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Sensitive detection of testosterone and testosterone prohormone administrations based on urinary concentrations and carbon isotope ratios of androsterone and etiocholanolone

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Accepted Article

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Abstract

The testing strategy for the detection of testosterone (T) or T-prohormones is based on the longitudinal evaluation of urinary steroid concentrations accompanied by subsequent isotope ratio mass spectrometry (IRMS)-based confirmation of samples showing atypical concentrations or concentration ratios. In recent years, the IRMS methodology focussed more and more on T itself and on the metabolites of T, 5α- and 5β-androstanediol. These target analytes showed the best sensitivity and retrospectivity, but their use has occasionally been challenging due to their comparably low urinary concentrations. Conversely, the carbon isotope ratios (CIR) of the main urinary metabolites of T, androsterone (A) and etiocholanolone (EITO), can readily be measured even from low urine volumes; those however commonly offer a lower sensitivity and shorter retrospectivity in uncovering T misuse.

Within this study, the CIRs of A and ETIO were combined with their urinary concentrations, resulting in a single parameter referred to as 'difference from weighted mean' (DWM). Both glucuronidated and sulphated steroids were investigated, encompassing a reference population (n = 108), longitudinal studies on 3 individuals, influence of ethanol in 2 individuals, and re-analysis of several administration studies including T, dihydrotestosterone, androstenedione, epiandrosterone, dehydroepiandrosterone, and T-gel. Especially DWM calculated for the sulphoconjugated steroids significantly prolonged the detection time of steroid hormone administrations when individual reference ranges were applied.

Administration studies employing T encompassing CIR common for Europe (-23.8 ‰ and -24.4 ‰) were investigated and, even though for a significantly shorter time period and less pronounced, DWM could demonstrate the exogenous source of T metabolites.

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Keywords

Isotope ratio mass spectrometry, testosterone, doping, endogenous carbon isotope ratios, steroid concentrations

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Introduction

The application of testosterone (T) and T-prohormones is forbidden in sports according to the List of Prohibited Substances issued by the World Anti-Doping Agency (WADA).[1] In order to detect potential misuse, urinary concentrations of T, epitestosterone (E), and T-metabolites are determined in each doping control urine sample. Both urinary concentrations and concentration ratios such as T/E are monitored on a longitudinal basis and combined to each individual athlete´s steroid profile.[2] If an atypical profile is detected, isotope ratio mass spectrometry (IRMS)-based confirmatory analyses are triggered to differentiate between naturally elevated urinary concentrations and a potential anti-doping rule violation (ADRV). Employing IRMS, the carbon isotope ratios (CIR) of T and its metabolites are determined and compared to endogenous reference compounds (ERC). If the difference between the ERC and the target analytes (TC) exceed relevant thresholds, an ADRV is substantiated.[3] CIR values are given in [‰] or [mUr] with reference to the primary isotopic reference material Vienna Pee Dee Belemnite (VPDB) based on the equation 1:[4]

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

The differences between TC and ERC are given as Δ-values:

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

Besides T itself, its minor metabolites 5α-androstanediol (5a) and 5β-androstanediol (5b) are commonly employed as TC due to their increased sensitivity and retrospectivity compared to the major metabolites androsterone (A) and etiocholanolone (ETIO).[3,5,6] Unfortunately, the urinary concentrations especially for T and 5a are generally low and can fall below the limit of detection of the IRMS method in female athletes, athletes comprising the UGT2B17 *del/del* polymorphism, or athletes providing diluted urine samples.[7-11] As the CIR determination of 5b alone as TC does not suffice as decisive criterion [2], ETIO and A may become relevant as TC despite their overall inferior utility to detect steroid administrations. Improving the sensitivity and retrospectivity of A and ETIO would therefore be beneficial to IRMS-based anti-doping testing strategies in general.

A first attempt to improve the application of ETIO and A in sports drug testing was demonstrated by switching towards longitudinal CIR profiles of athletes.[12] Within this study,

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the individual CIR were found stable over a prolonged time period, enabling the calculation of individual reference limits for both the δ- and Δ-values resulting in slightly improved detection capabilities for ETIO and A. Regarding the application of δ-values, changes in dietary habits may however complicate the application of this parameter in doping controls.[13,14]

The investigations presented herein are based on a combination of the urinary concentrations and CIR of ETIO and A for both glucuronidated (GLUC) and sulfated (SULF) phase-II-metabolites, waiving the use of any ERC. Fluctuations in absolute δ-values will therefore not show any impact on the results. Combining urinary concentrations and CIR mathematically to a single score referred to as 'difference from the weighted mean' (DWM) for GLUC and SULF separately, allows for a straightforward estimation if a sample is considered suspicious or not. For both the concentrations and the CIR (and the resulting DWM) it is expected that physiological ranges exist, which can be assumed to be unsuspicious; as soon as values fall outside these ranges, an application of T or T-prohormones is conceivable.[15] In a preliminary study it could be demonstrated that a significant impact was measurable, which was taken as a basis for the follow-up investigations presented here.[16]

The physiological ranges for DWM in GLUC and SULF have been established by the investigation of a reference population. In order to further improve the approach, individual ranges have also been implemented and investigated regarding their stability over time and possible confounding factors like ethanol intake, which constitutes one of the main confounders to the urinary steroid profile.[17,18] Numerous excretion studies have been investigated to evaluate both the reference based and the individual based ranges encompassing single oral administrations of T, 4-androstenedione (4EN), epiandrosterone (EpiA), dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), and multiple administrations of testosterone gel (T-Gel). As in principle this approach does not rely on absolute δ-values but only on differences between ETIO and A, administrations studies carried out with T showing endogenous CIR (when compared to CIR of European individuals) signatures of -23.8 ‰ and -24.4 ‰ were also investigated.[19]

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Experimental

Chemicals and steroids

All solvents and reagents were of analytical grade. Glacial acetic acid, sodium hydroxide (NaOH), methanol (MeOH), sulfuric acid (H_2SO_4), acetonitrile (ACN), *tert*-butyl methyl ether (TBME), ethyl acetate (EtOAc), ethanethiol, ammonium iodide (NH_4I), and cyclohexane were purchased from Merck (Darmstadt, Germany). *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Chemische Fabrik Karl Bucher (Waldstetten, Germany). Pyridine was from Sigma Aldrich (Steinheim, Germany), and acetic anhydride was a blend of reagents purchased from Sigma Aldrich and Merck and vacuum distilled in-house before use. Solid phase extraction (SPE) Chromabond® C18 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). 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).[20] Helium (purity 5.0) and oxygen (purity 5.0) were also from Linde.

Steroid reference material ETIO, A, T, DHEA, EpiA, DHT, PD and 4EN was supplied by Sigma Aldrich and 3β-hydroxy-5α-androstane (RSTD) by Steraloids (Newport, RI, USA). Deuterated internal standards for quantification, namely D4-androsterone_SULF, D4-androsterone_GLUC and D5-etiocholanolone were from the National Measurement Institute (Canberra, Australia), and T-preparations showing an endogenous CIR were a generous gift from the Seibersdorf Labor GmbH Doping Control Laboratory (Seibersdorf, Austria).[19]

Urinary steroid concentrations

Urinary concentrations of ETIO_GLUC, A_GLUC, and PD_GLUC were derived from the accredited routine doping control method of the Institute of Biochemistry at the German Sport University Cologne, employing deuterated internal standards and external calibration. [17,21] In order to determine the concentrations of ETIO_SULF, A_SULF, and DHEA_SULF, the

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aqueous residue of each sample was further processed including solid-phase extraction and acidic solvolysis.[22] The de-conjugation step was monitored by D4-androsterone_SULF, and for quantification the same external calibration curves as for the glucuronides were employed.

Sample preparation for CIR determinations

All samples were prepared following established protocols with a starting urine volume of 2 to 10 mL, using ≤ 5 mL for the majority of samples.[5,22-24] In brief, samples were applied on preconditioned SPE cartridges, washed with water, and eluted with MeOH. After adding 2 mL of aqueous phosphate buffer, the unconjugated steroids were extracted with 5 mL of TBME and discarded before glucuro-conjugated steroids were enzymatically hydrolysed employing β-glucuronidase. The liberated steroids were extracted with 5 mL of TBME, evaporated to dryness and transferred into autosampler-vials for further clean-up. The aqueous residue was acidulated with glacial acetic acid and applied to another preconditioned SPE, washed with water, and eluted with MeOH. After taking to dryness, the sulfoconjugated steroids were cleaved by adding EtOAc, MeOH and H_2SO_4. After adding methanolic NaOH, the samples were dried and reconstituted in 2 mL of water. Finally, the formerly sulphated steroids were extracted with TBME, dried and transferred into autosampler-vials.

High performance liquid chromatography sample clean-up

All samples were further purified on an Agilent 1100 HPLC system (Waldbronn, Germany) equipped with a XBridge™ Shield RP18 5 mm (4.6 x 250 mm) column purchased from Waters (Eschborn, Germany). Eluents were pure ACN and water, the gradient started from 50 % ACN to 65 % in 10 min, then to 98 % in 1 min, hold for 9 min followed by re-equilibration at 50 % ACN for 5 min. The column flow was set to 1 mL/min. Samples were reconstituted in 100 µL of ACN/water (50/50, v/v) and the injection volume was 100 µL. Fractions were collected using an automated fraction collector FOXY R1 (Axel Semrau, Sprockhövel, Germany). Typical fraction collection times were from 8.91 to 10.10 min for ETIO, from 10.11 to 11.50 min for A, and from 11.51 to 12.70 min for PD employed for the formerly glucuronidated steroids and from 7.90 to 8.90 min for DHEA and similar collection times for ETIO and A for

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the formerly sulfoconjugated steroids. All fractions were evaporated to dryness and acetylated by adding 75 µl of pyridine and 75 µL of acetic anhydride followed by 45 min at 70 °C in a heating block. Then samples were again evaporated and reconstituted in appropriate amounts of cyclohexane and transferred to autosampler-vials to be analysed on the IRMS system.

