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Urinary concentrations of ethyl glucuronide and ethyl sulfate as thresholds to determine potential ethanol-induced alteration of steroid profiles

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The suppression of steroid biotransformation resulting in a decrease of the major urinary metabolites – androsterone and etiocholanolone – and the elevation of testosterone/epitestosterone (T/E) ratios following ethanol administration is well described. At least the latter parameter T/E represents an important indicator for endogenous steroid abuse in doping control.

The quantitative correlation between ethanol consumption markers and steroid profile alteration was evaluated, aiming to differentiate between permitted ethanol administration and potential steroid abuse. Steroid profiles, ethanol, ethyl glucuronide (EtG), and sulfate (EtS) were quantified after administration of ethanol (intended maximum ethanol concentration in blood was 1 mg/g) to 21 male and 15 female volunteers.

EtG concentrations in urine (corrected by either specific gravity or creatinine concentration) were found to be most suitable for quantitative evaluations. Gender specific urinary EtG concentrations of 48 ug/ml (men) and 15.5 ug/ml (women) may be considered as useful thresholds for a potential ethanol-induced suppression of steroids biotransformation.

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

Introduction

The influence of ethanol intake on a variety of biotransformation reaction is widely acknowledged. Metabolic degradation of cocaine,[1] cannabinoids,[2] and in particular steroids[3–7] are known to be suppressed by co-administration of ethanol. Due to the high molar quantities of administered ethanol (1 litre of beer corresponds roughly to 1 mole ethanol and hence exceeds the total testosterone production in the life of a healthy man), even minor metabolic cross reaction may potentially influence many biotransformation reactions by competitive inhibition of enzymes (e.g. 17HSD, UGT2B17 or consumption of co-enzymes (NAD)). It is assumed that the general suppression of oxidative biotransformation reaction is responsible for the final observation in steroid profiles.[8–10] The predominant observation is a clear reduction of the most abundant terminal metabolites of testosterone, i.e. androsterone and etiocholanolone which may be reduced to less than 10% of the base value. The variations of absolute concentrations of testosterone (mild increase) and epitestosterone (decrease) is by far less diagnostic and often influenced by an elevated measurement uncertainty. This is due to the forced diuresis after ethanol consumption leading to very low absolute testosterone concentrations, particularly in women.

Testosterone is amongst the most frequently abused doping agents in sports. It belongs to the category of 'endogenous anabolic steroids' of the prohibited list of the World Anti-Doping Agency (WADA) and the proof of its exogenous administration requires laborious verification. According to WADA regulation,[11] significant deviations from population threshold or deviations of the individual steroid profile[12] require follow-up examination, for example, by carbon isotope ratio mass spectrometry.[13] Therefore, it appears to be essential for doping controls to sort out alternative sources for steroid profile alteration (e.g. ethanol intake) efficiently.

Ethylglucuronide (EtG)[14–16] and ethylsulfate (EtS)[17,18] are well-established ethanol consumption or abstinence control parameters in clinical and forensic toxicology. The former parameter was shown to be a robust marker for ethanol administration, although recently low concentrations were suspected to result from alternative sources and the long-term stability may be limited by bacterial contamination and subsequent degradation. Therefore, EtS gained relevance as a complementary parameter due to its increased stability with respect to enzymatic hydrolysis.

Various ethanol administration sub-projects were carried out to examine the correlation of steroid profiles with conventional ethanol consumption markers systematically to investigate:

- duration and extent of ethanol-induced elevation of testosterone/epitestosterone (T/E) ratios;
- relevance and options for normalizations of concentrations of EtG and steroids in urine;
- inter-individual variation and gender specificity of ethanol induced increase of T/E;

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- estimation of minimum ethanol amounts and concentrations which may cause significant effects in steroid metabolism; and
- suitability of urinary concentration of ethanol, ethylglucuronide or ethylsulfate as semi-quantitative markers (threshold concentration) for ethanol induced variation of steroid profiles.

