EtG/EtS in serum by UHPLC-MS-MS in suspected sexual assault cases

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Abstract

A method including semi-automated extraction of ethyl glucuronide (EtG) and ethyl sulfate (EtS) from serum followed by ultra-performance liquid chromatography tandem mass spectrometry (UHPLC-MS-MS) has been developed and validated. Sample preparation prior to UHPLC-MS-MS analysis consisted of protein precipitation and filtration through a phospholipid removal plate. Chromatography was achieved using an HSS T3 column and gradient elution with formic acid in water in combination with methanol. The mass spectrometer was monitored in the negative mode with multiple reaction monitoring (MRM). Two transitions were monitored for the analytes and one for the deuterated internal standards. The limits of quantification were 0.025 mg/L for EtG and 0.009 mg/L for EtS. The between-assay relative standard deviations were in the range of 3.8-9.1%, the recovery was 66-102% and matrix effects ranged from 88% to 97% (corrected with internal standard). Compared to previously published studies, the method presented is semi-automated, uses a simple method for phospholipid removal and has short run times and low LOQs. We analyzed serum samples from 49 female patients presenting to the Sexual Assault Centre at St. Olav University Hospital in Trondheim, Norway, for ethanol, EtG and EtS. EtG and EtS were detected longer than ethanol itself after intake of ethanol, with estimated maximum detection times of more than 24 hours. The ethanol, EtG and EtS concentrations were highly correlated ($p<0.001$), but with large inter-individual variations. This study suggests that analysis of EtG and EtS in serum or blood may complement ethanol analysis and shed light on the patient's recent ethanol intake after ethanol itself is no longer detectable.

Introduction

Ethanol is mainly oxidized to acetaldehyde in the liver, its elimination is rapid and the detection time in blood is limited. A small fraction (<0.1 %) of ethanol is conjugated to ethyl glucuronide (EtG) and ethyl sulfate (EtS) (1, 2). EtG and EtS have been shown to be highly specific markers of ethanol intake and are detectable in blood and urine for a longer period of time than ethanol itself (3-7). In blood, studies have shown detection times up to 10 h after ingestion of a single low dose of ethanol and up to 24 h after large and repeated doses (3, 5, 6, 8). In serum, EtS is detectable for about twice as long as ethanol, and EtG for even longer (3). In a study of heavy drinkers the median ratio between serum and whole blood were 1.69 for EtG and 1.30 for EtS (9), which may prolong the detection window of these analytes in serum compared to whole blood if the same analytical cut-off is used.

Drug and/or alcohol intake is often implied in cases of sexual assault, mostly voluntarily but occasionally as a result of suspected "spiking" of drinks. Ethanol, either alone or together with recreational/illicit drugs, has been the most common finding in previous surveys of alleged drug-facilitated sexual assault (10). It is often of great importance to establish the degree of intoxication of the victim, since many jurisdictions explicitly forbid the sexual exploitation of persons that are too inebriated to consent. A previous study from our group showed that analysis of EtG and EtS in urine greatly increases the detection window of alcohol ingestion in cases of sexual assault, corroborating or, alternatively, refuting the victim's self-reported alcohol ingestion prior to the assault (11). However, it should be noted that the absolute concentration of EtG and EtS in urine after a given dose of ethanol may vary considerably as it is influenced by factors such as urine dilution due to fluid intake, and time intervals of voiding (5). In addition, the detection window of EtG and EtS in urine may be several days; hence, the detection of EtG and EtS in urine from a sexual assault victim could stem from a previous alcohol intake with no relevance to the incident. EtG and EtS in serum, on the other hand, signify a recent alcohol intake, usually within the last 24 hours. Thus, serum testing of EtG and EtS may contribute to describe the timing and extent of alcohol intake more accurately, and could be used as supportive objective evidence to witness observations and statements regarding the victim's ethanol intake and inebriation.

Previously published liquid chromatography – tandem mass spectrometry (LC–MS-MS) methods for EtG and EtS in whole blood and serum have utilized protein precipitation with methanol or acetonitrile as sample cleanup (3, 8, 9, 12). Such precipitation does not remove phospholipids, which can build up on the analytical column and pollute the mass spectrometer source, thereby contributing to matrix effects and reduced sensitivity (13, 14).

