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Evaluation of endogenous steroid sulfates and glucuronides in urine after oral and transdermal administration of testosterone. Part I: Male participants

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ABSTRACT

The detection of the performance-enhancing drug testosterone (T) remains a significant challenge in doping control analysis. Longitudinal monitoring through the steroidal Athlete Biological Passport (ABP) is a valuable tool for T detection, but further research is needed to enhance its efficacy. Phase II metabolites of endogenous anabolic androgenic steroids (EAAS), including glucuronides and sulfates, have gained increasing interest as potential new biomarkers for the steroidal ABP. Notably, sulfate metabolites have demonstrated higher sensitivity to oral, transdermal, and intramuscular T administration, with extended detection windows compared to traditional biomarkers. However, before incorporating these promising biomarkers into urinary steroid profiling, it is essential to address the metabolic variations associated with different T administration methods, as well as differences related to ethnicity and sex. In this part of the study, we investigate the effects of oral and transdermal T administration on conventional biomarkers and phase II EAAS metabolites in male participants. Sulfate ratios indicated higher sensitivity to multiple administrations of testosterone undecanoate (TU) tablets and T gel, significantly prolonging detection times compared to conventional steroid profile biomarkers. Specifically, sulfate ratios such as androsterone sulfate (AS)/testosterone sulfate (TS) and epiandrosterone sulfate (EpiAS)/TS enabled detection for an average of 20 days following the last oral TU dose and at least 16 days after the last transdermal T application. These findings provide further evidence that incorporating sulfate EAAS metabolites into steroid profiling enhances detection capabilities. For advanced T doping detection, sulfate metabolites should be considered essential biomarkers in the steroid profile.

1. Introduction

The implementation of the steroidal module in the Athlete Biological Passport (ABP) in 2014 enabled more sensitive detection of doping with testosterone (T) and other endogenous anabolic androgenic steroids (EAAS) [1–3]. The steroid profile comprises T, its epimer epitestosterone (E), and key metabolites such as androsterone (A), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5a-Adiol), and 5β-androstane-3α, 17β-diol (5b-Adiol). These metabolites are quantified after enzymatic hydrolysis of glucuronidated EAAS and derivatization using gas chromatography – mass spectrometry (GC-MS) methods. To enhance stability and sensitivity, their concentrations are combined into specific ratios. Ratios included in the steroid profile as biomarkers are T/E, A/Etio, 5a-Adiol/E, 5a-Adiol/5b-Adiol, and A/T [4]. These urinary biomarkers and their individually calculated reference ranges are monitored longitudinally, allowing reliable discrimination between naturally elevated biomarkers and potential doping scenarios [2]. Samples indicating exogenous administration of T by suspicious fluctuations of the biomarkers are then further analyzed with gas chromatography – combustion – isotope ratio mass spectrometry (GC-C-IRMS) for the unambiguous confirmation of T misuse. Although the steroid profile improves T doping detection capabilities in sports drug testing, it

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still faces several challenges [2,5–7]. The detection window after oral administration of EAAS is short, while the sensitivity of the biomarkers to transdermal administration of EAAS is relatively low. Additionally, the sensitivity of the steroidal module is diminished for female athletes and individuals bearing the del/del genotype for the UGT2B17 enzyme. This deletion polymorphism, responsible for the glucuronide conjugation of T, leads to low urinary T levels obtained by GC-MS, which severely hinders the response of T/E to T administration [8].

These unresolved issues have contributed to the expansion of research on the urinary steroid profile toward the analysis of intact phase II metabolites of EAAS, comprising glucuronidated and sulfated steroids, by liquid chromatography-mass spectrometry (LC-MS). As the current steroidal ABP only incorporates glucuronidated and free forms of the EAAS, the investigation of sulfated metabolites of EAAS adds new insights into metabolic reactions. This complementary approach has aided in the discovery of new urinary biomarkers, focusing on sulfated metabolites of conventional and alternative EAAS metabolites [9–17]. Also, combined ratios of sulfated and glucuronidated metabolites have been investigated, showing great potential for expanding detection capabilities [18–21]. Various administration studies examining phase II metabolites after the administration of T have been conducted to assess the suitability of these alternative biomarkers. Studies from the last few years show promising but mixed results, highlighting high intra-individual variability among participants and strong dependence on the drug administration route and dose.

Several studies [9,12,20] investigating oral administration of testosterone undecanoate (TU), have proposed sulfate ratios, including testosterone sulfate (TS), epitestosterone sulfate (ES), androsterone sulfate (AS), and epitestosterone sulfate (EpiAS), as suitable biomarkers and have demonstrated significantly prolonged detection times (DTs) compared to the conventional T/E ratio [22,23]. Another study [21] could, however, not confirm these results after oral TU administration, pointing out differences in the administration dose.

Comparing different T administration routes, transdermal administration has shown generally lower sensitivity of conventional biomarkers [24,25]. T gel is often administered in even intervals, and low doses are absorbed, resulting in relatively stable steroid profiles. First investigations of phase II metabolites, especially sulfates, have shown moderate results regarding increased sensitivity and additional value compared to established biomarkers [9,21]. Nevertheless, a recent study [26] has proposed sulfate ratios of EAAS as sensitive biomarkers after multiple doses of T gel. The study has been evaluated in Caucasian and Asian male volunteers, gaining valuable information and prolonged DTs.

Phase II metabolites of EAAS have also been evaluated after intramuscular administration of different T esters. Sulfate ratios, for example, AS/TS, have significantly prolonged the DT after T cypionate administration in Caucasian and Asian male participants [27]. Another study has been investigating alternative biomarkers after T cypionate administration, focusing on glucuronidated phase II metabolites of EAAS [28]. Also, ratios between glucuronides and sulfates were investigated [21], showing similar or longer DT than conventional biomarkers. Two studies [18,19] proposed the “combined ratio” (CR) of testosterone glucuronide/testosterone sulfate to epitestosterone glucuronide/epitestosterone sulfate ((TG/TS)/(EG/ES)) as a promising biomarker for detecting various intramuscular T ester administrations, independent of UGT2B17 gene polymorphism. The authors have suggested further studies to prove the applicability of the CR for a wider application range to detect T doping, including different T administration forms and female steroid profiles.

Results of these various studies propose a great potential for including phase II metabolites in future steroid profiling. However, before implementing their quantification in routine doping control analysis, further research needs to be conducted. The applicability has to be tested for parenteral (transdermal, intramuscular) and enteral (oral) administration forms to gain insight into different metabolic pathways. Also, besides the comparison of Caucasian and Asian participants, female participants have to be included in further studies to build a robust monitoring system for T misuse. This work aimed to add value to the ongoing investigations by assessing the applicability of the CR and other ratios of phase II metabolites of EAAS as biomarkers for oral and transdermal T administration. The effect on the conventional steroid profile, glucuronidated, and sulfated metabolites of EAAS was evaluated and compared. The presented clinical study included 12 male and 6 female participants. In this first part, results for male participants are described.

2. Materials and methods

2.1. Chemicals and reagents

Methanol (HPLC grade) used to prepare standard solutions and for sample preparation was supplied by Chem-Lab (Zedelgem, Belgium). Water used for sample preparation was provided by a Milli-Q water purification system (Millipore, Reference A+, Burlington, Massachusetts, USA). Water and acetonitrile (ULC/MS-CC/SFC grade) as well as formic acid (99 %, ULC/MS-CC/SFC grade) used for HPLC analysis, were purchased from Bisolve (Valkenswaard, The Netherlands). Oasis HLB cartridges (60 mg/3 ml, 30 μm particle size) for solid phase extraction were obtained from Waters (Milford, MA, USA). Chemicals for the preparation of artificial urine and buffers were obtained as follows: potassium dihydrogen phosphate, sodium chloride, di-sodium hydrogen phosphate, and ammonium chloride, were obtained from Merck (Darmstadt, Germany). Urea was provided by GE Healthcare Life Sciences (Uppsala, Sweden) and creatinine was purchased from Sigma Aldrich (St. Louis, MI, USA). Further, methyl-t-butyl-ether, ammonium iodide (NH4I), and ethanethiol (97 %) were purchased from Sigma Aldrich (St. Louis, MI, USA). β-Glucuronidase (E. coli) for enzymatic hydrolysis was supplied by Roche (Mannheim, Germany). N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Macherey-Nagel (Düren, Germany). For silylation, a derivatization stock solution was prepared by dissolving 200 mg of NH4I in a mixture of 10 ml MSTFA and 600 μl ethanethiol. A derivatization working solution was prepared by mixing the stock solution with MSTFA (1:4, v/v) directly before sample preparation.

