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Androgen Sulfation in Healthy UDP-Glucuronosyl Transferase 2B17 Enzyme-Deficient Men

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Context: The conspicuous interindividual differences in metabolism and urinary excretion of testosterone and its metabolites make it challenging to reveal testosterone doping. The variation in testosterone glucuronide excretion is strongly associated with a deletion polymorphism in the uridine diphosphate-glucuronosyltranferase (*UGT*) *2B17* gene.

Objective: The objective of the study was to identify additional biomarkers to detect testosterone abuse and to elucidate alternative pathways for testosterone elimination in individuals devoid of the UGT2B17 enzyme. For this purpose a new ultraperformance liquid chromatographic tandem mass spectrometric method for simultaneous determination of 10 different sulfo- and glucuronide-conjugated steroids was developed.

Participants: Fifty-four healthy male volunteers with two, one, or no allele (*ins/ins*, *ins/del*, or *del/del*) of the *UGT2B17* gene participated in the study.

Intervention: Intervention included a single im dose of 500 mg testosterone enanthate.

Main Outcome Measures: Urinary sulfo- and glucuronide-conjugated steroids were measured.

Results: Testosterone sulfate levels decreased in all individuals after the dose. The individual differences in the excretion of all sulfated metabolites were large. Thus, these metabolites will not serve as appropriate biomarkers for testosterone abuse. However, androsterone glucuronide excretion increased in all of our study subjects after the testosterone dose. Etiocholanolone sulfate was excreted at significantly higher levels in *UGT2B17 del/del* individuals.

Conclusion: We propose that the androsterone glucuronide to epitestosterone glucuronide ratio may serve as a complementary biomarker to reveal testosterone abuse.

Testosterone and synthetic androgenic congeners, commonly known as anabolic androgenic steroids are misused to enhance muscle strength and performance (reviewed in Refs. 1 and 2). Two problems make the detection of testosterone doping an especially challenging analytical problem in drug testing, namely to differentiate between exogenous and endogenous testosterone, and the fact that there are large differences in metabolism and urinary excretion of testosterone metabolites. Today a urinary ratio between the glucuronides of testosterone and epitestosterone (T/E) is used as a screening method (3). Ratios above 4.0 are indicative of testosterone intake and the samples will be subject to further testing. The T/E ratio has a much higher inter than intraindividual variability

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Abbreviations: AG, Glucuronide of androsterone; AS, sulfate of androsterone; AUC, area under the curve; cr, creatinine; CI, confidence interval; DHEAS, dehydroepiandrosterone sulfate; DHTG, glucuronide of dihydrotestosterone; EPG, glucuronide of epitestosterone; EPS, sulfate of epitestosterone; ETG, glucuronide of etiocholanolone; ETS sulfate of etiocholanolone; ISTD, internal standard solution; MRM, multiple reaction mode; QCL, low-quality control sample; SPE, solid phase extracted; T/E, testosterone and epitestosterone ratio; TG, glucuronide of testosterone; TS, sulfate of testosterone; UGT, uridine diphosphate-glucuronosyltranferase; UPLC, ultraperformance liquid chromatographic; UPLC-MS/MS, UPLC tandem mass spectrometric method.

(4–6), and future test programs will be based on individual monitoring rather than population-based reference values (7, 8).

The overwhelming part of the interindividual variation in T/E ratios may be ascribed to genetic variability (9, 10). We have demonstrated that a common deletion polymorphism in the gene coding for uridine diphosphate-glucuronosyltranferase (UGT) 2B17 (11) is strongly associated with testosterone glucuronide levels in urine (4) and that 40% of the individuals lacking the *UGT2B17* gene would never reach the cutoff ratio of 4.0 used today after a single dose of 360 mg testosterone (6). We have also shown that a thymine greater than cytosine (A1 > A2) promoter polymorphism in the *CYP17* gene is associated with lower urinary epitestosterone excretion resulting in 64% higher T/E ratios in homozygotes, increasing the risk of false-positive doping test results (12).

Sottas *et al.* (reviewed in Ref. 13) have developed a statistical method for interpretation of doping tests based on empirical Bayesian inferential technique for longitudinal profiling (7, 8). The test progressively switches the focus from comparison with a population-based reference value to comparison of individual related values. This method drastically reduces both false-positive and false-negative doping test results. Other biological information about the athlete added to the method (*e.g.* age, gender, ethnic origin, *etc.*) reduces the between-subject variance (8). In addition, we have recently demonstrated that introduction of information on *UGT2B17* genotype to the method gives a substantial further improvement of the test (14).

Individuals homozygous for the *UGT2B17* deletion (*del/del*) have no or negligible amounts of urinary testosterone glucuronide (4), whereas the excretion of the two major testosterone metabolites, androsterone glucuronide, and etiocholanolone glucuronide is excreted at slightly lower or the same as in individuals with one (*ins/del*) or two (*ins/ins*) alleles of the *UGT2B17* gene (4). The *UGT2B17* polymorphism is not associated with any variation in serum testosterone concentration (4).

The metabolic inactivation of steroids by sulfation is important in the regulation of intracellular steroid activity (15, 16). Human cytosolic sulfotransferases constitute the major phase II drug metabolism superfamily responsible for the sulfation of numerous exogenous and endogenous compounds (17, 18). The major steroid precursor in humans is dehydroepiandrosterone, which is converted into both androgens and estrogens in peripheral tissues (15). It exists in the circulation predominantly as the sulfated form (19). Sulfation is not a major metabolic pathway for testosterone, but we hypothesized that the compromised glucuronidation capacity in *UGT2B17 del/del* individuals is compensated for by increased sulfation.

