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Quantitative Determination of Ethyl Glucuronide and Ethyl Sulfate in Postmortem and Antemortem Whole Blood Using Phospholipid Removal 96-Well Plate and UHPLC–MS-MS

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Abstract

Postmortem ethanol formation is a well-known problem in forensic toxicology. Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are ethanol metabolites that can be used to distinguish antemortem alcohol intake from postmortem formation of ethanol and in addition can be a helpful tool in assessment of the hip-flask defense. To an aliquot of 100 μL whole blood, internal standard (IS) and water was added before protein precipitation treatment (PPT) with ice-cold acetonitrile (ACN). The supernatants were filtered through a 96-well phospholipid removal plate, evaporated to dryness and reconstituted in 150 μL water/ACN/formic acid (FA). Identification of compounds was performed using multiple reaction monitoring (MRM) in negative mode. Gradient elution was performed on a C18 column with methanol (MeOH) and 0.1% FA. The run time was 4.5 min, and 0.5 μL was injected on an ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS-MS) instrument. Linearity was achieved (coefficient of determination (R^2) $\geq$ 0.999) for EtG in the range of 0.089 to 22 mg/L (0.40–100 μM) and EtS 0.025 to 6.3 mg/L (0.20–50 μM). The limit of quantification (LOQ) was 0.067 mg/L (0.30 μM) for EtG and 0.019 mg/L (0.15 μM) for EtS. Between assay accuracy was –15% to 8% and precision reported as relative standard deviation (RSD) was $\leq$ 4.5%. Precision, estimated as the RSD of the concentration difference between results from two independent analyses of authentic whole blood samples, was $\leq$ 6.7%. Recovery was $\geq$ 61% for EtG and $\geq$ 77% for EtS and matrix effects (ME) were 99% to 103%. Method comparison was carried out with a previously used UHPLC–MS-MS method, and satisfactory agreement was achieved, and external proficiency testing control samples had z-score < $\pm$ 1. The method has been used in routine work for more than 4 years analyzing about 6,000 antemortem and postmortem whole blood samples and has proven to be robust and reliable.

Introduction

The possibility of postmortem production of ethanol makes correct interpretation of ethanol detection in forensic autopsy samples difficult. Ethanol can rise as a putrefactive product formed by a wide range of species of microorganisms. The formation may occur as a part of postmortem degenerative processes. The substrate used in this reaction is often glucose. The level of glucose fermentation depends on microorganisms, and it might rise considerably after death, causing endogenous ethanol levels that are difficult to distinguish from those caused by ingestion (1–4). Severe trauma and high temperature on the location of the body can affect the probability of postmortem ethanol production (4). The concentration of ethanol measured in postmortem blood needs to be interpreted in relation to whether the detected ethanol is from postmortem production or ingestion prior to death. In the interpretation of a postmortem ethanol finding, information regarding each case, degree of

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putrefaction of the corpse, detection of other putrefactive products like n-propanol and comparison of ethanol levels found in blood, urine and vitreous humor are used, but are not always reliable (1, 3–5). In the driving under influence (DUI) of alcohol cases, the hip-flask defense (i.e., claiming ethanol intake after an incident) is a well-known problem in forensic toxicology. The hip-flask defense is difficult to refute by only ethanol analysis (6). The nonoxidative metabolites of ethanol, ethyl glucuronide (EtG) and ethyl sulfate (EtS) are direct ethanol metabolites and can be used to distinguish antemortem ethanol intake from postmortem formation of ethanol and in addition can be a helpful tool in assessment of the hip-flask defense (3, 6).

EtS and EtG represent only a small fraction of the consumed ethanol (i.e., < 0.1%). EtG and EtS are detectable in several body fluids and postmortem samples. Both of these metabolites remain in the body longer than ethanol itself, with detection window 10 to 14 hours for EtG, 4 to 10 hours for EtS and 5 to 7 hours for ethanol after ethanol ingestion of about 0.5 to 0.8 g/kg (7–10). Hence, EtG and EtS close the gap in the detection window between short-term direct ethanol markers (e.g., ethanol) and long-term marker phosphatidylethanol (PEth), which can be detected in blood up to about 2 to 3 weeks (11). EtG and EtS are also reported to be relatively stable compounds; however, under extreme conditions (heavy decomposition and high temperature), instability of EtG (12–14) and some instability for EtS (15) have been reported. Postmortem formation of EtS and EtG has been studied, and no formation of EtG (12, 16) and EtS (12, 14) in blood have been reported. In one recent report, small amount, < 0.1 mg/L EtG, was found in some of the ethanol spiked samples (0.8 g/kg) at 37°C (14). The other two direct ethanol markers, PEth and fatty-acid ethyl esters (FAEEs), have been reported to be less appropriate biomarkers for ethanol in postmortem whole blood due to in vitro formation of these compounds in the presence of ethanol, and also degradation after storage (14). Hence, EtG and EtS are considered to be sensitive and specific ethanol markers and are regarded as superior compared with other ethanol markers (3, 14).

As only a small fraction of the ingested ethanol dose is excreted as EtG and EtS, sensitive analytical methods are needed to be able to detect these compounds. The postmortem whole blood samples received for forensic toxicological analysis may have poor quality (i.e., putrefaction, high lipophilicity) and can also be contaminated with other matrixes particularly after severe trauma and high-temperature conditions. EtG and EtS are highly polar compounds requiring a very low proportion of organic modifiers (<5%) for elution from a reversed-phase column. The postmortem samples and the compounds chemical properties set demands on both the sample preparation and chromatographic separation. Several methods have been presented for EtG and EtS analysis in biological matrixes (2, 7, 8, 16–34). In forensic toxicology, postmortem whole blood is the matrix used in order to evaluate the finding of an ethanol concentration in relation to the detection of EtG and EtS. Some methods have been published for whole blood (2, 16, 22, 23, 25, 28, 29, 32, 34), and most of the whole blood methods use only protein precipitation treatment (PPT) as sample clean up before chromatographic analysis on ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS-MS). As reported by Hegstad et al. (22), PPT alone was not satisfactory, and poor chromatography was observed after analysis of putrefied postmortem whole blood samples. This problem was also seen in our former method (23). Several sample preparation products for cleanup of phospholipids in whole blood have become commercially available; however, only a few methods have been published (22, 29).