Testosterone glucuronide (TG; Batchnr.: 16S02), Epitestosterone glucuronide (EG; Batchnr.: 15S10), Androsterone glucuronide (AG; Batchnr.: 09S11), Etiocholanolone glucuronide (EtioG; Batchnr.: 15S14), Testosterone sulfate (TS; Batchnr.: 97000340), Epitestosterone sulfate (ES; Batchnr.: 97000341), Androsterone sulfate (AS; Batchnr.: 06S05), Etiocholanolone sulfate (EtioS; Batchnr.: 97001741) and the deuterated substances used as internal standard (ISTD) d3-TG (Batchnr.: 15S06), d4-EG (Batchnr.: 13S07), d5-EtioG (Batchnr.: 24S06), d3–5α-Dihydrotestosterone glucuronide (d3-DHTG; Batchnr.: 10S06), d3-TS (Batchnr.: 20S02), d3-ES (Batchnr.: 97000059), d4-AS (Batchnr.: 98000501), d5-EtioS (Batchnr.: 97001744) and d3–5α-Dihydrotestosterone sulfate (d3-DHTS; Batchnr.: 98001909) were purchased from National Measurement Institute (New South Wales, Australia). d5-AG was provided by synthesis from the Technical University of Vienna (Vienna, Austria). Epiandrosterone glucuronide (EpiAG; Batchnr.: C259) and Epiandrosterone sulfate (EpiAS; Batchnr.: B2387) were purchased from Steraloids (Newport, RI, USA). d5-EpiAS (Batchnr.: 2RRK261) was purchased from Toronto Research Chemicals Inc (Vaughan, Canada). The purity of all reference standards was 98 % or greater. In all cases, stock standard solutions of the analytes were prepared in methanol (100–200 μg/ml) and stored at −20 °C.

2.2. Clinical studies

Ethical approval of the studies was granted by the corresponding local Research Ethical Committees (Sports Medicine Association of Serbia, Belgrade, Serbia; Decision_0221). The studies were conducted in accordance with the Declaration of Helsinki.

All participants provided written informed consent before inclusion and underwent a general physical examination, blood test, urinalysis, and a 12-lead electrocardiogram with results within normal values. The medical evaluation was conducted again following the completion of the study. All participants declared not to consume any over-the-counter drugs or prescription drugs within 2 weeks before the start of the study. Participants were allowed to moderately consume alcoholic beverages. Twelve healthy male volunteers, aged between 18 and 40, with body weight not more than 10 % above or below the ideal weight for their height and frame, were included in the administration studies. Most of the participants were either medical students or practicing physicians, which contributed to high compliance with the sampling protocol and overall study procedures. Each participant received an oral T formulation in the first study period, and six participants, randomly assigned, received additionally a gel formulation in the second study period.

The oral formulation of T (Andriol® Testocaps, 10 times 40 mg TU, multiple dosing) was administered as follows: 4 capsules on day 1, 2 in the morning and the evening, respectively. Two capsules were administered in the morning on day 2, 3 and 4, resulting in a total T intake of 253 mg (400 mg TU). Six urine samples were collected during the week before administration to create baseline values. Urine samples were collected in the following periods, starting after the first T administration: all urine samples during week 1, one urine sample per day during week 2, and 2 urine samples per week for weeks 3 and 4. Four participants expanded the sample collection by one week.

2 months after the first period of the clinical study, six participants received a gel formulation in the second study part. The topical formulation of T (Testavan® gel, 5 times 46 mg of T (2 pumps each), multiple dosing) was applied to the upper arm in the morning on day 1–5, resulting in a total T administration of 230 mg. 3 urine samples were collected during the week before administration to create baseline values. Urine samples were collected in the following periods, starting after the first T administration: all urine samples during week 1, one urine sample per day during week 2, and 2 urine samples per week for weeks 3 and 4.

2.3. Determination of the conventional steroid profile

2.3.1. Sample preparation

The urinary steroid profile was measured according to the validated routine laboratory procedure compliant with World Anti-Doping Agency (WADA) guidelines [29,30], including hydrolysis of the glucuroconjugated part and derivatization before injection [31]. If the concentration was outside the calibration range using 1 ml of urine, a 100 µl urine aliquot was used.

2.3.2. GC-MS/MS analysis

Gas chromatography – tandem mass spectrometry (GC-MS/MS) analysis of the trimethylsilyl steroids was conducted on a Trace 1300 gas chromatograph coupled to a TSQ 8000 triple quadrupole mass spectrometer (Thermo Fisher, Waltham, MA, USA) according to the accredited procedure for quantifying the steroid profile in urine, as described elsewhere [31].

2.4. Determination of phase II metabolites

2.4.1. Sample preparation

To cover the broad concentration range of sulfates and glucuronides from sub-ng/ml to several µg/ml, two aliquots of urine were prepared. To cover lower concentration ranges, one aliquot was prepared using solid phase extraction (SPE), which was performed on Oasis HLB cartridges (60 mg, 30 µm) as follows: the cartridges were conditioned with 2 ml of methanol and equilibrated with 2 ml of water. The loading solution was prepared by the addition of 20 µl of ISTD solution (40 ng/ml d3-TG, 40 ng/ml d4-EG, 400 ng/ml d5-AG, 400 ng/ml d5-EtioG, 100 ng/ml d3-DHTG, 10 ng/ml d3-TS, 10 ng/ml d3-ES, 100 ng/ml d4-AS, 100 ng/ml d5-EtioS, 100 ng/ml d5-EpiAS and 100 ng/ml d3-DHTS) to 2 ml of urine and loaded onto the cartridges, followed by washing with 2 ml of water. Finally, the analytes were eluted with 2 ml of methanol. Samples were evaporated to dryness and reconstituted in loading solvent H2O: ACN (80:20) + 0.1 % FA. 2 µl were injected into the LC-High Resolution Mass Spectrometry (LC-HRMS) system. To cover the high concentration range for certain target substances, a second aliquot using 40 µl of urine was diluted with 360 µl aqueous ISTD solution (600 ng/ml d3-TG, 6000 ng/ml d5-AG, 6000 ng/ml d5-EtioG, 1500 ng/ml d4-AS, 1500 ng/ml d5-EtioS, 1500 ng/ml d5-EpiAS and 1500 ng/ml d3-DHTS in 96-deep well plates. 2 µl of the mixture was directly injected into the LC-HRMS system. If the concentration was outside the calibration range, a 10 µl urine aliquot was used.

2.4.2. LC-HRMS analysis

Analysis was carried out on a Q Exactive Focus Orbitrap MS analyzer coupled to a Vanquish Horizon UHPLC System (Thermo Fisher, Waltham, MA, USA). The LC system was equipped with a cooled sample tray (20 °C), a high-pressure binary pump, and a column oven set at 25 °C. Chromatographic separation was carried out using an InfinityLab Poroshell 120 EC-C18 column (150 ×2.1 mm i.d., 1.9 µm particle size, Agilent Technologies). To reduce non-defined adsorption, shielded fused silica nanoViper™ tubing sheathed in polyetheretherketone (PEEK) from Thermo Fisher was employed in the entire system. The mobile phase consisted of water containing 0.1 % formic acid (solvent A) and acetonitrile containing 0.1 % formic acid (solvent B). For the samples prepared with SPE (SPE method), a gradient elution program was employed at a constant flow rate of 0.3 ml/min with solvent B increasing over 10.5 min from 25 % to 39 %. Solvent B increased further to 98 % over 1 min. It was held there for 1.5 min before returning to 25 % B within 0.1 min. The column was re-equilibrated at 10 % B for 2.9 min, resulting in a total runtime of 16 min. Samples prepared by dilution and direct injection (dilute and shoot (DS) method) were analyzed on the same system with the same settings, only using a shorter gradient elution program. At a constant flow rate of 0.3 ml/min, solvent B increased from 10 % to 40 % in 0.2 min, followed by an increase to 51 % over 3.8 min. In 0.5 min, solvent B increased to 98 %, where it was held for 1.5 min, before returning to 10 % within 0.1 min. The column was re-equilibrated at 10 % B for 1.9 min, resulting in a total runtime of 8 min.