The aim of this study was to develop a semi-automated and robust routine method for the analysis of EtG and EtS in serum, using acidic acetonitrile protein precipitation and phospholipid removal plates in order to provide cleaner extracts due to the removal of endogenous phospholipids. A secondary aim was to apply the method on serum samples from female patients seeking health care after sexual assault to explore whether the EtG and EtS

findings could provide helpful supplementary information related to self-reported recent alcohol intake.

Material and methods
Chemicals and reagents
EtG, EtS, EtG-d_5 and EtS-d_5 were obtained from Lipomed GmbH (Weil am Rhein, Germany). LC-MS grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany) and formic acid Aristar 98% was from VWR (Leuven, Belgium). All water used was provided from a Millipore A10 Synthesis filtering system (Billerica, MA, USA).

Preparation of solutions
Two separate stock solutions (1 mg/mL) were prepared for each compound using the same lot numbers from Lipomed, identified as calibration and quality control (QC), respectively. Sample material was weighed and resolved in methanol. These solutions were further diluted with methanol and used for calibration and QC samples prepared in serum with concentrations of 0.08, 0.15, 0.30, 0.5, 1.0 and 2.0 mg/L for EtG and 0.025, 0.075, 0.15, 0.5, 1.0 and 2.0 mg/L for EtS. The internal standards EtG-d_5 and EtS-d_5 were diluted with water to a concentration of 2.5 mg/L. The stock solutions and standards in serum were stored at -20°C and the internal standard at 4°C. EtG and EtS purchased from Cerilliant (Round Rock, TX, USA) were used to control the concentrations of the Lipomed stock solutions. The solution from Cerilliant was diluted in water and compared to a corresponding dilution of Lipomed stock solution with good agreement.

Sample preparation

Automatic sample preparation was performed using a Tecan Freedom Evo pipetting robot (Tecan Nordic, Mölndal, Sweden). Aliquots of serum samples (100 µL) and internal standard (25 µL) were pipetted onto a phospholipid removal plate (Ostro Protein Precipitation & Phospholipid Removal Plate, 25 mg, Waters, Milford, MA, USA). Ice cold acetonitrile with formic acid (1% v/v, 375 µL) was added to the well. The mixture of serum samples and acetonitrile was mixed by aspirating thrice with the pipetting robot. The precipitate and the supernatant were separated using a positive pressure unit (Positive pressure-96, Waters) capturing the phospholipids and precipitated protein in the filtration plate. The eluates were collected in 2 mL sample collection wells (96-well Square collection plate, Waters), evaporated to dryness under compressed air at 40°C (Ultravap, Portvair science, North Wales, UK) and reconstituted in 100 µL water. After sealing the collection plate (Cap-mat square plugs, silicone/PTFE treated, pre-slit, Waters), the samples were mixed (Multi vortexer).

Instruments
A Waters Acquity UPLC I-Class FTN system (Waters) was used. Chromatographic separation was performed at 50ºC on an Acquity HSS T3 column (2.1 × 100 mm, 1.8 µm; Waters) . A pre-column (HSS T3, 2.1 × 5 mm, 1.8 µm; Waters) was used prior to the analytical column. A flow rate of 0.6 mL/min with the following binary solvent system was used: 0.1% formic acid in water (A) and 100% methanol (B). The gradient was run as

follows: 0 min, A 99%, B 1%; 1.2 min, A 80%, B 20%; 1.5 min, A 10%, B 90%; 1.9 min, A 99%, B 1%. Total run time was 2.5 min. The post-inject wash was performed with methanol/ acetonitrile/ isopropanol/ water/ formic acid (25/25/25/24/1, v/v) for 6 s. The injection volume was 1 µL.