Because of the shortcomings of the T/E ratio as marker for testosterone doping, it is important to identify new biomarkers that could be used for detecting testosterone abuse. Sulfated steroids may serve as additional biomarkers for androgen abuse.

Gas chromatography coupled to mass spectrometry used for the determination of endogenous anabolic steroids (*e.g.* T/E ratio) in human urine is a common method for World Anti-Doping Agency-accredited laboratories, even though the number of liquid chromatographic-mass spectrometric methods for direct measurement of steroid conjugates has increased in the past years (20–25). We have developed a new ultraperformance liquid chromatographic tandem mass spectrometric method (UPLC-MS/MS) for solid-phase extracted (SPE) human urine for simultaneous determination of 10 different sulfo- and glucuronide-conjugated steroids. The method is based on stable isotope dilution quantification.

The aim of our study was to identify additional biomarkers to reveal testosterone abuse and to elucidate alternative metabolic pathways for testosterone in *UGT2B17 del/del* individuals. For this purpose we used our new method to analyze urine samples from 54 healthy men, who received a single dose of testosterone enanthate.

Subjects and Methods

Subjects and design

Study subjects included 54 healthy male volunteers aged 18–50 yr (mean 30.6 ± 7.0 yr) with two (n = 13), one (n = 24), or no (n = 17) allele (*ins/ins*, *ins/del*, or *del/del*) of the *UGT2B17* gene. The study population has been described in more detail elsewhere (6). All participants gave informed consent consistent with the approval of the Ethics Review Board. The participants were given 500 mg testosterone enanthate as a single im dose of Testoviron Depot (kindly provided by Schering Nordiska AB, Solna, Sweden) equivalent to 360 mg testosterone. Before administration (d 0) urine samples were collected for analyses. Urine was further collected on d 1–9, 11, 13, and 15. All samples were collected between 0700 and 1100 h. Adverse drug reactions were monitored from the time of screening until d 15 after administration of testosterone. No major adverse drug reactions were registered. No follow-up was needed. The study was conducted according to the Helsinki Declaration and the ICH Harmonised Tripartite Guideline for Good Clinical Practice. The *UGT2B17* deletion polymorphism in these subjects had been genotyped by real-time PCR analysis in our previous study (6).

Materials

Glucuronides and sulfates of testosterone (TG, TS), epitestosterone (EPG, EPS), dihydrotestosterone (DHTG), androsterone (AG, AS), etiocholanolone (ETG, ETS), [$^2H_3$]testosterone, [$^2H_3$]epitestosterone, [$^2H_3$]dihydrotestosterone (sulfate of dihy-

drotestosterone) [$^2H_4$]androsterone, and [$^2H_5$]etiocholanolone were purchased from NMI (Pymble, Australia). Methanol was bought from Lab-Scan (Poch Sa, Swinskiego, Poland) and ammonium acetate, ammonia and acetic acid from Merck KGaA (Darmstadt, Germany). Two urine blanks were collected from a prepubertal boy and prepubertal girl, which were used for calibrators and control samples, respectively. The urine blank samples were analyzed both by Gas chromatography coupled to mass spectrometry and UPLC-MS/MS before use to quantify any sublevels of the endogenous steroids listed above. Standard stock solutions containing steroid glucuronides and sulfates were prepared in methanol. Intermediate solutions, stored in −20 C, were prepared by serial dilutions of stock solutions to adequate concentrations. Calibrators (n = 8) were freshly prepared at every batch analysis by diluting the intermediate solutions with urine blank (1:20 urine, from the prepubertal boy). Two quality control samples, low quality control sample (QCL) and a high-quality control sample (QCH), were prepared in urine blank (from the prepubertal girl) and stored in a freezer between batch analyses. Internal standard solution (ISTD) was prepared in water by diluting the deuterium-labeled conjugates listed above to relevant concentrations.

Sample preparation

Sample preparation, except for the centrifugation and evaporation step, was performed by a Xiril X-100 (Xiril AG, Hombrechtikon, Switzerland) pipetting robot. One milliliter sample and 20 μl ISTD was added to a SPE Oasis HLB 96-well plate, 10 mg (Waters Associates, Milford, MA). The SPE plate was washed with 1 ml 0.1% acetic acid, 1 ml 0.1% ammonia, and 0.5 ml 10% MeOH and centrifuged subsequently. The analytes were eluted from the SPE plate into a 96-well plate with 2 × 0.2 ml acetone. After evaporation, the residue was reconstituted with 200 μl aqueous solution of 20% MeOH. The injection volume was 5 μl.

Chromatographic separation

Waters Acquity ultraperformance liquid chromatographic (UPLC) system was used to perform the separation on Waters Acquity UPLC BEH RP18 column 50 × 2.1 mm with 1.7-μm particles. The mobile phases were 5 mM NH_4Ac adjusted by ammonia to pH 9.6 (A) and MeOH (B). Separation was performed with a flow rate at 0.75 ml/min and a column temperature at 50 C by the following gradient; initial mobile phase composition was 30% B with a linear gradient to 35% B at 4.0 min followed by another increasing gradient to 90% B at 7.5 min, which was maintained to 8.0 min. The organic modifier was decreased to initial condition (30%) at 8.1 min and the final equilibration time was 9.0 min.