The mass spectrometer was equipped with a heated electrospray ionization (HESI) source, operated in negative mode. Source parameters were as follows: spray voltage 3.7 kV, capillary temperature 320 °C, sheath gas, and auxiliary gas (nitrogen): 30 and 10 arbitrary units, respectively. The instrument was operated in single ion monitoring (SIM) mode at 70,000 resolving power.

2.5. Method validation

For accurate quantification of the target substances TG, EG, EtioG, AG, EpiAG, TS, ES, EtioS, AS, EpiAS, the methods were validated according to WADA guidelines [30]. For all target substances, their corresponding deuterated standards were used as ISTD. For EpiAG, where no deuterated standard was available, d3-DHTG was used as an internal standard. EpiAS was evaluated with its corresponding deuterated standard d5-EpiAS for participants O1-O6. Due to availability problems, EpiAS was quantified with the deuterated standard d3-DHTS for the rest of the samples. Artificial urine, prepared in the laboratory (2.5 g/L potassium dihydrogen phosphate, 9.0 g/L sodium chloride, 2.5 g/L disodium hydrogen phosphate, 3.0 g/L ammonium chloride, 25 g/L urea, and 2 g/L creatinine, prepared in water), was used as the matrix for method validation and preparation of calibration curves. To generate six different artificial urine samples, the amount of each ingredient was increased by 50 % from the standard protocol, once at a time. 10 urine samples from the laboratory's external quality assessment scheme (EQAS), allocated during the last 5 years, were used in method

validation.

The validation protocol included the determination of SPE extraction recovery, selectivity, linear range, limit of quantification (LOQ), repeatability, intermediate precision (IP), bias, measurement uncertainty (MU), robustness, extract stability, and carryover. All validation parameters were determined in compliance with the laboratory's standard procedures. The extraction recovery of the SPE method was calculated as the ratio of responses of 20 µl of ISTD solution, which was spiked in artificial urine before and after the extraction. Samples were prepared in triplicate. Selectivity was evaluated by analyzing 10 EQAS samples and the same set of samples spiked with a standard mixture of approximately 40 exogenous steroids (free forms and glucuronidated) to evaluate the presence of chromatographic interferences in the selected mass for all target analytes. Additionally, concentrations for AG, EtioG, TG, and EG were compared with the assigned value for A, Etio, T, and E from conventional steroid profile determination by GC-MS/MS. Samples were accepted if the deviation from the assigned value for the four target analytes was less than 20 %, and no notable interferences were detected in the elution region of each target analyte. Calibration curves were prepared by spiking six different artificial urine samples at seven different calibration levels. Samples were analyzed in triplicate. The linear range of the calibration curves was calculated from the peak area ratio of the analyte to that of the corresponding ISTD using a 1/x2 weighted regression. The calibration curves were accepted if the coefficient of determination (R2) > 0.99, and the concentration for all calibration levels did not differ more than +-20 % from the nominal concentrations. For LOQ determination, 6 additional calibration curves were prepared in the lower linear range and was determined according to DIN 32645 [32]. IP and repeatability were assessed by preparing three different batches, each consisting of six different artificial urines spiked with two different calibration levels (LOQ and 10xLOQ). Samples were prepared by two different analysts on three different days and analyzed in triplicate. The bias was evaluated as the difference from the assigned value of 10 EQAS samples for AG, EtioG, TG, and EG. For substances with no certified reference material available, bias was determined as the mean absolute percentage difference between the concentrations measured in the samples for linearity determination and their nominal concentration, taken as the reference. The MU was determined in accordance with the TD2022DL [33] by a quadratic combination of the IP and the root mean square of the bias. The acceptance criteria were set at 20 % of the mean result at 10xLOQ and at 30 % at LOQ [29]. The bias for AG, EtioG, TG, and EG determined with EQAS samples was used in the relevant MU calculation. Robustness was assessed by evaluating minor changes in the analytical measurement (e.g., fresh mobile phase preparation, SPE cartridges lot, LC column lot) during the three days of the quantitative validation protocol. Carryover was determined by the injection of blank water samples after the highest calibration sample and was regarded as negligible if it was less than 1 %. Extract stability was assessed by re-injection of samples from bias determination.

2.6. Data evaluation

The software Xcalibur Quan Browser (Thermo Fisher) was used to process raw data and for quantitation. Quantitative analysis was performed using an external calibration curve consisting of at least 5 points. For better comparison, urinary concentrations of endogenous steroids were corrected by specific gravity (SG) [33]. Samples that showed signs of extensive bacterial degradation (5α-Androstandione/A or 5β-Androstandione/Etio > 0.1, determined by GC-MS/MS) and ethyl glucuronide (ETG, determined by LC-HRMS) higher than 5 µg/ml were excluded [29]. Values below the LOQ of the analytical method were not considered. Ratios between endogenous steroid sulfate and glucuronide metabolites, which showed the highest sensitivity to T administration, were calculated and longitudinally evaluated. From the conventional steroid profile determined by GC-MS/MS, T/E was chosen as reference biomarker for oral administration, T/E and 5a-Adiol/E were chosen for transdermal administration. The ratios' average values obtained from the baseline samples before administration of the respective study phase, plus three times the standard deviation, which correspond to a 99 % confidence interval, were used to define the individual threshold (IT) limits [1]. The ratios obtained during administration and in the post-administration samples were compared with the calculated IT. Ratios outside the IT limits were considered as affected by the T administration. DT was calculated in post-administration samples from the last administration ($t = 0$). The last value outside the IT in the post-administration samples was defined as DT. If 5 consecutive values were below the IT, followed by a single value above the IT, this elevated value was considered an outlier and was not considered for further evaluation. As baseline values were determined again before the second study phase, a return to basal values of the ratios was ensured.

Of the 12 male participants receiving oral TU (O1-O12), one (O11) was excluded from further analysis due to labelling inconsistencies during the sampling process. All participants receiving T gel (D7-D12) were included in the data analysis.

3. Results and discussion

3.1. Method validation

Validation results for the two procedures for LC-HRMS analysis of phase II metabolites of EAAS are presented in Table A.1 (Supporting information). Both the SPE method and the DS method fulfilled the criteria according to WADA guidelines [30]. Regarding selectivity, no chromatographic interferences were observed in the elution region of the target analytes. Additionally, the concentrations for AG, EtioG, TG, and EG were compared with the assigned value for A, Etio, T, and E from conventional steroid profile determination by GC-MS/MS and met the acceptance criteria. The quantitative validation parameters were satisfactory for all target analytes. Repeatability and IP ranged from 2.7 % to 6.1 % and 2.8–6.2 %, respectively. The MU, combining random and systematic error, was calculated at LOQ and 10xLOQ with results below the acceptance criteria of 30 % and 20 %, respectively. Both procedures were linear ($r2 > 0.99$) for all target analytes in the concentration ranges described. Regarding the SPE method, the LOQ of TS and ES was 0.06 and 0.05 ng/ml, respectively. The LOQ for TG and EG was 0.1 ng/ml. The method was considered robust due to the minor variations during the three days of validation measurements, and negligible carry-over was observed for all target analytes, with values below 0.1 %. Extracts were stable after reinjection of bias samples 3 days later (stored at 10 °C), where values did not differ more than ± 20 % from the nominal concentrations. As substances TG, AG, EtioG, AS and EpiAS were present at high concentrations in many samples, they were evaluated with the DS method above the following concentrations: 400 ng/ml for TG and EpiAS, and 1000 ng/ml for AG, EtioG, and AS. To ensure consistent and accurate quantification of all target analytes, an in-house quality control (QC) sample was evaluated in each analytical batch. The QC for routine determination of the conventional steroid profile by GC-MS/MS, prepared in compliance with WADA guidelines [29,30], was used. Before the analysis of the clinical study samples, QC samples were prepared and measured 10 times for the determination of the concentration of the target analytes. TG, EG, AG, and EtioG were compared with target values for T, E, A, and Etio obtained from the routine GC-MS/MS analysis, which are monitored with control charts, and were within their acceptance criteria. The mean of the 10 measurements for each target analyte was set as the target value. The results were considered

acceptable if they were within a 20 % range of the target value. Results were consistent and reliable for the monitored compounds.