For the detection of EtG and EtS, a Xevo TQ-S tandem-quadrupole MS (Waters) equipped with a Z-spray electrospray interface was used. Negative electrospray ionization was used. The capillary voltage was set to 1.0 kV, the source block temperature was 120˚C, and the desolvation gas nitrogen was heated to 650˚C and delivered at a flow rate of 1000 L/h. The m/z 221.0 > 85.1 and 221.0 > 75.1 transitions (cone voltage: 40 V, collision energy: 15 eV) were monitored for EtG. The m/z 125.0 > 97.0 (cone voltage: 50 V, collision energy: 15 eV) and 125.0 > 80.0 (cone voltage: 50 V, collision energy: 22 eV) transitions were monitored for EtS. The m/z 226.0 > 85.1 transition (cone voltage: 40 V, collision energy: 15 eV) was monitored for EtG-d_5 and the m/z 130.0 > 97.8 transition (cone voltage: 50 V, collision energy: 15 eV) was monitored for EtS-d_5. System operation and data acquisition were controlled using the Mass Lynx 4.1 software (Waters). All data were processed with the Target Lynx quantification program (Waters).

Method validation

The four-point calibration curves (0.08-2.0 mg/L for EtG and 0.025-2.0 mg/L for EtS) with three replicates of each standard were based on peak-area ratios of the analyte relative to the internal standard using a weighted (1/x) linear line, which excluded the origin. Within-assay precision was estimated by analysis of ten separate replicates of QC samples at three concentrations in a single assay (EtG: 0.12, 0.6 and 1.5 mg/L, EtS: 0.04, 0.6 and 1.5 mg/L). Between-assay precision and accuracy were determined by analysis of one replicate at the same concentration levels on six different days. Extraction recoveries were determined at two concentration levels (lowest and highest QC sample) with six replicates at each level. Recovery was estimated by comparison of the peak areas obtained when the analytes were added before sample preparation with those obtained when the analytes were added after the extraction step. In both cases, the internal standards were added after the extraction step. Matrix effects (ME) were evaluated at the lowest and highest QC level, the analyte signal in spiked water was compared with the analyte signal in the matrix, and the ME was defined as ME% = (matrix area/water area) x 100. Six replicates of serum samples (from six different individuals) extracts were analyzed.

Limit of detection (LOD) was determined by evaluation of signal-to-noise (S/N) for extracted dilution of a low calibrator with a criterion of S/N ≥ 3 for both transitions. Limit of quantification (LOQ) was determined by spiking serum with various concentrations with a criterion of S/N ≥10. Samples of 0.025 mg/L of EtG and 0.009 mg/L of EtS were found to fulfill the LOQ criterion and were run in one replicate on six different days, and the concentration was calculated using a five-point calibration curve in the range of 0.025-2.0 mg/L (EtG) and 0.009-2.0 mg/L (EtS). Precision and bias of calculated concentrations were CV < 20% and within ± 20%, respectively.

During the validation process, four-point calibration curves of 0.08-2.0 mg/L for EtG and 0.025-2.0 mg/L for EtS proved to be linear (r>0.998) with intercepts insignificantly different from origin (<0.01). The concentrations in patient samples analyzed after the validation process were calculated using these four-point calibration curves of 0.08-2.0 mg/L for EtG and 0.025-2.0 mg/L for EtS. Thus, concentrations between LOQ and Std1 were quantified and considered positive.

Analysis of ethanol in urine and serum and EtG/EtS in urine

Ethanol in serum was analyzed with headspace gas chromatography using a flame ionization detector (GC Trace 1310, Thermo Scientific, Passau, Germany). The analytical cut-off was 0.05 g/L. Ethanol in urine was determined by the test kit DRI$^{®}$ Ethyl Alcohol Assay on a Beckman Coulter AU680 (Thermo Scientific) with an analytical cut-off of 0.1 g/L. EtG and EtS in urine were determined by a previously described method, with a cut-off of 0.2 mg/L for both analytes. (11).

Patients and sampling

Serum and urine samples were obtained from all female patients who were examined and consented to sampling at the Sexual Assault Centre at St. Olav University Hospital, Trondheim, Norway, between September 2014 and September 2015. Only patients with positive analysis of EtG, EtS or ethanol in at least one matrix were included in the study. According to instructions from the Regional Committee for Research Ethics, which approved the study, the patients received a letter of information about the study. Those who declined to participate on the basis of this letter were excluded.

This procedure resulted in a sample of 49 patients aged 15-58 years (mean 24 years). Self-reported alcohol consumption in relation to the assault, as well as the time lapse from the end of alcohol intake to serum sampling, were estimated from the patients' records. Intake of alcohol was converted to standard alcohol units. We used the Norwegian definition of one alcohol unit, i.e. 12.8 g ethanol, which corresponds to approximately one standard can or bottle (33 cl) of 4.7% beer, one standard glass (12.5 cl) of 13% wine or one standard size (4 cl) drink with 40% spirits (15).

