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RESEARCH ARTICLE

Development and validation of a fast gas chromatography combustion isotope ratio mass spectrometry method for the detection of epiandrosterone sulfate in urine

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Abstract

In doping control, to confirm the exogenous origin of exogenously administered anabolic androgenic steroids (AAS), a gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) analysis is performed. Recently published work suggests that epiandrosterone sulfate (EpiAS) is a promising IRMS target compound for the detection of AAS, capable of prolonging the detection window. However, EpiAS is only excreted in urine in its sulfoconjugated form, while all other IRMS target compounds are excreted glucuronidated, meaning that EpiAS cannot be incorporated in the existing IRMS methods. A separate extensive sample preparation needs to be performed on this compound with a different hydrolysis and extraction procedure and a different liquid chromatography (LC) clean-up.

The current work presents a new, fast, and easy to implement EpiAS IRMS method. The approach was based on the direct GC analysis of non-hydrolyzed EpiAS, making the solid phase extraction, hydrolysis, and acetylation step redundant. Sample preparation consisted of a simple liquid–liquid extraction, followed by LC fraction collection. A population study was performed to check compliance with the criteria drafted by the World Anti-Doping Agency (WADA). To verify the applicability of the developed approach, the method was applied to the samples of four administration studies (i.e. dehydroepiandrosterone (DHEA), testosterone gel (T gel), androstenedione (ADION), and intramuscular testosterone undecanoate. In contrast to previously published data, the strength of EpiAS as the target compound and the prolongation of the detection window in comparison with the conventional IRMS target compounds was less pronounced.

KEYWORDS

doping, epiandrosterone sulfate, isotope ratio mass spectrometry, steroids, urine

1 | INTRODUCTION

Testosterone (T) and its prohormones are endogenous anabolic androgenic steroids (AAS) that can be misused for their anabolic effects and are therefore on the prohibited list of the World Anti-Doping Agency (WADA).[1] The detection of endogenous AAS misuse is challenging. Determination of endogenous steroid concentrations and their ratios, the so-called steroid profile, is conducted during the gas chromatography mass spectrometry (GC–MS) initial testing procedure (i.e. GC–MS screening).[2,3] Quantification can point out suspicious samples, but it cannot distinguish whether an elevation of steroid concentrations and/or ratios is the result of the intake of a prohibited steroid, the

influence of a confounding factor (e.g. ethanol intake, microbial degradation), or intra-individual variation.[4,5] To confirm whether there has been an administration of testosterone or its precursors, a gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) analysis needs to be performed.[6] This technique is based on the difference between the carbon isotope ratio (CIR) of synthetic analogs and the CIR of endogenously produced steroids. As endogenous CIR values are subject to intra- and inter-individual variation and are diet-dependent, the CIRs of the target compounds (TC) are compared with the CIR of an endogenous reference compound (ERC). An ERC (e.g. pregnanediol) is part of a different metabolic pathway from the target compounds and is not affected by the intake of a synthetic AAS. Exogenous administration of endogenous AAS will lead to CIRs for the target compounds being significantly different from the CIR of the endogenous reference compound, allowing the confirmation of an exogenous origin.[7–13]

For IRMS, an extensive sample preparation is necessary to remove all the interferences, as pure compounds need to be analyzed for reliable CIR measurements. Such a sample preparation consists of a solid-phase extraction (SPE), cleavage of the glucuronide (i.e. enzymatic hydrolysis), liquid–liquid extraction (LLE), and one or multiple liquid chromatography (HPLC) fraction collection steps. In some IRMS methodologies, the steroids are also acetylated.

Recently, Piper et al. studied epiandrosterone sulfate (EpiAS) as a new target compound for testosterone, 4-androstenedione (ADION), dihydrotestosterone (DHT), and dehydroepiandrosterone (DHEA) abuse, capable of extending the detection window.[9–11] In some cases, it prolonged the detection of a single testosterone or testosterone prohormone administration (using the conventional target compounds) from 24 h to more than 100 h. Unfortunately, EpiAS is only excreted in urine in its sulfoconjugated form, while all other IRMS target compounds are excreted glucuronidated. This means that EpiAS cannot be incorporated in the existing IRMS methods and a separate extensive sample preparation needs to be performed on this compound with a different hydrolysis procedure (i.e. enzymatic hydrolysis using a sulfatase), extraction procedure, and HPLC clean-up. On top of that, the cleavage of the sulfate group is not always straightforward.[10,14–17] As a result, doping control laboratories do not perform IRMS analyses on EpiAS in routine practice.

Recently, our laboratory discovered that non-hydrolyzed sulfated steroids can be analyzed directly on gas chromatography.[18] Direct injection of a non-hydrolyzed sulfated steroid results in cleavage of the sulfate group in the GC injection port with the formation of two isomers. This approach of directly injecting non-hydrolyzed EpiAS enables a fast and easy to implement EpiAS IRMS method by omitting the solid phase extraction, hydrolysis, and acetylation step (Figure 1).

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Potassium carbonate (K_2CO_3), sodium hydrogen carbonate ($NaHCO_3$), and sodium chloride (NaCl) were purchased from Merck (Darmstadt, Germany). Ethyl acetate and ammonium formate (NH_4OOCH) were bought from Fisher Scientific (Leicestershire, UK) and formic acid (HCOOH) from Fisher Scientific (Geel, Belgium).

LC–MS grade water and LC–MS grade methanol were purchased from J. T. Baker (Deventer, the Netherlands). 5β-Pregnan-3α,20α-diol (PD) and 17β-trenbolone (β-Tren) were bought from Sigma Aldrich

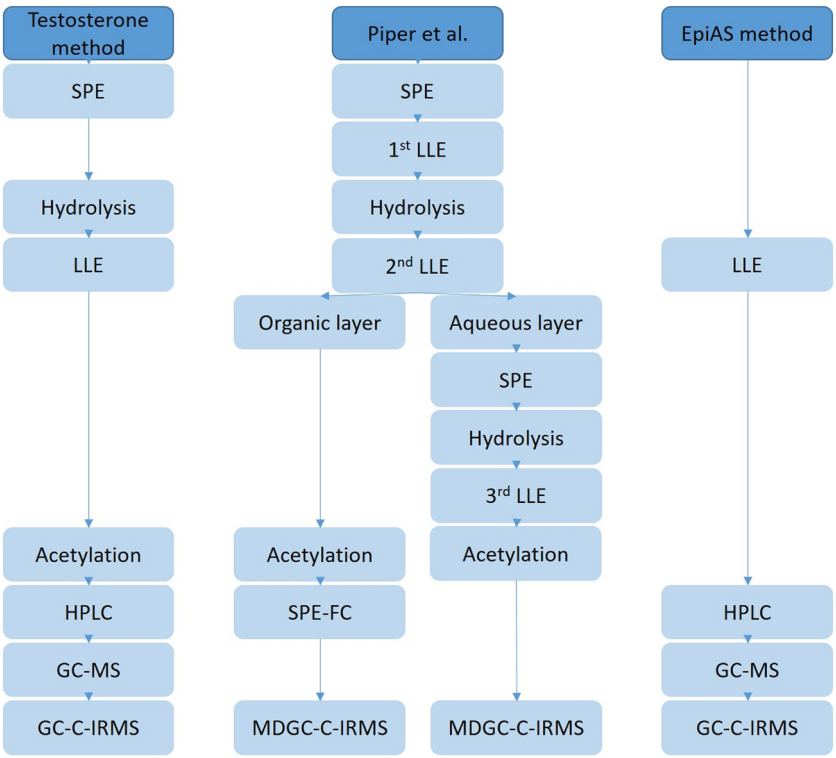

FIGURE 1 Sample preparation flowchart for testosterone, the study of Piper et al. and the EpiAS method [Colour figure can be viewed at wileyonlinelibrary.com]

(St Louis, MO, USA). 11-oxo-Etiocholanolone (11-oxo) was purchased from Steraloids (Newport, USA), epiandrosterone sulfate was obtained from Toronto Research Chemicals (Toronto, Canada), and the certified standards 5α-androstane and CU-USADA 33–1 were bought from Indiana University (Bloomington, IN, USA). Helium, carbon dioxide, and oxygen were purchased from Air Liquide (Bornem, Belgium).