3.2. Oral administration

3.2.1. General

The steroid profile determined by GC-MS/MS and phase II metabolites, including glucuronides and sulfates, determined by LC-HRMS, were measured in urine samples collected from eleven male participants before, during, and after oral TU administration.

In general, for oral TU administration, a fast and notable increase in the conventional T/E ratio is expected [2,34]. As T/E is the most sensitive biomarker of the conventional steroid profile, it was selected for comparison to phase II metabolite ratios.

The phase II metabolite target substances TG, EG, EtioG, AG, EpiAG, TS, ES, EtioS, AS, and EpiAS were present mainly with concentrations above the LOQ and could be quantified in all samples. A small number of samples of participants O2, O5, and O8 showed interferences or concentrations < LOQ for TS. A few samples of participant O10 showed EpiAG < LOQ. These substances were excluded from further evaluation in the affected samples. Possible ratios between the target substances were calculated and evaluated regarding their sensitivity to oral TU administration. After exclusion of ratios, without sensitivity, the following ratios were selected for further evaluation: the CR ((TG/TS)/(EG/ES)), and all combinations with EpiAS, AS, or EtioS in the numerator and EG, ES, or TS in the denominator. The glucuronides AG and EtioG were not considered further, as their applicability is already demonstrated by the conventional steroid profile, and no ratio combining AG or EtioG with sulfates exceeded the results obtained by conventional steroid profiling. Also, the glucuronide EpiAG was not evaluated further, as the substance was not affected by oral TU administration, and no ratio including this substance was suitable for monitoring.

3.2.2. Comparison of conventional and novel steroid profile biomarkers

In Table A.2 (Supporting information), the mean of the basal values and the corresponding relative standard deviation (RSD) used to determine the IT for the investigated ratios are presented. High intra-individual variability obtained from basal values could be observed for all phase II metabolite ratios, including the CR. RSD varied from 4.6 % to 89 %, leading to a high IT for certain substances. The variability differed for each participant, with different ratios affected. Conventional T/E showed similar variability as some of the more stable sulfate ratios, such as AS/TS or EtioS/ES.

In our study, TU was administered orally multiple times over 4 consecutive days. Samples were collected for four to five weeks. In Table 1, the number of samples affected by oral TU administration (values above the IT) is presented as a percentage of the total number of samples since the first administration. For each participant, the two ratios showing the most samples above the IT are highlighted. The ratios showing the greatest response to oral T administration among most participants are also highlighted.

All participants showed higher sensitivity of sulfate ratios compared to the conventional steroid profile biomarker T/E and combined ratios between sulfates and glucuronides. The CR was similar to or less affected by oral TU administration than T/E. Participant O10 generally shows a steroid profile different from other participants, as the basal T/E value is very low (0.12), corresponding to an Asian profile, which is most probably correlated to a UGT2B17 gene polymorphism [5,8]. Although the sensitivity of T/E and the CR is very low for this participant, the other investigated ratios do not show any differences from those of other participants. These results suggest that ratios without TG do not show reduced sensitivity to T administration in Asian steroid profiles, supporting the findings in similar studies [11,12,20]. However, as our study only included one participant with low T/E and no genotyping of the UGT2B17 deletion was conducted, differences compared to other participants cannot be related to this condition with certainty.

Most values above the IT were achieved by the ratios AS/TS, AS/ES, EpiAS/TS, EpiAS/ES, and EtioS/ES. No ratio could clearly be selected as a superior biomarker. However, sulfate ratios were found to be more affected by oral TU administration compared to conventional steroid profile biomarkers and the CR.

As examples, the ratios T/E, CR, EpiAS/TS, and AS/TS are displayed in Figs. 1 and 2 during the administration phase and the first week after administering TU orally for participants O7 and O10, respectively.

For participant O7 with a basal T/E ratio of 2.2, a sharp increase in T/E after administration of oral TU, followed by a fast decline after the last administration, can be observed, as expected [2]. Also, in the 24 h interval between TU capsule intake, ratios fall back below the IT, demonstrating the short DT for oral TU administration. The CR follows

Table 1
Number of samples affected by oral TU administration. All samples since the first administration were considered and are presented in % of the total number of samples. The overall mean of the detection sensitivity of each biomarker is calculated at the end of the table.

Ratio	O1	O2	O3	O4	O5	O6
T/E	46 %	69 %	40 %	54 %	48 %	33 %
CR	29 %	28 %	43 %	36 %	20 %	24 %
EpiAS/EG	56 %	75 %	32 %	52 %	15 %	52 %
EpiAS/ES	53 %	***78 %***	53 %	60 %	***65 %***	65 %
EpiAS/TS	65 %	65 %	66 %	52 %	17 %	***76 %***
AS/EG	60 %	***78 %***	45 %	48 %	7 %	46 %
AS/ES	65 %	***83 %***	64 %	***66 %***	***65 %***	67 %
AS/TS	***78 %***	71 %	***83 %***	***66 %***	10 %	***70 %***
EtioS/EG	60 %	74 %	49 %	44 %	28 %	52 %
EtioS/ES	***75 %***	55 %	***68 %***	***72 %***	62 %	69 %
EtioS/TS	26 %	2 %	60 %	24 %	25 %	50 %
Ratio	**O7**	**O8**	**O9**	**O10**	**O12**	**Overall detection sensitivity**
T/E	44 %	68 %	36 %	10 %	24 %	43 %
CR	38 %	49 %	33 %	6 %	25 %	30 %
EpiAS/EG	66 %	82 %	39 %	65 %	69 %	55 %
EpiAS/ES	***75 %***	90 %	***48 %***	***69 %***	***76 %***	***67 %***
EpiAS/TS	***81 %***	63 %	***48 %***	***79 %***	75 %	62 %
AS/EG	63 %	88 %	27 %	63 %	73 %	54 %
AS/ES	66 %	***95 %***	30 %	63 %	***76 %***	***67 %***
AS/TS	***75 %***	63 %	42 %	62 %	***86 %***	64 %
EtioS/EG	31 %	91 %	27 %	54 %	58 %	52 %
EtioS/ES	50 %	***95 %***	30 %	48 %	68 %	63 %
EtioS/TS	38 %	55 %	18 %	31 %	***76 %***	37 %

