−21.1	1.5	1.7
J	−22.2	−22.8	−24.5	0.6	2.3
K	−22.8	−23.9	−23.7	1.1	0.9
QCP	−22.1	−32.4	−30.9	10.3	8.9

lower peak heights tend to show more ^{13}C-depleted values and should therefore not be considered.

3.2.5 | Population samples

In Table 3, the results obtained for the population of presumably negative samples are summarized. Again, due to the way of selecting these samples, they cannot serve as a reference population to derive any thresholds. Nevertheless, these measurements clearly demonstrate that for samples showing only slightly elevated concentrations of 7β-OH- and 7-oxo-DHEA, the CIRs fit perfectly into the endogenous range and the Δ-values calculated using the ERC PD do not exceed 3‰. According to the Technical Document on IRMS issued by WADA, we suggest applying a preliminary threshold of 4‰ as has been established for other pseudo-endogenous steroids for which a population-based threshold can also not be derived.[7]

3.2.6 | Specificity

Due to the two-fold HPLC clean-up, most of the GC peaks were found to consist of the pure TC only. In the population samples reflecting very low concentrations, a co-elution for 7-oxo-DHEA was occasionally observed. Interestingly, during the elimination studies this co-elution was identified as arising from a metabolite of 7-oxo-DHEA and should therefore have a comparable CIR.

3.3 | Elimination study samples

The samples collected after the administration of 16,16-$^2H_2$-7-oxo-DHEA were solely used to ascertain if a found metabolite was directly attributable to 7-oxo-DHEA and to enable some considerations on the structure of selected metabolites. The elimination study carried

TABLE 4 Summary of urinary metabolites identified after the administration of 100 mg of 7-oxo-DHEA. Elemental compositions were calculated with a mass error of ±6 ppm. Excretion forms under investigation were F – unconjugated, G – glucuronidated and S – sulfo-conjugated. Further information can be found in the text

#	Retention time	Measured mass	Calculated elemental composition	Excreted as
1	9.9	428.2561	$C_{25}H_{40}O_2Si_2$	G + S
2	11.6	430.2719	$C_{25}H_{42}O_2Si_2$	G + S
3	11.8	428.256	$C_{25}H_{40}O_2Si_2$	G + S
4	11.9	432.2873	$C_{25}H_{44}O_2Si_2$	G + S
5	12.3	520.3221	$C_{28}H_{52}O_3Si_3$	G + S + F
6	13.1	522.3386	$C_{28}H_{54}O_3Si_3$	G + S
7	13.8	520.3230	$C_{28}H_{52}O_3Si_3$	F
8	14.1	520.3219	$C_{28}H_{52}O_3Si_3$	G + S
9	14.8	522.3884	$C_{28}H_{54}O_3Si_3$	G + S
10	14.9	517.2998	$C_{28}H_{49}O_3Si_3$	G + S
11	15.3	520.3230	$C_{28}H_{52}O_3Si_3$	G + S
12	15.6	519.3152	$C_{28}H_{51}O_3Si_3$	S
13	16.3	608.3602	$C_{31}H_{60}O_4Si_4$	G
14	16.8	608.3602	$C_{31}H_{60}O_4Si_4$	F
15	17.2	518.3067	$C_{28}H_{50}O_3Si_3$	G + S
16	17.4	606.3443	$C_{31}H_{58}O_4Si_4$	G + S
17	17.6	606.3444	$C_{31}H_{58}O_4Si_4$	F
18	17.7	520.3224	$C_{28}H_{52}O_3Si_3$	G + F
19	18.3	520.3228	$C_{28}H_{52}O_3Si_3$	G + F
20	18.4	608.3602	$C_{31}H_{60}O_4Si_4$	G
21	18.8	520.3228	$C_{28}H_{52}O_3Si_3$	G
22	19.0	606.3445	$C_{31}H_{58}O_4Si_4$	G + F
23	19.4	607.3521	$C_{31}H_{59}O_4Si_4$	G
24	19.8	607.3521	$C_{31}H_{59}O_4Si_4$	G+ S

out with product **1** was investigated most thoroughly, for both the steroid profile and the CIR of all collected samples. The samples collected after the administration of product **2** were used as proof-of-concept that the found results are applicable to other individuals.