135 g of K_2CO_3 and 111 g of $NaHCO_3$ were dissolved in 900 mL of distilled water to obtain a carbonate buffer with a pH of 9.5. Steroid stripped urine was prepared by pouring negative urine of an infant onto a preconditioned XAD-2 column.

2.2 | Instruments

2.2.1 | High performance liquid chromatography – fraction collection (HPLC-FC)

High performance liquid chromatography (HPLC) was performed on a Thermo Scientific Surveyor (Bremen, Germany) using a Hypersil Gold C18 column (Thermofisher Scientific, 150 × 4.6 mm; 5 μm). 100 μL of sample was injected. The mobile phases consisted of water (A) and methanol (B), both containing 0.01% formic acid and 20 mM NH_4OOCH. A gradient elution program was applied at a constant flow rate of 1 mL/min. The gradient started at 100% A and decreased for 1 min to 50% A. Subsequently, it decreased for 14 min to 36% after which it reached 0% in 0.1 min, where it was kept for 2.4 min. The column was then equilibrated to starting conditions for 3.9 min.

The HPLC was coupled to a Gilson FC 204 fraction collector (FC, Gilson, Middleton, WI, USA). 17β-Trenbolone served as a retention time marker as both EpiAS and β-Tren elute very close to each other. Collection started 0.5 min before the t_R of β-Tren (12.6 min) and stopped 0.9 min after the t_R of β-Tren.

2.2.2 | GC–MS

An Agilent 6890 GC, equipped with a Gerstel PTV-injector (Mulheim an der Ruhr, Germany) was coupled to a 5975B VI MSD from Agilent Technologies (Palo Alto, CA, USA). An Agilent J&W DB17-ms column with a length of 30 m, internal diameter of 0.25 mm and a film thickness of 0.25 μm was installed to perform separation, using helium as a carrier gas. For the EpiAS method, a 1 μL splitless injection was used. The injector was set at 60°C. After holding at 60°C for 0.15 min, the temperature was increased at a rate of 720°C/min to 280°C, where it was held for 3 min. Subsequently, the temperature was further increased (720°C/min) to 400°C, where it was held for 8 min. Then the temperature was decreased to 310°C. The temperature of the column was held at 70°C for 1 min before increasing the temperature to 250°C with 30°C/min and immediately heating to 258°C at a rate of 2°C/min. The final temperature of 310°C, which was held for 4 min, was reached by increasing the temperature at 60°C/min. Data were acquired using Chemstation software (Agilent, Waldbronn, Germany).

2.2.3 | GC-C-IRMS

An Agilent 7890A GC (Palo Alto, CA, USA) was coupled to a Thermo Scientific MAT253 IRMS (Bremen, Germany), using a Thermo GC-Isolink and a Thermo ConfloIV interface. The same GC conditions were used as for the GC–MS analysis, but with an injection volume of 5 μL. The temperature of the combustion reactor was held at 940°C. Calibration was performed with the certified standard CU-USADA 33–1.

2.3 | Sample preparation

2.3.1 | Testosterone method

The conventional testosterone method was described elsewhere.[8,13] The sample preparation consisted of a solid-phase extraction, a hydrolysis with β-glucuronidase (*E. coli*), a liquid–liquid extraction with n-pentane and an acetylation with acetic anhydride and pyridine. Subsequently, HPLC-FC, GC–MS, and GC-C-IRMS were performed (Figure 1).

2.3.2 | EpiAS method

Two 5 mL aliquots of urine were taken after which NaCl, 5 mL of ethyl acetate, and 1 mL of carbonate buffer were added to both aliquots. After rolling and centrifuging the samples (2800 rpm, 5 min), the ethyl acetate of both aliquots was transferred and combined in a new tube where 50 μL of 17β-trenbolone was added. Then the samples were evaporated to dryness at 60°C, the residue was reconstituted in 110 μL of 10% methanol/water, containing 0.01% HCOOH and 20 mM NH_4OOCH, and transferred to a microfilter. After centrifugation for 5 min, the sample was transferred to a vial and 100 μL was injected into the HPLC-FC. The collected fraction was dried, reconstituted, and transferred to a GC vial using 2 times 150 μL ethyl acetate. Afterwards, the solvent was evaporated to dryness at 40°C and reconstituted in 25 μL of 5α-androstane (5 μg/mL, ethyl acetate). Then the samples were analyzed on GC–MS to check for impurities. Then GC-C-IRMS analysis was performed for CIR determination.

2.3.3 | Quality control

Quality control samples were analyzed with every batch of samples. A system blank (water) was analyzed to check for interferences in the system. A negative control urine sample (concentration EpiAS 567 ng/mL) was prepared by aliquoting 5 mL of the negative control urine sample used for the conventional testosterone method. A positive control urine sample was prepared by aliquoting 1 mL of the negative control urine sample used for the testosterone method and adding 4 mL of purified water and

50 μL of EpiAS methanolic stock solution (100 μg/mL). A fourth quality control sample was prepared by adding 50 μL of EpiAS methanolic stock solution (100 μg/mL) to 5 mL steroid stripped urine. Analyzing this extra spiked control sample aimed to exclude that fractionation occurred during the sample preparation procedure, as the CIR of this sample should have the same CIR as the directly injected standard because there is no endogenous dilution of EpiAS. At the beginning and end of every batch on the GC-C-IRMS instrument, an EpiAS standard was analyzed also. This standard was prepared by pipetting 10 μL of EpiAS methanolic stock solution (100 μg/mL) to a vial and evaporating to dryness using oxygen-free nitrogen. The dry residue was then reconstituted in 50 μL of internal standard (IS) solution 5α-androstane in ethyl acetate (5 μg/mL). The $\delta^{13}C$ value of the internal standard 5α-androstane was checked in every analysis to ensure that the $\delta^{13}C$ values generated by the IRMS were correct. The certified $\delta^{13}C$ value of the internal standard was −31.64‰.

2.4 | Validation

The linear range of the instrument was assessed by the analysis of aliquots of EpiAS standards over a range of approximately 0.3–5 V.

To evaluate the within-batch and between-batch precision of the method, four batches were prepared and analyzed as specified in Table 1. Each batch was prepared by spiking steroid stripped urine (six replicates of a set concentration level per batch). Two batches of the lowest concentration level were prepared and analyzed by two different operators. Hence, in total, 24 samples were analyzed to evaluate the within-batch and between-batch precision of the method.

