Forensic Confirmatory Analysis of Ethyl Sulfate—A New Marker for Alcohol Consumption—by Liquid-Chromatography/ Electrospray Ionization/Tandem Mass Spectrometry

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Ethyl sulfate (EtS)—a new direct marker for ethanol intake besides ethyl glucuronide (EtG) and others—was detected in urine samples by electrospray ionization tandem mass-spectrometry (LC-ESI-MS/MS). Ethyl sulfate sodium salt was used for method development, yielding a precursor $[M - H]^-$ *m/z* 125 and product ions *m/z* 97 $[HSO_4]^-$ and *m/z* 80 $[SO_3]^-$. Pentadeuterated EtS (D_5-EtS) was synthesized by esterification of sulfuric acid with anhydrous hexadeutero ethanol ($[M - H]^-$ *m/z* 130, product ions *m/z* 98 $[DSO4]^-$ and *m/z* 80 $[SO3]^-$). After addition of D_5-EtS and D_5-EtG, urine samples were analyzed by direct injection into the gradient LC-MS/MS system. Analysis was performed in accordance with forensic guidelines for confirmatory analysis using one precursor and two product ions. EtS has been detected (in addition to EtG) in the urine samples of nine volunteers after drinking sparkling wine containing between 9 and 49 g of ethanol. Both EtS and EtG could be detected up to 36 h after consumption of alcohol. The excretion profile was found to be similar to that of EtG. No EtS was found in teetotalers' urine samples. Method validation parameters are presented. EtS was stable in urine upon storage up to twenty days at room temperature. In addition to EtG, EtS can be used to detect recent alcohol consumption, thus providing a second marker for the time range of up to approximately one day after elimination of ethanol from urine samples. The determination of EtS can be used in addition to EtG as proof of ethanol consumption in workplace monitoring programs. (J Am Soc Mass Spectrom 2004, 15, 1644–1648) © 2004 American Society for Mass Spectrometry

Ethyl sulfate (EtS) is a newly discovered direct ethanol metabolite. The formation of ethyl sulfate by conjugation of activated sulfate and ethanol by rat liver was reported in 1959 [1], and its detection in rat urine after dosing with ^{35}S-sulfate and ethanol, was performed by thin-layer chromatography and by autoradiographic detection on X-ray films [2]. Lung tissue was found to have the ability to metabolize ethanol via glucuronidation [3] and by sulfation [4, 5]. Later, Manautou and Carlson compared the hepatic and pulmonary metabolism via glucuronidation and sulfation in rats and rabbits [6]. Meanwhile, for sulfate conjugation a superfamily of cytosolic sulfotransferases has been described that shows a genetic polymorphism [7]. The conjugation of aliphatic alcohols in humans has been mentioned by Bonte et al. when investigating metabolites of higher aliphatic alcohols [8]; detection was performed indirectly via cleavage by sulfatase followed by analysis of the alcohols. However, no direct analytical method to detect and quantify EtS as a marker for recent ethanol consumption by humans has been available until very recently. Parallel to our work, Helander and Beck have developed an LC-ESI-MS assay using single-quadrupole mode and D_5-ethyl glucuronide as internal standard for quantitation of EtS in urine samples [9]. However, selected ion monitoring of the deprotonated molecule with a single-stage quadrupole MS cannot be applied to forensic samples as the only method for compound detection, since it does not fulfill forensic criteria to prove compound identity [10]. When the results of urine drug testing can affect an individu-

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al's reputation, job status, or freedom, forensically-acceptable analytical procedures must be utilized, and the findings legally defensible. For a confirmatory analysis in forensic, veterinary, or environmental toxicology, international guidelines require chromatographic separation and at least one precursor ion with two product ions for compound detection and identification by LC-MS/MS or monitoring of three selected ions with single-stage MS instruments [11–13]. LC-ESI-MS/MS seems to be the method of choice for forensic confirmatory analysis of EtS. Our aim was to develop and to validate an LC-MS/MS method with two transitions for EtS which would fulfill these requirements for forensic confirmatory analysis, thus being suitable for the detection of relapse in alcohol-withdrawal treatment, in workplace monitoring programs, and for the differentiation of ethanol derived from intake of ethanol or generated post-mortem or post-sampling by fermentation in forensic cases.

Experimental

Chemicals and Sample Preparation

Ethyl sulfate sodium salt was obtained from ABCR (Karlsruhe, Germany) (purity 99.8%), hexa-deutero ethanol (anhydrous, purity >99.5%) from Sigma-Aldrich, (Deisenhofen, Germany), sulfuric acid (95–97%), HPLC-grade acetonitrile and formic acid (analytical grade) from Merck, (Darmstadt, Germany). EtG and D_5-EtG standards were obtained from Medichem (Stuttgart, Germany). Deionized water was prepared with a cartridge-deionizer from Memtech (Moorenweis, Germany).