3.3.1 | 16,16-$^{2}H_2$-7-oxo-DHEA administration

A substantial number of metabolites was detected within the first 24 h post-administration, some of which have recently been reported, and others that had not yet been described.[11] In total, seven metabolites were found in the fraction of unconjugated steroids, 20 in the fraction of glucurono-conjugated steroids, and 14 in the fraction of sulfated steroids. All these were also identified after the administration of unlabeled 7-oxo-DHEA (Table 4) and further investigated regarding their potential for sports drug testing purposes. Unfortunately, it was necessary to focus on the unlabeled excretion study samples, as traces of all metabolites were also detected in the blank urine samples and these have to be considered as endogenous like 7-oxo-DHEA itself.

3.3.2 | 7-oxo-DHEA administration – Urinary concentrations

Taking into consideration the different urinary excretion forms, 24 different metabolites were detected, several of which seem to be present as isomers (Table 4). All these metabolites were found in blank urine samples, especially when parallel reaction monitoring (PRM) was applied for data acquisition. Albeit some of these metabolites were only present in trace amounts, they can only serve to identify a routine doping control sample as being suspicious for 7-oxo-DHEA administration but they cannot be considered as undisputable evidence for an administration, in addition to the 7βOH- and 7-oxo-DHEA markers already implemented. Nevertheless, some of the found metabolites might be of interest for sports drug testing

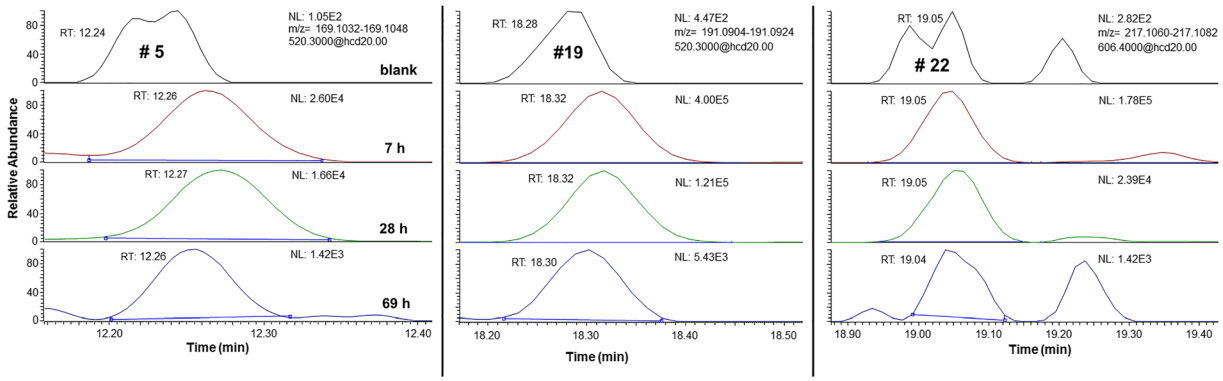

FIGURE 2 Chromatograms obtained for three different metabolites over time employing PRM experiments [Color figure can be viewed at wileyonlinelibrary.com]