Gas chromatography-combustion-isotope ratio mass spectrometry set up

The gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) system employed consisted of a Delta V Plus isotope ratio mass spectrometer coupled to a Trace GC 1310 equipped with a TriPlus RSH Autosampler (ThermoFisher, Bremen, Germany). Both systems were coupled via the GC IsoLink CNH combustion unit operated at 950°C and the ConFlo IV interface (ThermoFisher). Isodat 3.0 (ThermoFisher) was used for data acquisition and evaluation. To ensure specificity and peak purity, a Thermo ISQ single quadrupole mass spectrometer 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 Thermo Xcalibur (version 2.2).
The GC column was an Agilent J&W Scientific DB-17MS (length 30 m, i.d. 0.25 mm, film thickness 0.25 µm). The initial oven temperature was 100°C, held for 2 min and increased with 40°C/min to 273°C, then with 2°C/min to 301°C, then with 40°C/min to 320°C and finally held for 2 min. Carrier gas was He (purity grade 5.0) with a programmable flow starting with 2.5 mL/min during injection and then with 1.5 mL/min during the analytical run. Injections were performed using the internal standard double method with 1 µL of a cyclohexane solution containing 40 µg/mL RSTD and 2 to 4 µL of sample reconstituted in cyclohexane.

Calculation of the difference from the weighted mean

While several factors have been identified that may have an impact on the CIR of urinary steroids like the individual´s diet or the use of contraceptives, no correlation has been found between the urinary concentration of any steroid and its CIR.[5,6,25-27] But interestingly, a significant correlation was established between the urinary concentrations of ETIO_GLUC and A_GLUC summarized in the fractional yield and related CIR.[16] While this sophisticated

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approach was aiming towards the calculation of metabolic fluxes, a simplified approach was chosen here to establish empirically derived correlations between concentrations and CIR and to evaluate their usefulness for sports drug testing.

The mass fraction of ETIO and A was calculated using equation 3 and 4:

(Eq 3) $$f(ETIO) = \frac{conc(ETIO)}{conc(ETIO)+conc(A)}$$

(Eq 4) $$f(A) = 1 - f(ETIO)$$

The weighted mean (WM) then took into account the individual CIR:

(Eq 5) $$WM = CIR(ETIO) * f(ETIO) + CIR(A) * f(A)$$

This enabled to calculate the DWM for ETIO following equation 6:

(Eq 6) $$DWM(ETIO) = WM - CIR(ETIO)$$

These calculations were performed separately for both the GLUC and SULF steroids enabling to combine four different parameters into one meaningful score. The calculated DWM(ETIO)_GLUC and _SULF has been investigated taking into consideration different aspects like intra-individual stability over time, influence of ethanol intake and inter-individual variation by means of a reference population.

Intra-day reproducibility

A pooled blank urine sample provided by one male volunteer was processed with each batch prepared for the reference population over a time period of 3 months resulting in 10 individual preparations. Based on these measurements, the analytical error of the approach for DWM(ETIO) was evaluated.

Intra-individual stability of DWM(ETIO) over time

In order to evaluate the short- and mid-term stability of individual DWM(ETIO) values, urine samples collected during a clomiphene micro-dose administration trial have been re-investigated.[28] The volunteers provided 4 samples collected on the same day at different time spots and additional 4 morning urines collected over a period of 3 weeks. All samples were stored frozen until preparation and evaluated regarding the inter- and intra-day repeatability of

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individual DWM(ETIO) values. The steroid profiles of all volunteers did not show any response to the microdose of the administered clomiphene.

Investigated reference populations

In order to estimate the naturally occurring distribution of DWM(ETIO), a reference population encompassing n = 110 volunteers (55 females and 55 males) was investigated concerning both their GLUC and SULF steroids. The samples were collected from employees and students at the German Sport University Cologne and stored frozen at -20°C until analysis. The GLUC values obtained here were compared to the results obtained on a formerly investigated reference population encompassing n = 67 males and females.[24] The DWM(ETIO)_SULF were compared to another population investigated already in 2008.[22] All volunteers gave written consent and the sample collection was approved by the local ethics committee of the German Sport University Cologne.

Possible influence of ethanol intake

As the impact of ethanol on the steroid profile is well known, its impact on the DWM(ETIO) values was investigated exemplarily in 2 volunteers. One female and one male participant provided urine samples prior, during the intake of ethanol in the evening, and on the next morning. Samples were investigated regarding their steroid profile and the DWM(ETIO) values. Samples were stored frozen until preparation.

Excretion studies under investigation

Numerous excretion studies already available at the Cologne anti-doping laboratory were re-investigated. Regarding the administration of T encompassing an endogenous CIR, additional trials were conducted with the support of the local ethics committee at the German Sport University Cologne.

Oral application of testosterone

The first administration study under investigation employed samples collected after the administration of 100 mg of T-undecanoate ($\delta^{13}C_{VPDB}$ = -27.0 ± 0.32 ‰) to one healthy male

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volunteer.[23,24] Here only the CIR values and urinary concentrations of the GLUC already determined in 2012 were re-evaluated. In order to complement the findings, urine samples derived from an additional excretion study after the oral application of 40 mg of T ($\delta^{13}C_{VPDB}$ = -29.7 ± 0.39 ‰) were re-prepared and investigated on both the GLUC and SULF steroids.[29] Ethical approval was obtained by the ethics committee of the canton Vaud (Protocol 155/11) and Swissmedic (Ref. No. 2011DR3149) and ethics committee of the German Sport University Cologne (#27112014) and written informed consent was obtained from both participants

Topical application of testosterone gel

One male volunteer applied 100 mg of T-gel (two bags of Testogel® containing 50 mg of non-esterified T each, $\delta^{13}C_{VPDB}$ = -27.3 ± 0.30 ‰) on 5 consecutive days and collected samples prior, during and until 3 days after the last administration.[30] Samples were stored frozen until reprocessing in order to obtain values on both DWM(ETIO) for GLUC and SULF. The volunteer gave written consent and the study was approved by the local ethics committee of the German Sport University Cologne (ethical_approval_130404).

Oral application of 4-androstenedione

One male volunteer applied 80 mg of 4EN ($\delta^{13}C_{VPDB}$ = -31.8 ± 0.33 ‰) orally and collected samples for up to 12 days after the administration.[31] Samples were stored frozen until reprocessing in order to obtain values on both DWM(ETIO) for GLUC and SULF. The participant provided written informed consent and study approval was obtained from the Ethical Committee of the National Institute of Sports of Romania (Ethical approval number 124).

Oral application of dihydroepiandrosterone

After the application of 100 mg DHEA ($\delta^{13}C_{VPDB}$ = -29.7 ± 0.14 ‰) to one male volunteer, urine samples were collected for 66 h.[22] As this study was focusing on steroid SULF, both DWM(ETIO)_GLUC and SULF could be re-evaluated based on the already collected data.

Oral application of dihydrotestosterone

One male volunteer administered 50 mg of DHT ($\delta^{13}C_{VPDB}$ = -28.9 ± 0.14 ‰) and collected urine specimen for 7 days.[31] Here, only samples from the first 5 days were re-evaluated considering only the CIR values and urinary concentrations of the GLUC. The participant

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provided written informed consent and study approval was obtained from the Ethical Committee of the National Institute of Sports of Romania (Ethical approval number 124).

Oral application of epiandrosterone

One male volunteer administered one capsule of a dietary supplement (ANDROVAR, Hardrock Supplements, USA) containing 100 mg of EpiA ($\delta^{13}C_{VPDB}$ = -30.2 ± 0.12 ‰) and collected samples for up to 15 days.[31] Only samples from the first 5 days were re-evaluated considering only the CIR values and urinary concentrations of the GLUC. Unfortunately, the urinary concentrations of SULF were not determined which impeded the calculation of DWM(ETIO)_SULF. The participant provided written informed consent and study approval was obtained from the Ethical Committee of the National Institute of Sports of Romania (Ethical approval number 124).

Oral application of testosterone exhibiting endogenous carbon isotope ratios

Three different administration trials were conducted by the same healthy male volunteer (46 years, 180 cm, 85 kg) with wash out periods of two weeks in-between. The first study comprised a single oral administration of 40 mg of T-propionate ($\delta^{13}C_{VPDB}$ = -23.8 ± 0.13 ‰) and urine sample collection for 4 days. In a second trial, 40 mg of T-enanthate ($\delta^{13}C_{VPDB}$ = -24.4 ± 0.21 ‰) were administered and urine specimen were collected for 7 days. As these single applications of T most probably do not reflect a real doping scenario, a third administration study was conducted employing eight oral administrations of 50 mg of the above mentioned T-propionate on eight consecutive days. Three samples were collected prior the first administration, than 3 samples each day during the administration phase (one in the morning, one in the afternoon and one before sleep) and for the first 2 days after the last administration al samples were collected, then 4 samples per day for the next 2 days and finally each morning urine until day 11 after the last administration. All samples collected during the different administration trials were stored frozen until preparation. The volunteer gave written consent and the study was approved by the local ethics committee at the German Sport University Cologne (ethical_approval_130404).

Case study on DWM(ETIO)

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The case of a male powerlifter, which will be presented as an example here, was one of the main triggers for the investigations in DWM(ETIO). The athlete was tested for more than 90 times during a period of 5 years, presenting a T/E ratio of ca. 0.5. Several samples however yielded a significantly elevated T/E ratio between 1.2 and 3.4, all of which were collected at out-of-competition occasions. All of these samples were classified as suspicious, and further investigations demonstrated the presence of ethanol glucuronide (EtG) between 50 to 190 µg/mL. Several samples were subjected to IRMS analysis and were found to fall well within WADA-established criteria for negative samples. The coincidence of significantly elevated EtG findings and elevated T/Es together with the absence of traces of EtG in all other samples from the athlete raised the suspicion that EtG was administered as a masking agent together with T encompassing an endogenous CIR.

Statistical analysis

The data obtained on the reference populations was tested for Gaussian distribution employing Shapiro-Wilks test using R.[32] Population based reference limits were calculated by adding the threefold standard deviation (SD) to the mean value following the recommendation of the International Federation of Clinical Chemistry.[33] Individual thresholds employed during the evaluation of administration trials were calculated by adding the threefold estimated measurement uncertainty for DWM(ETIO) to the mean value of the pre-administration samples. For differences between inter- and intra-day values was tested by Student´s t-test ($p < 0.05$) applying Bonferroni correction if applicable.

Results and discussion

Intra-day reproducibility

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The results of blank urine reference population study samples are listed in Table 1. From the fraction of glucuronidated steroids, PD was also included besides ETIO and A, and from the fraction of sulfoconjugated analytes, DHEA was additionally considered. Both may serve as regular ERC. The SD found for all CIR under the tested repeatability conditions was in the expected range < 0.4 ‰ for all steroids under investigation. The combined DWM(ETIO) values were found to be more stable with SDs < 0.1 ‰. This decreased variability may be due to the fact that CIR are somehow correlated, i.e. small drifts of the IRMS will be reflected by both ETIO and A and will therefore not increase the SD.