D_5-EtS was synthesized by adding D_6-ethanol (116 μL; 1.96 mMol) to sulfuric acid (106 μl, 1.93 mMol) in an autosampler vial and heating for 60 min at 80 °C. A dilution of the reaction mixture with water (1: 100,000; vol/vol) was stored, and 20 μl of this D_5-EtS solution were added to each urine sample prior to analysis. For calibration, the same amount of this internal standard solution was added to EtS-free urine samples, which had been spiked with 0.05–50 mg L^{-1} EtS (prepared from a stock solution with 1.18 g L^{-1} EtS sodium salt, equal to 1 g L^{-1} EtS). For determination of EtG, parameters of the previously published method were used [10]. After the addition of deuterated standards, 0.1 ml urine samples were diluted to 0.2 ml with 0.1% formic acid and 10 μL were injected into the LC-MS/MS system.

Controlled drinking experiments with healthy volunteers have been approved by the ethics commission of the University Hospital Freiburg. Four male and four female volunteers (age: 21–63) drank 0.1 to 0.2 L of sparkling wine (9 or 18 g ethanol) with a brunch. Spot urine samples were collected up to 44 h. Furthermore, one volunteer drank wine (0.54 L; 49 g ethanol) in the evening, spot urine samples were collected for 36 h at variable intervals. Urine samples were stored at −20 °C until analysis.

Creatinine values have been determined by the Jaffe reaction with picrinic acid and urine ethanol concentrations (UAC) by a standard headspace gas chromatographic method with a flame-ionization detector (HS-GC/FID; cut-off: 0.004 g dL^{-1}).

Instrumentation

The LC-MS/MS system consisted of an API 365 triple-quadrupole mass-spectrometer fitted with a turbo ionspray interface (Applied Biosystems/Sciex, Darmstadt, Germany) and a Shimadzu HPLC system (three pumps LC10AD Shimadzu, Duisburg, Germany). Analyses were performed with electrospray ionization using a turbo ionspray source in the negative mode. EtS and EtG were separated at 40 °C on a polar-endcapped phenylpropyl reversed phase column (Synergy Polar-RP 250 × 2 mm, 4 μm) with a guard column (4 mm × 2 mm, same packing material) (Phenomenex, Aschaffenburg, Germany). The mobile phase consisted of solvent A [aqueous 0.1% formic acid (vol/vol)] and Solvent B (acetonitrile). The following gradient elution was used at a flow-rate of 0.2 mL min^{-1}: 0–1 min: 0% B, 1–3 min: 0–30% B linear, 3–7 min 30–95% B linear, 7–8: 95%–0% B linear, 8–15 min: 0% B for re-equilibration. To enhance signal intensity, acetonitrile was added with a post-column "tee" before the effluent enters the turbo ionspray interface. With a six-port/two-way switching valve (Labpro, Rheodyne) the LC-effluent was admitted to the MS interface only between 3 and 6 min of the chromatographic retention time. The turbo ionspray source was operated at 400 °C with an ionization voltage of −4 kV, and nitrogen as curtain gas and nebulizer gas. Analysis was performed by multi-reaction monitoring, using the precursor ion at *m/z* 125 and the product ions at *m/z* 80, 97 for EtS, and precursor ion at *m/z* 130 and product ions at *m/z* 80 and 98 for the internal standard D_5-EtS (dwell-times: 200 ms for each transition, defragmentation potential: −20 V, focussing potential: −150 V, entrance potential: −10 V, collision cell entrance potential: −10 V, collision energy: −20 eV (for monitoring the non-fragmented precursor ions with *m/z* 125 and 130, respectively) and −35 eV (for the product ions), collision cell exit potential: −15 V, analyzer gas pressure: 2.4–0.7 × 10^{-5} torr nitrogen; unit resolution for Q1 and Q3). For EtG determination the previously described isocratic LC-method was used, but EtG was also monitored in the gradient method for EtS (MRM-transitions for EtG: Precursor *m/z* 221, product ions: *m/z* 75, 85, 113, and 159, precursor ion at *m/z* 226 and product ion at *m/z* 75 for the internal standard D_5-EtG); (instrument parameters see [10]).