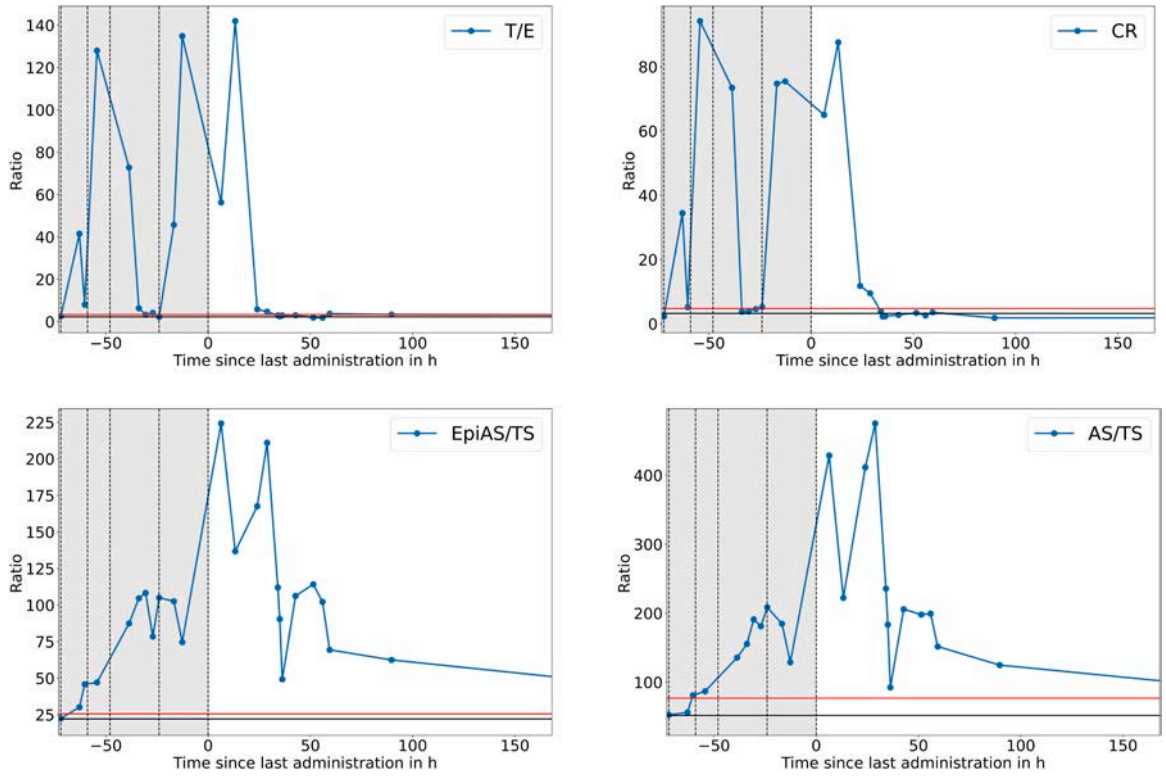

Fig. 1. Comparison of conventional T/E, CR, and two sulfate ratios for participant O7 (basal T/E = 2.2). The administration phase (grey) and the first week after the last administration of TU tablets (t = 0–168 h) are displayed. The mean of baseline values (black) and the IT (red) are displayed horizontally; administration of TU is displayed vertically (black, dotted).

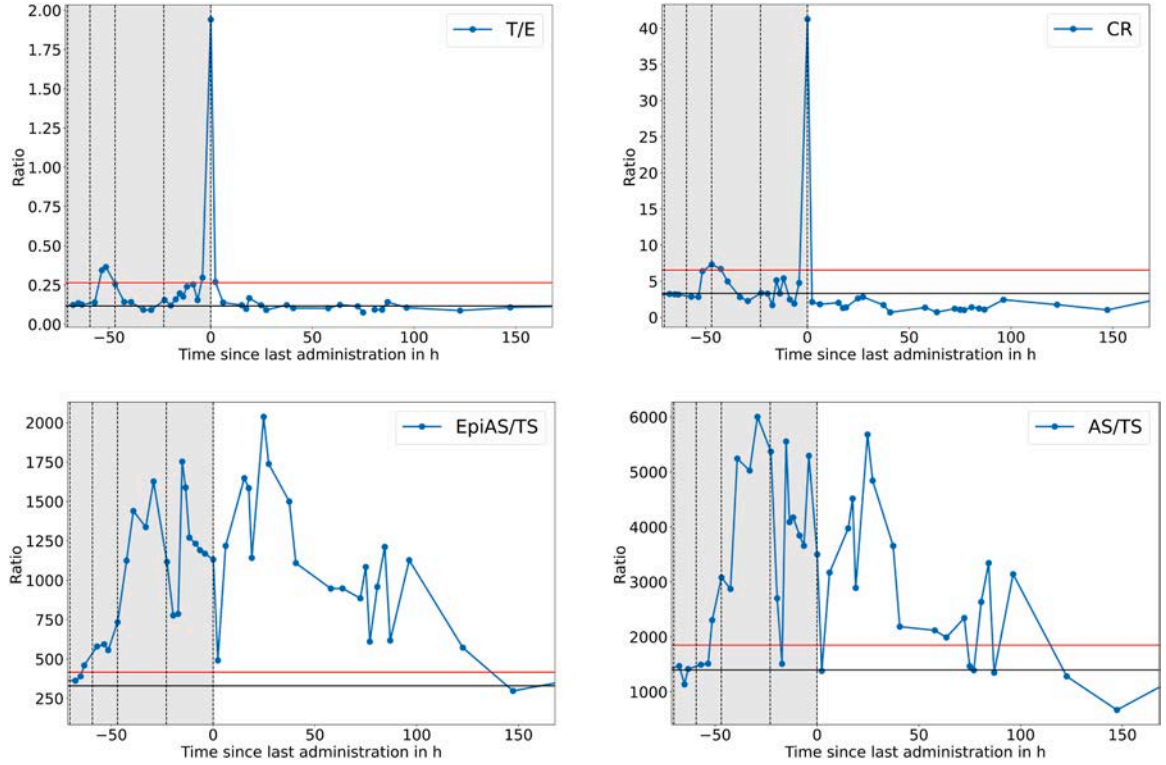

Fig. 2. Comparison of conventional T/E, CR, and two sulfate ratios for participant O10 (basal T/E = 0.12). The administration phase (grey) and the first week after the last administration of TU tablets (t = 0–168 h) are displayed. The mean of baseline values (black) and the IT (red) are displayed horizontally; administration of TU is displayed vertically (black, dotted).

this behavior, although the increase is less prominent than for the T/E. The sulfate ratios EpiAS/TS and AS/TS show a less rapid increase compared to T/E and CR. Ratios exceed the IT continuously during the administration phase and stay elevated after the last administration. The same general trends were observed for all other participants with Caucasian steroid profiles.

In comparison, the T/E ratio for participant O10, with a basal T/E ratio of 0.12, demonstrates the poor sensitivity of this biomarker for T administration for individuals showing an Asian profile [2]. Only 10 % of all samples were affected by the oral TU administration, only slightly exceeding the IT. One sample, directly collected after oral intake of the last capsules, resulted in a sharp increase in T/E, still far less prominent than in other participants, and the ratio came back below the IT after 6 h. The CR depicts the same behaviour as the T/E ratio, with even fewer values exceeding the IT (6 % of all samples). In contrast, sulfate ratios, demonstrated on EpiAS/TS and AS/TS, did not show any differences compared to participants with higher basal T/E ratios.

One of the most interesting parameters in evaluating the suitability of a biomarker for T misuse is the DT. The DT describes the timeframe in which a ratio would be highlighted as suspicious in the steroidal ABP and could therefore lead to further analysis and confirmation of a doping scenario. DTs were calculated after the last administration of TU on day 4. In Fig. 3, the DT for T/E, the CR, and the two sulfate ratios, which demonstrated the longest DT for most participants, are displayed. For participant O5, who showed very low sensitivity of ratios with TS in the denominator, the ratio EpiAS/ES providing the longest DT is depicted for comparison.

The ratio AS/TS showed the longest DT, longer than 22 days (530 h), for 7 out of 11 participants. In all cases, sulfate ratios expanded the DT compared to T/E many times over. O10 showed the shortest DTs for T/E and the CR, with sulfate ratios expanding the DT from 2 h to 23 days (555 h). The CR showed the same or shorter DTs compared to T/E for most participants; only for O6 and O12, the DT could be expanded. However, the CR was exceeded by other sulfate ratios in any case.

In general, EG and ES are known to decrease after oral T administration [16,35]. EG is secreted mainly by the testis, and its excretion rate is repressed by negative feedback of the hypothalamus-pituitary-gonadal axis exerted by the exogenous T administration. Even bigger proportions of ES, as well as TS, are secreted by the testis and are therefore expected to be suppressed as well [36]. Nevertheless, compared to parenteral administration forms of T (intramuscular and transdermal), enteral administration, as oral administration, is known to display no decrease in TS [12,37]. A possible explanation for this behavior is first-pass metabolism, occurring in the liver after oral drug intake. Although TU is known to bypass first-pass metabolism to a certain extent, the effect of extensive biotransformation in the liver is still observed [38,39]. Due to the high concentration of the orally administered drug, metabolism is most likely switching from glucuronidation to sulfation in the liver due to enzyme saturation [12,37,40]. In our study, no decrease in TS was observed, supporting the theory of sulfation occurring in the liver. Still, EpiAS/TS and AS/TS are the best biomarkers in our study, probably caused by the lower RSD of basal values for TS compared to ES and EG.

