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Quantification of endogenous steroid sulfates and glucuronides in human urine after intramuscular administration of testosterone esters

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ABSTRACT

For an effective detection of doping with pseudo-endogenous anabolic steroids, the urinary steroid profile is of high value. In this work, the aim was to investigate steroid metabolism disruption after exogenous intramuscular administration of different testosterone esters. The investigation focused on both sulfo – and glucoro conjugated androgens. A single intramuscular injection of either 1000 mg testosterone undecanoate (Nebido®) or a mixture of 30 mg testosterone propionate, 60 mg testosterone phenylpropionate, 60 mg testosterone isocaproate, and 100 mg testosterone decanoate (Sustanone®), was given to six healthy volunteers. Urine was collected throughout a testing period of 60 days. A LC-MS method was developed and validated for the analysis of eight conjugated steroids in their intact form. The results show that urinary changes in both sulfo – and glucuro conjugated steroid levels are prominent after the injection of testosterone esters. A promising potential marker for the intake of exogenous testosterone is the combined ratio of epitestosterone sulfate/epitestosterone glucuronide to testosterone sulfate/testosterone glucuronide ((ES/EG)/(TS/TG)) as a complementary biomarker for testosterone abuse. This represents a new piece of evidence to detect testosterone doping, representing a new approach and being independent from the metabolic connections of the markers in the steroid passport.

1. Introduction

The most frequently banned substances detected in anti-doping laboratories are anabolic steroids [1]. The intake of anabolic steroids alters the endogenous steroid profile, especially when pseudo-endogenous steroids like testosterone or testosterone derivatives are used. Hence, urinary steroid profiling is a powerful tool to detect the misuse of pseudo-endogenous anabolic steroids in sports [2]. Today, the profile consists of concentrations and ratios of various endogenously produced steroidal hormones, as well as their precursors and metabolites [3]. In 2014, the steroidal module of the Athlete Biological Passport (ABP) was implemented in doping control procedures [4,5]. This includes a longitudinal follow up of the individual athlete's steroid profile, which increases the sensitivity for detecting alterations, compared to the use of solely population reference values.

Most anabolic steroids are extensively metabolized in the human body [6,7]. The metabolism includes phase I, as well as phase II transformations, and most endogenous steroids are excreted in urine in conjugated form. Two major pathways are involved in the conjugation; one leads to the formation of glucuronides and requires uridine diphosphoglucuronic acid (UDPGA) and a glucuronyl transferase. The other pathway leads to the formation of sulfates and a family of enzymes called sulfotransferases (SULTs) mediates the reaction. SULTs catalyze the transfer of sulfonate ($-SO_3^-$) from the universal sulfonate donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to the hydroxyl or amino group of an acceptor molecule [8]. The subfamilies of the cytosolic SULTs are responsible for the conjugation of steroids.

Today, the steroid profiling mainly relies on the analysis of glucuronide metabolites. After the selective hydrolysis of the glucuronide moiety, the corresponding free steroid is analysed by gas chromatography – mass spectrometry (GC–MS) subsequent to silylation of hydroxyl – and keto-groups [2,3]. The inclusion of sulfate metabolites has previously been difficult due to a non-efficient hydrolysis to the corresponding phase I metabolites. Consequently a potenially important

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Table 1
Validation data for TS, ES, AS, EtioS, TG, EG, AG, and EtioG.

	TS	ES	AS	EtioS	TG	EG	AG	EtioG
Linearity (r^2)	0.9995	0.9994	0.9992	0.9982	0.9992	0.9992	0.9927	0.9984
LOQ (ng/ml)	0.4	0.4	9	8	1	0.6	30	29
Recovery (%)	99	99	99	99	99	99	99	98
Carry-over (%)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Repeatability (RSD%)								
Calibration level 1	4.0	4.2	2.8	3.5	3.9	5.2	3.3	3.8
Calibration level 3	1.0	0.9	3.4	1.0	1.4	1.8	1.9	1.3
Inter-assay precision (RSD%)								
Calibration level 1	9.6	9.4	7.1	8.0	9.5	10.6	11.3	10.3
Calibration level 3	3.1	3.3	3.2	2.4	3.2	3.8	3.1	3.1
Robustness (RSD%)								
Precision	0.6	1.0	0.8	0.7	1.5	1.8	2.7	1.3
Retention time	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Accuracy (% recovery)								
Calibration level 3	96	97	103	100	99	100	101	101

piece of information about alterations in the steroid metabolism after testosterone doping is not monitored so far. However, during the last years, the direct analysis of phase II steroid metabolites by liquid chromatography – mass spectrometry (LC-MS) has been described in numerous reports [9–23].

Although the current strategy in steroid profiling represents an efficient detection of doping with endogenous steroids, there are still challenges in revealing for instance doping with some low-dose administration forms. Hence, additional approaches are needed to improve the detection capabilities within the steroidal passport.

The aim of this study was therefore to explore the impact of endogenous sulfate steroid metabolites on the steroid profile after the intramuscular administration of a single dose of testosterone esters to six healthy volunteers. We wanted to investigate how both android sulfates and android glucuronides are affected by the intake of exogenous testosterone, including the ratios between them. For this purpose, a fully validated LC-MS method for the simultaneous determination of 8 different sulfate- and glucuronide-conjugated steroids was developed and applied. The steroids included in the study were: testosterone glucuronide (TG), epitestosterone glucuronide (EG), androsterone glucuronide (AG), etiocholanolone glucuronide (EtioG), testosterone sulfate (TS), epitestosterone sulfate (ES), androsterone sulfate (AS), and etiocholanolone sulfate (EtioS).

2. Experimental

2.1. Chemicals and reagents

Reference conjugated steroid analytes and internal standards were purchased from The National Measurement Institute (NMI) (Sydney, Australia), except D5-AG, which was synthesized at the Vienna University of Technology (Vienna, Austria). All steroid solutions were prepared in isopropanol and stored at −20 °C. Working solutions were obtained by diluting stock solutions. Water and acetonitrile were of HPLC grade and was provided by Biosolve Chimie (Dieuze, France). Methanol of HPLC grade was obtained from Chem Lab (Zedelgan, Belgium) and Formic acid (99%, ULC/MS-CC/SFC) was purchased from Bisolve (Valkenswaard, The Netherlands). 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). SPE Oasis HLB cartridges (60 mg, 30 μm particle size) was obtained from Waters (Milford, MA, USA).