Methods and materials

Examination of steroid profile parameters

All relevant steroid concentrations were examined by gas chromatography coupled to mass spectrometry (GC-MS) according to analytical procedures commonly used in doping analysis.[9,19] Steroid standards used for quantification were certified reference compounds (95% minimum purity, National Measurement Institute, Pymble, Australia). Briefly, a mixture of internal standards (epitestosterone-d3 and androsterone-d4-glucuronide at concentrations of 100 and 1000 ng/ml, respectively) was added to urine aliquots of 2 ml prior to hydrolysis by β-glucuronidase from *Escherichia coli* (Roche, Mannheim, Germany). Subsequently, a liquid-liquid extraction was carried out using methyl tert-butyl ether (analytical grade, Sigma-Aldrich, Munich, Germany) at a pH value of 9. The dried residues were derivatized by addition of 50 µl of the silylation reagent consisting of N-methyl-trimethylsilyl-trifluoroacetamide (MSTFA, GC grade, Macherey & Nagel, Duren, 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 analyzed on a 6890/5975 GC/MSD system (Agilent Technologies, Waldbronn, Germany). One microlitre of the extract was injected in split mode (1:10) 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. When required (i.e. low epitestosterone concentrations), samples were re-injected in splitless mode. The oven temperature was ramped from 130 °C to 185 °C (0–1 min), increased to 235 °C at a rate of 5 °C/min and adjusted to a final level of 325 C (30 °C/min), which was held constant 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 trimethylsilyl-derivatives of testosterone and epitestosterone (m/z = 432) or androsterone (m/z = 434) extra to the deuterated internal standards epitestosterone-d3 (m/z = 435) and androsterone-d4 (m/z = 438). The intermediate precision for the T/E and androsterone/ testosterone (A/T) ratios was 7.5% and 11.1%, respectively.

Determination of EtG and EtS in urine

Quantitation of EtG and EtS in urine is well established in clinical and forensic toxicology and analytical procedures are published elsewhere in more detail.[9,14] Ethylglucuronide, ethylsulfate, EtG-d5, and EtS-d5 were purchased from Medichem Diagnostica (Steinenbronn, Germany). The internal standards (EtG-d5 and EtS-d5, final concentrations of 20 µg/ml), were added to a diluted centrifuged urine sample (diluted 1 : 1 *v/v* by aqueous LC eluent A). Mixtures of 2 mM aqueous ammonium acetate solution and acetonitrile 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*). Five µl of the sample was directly injected onto a Zorbax Eclipse XDB-C8 column (100 × 2 mm, 3 micron) and separated using isocratic elution with 60% B at a flow rate of 0.250 ml/min.

An API 3200 triple quadrupole mass spectrometer (Applied Biosystems, Darmstadt, Germany) was applied in negative electrospray ionization mode. Multiple reaction monitoring acquisition of the following fragmentation reactions was carried out at a declustering potential of -60 V and dwell times of 100 ms:

EtG-d5: 226 ➔ 85 (collision energy CE −25 eV),

EtG: 221 ➔ 113 (CE −20 eV),
221 ➔ 85 (CE −25 eV),
221 ➔ 75 (CE −25 eV)

EtS-d5: 130 ➔ 80 (CE −42 eV) and

EtG: 125 ➔ 97 (CE −22 eV).

Upper calibration limits of EtG and EtS were 80 µg/ml. The intermediate precision at the level of the QC sample (i.e. EtG = 20 µg/ml) was 5.2%.