Mass spectrometric method

A Waters Micromass Quattro Premier triple-quadrupole instrument (Waters Associates, Manchester, UK) operating with fast polarity switching in multiple reaction mode (MRM) was used to detect the target analytes in the method. An electrospray probe was interfaced between the UPLC and the mass spectrometry system. The electric potential was set to 0.5 kV and the nitrogen desolvation gas flow and temperature was 1100 liters/h and 375 C, respectively. The ion source was heated to 120 C. Argon gas at a pressure of 3.5–4.5 × 10^{-3} mbar was used for product ion fragmentation in the T-wave collision cell. The method uses 20 msec dwell time for all MRM channels and 20 msec time between the polarity switching, giving at least 10 data points for all substances. Precursor (protonated, deprotonated, or ammonium adduct of the molecular ion) and product ions of each analyte were obtained by infusing 5–10 μl/min of solutions of the reference substances (~2 μg/ml) to a tee connector between the outlet of the UPLC flow and the electrospray probe. The selected precursors, precursor ions, product ions, and collision offset are listed in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org.

Batch analysis

One batch consisted of samples, calibrators, and blank and control samples, which were injected from the 96-well plate to the UPLC system. Urine blank and control samples, low and high, were analyzed every 13 samples. All samples were analyzed within 1 month, divided into 10 batches.

Data evaluation

Integration, calibration, and data evaluation was performed by the TargetLynx software (Waters Associates, Manchester, UK).

The between-subject variation in urine dilution was corrected for by dividing the concentration values by the urinary creatinine (cr) concentration, which was determined by colorimetric analysis (DRI Creatinine-Detct Test; Thermo Fisher Scientific, Waltham, MA). The areas under the curves (AUC) of the different urinary steroids were calculated using the trapezoidal rule. Statistical analyses were performed by one-way ANOVA, the Student's *t* test, or, when the data were not normally distributed, Kruskal Wallis ANOVA or the Mann-Whitney *U* test, if not specified otherwise.

Results

The UPLC-MS/MS method

The UPLC method was developed to analyze one batch within 24 h to avoid possible changes of the sample pending injection, *e.g.* evaporation, degradation, and/or adsorption to vial surface. The chromatographic separation is demonstrated in Fig. 1, with three stacked integrated ion chromatograms, of 10 different analytes, from injections of a quality control sample (QCL) and two samples from the study (*ins/ins* and *del/del*) 5 d after the testosterone dose.

During method development dehydroepiandrosterone sulfate (DHEAS) was observed as a coeluting peak with EPS when detected in electrospray negative mode. To exclude the DHEAS as interference, detection of EPS was performed in positive mode and a longer gradient (20 min), lower flow rate (0.25 ml/min), and a longer column (10 cm) was used to baseline resolve DHEAS from EPS.

Ion suppression/enhancement setup according to Annesley (26) was investigated by postcolumn continuous infusion of compounds of interest with simultaneous injection of a solid-phase extracted urine sample. There were

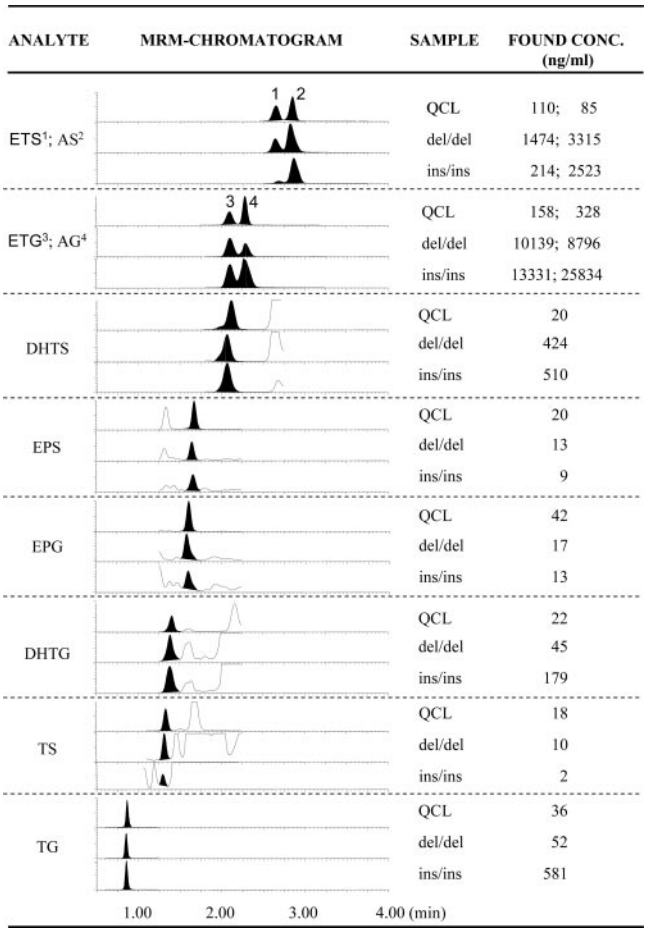

FIG. 1. Chromatographic separation of 10 targeted analytes listed in the *left column*. Three different sample injections [QCL, *del/del* and *ins/ins* sample (5 d after testosterone dose)] showed as stacked, integrated MRM-chromatogram windows, with the quantified results listed in the *right column*.

no indications of ion suppression in which the target analytes were eluted. In any case of matrix effect, the stable isotope internal standard will correct any suppression/enhancement for the corresponding analyte.