An internal standard (IS) solution was prepared in methanol with the following concentrations: 1.8 μg/ml D_3-TG, 1.8 μg/ml D_4-EG, 56 μg/ml D_5-AG, 51 μg/ml D_5-EtioG, 0.7 μg/ml D_3-TS, 0.7 μg/ml D_3-ES, 15 μg/ml D_4-AS, and 15 μg/ml D_5-EtioS. The solution was stored in the dark at −20 °C.

2.2. Sample preparation

A loading solution for solid phase extraction (SPE) was prepared by adding 25 μl of the IS solution to 2 ml of urine. The extraction was performed on Oasis HLB cartridges (60 mg, 30 μm particle size) and carried out as follows: cartridges were first conditioned with 2 ml of methanol and equilibrated with 2 ml of water. The loading solution was added, followed by washing with 2 ml of water. Finally, the analytes were eluted with 2 ml of methanol. The eluate was evaporated to dryness and reconstituted with 150 μl Acetonitrile/Water (20:80, v/v).

2.3. Instrumentation

The analysis was carried out on a Q Exactive orbitrap mass spectrometer coupled to a Vanquish Horizon UHPLC System (Thermo Fisher, Austin, TX, USA). The system was equipped with an autosampler with a temperature controlled sample tray set at 20 °C and a column oven set at 25 °C. Chromatographic separation was performed using a Phenomenex Kinetex C18 column (150 × 2.1 mm i.d., 1.7 μm particle size). The mobile phase was composed of water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). A gradient elution program was employed at a constant flow rate of 0.3 ml/min with solvent B starting at 25% for 2 min; 2–12.5 min, 25–39% B; 12.5–13.5 min, 39–98% B; 13.5–15 min, 98% B. The column was finally re-equilibrated at 25% B for 3 min.

The mass spectrometer was equipped with a heated electrospray ionization (HESI) source, operated in the negative mode. Source parameters were as follows: spray voltage 2.8 kV, capillary temperature 320 °C, sheath gas and auxiliary gas (nitrogen): 30 and 10 arbitrary units, respectively. The instrument was operated in the FS mode from m/z 100–700 at 70 000 resolving power.

2.4. Validation

Validation of the analytical assay was carried out in compliance with the International Standard for Laboratories (ISL) of the World Anti-Doping Code [24]. The matrix used for the validation study and for the preparation of calibration curves was an artificial urine prepared in the laboratory (2.5 g/L potassium dihydrogen phosphate, 9.0 g/L sodium chloride, 2.5 g/L di-sodium hydrogen phosphate, 3.0 g/L