The purpose of the following study was, first, to explore sample preparation procedures which could be simple and fast with a minimal number of steps. The procedure should also provide clean extract and eliminate interferences. Second, it was to develop and validate a high throughput, specific and sensitive UHPLC–MS-MS method for the quantitative determination of EtG and EtS in postmortem and antemortem whole blood covering the concentrations found in forensic samples.

Experimental

Chemicals and reagents

The reference substances for calibrators and quality control (QC) samples were for both EtG and EtS obtained from Lipomed (Arlesheim, Switzerland) and Cerillant (Round Rock, TX, USA). EtG-d_5 was obtained from Cerillant and EtS-d_5 from Lipomed. High-performance liquid chromatography (HPLC)-grade acetonitrile (ACN) and methanol (MeOH), ammonium acetate, 32% ammonia and acetic acid were obtained from Merck (Darmstadt, Germany). Ammonium format was provided from Prolab (Briare, France), heptane and formic acid (FA) were purchased from BHD Prolab (Briare, France). Type-1 water (18.2 MΩ cm) was obtained by filtering deionized water on an in-house Milli-Q filtration system (Millipore, Bedford, MA, USA).

Phospholipid removal plates tested in the method development were Captiva ND Lipids from Agilent (Thermo Fisher Scientific, Cheshire, UK), Supelco Hybrid-SPE-Phospholipid-Technology from Sigma (Darmstadt, Germany), Ostro Protein Precipitation & Phospholipid Removal from Waters (Milford, MA, USA), Phree Phospholipid Removal from Phenomenex (Torrance, CA, USA), ISOLUTE® PLD + Protein and Phospholipid Removal from Biotage (Charlotte, NC, USA).

Biological Samples

Whole blood containing 2 g sodium fluoride, 6 mL heparin and 10 mL water per 450 mL blood was used for development and validation of the method and was purchased from the Blood Bank at Oslo University Hospital (OUS) (Oslo, Norway). Confirmation analysis of EtG and EtS in whole blood samples at OUS are utilized in DUI cases and forensic autopsy cases. The DUI samples were received in 5 mL BD Vacutainer® Blood Collection glass tubes (BD Vacutainer Systems, Franklin Lakes, NJ, USA) containing 4 mg/mL sodium fluoride and 28 IU/mL sodium heparin. The forensic autopsy samples were received in 25 mL Sterilin tubes (Sterilin, Caerphilly, UK) containing 200 mg potassium fluoride. Collected samples were stored at 4°C prior to processing. Aliquots of 100 μL were transferred to 5-mL polypropylene tubes (Sarstedt AG, Nümbrecht, Germany), which were stored at 4°C until the time of analysis.

Preparation of Solutions

Two separate stock solutions, identified as calibrator and QC, were prepared in MeOH for both EtG and EtS. Stock solutions were further diluted to prepare working solutions in MeOH. Calibrator and QC samples were prepared by diluting suitable amounts of each working solution with Type-1 water. The concentrations of the calibrators were 0.089, 0.22, 1.1, 7.8 and 22 mg/L (0.40–100 μM) for

EtG and 0.025, 0.063, 0.31, 2.2 and 6.3 mg/L (0.20–50 μM) for EtS. Four QC levels were distributed from the low to the high calibration range. All stock solutions were stored at –20°C. Calibrators and QC samples were stored at 4°C and used for up to 12 months.

Internal standard (IS) stock solutions of EtG-d_5 and EtS-d_5 (1 mg/mL) were prepared in MeOH for each compound and stored at –20°C. From the stock solutions, a IS solution was prepared by dilution with Type-1 water to a final concentration of 2.3 mg/L (18 μM) EtS and 7.8 mg/L (35 μM) EtG, and stored at 4°C.

Sample Preparation

To an aliquot of 100 μL whole blood (authentic samples), 50 μL Type-1 water was added. The calibrators and QC samples were prepared by adding 50 μL of calibrator/QC solutions to 100 μL whole blood. The samples were added 50 μL of IS-solution and mixed. Four hundred microliters of ice cold ACN was added to precipitate the proteins. Following centrifugation at 4,500 x g (4,500 rpm on Heraeus, Multifuge X3R centrifuge, Osterode, Germany) for 5 min, the supernatants were filtered through a 96-well phospholipid removal Phree plate, 30 mg/well and collected into a 96-well collection plate using AV vacuum manifold all from Phenomenex. The filtered samples were evaporated to dryness under a stream of nitrogen at 65°C using a Zymark TurboVap (Caliper life sciences, Hopkinton, MA, USA). The residue was dissolved in 150 μL water/ACN/FA (100/0.2/0.1, v/v) solution, sealed with 96-square well silicone sealing mat (Phenomenex) and shaken for 1 min before UHPLC–MS-MS analysis.

Instrumentation

UHPLC conditions An Acquity UPLC module (Waters) was used for separation. Chromatographic separation was carried out using an ACQUITY UPLC HSS T3 C18 Column (2.1 mm x 100 mm, 1.8 μm) coupled to a guard column (2.1 mm × 50 mm) with column temperature of 65°C. A linear gradient with flow rate of 0.4 mL/min with 0.1% FA in H_2O (A) and MeOH (B) was used: initial 99% A, 2.0 min 80% A, 2.01 min 10% A, 3.0 min 10% A, 4.0 min 99% A, 4.5 99% A. The total cycle time of the method was 4.5 min. The injection volume was 0.5 μL using partial loop with needle overfill. A weak wash and strong wash were performed with 600 μL 5% MeOH and 200 μL 90% MeOH, respectively.