The recovery of the SPE method was investigated by two experiments: 1) extraction of one calibrator together with the ISTD and 2) extraction of a urine blank together with the ISTD into a test tube containing intermediate solution (corresponding to the concentrations in the calibrator used in experiment 1). The recovery ranged between 72.7% (DHTG) and 99.9% (ETS).

The sensitivity of the method for all substances, calculated by a signal-to-noise ratio of 3:1, was estimated from 0.03 ng/ml (EPS) to 1 ng/ml (AS). To achieve better method sensitivity, the reconstitution volume (200 μl) may be reduced in the final sample preparation step.

The quality control data are presented in Supplemental Table 2. The between-batch variances for the control samples were satisfactory and ranged from 4.17% (TS, high quality control sample) to 16.6% (EPS, QCL). The explanation of the high bias values of the QCL for ETG (+59.6%), AG (+63.2%) and AS (+40.4%) may be due to the different steroid profile from the two different urine blanks (the concentration of the three steroid conjugates in the prepubertal girl blank were found to be approximately 120, 75, and 20 ng/ml, respectively, higher than the urine blank from the prepubertal boy). Supplemental Fig. 1 shows the graph of quality control samples of TG demonstrating the inter- and intraday variation during the complete batch analysis. The precision and accuracy of the method was ±15% or less, indicating an acceptable level for bioanalytical quantitative methods.

Baseline urinary steroids

The individuals were divided into three groups according to *UGT2B17* genotype: *ins/ins*, *ins/del*, and *del/del*. There was no difference in TS levels between the groups ($P = 0.3$), whereas, as shown before, the *del/del* group had significantly lower baseline TG levels (Table 1). Only a minor part of the urinary testosterone was sulfated except in the *del/del* individuals, in whom it accounted for 50% of the testosterone conjugates. In contrast, EPS corresponds to 25% on average (minimum 2% and maximum 60%) of the total urinary epitestosterone in all genetic

TABLE 1. Baseline urinary androgen conjugate levels in three volunteer panels with respect to the *UGT2B17 insertion (ins)* and *deletion (del)*

Androgen conjugate (nmol/μmol cr)	*UGT2B17*genotype			*P* value
	***del/del* (n = 17)**	***ins/del* (n = 24)**	***ins/ins* (n = 13)**	
TG	1.2 (0.92–1.4)	9.5 (7.8–11.2)	13.0 (10.5–15.5)	<0.001
TS	1.7 (0.45–2.9)	1.4 (0.45–2.3)	0.61 (0.14–1.1)	0.30
EPG	8.9 (4.3–13.5)	8.4 (6.3–10.4)	7.0 (4.8–9.1)	0.53
EPS	2.9 (1.8–4.1)	3.3 (2.6–3.9)	3.2 (2.4–3.9)	0.80
AG	667 (440–895)	680 (563–797)	660 (565–755)	0.42
AS	140 (111–169)	125 (88.5–162)	126 (81.9–170)	0.43
ETG	413 (266–560)	446 (377–515)	529 (419–638)	0.054
ETS	64.0 (50.0–78.4)	32.1 (20.9–43.4)	29.7 (13.8–45.6)	0.0031

The values are given as the mean with the 95% CI within *parentheses*. The *P* values were calculated using ANOVA or, when the data were not normally distributed, Kruskal Wallis ANOVA.

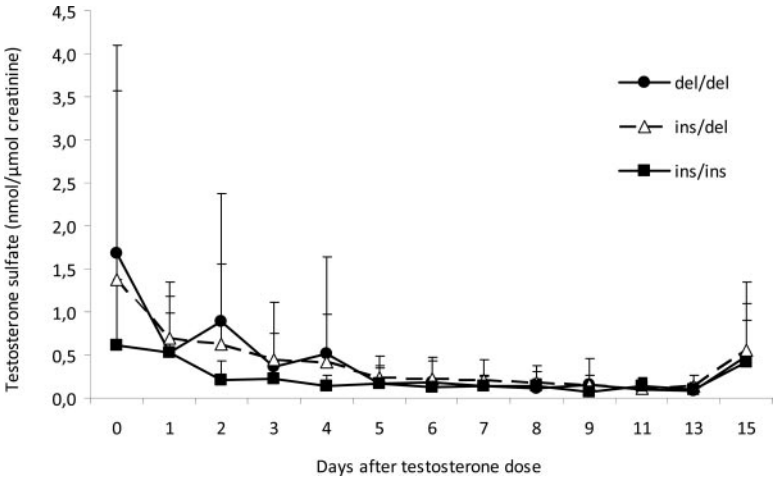

FIG. 2. Urinary testosterone sulfate excretion (nanomoles per micromole cr) for 15 d in the different *UGT2B17* genotype groups after an im injection of 500 mg testosterone enanthate, equivalent to 360 mg testosterone, on d 0. *Vertical bars* denote SD.

panels. The ETG levels tended to be lower in the *del/del* group ($P = 0.054$), whereas the ETS levels were significantly higher in the *del/del* group ($P = 0.0031$) compared with the other genotype panels (Table 1). The glucuronidated and sulfated metabolites of DHT were not investigated in this study.

