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Ethylglucuronide as a potential marker for alcohol-induced elevation of urinary testosterone/epitestosterone ratios

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The potential influence of alcohol consumption on endogenous steroids has already been described in the literature. In those studies the ethanol level after ingestion was monitored using its concentration in blood, urine or saliva. Corresponding methods are not commonly used in anti-doping laboratories. Ethylglucuronide (EtG), which can be easily detected by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), appears to be a more suitable parameter for this purpose. It is slowly excreted into the urine and indicates alcohol intake for a much longer period than blood or urinary alcohol and it is therefore routinely used for legal purposes as an alcohol consumption marker. In pharmacokinetic studies that aimed to establish calculation models after ethanol intake, the formation of EtG was observed to coincide with elevated urinary testosterone/epitestosterone (T/E) ratios. Similarly, large amounts of EtG were correlated with abnormal steroid profiles found in routine doping samples. In this pilot study, several cases with significantly elevated T/E ratios were associated with urinary EtG concentrations higher than 50 μg/mL. These findings confirmed recent intake of ethanol in considerable amounts and suggest a connection to changes in specific steroid profile parameters. Owing to the ease with which procedures to determine EtG can be carried out, and the potential for such procedures to be introduced into screening schemes, the inclusion of this marker in the final evaluation of suspicious outliers in T/E ratio longitudinal studies would seem to be very useful.

Keywords: doping analysis; ethylglucuronide; testosterone/epitestosterone ratio; steroid profile; ethanol consumption

Introduction

The potential influence of excessive ('inebriating') alcohol consumption on the urinary testosterone/epitestosterone (T/E) ratio was first investigated in males. It has been shown that the intake of ethanol (1 to 2 g per kg body weight) may raise not only the T/E ratio in urine but also the plasma testosterone concentration.[1] Increases in the T/E ratio ranging from 30% to 90% were observed for several hours after ingestion. Further studies indicated the possibility of even higher rates (about threefold) in individual male subjects and suggested a more distinct effect in females, with the increase in the T/E ratio reaching more than 500% in a few cases.[2,3,4] However, the guideline that requires a mandatory longitudinal study if a T/E ratio exceeding the threshold of 4 has been reported assumes a normal variation of less than 30% in males and 60% in females.[5,6]

In 2008 our laboratory was requested by result management authorities to provide evaluation reports for 30 such longitudinal studies. In two cases the atypical finding that had triggered the study proved to be a significant outlier in the T/E time course of three samples collected over approximately one year. Compared to the basal value resulting from a previous and a subsequent test, an increase of 174% was found for a male athlete and another one of 238% for a female athlete (for more details see Table 3, cases 1 and 4, respectively). The carbon isotope ratio (CIR) determination gave no evidence of an exogenous source of testosterone and/or related prohormones so the possible effect of ethanol was taken into consideration. In the investigations mentioned above the concentration of ethanol in blood, urine or saliva was monitored. Since corresponding methods are not commonly used in anti-doping laboratories the samples were checked for the presence of ethylglucuronide (EtG), which can be easily detected by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The benefits and implications of EtG as biomarker have been reviewed in the literature.[7,8] Ethylglucuronide is slowly excreted into the urine and indicates alcohol intake for a much longer period (up to 80 hours after intake) than blood or urinary ethanol and is therefore routinely used for legal purposes as an alcohol consumption marker.

The aim of this pilot study was to evaluate the suitability of EtG as marker for alcohol-induced changes of the steroid profile monitored in doping analysis.

Experimental

Ethanol ingestion study

Urine samples originally taken for a study designed to examine the kinetics of formation and elimination of EtG (as a tool for modelling the pattern of ethanol ingestion by retrograde calculation) were subjected to the steroid profile analysis used in doping control

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Table 1. Relevant data for the volunteers participating in the ethanol ingestion study

volunteer	gender	age	body mass kg	body height cm	ethanol intake g/kg b. m.
1	male	47	78	174	1.92
2	male	47	98	190	1.53
3	male	32	67	171	2.24
4	male	50	72	180	2.08

in order investigate a possible correlation of EtG elimination with changes of steroid profile parameters.