Results and Discussion

Method Development and Validation

The characteristic precursor ions with ESI in the negative mode of EtS and D_5-EtS are $[M - H]^-$ 125 and 130, respectively. The product ion spectra (summed spectra obtained at three collision energies: 20, 35, and 50 eV,

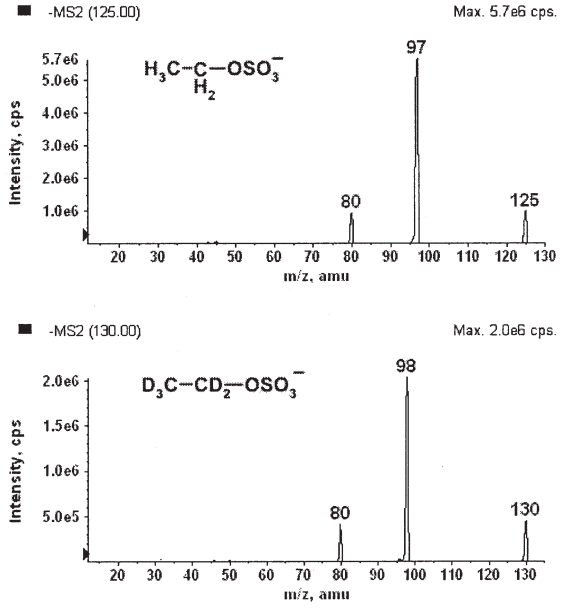

Figure 1. Product-ion spectra of EtS and D_5-EtS (precursor ions *m/z* 125 and 130).

respectively) are shown in Figure 1, with *m/z* 80 $[SO_3]^-$, *m/z* 97 $[HSO_4]^-$ and *m/z* 98 $[DSO_4]^-$.

For determination of the limit of detection (LOD) and the limit of quantitation (LOQ) B.E.N.-software 2.0 [14] has been used, based on a linear regression model according to the German Industrial Norm DIN 32645 with equidistant concentration levels in the low concentration range (0, 0.1, 0.2, 0.3, 0.4, and 0.5 mg L^{-1} EtS), with an α-error of 1% and a relative confidence interval of 33% (k = 3) for the quantifier transition (125/97). The transitions 125/80 and the non fragmented precursor ion have been monitored as qualifiers for identification of EtS. For quantitation of samples and for method validation a weighted linear regression (1/×) was used by the quantitation wizard of the Analyst software. A calibration curve with a spiked urine sample—which was free of EtS prior to spiking—is shown in Figure 2. By use of a weighted linear regression (1/×) good correlation was obtained (R^2 = 0.9997) over the whole concentration range. Repeatability (intra-day precision and inter-day precision) and accuracy (bias) were determined by replicate analyses of spiked urine samples used as quality control samples with three concentration levels of EtS (0.2, 5.0, and 25 mg L^{-1}, respectively). Analyses were performed five times on one day and once on five different days, respectively. The validation data for low, medium, and high concentration levels were: intra-day precision: 7.21%, 3.51%, 4.40% relative standard deviation (RSD); inter-day precision: 9.07%, 4.41%, 2.54% RSD; bias (inter-day): 6.6%, −6.8 and −0.32%. The LOD and LOQ were 0.05 and 0.11 mg L^{-1} EtS in urine, respectively.

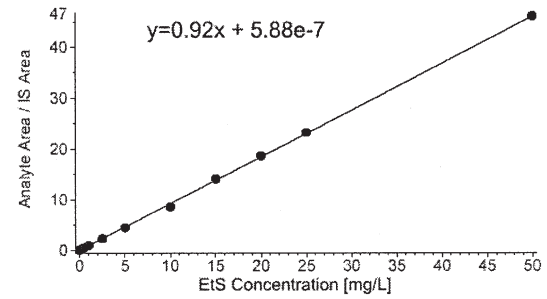

Figure 2. Calibration with spiked urine samples (range: 0.1–50 mg L^{-1} EtS).