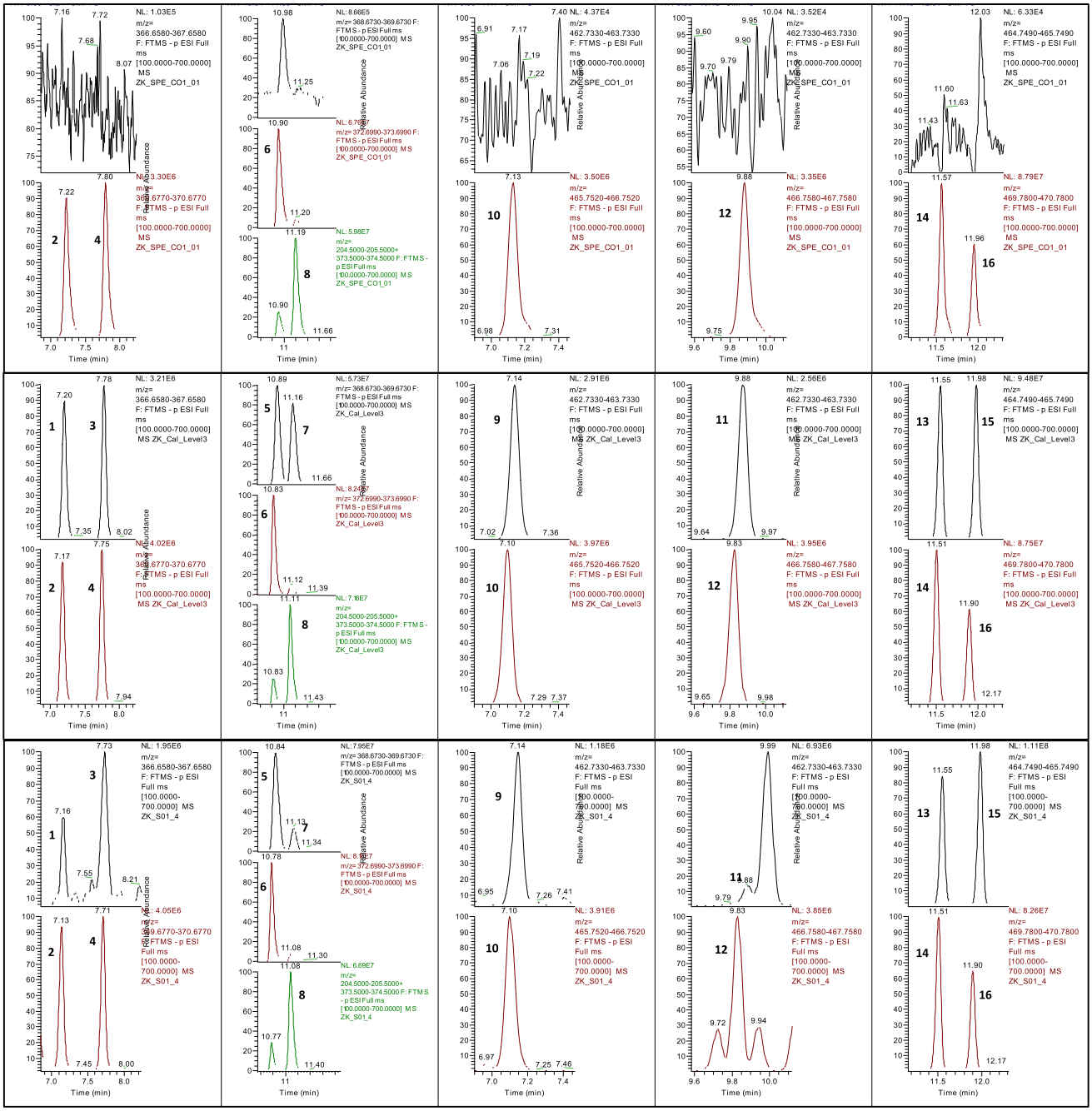

Fig. 1. UPLC-MS extracted chromatograms of A: Extracted blank artificial urine sample, B: Extracted spiked artificial urine sample (level 3), C: Extracted urine sample collected 1 h after intramucular administration of Sustanon®. 1: Testosterone sulfate (TS), 2: D_3-Testosterone sulfate (D_3-TS), 3: Epitestosterone sulfate (ES), 4: D_3-Epiestosterone sulfate (D_3-ES), 5: Androsterone sulfate (AS), 6: D_4-Androsterone sulfate, 7: Etiocholanolone sulfate (EtioS), 8: D_5-Etiocholanolone sulfate (D_5-EtioS), 9: Testosterone glucuronide (TS), 10: D_3-Testosterone glucuronide (D_3-TG), 11: Epitestosterone glucuronide (EG), 12: D_4-Epitestosterone glucuronide, 13: Androsterone glucuronide, 14: D5-Androsterone glucuronide (D_5-AG), 15: D5-Etiocholanolone glucuronide (EtioG), 16: D_5-Etiocholanolone glucuronide (D_5-EtioG).

ammonium chloride, 25 g/L urea, and 2 g/L creatinine, prepared in water).

The calibration was established over the range of 1–600 ng/ml for TG, 0.6–372 ng/ml for EG, 0.5–300 ng/ml for TS and ES, 30–3000 ng/ml for AG and EtioG, and finally 10–6000 ng/ml for AS and EtioS. Each calibration curve consisted of seven calibration levels. The calibration curves were built from the peak area ratio of the conjugated steroids versus the deuterated internal standard. The limit of quantification (LOQ) was determined as the lowest calibration point for which an acceptable precision was tested and proven.

Matrix effects were assessed by comparison of the slopes of the calibration curves of artificial urine standards with the slopes of the calibration curves of standards in water. In addition, chromatograms generated from urine samples were visually inspected for interfering signals, in order to evaluate matrix interferences and specificity.

Repeatability was evaluated at two different concentration levels (level 1 and level 3) with 10 independent spiked artificial urine samples on each level (Level 1 (ng/ml); TS: 0.4, ES: 0.4, AS: 9, EtioS: 8, TG: 1, EG: 0.6, AG: 30, EG: 29. Level 3 (ng/ml); TS: 8, ES: 8, AS: 189, EtioS: 157, TG: 20, EG: 12, AG: 600, EG: 573). The analysis was performed on the same day. Inter-day precision was performed by repeating the experiment on three consecutive days.

Table 2
Statistical significance of tested parameters in the clinical study.

	Wilcoxon signed-rank test: Base-line values compared to post administration values (11 days)	Direction of change
Testosterone sulfate (TS)	significant change	decrease
Epitestosterone sulfate (ES)	significant change	decrease
Androsterone sulfate (AS)	–	–
Etiocholanolone sulfate (EtioS)	–	–
Testosterone glucuronide (TG)	significant change	increase
Epitestosterone glucuronide (EtioG)	significant change	decrease
Androsterone glucuronide (AG)	–	–
Etiocholanolone glucuronide (EtioG)	–	–
TG/EG	significant change	increase
TS/TG	significant change	decrease
ES/EG	significant change	increase
AS/AG	–	–
EtioS/EtioG	–	–
AG/TG	significant change	decrease
AS/TS	significant change	increase
(ES/EG)/(TS/TG)	significant change	increase

Accuracy was assessed by comparing the theoretical amount of analyte to the actually quantified value in the repeatability studies.

To ensure that the developed method was robust to small variations in various sample – and preparation conditions, robustness was tested by extracting artificial urine samples at different pH values (pH 5, pH 7, and pH 9). Each variation was tested on two different concentration levels (level 1 and level 3).

Carry-over during instrumental analysis was evaluated by injecting a high concentrated artificial urine sample prior to the injection of two consecutive blank water samples.

Extraction recoveries were investigated in urine samples by comparing responses of deuterated internal standards (D_3-TG, D_4-EG, D_5-AG, D_5-EtioG, D_3-TS, D_3-ES, D_4-AS, D_5-EtioS) that were spiked in urine samples before and after the extraction (25 µl of the IS solution).

2.5. Clinical study design

The study was an open-label, parallel group investigation with six healthy volunteers. All participants were male, in good health, and submitted to a clinical evaluation before they were included in the study. Written consent was obtained, allowing the use of collected samples for research purposes.

Each volunteer was randomly assigned to either a single intramuscular injection of testosterone undecanoate 1000 mg (Nebido®) or a single intramuscular injection of a mixture of four testosterone esters: testosterone propionate 30 mg, testosterone phenylpropionate 60 mg, testosterone isocaproate 60 mg and testosterone decanoate 100 mg (Sustanon®). Three subjects were assigned to each study group.

The collection of urine samples was performed predose (3 samples; day -2, day -1 and day 0) and at the following times after dose administration: 1, 4, 8 and 12 h (day 0), followed by sampling at day 1, 2, 3, 4, 5, 6, 8, 11, 15, 18, 22, and 60.

The study was approved by the Local Review Board and Ethics Committee of Children's Hospital Srebrnjak (Zagreb, Croatia) and the Croatian Central Ethics Committee, and conducted according to the Helsinki declaration.

2.6. Data analysis

All urinary concentrations were corrected for specific gravity [3]. Distribution of data was assessed using the normality tests Shapiro-Wilks and Kolmogorov-Smirnov. Furthermore, histograms were used to evaluate model assumptions. Non-parametric Wilcoxon signed-rank test was used to assess changes after treatment with testosterone esters. Base-line values (average of three values), recorded before the testosterone ester application, were compared to post-administration values. Post-administration values were recorded from the samples collected during the first 11 days subsequent to drug administration (average of twelve values). A *p*-value below 0.05 was regarded as statistically significant. Data analysis were performed using SPSS statistical software version 25 (IBM Corporation, New York, USA) and Xcalibur (Thermo Fisher, Austin, TX, USA)

3. Results and discussion

3.1. Validation

The method validation performance criteria were evaluated by assessing linearity, LOQ, specificity, matrix effects, repeatability (% RSD), inter-assay precision (% RSD), accuracy (as % recovery of theoretical value), carry-over (%), and extraction recovery. In general, the assay validation demonstrated good performance for all tested analytes. The results are summarized in Table 1.