MS-MS conditions Xevo TQ-S tandem mass spectrometer with an electrospray ionization source (ESI) from Waters Corp. was employed for MS-MS analysis. Negative ESI-MS-MS-detection in multiple reaction monitoring (MRM) mode with two transitions for EtG and EtS and one transition for the ISs was performed. Fifteen data-points were collected over the chromatographic peaks. Nitrogen (>99% N_2) from a nitrogen generator from Oxymat (Helsinge, Denmark) was used as desolvation gas, delivered at a temperature of 500°C and a flow rate of 900 L/h. The cone gas (N_2) flow rate was 150 L/h. The capillary voltage was 1.0 kV and the source block temperature was 150°C. Argon (99.999%) from Yara (Oslo, Norway), was used as collision gas, and collision gas pressure in the collision cell was maintained at approximately 3.5×10^{-3} mbar. Ion energy was 1.5 V. Other parameters such as MRM transitions, cone voltage, collision energy and dwell times are displayed in Table I. System operation and data acquisition were controlled using MassLynx 4.1 software from Waters Corp.

Method Validation Validation of the method was done according to international guidelines (35) and included linearity, precision and accuracy, limit of quantification (LOQ), extraction recovery, matrix effects (ME), selectivity, carryover, stability, comparison with other methods and external proficiency testing control samples.

Results and Discussion

To our knowledge, only a few HPLC or UHPLC–MS-MS methods for determination of EtG and EtS in postmortem whole blood samples utilizing phospholipid removal in the sample preparation have been published (22,29). Santunione et al. (29) have published a method for EtG determination in whole blood and other postmortem matrixes. The sample preparation used about 200 mg blood, dilution and precipitation with MeOH before phospholipid removal on Phree cartridges. Analysis was done on an HPLC–MS-MS with a run time of 20 min, whereas in the presented work, the UHPLC–MS-MS method has a run time of 4.5 min including equilibration. The cutoff was higher, and the measurement range was much narrower than the presented method, and EtS was not included. The presented method has many similarities with the method of Hegestad et al. (22). The PPT was based on the use of ACN and phospholipid removal on Phree filter plates, while Hegstads' method was made with MeOH, freezing of the sample for 10 min and Ostro 96-well filter plate was used to remove the phospholipids. The reported LOQs were slightly higher, and the measurement range was narrower, with calibration range up to 2 mg/L for both compounds whereas in the presented method the calibration range was up to 22 mg/L EtG and 6.3 mg/L EtS. In the method development, much effort has been done exploring the effects of different PPT and phospholipid removal 96-well filter plates on the recovery of EtG and EtS. As a result, it was achieved higher recoveries as compared to Hegstad et al. (22). The analysis of EtG and EtS is used in postmortem forensic toxicology in order to interpret ethanol positive samples. In the presented work, the concentration distribution in authentic samples and the portion of EtG- and EtS-negative samples that tested positive for ethanol is reported. Long-term precision is reported for QC samples, and the

Table I. MRM Transitions, Cone Voltages, Collision Energies and Dwell Times for EtG, EtS and their Deuterated Analogs

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)	Dwell time (sec)
EtG (Qualifier)	221.0	75.1	40	15	0.1
EtG (Quantifier)	221.0	85.1	40	15	0.1
EtG-d_5	226.0	85.1	40	15	0.1
EtS (Qualifier)	125.0	80.0	50	22	0.1
EtS (Quantifier)	125.0	97.0	50	22	0.1
EtS-d_5	130.0	97.8	50	22	0.1

reproducibility of the method was determined with a large number of authentic postmortem samples.

Chromatographic Separation

UHPLC columns with small particles are expected to give better resolution and narrow peaks are obtained, which should translate to better sensitivity. EtG and EtS are polar compounds and are poorly retained in conventional reversed-phase (RP) chromatography. In order to enhance the retention and the sensitivity of EtG and EtS, three different RP C18 columns reported to have increased retention of polar compounds were evaluated using various chromatographic conditions. The columns tested were a polar end-capped CORTECS C18 (2.1 mm ID X 100 mm, 1.6 μm; Waters), Fortis C18 (2.1 mm ID X 100 mm, 1.7 μm; Thermo Fisher Scientific, Cheshire, UK) and HSS T3 C18 (2.1 mm ID X 100 mm, 1.8 μm; Waters). All the columns were tested with a mobile phase of using 0.1% FA in water and MeOH. For all the columns, satisfactory retention was obtained for EtG and EtS using the validated gradient. For both the CORTECS and Fortis columns, the peak intensity for EtS was lower when compared to the HSS T3 column. Compared with the Cortecs and Fortis C18 columns, a better separation was obtained by using the HSS T3 C18 column. Based on these observations and the fact that HSS T3 was used on several methods at our laboratory, the HSS T3 column was chosen.

Separation of EtG and EtS on HSS T3 C18 column was further examined with different acidified mobile phases; 0.1% and 0.2% FA, ammonium formate pH 3.1 and ammonium acetate pH 5.0. No major changes in the peak shape or retention time were observed using FA mobile phases in combination with MeOH. Small alterations in retention time were observed using ammonium acetate and ammonium formate buffer compared with FA mobile phases. A significant reduction in peak intensities was observed when using buffer as pH adjustment. The reason for the dramatic reduction in the peak intensity was not further investigated. Based on these observations, a mobile phase containing 0.1% FA and MeOH was chosen. EtG and EtS were baseline separated within 1.6 min by using 0.1% FA in water and MeOH with a flow rate of 0.400 mL/min (Figure 1).

Sample Preparation

Postmortem whole blood is a complex matrix containing many compounds that may co-elute with analytes of interest when using traditional sample preparation procedures. As reported by Hegstad et al. (22), poor chromatographic performance of EtG and EtS was observed on RP column after only PPT. Phospholipid removal plates have been reported to efficiently remove phospholipids; however, clogging of the plate is a problem when analyzing postmortem whole blood samples. PPT should be carried out to prevent this. Different PPT solvents in combination with phospholipid removal 96-well plates were evaluated in the method development.