Intra-individual stability of DWM over time

Only a few studies have investigated the intra-individual stability of CIR over time, mainly focusing on possible impact of dietary changes.[5,34,35] The only study focusing on uninfluenced stability over time presented longitudinal IRMS data on 3 different individuals and demonstrated stable CIR values over the tested period of approximately 2 years.[12] Unfortunately, SDs are not explicitly given but can be estimated to be around 0.4 ‰ as derived from the presented graphs.

The DWM(ETIO) results obtained in this study are listed in Table 2. For both intra-day (samples 1-4) and inter-day (samples 5-8) analyses, the values were found stable and not significantly different. The combined SDs were < 0.1‰ for the GLUC and, again slightly higher, but well below 0.2 ‰ for the SULF. As these samples were derived from a clomiphene microdosing study, the individual steroid profiles were carefully checked for any influence attributable to the administration, but no effect was noted. Extended time frames including samples collected over years should be investigated in order to estimate the long-term stability with more confidence and to elucidate possible confounding factors to DWM(ETIO). But the preliminary results obtained here were considered sufficient to apply a longitudinal approach to the investigated administration trials presented later on.

Investigated reference populations

Despite the high intra-individual stability found, the inter-individual variation for DWM(ETIO) was pronounced. Within the reference population encompassing n = 110

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individuals for both GLUC and SULF, a broad and Gaussian shaped distribution of values was established as shown in Figure 1. Mean values and SDs were comparable as listed in Table 3. Population-based upper and lower reference limits were calculated in parallel to CIR and are also listed in Table 3. The lower number (n = 95) of individual values available for calculating the limits for DWM(ETIO)_SULF was mainly due to a HPLC failure (11 samples) and to incomplete acidic solvolysis reactions during concentrations determinations (4 samples), resulting in disregarding a total of 15 specimens.

An interesting finding was the highly significant correlation found between DWM(ETIO)_SULF and _GLUC. Usually, urinary concentrations and CIR of steroids are not correlated at all and this was corroborated by the reference population investigated here.[5,14] The absolute $\delta^{13}C$-values of steroids excreted as GLUC or SULF are of course well correlated, but as soon as Δ-values are investigated, the correlation cancels out.[22,24,36] Urinary concentrations of GLUC and SULF are also not correlated but the amount of steroid excreted either as the one or the other phase-II-metabolite seems to be a highly individual and usually stable parameter over time. So the found correlation here seems be attributable to a stable enzymatically derived isotopic fractionation between ETIO and A, regardless of the phase-II-metabolism and the intermediate steroids synthesized within the body from cholesterol as the starting point towards ETIO and A as end products. Even the relatively stable offset between steroids excreted as GLUC and as SULF (with the SULF being the more enriched ones), does not seem to have any impact on the individuals difference between both steroids.[36]

Even if it may be advisable to investigate a larger number of individuals prior to establish reference limits, investigations in CIR demonstrated that population-derived thresholds of smaller populations are usually well reflected by larger populations.[14,27] Therefore it was found suitable to apply these preliminary limits during the evaluation of the administration trials under investigation. This was additional corroborated by a comparison of the results obtained on this population to a reference population already investigated in 2011.[23,24] With the mean value for DWM(ETIO)_GLUC obtained at 0.53 ‰ and a SD of 0.28 ‰ the numbers were perfectly in agreement compared to the results in Table 3. Regarding DWM(ETIO)_SULF, another population investigated in 2008 was considered and, with values of 0.69 ‰ ± 0.37, again a good agreement was noted. As only the samples of the new reference population investigated here were prepared with the IRMS method applied to other experiments, only these data was used to calculate the reference limits as presented in Table 3.

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Possible influence of ethanol intake

No measureable impact on either DWM(ETIO)_GLUC or _SULF was noted after the administration of ethanol. Within the female volunteer, a slight increase in the T/E ratio from < 2 to > 3 was detectable, but no changes in the urinary concentrations of ETIO or A or in CIR were ascertained. Considering again the preliminary character of these observations, the intake of ethanol does not seem to be a strong confounding factor for DWM(ETIO).

Excretion studies under investigation

The administration trials presented here were not only investigated in order to elucidate the potential of DWM(ETIO) for sports drug testing but additionally to understand and retrace the impact of different applications on this new diagnostic item.

Oral administration of testosterone

Two different trials with orally administered T exhibiting exogenous CIR were considered. During the first experiment, urine sample were collected at short intervals resulting in a good temporal resolution over a time period of 92 hours. Results are shown in Figure 2. Directly after the administration, both the CIR and DWM(ETIO)_GLUC respond instantaneously. As expected, the CIR of both ETIO and A are strongly depleted but do not reach the δ^{13}C-value of the administered T at -27 ‰ due to endogenous dilution (Figure 2, part B). The DWM(ETIO)_GLUC values show a time-dependent swing starting with decreased values directly and up to 10 h after the administration, followed by increased values between 20 and 35 h and again decreased values beyond 60 h after application. (Figure 2, part A). This behavior can be explained by the underlying metabolism of the exogenous T. A closer look at the lower part of Figure 2 reveals that ETIO and A are impacted in a slightly different way by the exogenous T. Directly after the application, A tends to result in more depleted values compared to ETIO which results in an un-physiological approximation of both, i.e. the Δ-value of ETIO-A is minimal as also shown in Figure 2 (lower part). Approximately 20 h after administration, this effect is turned around with ETIO showing more depleted values compared to A. This might be due to a larger proportion of ETIO derived from the exogenous T in this time frame. It would also be explainable by a slower turn-over of the ETIO_GLUC pool being more depleted over a longer time interval. Based on the presented data these are only potential

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explanations and other factors influencing the steroid metabolism like any kinetic isotope effects cannot be ruled out. Finally, after a short period where an apparent equilibrium was again reached, the CIR of A were apparently slightly too depleted again resulting in diminished DWM(ETIO)_GLUC values until the end of the study. A comparable, albeit less pronounced, trend is visible in the urinary concentrations. Before the T application, a higher concentration of ETIO compared to A is found in urine resulting in a ratio of ETIO/A of 1.3. Directly after the T application, more A than ETIO is excreted (the ETIO/A ratio drops to 0.7) followed by a phase of increased ETIO concentrations (the ETIO/A ratio increases to 1.8) and back to starting values after 35 h. The combination of both effects on CIR and urinary concentrations results in the amplitude of DWM(ETIO)_GLUC.

From the physiological point of view, another explanatory approach is conceivable: Without the administration of an exogenous steroid, the human steroid metabolism is in an equilibrium that only shows minor deviations due to, for example, diurnal variations in steroid production. As soon as this equilibrium is disturbed by an administration of an exogenous steroid, the DWM(ETIO) value will be impacted and may even reach un-physiological states. Depending on the preferred metabolism of the exogenous steroid (either more in the direction of 5α-steroids like A or more towards 5β-steroids like ETIO), DWM(ETIO) will decrease or increase, respectively. That the physiological state may be disturbed for a significant time period was demonstrated by the second administration trail depicted in Figure 3. For DWM(ETIO)_GLUC again the swing within the first day after administration is noted, and values remain influenced for up to 120 h after administration. Evaluation of the data in a longitudinal manner based on the estimated measurement uncertainty and intra-individual variation of the data resulting in adding ± 0.3 ‰ to the individual mean value demonstrated only 2 samples within the first 20 h to be outside the individual limits. Population-based limits are by far not exceeded. The DWM(ETIO)_SULF values instead are significantly decreased from 20 h after application on until the end of the study 210 h after administration. In-between, the measured values were even found below the population-based threshold of -0.41 ‰ for approximately 1 day. These are very promising results for sports drug testing taking into consideration that they were based on a single oral administration of only 40 mg T. Considering the currently applied WADA criteria, samples would have been declared negative after approx. 24h.

Topical application of testosterone gel

The preferred metabolic conversion of T-Gel towards 5α-steroids was also observed for DWM(ETIO) as shown in Figure 4.[5] During the application phase, DWM(ETIO)_GLUC

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shows a constant and significant decrease as the influence of the exogenous T is stronger on A compared to ETIO. Already 25 h after the last application, the value for DWM(ETIO)_GLUC is found to be back to starting values and individual thresholds are only exceeded for 14 h. For the steroids excreted as sulfo-conjugates, the picture is completely different. DWM(ETIO)_SULF shows a very slow and moderate decrease during the application phase and exceeds the individual threshold only after the five-fold administration of T-Gel. Then the values are decreased until the end of the study after 105 h. All results obtained fall within the population-based thresholds and only long-term investigations can prove the administration here. Applying the current WADA criteria would result in adverse analytical finding for 18 hours after the last administration.

Oral application of 4-androstenedione

A different and unexpected picture was obtained after the single oral administration of 80 mg of 4EN. As depicted in Figure 5, the impact on DWM(ETIO)_GLUC seems to be negligible. Only some minor fluctuation was noted and the individual reference ranges were hardly exceeded. This may be explainable by the metabolism of 4EN which neither seems to prefer the 5α- nor the 5β-pathway after oral administration leading to strongly increased urinary concentrations of both A and ETIO.[37,38] The CIR are influenced in parallel and the resulting effect on DWM(ETIO)_GLUC becomes very small. In contrast, the steroids excreted sulfo-conjugated are apparently influenced significantly differently with a much higher impact on A_SULF. After a short increase of DWM(ETIO)_SULF directly after the administration, reflecting a short increase in urinary concentrations of ETIO_SULF, the measured values drop below the individual threshold for more than 200 h after application. Even the population-based limits are exceeded between 58 and 140 h after administration compared to a detection window of up to 26 h if current WADA criteria are applied. To the best of our knowledge, this strong and prolonged effect on urinary A_SULF has not been described for the 4EN metabolism so far, which may be simply due to the fact that urinary sulfo-conjugated steroids in general have not been investigated as extensive as glucurono-conjugated metabolites. Further investigations including different volunteers will be necessary to elucidate the described impact of 4EN administrations unambiguously.

Oral application of dehydroepiandrosterone

Switching from 4-ene-steroids to the 5-ene-steroid DHEA resulted again in a different pattern of DWM(ETIO) response after administration as demonstrated in Figure 6. As expected, a

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stronger impact on the 5β-steroid ETIO in comparison to A was noted, which resulted in an increase of DWM(ETIO).[39] Regarding DWM(ETIO)_GLUC, this impact was not very pronounced, which was also reflected by the concentrations of both urinary steroids: A increased nearly as significantly as ETIO and the ratio between both showed only slight variations. The generally stronger impact on ETIO was better reflected by DWM(ETIO)_SULF exceeding not only individual but also the population-based limit at least for a short time period. Here the current WADA criteria allowed for detection of DHEA administration for up to 50 h.