Enzymes responsible for the sulfation of steroids in the liver are cytosolic sulfotransferases (SULT), with dehydroepiandrosterone SULT conjugating 3-hydroxysteroids and T. The activity of the enzyme has been investigated by *Falany et al.* [41,42] and showed significantly higher activity towards A, Etio, and EpiA compared to T. This could be a reason why a higher increase in AS, EtioS, and EpiAS was observed compared to other sulfates, and therefore explains the valuable contribution of these substances for the detection of T administration.

3.3. Transdermal administration

3.3.1. General

Urine samples collected from six male participants before, during, and after transdermal T gel administration were evaluated by determination of the conventional steroid profile by GC-MS/MS, and phase II metabolites, including glucuronides and sulfates, by LC-HRMS.

In general, conventional steroid profile determination has proved to show low sensitivity for transdermal administration of T, leading to a low increase in concentrations and a short DT [9,24,43]. However, T/E and 5a-Adiol/E ratios are proposed as best biomarkers, showing low but noticeable sensitivity to T gel administration [16,44]. Also, 5a-Adiol/5b-Adiol or A/Etio can be affected, as 5α-metabolites are produced favorably after transdermal T administration, caused by high levels of 5α-reductase in the skin [2,45].

In our study, participants D7 and D11 showed the expected increase in 5a-Adiol/5b-Adiol or A/Etio, demonstrating the favored 5α-metabolite pathway. Others only showed an increase in 5a-Adiol/E and T/E, but stable 5a-Adiol/5b-Adiol and A/Etio ratios. As T/E and 5a-Adiol/E were most affected for all participants, these two ratios were selected for comparison to phase II metabolites and their ratios.

All phase II metabolite target substances TG, EG, EtioG, AG, EpiAG, TS, ES, EtioS, AS, and EpiAS were present with concentrations above the LOQ and could be quantified in all samples. Ratios between these substances were calculated and evaluated regarding their sensitivity to T

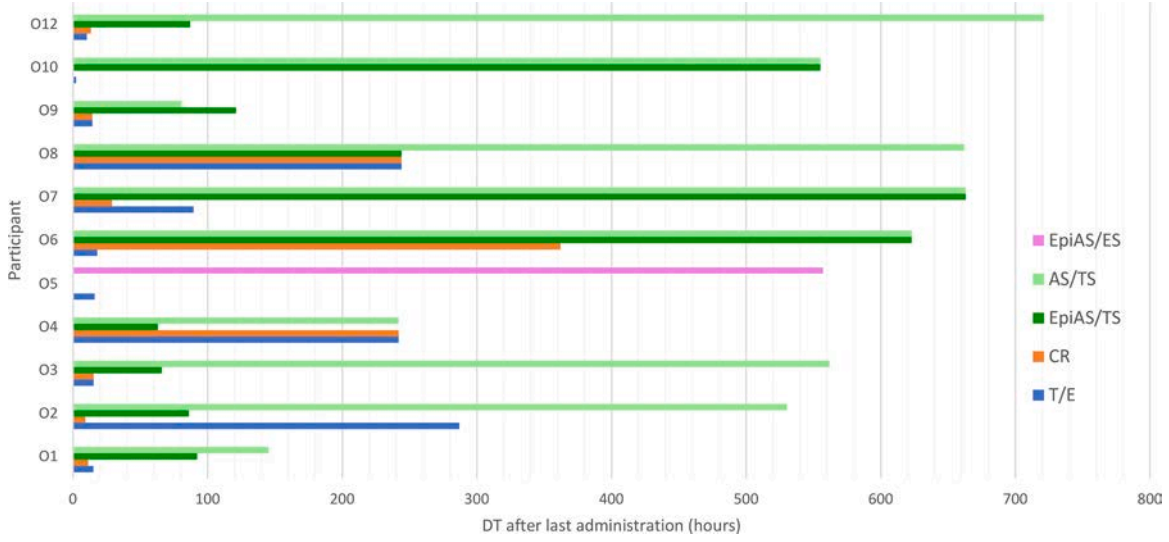

Fig. 3. DT after the last administration of TU tablets. Comparison of T/E, CR, and the sulfate ratios AS/TS and EpiAS/TS (EpiAS/ES for participant O6) for all eleven participants.

Table 2
Number of samples affected by transdermal T gel administration. All samples since the first administration were considered and are presented in % of the total number of samples. The overall mean of the detection sensitivity of each biomarker is calculated at the end of the table.

Ratio	D7	D8	D9	D10	D11	D12	Overall detection sensitivity
T/E	71 %	49 %	***39 %***	***67 %***	27 %	56 %	***51 %***
5a-Adiol/E	50 %	70 %	***39 %***	38 %	37 %	67 %	50 %
CR	88 %	5 %	***39 %***	21 %	77 %	27 %	43 %
EpiAS/EG	0 %	65 %	11 %	31 %	7 %	13 %	21 %
EpiAS/ES	0 %	81 %	0 %	44 %	37 %	31 %	32 %
EpiAS/TS	67 %	***86 %***	5 %	18 %	***90 %***	62 %	***55 %***
AS/EG	4 %	70 %	13 %	33 %	23 %	4 %	25 %
AS/ES	8 %	78 %	0 %	***51 %***	53 %	15 %	34 %
AS/TS	***92 %***	57 %	3 %	0 %	***83 %***	21 %	43 %
EtioS/EG	8 %	68 %	13 %	28 %	0 %	***85 %***	34 %
EtioS/ES	25 %	***92 %***	5 %	33 %	37 %	***79 %***	45 %
EtioS/TS	***96 %***	27 %	3 %	0 %	***83 %***	42 %	42 %

administration. After exclusion of ratios without sensitivity, the same ratios as for oral administration were selected for further evaluation: the CR ((TG/TS)/(EG/ES)), and all combinations with EpiAS, AS, or EtioS in the numerator and EG, ES, or TS in the denominator. As already described for the oral administration phase, the glucuronides AG, EtioG, and EpiAG were not considered further for the same reasons.

3.3.2. Comparison of conventional and novel steroid profile biomarkers

In Table A.3 (Supporting information), the mean of the basal values and the corresponding RSD used to determine the IT for the investigated ratios are presented. Like the basal values collected before oral TU administration, high intra-individual variability was observed for all phase II metabolite ratios (RSD between 2.6 % and 69 %). In comparison, the conventional T/E ratio was more stable, with an overall intra-individual variability of 11 % (±5 %).

In our study, multiple administration of T gel was carried out over 5 days. Samples were collected over a four-week period following the first administration. In Table 2, the number of samples affected by the transdermal T administration (values above the IT) is presented as a percentage of the total number of samples since the first administration. For each participant, the two ratios showing the most samples above the IT are highlighted. The ratios showing the greatest response to transdermal T administration among most participants are also highlighted.

Participants D7, D8, D11, and D12 showed higher sensitivity of sulfate ratios compared to the conventional steroid profile. Participant D9 showed no sensitivity of sulfate ratios in post-administration samples, including EpiAS and AS. The high variability of these ratios in baseline values (RSD 31 – 69 %) leads to a high IT, which contributes to the poor sensitivity. However, ratios including EtioS in the numerator showed lower RSD, but still, not many values exceeded the IT. The ratios 5a-Adiol/E and CR showed the lowest RSD (13 %, 14 %, respectively) for this participant, leading to a moderate number of samples exceeding the IT. Participants D10 showed moderate sensitivity of sulfate ratios. No different behavior is expected for Asian steroid profiles, as Bressan