Urinary steroid profile after testosterone administration

To see whether TS could be used as a biomarker for testosterone abuse, the TS levels were measured before and on d 1–9, 11, and 13 and 15 d after an injection of 500 mg testosterone enanthate. The TS levels decreased to almost undetectable levels in less than a week for all three genotype groups without difference between the groups (Fig. 2).

The rate of excretion increased for TG, AG, AS, ETG, and ETS (Fig. 3A). As expected, the TG excretion increased much less in the *del/del* group compared with the *ins/ins* group. There was also a conspicuous decrease in urinary excretion of EPS and EPG after a testosterone dose (Fig. 3B).

One metabolite, ETS, was always significantly higher in the *del/del* individuals compared with the other genotypes throughout the study period after the testosterone dose (Fig. 4). The AUC during 15 d for ETS in the *del/del* individuals was 1600 [95% confidence interval (CI) 1160–2010] nmol/µmol cr compared with 770 (95% CI 490–1080) nmol/µmol cr in the *ins/del* and 720 (95% CI 340–1140) nmol/µmol cr in the *ins/ins* group ($P = 0.0015$). The maximum difference compared with d 0 was 125 (95% CI 78.1–172) nmol/µmol cr, 58.1 (95% CI 26.3–90.0) nmol/µmol cr and 62.8 (95% CI 24.0–102) nmol/µmol cr in the *del/del, ins/del*, and *ins/ins* group, respectively ($P = 0.015$).

The sum of the glucuronide metabolite AUC (androsterone + etiocholanolone + testosterone + epitestoster-

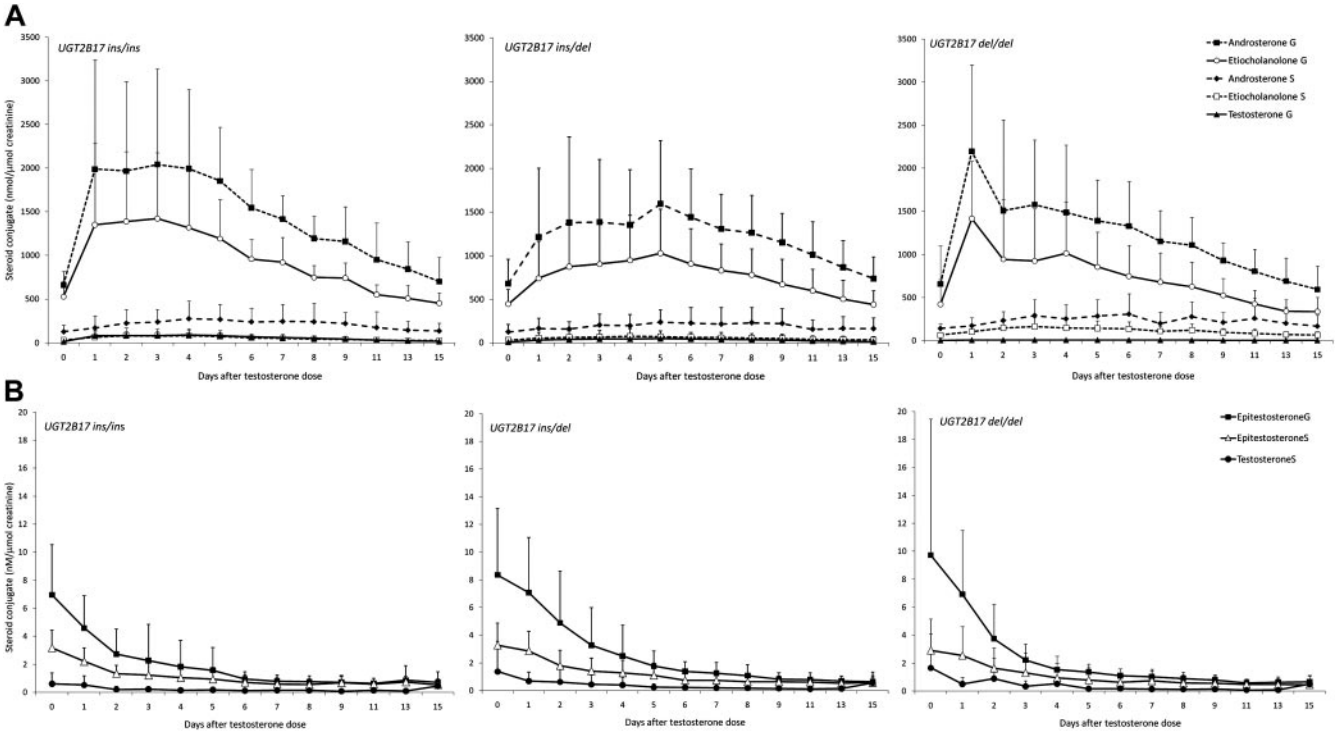

FIG. 3. A and B, Average urinary androgen conjugate excretion (nanomoles per micromole cr) during 15 d in the *UGT2B17 ins/ins* (*left panel*), *ins/del* (*middle panel*), and *del/del* group (*right panel*) after an im injection of 500 mg testosterone enanthate on d 0. Day 0 values represent baseline levels. *Vertical bars* denote SD.