Oral application of dihydrotestosterone and epiandrosterone

Both steroids already encompass a 5α-configuration and will therefore only result in 5α-metabolites as an interconversion from 5α- to 5β-steroids does not take place in the human metabolism of steroids.[31,40-42] This is perfectly reflected by DWM(ETIO)_GLUC for both administration trials (Figure 7). The administration of 50 mg of DHT induces a short but very intense drop in DMW(ETIO)_GLUC even far beyond the population-based limit. As described for the response found in the steroid profile or $\delta^{13}C_{VPDB}$-values, after 24 h the values are unsuspicious again.[31,40,41] After the application of EpiA, the influence on DWM(ETIO)_GLUC was also very pronounced and significantly longer compared to the DHT-administration. Here the population based threshold was exceeded for nearly 2 days and the last sample collected after 120 h was still not at starting level. This is in agreement with the comparably prolonged influence on sulfo-conjugated EpiA that may explain the constant influence in the A-pool resulting in diminished values for DWM(ETIO)_GLUC for such a long time period.[31] Unfortunately, urinary concentrations of A_SULF and ETIO_SULF have not been determined within the investigations on EpiA administrations, circumventing the calculation of DWM(ETIO)_SULF. But as the $\delta^{13}C_{VPDB}$-values of A-SULF were still found at -23.7 ‰ 120 h after compared to -21.5 ‰ before administration, it can be speculated that DWM(ETIO)_SULF will prolong the detection time compared to DWM(ETIO)_GLUC as described for 4EN (*vide supra*). But if this impact lasts longer than the direct detection using EpiA (found depleted for more than 250 h after administration) will have to be investigated in the future.

Oral application of testosterone employing endogenous carbon isotope ratios

The results obtained after the single oral administrations are summarized in Figure 8. If T indistinguishable from the endogenous CIR is administered, the fluctuations in both DWM(ETIO)_GLUC and _SULF are visible, but the values do not exceed the individual

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thresholds. The endogenous CIR of T was found at -23.6 ‰ and the exogenous T was at -23.8 ‰. Within DWM(ETIO)_SULF, a slight trend toward decreased values was visible over time. This trend is significantly pronounced as soon as the CIR of the exogenous T is slightly more depleted at -24.4 ‰ (Figure 8, b). Now the values for DWM(ETIO)_SULF exceed the individual threshold between 20 and 60 h after application. The short decrease in DWM(ETIO)_GLUC also noted during the first administration study is again more pronounced in the second resulting in two measurements falling beyond the individual threshold shortly after administration. As soon as the CIR of the exogenous T is approximately 1 ‰ away from the endogenous T, the application of the DWM(ETIO) approach seems to result in values outside the individual reference ranges at least for several hours.

As a doping scenario with T would not only encompass a single oral administration but multiple administrations, especially if the athlete is aware of the fact that the CIR of the applied T is similar to endogenous values, a multi-dose administration trial was carried out with the "worst-case" T showing a $\delta^{13}C_{VPDB}$-value of -23.8 ‰. Figure 9 combines the results obtained for both DWM(ETIO)_GLUC and _SULF (upper part) together with the absolute CIR obtained after administration (lower part). DWM(ETIO)_GLUC still shows only a weak response and the values hardly exceed the individual limit. For DWM(ETIO)_SULF, a slow but constant decrease of values is noted that results temporarily in values significantly below the individual threshold during application and for up to 80 h afterward. But is has to be mentioned that several measurements in between fall within the individual limits and would not be able to proof an administration of T. If the exogenous T shows identical CIR to the endogenous one, the detection of administrations seems to be very complicated even after multiple administrations. This phenomenon may be explained by taking a look at the absolute $\delta^{13}C_{VPDB}$-values obtained after administration (Figure 9, lower part). The steroids excreted glucurono-conjugated show a depletion in their CIR from -22.5 to -23.5 ‰ for A and from -23.2 to -24.0 ‰ for ETIO, but this small shift of 1 ‰ is more or less in parallel for both steroids resulting in fluctuation in DWM(ETIO)_GLUC but not in a clear trend. Furthermore, only samples collected directly after the administration show significant depleted CIR, samples collected 12 h later are already considerably enriched again. No accumulation is taking place during the investigated eightfold application of T. Steroids excreted sulfo-conjugated show a slightly different pattern (Figure 9, lower part). A_SULF is slightly more depleted than ETIO_SULF resulting in the significant decrease in DWM(ETIO)_SULF. The pattern shows less fluctuation compared to the GLUC-steroids and as already described in literature, after cessation of administration, the steroids excreted sulfo-conjugated show a slower return to baseline

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values.[31] As long as the CIR of the preparation administered is very close to endogenous CIR, the measurement uncertainty has a strong impact on the results of DWM(ETIO). All samples falling within the individual limits after administration are as depleted in their CIR as the samples showing decreased values but based on a measurement uncertainty for CIR of ± 0.3 ‰ the values of ETIO_SULF and A_SULF will by chance become separated enough to show normal values in their DWM(ETIO)_SULF values. Here a careful data evaluation, preferentially based on several and not only one sample seems to be a prerequisite for applying this approach in sports drug testing.

Taking a look at the ERCs employed in this study demonstrated that the exogenous T seems to have an impact on endogenous steroid production. The CIR obtained for PD (Figure 9) show extraordinary fluctuations during the application phase and shortly after and take approximately 2 days after cessation before an undisturbed state is reached again. An interesting trend is also visible in the ERC DHEA_SULF, which shows some slightly enriched values over the course of the study. Again, a negative feedback effect on endogenous steroid production may be responsible for this artefact.

However, as soon as the CIR of the exogenous steroids comes extremely close to endogenous CIR, the DWM(ETIO) approach seems to be limited. If there is a small but significant difference in the CIR, the approach seems to be suitable to identify suspicious samples as may be demonstrated exemplarily in the following paragraph.

Case study on DWM(ETIO)

A male athlete, which was tested numerous times over the recent years, showed some anomalies in his longitudinal steroid profile that triggered several IRMS confirmations. For most of these IRMS measurements, the CIR of A and ETIO were determined and reported, which enabled to plot the DWM(ETIO)_GLUC as shown in Figure 10. If the first samples collected in 2016 and 2017 are considered to represent the equilibrated and uninfluenced state of the athlete, samples collected from mid 2018 onwards show significantly different values. All of these samples yielded elevated T/Es, and the majority of samples exhibited elevated concentrations of EtG as given in Figure 10. The EtG was considered as confounding factor, which invalidated the steroid profile, but in line with WADA regulations, IRMS was triggered to substantiate the endogenous origin of T and T-metabolites. None of the determined Δ-values

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exceeded the thresholds established by WADA and all samples were reported as negative. Due to the repeatedly found atypical steroid profiles of this athlete, further investigations were carried out. Additional samples were collected out-of-competition in a short time interval of 2 weeks (the last three samples in Figure 10) and hydrogen isotope ratio mass spectrometry analysis was carried out on these samples together with the last sample found with elevated T/E collected at the end of 2019. The hydrogen isotope ratios in all 4 samples were found to fall within published population-based reference limits.[23]

Evaluation of the results taking DWM(ETIO)_GLUC into consideration does not necessarily support the interpretation of the test results as 'negative'. The three samples collected as negative controls within two weeks indeed showed comparable values for DWM(ETIO)_GLUC as the first two samples collected. Calculation of an individual threshold based on these five negative samples demonstrated clearly that all samples collected in-between showed decreased DWM(ETIO) values, and especially those containing significant amounts of EtG exceed the threshold (Figure10). Taking into account our preliminary results after ethanol ingestion, it is unlikely that ethanol is responsible for this finding. The most probable doping scenario derived from these values is the administration of T with a CIR close to the CIR of the athlete. This hypothesis is supported by the $\delta^{13}C_{VPDB}$-values of A: The three negative controls show a value of -19.3 ± 0.2 ‰ while the three samples with the lowest DWM(ETIO) values show a mean of $\delta^{13}C_{VPDB}$ = -21.0 ± 0.4 ‰. For ETIO, the difference is not significant with -20.1 ± 0.2 ‰ vs. -20.4 ± 0.4 ‰.

It has to be clearly stated that the data gathered support the hypothesis of an administration of T with a CIR around -21‰, but that due to the preliminary character of all these investigations it cannot be taken as proof of an administration. Here, further studies will be necessary and especially a closer look on possible confounding factors will be required.

Conclusions

A novel concept of data interpretation has been introduced combining the urinary concentrations and CIR of ETIO and A. The combined value named DWM(ETIO) showed a broad and Gaussian-shaped distribution within the investigated reference population and intra-individual very stable and reproducible values over short and mid-term periods. By the

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numerous excretions studies investigated here, it was possible to estimate the diagnostic value of DWM(ETIO) for both, steroids excreted glucurono- and sulfo-conjugated. The different and time-dependent influence of an administered steroids is nicely reflected by the differences found between the 5β-steroid ETIO and the 5α-steroid A. Under the perspective of doping controls, individually derived thresholds for DWM(ETIO)_GLUC and _SULF seem to be more promising compared to population-based limits, but even these are sometimes clearly exceeded depending on the steroid administered like demonstrated for 4EN, DHT, DHEA and EpiA. Oral applications of T were mainly detectable by individual thresholds and employing DWM(ETIO)_SULF also even by reference population derived limits, demonstrating once again the potential of steroids excreted sulfated to improve doping control analysis.

Regarding the administration of T encompassing an endogenous CIR, which still remains an unsolved challenge in sports drug testing, DWM(ETIO) demonstrated that it might add value as a possible solution as long as the CIR of the exogenous T is not perfectly identical to the endogenous CIR. In these cases, even multiple administrations only showed a minor, but still significant, impact on DWM(ETIO)_SULF values. The administration of a T-preparation approximately 1 ‰ more depleted than the endogenous steroids was detectable even after a single administration.

Considering these findings for the interpretation of real data obtained on a male athlete supported the hypothesis that this athlete may have administered T with a CIR less than 2 ‰ depleted compared to his own values. It has to be emphasized that this hypothesis cannot be taken as a proof for steroid administration based on the already gathered data. Nevertheless, this promising finding on the possible detection of administrations of steroids with pseudo-endogenous CIR needs to be corroborated by additional supporting data in the near future, especially considering possible confounding factors to DWM(ETIO) values.