Protein precipitation PPT does not result in a very clean final extract, and none of the employed precipitation agents provides complete protein removal. The amount of remaining proteins in the final extract depends on the PPT solvent used and the solvent ratio (36). The PPT solvents tested were ACN, 1% FA in ACN, ACN and heptane (85 + 15), MeOH, ACN and MeOH (85 + 15). The precipitation procedures were carried out by using increasing volume of ACN and MeOH or mixtures, from 300 to 1,000 μL. The sample volume, 100 μL, was kept constant in all the experiments. As expected less ACN was needed for efficient precipitation when compared to MeOH (36,37). At least, five volumes of MeOH were required for one volume of blood compared with three volumes of ACN. Acidification of blood samples with 1% FA in ACN is typically used in combination with phospholipid removal filter plates. The presence of FA caused low recovery of both EtG and EtS. Using 1% FA in ACN as precipitation agent also required a higher ratio of solvent (5:1, v/v) when compared to pure ACN. PPT with MeOH or a mixture containing MeOH and ACN indicated lower recovery for EtS when compared to PPT with ACN or ACN and heptane.

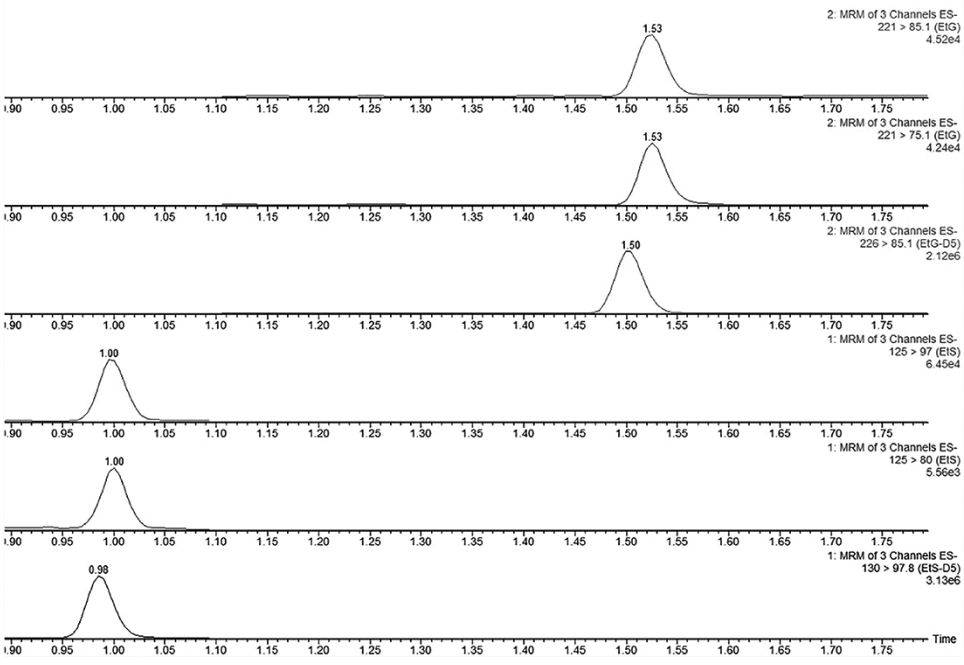

Figure 1. MRM chromatograms of EtG and EtS for the lowest calibrator separated on HSS T3 C18 column.