Four healthy male volunteers (Table 1) had participated in this study, which had been approved by the ethical review board of the Ludwig Maximilian University of Munich (project no. 355/03). A total of 150 g of ethanol was gradually ingested starting with one portion of 45 g at once and then, after the blood alcohol concentration had dropped below 0.15 mg/g, three-and-a-half hours later another 105 g in smaller portions over two hours. Urine was collected before intake and, spontaneously as needed up to 32 hours afterwards, yielding in total 50 samples. The maxima of corresponding blood alcohol concentrations ranged from 0.8 to 1 mg/g.

Steroid profile parameters

The sample preparation method and instrumental conditions of gas chromatography coupled to mass spectrometry (GC/MS) for the measurement of the relevant steroid profile data were adapted from procedures commonly used in doping analysis.[9] All steroids used for quantification were certified reference substances (95% minimum purity, National Measurement Institute, Pymble, Australia). In brief, a mixture of internal standards (epitestosterone-d3, androsterone-d4-glucuronide, etiocholanolone-d5 at concentrations of 100, 1000 and 600 ng/mL, respectively) was added to urine aliquots of 2 mL adjusted to pH 6.5 by phosphate buffer solution (KH_2PO_4 0.5 M + Na_2HPO_4 0.5 M, 8 : 5 (v/v), reagent grade) before enzymatic hydrolysis by β-glucuronidase from *Escherichia coli* (Roche, Mannheim, Germany) at 50 °C for 1 h. Subsequently, the pH value was adjusted to 9 by adding solid carbonate buffer ($NaHCO_3$ + K_2CO_3, 84 : 138 (m/m), reagent grade) and a liquid-liquid extraction was carried out using 2 × 2 mL methyl *tert*-butyl ether (analytical grade, Sigma-Aldrich, Munich, Germany). After removal of the organic solvent under a nitrogen stream the dried residues were derivatized by addition of 50 µL of the silylation reagent consisting of N-methyl-trimethylsilyl-trifluoro-acetamide (MSTFA, GC grade, Macherey & Nagel, Düren, Germany), ammonium iodide (analytical grade, Merck, Darmstadt, Germany) and *n*-propanethiol (reagent grade, Merck, Darmstadt, Germany) 1000 : 5 : 1 (v/w/v) for 30 min at 55 °C. The silylated extracts were then analysed on a 6890/5975 GC/MSD system (Agilent Technologies, Waldbronn, Germany). In split mode (1 : 10) 1 µL of the extract was injected at 260 °C onto a Zebron ZB-1 ms capillary column (10 m, 0.18 mm i.d., 0.18 µm film, methyl silicone) operated in constant pressure mode giving a helium flow of 0.7 mL/min @ 130 °C. The oven temperature was ramped from 130 °C to 185 °C within one minute, further increased at a rate of 5 °C/min to 235 °C and then with 30 °C/min to the final level of 325 °C, which was held for 2 min. Mass spectrometric detection was performed in selected ion monitoring (SIM) mode after electron impact ionization (EI) at 70 eV electron energy. The following target ion signals using a dwell time of 20 ms were recorded to determine the relevant steroids as their trimethylsilyl (TMS) derivatives: 432 (testosterone and epitestosterone), 434 (androsterone and etiocholanolone) and 435 (epitestosterone-d3) for quantification, 438 (androsterone-d4) and 439 (etiocholanolone-d5) for checking the hydrolysis yield and 272 (androsterone-mono-TMS) to ensure a complete formation of TMS-enol ethers. A pooled urine collection was used as quality-control sample. The intermediate precision ranged from 10.7% to 13.7% for the concentration of all target analytes; for the T/E and androsterone/testosterone (And/T) ratios it was 7.5% and 11.1%, respectively.