Ethanol administration studies

There were three distinct phases of the study. The first part was designed to estimate the influence of the total ethanol dosages and useful time windows and frequency of sampling, whereas the goal of the second was mainly the examination of differences due to maximum blood concentrations (BAC). Due to the high analytical effort, the number of participants in these sub-project was limited to five. The administration was carried out based on the evaluation of BAC according to Widmark's Equation and controlled in a timely manner by breath analysis and retrospectively by examination of BAC. Maximum ethanol administrations equivalent to 1 mg/g BAC were assumed to be sufficient for a significant influence on steroid concentrations. The intended sampling duration of blood was the time-span to total elimination of ethanol, urine samples should be collected at least 24 h. In stage 2, similar amounts of ethanol were administered either at once (sculling) or maintaining a constant BAC of 0.3 mg/g in order to differentiate the relevance of total accumulated ethanol dosages versus maximum blood alcohol concentrations.

The final part consisted in the determination of the statistical significance of ethanol-induced elevation of T/E ratios and resulting inter-individual variations and gender specificities. The number of test persons included in this module was 31. The sampling time was limited to 3–5 h for blood and urine samples. The intended maximum BAC was 1 mg/g and sampling could be stopped when the maximum BAC was exceeded. The amount of ethanol was theoretically predicted and controlled by breath as well as blood alcohol examination.

Single administrations of larger amounts of ethanol (i.e. > 0.4 g/kg) were accomplished by drinking defined volumes of vodka (37.5 Vol-%) at once. Long-term administrations of maintenance doses of ethanol was carried our empirically, i.e. incorporation of estimated amounts of any alcoholic drink was simultaneously controlled by breathalyzer tests and verified by blood ethanol testing.

All administration studies were approved by the ethics committee of the University of Munich.

Results and discussions

All steroid profiles collected in the scope of the first sub-project revealed significant variations of T/E ratios and concentrations of androsterone (A) and etiocholanolone after ethanol administration. Due to the forced dieresis after ethanol administration, it is essential to compensate absolute concentrations of EtG and EtS appropriately, while UAC concentrations are not affected by renal excretion[20] and can remain uncorrected. The application of a specific gravity correction (which is a default procedure in doping control) as well as reference to urinary creatinine concentration is apparently adequate (Figure 1). The suitability of analytical procedures covering the low density (low concentration) range is essential for a successful compensation of renal clearance.

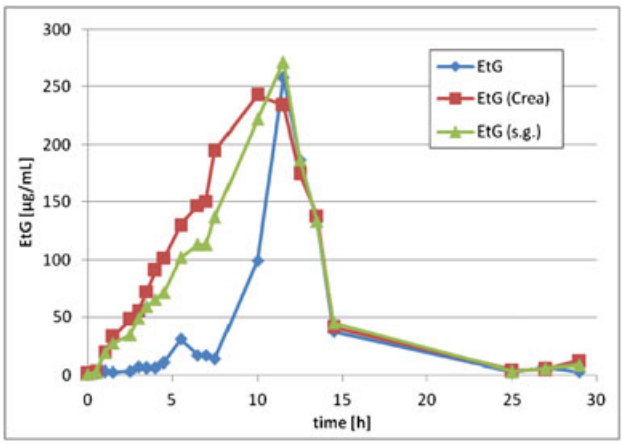

Figure 1. Specific gravity or creatinine correction of urinary concentrations of EtG and EtS are equally suitable and essential to compensate for ethanol induced diuresis and resulting significant sample dilution. The accuracy of the respective technique in highly diluted urine samples should be determining for the selection of either specific gravity or creatinine as dilution marker.