All calibration curves had a coefficient of determination (R^2) > 0.99, which indicated excellent linearity within the respective calibration range for each validated compound. A narrower calibration range was applied for AG and EtioG, compared to AS and EtioS, due to a lower linear range. LOQ was between 0.4 ng/ml and 30 ng/ml. The assay showed good specificity with no interfering peaks observed at the expected retention times of TS, ES, AS, EtioS, TG, AG, and EtioG. An interfering peak was, however, observed close to the EG retention time in urine. Nevertheless, the quantification could be carried out with sufficient accuracy and precision, and the values matched corresponding values generated by previous GC–MS measurements. By comparing slopes of the calibration curves from calibration standards in artificial urine and water, only a slight to moderate variation could be observed. Nevertheless, all calibrations in the study were performed in artificial urine and exclusively isotopically labelled internal standards (IS) were utilized for the quantification. The calculated values for repeatability and inter-assay precision are within acceptable ranges for all substances. Furthermore, the method demonstrated sufficient accuracy, no sample carry over and excellent recoveries. Extracting samples with different pH-values yielded low RSD variation in measured urine concentrations, as well as in retention times (RT). Hence, robustness of the procedure is ensured.

In Fig. 1, extracted ion chromatograms generated during the validation procedure, as well as during the analysis of urine samples from the clinical study, are shown. They represent a blank artificial urine

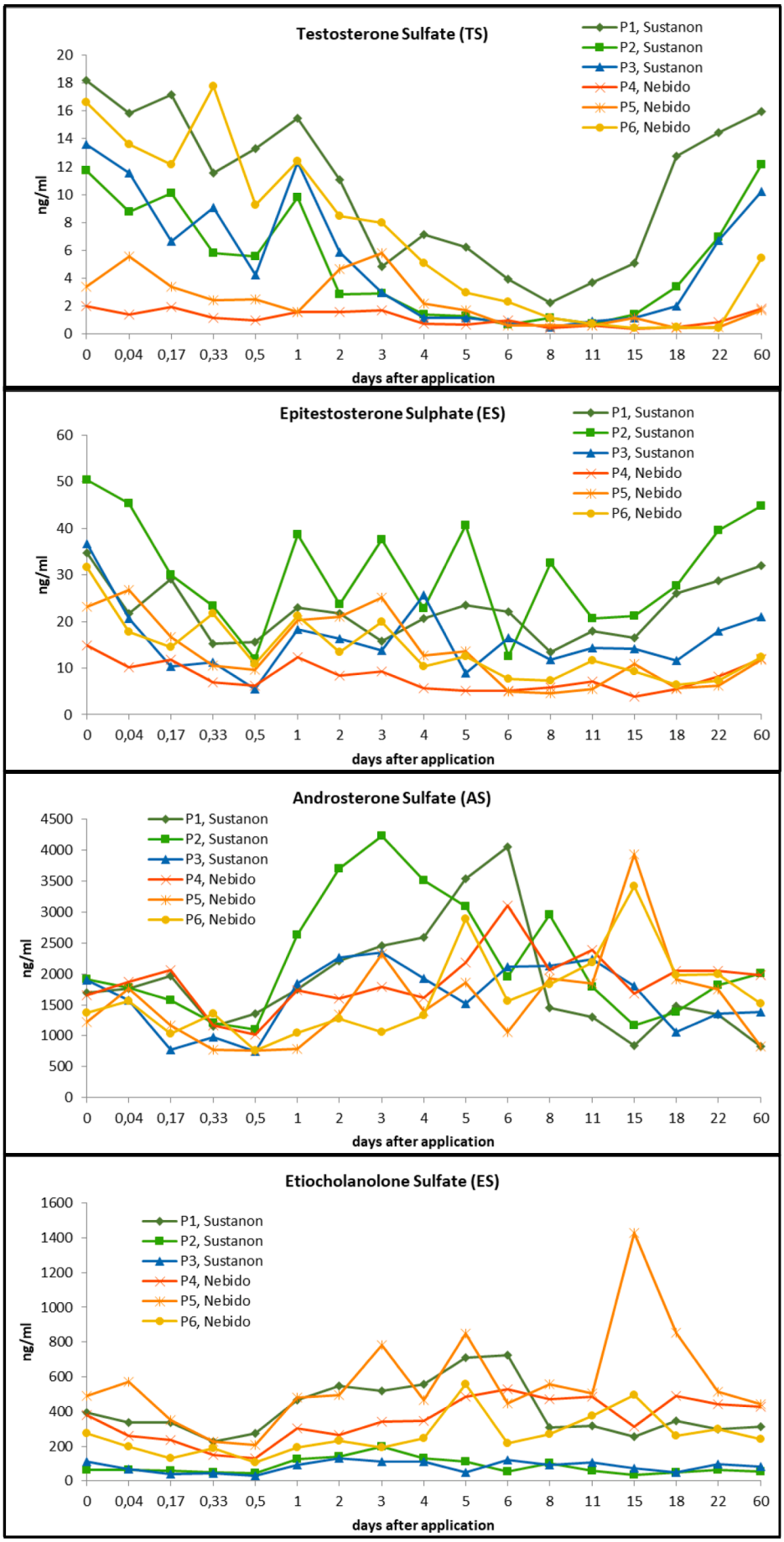

Fig. 2. Urinary androgen sulfate excretion during 60 days after administration of Sustanon or Nebido. P1: Participant 1, P2: Participant 2, P3: Participant 3, P4: Participant 4, P5: Participant 5, P6: Participant 6.