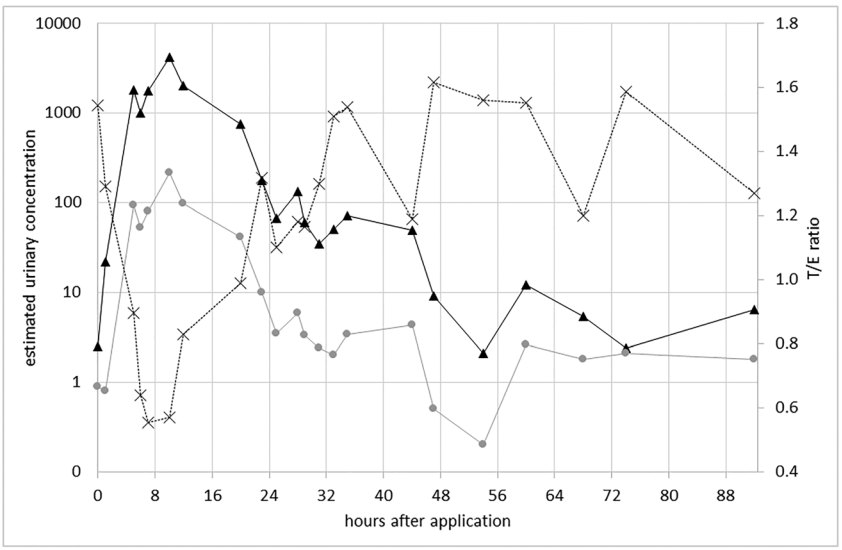

FIGURE 3 Estimated urinary concentrations of 7-oxo-DHEA (grey circles) and 7β-OH-DHEA (black triangles) excreted glucuronidated after the administration of 100 mg of 7-oxo-DHEA on a logarithmic scale (left). In addition, the T/E ratio is depicted (black crosses, secondary axis)

purposes as they increase significantly after administration or may be elevated for a longer time period as suggested previously.[11] Exemplarily, three metabolites are shown in Figure 2. Metabolite # 5, found as conjugated and unconjugated species, elutes relatively early from the GC column, and had significantly elevated responses for up to 3 days after administration (Figure 2, left). Similar retrospectivity was obtained by the metabolite # 19 (Figure 2, middle), which might correspond to the already described metabolite androst-5-ene-3α,7ξ-diol-17-one.[11] Metabolite # 22 (Figure 2, right) was formerly not reported and may also be useful to prolong detection times. Interestingly, while DHEA and the majority of its metabolites are preferentially excreted sulfo-conjugated in both their quantity and their urinary concentrations, the herein observed metabolites of 7-oxo-DHEA were predominantly detected in the fraction of glucuronic acid conjugates.

7-Oxo-DHEA (measured as its thermal degradation product) itself (metabolite # 1) was only moderately elevated after a dose of 100 mg. The urinary concentration of 7β-OH-DHEA (metabolite # 8) increased considerably in the glucurono-conjugated fraction, but decreased rapidly afterwards and returned to natural abundance after 2 days (Figure 3). As already mentioned, the concentration for 7α-OH-DHEA did not increase significantly (retention time of 11.8 min with *m/z*

TABLE 5 Mean values (n = 22) and standard deviations for all endogenous steroids under investigation. All values are given in $\delta^{13}C_{VPDB}$ [‰]

Steroid	PD	16EN	T	E	5a	5b	A	ETIO	DHEA	DHEA_S	EpiA_S
Mean	−22.8	−23.9	−24.0	−24.7	−23.4	−23.5	−22.7	−22.9	−21.7	−21.1	−23.5
SD	0.31	0.31	0.44	0.23	0.30	0.27	0.46	0.18	0.56	0.35	0.38