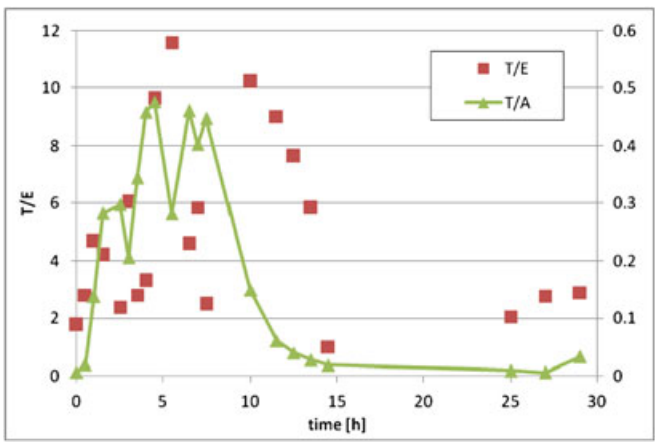

Figure 2. The accuracy of T/E ratios is critically dependent on the absolute epitestosterone concentration in urine. The figure represents urine samples of a female test person. In particular T/E ratios examined in diluted samples (1–8 h) are significantly associated with high analytical uncertainties. Alternatively, the T/A-ratio of testosterone to androsterone (which is much more concentrated yet significantly depleted after ethanol intake) may be used as more robust parameter for correlation of ethanol related steroid interference.

More problematic was the accurate determination of epitestosterone (and hence of T/E ratios) in case of extremely low absolute concentrations. The time frame of 1–5 h post-administration was particularly critical in case of female test persons. The examination of the ratio T/A is apparently more selective and robust due to the fact that concentration of A is significantly higher and resulting analytical uncertainties are lower than those of E (Figure 2). Moreover, the decrease of A concentrations is far more pronounced than the reduction of epitestosterone. However, the following considerations were focused to the T/E ratios which are the best

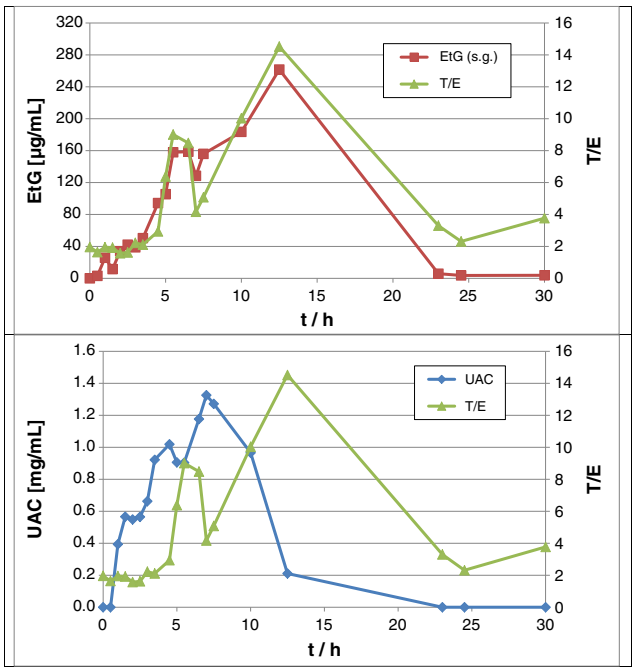

Figure 3. Comparison of temporal correlations between urinary T/E ratios to specific gravity corrected EtG (female volunteer TP5, comp. Table 1) and ethanol concentrations.

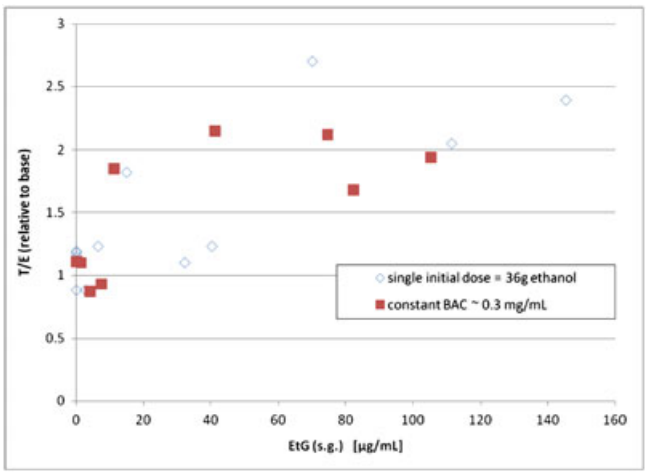

Figure 4. Comparison of ethanol induced increase of relative T/E ratios (calculated relative to base values prior to ethanol intake) after one single high dosage and subsequent low dosages (similar total amount, constant BAC of ~0.3 mg/g). Significant and similar elevation of T/E ratios is observed in each case (both referring to TP 1).

established conventional parameters in evaluation of steroid profiles.