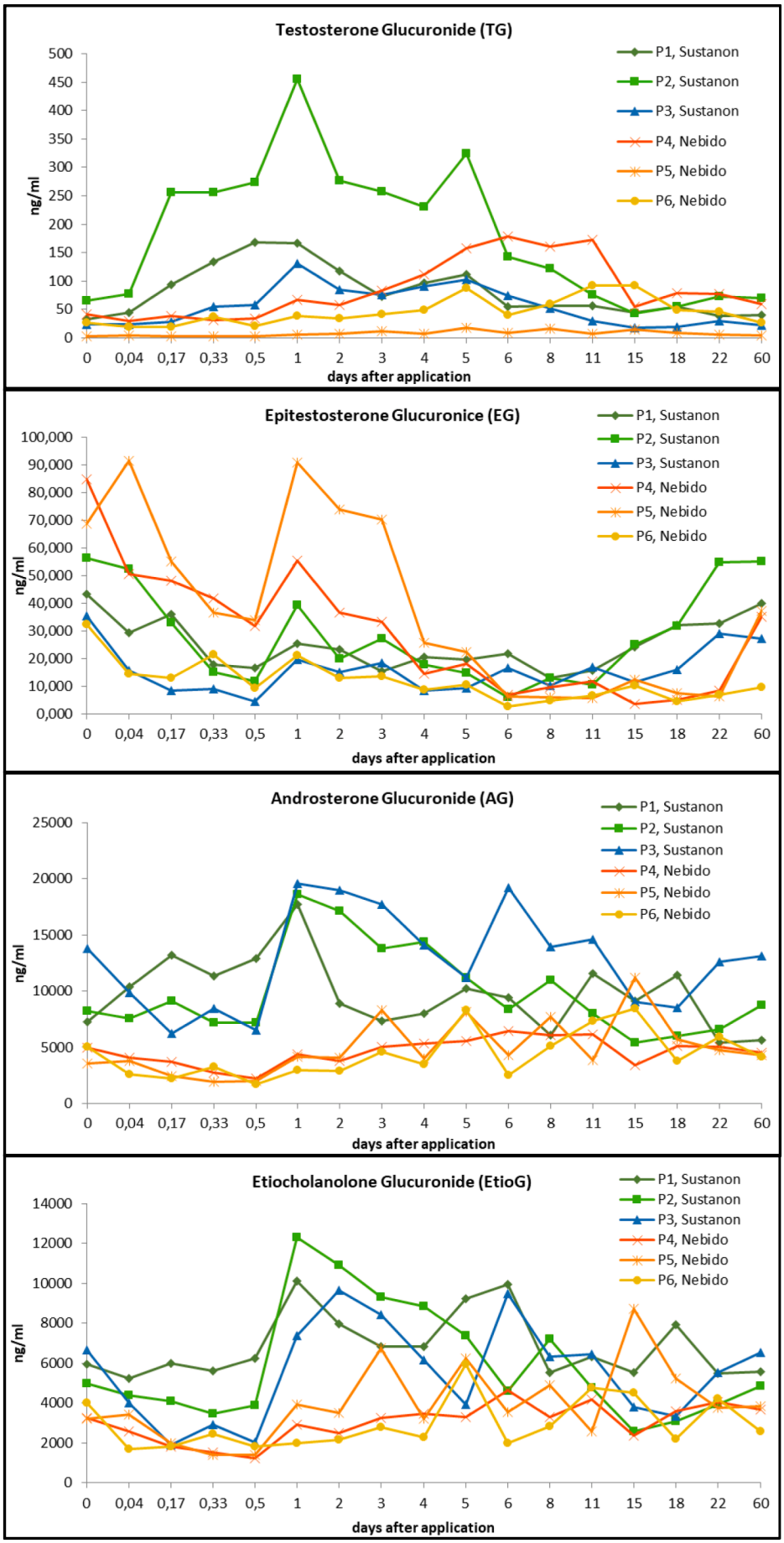

Fig. 3. Urinary androgen glucuronide excretion during 60 days after administration of Sustanon or Nebido. P1: Participant 1, P2: Participant 2, P3: Participant 3, P4: Participant 4, P5: Participant 5, P6: Participant 6.