The group of Esquivel et al.[19] described concentration ranges for EpiAS in Caucasian females, Caucasian males, and Asian males. The 10th percentile was 38.6 ng/mL, 47.8 ng/mL, and 73.8 ng/mL for the Caucasian female, Caucasian male, and Asian male population, respectively. Our LOD was set at the lowest concentration used for the precision experiments (50 ng/mL). This is a conservative LOD. It is possible to detect lower concentrations but extraction recovery differs between samples. Taking the results of Esquivel et al.[19] into account, the LOD of the developed method was considered fit for purpose.

Possible fractionation during the sample preparation process was evaluated by analyzing the 24 samples used for the precision experiment and comparing the $\delta^{13}C$ values with the $\delta^{13}C$ values of enzymatically hydrolyzed EpiAS standards and the $\delta^{13}C$ values of chemically hydrolyzed (acidic solvolysis) EpiAS standards. The enzymatically hydrolyzed standards were prepared by pipetting 100 μL of the EpiAS methanolic stock solution (100 μg/mL) and evaporating to dryness. 1 mL of acetate buffer (pH 5.2) and 50 μL of β-glucuronidase/aryl sulfatase (*Helix pomatia*) were added. Samples were incubated at 56°C for 1.5 h. Afterwards, 1 mL carbonate buffer (pH 9.5) and 5 mL methyl-tert-butyl ether were added to perform the liquid–liquid extraction. After evaporation to dryness, the samples were reconstituted as already described above, after which GC–MS and GC-C-IRMS analyses were performed. The chemically hydrolyzed standards were prepared according to the protocols described in the works of Talbot et al.[20] and Burstein et al..[21] The acidic solvolysis was carried out with ethyl acetate/H_2SO_4 (10 mL/20 mg) for 1 h at 56°C. Subsequently, methanolic NaOH was added and the samples were evaporated to dryness, after which they were dissolved in 2 mL of water and extracted with 5 mL methyl-tert-butylether (MTBE).

To assess the specificity of the method, the 24 urine samples from the precision experiments and an additional 61 urine samples were extracted and analyzed with the developed method.

Bias was assessed with the linear mixing model according to the equation $\delta^{13}C_m = (\delta^{13}C_e - \delta^{13}C_a)$. $c_e/c_m + \delta^{13}C_a$ with $\delta^{13}C_m$ as the CIR of the mixture, $\delta^{13}C_e$ the endogenous CIR, and $\delta^{13}C_a$ the CIR of the added standard.[12] Two batches of six aliquots were prepared with a concentration ratio c_e/c_m range of approximately 0.3 to 1.0. The intercept of the line of best fit was compared with the average CIR value obtained by the direct injection and analysis of 56 EpiAS standards over a period of 1 year. The difference between these two values is defined as the bias.

Measurement uncertainty had to be lower than 1.0‰ according to WADA's technical document on IRMS[6] and is calculated by the formula $u(\delta^{13}C) = \sqrt{u_{Precision}{}^2 + u_{Bias}{}^2}$, which is described in WADA's technical document on decision limits.[22] $u_{Precision}$ is the standard deviation of the analysis of the negative quality control urines prepared with every batch. u_{Bias} is calculated according to the linear mixing model.[12]

2.5 | Population study

Sixty-one samples (41 male, 20 female), previously declared negative based on IRMS results, were analyzed with the EpiAS and conventional testosterone IRMS method to establish population reference

TABLE 1 Concentration levels, the average δ values and standard deviations for the evaluation of within-batch and between-batch precision

Operator	Batch	Concentration EpiAS (ng/mL)	Concentration free EpiA (ng/mL)	Average (‰)	SD (‰)
A	Low 1	50	39.2	−30.31	0.33
B	Low 2	50	39.2	−30.69	0.38
A	Medium	300	235.2	−30.79	0.27
A	High	1000	783.9	−30.62	0.08
	Total			−30.60	0.33

limits and to check the conformity with WADA's criteria for IRMS analysis.[6] These samples were selected randomly. To obtain a broad variety of samples and baseline CIR values, different populations (Western Europe, Northern Europe, and South-Africa) were included. 67 samples were analyzed of which 61 samples resulted in detectable concentrations. Of these 61 samples, 7 samples (5 males, 2 females) were from Northern Europe. Eight samples (7 males, 1 female) were from South Africa. The values were tested for Gaussian distribution using the Shapiro Wilk test (SPSS).

2.6 | Administration studies

Previous work has indicated a prolonged detection time, in comparison with the conventional IRMS target compounds, when using EpiAS as a marker for AAS abuse.[9–11] To verify these claims and prove that the developed EpiAS IRMS method is fit for purpose, samples from four administration studies from our in-house collection were analyzed.

Dehydroepiandrosterone (DHEA), testosterone gel (T gel), androstenedione (ADION), and NEBIDO were administered to four different volunteers. These administration studies were approved by the ethics committee of the Ghent University Hospital (B67020064707). All volunteers signed an informed consent prior to the study. Samples were taken at appropriate collection times depending on the duration of activity of the administered steroid and stored frozen (−20°C) until analysis.

2.6.1 | DHEA

A healthy male volunteer (23 years old, 78 kg) was administered an oral dose of a therapeutic preparation of 50 mg DHEA. A negative control sample was collected prior to the administration. Post-administration samples were collected at 4, 12, 24, 36, 48, 52, 60, 72, 76, and 84 hours.

2.6.2 | T gel

After collecting a negative control sample, T gel (Androgel, 50 mg, CIR: −29.0‰) was administered topically to a healthy male volunteer (25 years old, 80 kg). Post-administration samples were collected after 4, 8, 12, 24, 36, 48, 52, 60,72, 76, and 84 hours.

2.6.3 | ADION

For the ADION administration study, a healthy male volunteer (33 years old, 92 kg, CIR: −30.7‰) was administered an oral dose of 50 mg of ADION (Androstene Power). Post-administration samples were collected after 2, 4, 6, 9, 12, 14, 23, 36, and 47 hours.

2.6.4 | NEBIDO

A fourth volunteer (41 years old, 72 kg, CIR: −30.37‰) was administered a single dose of 1 g of NEBIDO intramuscularly. As this is a long-acting testosterone preparation, post-administration samples were collected over 87 days. A pre-administration sample and post-administration samples after 1, 3, 4, 5, 6, 7, 9, 11, 13, 21, 27, 38, 45, 52, 59, 66, 73, 80, and 87 days were included in the study.

3 | RESULTS AND DISCUSSION

3.1 | Sample preparation

The compounds analyzed with the conventional testosterone method are all excreted as glucuronides. The sulfated fraction is not taken into account as they cannot be hydrolyzed by β-glucuronidase. As EpiAS is only excreted in a sulfoconjugated form,[9] it cannot be incorporated in the conventional testosterone method. Therefore, a different and fast extraction procedure and HPLC clean-up were developed. Figure 1 presents an overview of the conventional testosterone method, the developed EpiAS method, and the developed method by Piper et al..[10] Solid-phase extraction was a common step between the conventional testosterone method and the method of Piper et al.[10] followed by hydrolysis, which in the work of Piper et al.[10] was preceded by a first LLE step. Previous work had demonstrated the direct injection of non-hydrolyzed sulfated compounds on GC–MS,[18] so hydrolysis could be omitted. Another difference between the conventional testosterone method and the method of Piper et al.[10] is the use of ethyl acetate in combination with adding salt to extract EpiAS, instead of n-pentane or TBME, which are not suitable due to the polarity of EpiAS. In the conventional testosterone method and the method of Piper et al.[10] formerly glucuronidated steroids were acetylated before performing HPLC or SPE-FC, respectively. While Piper et al.[10] used a second SPE, a second hydrolysis, a third LLE, and an acetylation step before performing multidimensional GC-IRMS (MDGC-C-IRMS), the samples were evaporated to dryness, reconstituted in mobile phase and filtered prior to HPLC, GC–MS, and GC-C-IRMS in the EpiAS method used in this study.