Determination of EtG in urine

An established LC-MS/MS method was applied for this purpose.[10] Ethylglucuronide and EtG-d5 were purchased from Medichem Diagnostica (Steinenbronn, Germany). In the first approach a method dedicated to detect low levels of EtG in the context of abstinence control was performed for all samples in the ingestion study. After addition of the internal standard (EtG-d5, final concentration 400 ng/mL) 5 µL of the centrifuged urine sample (diluted 1 : 1 v/v by LC eluent A) was directly injected onto a Zorbax Eclipse XDB-C8 column (150 × 3 mm, 5 micron). Mixtures of 2 mM aqueous ammonium acetate solution and acetonitile were used as mobile phases, the mixing ratio of eluent A was 95 : 5 (v/v) and that of eluent B 5 : 95 (v/v). Gradient elution from 20% B (held for 1 min) to 80% B (after 5 min) was employed at a flow rate of 700 µL/min and ambient temperature. An API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Darmstadt, Germany) with electrospray ionization (TurboIon Spray) in multiple reaction monitoring (MRM) acquisition mode after negative ionization was utilized as detector. MRM transitions of interest using a dwell time of 100 ms were 226 > 85 (collision energy CE −25 eV) for EtG-d5 and 221 > 113 (CE −20 eV), 221 > 85 (CE −25 eV), and 221 > 75 (CE – 25 eV) for EtG. Alternatively, for EtG determination in real doping-control samples, an API 3200 Q Trap triple quadrupole mass spectrometer (Applied Biosystems, Darmstadt, Germany) was employed. To achieve a higher throughput an isocratic elution with 60% B was performed on a Zorbax Eclipse XDB-C8 column (100 × 2 mm, 3 micron) at a flow rate of 250 µL/min and a column temperature of 30 °C. Because the level of EtG associated with alterations of the steroid profile proved to be distinctly higher, the method was modified accordingly: the final concentration of the internal standard was set to 20 µg/mL and the calibration range for EtG was extended to 80 µg/mL. An EtG concentration of 20 µg/mL was chosen as appropriate level for the quality control sample (see Figure 1). The intermediate precision at this concentration was 5.2%.

Results and Discussion

EtG elimination and steroid profile after controlled ethanol ingestion

For all subjects the increase in the urinary T/E ratio after ethanol intake is clearly correlated ($p < 0.01$) with the elimination of EtG (half-life 4.4 hours) whereas the And/T ratio shows an inverse relationship (Figure 2a–d). A T/E increase of more than 30% (i.e. above the margin set as normal variation for males in the WADA guideline) was observed from 1 hour and 40 minutes (subject 2) up to 24 hours (subject 4) after starting ethanol ingestion. Compared