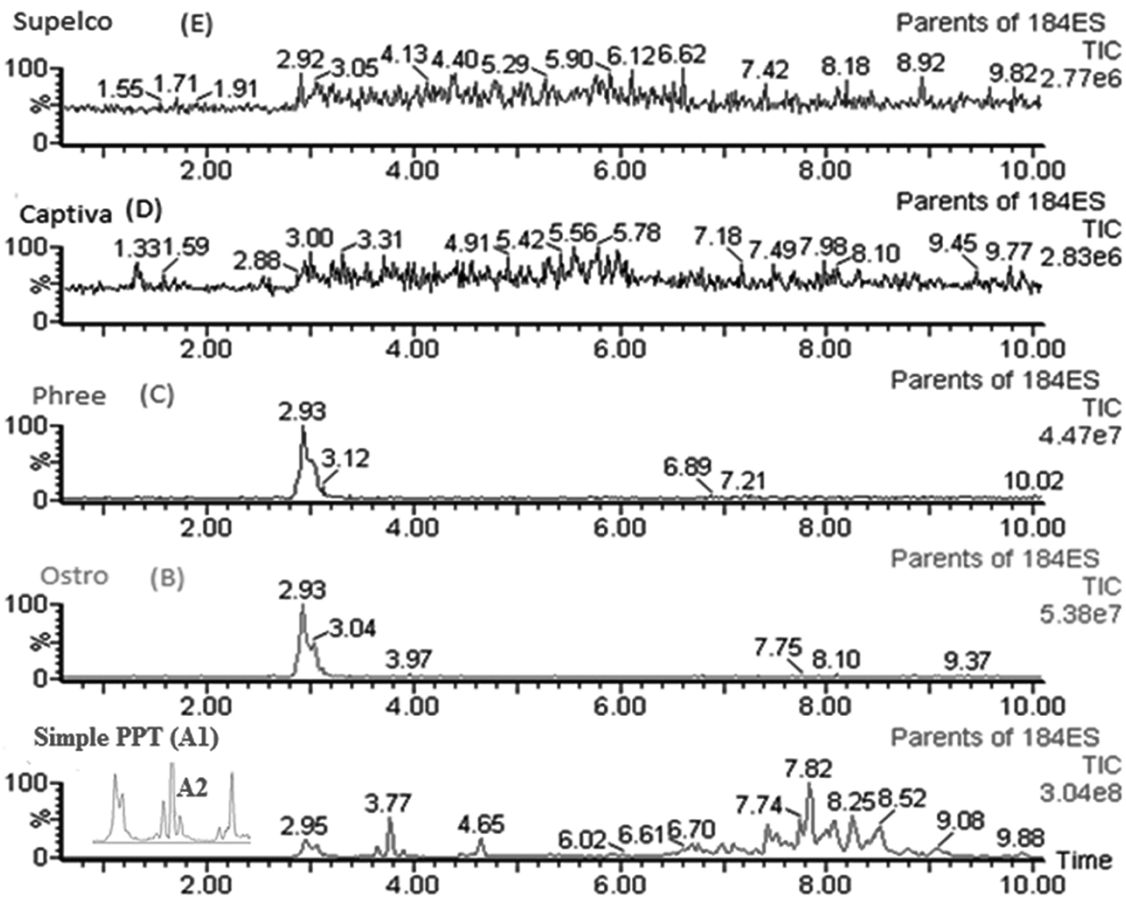

Figure 2. Phospholipids monitoring using ESI + total ion chromatograms of *m/z* 184. ACN protein precipitated blood (A1, A2 showing the phospholipids eluting in the same time frame as EtG and EtS), ACN protein precipitated blood and filtered through hybrid-SPE-PPT Ostro plate (B), Phree plate (C), Captiva plate (D) and Supelco plate (E).

EtS recovery was very sensitive to the amount of organic solvent and increased with decreased amount of MeOH. Based on these observations, ACN was selected as PPT solvent.

Phospholipid removal 96-well plates Five different phospholipid removal plates were evaluated: Captiva ND Lipids, Supelco Hybrid-SPE-Phospholipid-Technology, Ostro Protein Precipitation & Phospholipid Removal, Phree Phospholipid Removal and ISOLUTE® PLD + Protein and Phospholipid Removal plates. The results revealed that the recovery and phospholipid removal was dependent on the filter plate used. This may be explained by different interactions between analyte and filter plate sorbent.

In order to evaluate the efficiency of removal of phospholipids, the presence of phospholipids was studied after ACN precipitation of whole blood obtained from the Blood Bank at OUS, and ACN precipitation and further sample preparation on the different filter plates (Figure 2). All phospholipids and lysophospholipids generate an intense fragment ion at *m/z* 184 for the trimethylammonium methyl phosphate in the source, due to the collision-induced dissociation (38). Total phospholipids in blood samples were monitored using this characteristic MRM transition. The blank blood sample prepared by using only PPT demonstrated a significant amount of phospholipids. There was a cluster of compounds that co-eluted with EtG and EtS (Figure 2, A2). Supelco and Captiva filter plates showed most effective removal of phospholipids. Ostro and Phree filter plates provided also a significant elimination of phospholipids but with one peak remaining, which eluted after EtG and EtS. The Isolute plate was not included in the phospholipids monitoring experiments due to late delivery.

The recoveries, represented by the obtained peak area of the lowest calibrator, of EtG and EtS on the five phospholipid removal plates were evaluated. Three replicates of the lowest calibrator was precipitated and added to the phospholipid removal plates as described in the sample preparation section. The EtG recovery on Supelco filter 96-well plate was unsatisfactory as EtG was strongly retained on the Supelco filter plate and very low recovery was obtained (Figure 3). EtS recovery was satisfactory. Of the four remaining filter plates, the EtG recovery was satisfactory. For EtS, highest recovery was obtained on the Phree filter plate. Phree filter plate in combination with ACN as precipitation agent was selected for method validation and further EtG and EtS analysis.

Method Validation

The UHPLC–MS-MS method was validated according to international guidelines (35). All validation parameters were evaluated using spiked blood samples, and calculations were performed using Excel and SigmaPlot 14 software.

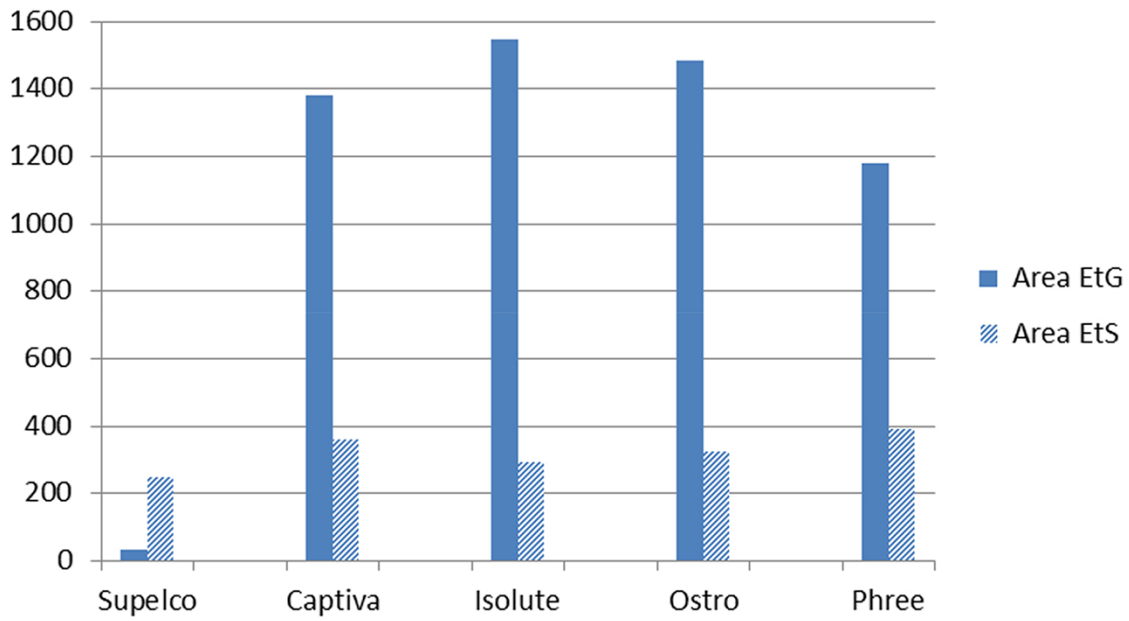

Figure 3. EtG and EtS peak area based on filtration through different phospholipids removal filter plates after ACN precipitation.