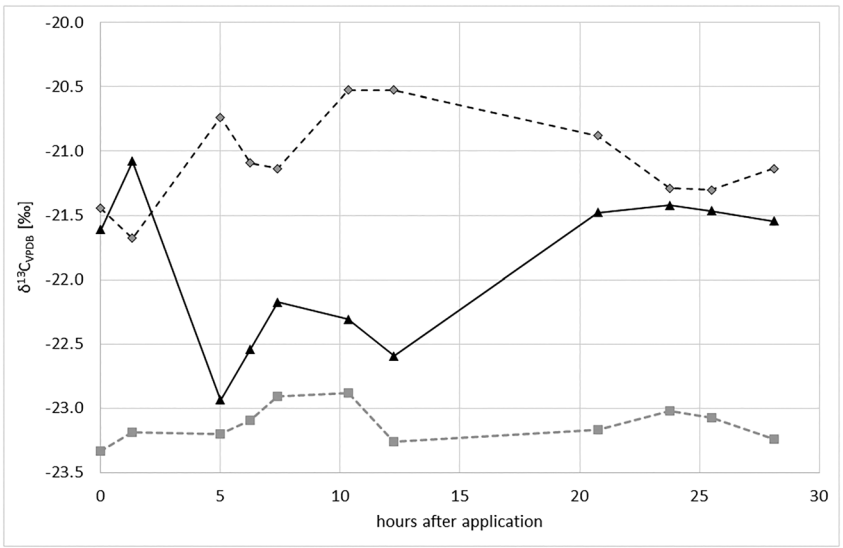

FIGURE 4 Carbon isotope ratios determined for DHEA excreted glucuronidated (black triangles), DHEA excreted sulfo-conjugated (grey diamonds) and pregnanediol excreted glucuronidated (grey squares) after application of 100 mg of 7-oxo-DHEA

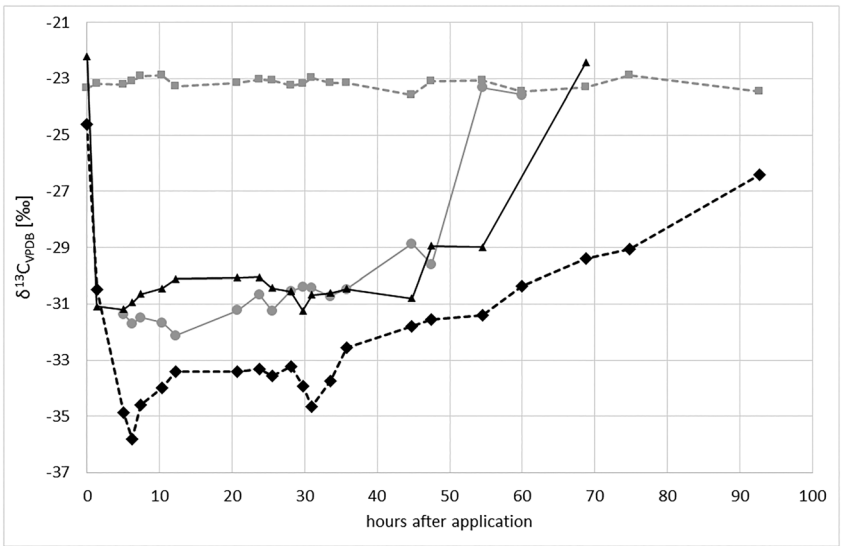

FIGURE 5 Carbon isotope ratios determined for pregnanediol (grey squares), 7β-OH-DHEA (black triangles), 7-oxo-DHEA (grey circles) and the novel metabolite 5α-androstane-3β,7β-diol-17-one (black diamonds) after application of 100 mg of 7-oxo-DHEA. All steroids have been excreted glucuronidated

520.3226) and the small amounts present were probably overlaid by other metabolites eluting at the same time from the GC column (vide supra). Another metabolite was unambiguously identified (metabolite # 9) by means of a reference material to be 5α-androstane-3β,7β-diol-17-one (5aM) and will be further discussed with regard to its CIR as it was demonstrated to be a very promising candidate for doping controls.

All other steroids encompassed in the urinary steroid profile did not show any response to the administration of 7-oxo-DHEA. Only two exceptions were identified: the first was the T/E ratio that exhibited a stable mean value in the unaffected urines of 1.4 ± 0.16 and dropped to values as low as 0.6 within the first 8 h after administration, before returning to baseline level within 24 h (Figure 3). The reason for this phenomenon remains unclear but the effect was observed and described in the literature in a comparable way previously.[8] Furthermore, an increase in the urinary concentration of 6α-OH-androstenedione was noted from 0.2 ng/mL up to 9.6 ng/mL within the first 10 h after administration, returning back to normal concentrations after 24 h.