In contrast to the sample preparation steps described in the work of Piper et al.[10] the sample preparation in the developed EpiAS method is easy to implement, less extensive, and takes less time compared with the conventional testosterone method and the method of Piper et al.[10] as no SPE, hydrolysis, and acetylation are needed because EpiAS is injected in its intact form on the GC. Subsequently, the sulfate group is cleaved off in the injection port of the GC with the formation of two isomers,[18] as can be seen in Figure 2A. The only disadvantage is the need for an extra aliquot of urine, besides the aliquot needed for the testosterone method.

Besides differences in sample preparation, the choice of mobile phases and the column was also important in order to be able to collect the appropriate fraction. The HPLC must be able to separate

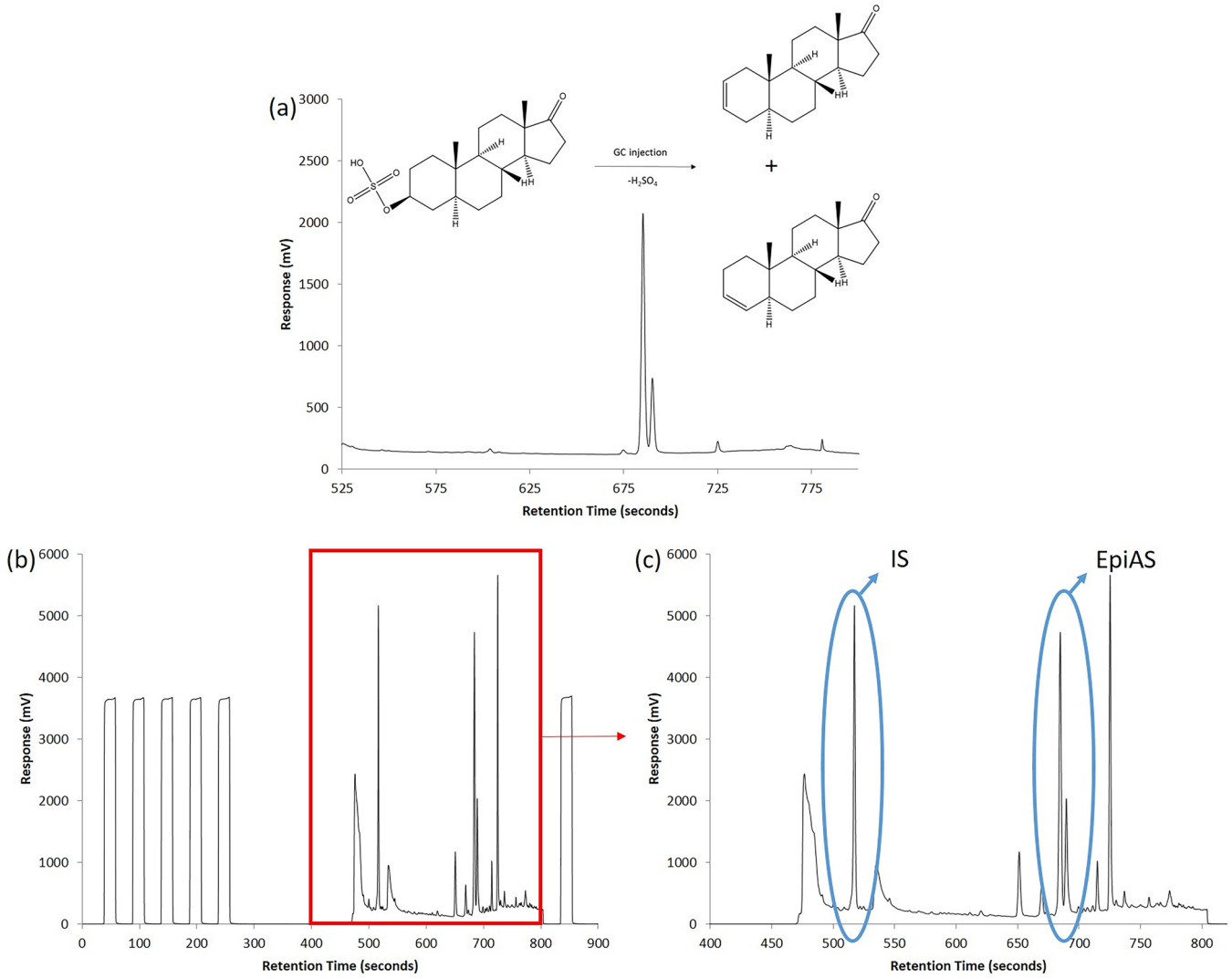

FIGURE 2 (a) Structure and IRMS analysis of EpiAS. (b) Chromatogram of a dirty sample. (c) Zoomed in chromatogram of a dirty sample [Colour figure can be viewed at wileyonlinelibrary.com]

EpiAS and androsterone sulfate (AS) because they lead to the same isomers on GC. Etiocholanolone sulfate (EtioS) and epi-etiocholanolone sulfate (EpiEtioS) elute at retention times that differ from EpiAS and AS. On GC, the combination of the chosen column and temperature program ensures that both isomers coelute. By integrating their peaks together on the IRMS, the right CIR is obtained. If the peaks were integrated separately, a difference of 0.5–1‰ could be observed. When the results of the integration of both peaks were compared with that from a hydrolyzed standard, the CIR was the same.

Because acetylation takes place in the conventional testosterone method, the $\delta^{13}C$ values of those steroids have to be converted to their underivatized values using the equation $\delta^{13}C_s = (n_{cd}.\delta^{13}C_{cd} - n_d.\delta^{13}C_{d\_corr})/n_s$. In this equation, n represents the number of carbon atoms, s the native steroid (underivatized form), d the derivative group, and c_d the derivatized compound. The $\delta^{13}C$ values of EpiAS were then compared with those of PD and 11-oxo by the equation $\Delta\delta^{13}C = \delta^{13}C_{ERC} - \delta^{13}C_{TC}$.

3.2 | Validation

A summary of the results is presented in Table 2. The slope of the regression line is acceptable (−0.01‰/V) within the range of 0.3 V to 5 V, indicating that 0.3–5 V is a suitable interval for appropriate $\delta^{13}C$ determination.

The within-batch precision (repeatability) ranged from 0.08‰ to 0.38‰. The between-batch precision (reproducibility) was 0.33‰ (Table 1).

As the sulfate group of EpiAS is cleaved off during the injection, it is important to verify that there is no fractionation when the two isomers are formed. The average $\delta^{13}C$ value in the spiked steroid stripped urine samples was −30.60‰ with a standard deviation of 0.33‰. The average $\delta^{13}C$ value of the enzymatically hydrolyzed standards was −30.58‰ with a standard deviation of 0.07‰. The average $\delta^{13}C$ value of the chemically hydrolyzed standards was −30.64‰ with a standard deviation of 0.29‰. As the values in the urine samples are in agreement with those of the hydrolyzed standards and it is highly

TABLE 2 Linearity results within a range of 0.3–5 V

	300–500 mV	500–1500 mV	1000–5000 mV
Number of measurements	5	9	4
Average (‰)	−30.65	−30.60	−30.64
SD (‰)	0.09	0.06	0.02
Slope (‰/V)	−0.01		

unlikely that three different procedures would lead to the same amount of fractionation, no significant fractionation occurs during the sample preparation procedure and the injection leading to the formation of two isomers. In addition, using linear mixing models, Piper et al.[11] provided evidence that acidic solvolysis does not result in significant fractionation.