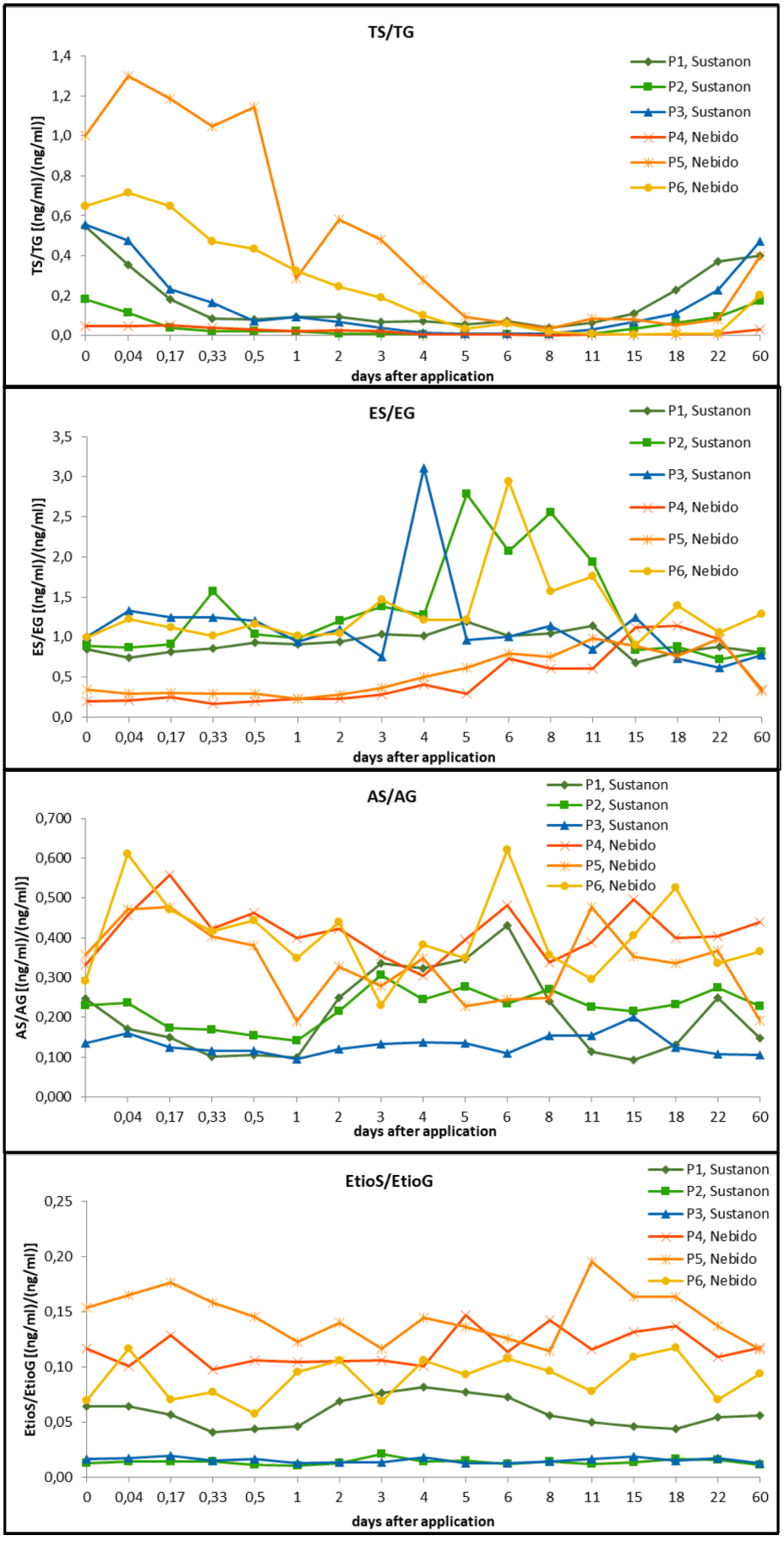

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Fig. 4. Urinary androgen conjugate excretion ratios during 60 days after administration of Sustanon or Nebido. TS: Testosterone sulfate, TG: Testosterone glucuronide, ES: Epitestosterone sulfate, EG: Epitestosterone glucuronide, AS: Androsterone sulfate, AG: Androsterone glucuronide, EtioS: Etiocholanolone sulfate, EtioG: Etiocholanolone glucuronide.

sample, a spiked artificial urine sample at calibration level 3, and a urine sample from the clinical study. In conclusion, the analytical assay proved to be fit for purpose.

3.2. Steroid profiles

Intramuscular administration of testosterone esters resulted in numerous changes in the steroid profile. This includes both ratios and concentrations of conjugated steroids. The investigated parameters were the urinary concentrations of TG, EG, AG, EtioG, TS, ES, AS and EtioS, in addition to the ratios TG/EG, TS/TG, ES/EG, AS/AG, EtioS/EtioG, AG/TG, AS/TS, and finally the combined ratio (ES/EG)/(TS/TG). Altogether, 10 of the investigated parameters showed significant changes after administration of testosterone. A significant increase was observed for the urinary levels of TG, TG/EG, (ES/EG)/(TS/TG), and AS/TS, whereas the urinary levels of EG, TS, ES, TS/TG, and AG/TG were significantly decreased. The results are summarized in Table 2. In Figs. 2–5, the excretion profiles for all tested conjugated steroids, in addition to ratios are illustrated. The baseline values are average values from the three urine samples collected before the administration of testosterone esters.

The changes observed for the urinary levels of the measured glucuronides are expected [2,25,26]. In abusers of testosterone, the concentration of TG in urine is normally increased. EG, on the other hand, is not a metabolite of exogenous testosterone and it is not unusual to see a diminished excretion by negative feedback. Hence, the testosterone/epitestosterone ratio of the combined free and glucuronide fractions is one of the most important markers of testosterone abuse in doping analysis today [3,27]. The excretion of free testosterone and epitestosterone in urine is negligible, thus the ratio TG/EG is equivalent to the ratio applied in routine doping analysis. Not surprisingly, this ratio increased significantly in our study. Furthermore, the testosterone metabolites AG and EtioG showed an increase in urinary excretions after the administration of Sustanon®, but not for Nebido®. The reason for this is that these two preparations have quite different absorption characteristics. The depot effect of the testosterone ester preparations increases in proportion to the length of the ester side chains [28]. Sustanon® is a mixture of fast – and long acting testosterone esters. Nebido®, on the other hand, only contains the long-acting ester testosterone undecanoate. Hence, a peak in AG and EtioG concentrations could not be detected. Not surprisingly, however, more prominent changes were observed for the AG/TG ratio, which significantly decreased.

The aim of this study was to explore the implementation of sulfate androgen metabolites in the steroid profile. One of the most abundant changes in sulfate metabolites could be observed in the concentration levels of TS. Interestingly, the levels decreased after intramuscular administration of the preparations. This can most likely be explained by a low sulfatation of exogenous testosterone in the human body. The reason is that endogenous testosterone is mainly sulfated by subfamilies of SULTs that are present in the testis, which is the main production site for testosterone in the male human body. It has previously been shown that approximately 95% of TS and 70% of ES are of testicular origin [29]. The decreased concentration levels of TS might be due to a feedback inhibition of luteinizing hormone (LH), initiated by the exogenous testosterone intake. This has also been suggested by Schulze et al in 2011 who examined androgen sulfatation in association with gene polymorphism [30]. Like in our study, they also observed a decrease in TS levels subsequent to an intramuscular administration of a testosterone ester.

In another study, though, comprising a single oral dose of testosterone undecanoate, Esquivel et al reported an increase of urinary TS concentration values [12]. This result is contrary to our observation. Nevertheless, a crucial difference between the two studies is the route of administration. In our study, all testosterone esters were administered as intramuscular injections, whereas in the study of Esquivel et al, testosterone undecanoate was given orally. By oral administration, drugs are absorbed from the gastrointestinal tract and pass via the portal vein to the liver where some drugs are metabolized (first pass metabolism). During the gastrointestinal absorption of a drug, the concentration in the liver is high. Hence, liver passage and first pass metabolism might be one explanation for the different observations. In the case of oral administration, the metabolism could switch towards sulfatation due to saturation processes in the liver caused by the high concentration of the drug.