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Table 1: Results obtained on the tenfold preparation of the blank urine over the course of the reference population study. Carbon isotope ratios are given in $\delta^{13}C_{VPDB}$ [‰], the measurement unit of DWM(ETIO) is logically derived also [‰]. SD stands for standard deviation.

	PD GLUC	ETIO GLUC	A GLUC	DHEA SULF	ETIO SULF	A SULF	DWM (ETIO) GLUC	DWM (ETIO) SULF
BW1	-22.1	-22.9	-22.5	-20.5	-21.9	-21.5	0.15	0.18
BW2	-22.0	-23.3	-22.6	-21.1	-22.3	-21.6	0.24	0.27
BW3	-22.3	-23.6	-23.0	-20.3	-22.2	-21.6	0.24	0.22
BW4	-21.8	-23.0	-22.4	-20.3	-22.0	-21.4	0.20	0.22
BW5	-21.8	-23.1	-22.8	-20.2	-22.1	-21.4	0.11	0.26
BW6	-21.7	-22.9	-22.3	-20.5	-22.3	-21.0	0.22	0.48
BW7	-21.5	-23.6	-23.1	-20.3	-22.5	-21.4	0.19	0.43
BW8	-22.4	-22.9	-22.7	-20.7	-22.1	-21.3	0.08	0.29
BW9	-21.8	-22.9	-22.6	HPLC failed			0.12	
BW10	-21.6	-23.4	-22.8	-19.8	-22.1	-21.4	0.21	0.26
mean	**-21.9**	**-23.2**	**-22.7**	**-20.4**	**-22.2**	**-21.4**	**0.18**	**0.29**
SD	0.27	0.28	0.24	0.34	0.16	0.16	0.05	0.09

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Table 2: Intra-individual variation of DWM(ETIO) for three different individuals. Samples 1-4 (collected on the same day) and samples 5-8 (over a time period of three weeks) were found to be equal. All values in [‰].

		GLUC			SULF	
volunteer	**samples**	**mean**	**SD**	**samples**	**mean**	**SD**
P4	1-4	0.31	**0.060**	1-4	0.35	**0.077**
	5-8	0.35	**0.086**	5-8	0.51	**0.093**
	1-8	0.33	**0.077**	1-8	0.43	**0.116**
P5	1-4	0.99	**0.098**	1-4	1.15	**0.072**
	5-8	0.99	**0.091**	5-8	1.21	**0.159**
	1-8	0.99	**0.095**	1-8	1.18	**0.127**
P6	1-4	0.28	**0.040**	1-4	0.53	**0.033**
	5-8	0.39	**0.093**	5-8	0.61	**0.096**
	1-8	0.33	**0.091**	1-8	0.57	**0.080**

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Table 3: Population based reference limits calculated by adding the threefold SD to the mean value resulting in a 99.7 % confidence interval.

	DWM(ETIO)_GLUC	DWM(ETIO)_SULF
Mean	0.46	0.56
SD	0.31	0.33
Lim_up	**1.39**	**1.54**
Lim_down	**-0.48**	**-0.41**
n	**110**	**95**

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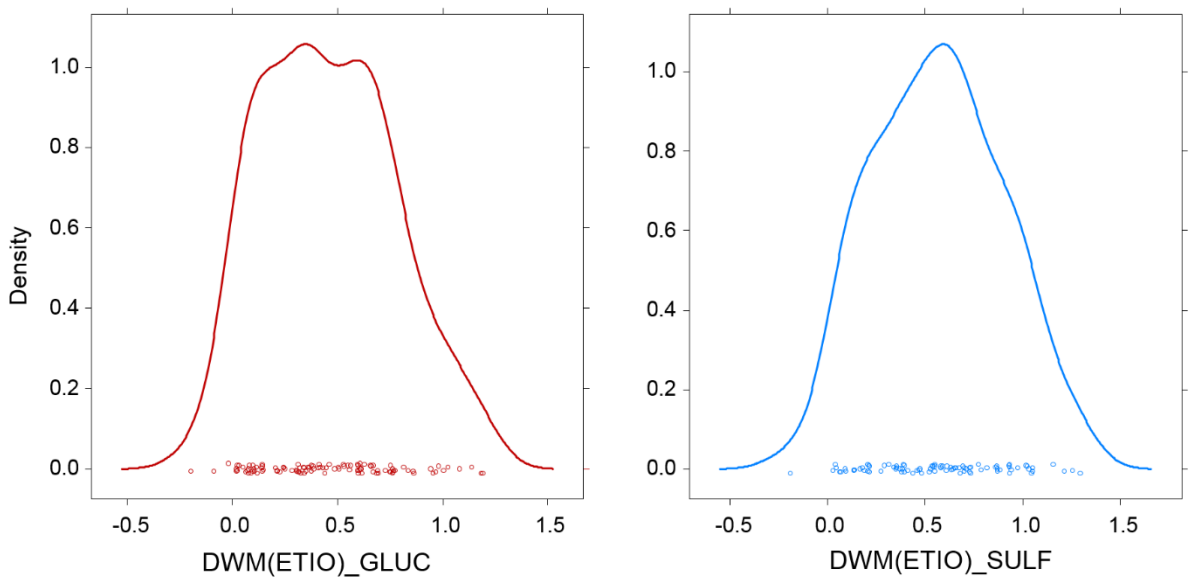

Density plot for DWM(ETIO)_GLUC and _SULF found in the investigated reference population.

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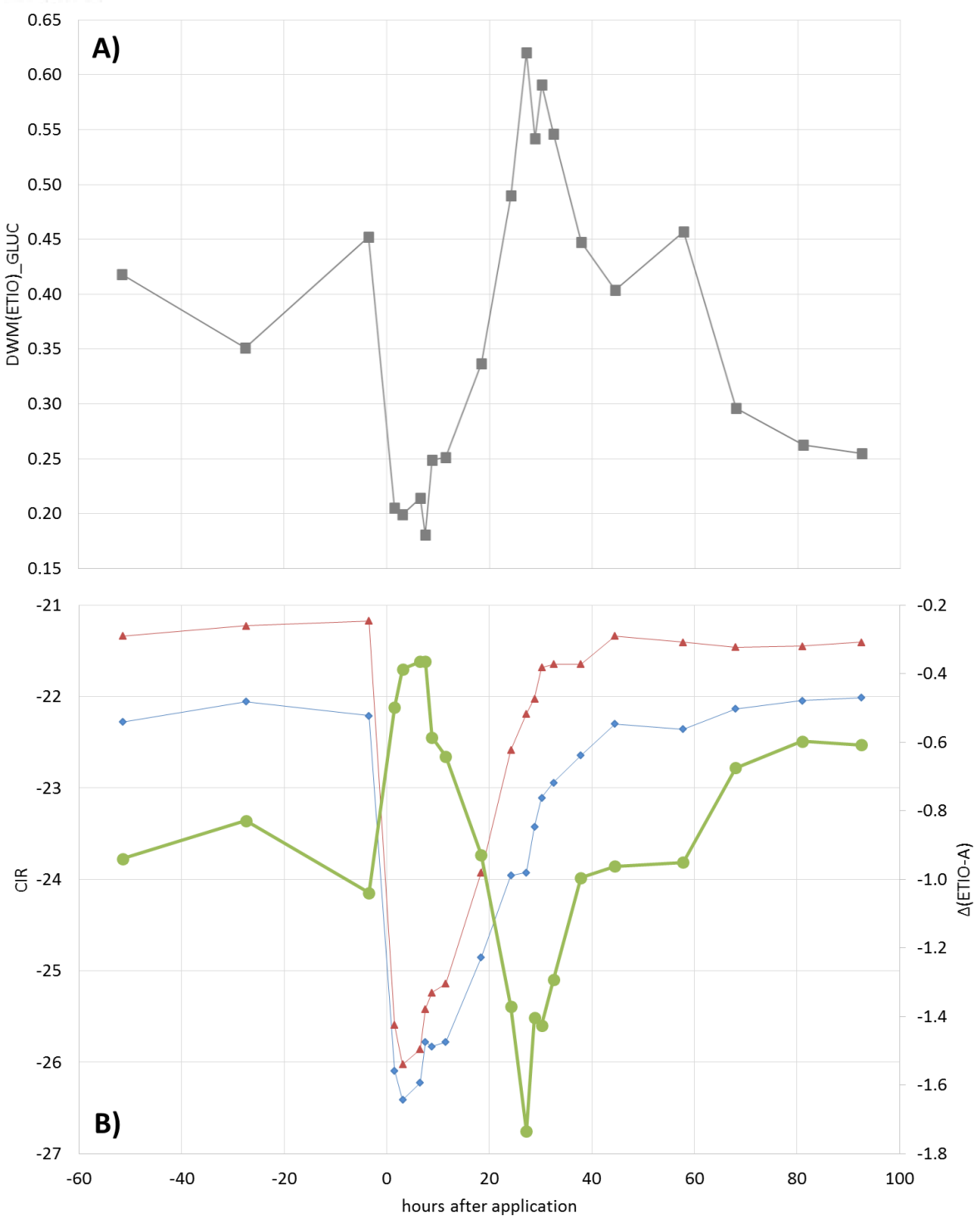

Results obtained after oral administration of 100 mg T-undecanoate. A) DWM(ETIO)_GLUC, B) δ^{13}C-values of A (red triangles) and ETIO (blue diamonds) and the Δ-values of ETIO-A (green dots). Further information in the text.