The obtained GC chromatograms showed no major interferences in the fraction containing the analyte of interest. In none of the samples, was coelution of EpiAS and an interference observed. In Figure 2B, C, the chromatograms of a dirty urine sample (i.e. high urinary density and high concentrations of endogenous steroids) are presented. As this is a worst-case scenario and the peaks for EpiAS showed no major interferences, the developed sample preparation method was considered fit for purpose.

The $\delta^{13}C$ values of the two batches for the linear mixing model are presented in Figure 3. The point of intersection with the Y-axis is −30.57‰ and the slope of the line of best fit 6.13‰. The value obtained from the injection of 56 EpiAS standards is −30.56‰ with a standard deviation of 0.22‰. Consequently, the bias of the method is −0.01‰. Overall, the standard deviations for the point of intersection of the parameters (0.20‰) and the slope of the line of best fit (0.10‰) are satisfactory. The average scatter of the residuals is 0.00‰ with a standard deviation of 0.23‰.

The standard deviation of EpiAS in the negative quality control urines, prepared with every batch, was 0.45‰. Consequently, the measurement uncertainty, calculated with u_{bias} and $u_{precision}$, was 0.45‰, which is lower than the allowed WADA maximum of 1‰.[6]

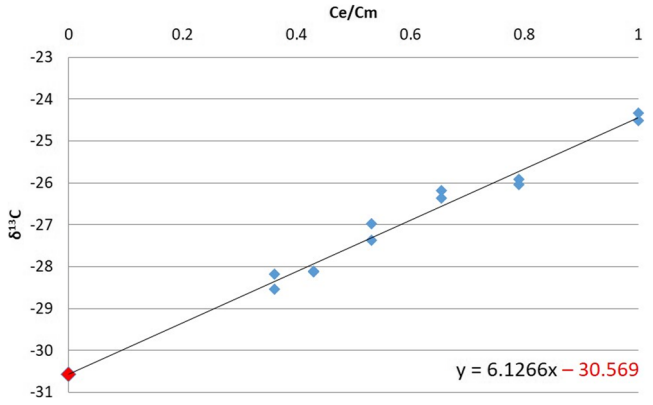

FIGURE 3 Application of the linear mixing model [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | Population study

Samples were subjected to both the conventional testosterone method and the EpiAS method.

The Shapiro–Wilk test of the $\Delta\delta^{13}C$ values of PD-EpiAS and 11-oxo-EpiAS, with a confidence interval for the mean at 95%, resulted in significance levels of 0.391 and 0.886, respectively, meaning that the population values are normally distributed ($P > 0.05$).

Criteria have been established by WADA for the conventional testosterone method. To meet these criteria, the mean $\Delta\delta^{13}C$ + 2SD value for ERC-TC combinations containing A, testosterone, or the androstanediols (Adiols) as the target compounds should be lower than, or equal to, 3‰ and the mean $\Delta\delta^{13}C$ + 2SD value for ERC-TC combinations containing Etio or epitestosterone (E) as TCs should be lower than or equal to 4‰. The standard deviation of all $\Delta\delta^{13}C$ values should be lower than or equal to 1.2‰ for each ERC-TC combination.[6]

The average of absolute EpiAS CIR values in our studied population was −22.78‰ and the average absolute PD CIR value was −21.88‰. The most depleted EpiAS CIR value was −25.40‰ (CIR_{PD}: −24.36‰) and the least depleted was −17.71‰ (CIR_{PD}: −17.27‰). This broad range was expected, as different populations were included in our study.

In Figure 4, the $\Delta\delta^{13}C$ values of PD-EpiAS and 11-oxo-EpiAS are presented together with the absolute CIR values for EpiAS. The average $\Delta\delta^{13}C$ + 3SD value of PD-EpiAS was 3.15‰, meaning that 3‰ would not be sufficient as a population limit. A reference population limit of 3.30‰ will be used. The average $\Delta\delta^{13}C$ + 3SD value of PD-EpiAS was 3.24‰ and 2.94‰ for males and females, respectively.

The mean $\Delta\delta^{13}C$ + 3SD value of 11-oxo-EpiAS was 3.17‰, so a reference value of 3.20‰ will be used as reference population limit. The average $\Delta\delta^{13}C$ + 3SD value of 11-oxo-EpiAS was 3.16‰ and 2.90‰ for males and females, respectively.

3.4 | Administration studies

3.4.1 | DHEA

Figure 5 shows the $\Delta\delta^{13}C$ data of the conventional ERC-TC combination with the longest detection time (PD-T) and PD-EpiAS after the administration of DHEA and the absolute CIR values. The population limits of 3‰ (T) and 3.30‰ (EpiAS) are also indicated. The