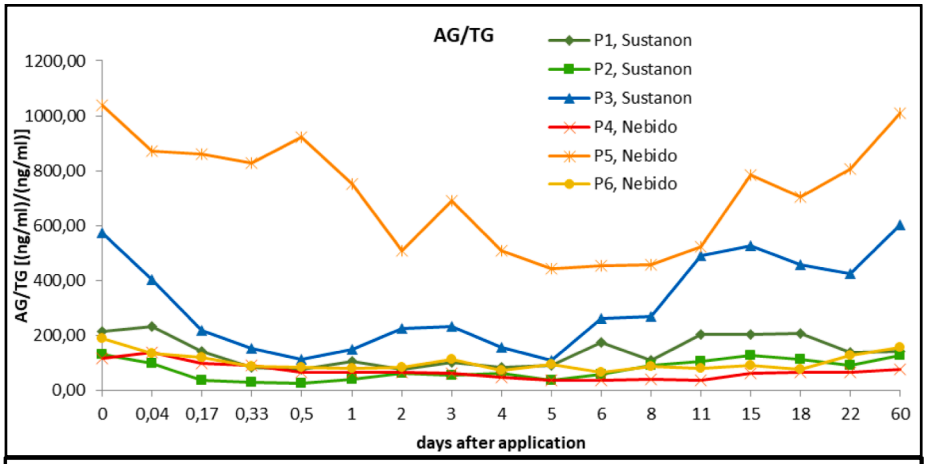
In our study, both EG and ES significantly decreased subsequent to testosterone administration. Already in 1996, Dehennin et al proposed to include ES in the steroid profile in sports drug testing. They suggested that the ratio of TG and total epitestosterone (glucuronides and sulfates) would better discriminate between physiologically high and pharmacologically high T/E-ratios [31]. It was proposed that naturally low levels of EG could be attributed to a dysregulation in the secretion of epitestosterone by the testis which implies that the secretion of EG is decreased and the secretion of ES is normal or increased [32]. An alternative explanation was suggested to be a deficiency of a specific sulfatase, leading to less circulating epitestosterone available for hepatic glucuronidation [32].

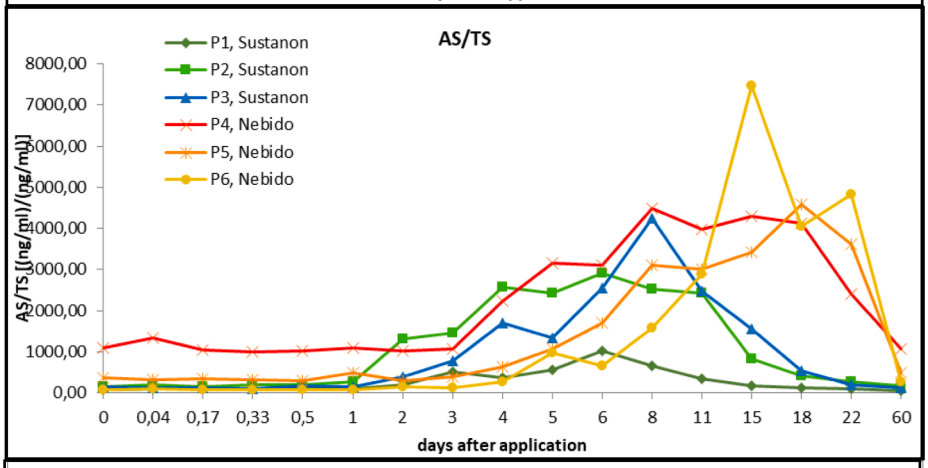
Nevertheless, in steroid profiling, steroid ratios seem to be superior to steroid concentrations. In this study, a number of ratios were explored. After intramuscular administration of testosterone esters, the most abundant decrease was seen in the ratio TS/TG, in addition to an increase in the well characterized ratio of TG/EG. As already outlined, it seems to be a shift towards glucuronidation of testosterone. Furthermore, as already mentioned, a decrease in the urinary excretion of ES, as well as EG, was observed. Since the ratio ES/EG is decreased, the suppression of EG seems to be more pronounced than ES.

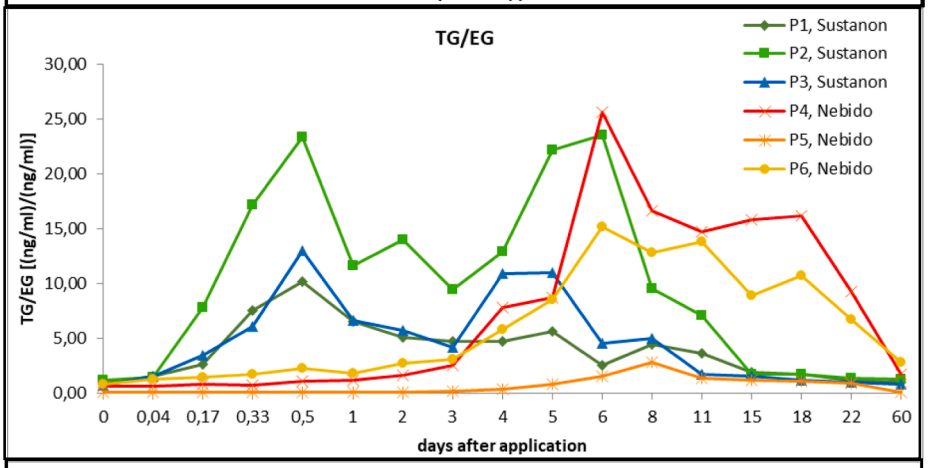
In the light of these results, we propose the combination of the ratios ES/EG and TS/TG ((ES/EG)/(TS/TG)) as a complementary biomarker for testosterone abuse. After injection of Sustanon®, the ratio reached its maximum value between 3 and 11 days. The changes in the ratio was, however, already detectable from the same day of injection of the ester. Subsequent to the administration of Nebido®, the raise in the (ES/EG)/(TS/TG) ratio was slightly delayed, compared to the results from the Sustanon® study. In this case, the maximum value was recorded between 6 and 22 days.

In Fig. 6, T/E-ratios are compared to (ES/EG)/(TS/TG)-ratios for all six participants. In window A, the calculated ratios are shown, whereas normalized values are pictured in window B. For each participant, the values in window B are normalized to calculated ratios at time point zero, which are considered the baseline values. Elevated ratios were observed in approximately the same time window, comparing T/E - and (ES/EG)/(TS/TG)- ratios. It is, however, of high interest that the increase in normalized (ES/EG)/(TS/TG) ratios are clearly higher than normalized T/E- ratios for participants 1, 2, 3 and 6. This might suggest the (ES/EG)/(TS/TG)- ratio to be a more sensitive marker than the T/E-ratio for some individuals.