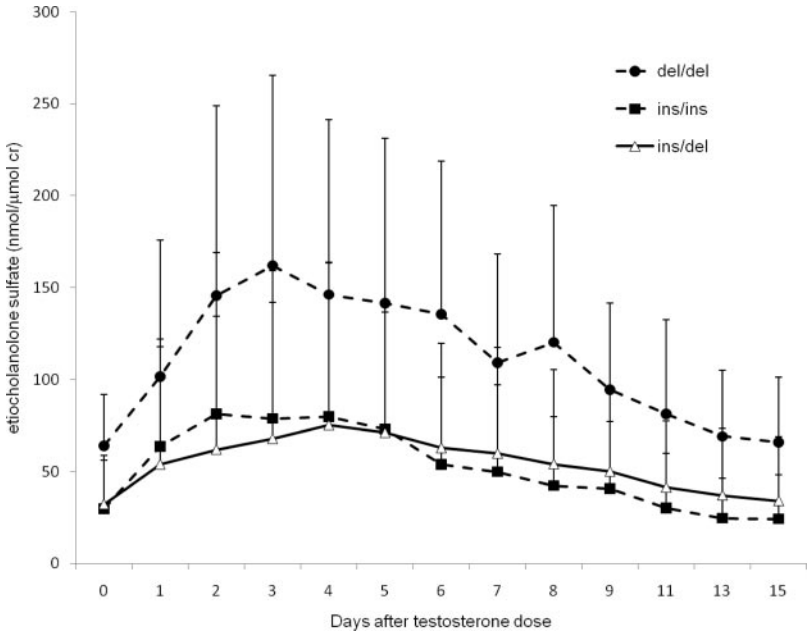

FIG. 4. Average urinary etiocholanolone sulfate excretion (nanomoles per micromole cr) for 15 d in the *UGT2B17 ins/ins*, *ins/del*, and *del/del* groups after an im injection of 500 mg testosterone enanthate on d 0. *Vertical bars* denote SD.

one) during 15 d after the testosterone dose was significantly higher in the *ins/ins* group compared with the *del/del* group [34.2 (95% CI 29.5–38.9) μmol/μmol cr *vs.* 25.5 (95% CI 21.2–29.9) μmol/μmol cr, $P = 0.008$]. If AS and ETS excretion is also added to the glucuronide metabolites after a testosterone dose, the difference is smaller between the *ins/ins* and the *del/del* group for the sum of AUC, but it is still statistically significant [38.0 (95% CI 32.7–43.3) μmol/μmol cr *vs.* 30.7 (95% CI 26.0–35.4) μmol/μmol cr, $P = 0.034$].

New biomarkers

A suitable biomarker that reveals testosterone abuse could be any ratio between metabolites that increases and decreases respectively after testosterone intake. AG and ETG levels had the highest increase among the metabolites measured; however, the interindividual differences were large. In four of the subjects (two *del/del* and two *ins/del*), the levels of ETG did not increase after the testosterone dose. The only metabolite that increased notably in all of our study subjects after a testosterone dose was AG. EPG decreases in all study subjects and is a suitable denominator of the ratio. During the study period of 15 d, the average increase in the AG to EPG ratio was 1470, 1040, and 1210 in the *ins/ins*, *ins/del*, and *del/del* group, respectively. This is to be compared with an average increase in the TG to EPG ratio of 58 in the *ins/ins* group, 32 in the *ins/del* group, and 4.1 in the *del/del* group.

Discussion

We hypothesized that after a dose of testosterone enanthate, the TS levels would increase and serve as a good biomarker for doping with this steroid in individuals devoid of the glucuronidation enzyme, UGT2B17. However, the TS levels were markedly decreased to negligible levels about 5 d after the dose (Fig. 2). Exogenous testosterone does not seem to be sulfated at all. Dehennin (27) showed that 95% of the TS originates from the testis and the decrease in testosterone sulfate levels may be the result of an endocrine feedback suppression of the secretion of LH (28, 29) with subsequent decrease in sulfation of endogenous testosterone. Testosterone is not a preferred substrate for liver sulfotransferase enzymes, with a 148-fold higher glucuronidation activity compared with sulfation activity for testosterone (30). It is possible that an increase in urinary TS may be observed for oral formulations of testosterone, as indicated elsewhere (31), in which the concentrations in the liver are very high for the first hours after administration. However, TS will not serve as an appropriate biomarker for testosterone abuse.

Any ratio between an increasing and a decreasing testosterone metabolite has the potential to be used as a biomarker for testosterone abuse. The individual differences in the excretion of the sulfated metabolites were large. The maximum increase in the ETS excretion after a testosterone dose during 15 d was on average 31 ± 32 nmol/μmol cr and ranged between 1 and 130 nmol/μmol cr with similar variation in all three genotypes. The variability in

TABLE 2. The ratio between AG and EPG in all subjects of the *UGT2B17 ins/ins*, *ins/del*, and *del/del* groups before (d 0), and 3, 7, and 15 d after an im injection of 500 mg testosterone enanthate, equivalent to 360 mg testosterone

	***del/del* subjects AG to EPG ratio median (range)**	***ins/del* subjects AG to EPG ratio median (range)**	***ins/ins* subjects AG to EPG ratio median (range)**
Day 0	75 (27–308)	82 (35–224)	102 (53–285)
Day 3	680 (220–2763)	525 (71–21179)	1718 (117–3068)
Day 7	1154 (483–2629)	1404 (326–2872)	2098 (771–3613)
Day 15	1137 (251–9714)	1203 (428–2396)	1359 (230–3799)

the AS excretion was almost as large. Glucuronide conjugated androgens seem to be more appropriate biomarkers for testosterone abuse because the interindividual variation in excretion of glucuronide-conjugated metabolites is generally smaller, with the exception of testosterone glucuronide.