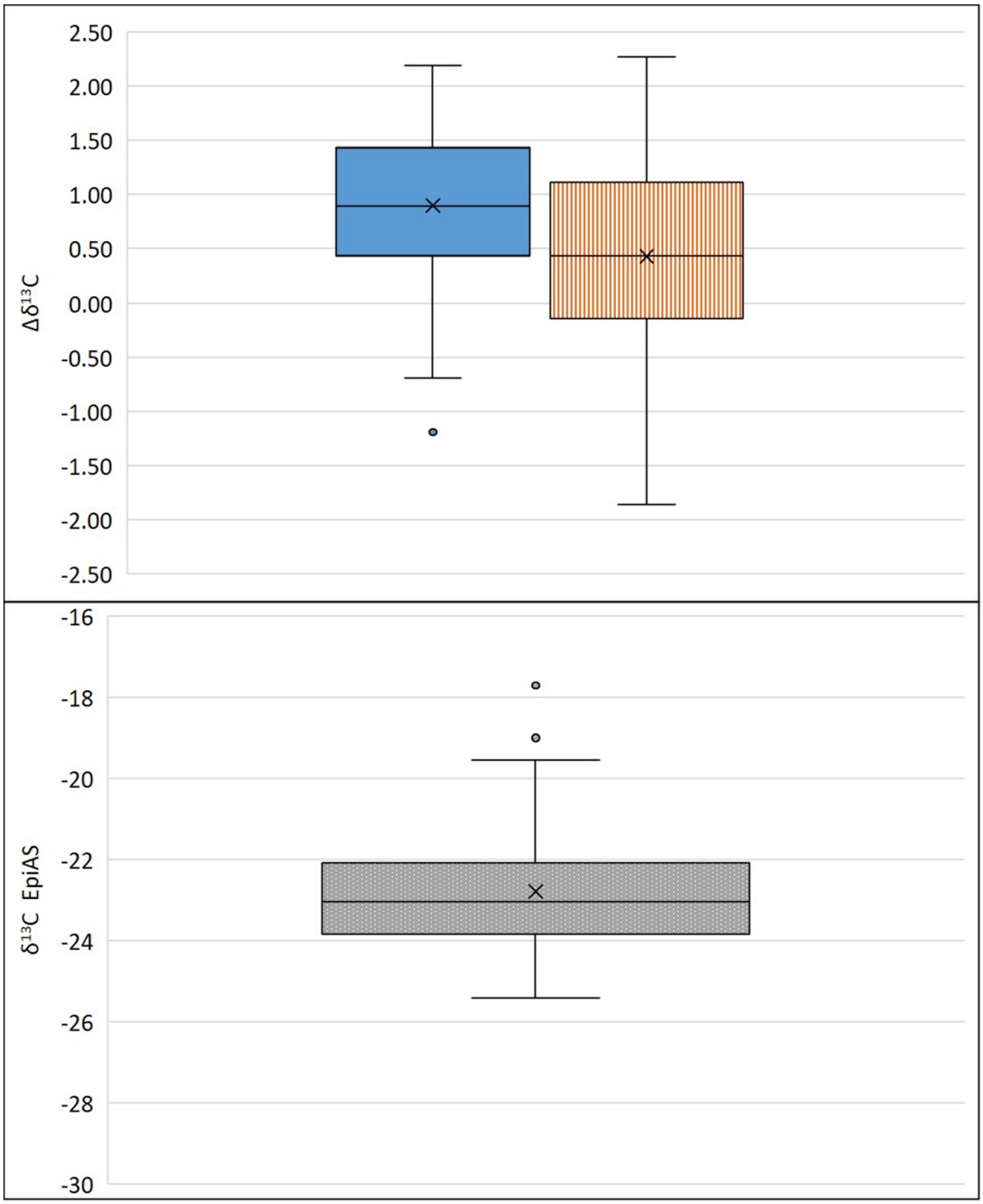

FIGURE 4 Boxplots of Δδ13C values for PD-EpiAS (blue) and 11-oxo-EpiAS (orange, striped) of the 61 samples from the population study. The black, dotted boxplot represents the absolute EpiAS CIR values [Colour figure can be viewed at wileyonlinelibrary.com]

$\Delta\delta^{13}C$ values of PD-Etio, PD-βαβ (5β-androstane-3α,17β-diol), and PD-ααβ (5α-androstane-3α,17β-diol) have values higher than 3‰ up to 36 hours post-administration. The $\Delta\delta^{13}C$ value of PD-T exceeds the threshold until 48 hours post-administration. The $\Delta\delta^{13}C$ value of PD-EpiAS is still higher than the threshold at 52 hours post-administration, meaning that the detection time is prolonged by 4 hours compared with the conventional method, which is a rather small advantage compared with the conventional method.

3.5 | T gel

The $\Delta\delta^{13}C$ values of PD-ααβ, which was the longest detectable conventional TC, PD-T, and PD-EpiAS of the T gel administration study are depicted in Figure 6, as well as the absolute CIR values. PD-T and PD-ααβ are the only ERC-TC combinations that result in $\Delta\delta^{13}C$ values higher than 3‰. For PD-T, the values remain above 3‰ up to 12 hours post-administration. The $\Delta\delta^{13}C$ values of PD-ααβ exceed the limit of 3‰ up to 48 hours post-administration.

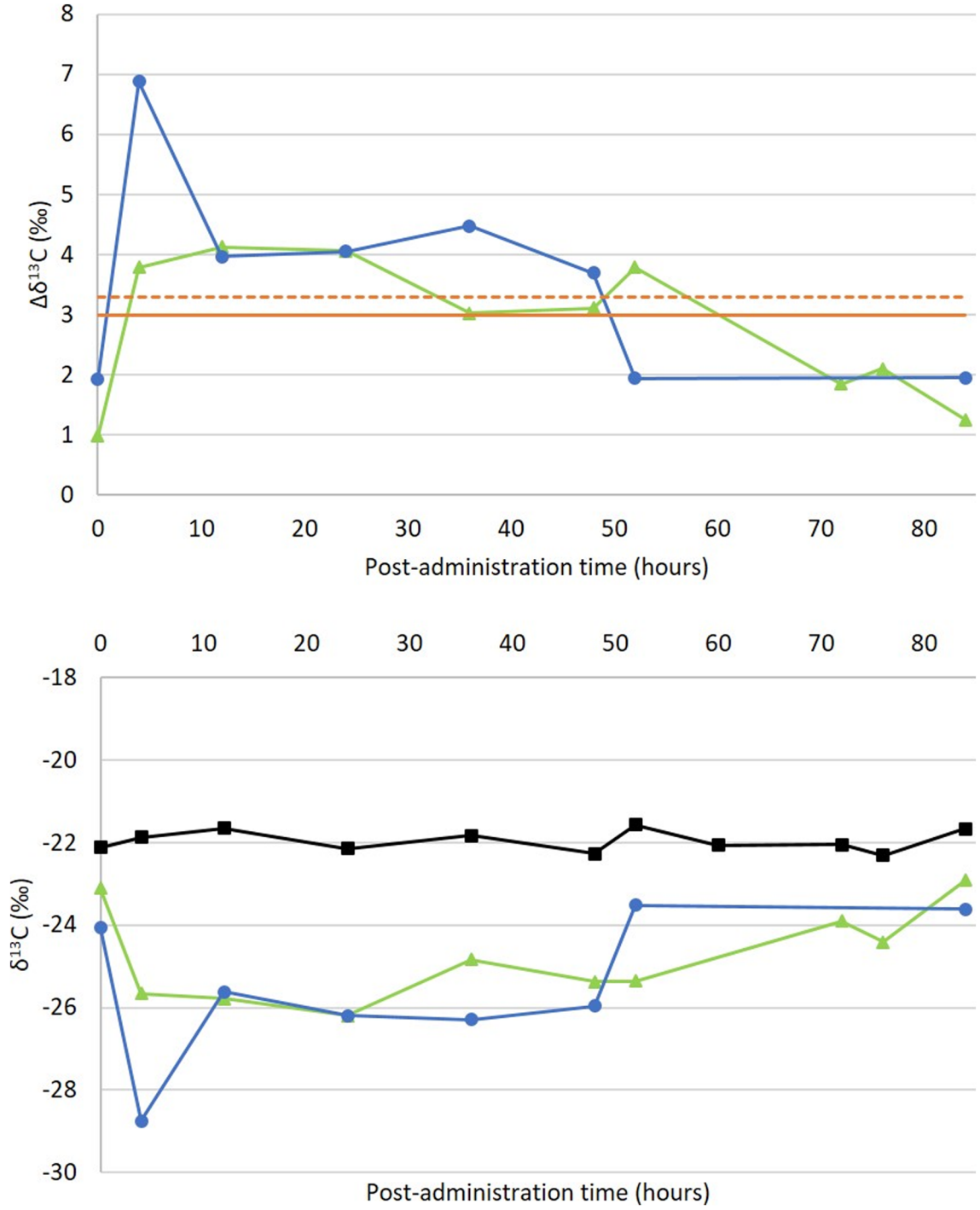

FIGURE 5 Δδ13C results of PD-T (blue dots) and PD-EpiAS (green triangles) and absolute CIR values for testosterone (blue dots), EpiAS (green triangles), and PD (black squares) after the administration of DHEA. The upper graph represents the Δδ13C results. The orange lines represent the thresholds of 3‰ and 3.30‰. The lower graph represents the absolute CIR values [Colour figure can be viewed at wileyonlinelibrary.com]

The EpiAS $\Delta\delta^{13}C$ values remain relatively stable and unaltered. Based on these data, EpiAS seems unsuitable as a marker for detecting a T gel misuse. However, in this study, T gel was only applied once, which is not representative for its normal daily way of application. In addition, due to intra-individual variation, it is possible that for other individuals EpiAS might be a useful marker. For example, in the study of Piper et al.[10] T gel was applied for 7 consecutive days and for volunteer 1, EpiAS prolonged the detection time by 1 day compared with ααβ-diol, which was the longest detectable conventional target compound. For volunteer 2, the detection time was prolonged by 1 day compared with testosterone, which was the longest detectable conventional TC. These results indicated that EpiAS is a good marker for the detection of repeated T gel misuse.