3.3.3 | 7-oxo-DHEA administration – Carbon isotope ratios

Even if the urinary concentrations of all components of the commonly monitored steroid profile did not indicate any alteration caused by the administration of 7-oxo-DHEA, the CIRs of all the endogenous steroids were determined for the 22 post-administration samples. All steroids under investigation are listed in Table 5 showing SDs in the expected range for samples collected by the same individual over a short-term period. Interestingly, neither T nor E showed any trend and influenced the CIR after administration. The only small influence that was detected is depicted in Figure 4 for DHEA – excreted glucuronidated (G) and sulfated (S). While DHEA_G seems to be slightly ^{13}C-depleted, DHEA_S shows somewhat ^{13}C-enriched values. This does not seem to be due to an endogenous interconversion of 7-oxo-DHEA to DHEA (as already excluded by others[11]) but may be due to an intracrine or general regulatory effect that the exogenous steroid has on the steroid biosynthesis. Here, further studies would be necessary to elucidate the source of this phenomenon. Regarding doping controls, it can be stated that no impact of 7-oxo-DHEA on the CIRs of endogenous steroids is expected.

In contrast, the CIRs of 7-oxo-DHEA and its metabolites 7β-OH- and 7-oxo-DHEA perfectly reflect the CIR of the administered steroid ($\delta^{13}C_{VPDB}$ = −31.2 ± 0.18‰, n = 6) directly after administration, and the values are stable for up to 36 h after application (Figure 5). This is due to the low urinary endogenous production of these steroids, i.e. the dilution of CIR values by endogenously produced counterparts, as known from testosterone applications, is negligible here. Afterwards, the urinary concentrations return back to low normal values and, for a few samples, an endogenous impact on CIR is visible before the signals were found to be too low for CIR determinations. It has to be stated that some of the values determined in the pre-application urine and at the end of the excretion study (around 50 h) were derived from urinary concentrations and signal heights below the validated range of the method and are only added here for the sake of completeness.

In the course of this study, a novel metabolite (5aM) of 7-oxo-DHEA was identified and considered to be favorable for IRMS determinations as it seemed to be present in higher endogenous urinary concentrations and like the acetate derivatives was stable