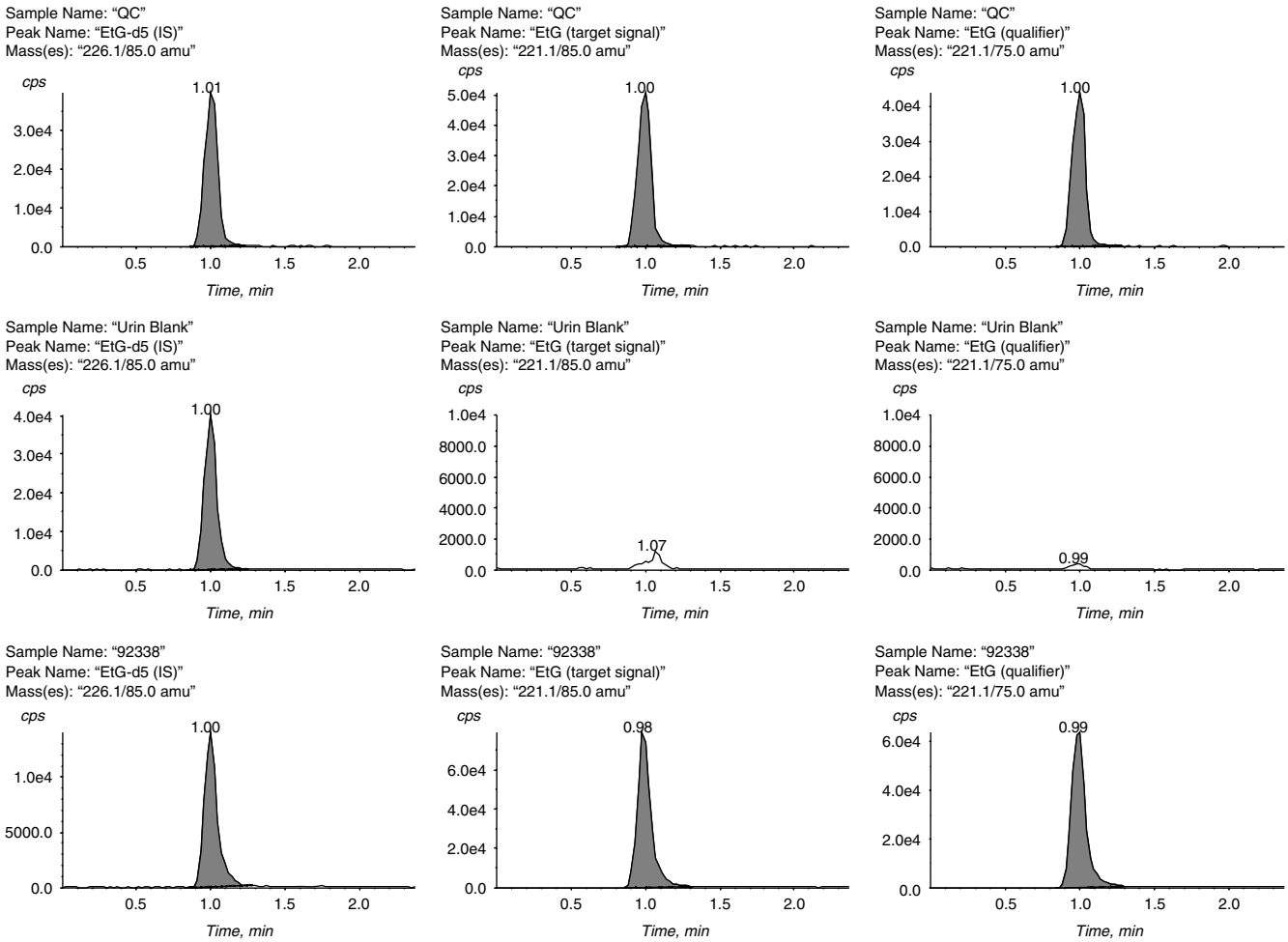

Figure 1. Determination of EtG by LC-MS/MS: Quality control sample at 20 µg/mL EtG (upper row), blank urine (middle row) and one athlete's sample containing 104 µg/mL (lower row).

to the basal values, the highest T/E ratios represent an increase of 80% ± 22% and the lowest And/T ratios a decrease of 58% ± 12% (Table 2). As described before, the volunteers were recruited for a controlled ethanol ingestion study and not selected for their basal T/E ratio, which turned out to be around 1 for all of them. However, the T/E ratio is a relative value and therefore the monitoring of the proportional change of this parameter appeared to be indicative to demonstrate the resulting impact. It is obvious that, applying comparable rates, higher basal values would yield higher ethanol-affected values; for instance, starting from a value of 3, an increase of 80% would give a T/E of 5.4, which would then clearly exceed the threshold of 4.

One explanation for the observed phenomenon could be diminished capacity of the enzymes involved in phase I metabolism of testosterone – especially 17β-hydroxysteroid-dehydrogenases – in the presence of large amounts of ethanol, which is itself a substrate of oxido-reductive conversion by alcohol dehydrogenase (ADH). This competitive enzyme inhibition leads to higher levels of testosterone and lower levels of androsterone (and etiocholanolone) available for glucuronidation in phase II metabolism. However, the concentration of epitestosterone as 17α-isomer of testosterone is not influenced in this way because an analogous 17α-hydroxysteroid-dehydrogenase is lacking (Figure 3).

In phase II metabolism, all enzymes of the UGT (UDP-glucuronosyltranferases) family relevant for steroid glucuronidation are also involved in ethanol conjugation and competitive enzyme inhibition could also be expected at this stage.[11] While the glucuronidation of testosterone is primarily under the control of the UGT2B17 isoform, epitestosterone is conjugated mainly by UGT2B7.[12] The findings do not indicate that the presence of ethanol would cause a discrimination of these different isoforms. In the same way, so far nothing could be found to suggest that the reciprocal behaviour of the 17β-hydroxysteroid testosterone and the 3α-hydroxysteroid androsterone would be a result of such theoretically possible differences in the glucuronidation process. For the observed effect this route is therefore obviously not critical due to a sufficient capacity of the participating enzymes. Consequently, the detected changes in the steroid profile after inordinate alcohol consumption are most likely to be triggered by competitive enzyme inhibition in phase I metabolism.

Elevated T/E ratios and decreased And/T ratios in doping control samples associated with alcohol consumption

Samples identified as significant outliers in T/E longitudinal studies and standalone findings of elevated T/E ratios without any indication regarding exogenous origin after CIR analysis