Evaluating the time-courses of UAC, T/E, and urinary EtG, the correlation between T/E ratios and EtG is apparently superior to the association between T/E and UAC. This is depicted in Figure 3 and results from statistical evaluations of the five test persons (TP1-5) involved in the long-term examination. The 2-tailed significance levels of a positive correlation between T/E ratios and EtG [UAC] are: TP1 = 0.0011 [0.38], TP2 = 0.096 [0.59], TP3 = 0.000012 [0.81], TP4 = 0.0008 [0.53] and TP5 = 0.001 [0.31].

Urinary concentrations of EtG are obviously more eligible for quantitative estimation of ethanol induced steroid variation than UAC, which tends to overestimate the likelihood of ethanol interference in the early phase of administration and ignores prolonged effects which are regularly observed after complete ethanol elimination. The statistical significance of ethylsulfate is only slightly lower than EtG, and EtS could be considered another optional parameter, in particular if microbial contamination and subsequent sample deterioration maybe assumed.

The influence of ethanol on steroid profiles was initially discussed after administration of high dosages[4,6] and is often associated with 'heavy' or 'inebriate' drinking behaviour,[21] while increased testosterone and depletion of A was already observed after low dosages of ethanol.[7] To compare the relevance of high peak concentrations of ethanol with repeated applications of low ethanol dosages, a similar amount of ethanol (0.5–0.6 g/kg) was administered to TP1-5 following different protocols. The first scenario consisted in a rapid intake of the total amount of ethanol and subsequent evaluation of ethanol, EtG and T/E ratios to the end of ethanol and EtG elimination. In a second experiment, a similar amount of

Table 1. Body weight, height, maximum blood alcohol concentration, base and maximum values of T/E ratios of test persons involved in the statistical sub-project. Three individuals (TP15, 23, and 33) did not exhibit a significant variation of T/E-values after ethanol administration. TP 33 had to be discarded due to an insufficient sampling interval. TP15[*)] represents a repetition of TP15.

TP	Sex	weight [kg]	height [cm]	T/E base	T/E max	BAC max [mg/g]
1	m	75.0	174		multiple studies	
2	m	109.0	190		multiple studies	
3	m	69.0	171		multiple studies	
4	m	72.0	180		multiple studies	
5	f	61.0	172	1.9	14.7	1.272
6	m	84.0	185	1.92	2.67	0.954
7	m	77.0	178	0.62	0.98	0.905
8	f	54.0	164	1.02	3.11	0.732
9	m	82.0	186	2.21	2.80	0.869
10	f	68.0	178	3.23	5.89	1.054
11	f	67.0	180	2.82	3.36	1.144
12	f	70.0	169	1.89	11.20	0.695
13	f	60.0	171	2.42	3.22	0.692
14	m	79.0	180	0.62	0.84	0.841
15	m	82.0	190	*0.10*	*0.12*	*0.681*
16	m	79.0	189	3.29	4.13	0.783
17	m	81.0	180	1.08	1.92	1.385
18	m	98.0	190	1.24	2.01	0.941
19	m	74.0	195	1.26	1.46	0.998
21	m	72.0	180	0.17	0.22	0.522
20	m	76.0	177	3.90	7.70	1.053
22	m	73.0	176	2.00	2.60	1.037
23	m	78.0	197	*0.10*	*0.10*	*1.052*
24	m	75.0	193	1.40	2.20	1.032
25	m	68.0	175	0.60	1.00	0.932
26	m	68.0	174	0.90	1.20	0.907
27	f	61.0	164	1.80	5.00	0.734
28	m	79.0	174	0.70	1.20	1.123
29	m	78.0	185	0.90	1.70	1.005
30	f	63.0	181	2.30	4.50	0.773
31	f	59.0	160	0.80	2.95	0.788
32	f	53.0	163	2.20	9.82	0.795
33	f	47.0	163	*0.30*	*0.30*	*0.529*
34	f	65.0	168	1.65	1.98	1.214
35	f	70.0	178	0.13	0.47	0.579
36	f	54.0	164	0.36	0.79	0.826
15[*)]	m	84.5	190	*0.10*	*0.13*	*0.75*