Table II. Calibration Range, Determination Coefficients (R^2), LOD and LOQ[a]

Compound	Calibration range		R^2	LOD		LOQ	
	µM	mg/L		µM	mg/L	µM	mg/L
EtS	0.20–50	0.025–6.3	0.999	0.020	0.0025	0.15	0.019
EtG	0.40–100	0.089–22	0.999	0.040	0.0089	0.30	0.067

[a]The cut-off at which the results were reported positive corresponds to the lowest calibrator.

Table III. Within-Assay (n = 10) and Between-Assay (n = 8) Accuracy and Precision

Compound	Concentration		Within assay precision (n = 10)		Between assay precision (n = 8)	
	µM	mg/L	RSD %	Bias %	RSD %	Bias %
EtS	0.15	0.019	3.2	1	2.4	–1
	0.30	0.038	2.6	–1	3.2	0
	20	2.5	2.3	–15	4.5	–6
	40	5.0	1.9	–9	1.6	–9
EtG						
	0.30	0.067	3.7	5	4.0	4
	0.60	0.13	2.4	8	2.9	7
	40	8.9	1.9	–14	2.9	2
	80	18	1.6	–10	1.8	1

Linearity

A total of nine calibrators were used to evaluate linearity. The lowest calibrator was prepared to 1/10 of the cut-off concentration level for which EtG and EtS are reported as positive to the customers of OUS (Table II). The calibration curves were evaluated based on one assay with six replicates of each of the nine calibrators. The difference between the calculated calibrator value and its nominal value was evaluated. The residuals should be < 15%. The linear ranges were based on the measurement of the peak area of the compounds versus the peak area of the corresponding deuterated IS analog. Linear calibration curves with R^2 values ≥ 0.999 were achieved using a weighting factor (1/x) for both compounds and including the origin point for the concentration range (0.089–22 mg/L for EtG and 0.025–6.3 mg/L for EtS). Increased residuals were observed at the lowest concentration range (below cut-off). The concentration range using five calibrators of each compound was able to cover the concentration range required for forensic toxicology samples.

Table IV. ME Corrected Without and With IS and Extraction Recovery

Compound	Concentration		Recovery %	Without IS correction		Corrected with IS	
	μM	mg/L		ME %	RSD %	ME %	RSD %
EtS	0.30	0.038	77	62	10	103	3
	20	2.5	78	70	7	100	3
	40	5.0	79	70	13	100	2
EtG	0.60	0.13	62	62	11	99	3
	40	8.9	62	76	7	100	3
	80	18	61	74	12	100	4

Limits of Detection and Quantification

The LOQ was calculated as the QC concentration where relative standard deviation (RSD) was ≤ 20%, bias was within ± 20%, and with signal to noise higher than 10 for both transitions (Tables II and III). The limit of detection (LOD) was determined by extracting dilutions of a low calibrator using five different lots of human blood. The calculation was based on signal to noise better than three for both transitions. In order to enhance LOD or to achieve lower LOQ, it is possible to increase the injection volume.

Within and Between Assay Accuracy and Precision

Precision and accuracy were determined by analysis of QC samples at four different concentration levels in blood (Table III). Within assay was obtained by analyzing 10 replicates at each of the concentration levels in one assay. Between assay was based on eight independent assays with one replicate on each assay, performed by three technicians. Accuracy given as bias was calculated as the percent deviation of the measured mean of the QC samples from the nominal concentration. Satisfactory accuracy –15% to 8% and precision ≤ 4.5% were obtained (Table III).

Specificity The specificity of the method was investigated by analysis of blank whole blood in five different lots. In addition the method was tested analyzing frequently found drugs in forensic samples. The chromatograms were evaluated for interfering peaks at the same retention time as the compounds. Only gamma-hydroxybutyrate (GHB) eluted at the same retention time as EtS. However, GHB (*m/z* 105–> 87.05) and EtS (*m/z* 124.9–> 79.9) have different molecular masses and MRM transitions, and GHB did not interfere with the EtS determination. No interfering peaks from the investigated compounds were observed at or close to the retention time of EtG and EtS. After analyzing about 6,000 authentic samples, problems with interfering peaks have not been observed. Hence, the method is selective for determination of EtG and EtS in forensic samples.

Matrix Effects and Recovery

The ME corrected with IS was evaluated using the post extraction approach at three different QC concentrations levels. Blank blood from six different lots of human blood obtained from the Blood Bank at OUS was used. Sample preparation was based on two sets. Set A consisted of six extracts of the blank matrices with compounds of interest, added post extraction and compared with set B, which consisted of four replicates with neat solutions containing equivalent amounts of compounds of interest prepared in the solution used for reconstitution. IS was added after extraction and prior to evaporation. ME was calculated by comparison of peak area from samples spiked before (set A) and after (set B) extraction:

$$ME = \frac{[A]}{[B]} \times 100$$

ME > 100 indicates ion enhancement and ME < 100 ion suppression.

As it is demonstrated in Table IV, MEs were observed without IS. The deuterated ISs corrected well for the MEs, which were between 99 and 103% with RSDs ≤ 4%. Recovery was evaluated at three different QC levels. Recovery was calculated by comparison of the peak area obtained when the compounds were added before extraction and the IS were added after (n = 6), with those obtained when both the compounds and IS were added after the extraction, but before the evaporation (n = 6). Satisfactory recoveries ≥ 61% EtG and ≥ 77% for EtS were obtained (Table IV).

Carryover The carryover for the method was evaluated by preparing a calibrator with a concentration 3-fold the concentration of the highest calibrator followed by injection of two extracted matrix blanks analyzed consecutively after the calibrator. The carryover was calculated by measuring concentration of the blank sample versus concentration of the standard solution with 3-fold calibrator concentration. The carryover was found to be < 0.001% for EtS and EtG, corresponding to < 5% of the lowest calibrator. No false-positive result due to carryover has been observed in the use of the method.