a–2d

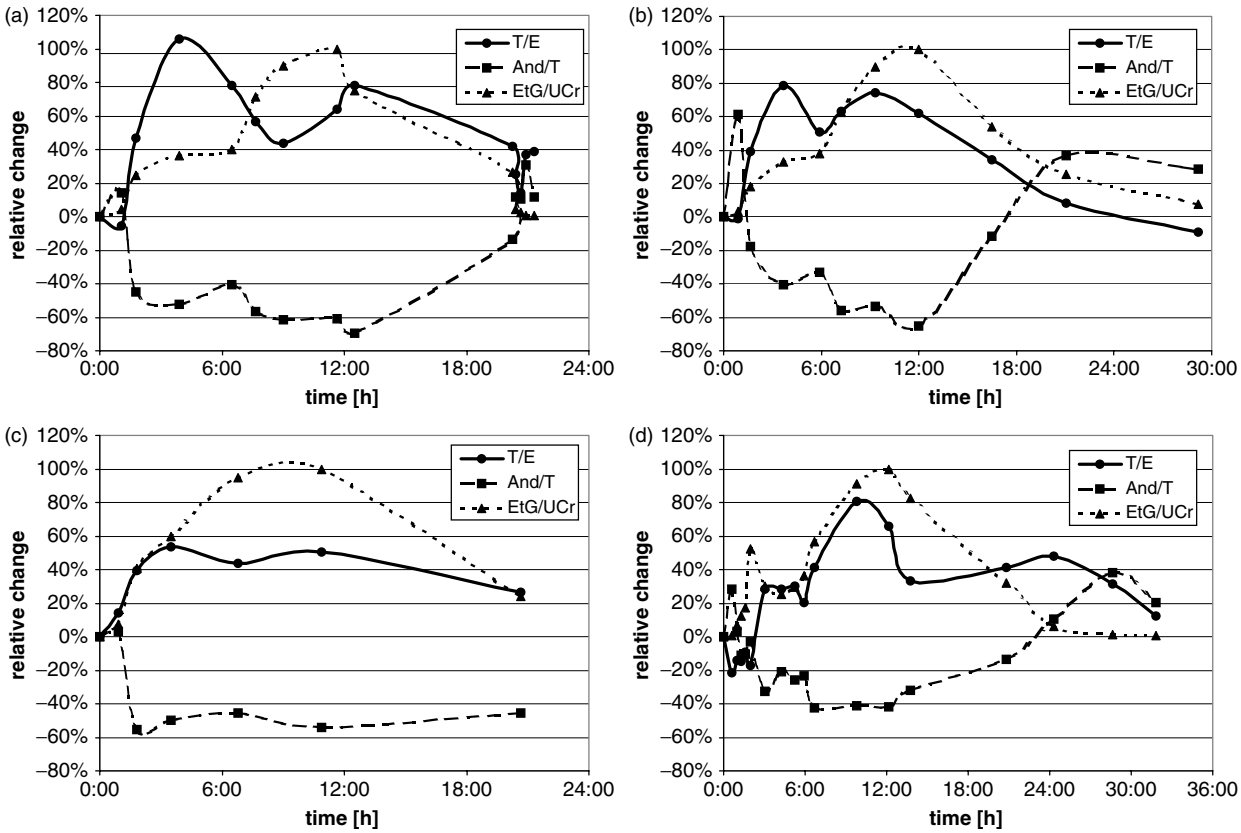

Figure 2. Ethanol ingestion studies: Relative changes of the T/E ratio and, inversely, the And/T ratio appear clearly correlated to the time course of the excretion of EtG corrected for urinary creatinine (UCr). (2a for volunteer 1 etc).

Table 2. Individual values and time for maximal EtG concentration and most distinct relative changes of the ratios T/E and And/T obtained from the ethanol ingestion study

	EtG_{max}			T/E				And/T			
volunteer	µg/mL	µg/µg UCr	time after start [h]	basal	max	time after start [h]	change	basal	min	time after start [h]	change
1	197	1.49	12:30	0.94	1.94	3:55	106%	56.5	17.1	12:30	−70%
2	345	2.41	12:00	0.78	1.40	3:40	79%	121.1	42.1	12:00	−65%
3	154	0.91	10:50	0.75	1.15	3:30	53%	50.0	22.9	10:50	−54%
4	354	3.85	12:05	1.00	1.80	9:50	80%	23.8	13.8	12:05	−42%

yielded high amounts of EtG, thus confirming a recent ingestion of ethanol (Table 3). Moreover, compared to existing basal values the And/T ratios were lowered in the same manner as observed in the ethanol intake study. All samples were taken out-of-competition between 7:00 and 14:00. Assuming an excessive alcohol consumption the evening or the night before, ethanol-actuated changes of the steroid profile would be conspicuous in exactly this interval of time. Even though the probability of similar findings from in-competition tests is much lower because most athletes will abstain from ethanol consumption around such an event, it cannot be excluded in general because the induced alterations may last longer than the acute 'attenuating' effect of ethanol. On the other hand, it should be noted that EtG can be detected much longer after a heavy intake of alcohol (about three days) compared to the period of time when ethanol is present in the body at a level that would cause the observed effects on the steroid profile (less than 24 hours). To avoid inadequate interpretations in connection with low EtG levels, further studies should be performed to produce recommendations that may include different threshold values taking into account gender and epitestosterone concentration.