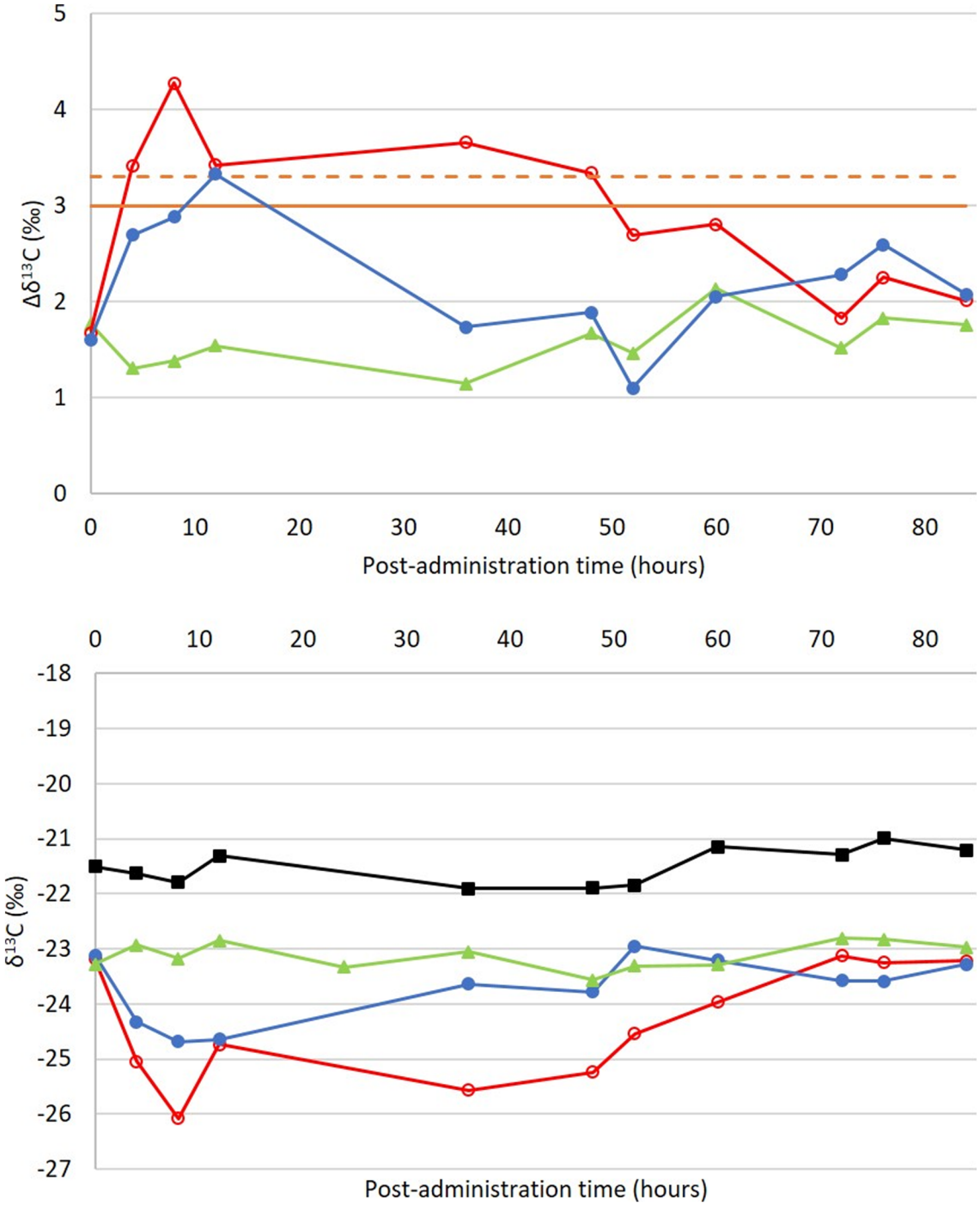

FIGURE 6 Δδ13C data of PD-ααβ (red open circles), PD-T (blue dots), and PD-EpiAS (green triangles) and absolute CIR values for ααβ (red open circles), testosterone (blue dots), EpiaS (green triangles), and PD (black squares) after the administration of T gel. The upper graph represents the Δδ13C results. The orange lines represent the thresholds of 3‰ and 3.30‰. The lower graph represents the absolute CIR values [Colour figure can be viewed at wileyonlinelibrary.com]

3.5.1 | ADION

After the administration of a single dose of ADION, $\Delta\delta^{13}C$ values of PD-T exceed the threshold until 23 hours post-administration. For PD-Etio and PD-ααβ, which were the longest detectable conventional TCs, the $\Delta\delta^{13}C$ values were higher than 3‰ until 36 hours post-administration. PD-βαβ and PD-EpiAS remained above 3‰ until the end of the study (Figure 7). From 12 hours up to the end of the study, the $\Delta\delta^{13}C$ value of PD-EpiAS remains relatively stable (i.e., around 4‰), whereas the $\Delta\delta^{13}C$ value of PD-βαβ decreases from 7.2‰ to 3.7‰. It is likely that EpiAS remains above its population reference limit for a longer period of time than PD-βαβ and, as such, prolongs the detection time. Unfortunately, this could not be verified as 47 hours was the last collection time. A longer detection time was

reported by Piper et al.[10] The CIR of the conventional metabolites returned to baseline values within 24 hours post-administration while the CIR of EpiAS remained depleted up to 130 hours.

3.5.2 | NEBIDO

For the NEBIDO excretion samples, the $\Delta\delta^{13}C$ values of PD-ααβ and PD-T exceeded the reference threshold until the end of the study (i.e. 87 days). PD-Etio and PD-βαβ exceeded the reference threshold until 52 days. PD-A returned to unsuspicious values after 21 days. As visualized in Figure 8, EpiAS was less depleted than ααβ and T and remained above its reference threshold from day 5 until day 73. A final conclusion cannot be drawn based on these data, but it seems that the conventional TCs and EpiAS have the same trend of returning to baseline values and that EpiAS will probably not extend the detection time. However, additional data are needed to confirm this conclusion.