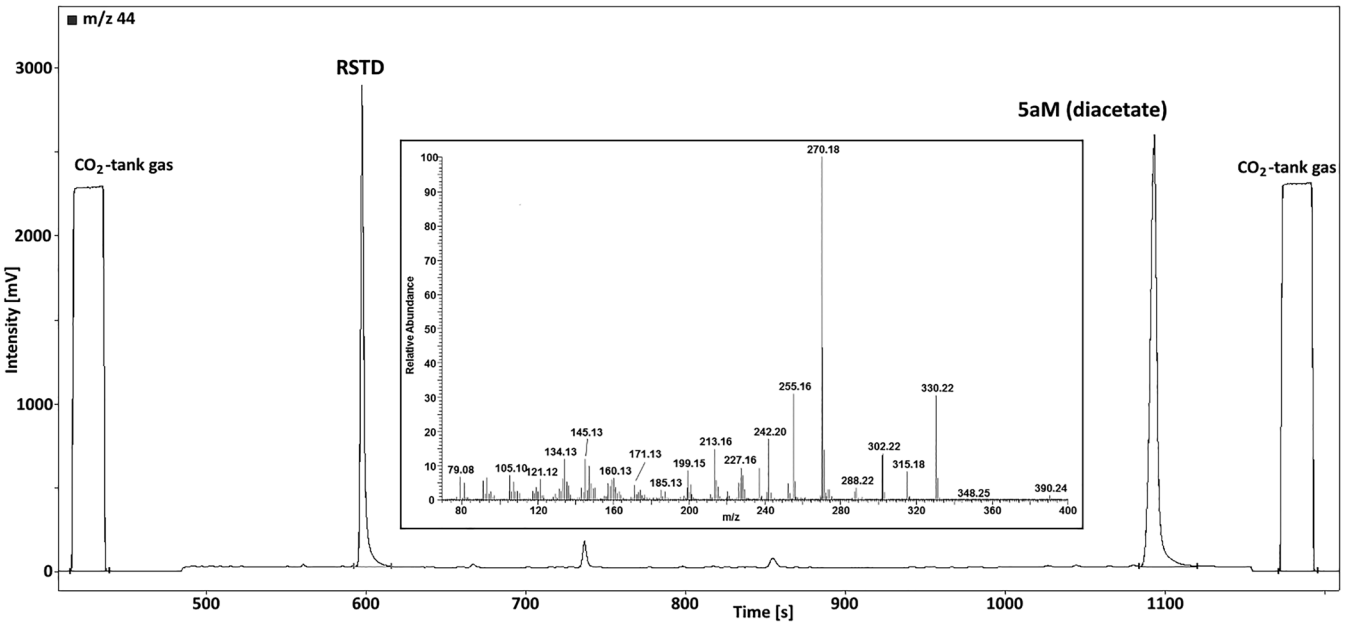

FIGURE 6 IRMS chromatogram of a standard containing androstanol (RSTD) and the new metabolite 5α-androstane-3β,7β-diol-17-one (5aM) as diacetate together with the corresponding mass spectrum. Further information can be found in the text

under the described GC conditions. An IRMS chromatogram and the corresponding mass spectrum are shown in Figure 6. In addition to the di-acetylated steroid eluting at 1090 s, small degradation product peaks are visible at retention times of 860 s (minus $C_2H_4O_2$) and 730 s (minus $C_4H_8O_4$). Taking into account the area under the peak both contribute less than 5% to the amount of CO_2 measured. During the HPLC clean-up, 5aM was found in the same fraction as 7β-OH-DHEA, both underivatized (during the first HPLC clean-up) and acetylated during the second run. As, however, 5aM elutes very late from the GC column under the described chromatographic conditions, it is perfectly separated from 7β-OH-DHEA (retention time of 600 s) and both analytes can easily be determined even if they are present in urine at substantially different concentrations. This only necessitates a two-fold injection of the same HPLC fraction at different dilutions. Regarding its potential to identify the administration of 7-oxo-DHEA, the new metabolite shows clearly influenced CIR values until the end of the study (94 h, Figure 5).

In general, 5aM shows more ^{13}C-depleted ratios than expected after the administration (−33‰ and even more ^{13}C-depleted values) pointing towards a possible isotopic fractionation taking place during the metabolism towards 5aM. In order to gain knowledge on the endogenous CIR of this metabolite, the population samples were reinvestigated; 5aM could be measured in all samples and the results are summarized in Table 6. Here, no significant isotopic fractionation was visible. Due to these promising results, 5aM will be implemented in the IRMS method to detect the misuse of 7-oxo-DHEA in the near future and the potential to investigate a real reference population on this steroid will be evaluated.

TABLE 6 CIR values of DHEA and the novel metabolite of 7-oxo-DHEA 5aM determined in the routine doping control samples investigated as population of negative samples. All values are given in $\delta^{13}C_{VPDB}$ [‰]

Sample	DHEA	5aM
A	−19.4	−20.0
B	−21.1	−21.8
C	−21.7	−20.5
D	−21.8	−21.3
E	−22.5	−21.8
F	−19.7	−18.9
G	−20.2	−21.9
H	−21.8	−22.6
I	−20.0	−18.9
J	−21.3	−23.2
K	−22.0	−22.6
QCP	−23.7	−34.7