Stability

In order to determine a potential decrease of EtG and EtS concentrations, stability of the EtG and EtS in extracted blood samples were examined. Calibrators and QC samples were analyzed by the UHPLC–MS-MS method when the samples were extracted. Then, the samples were kept for three days in the auto-sampler at 10°C as well as one week in a freezer at –20°C and then re-injected. The concentrations were compared and found to be within ± 5% for both compounds.

Method Comparison

Bland-Altman method comparison was carried out during routine analysis: one replicate was analyzed on the former method (23), and one on the presented method. All together 120 forensic autopsy samples were analyzed. No false positives or negatives were found for the presented method. In general, good agreement of quantified concentrations was found (Figure 4). A statistically significant negative bias was found for both compounds when compared with the former method. This negative bias seems to be due to measurements in the higher measurement range. For EtS, deviations were found for samples with concentrations above the calibration range. For EtG, deviations were found also within the calibration range. For

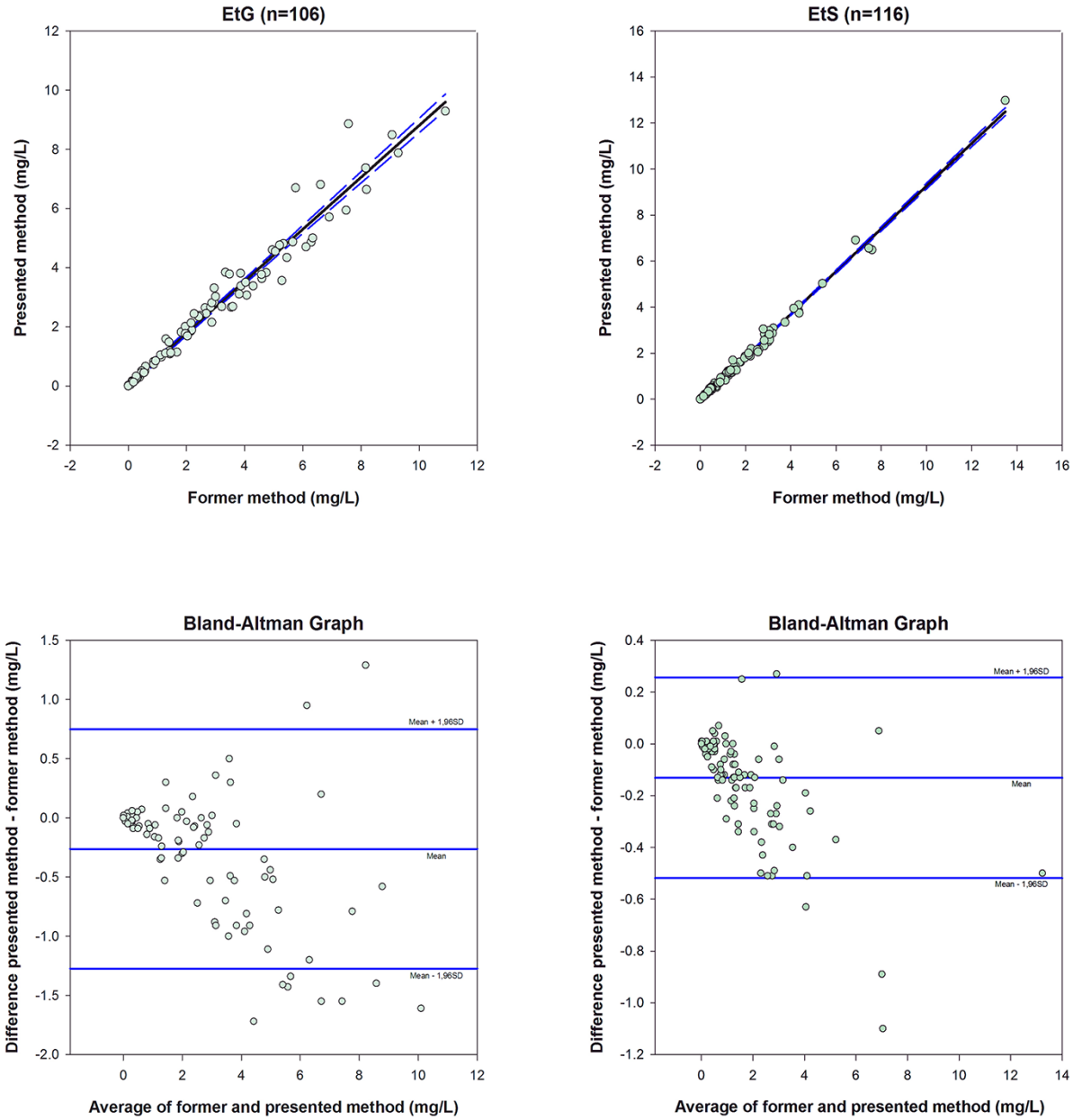

Figure 4. Bland Altman method comparison between EtG and EtS concentrations obtained by the previously used method (x-axis) and the presented method (y-axis). Bias was –0.26 and –0.13 with SD 0.52 and 0.20 for EtG and EtS, respectively.

lower concentrations, data are close to each other. In the method comparison, one external proficiency sample was also analyzed, The result revealed better accuracy on the presented method with z-score –0.02 EtG and 0.50 EtS, and z-score 0.77 EtG and 1.68 EtS on the former method. In the former method, the samples were PPT with MeOH, evaporated, re-dissolved in 65 µL ACN + FA (1 + 99), and 5 µL was injected. The chromatographic conditions were otherwise identical as in the presented method. In the previously used method, occasionally split peaks of EtG and EtS were observed in postmortem samples, and for some samples, there was not possible to report results (Figure 5). Deteriorated chromatography and peak splitting were not observed with the presented method; this supports the findings reported by Hegstad et al. (22) that PPT alone may not be applicable as sample preparation of postmortem whole blood samples.

Method Performance

A study of the long-term precision of EtG and EtS was carried out for a period of 19 months. On each assay (n = 67), two replicates of each QC-level were analyzed. During this period, there were two changes in the QC batch and two in calibrator batch. The preparation was done independently within different time periods. This minimizes the possibility that any stability problems would go unnoticed due to concurrent changes in solutions made at the same period of time. The long-term precision and accuracy were found to be very good.