Calculations and statistics

Statistical analyses were performed with SPSS version 21.0. Spearman's rank correlation was used to test the relationships between concentrations of ethanol, EtG and EtS in serum as well as the relationship between EtG and EtS concentrations and the time interval from end of alcohol intake to serum sampling. P values < 0.05 were considered statistically significant.

Results and discussion

The presented method is semi-automated where all pipetting steps are performed by a robot, thereby reducing manual workload. The analysis time on the instrument is short (2.5 min), as compared to 4 to 45 min in previously published studies. The LOQ of the present method is also lower compared to the methods published previously (3, 8, 9, 12).

Method validation

The calibration range, LOD, LOQ, within-assay precision, between-assay precision, bias and recovery for EtG and EtS are presented in Table 1. The within-assay coefficients of variation (CVs) were 5.6%-8.4%, and the between-assay CVs were 3.8%-9.1%. The bias was in the range of -5.2% to 4.7%. The recoveries varied between 66% and 74% for EtG and 94% and 102% for EtS. The matrix effect ranged from 88% to 97% (Table 2), indicating some ion suppression for both analytes. However, when corrected with the internal standard, the observed matrix effects were reduced for both compounds. In cases with a concentration higher than 2.0 mg/L, the samples were diluted and reanalyzed. The MRM chromatograms of a patient sample are shown in Figure 1.

Application
Of the 49 female patients, 46 (94 %) reported intake of alcohol in relation to the assault. Their self-reported intake ranged from 1 to 18 standard alcohol units, with a median of 6.5 units corresponding to 83 g ethanol. One patient denied intake, whereas in two cases, information on alcohol intake was missing. The median time from alcohol intake to serum sampling was 11.0 hours (range 1.5-121 hours).

Ethanol was found in serum in 26 samples and in urine in 30 samples, in concentrations up to 2.8 g/L and 3.1 g/L, respectively. EtG and EtS were found in serum in 36 samples, with concentrations ranging up to 8.2 mg/L and 2.1 mg/L, respectively. In urine, EtG and EtS were found in 48 and 49 of the 49 samples, respectively. The median urine-to-serum ethanol concentration ratio was 1.39, with 80% within the range of 1.08-2.15, which is in accordance with most of the samples having been taken during the ethanol elimination phase (16).

All serum samples positive for EtG were also positive for EtS and vice versa. There was a linear and highly significant relationship (N = 36; r = 0.94; p < 0.001) between the concentrations of EtG and EtS in serum, with a mean EtG/EtS ratio of 2.9. There were, however, large individual variations in this ratio, ranging from 1.0 to 5.2. In a previous study inter-individual EtG/EtS ratios varied between 1.5 and 3.9 (9).There was a significant decrease in serum EtG and EtS concentrations with time after alcohol intake (N =31; r = 0.60 and r = 0.66, respectively; p < 0.001 for both) (Figure 2). By employing a cut-off at the method's LOQs of 0.025 mg/L for EtG and 0.009 mg/L for EtS, approximate maximum detection times slightly above 24 hours could be anticipated (Figure 2). The present study demonstrates that a low LOQ is essential to achieve a detection time of more than 24 hours.

Our LOQ is significantly lower than in other studies, where LOQs in the range of 0.044 to 0.1 mg/L for EtG and0.03 to 0.1 mg/L for EtS have been reported (3, 8, 9, 12).

In the samples positive for EtG and EtS in serum that were obtained during the elimination phase (i.e., more than 4 h after cessation of alcohol intake), there was a highly significant correlation between the ethanol and the EtG and EtS concentrations (N = 29; r = 0.90 for EtG and r = 0.89 for EtS; p < 0.001 for both). As shown in Figure 3, there was a large variability in the EtG/ethanol and EtS/ethanol ratios, even though we only included samples taken in the elimination phase.