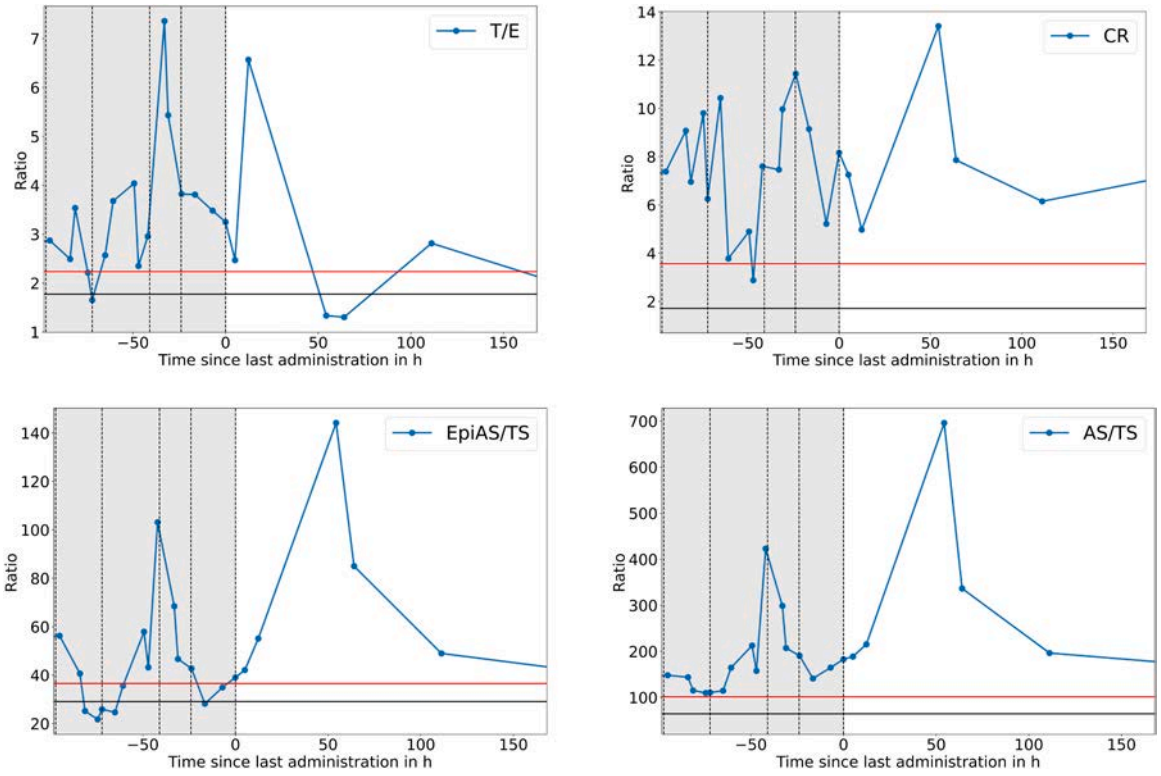

Fig. 4. Comparison of conventional T/E, CR, and two sulfate ratios for participant D7 (basal T/E = 1.8). The administration phase (grey) and the first week after the last administration of T gel (t = 0–168 h) are displayed. The mean of baseline values (black) and the IT (red) are displayed horizontally; administration of T gel is displayed vertically (black, dotted).

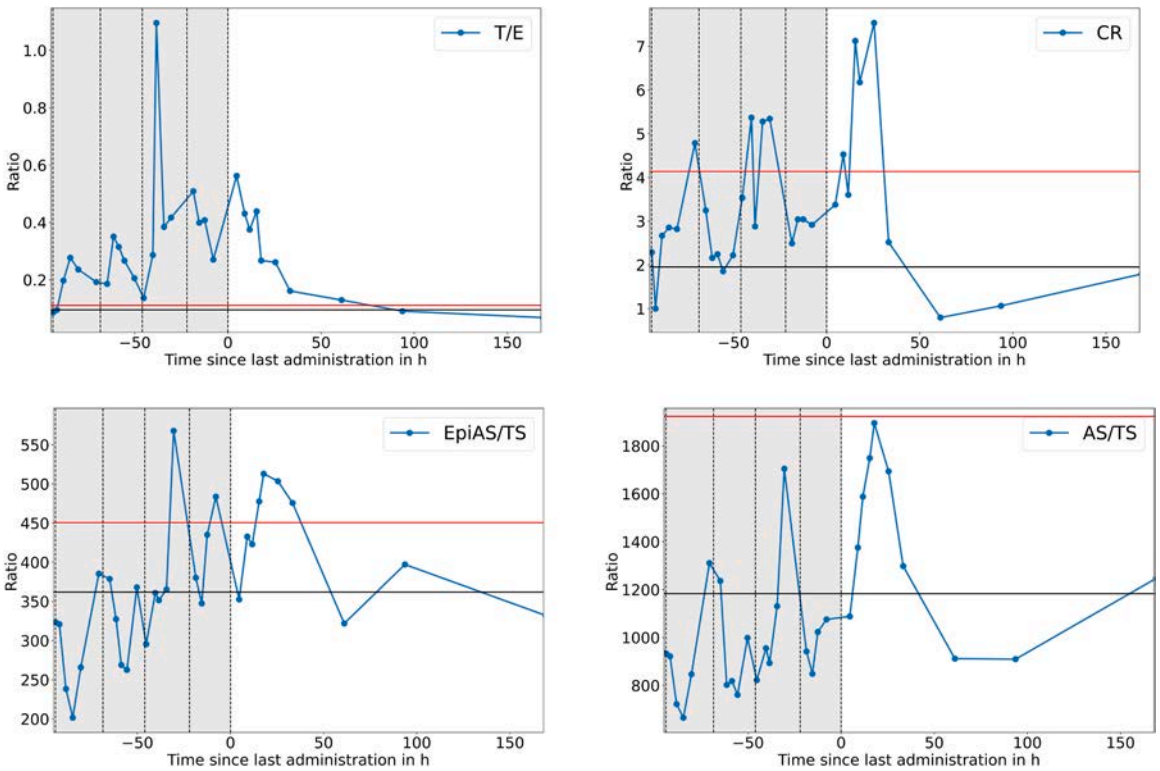

Fig. 5. Comparison of conventional T/E, CR, and two sulfate ratios for participant D10 (basal T/E = 0.10). The administration phase (grey) and the first week after the last administration of T gel (t = 0–168 h) are displayed. The mean of baseline values (black) and the IT (red) are displayed horizontally; administration of T gel is displayed vertically (black, dotted).

et al. [26] investigated sulfate ratios of Caucasian and Asian participants after transdermal T administration, showing no differences in sensitivity between ethnicities.

Most values above the IT were achieved by the ratios AS/TS, EpiAS/TS, EtioS/TS, and EtioS/ES. There was no ratio that could clearly be selected as a superior biomarker. However, sulfate ratios were found to be more affected by transdermal T administration compared to conventional steroid profile biomarkers and the CR.

As examples, the ratios T/E, CR, EpiAS/TS, and AS/TS are displayed in Figs. 4 and 5 during T gel administration and the first week after the last administration for participants D7 and D10, respectively.

For participant D7 with a basal T/E ratio of 1.8, a moderate increase in T/E could be observed after multiple T gel administrations. Compared to oral administration of TU, the increase is moderate, and values