The same tendency for steroid profiles to change in doping control samples in conjunction with ethanol intake was described in another study employing urinary ethanol as an alcohol consumption marker.[13]

Conclusions

In routine doping analysis, large amounts of EtG proved repeatedly to be correlated with abnormal steroid profiles. In our observations, several cases with significant elevation of T/E ratios were associated with urinary EtG concentrations typically found after excessive

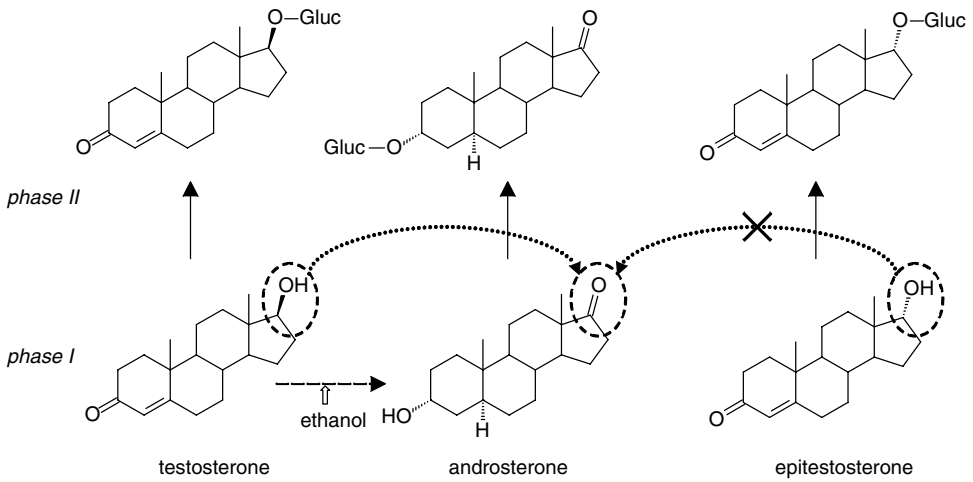

Figure 3. Proposed steroid profile changes associated with ethanol consumption. Competitive enzyme inhibition in phase I metabolism reduces the oxido-reductive conversion of testosterone to androsterone while the 17α hydroxyl group of epitestosterone remains unchanged. Steroid glucuronidation as major phase II process is not noticeably affected.

Table 3. Examples of elevated T/E ratios from routine doping control samples associated with recent alcohol consumption, either as part of a longitudinal study (cases 1 to 5) or as standalone finding (cases 6 and 7)

			T/E			And/T			
case	gender	sampling clock time	finding	basal mean	change	finding	basal mean	change	EtG µg/mL
1	male	08:30	7.4	2.7	174%	32.6	125.7	−74%	155
2	male	08:30	6.9	3.3	109%	12.7	64.7	−80%	168
3	male	07:30	6.5	3.8	71%	19.7	66.3	−70%	314
4	female	14:00	8.1	2.4	238%	156.8	331.7	−53%	56
5	female	10:50	6.8	1.4	386%	45	457.6	−90%	96
6	male	07:00	5.9	n/a	n/a	7.3	n/a	n/a	104
7	male	11:00	12.6	n/a	n/a	1.2	n/a	n/a	80

alcohol consumption. These findings proved a recent intake of ethanol in considerable amounts and suggest a connection to changes in the steroid profiles presumably caused by competitive enzyme inhibition in phase I metabolism. The possibility of such ethanol-induced alterations has to be taken into consideration particularly for out-of-competition tests because in this period of time alcohol consumption in considerable amounts may occasionally happen. Owing to the ease of EtG determination procedures, which may potentially be introduced into routine screening, the inclusion of this marker into the final evaluation of suspicious outliers in T/E ratio longitudinal studies seems to be very useful. Further studies will have to focus on the evaluation of robustness and applicable thresholds for different alcohol consumption markers in urine (ethanol, EtG and ethylsulfate) in relation to the significance of steroid profile changes.

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