ethanol was spread throughout the same timeframe, i.e. a BAC of 0.2–0.3 mg/g was maintained (controlled by breath analyses) over a time-span of 5 h.

Similar effects were observed following both administration protocols and in particular the T/E ratios were similarly affected by single high dosages as well as by repeated low dosages of ethanol (Figure 4). It is likely that a suppression of steroid biotransformation maybe observed after low ethanol dosages and the elevated T/E or T/A ratios depend on total accumulation of subsequent alcohol administration cycles rather than maximum peak concentrations of ethanol in blood.

The evaluation of statistical significance was carried out based on ethanol administration studies in 31 volunteers. Individual parameters and certain analytical data are summarized in Table 1. The average maximum of BAC was 0.88 mg/g (stdev = 0.20 mg/g). Significantly increased T/E values were observed in 28 cases, data from one female were discarded due to insufficient sampling time. Two male 'non-responders' were characterized by very low T/E ratios which remained unaffected after ethanol intake. This lead to the suspicion that the low T/E ratio might result from the well described polymorphism of the UGT 2B17 gene.[22] One of the two candidates was available for re-testing of the T/E ratios (tab.1, TP15 and TP15*)) and subsequent UGT2B17 genotyping. Following a standard procedure,[23] the genotype could be identified as del-del resulting in significant depletion of T/E ratios.

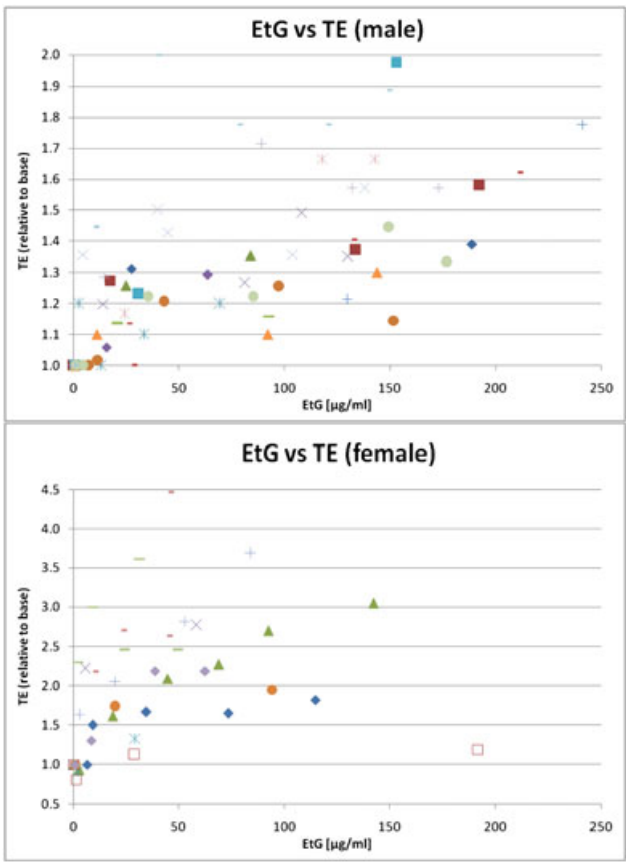

Figure 5. Correlation of T/E ratios (calculated relative to base values prior to ethanol intake) with specific gravity corrected EtG concentration in urine. A significant positive correlation was observed in all cases except two male test persons (Table 1).