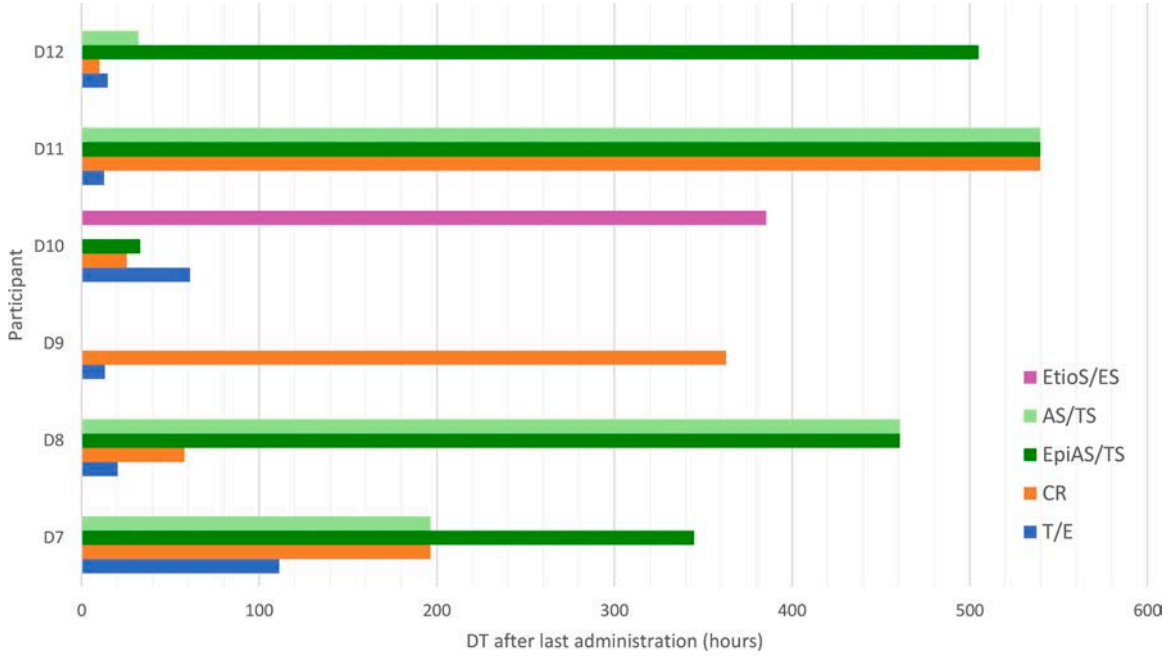

Fig. 6. DT after the last administration of T gel. Comparison of T/E, CR, and the sulfate ratios AS/TS and EpiAS/TS (EtioS/ES for D10) for all six participants.

continue to be elevated after the last administration. This behavior is expected [2], caused by the low absorption of T and the even diffusion through the skin of the administration form [24]. The other three ratios containing sulfates also showed a moderate increase during the administration phase, reaching the highest value after the last administration, up to 2.5 days later. Values stayed elevated for a longer period compared to the T/E ratio. The same general trends were observed for participants D7, D8, D11, and D12. Participant D9 showed very low sensitivity to transdermal T administration in post-administration samples.

Participant D10, with a basal T/E ratio of 0.10, also shows a moderate increase in the T/E ratio as observed in other participants. The increase is moderate, and values continue to be elevated after the last administration. However, the other ratios, including sulfates, show a less prominent increase of 1.5–2.5 x compared to other participants, which show approximately a 3–4 x increase in ratios.

For further evaluation of the applicability of the biomarkers, DTs were calculated after the last administration of T gel on day 5. In Fig. 6, the DT for T/E, the CR, and the two sulfate ratios, which demonstrated the longest DT for most participants, are displayed. For D9, only the T/E and the CR are displayed, as other sulfate ratios showed no sensitivity in post-administration samples. For participants D10, who showed low sensitivity of ratios with TS in the denominator, the ratio EtioS/ES providing the longest DT is depicted for comparison.

The ratio EpiAS/TS showed the longest DT for all participants except D9 and D10. Compared to T/E, EpiAS/TS increased the DT threefold for D7 and 20–40 times for participants D8, D11, and D12. Participant D10's DT was extended by the ratio EtioS/ES by six times compared to T/E. AS/TS also increased DT for most participants. The CR resulted in longer DTs for D7, D8, D9, and D11 compared to T/E (2x, 3x, 27x, and 42x longer, respectively), but other sulfate ratios exceeded it in all cases except for D9. The longest DT for T/E was observed in participant D7 at 4.5 days (111 h after the last administration). In contrast, the DT achieved with a sulfate ratio was a minimum of 16 days (345 h after the last administration, participant D7). This demonstrates the significant extension of the DT after transdermal administration of T.

Enzymes responsible for sulfation (SULT) are not only present in the liver, but also widely distributed in other tissues such as the adrenal glands, prostate, and small intestine, and are released directly into the bloodstream [46]. Drugs administered by parenteral routes reach systemic circulation before extensive hepatic metabolism, explaining the moderate and slow increase in ratios. Sulfation likely occurs at various sites, with DHEA-SULT, which has higher activity toward A, Etio, and EpiA compared to T, being responsible for sulfation. This explains the higher increases in AS, EtioS, and EpiAS [41,42]. EG and ES remained stable or slightly decreased during T gel administration, as expected. Similar to intramuscular administration, TS is expected to decrease via negative feedback. Sulfation of T mainly occurs in the testis, and for parenteral routes like intramuscular or transdermal, the slow-release kinetics result in minimal additional sulfation. However, a decrease in TS was only confirmed in one participant (D7). In other participants, TS did not follow a clear trend after T gel administration, showing only fluctuations. Nonetheless, the CR, which includes sulfates TS and ES, showed better sensitivity to transdermal T administration than the T/E ratio. Unlike oral administration, the CR prolonged DT in most participants, supporting the idea that the CR is a better biomarker for parenteral T administration compared to the conventional T/E ratio. Still, other sulfate ratios demonstrated higher sensitivity.

3.4. Overall applicability

The representation of values above the IT and the calculation of DTs provide valuable insights into the sensitivity and consistency of the proposed biomarkers. By combining these parameters, ratios obtained from combining EpiAS, AS, and EtioS in the numerator and TS and ES in the denominator demonstrated a significant improvement in detection compared to the conventional steroid profile biomarker T/E. Especially, the ratios AS/TS and EpiAS/TS showed great potential to be included in future steroid profiling. The CR did not show great improvements in sensitivity, as it was proposed after intramuscular administration of T [18,19], which differs from the administration routes examined in this study. The evaluation of phase II metabolites of EAAS after oral and transdermal administration of T revealed important metabolic differences between enteral and parenteral administration methods. Specifically, the enteral administration of TU as tablets resulted in a rapid increase in drug concentration, with liver metabolism playing a significant role. In contrast, the parenteral administration of T gel demonstrated a slower, more sustained drug release through the skin, leading to lower peak concentrations and a more systemic metabolic pattern. Despite the higher intra-individual variability of the phase II metabolite biomarkers —and consequently a higher IT—the described ratios have shown clear advantages over conventional steroid profile biomarkers. Interestingly, each participant exhibited some ratios with low variability and others with high variability. Although this variability is highly individual-dependent, monitoring a diverse set of biomarkers enhances the reliability of this novel approach. The results of this T administration study confirm the findings of recent studies [20,26], where ratios containing the biomarkers AS, EpiAS, and EtioS in the numerator are also proposed to be monitored in the steroidal ABP. These results underscore the potential of these novel biomarkers to improve the detection and monitoring of steroid use in sports.

4. Conclusions

In this study, conventional steroid profile biomarkers were compared with the CR and various ratios of phase II metabolites of EAAS for detecting oral and transdermal T administration. Sulfate ratios, particularly EpiAS/TS and AS/TS, showed their potential for inclusion in future steroid profiling. Also, other ratios, including AS, EpiAS, and EtioS in the numerator and TS, ES, and EG in the denominator, are proposed to be monitored in the steroidal ABP. Although the CR did not reveal significant improvements like those observed for intramuscular administration in other studies, it was shown that the CR is more sensitive to parenteral T administration, as it extended the DT for more participants after transdermal administration compared to oral TU administration. Results indicate high variability in sulfate concentrations among most participants and no clear indication of a universal biomarker, confirming previous findings and highlighting the heterogeneity in metabolism among participants. Until now, phase II metabolites of EAAS, particularly sulfates, have been studied in male participants of different ethnicities and after various administration forms. However, additional data is needed before these promising biomarkers can be implemented in urinary steroid profiling. A key area for further research is investigating phase II metabolites of EAAS after T administration in female participants. The second part of this study will describe oral and transdermal T administration in women. Incorporating these biomarkers into the steroidal ABP could provide an additional layer of evidence in challenging T doping cases. In particular, for cases with inconclusive results, these novel biomarkers could enhance the interpretation of conventional steroid profiles, potentially triggering further sample analysis, additional testing, or deeper investigations into athletes.

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CRediT authorship contribution statement

Günter Gmeiner: Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition,

Conceptualization. **Sandra Pfeffer:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Marija Andjelkovic:** Resources, Project administration, Methodology, Investigation, Conceptualization. **Nenad Dikic:** Resources, Project administration, Methodology, Investigation, Conceptualization. **Guro Forsdahl:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Gmeiner, Günter reports financial support was provided by World Anti-Doping Agency. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jsbmb.2025.106870.

Data availability

Data will be made available on request.

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