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Identification and quantitative determination of long-term alcohol markers ethylglucuronide and ethylsulfate in human urine by LC-MS/MS in doping control analysis

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Abstract

Alcohol intake can influence the urinary testosterone/epitestosterone ratio and may consequently lead to misinterpretation of the athlete's urinary steroid profile. The aim of this study was to develop a fast and reliable LC-MS/MS method to determine ethylglucuronide and ethylsulfate as long-term alcohol metabolites to indicate alcohol related elevation of the testosterone/epitestosterone ratio.

Two studies with selected urine samples (n_1=528, n_2=120) from different classes of sports indicated that alcohol consumption is fairly common in sports. Within the investigated urine specimens, out of competition samples and samples from teamsports show elevated EtG and EtS values most frequently (n_1 positive findings: *total = 8.1%, out of competition = 4.7%, team sports = 4.0%*; n_2 positive findings: *total = 14.2%, out of competition = 9.2%, team sports = 6.7%*)

An excretion study was performed to estimate the duration and magnitude of the alcohol-related testosterone/epitestosterone increase. For all post-administration samples relevant urinary amounts of ethylglucuronide and ethylsulfate were detectable up to 40 hours after alcohol intake. Furthermore a good correlation between ethylglucuronide-, ethylsulfate-concentrations and T/E ratio could be demonstrated, so that detection of these long-term alcohol markers is a suitable method to indicate alcohol related changes in steroid profile.

Introduction

In recent years numerous studies have demonstrated that alcohol consumption can influence the urinary steroid profile. The alcohol related increase of urinary testosterone/epitestosterone (T/E) ratio is of particular interest for doping control laboratories, as it can lead to misinterpretation of the steroid profile. The main problem in measuring ethanol levels in blood or urine is the short half-life of ethanol in these body fluids, thus a few hours after intake, alcohol consumption is no longer detectable. However, to detect changes in the

steroid profile after alcohol consumption long-term alcohol markers, such as ethylglucuronide (EtG) and ethylsulfate (EtS) are most promising. [2]

The present study was initiated to set reference values of urinary excreted EtG and EtS and to investigate the incidence of alcohol intake in elite athletes. Therefore, a quantitative analytical method by means of liquid chromatography coupled to tandem mass spectrometry utilizing direct injection of urine was developed.

Doping control samples of 528 athletes covering different classes of sports were analysed, and both EtG and EtS concentrations in urine were determined. Furthermore, 120 samples with atypical T/E-findings were investigated for the presence of alcohol markers. In addition an excretion study was performed to collect pharmacokinetic parameters of EtG and EtS to define the magnitude and duration of ethanol effects on the steroid profile.

Materials and Methods

A volume of 100 µL urine were fortified with 0.1 µg D5-EtG and D5-EtS (IS). After shaking 5 µL were injected into the LC-MS/MS system. While EtG, D5-EtG, and EtS were obtained from commercial sources, D5-EtS (IS) was prepared in-house.

Table 1: Summary of LC-MS/MS parameters

LC-MS/MS	Thermo Scientific TLX(Thermo Fisher) TSQ Vantage (Thermo Fisher)
Column:	Gemini 3u C6-Phenyl 110 A (150 x 4.60 mm; particle size 3 µm) (Phenomenex, Aschaffenburg)
Mobile phase:	**A:** ammonium acetate buffer (5 mM/L, 0.1 % glacial acetic acid) **B:** acetonitrile gradient: 5 % B 0 min flow rate: 0.8 ml/min 100 % B 7 min 95 % B 11 min
Injection Volume:	5 µl
Ionization:	ESI (350°C), in negative Mode Spray Voltage -3500 V
MS/MS Mode:	MRM

Validation

The method was fully validated for quantitative purposes considering the parameters specificity, intra- and interday precision, accuracy (recovery), linearity, limit of detection, limit of quantitation, stability and ion suppression effects.

Results and Discussion

Based on the validation data (Table 2) the LC-MS/MS method was suitable for the quantitative determination of urinary EtG and EtS.

Table 2: Summary of MS parameters and validation results

	MS/MS parameters	Validation results					
	precursor/production (m/z)	**collision energy (eV)**	**intraday precision CVs (%, n=6+6+6)**	**interday precision CVs (%, n=18+18+18)**	**recovery (%)**	**LOD (S/N>3, ng/mL)**	**LOQ (S/N>10, ng/mL)**
EtG	**quantifier ion: 221→85**	24	2.9 – 6.1	4.4 – 14.1	102	4.7	15.5
EtS	**quantifier ion: 125→97**	22	1.2 – 2.6	5.4 – 18.6	99	0.8	2.5

Studies with selected urine samples

EtG and EtS determination of 528 urine samples from different classes of sports demonstrated that approximately 25% of the examined samples were positive for the determined alcohol markers. Corresponding to a previous study from Helander *et al*. a good correlation between EtS and EtG with somewhat higher mean concentrations for EtG (mean EtG:EtS ratio: 1.8) were monitored [4]. The highest concentration was determined in one sample (team sport) with 472 µg/mL EtG and 153 µg/mL EtS (specific gravity corrected).