We propose that the AG to EPG ratio may serve as a complementary biomarker for testosterone abuse. AG levels increased after testosterone administration in all of our study subjects irrespective of the *UGT2B17* genotype. The ratio was not significantly higher in the *ins/ins* and the *ins/del* group compared with the *del/del* group, neither before nor after the testosterone dose (Table 2), which shows that this ratio is largely independent of the *UGT2B17* polymorphism. However, the interindividual differences in these ratios were large. If shown to have use, it will be difficult to determine general cutoff ratios for this biomarker. Because of the marked increase of the ratio after the challenge with testosterone, the AG to EPG ratio has the potential to be used as a complement to the T/E ratio in the athlete's biological passport (13), particularly in the *del/del* population in which the T/E ratio is an unsatisfactory marker of doping. Further studies in larger population samples are required to determine the usefulness of this biomarker approach.

Baseline urinary testosterone sulfate levels are low (22, 32). One study suggested that individuals with low urinary TG also have low urinary TS (32). In our study we observed a tendency of slightly higher TS levels in the *del/del* population compared with the other genotype groups, albeit not statistically significant (Table 1). About 25% of the epitestosterone was sulfated, with no difference between the *UGT2B17* genotypes. This is in accordance with previous studies (22, 32).

Interestingly, the *del/del* group had significantly higher ETS levels than both the *ins/del* and the *ins/ins* group (Fig. 4). This difference was maintained after a challenge with testosterone. Etiocholanolone is a 5β-reduced metabolite of testosterone. This is the only metabolite we know of, so far, that is consistently higher in the *del/del* group. The higher levels may be a compensatory way for the *del/del* group to excrete androgens, even though this difference did not fully explain the difference between genotypes when all major metabolites of testosterone (androsterone and etiocholanolone glucuronide and sulfate) were accounted for.

There are a number of minor metabolites that were not measured in this study. Testosterone is also metabolized to estrogens catalyzed by CYP19, but no estrogens were included in the analytical work in this study. If these are considered, the difference between the *ins/ins* and the *del/del* individuals may be altered, but this remains to be studied.

In summary, the ratio between androsterone glucuronide and epitestosterone glucuronide may serve as a complement to the testosterone/epitestosterone glucuronide ratio in identification of subjects with intake of exogenous testosterone, preferentially in individuals devoid of the UGT2B17 enzyme (*del/del* genotype). Our studies also indicate that these individuals partly compensate for their compromised capacity to excrete testosterone by excreting higher levels of an alternative testosterone metabolite, etiocholanolone sulfate.

Acknowledgments

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References

1. **Sjöqvist F, Garle M, Rane A** 2008 Use of doping agents, particularly anabolic steroids, in sports and society. Lancet 371:1872–1882
2. **Handelsman DJ, Heather A** 2008 Androgen abuse in sports. Asian J Androl 10:403–415
3. **Donike M, Bärwald K-R, Klostermann K, Schänzer W, Zimmermann J** 1983 Nachweis von exogenem testosteron. In: Heck H, Hollmann W, Liesen H, Rost R, eds. Sport: Leistung und Gesundheit, Köln, Deutscher Ärzte Verlag; 293–298
4. **Jakobsson J, Ekström L, Inotsume N, Garle M, Lorentzon M, Ohlsson C, Roh HK, Carlström K, Rane A** 2006 Large differences in testosterone excretion in Korean and Swedish men are strongly associated with a UDP-glucuronosyl transferase 2B17 polymorphism. J Clin Endocrinol Metab 91:687–693
5. **Donike M, Mareck-Engelke U, Rauth S** 1995 Statistical evaluation of longitudinal studies. Part 2. The usefulness of subject-based reference ranges. In: Donike MHG, Gotzmann A, Mareck-Engelke U, Rauth S, eds. Proceedings of the 12th Cologne Workshop on Dope Analysis. Koln, Germany: Sport und Buch Strauß; 157–165
6. **Schulze JJ, Lundmark J, Garle M, Skilving I, Ekstrom L, Rane A** 2008 Doping test results dependent on genotype of uridine diphospho-glucuronosyl transferase 2B17, the major enzyme for testosterone glucuronidation. J Clin Endocrinol Metab 93:2500–2506
7. **Sottas PE, Baume N, Saudan C, Schweizer C, Kamber M, Saugy M** 2007 Bayesian detection of abnormal values in longitudinal biomarkers with an application to T/E ratio. Biostatistics (Oxford, England) 8:285–296
8. **Sottas PE, Saudan C, Schweizer C, Baume N, Mangin P, Saugy M** 2008 From population- to subject-based limits of T/E ratio to detect testosterone abuse in elite sports. Forensic Sci Int 174:166–172
9. **Schulze JJ, Rane A, Ekstrom L** 2009 Genetic variation in androgen disposition: implications in clinical medicine including testosterone abuse. Expert Opin Drug Metab Toxicol 5:731–744
10. **Bowers LD** 2008 Testosterone doping: dealing with genetic differ-