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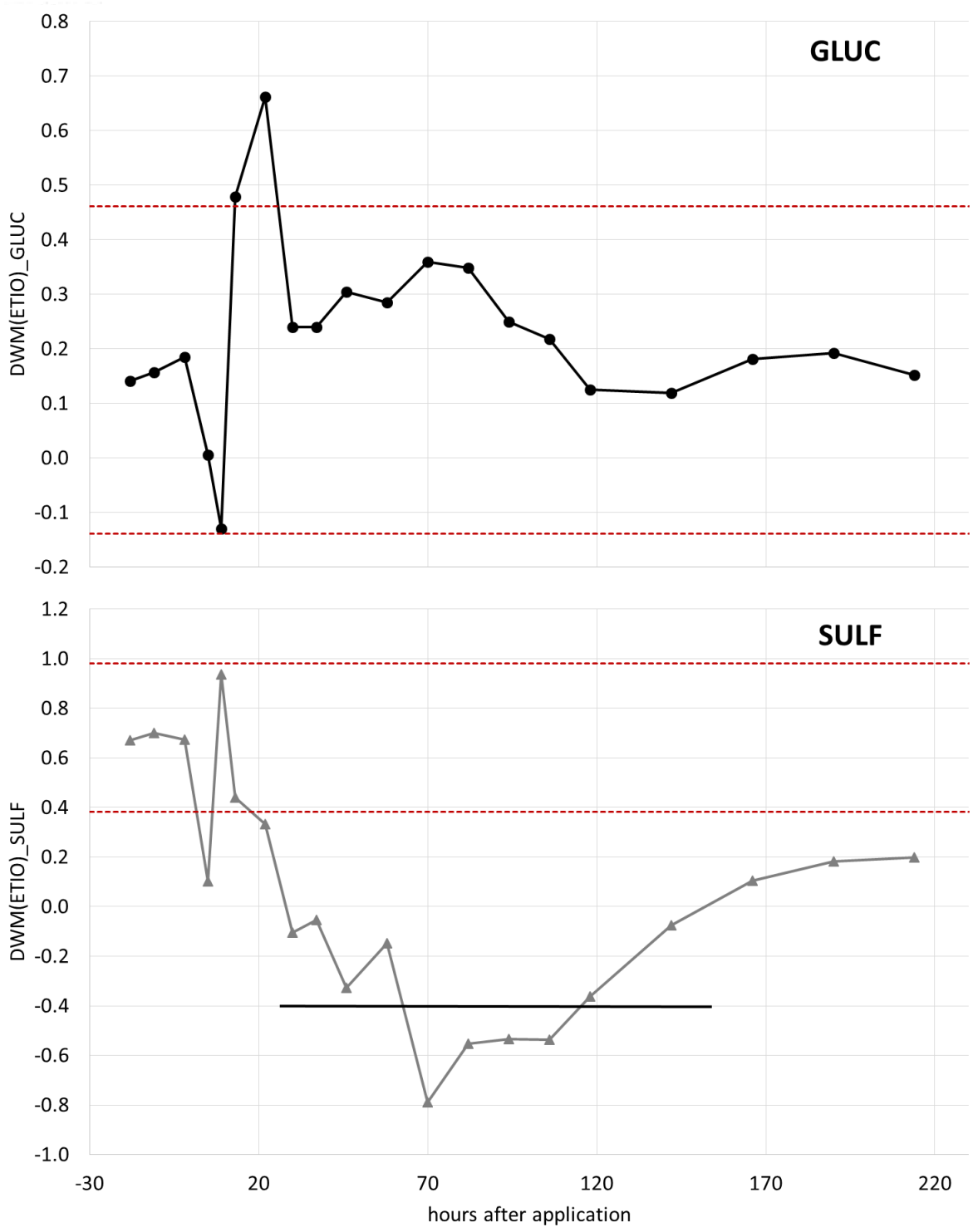

Impact on DWM(ETIO)_GLUC (upper part) and DWM(ETIO)_SULF (lower part) after oral administration of 40 mg T. The dashed lines represent the individual limit for the respective DWM(ETIO) value, the bold line in the lower graph the reference population derived threshold.

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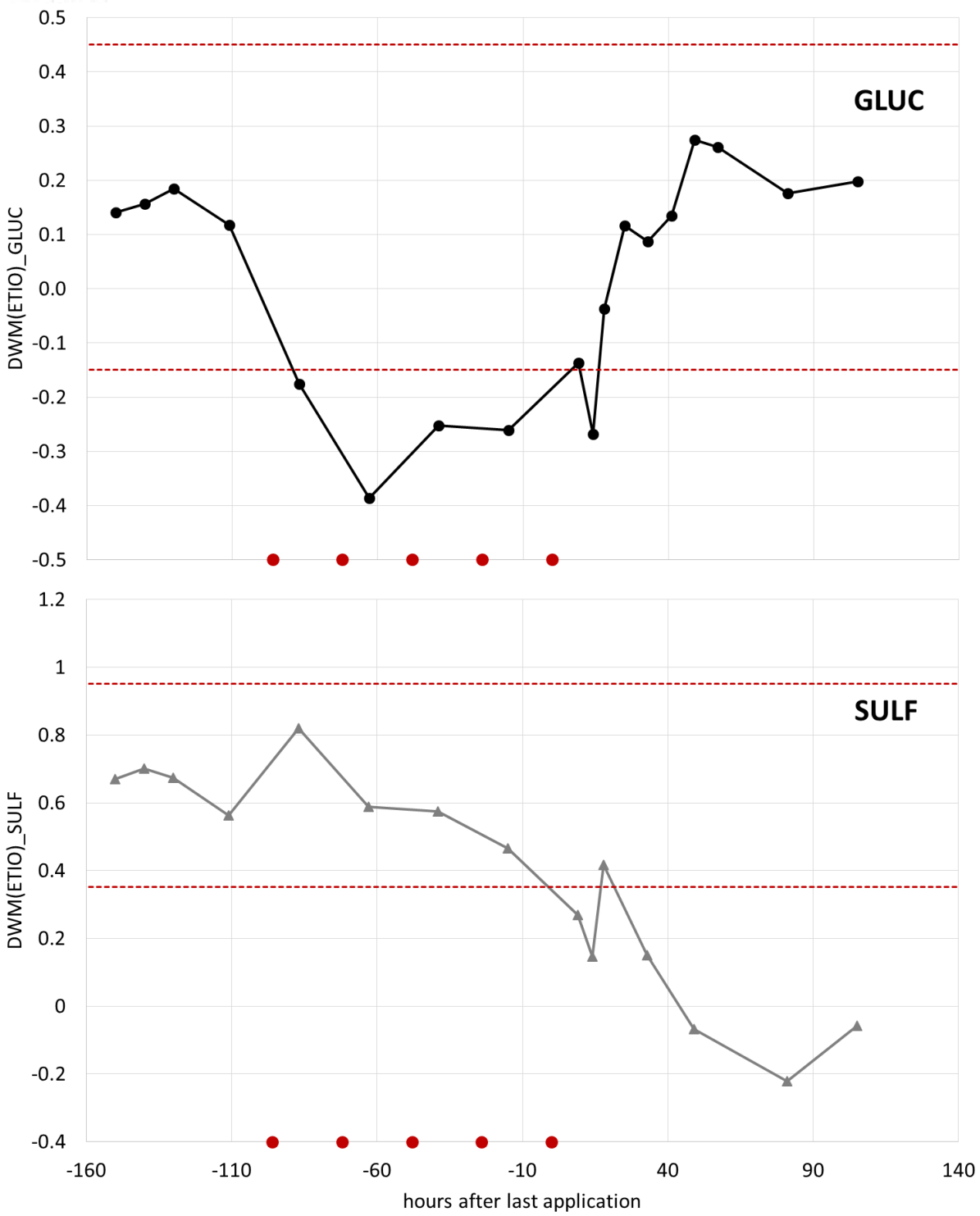

Results obtained on DWM(ETIO)_GLUC (upper part) and DWM(ETIO)_SULF (lower part) after administration of 5-times 100 mg T-gel. Time points of administration are shown by red circles on the x-axis. The dashed lines represent the individual limit for the respective DWM(ETIO) value.

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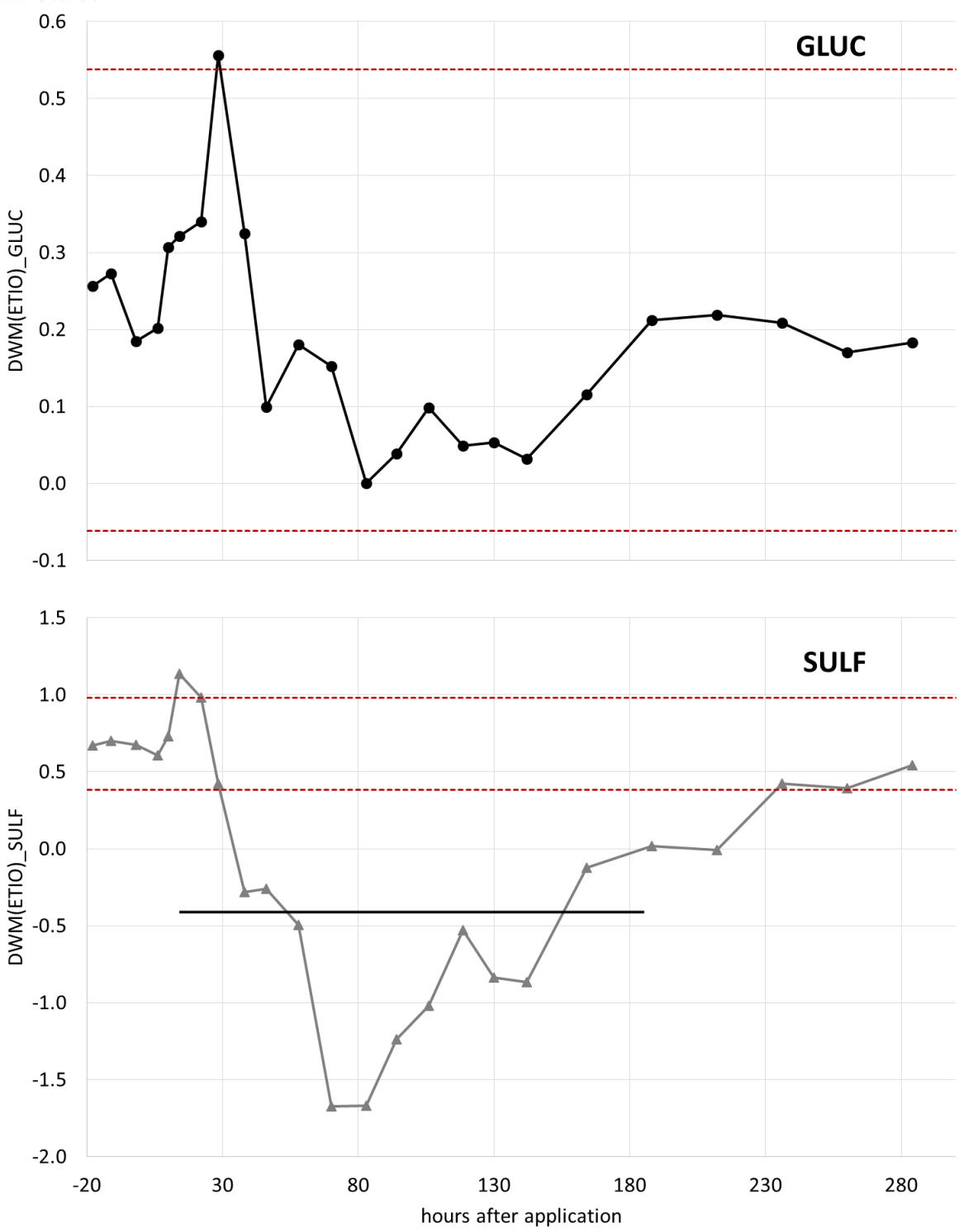

Unexpected effects of a single oral 4EN administration on DWM(ETIO)_GLUC (upper part) and DWM(ETIO)_SULF (lower part). The dashed lines represent the individual limit for the respective DWM(ETIO) value, the bold line in the lower graph the reference population derived threshold.