3.3.4 | Product 2 administration

The increase in urinary concentrations of 7-oxo-DHEA was dose-dependent, showing urinary concentrations of 35 ng/mL after one capsule and up to 200 ng/mL after four capsules. Maximum concentrations were reached 3 to 5 h after administration, similar to 7β-OH-DHEA, increasing more than twenty-fold even for the single dose administration. While no effect on the T/E ratio was visible, especially after the four-capsule intake, a significant increase in the urinary concentrations of 6α-OH-androstenedione was noted. CIRs were exemplarily determined in samples collected prior to administrations and at two different time points afterwards for both

TABLE 7 CIR values obtained after the administration of 7-oxo-DHEA-3-acetate (product **2**). All values are given in $\delta^{13}C_{VPDB}$ [‰]

Trial	Hours	16EN	PD	7-oxo-DHEA	7β-OH-DHEA	5aM
1 capsule	0	−23.4	−23.2	−22.6	−23.9	−24.8
	13	−22.2	−23.6	−32.8	−30.0	−32.0
	26	−23.3	−23.4	−25.9	−27.0	−30.0
4 capsules	0	−22.8	−23.3	Nd	−24.0	−25.2
	13	−22.5	−23.0	−31.5	−29.8	−32.8
	28.5	−23.1	−23.1	−24.4	−29.3	−28.8

TABLE 8 CIR values obtained in doping control samples suspicious for the administration of 7-oxo-DHEA. All values are given in $\delta^{13}C_{VPDB}$ [‰]

	PD	16EN	7-oxo-DHEA	7β-OH-DHEA	5aM
QCN	−23.3	−23.7	Nd	−21.2	−24.9
Cologne	−20.4	Nd	−30.3	−30.6	−30.1
Bucharest	−22.3	−23.0	−28.5	−28.8	−31.7
QCP	−23.1	−23.5	−31.5	−29.9	−33.3

administration trials. The results are summarized in Table 7. The CIR of product **2** itself was found to be $\delta^{13}C_{VPDB}$ = −31.4 ± 0.15‰ (n = 6).

3.4 | Routine doping control samples suspicious for 7-oxo-DHEA administration

Both samples were analyzed using the described method and in both cases the obtained results indicate the exogenous origin of 7-oxo-DHEA and its metabolite, taking into account both the Δ-values (>4‰) and the absolute δ-values falling below −27‰ (Table 8).[7] Due to the novelty of the method and the fact that no clear regulations exist on IRMS for urinary concentrations of 7-oxo-DHEA and its metabolites, further studies on both athletes seem advisable to corroborate the findings before drawing a final conclusion.

4 | CONCLUSIONS

The development of an IRMS method to elucidate the source (endo- or exogenous) of urinary 7-oxo-DHEA and its metabolites is deemed necessary as no urinary concentration threshold has been established so far and as – in parallel to other endogenous and pseudo-endogenous steroids – IRMS is the method of choice here to separate doping offences from naturally elevated urinary concentrations. The developed method was fully validated for 7-oxo-DHEA and 7β-OH-DHEA and was successfully applied to both an excretion study and routine doping control samples. In contrast to other publications, 7α-OH-DHEA was found to be of less importance for doping controls and was not considered here in detail. In addition, a novel metabolite of 7-oxo-DHEA was unambiguously identified as 5α-androstane-3β,7β-diol-17-one by means of a certified reference material. This metabolite was excreted both sulfo- and glucurono-conjugated and the latter especially proved to be a valuable addition to the IRMS method.

By means of an excretion study performed with deuterated 7-oxo-DHEA, 24 different metabolites were identified and confirmed, taking into consideration the non-deuterated excretion studies. Unfortunately, and in contrast to reports from earlier publications, all these metabolites were also present in blank urine samples at trace levels. Therefore, a urinary concentration threshold for 7-oxo-DHEA or one of its metabolites will be required to identify the misuse of 7-oxo-DHEA and to trigger IRMS determinations on such samples. Here, further investigations will be necessary.

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