ences in metabolism and excretion. J Clin Endocrinol Metab 93: 2469–2471
11. **Wilson 3rd W, Pardo-Manuel de Villena F, Lyn-Cook BD, Chatterjee PK, Bell TA, Detwiler DA, Gilmore RC, Valladeras IC, Wright CC, Threadgill DW, Grant DJ** 2004 Characterization of a common deletion polymorphism of the UGT2B17 gene linked to UGT2B15. Genomics 84:707–714
12. **Schulze JJ, Lorentzon M, Ohlsson C, Lundmark J, Roh HK, Rane A, Ekström L** 2008 Genetic aspects of epitestosterone formation and androgen disposition: influence of polymorphisms in CYP17 and UGT2B enzymes. Pharmacogenet Genomics 18:477–485
13. **Sottas PE, Saugy M, Saudan C** 2010 Endogenous steroid profiling in the athlete biological passport. Endocrinol Metab Clin North Am 39:59–73, viii-ix
14. **Schulze JJ, Lundmark J, Garle M, Ekström L, Sottas PE, Rane A** 2009 Substantial advantage of a combined Bayesian and genotyping approach in testosterone doping tests. Steroids 74:365–368
15. **Labrie F, Dupont A, Simard J, Luu-The V, Belanger A** 1993 Intracrinology: the basis for the rational design of endocrine therapy at all stages of prostate cancer. Eur Urol 24(Suppl 2):94–105
16. **Reed MJ, Purohit A, Woo LW, Newman SP, Potter BV** 2005 Steroid sulfatase: molecular biology, regulation, and inhibition. Endocr Rev 26:171–202
17. **Falany CN** 1997 Enzymology of human cytosolic sulfotransferases. FASEB J 11:206–216
18. **Gamage N, Barnett A, Hempel N, Duggleby RG, Windmill KF, Martin JL, McManus ME** 2006 Human sulfotransferases and their role in chemical metabolism. Toxicol Sci 90:5–22
19. **Bovenberg SA, van Uum SH, Hermus AR** 2005 Dehydroepiandrosterone administration in humans: evidence based? Neth J Med 63: 300–304
20. **Bowers LD, Sanaullah** 1996 Direct measurement of steroid sulfate and glucuronide conjugates with high-performance liquid chromatography-mass spectrometry. J Chromatogr B Biomed Appl 687: 61–68
21. **Bean KA, Henion JD** 1997 Direct determination of anabolic steroid conjugates in human urine by combined high-performance liquid chromatography and tandem mass spectrometry. J Chromatogr 690:65–75
22. **Borts DJ, Bowers LD** 2000 Direct measurement of urinary testosterone and epitestosterone conjugates using high-performance liquid chromatography/tandem mass spectrometry. J Mass Spectrom 35:50–61
23. **Hintikka L, Kuuranne T, Leinonen A, Thevis M, Schänzer W, Halket J, Cowan D, Grosse J, Hemmersbach P, Nielen MW, Kostiainen R** 2008 Liquid chromatographic-mass spectrometric analysis of glucuronide-conjugated anabolic steroid metabolites: method validation and interlaboratory comparison. J Mass Spectrom 43:965–973
24. **Strahm E, Kohler I, Rudaz S, Martel S, Carrupt PA, Veuthey JL, Saugy M, Saudan C** 2008 Isolation and quantification by high-performance liquid chromatography-ion-trap mass spectrometry of androgen sulfoconjugates in human urine. J Chromatogr A 1196:153–160
25. **Pozo OJ, Van Eenoo P, Van Thuyne W, Deventer K, Delbeke FT** 2008 Direct quantification of steroid glucuronides in human urine by liquid chromatography-electrospray tandem mass spectrometry. J Chromatogr A 1183:108–118
26. **Annesley TM** 2003 Ion suppression in mass spectrometry. Clin Chem 49:1041–1044
27. **Dehennin L** 1993 Secretion by the human testis of epitestosterone, with its sulfoconjugate and precursor androgen 5-androstene-3β,17α-diol. J Steroid Biochem Mol Biol 44:171–177
28. **Dehennin L, Matsumoto AM** 1993 Long-term administration of testosterone enanthate to normal men: alterations of the urinary profile of androgen metabolites potentially useful for detection of testosterone misuse in sport. J Steroid Biochem Mol Biol 44:179–189
29. **Palonek E, Gottlieb C, Garle M, Björkhem I, Carlström K** 1995 Serum and urinary markers of exogenous testosterone administration. J Steroid Biochem Mol Biol 55:121–127
30. **Pacifici GM, Gucci A, Giuliani L** 1997 Testosterone sulphation and glucuronidation in the human liver: interindividual variability. Eur J Drug Metab Pharmacokinet 22:253–258
31. **Strahm E** 2010 Étude du métabolisme des androgènes et implications dans la lutte contre le dopage. PhD thesis, Faculté des Sciences, Section des Sciences Pharmaceutiques, University of Geneva, Geneva, Switzerland
32. **Sten T, Bichlmaier I, Kuuranne T, Leinonen A, Yli-Kauhaluoma J, Finel M** 2009 UDP-glucuronosyltransferases (UGTs) 2B7 and UGT2B17 display converse specificity in testosterone and epitestosterone glucuronidation, whereas UGT2A1 conjugates both androgens similarly. Drug Metab Dispos 37:417–423