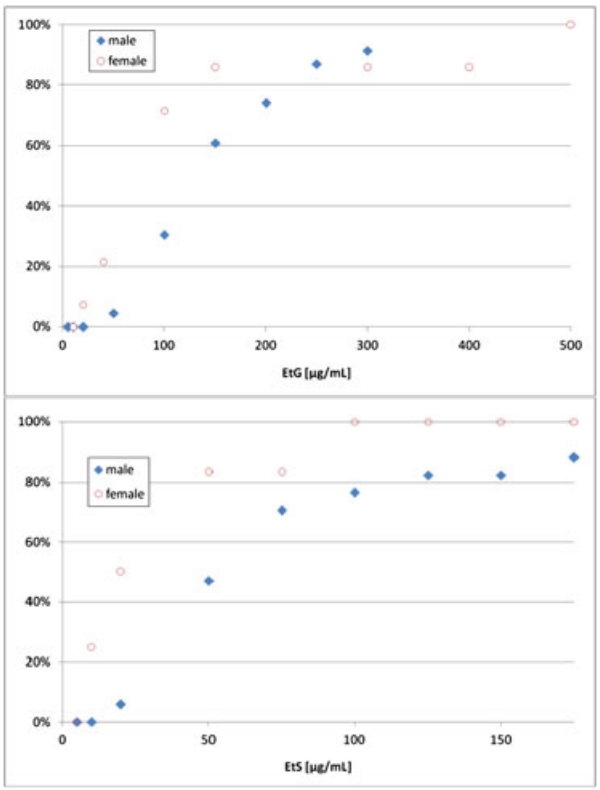

Figure 6. Histograms of the relative frequency of a significant increase of T/E ratios (i.e. deviation from base value of 30% in males and 60% in females) depending on corrected urinary concentrations of EtG and EtS, respectively. The characterization of the cohorts is described in Table 1.

Significant increases of T/E ratios were observed in all remaining cases (Figure 5). The slope of the T/E – EtG curve is significantly higher in female individuals. This is in good accordance to WADA recommendations for identification and management of elevated T/E ratios,[11] defining a 30 % deviation from base-values in men and a 60 % increase of T/E ratios in women as critical variation which should result in follow up examinations. Based on the slope of individual curves in Figure 5, the critical EtG concentration for an atypical (i.e. 30% in men or 60% in women) was identified for each subject. A significant T/E-increase was observed in 93% of the cases. Minimum s.g. corrected urinary EtG concentration 48 ug/ml in women and 15.5 ug/ml were compatible with significant alterations of T/E ratios. Therefore, corresponding concentrations – as appropriate after inclusion of safety margins – would be suitable as minimum threshold values for presumptive ethanol-induced variation of steroid profiles. In contrast, the lowest sensitivity -corresponding to a maximum EtG concentration causing a critical increase of T/E ratios was observed in TP16 (male, 274 µg/ml) and TP11 (female, 436 µg/ml). Figure 6.

Conclusion

There is a clear quantitative and reproducible correlation between ethanol intake and steroid profiles, in particular a significant increase of T/E and T/A ratios was observed. The phenomenon was proven in 33 (out of a total number of 36) subjects. The duration of T/E-elevation is correlated best with urinary EtG concentrations which are therefore potential

markers for ethanol-induced alteration of steroid profiles in doping control analyses. Significant total ethanol dosages – for example, by long-term accumulation of moderate quantities – are apparently sufficient for inhibition of steroid biochemistry whereas high peak concentration of ethanol in blood is not mandatory.

The quantitative correlation between concentration of EtG and T/E-increase is significantly higher in females. Urinary EtG-concentrations greater than 48 ug/ml in man or greater than 15.5 ug/ml in women could be regarded as threshold values for potential ethanol induced variation of T/E ratios.

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