Except in one patient, the detection of ethanol and ethanol metabolites in serum and urine followed the predicted pattern: Ethanol in serum had the shortest detection time, followed by ethanol in urine, EtG/EtS in serum, and EtG/EtS in urine. One patient had a low concentration of ethanol as well as high concentrations of EtG and EtS in urine, whereas no ethanol or ethanol metabolites could be detected in serum. Urine and serum samples were collected 8 hours after the cessation of a self-reported intake of 10 standard alcohol units. These results do not fit the expected pattern of elimination. Possible explanations include a previous ethanol intake combined with formation of or inadvertent addition of ethanol in the urine.

In seven patients who reported alcohol ingestion in relation to the alleged assault, no ethanol was detected in serum or urine, whereas EtG and EtS were detected in serum. The time interval between the end of alcohol intake and the serum sampling was 11 to 21 h in six of these patients. In the last patient, the time interval was unknown. The EtG and EtS concentrations in these patients were rather low, in the range of 0.030-0.60 mg/L and 0.012-0.20 mg/L, respectively. These results show that in a subset of patients, the analysis of EtG and EtS in serum may confirm (or, alternatively, refute) the recent intake of ethanol when ethanol itself can no longer be detected.

In conclusion, this study reports the development and validation of a fast and reliable method for the quantification of EtG and EtS in serum. Compared to previously published studies, the method presented has the advantages of being semi-automated, using a simple method for phospholipid removal and having short run times and low LOQs. The results from our application indicate that EtG and EtS in serum could be a valuable supplement to ethanol measurements in patients seeking health care after sexual assault, to prolong the detection time, improve the assessment and provide objective evidence of recent alcohol intake.

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Table 1. Calibration range, correlation coefficient, limit of detection (LOD), limit of quantification (LOQ), within-assay and between-assay precisions, bias and recovery for EtG and EtS in serum.

Analyte	Calibration range (mg/L)	Correlation coefficient (r value) (n=3)	LOD (mg/L)	LOQ (mg/L) (n=6)	Spiked QC sample concentration (mg/L)	Within-assay CV (%) (n=6)	Between-assay CV (%) (n=6)	Bias (%) (n=10)	Recovery (%) (n=6)
EtG	0.08-2.0	0.993	0.003	0.025	0.12	7.4	3.8	4.2	66
					0.6	6.2	8.1	-5.2	-
					1.5	5.7	6.2	-4.2	74
EtS	0.025-2.0	0.997	0.005	0.009	0.04	8.4	3.9	4.7	94
					0.6	6.0	9.1	-5.0	-
					1.5	5.6	5.6	-4.4	102

Table 2. Evaluation of matrix effects (ME) for EtG and EtS in serum.

Analyte	Concentration (mg/L)	ME (%)	Relative ME (CV %)	ME corrected with IS[a] (%)	Relative ME corrected with IS (CV %)
EtG	0.12	91	2.1	98	3.2
	1.5	91	1.9	101	3.5
EtS	0.04	97	2.5	101	5.2
	1.5	88	0.7	97	2.3

[a] IS = internal standard

Figure legends

Figure 1

MRM-chromatograms of ethyl glucuronide (EtG; left panel) and ethyl sulfate (EtS; right panel) of an authentic sample. Determined concentrations: EtG = 0.29 mg/L, EtS = 0.06 mg/L.

Figure 2

Scatterplot showing the relationship between time since alcohol intake and concentrations of ethyl glucuronide (EtG; red squares) and ethyl sulfate (EtS; blue circles) in serum. Note that the plot is semi-logarithmic. The correlations between time after intake and concentrations are highly significant ($p < 0.001$ for both). Two patients with missing information on time lapse between alcohol intake and sampling, and three patients who obviously ingested ethanol after the assault (signified by high ethanol concentrations in urine and serum > 24 hours after the reported cessation of ethanol intake) were excluded; therefore the total number of subjects included is 31. From extrapolation of the regression lines to the intercept with the LOQs for EtG and EtS (shown with dotted lines), maximal detection times slightly longer than 24 hours could be expected for both analytes.

Figure 3

Scatterplot showing the relationship between the concentration of ethanol and ethyl glucuronide (EtG; red squares) and ethyl sulfate (EtS; blue circles) in serum. The correlations are highly significant ($N = 29$; $p < 0.001$ for both). Only serum samples positive for EtG and EtS and obtained during the elimination phase (i.e. more than 4 hours after cessation of ethanol intake) are included.