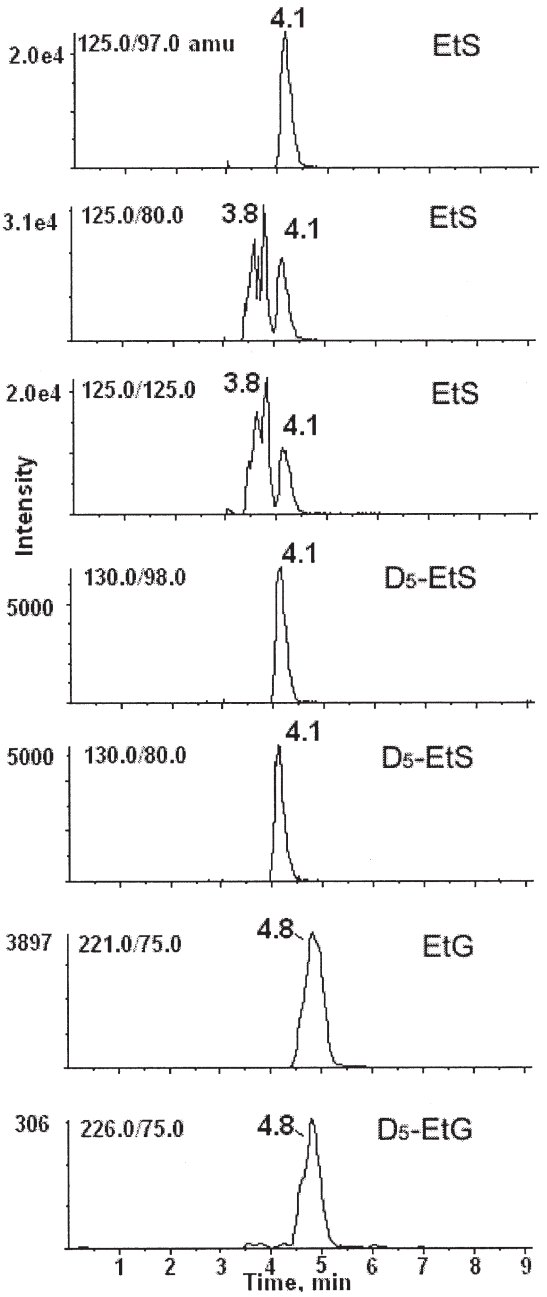

Figure 3. Ion chromatograms of a urine sample of a female volunteer 1.5 h after drinking sparkling wine with 9 g ethanol content (7.6 mg L^{-1} EtS and 5.9 mg L^{-1} EtG were determined).

Figure 3 shows the typical ion chromatograms of the

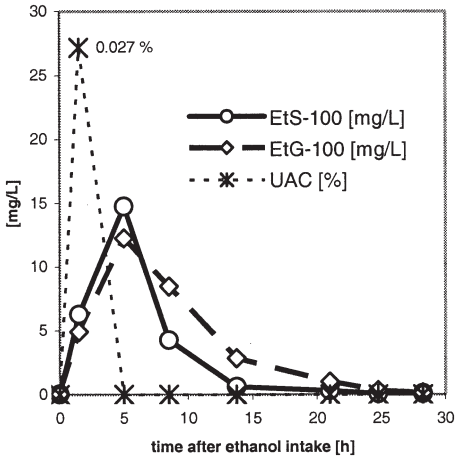

Figure 4. Time course of urine EtS-100 and EtG-100 (after normalizing to creatinine concentration of 100 mg dL^{-1}) of the female volunteer from Figure 3.

quantifier of EtS (125/97) and two additional qualifiers for EtS (125/80 and 125/125) at 4.1 min in a urine sample obtained from a female volunteer 1.5 h after drinking 0.1 L of sparkling wine (9 g ethanol). Additionally, the ion chromatograms of D_5-EtS (130/98—used for quantitation—and 130/80) and for EtG (221/75) and D_5-EtG (226/75) at 4.8 min are shown.

Stability of the D_5-EtS stock solution during storage at 4 °C was confirmed by comparison of ion ratios (EtS/D_5-EtS) with freshly prepared EtS solutions for 26 days. For stability tests upon storage, a positive urine sample from a volunteer study was stored at room temperature on the lab bench for twenty days. No significant degradation was detected during this time in both experiments.

Urinary Excretion Profile of EtS and EtG

The time course of urinary excretion of EtS and EtG of a female volunteer after drinking 9 g ethanol is presented in Figure 4. The excretion profiles of the other volunteers showed similar characteristics, with longer excretion times after consumption of higher amounts of ethanol (up to 36 h after 49 g ethanol uptake). Due to the correlation of EtG concentration and creatinine concentration [15], EtS-100 and EtG-100 were calculated by normalizing to a creatinine concentration of 100 mg dL^{-1}.

The determination showed high specificity: No EtS or EtG were detected in the baseline urine samples of the volunteers collected after one week of abstinence, and no interferences with other compounds were found in the retention time window of EtS (4.1 ± 0.2 min) for the quantifier ion (125/97). However, in some cases with low EtS concentrations the qualifier ions were not chromatographically baseline separated from earlier eluting compounds.

Conclusions

In these first drinking experiments with healthy volunteers, EtS was detected in urine for up to 26 and 36 h after drinking 9 and 49 g ethanol, respectively, and was detectable up to 16 to 27 h longer than ethanol in urine. Similar detection times were found for EtG and EtS. EtS, therefore, can be used as a marker for ethanol consumption in a similar way as ethyl glucuronide. The need for a second specific short-term marker for alcohol consumption arises from the growing legal relevance of EtG-testing especially in workplace-monitoring programs in the United States [16]; therefore, the determination of EtS can be used for confirmation of an EtG positive urine sample. When the question of recent alcohol consumption has to be answered by yes or no, like for the question of lapses, the use of EtG or EtS in urine can be suggested as a test of first choice. The presented method fulfills the requirements for forensic confirmatory analysis of EtS.

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