To verify alcohol consumption as a possible cause of elevated T/E ratios, 120 urine samples with atypical T/E ratios were examined for the presence of the two alcohol markers EtG and EtS. In 14.2% increased EtG and EtS concentrations were found, indicating increased T/E values of the concentration range probably caused by alcohol intake. Comparable to the athletes' group (n=528) relevant concentrations of alcohol markers (conc. > 1µg/mL) were detected more frequently in out-of competition samples. Furthermore in both studies most positive alcohol findings were detected for team sports.

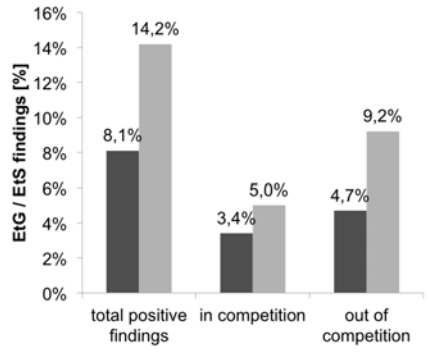

Figure 2: Distribution of EtG and EtS findings between out of- and in-competition samples (athletes, n=528; atypical T/E, n=120).

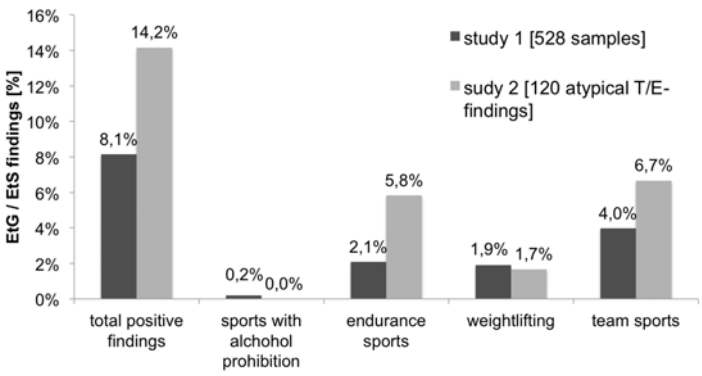

Figure 3: Distribution of EtG and EtS findings between different classes of sport. In both studies most positive alcohol findings were detected for team sports.

Excretion study

After at least five days of abstinence three male and three female test persons ingested 1.0 to 1.7 g ethanol per kg body weight over 5 hours to simulate common alcohol consumption. Urine was collected before intake and spontaneously as needed up to 40 hours afterwards.

The excretion study shows a good correlation between alcohol markers and elevated T/E ratios for all test persons. Two urine samples of volunteer P2 demonstrated increased T/E ratios above the WADA threshold of 4.

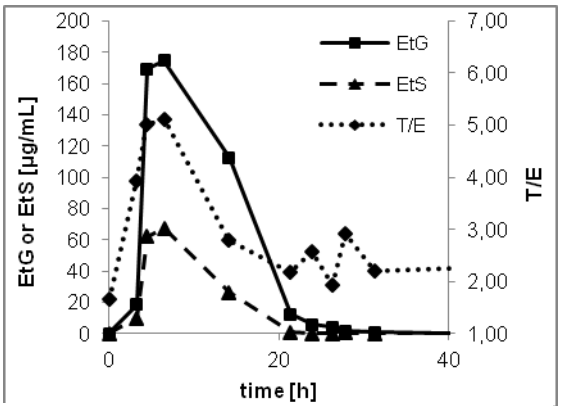

Figure 1: Comparison of the urinary T/E ratio and corresponding EtG and EtS concentrations as a function of time for volunteer P2.

Table 3: Maximum T/E values and max. concentrations for EtG and EtS (specific gravity corrected) for the 6 volunteers

test person	sex	T/E (basal)	EtG (max) [µg/mL]	EtS (max) [µg/mL]	T/E (max)
P1	m	0.59	288.5	123.5	1.32
P2	f	1.67	174.5	67.3	5.11
P3	m	1.25	75.8	20.3	1.95
P4	f	1.07	56.1	11.4	1.81
P5	m	1.89	197.1	91.9	3.00
P6	f	1.48	30.8	10.6	3.74

Conclusion

The developed LC-MS/MS method allows a fast and reliable determination of ethylglucuronide (EtG) and ethylsulfate (EtS) with limits of quantitation in the sub ng/mL range. As demonstrated in other studies T/E ratios and associated EtG / EtS concentrations highly correlated, and thus, can be used as reliable markers for alcohol-consumption to identify alcohol-related T/E increase [2]. Investigated urine specimens, both athletes and those with elevated T/E ratios demonstrated, that alcohol-related T/E increase was detected more frequently in out-of competition samples. Within investigated classes of sport positive alcohol findings were found to a greater extend in team-sport.

In post-administration samples relevant urinary amounts of EtG and EtS were detectable up to 40 h after alcohol intake. In agreement to other studies, a high correlation between EtG and EtSconcentrations and T/E ratios could be demonstrated. [4] A significant elevation in T/E ratio was observed in all subjects, while the magnitude of increase differ strongly in gender.

References

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