Noteworthy, the urinary AS/TS ratio also increased for all participants. The increase was in particular remarkable between 2 and 22 days after application of the preparations. Nevertheless, we do not consider this ratio as suitable for the detection of testosterone misuse as the combined ratio (ES/EG)/(TS/TG). AS/TS is most likely not sensitive after oral intake of testosterone, since an increase in TS is expected. The (ES/EG)/(TS/TG) ratio, on the other hand, also includes the glucuronated steroids, and consequently takes into account changes in both







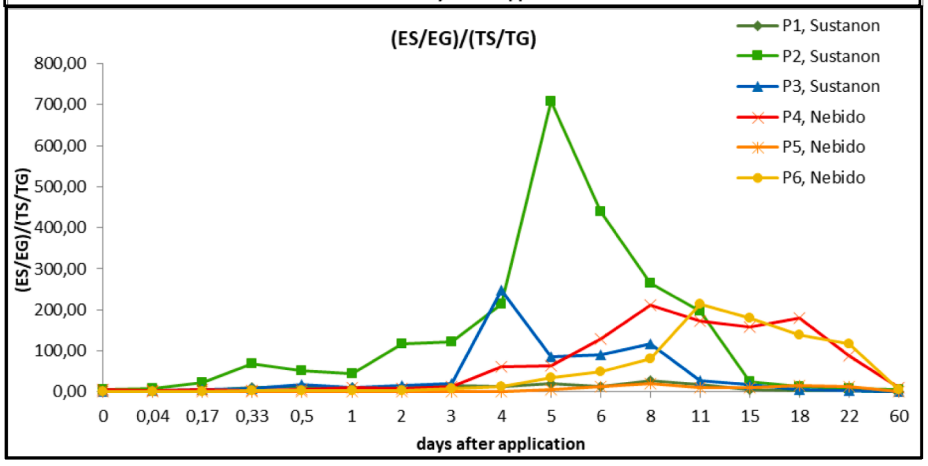


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Fig. 5. Urinary androgen conjugate excretion ratios during 60 days after administration of Sustanon or Nebido. TS: Testosterone sulfate, TG: Testosterone glucuronide, ES: Epitestosterone sulfate, EG: Epitestosterone glucuronide, AS: Androsterone sulfate, AG: Androsterone glucuronide.

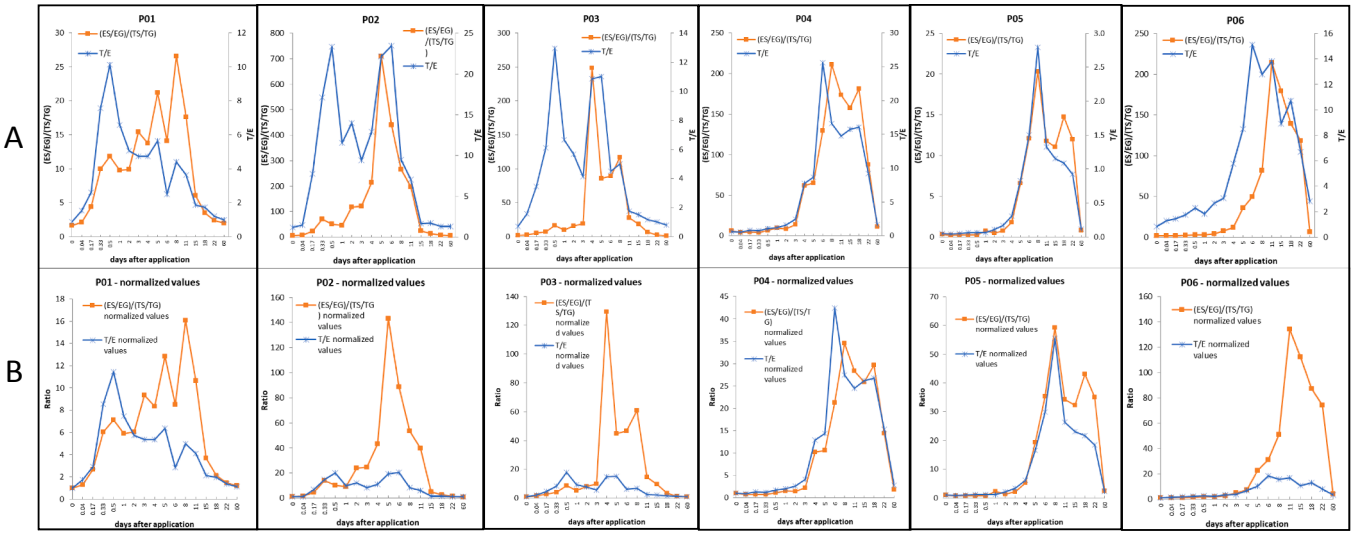

Fig. 6. Comparison of T/E-ratios to (ES/EG)/(TS/TG)-ratios for all 6 participants (P1-P6). P1, P2 and P3 adminstrated Sustanon© (testosterone propionate 30 mg, testosterone phenylpropionate 60 mg, testosterone isocaproate 60 mg and testosterone decanoate 100 mg) and P4, P5 and P6 administrated Nebido© (testosterone undecanoate 1000 mg). In window A, calculated ratios from urine concentrations are shown, whereas normalized values are pictured in window B. For each participant, the values in window B are normalized to calculated ratios at time point zero, which are considered the baseline values.

sulfatation and glucuronidation.

This study only included one route of administration (intramuscular) and exclusively male participants. Important for future investigations is to focus on additional routes of administration, like for instance oral and dermal applications. Additionally, the influence of potential confounding factors [25] on the (ES/EG)/(TS/TG) ratio should be explored. Furthermore, female steroid profiles should be included in future studies.

4. Conclusion

In conclusion, sulfoconjugated metabolites are relevant for the interpretation of steroid profiles. In this study, a LC-MS method was developed and validated, and allowed for the quantification of eight conjugated steroids within the same run. Among the targeted glucuro -or sulfo -conjugated steroids, their ratios seem to be superior to absolute urinary concentrations as indicators for testosterone ester consumption. The ratio (ES/EG)/(TS/TG) is suggested as a potential biomarker for exogenous intake of testosterone.

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