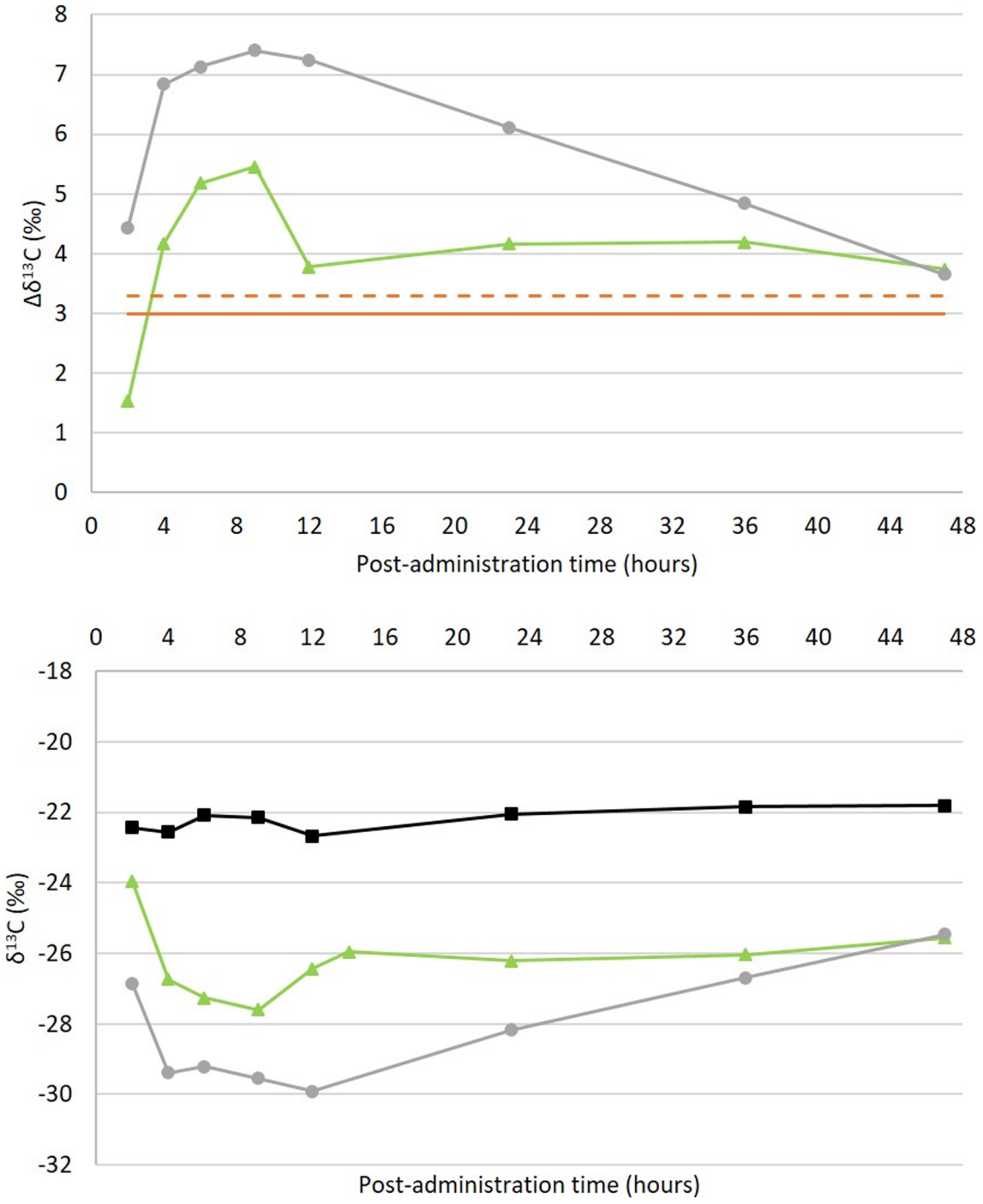

FIGURE 7 Δδ13C results of PD-βαβ (grey dots) and PD-EpiAS (green triangles) and absolute CIR values for βαβ (grey dots), EpiAS (green triangles), and PD (black squares) after the administration of ADION. The upper graph represents the Δδ13C results. The orange lines represent the thresholds of 3‰ and 3.30‰. The lower graph represents the absolute CIR values [Colour figure can be viewed at wileyonlinelibrary.com]

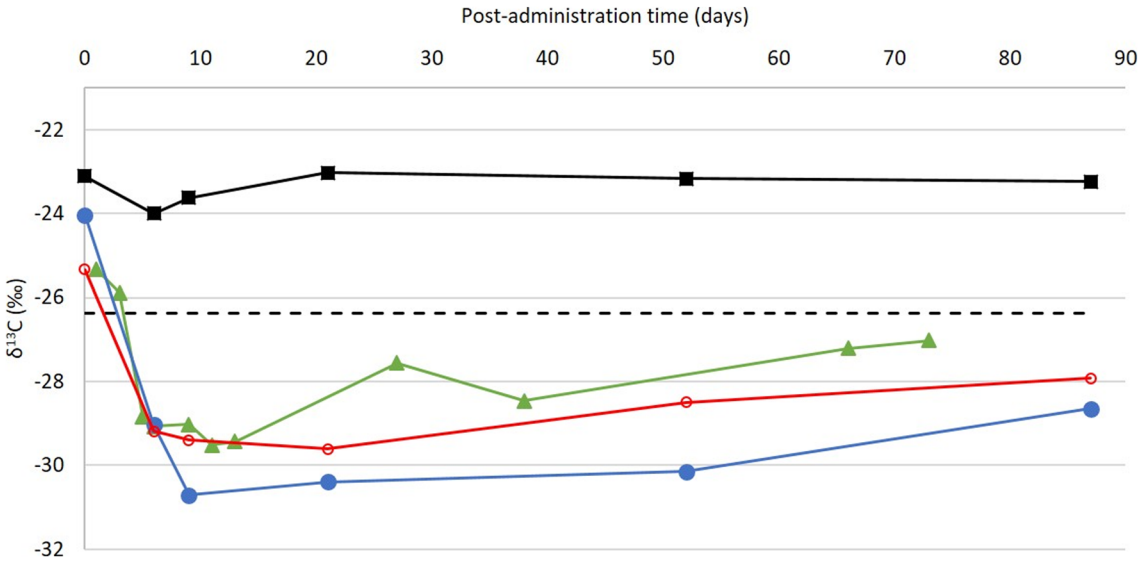

FIGURE 8 δ13C results of PD (black, squares), testosterone (blue circles), ααβ (red, open circles), and EpiAS (green, triangles) after the administration of NEBIDO. The reference limit (−26.36‰) is represented by the dashed line [Colour figure can be viewed at wileyonlinelibrary.com]

4 | CONCLUSION

A fast and easily implementable GC-C-IRMS method for EpiAS was developed and validated. The approach is based on the direct GC analysis of non-hydrolyzed EpiAS, making the solid phase extraction, hydrolysis, and acetylation step redundant. Sample preparation consisted of a simple liquid–liquid extraction, followed by LC fraction collection, which was an appropriate sample preparation as there were no interferences observed.

$\Delta\delta^{13}C$ population reference limits of 3.30‰ and 3.20‰ were established for PD-EpiAS and 11-oxo-EpiAS, respectively. The conducted administration studies confirm, to a certain extent, previous studies and show the usefulness of EpiAS as an extra TC to prolong the IRMS detection times. After administration, EpiAS $\delta^{13}C$ values depleted more slowly in comparison with the conventional TCs, but also remained depleted for a longer time period, leading to longer detection times. However, in the current study, this was less pronounced than in previous studies. Slightly longer detection times were assessed for DHEA and most likely for ADION as well. EpiAS did not result in longer detection times for a single administration of T gel. For NEBIDO, it is most likely that EpiAS will not extend the detection time compared with the conventional TCs but additional data are needed to confirm this statement. As such, the collected data do suggest that more administration studies are required to map comprehensively the advantages of EpiAS as TC in comparison with the conventional TCs.

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