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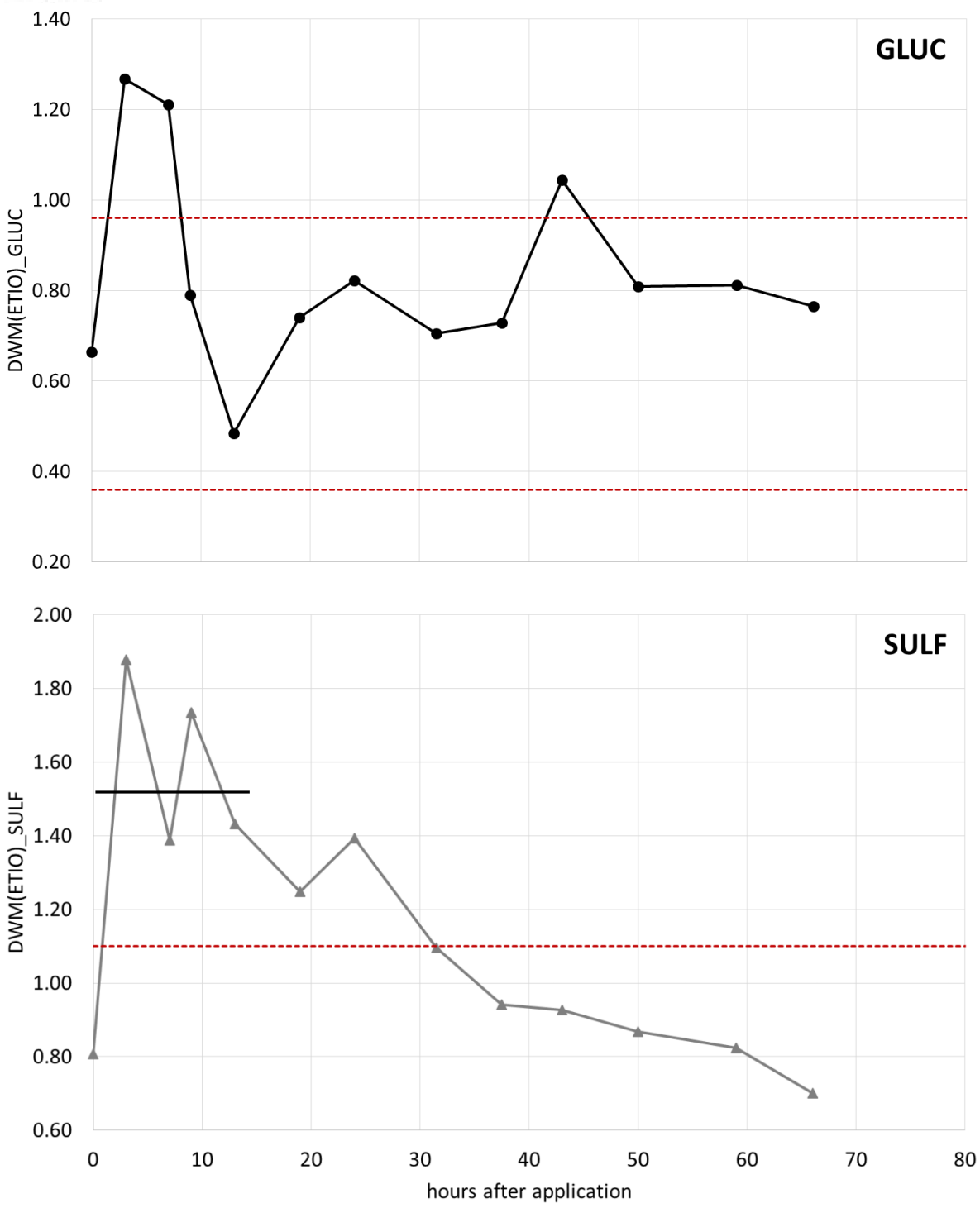

Results obtained on DWM(ETIO)_GLUC (upper part) and DWM(ETIO)_SULF (lower part) after administration of 100 mg DHEA. The dashed lines represent the individual limit for the respective DWM(ETIO) value, the bold line in the lower graph the reference population derived threshold.

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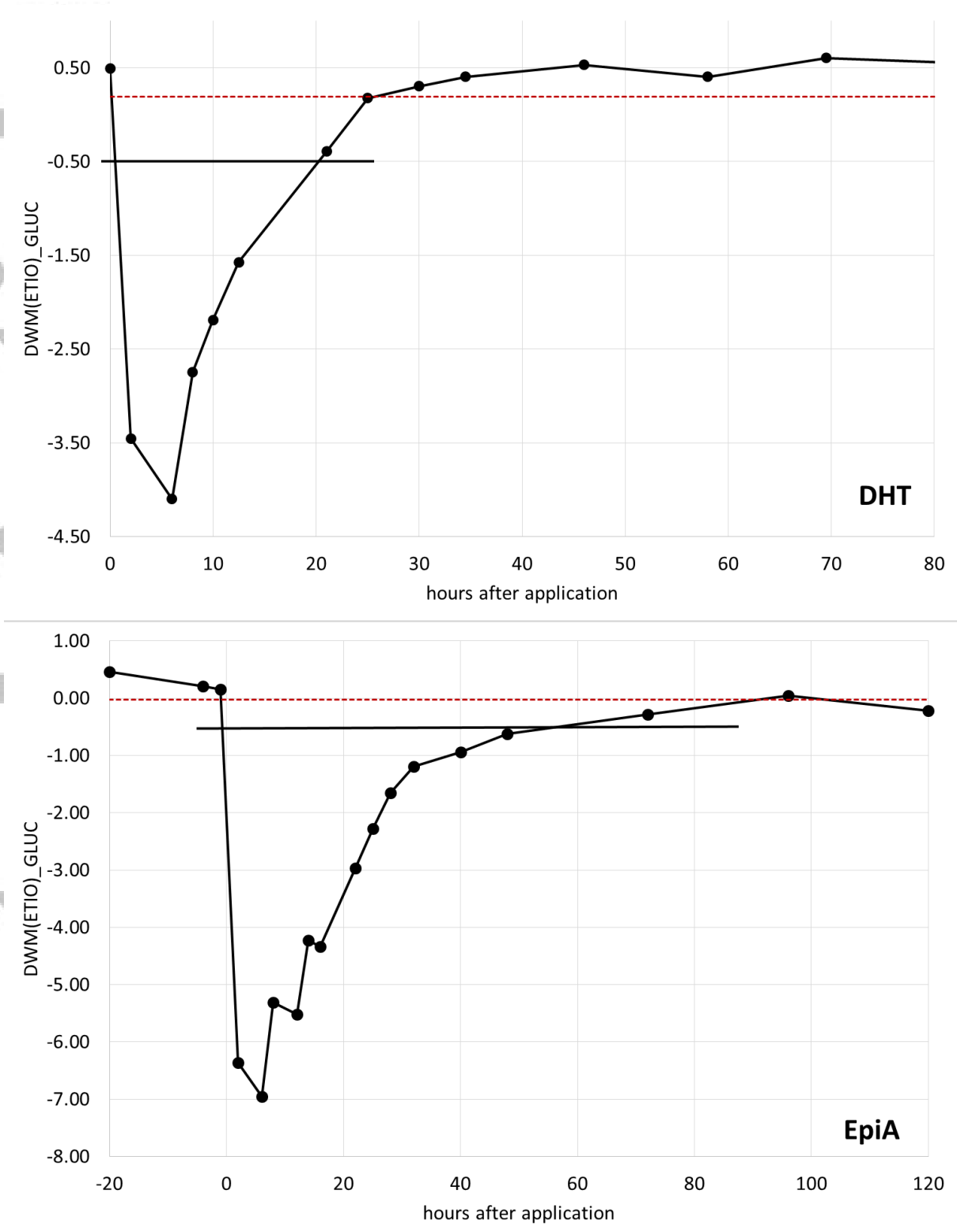

Results obtained on DWM(ETIO)_GLUC after oral administration of 50 mg DHT (upper part) and after oral administration of 100 mg EpiA (lower part). The dashed lines represent the individual limit for DWM(ETIO)_GLUC, the bold line the reference population derived threshold.

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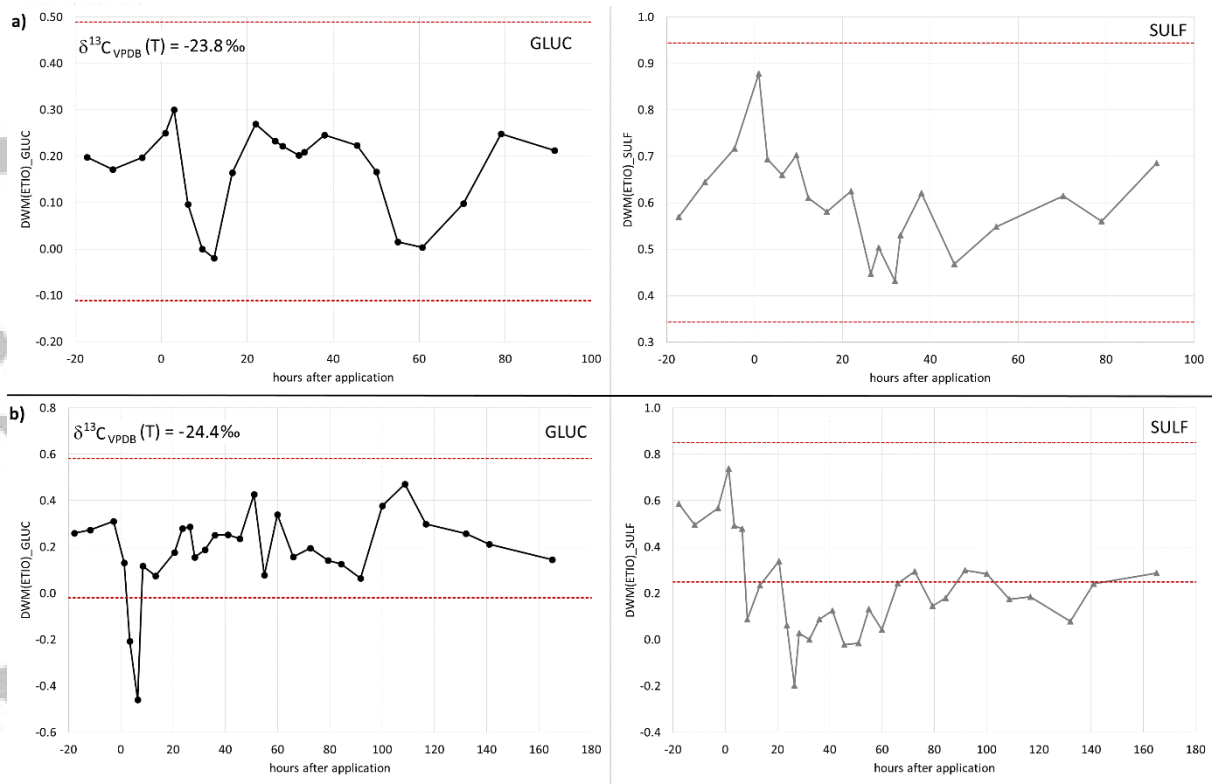

DWM(ETIO)_GLUC (left) and DWM(ETIO)_SULF (right) after single oral applications of testosterone preparations with CIR close to endogenous values (a = -23.8 ‰, b = -24.4 ‰). The ERC PD was at -22.4 ± 0.41 ‰ (n = 57). The dashed lines represent the individual limit for the respective DWM(ETIO) value.

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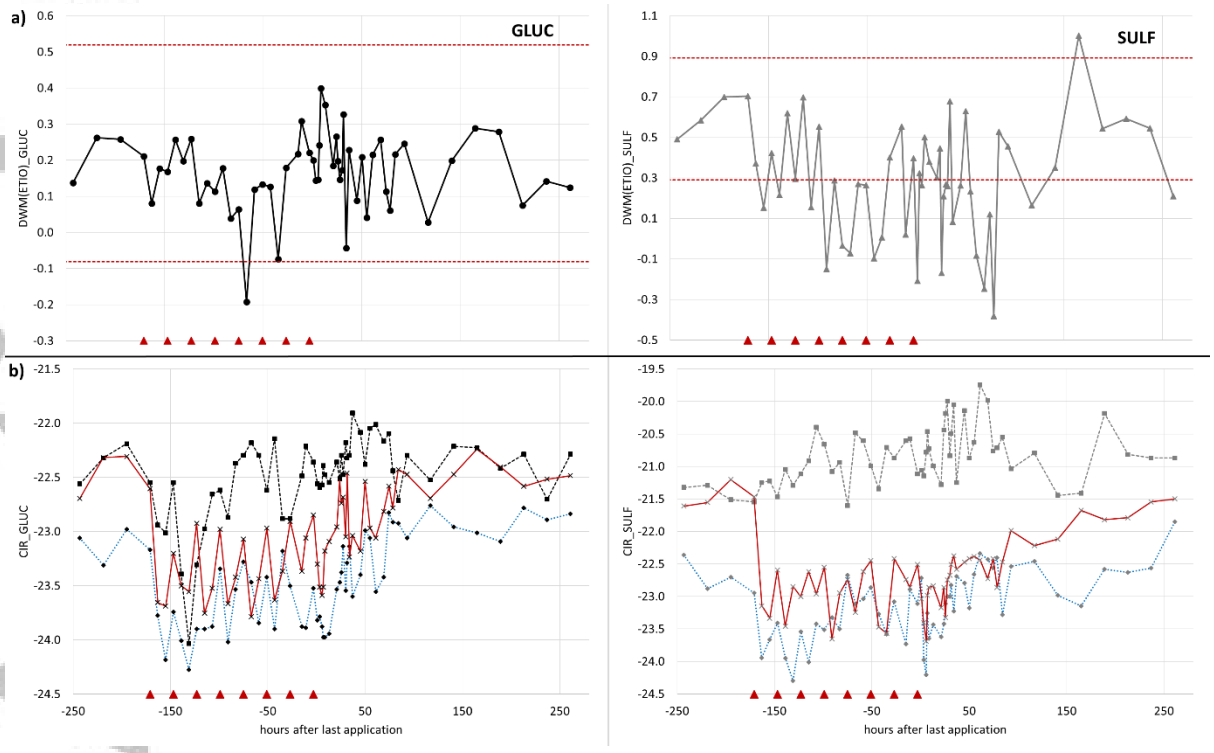

DWM(ETIO)_GLUC and _SULF (a) and corresponding CIR (b) after multiple oral administrations (red triangles) of testosterone encompassing endogenous CIR at -23.8 ‰. The dashed lines represent the individual limit for the respective DWM(ETIO) value. In the lower part the black squares represent PG_GLUC, black crosses A_GLUC and black diamonds ETIO_GLUC, grey squares DHEA_SULF, grey crosses A_SULF and grey diamonds ETIO_SULF.

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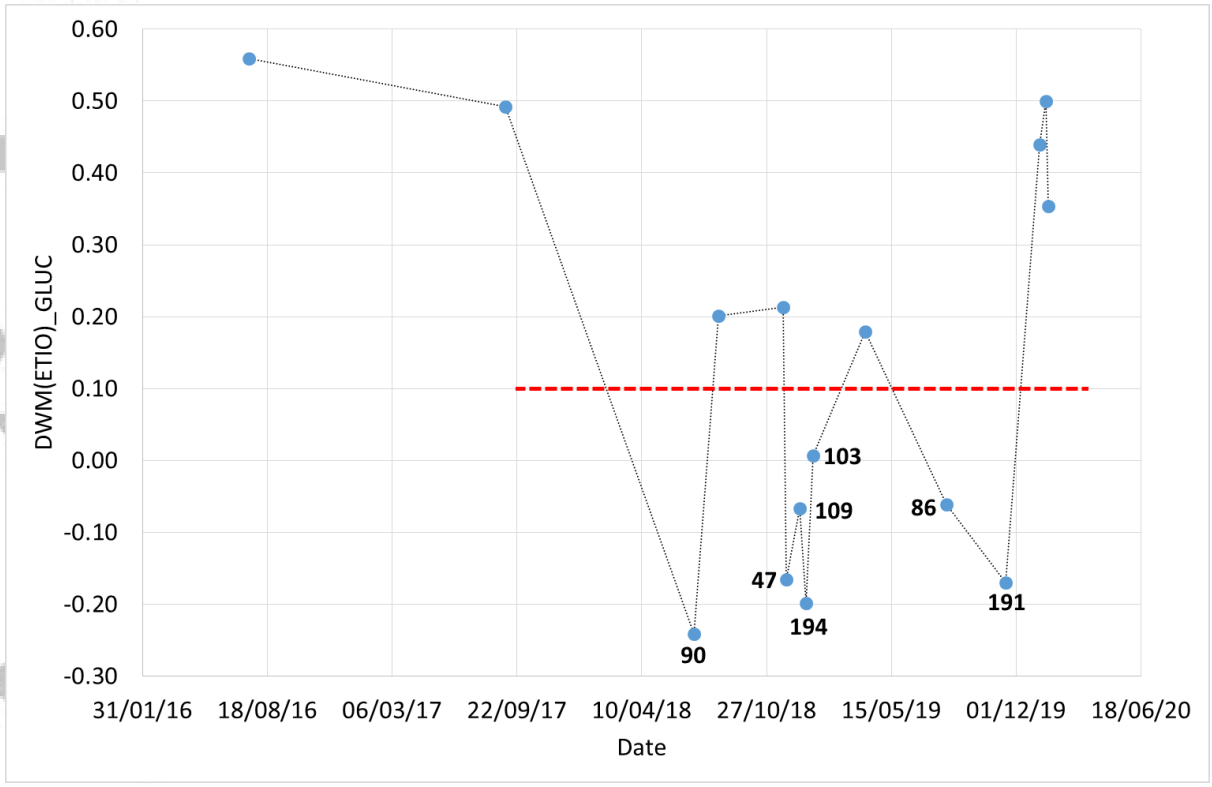

DWM(ETIO)_GLUC data of a male athlete collected over a time period of 3 years. The bold numbers represent the urinary EtG concentration in µg/mL, the dashed red line the presumptive individual threshold. Further information in the text.

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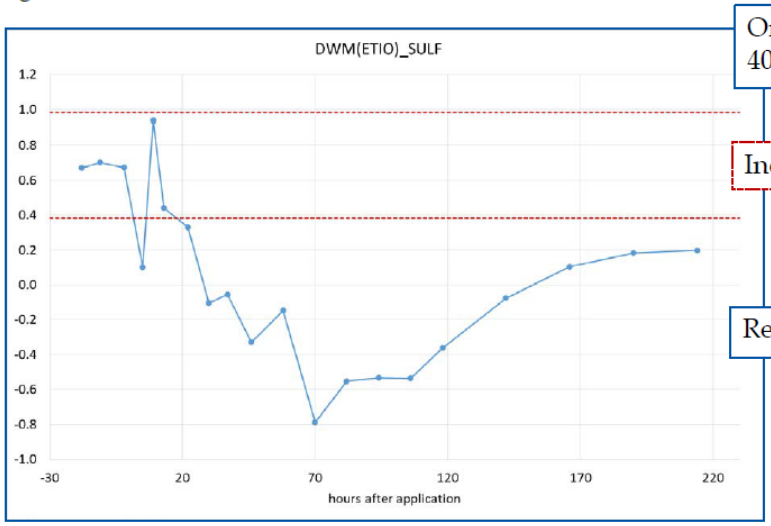
Sensitive detection of testosterone and testosterone prohormone administrations based on urinary concentrations and carbon isotope ratios of androsterone and etiocholanolone

Thomas Piper*, Nadine Haenelt, Gregor Fusshöller, Hans Geyer, Mario Thevis

A novel concept of data interpretation has been introduced, validated and tested combining the urinary concentrations and carbon isotope ratios of etiocholanolone and androsterone excreted glucuronidated or sulphated. The combined value named difference from the weighted mean (DWM) allowed for a prolonged and sensitive detection of testosterone and testosterone prohormone administrations. Even the detection of testosterone administrations encompassing a pseudo-endogenous carbon isotope ratio signature seems feasible.



	GLUC	SULF
Mean	0.46	0.56
SD	0.31	0.33
Lim_up	1.39	1.54
Lim_down	-0.48	-0.41

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