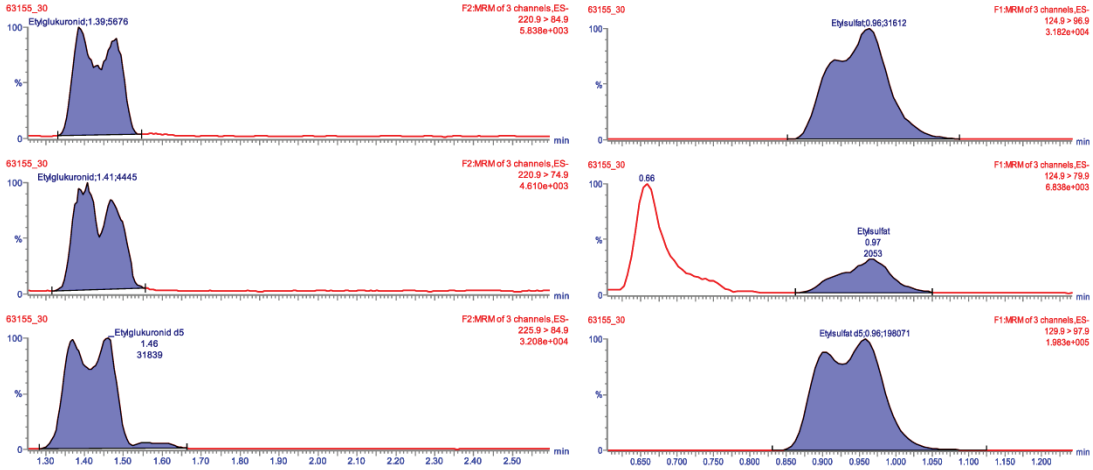

Figure 5. Examples of peak splitting and deteriorated chromatography for EtG on the former method.

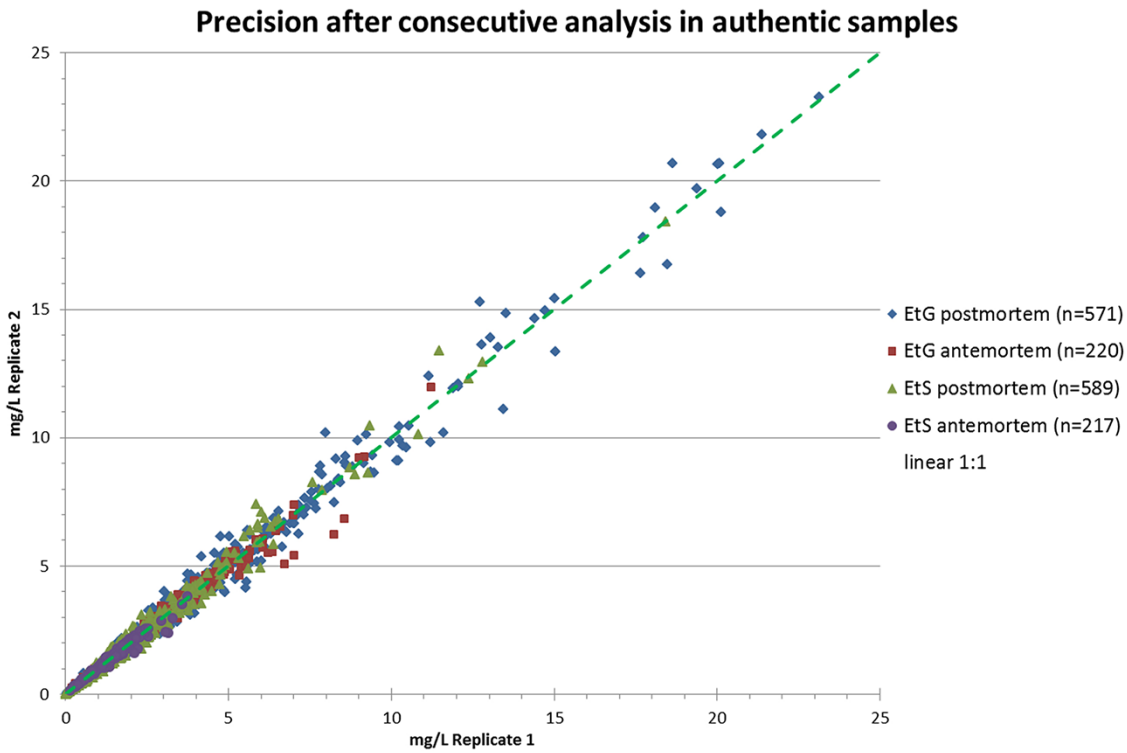

Figure 6. Precision after consecutive analysis in authentic samples.

For the low concentration level, the RSD were 8% for EtS and EtG, bias was 9% and –7% for EtS and EtG, respectively. For the high concentration level, the RSD was 4% and 3% and bias was 1% and –10% for EtS and EtG, respectively.

The method has been run on routine basis since November 2015. All together a total of about 4,500 postmortem whole blood and 1,500 antemortem (DUI and other forensic samples) samples have been analyzed with the method, corresponding to about 3,000 individual samples. For each authentic sample, two replicates (R_1 and R_2) were analyzed on the same work list. The sample withdrawal from the authentic sample was independent of each other either by assuring sample withdrawal of R_1 and R_2 made by two different analysts, or at different times. The assay precision (σ_{WR}), was obtained by determination of the concentration differences (δ_i-s) from the two replicates (R) of authentic samples analyzed on the same work lists (W) as described by Kristoffersen et al. (39), and according to the equations (1.1 and 1.2):

$$\sigma^2_{WR} = \frac{1}{2(N-1)} \sum_{i=1}^{i=N} (\delta_i - \bar{\delta})^2 \tag{1.1}$$

N was the number of pairwise samples (individuals, i) and $\bar{\delta}$ was the average of the δ_i differences. Natural logarithmic scale (ln) was used. The precision, expressed for a single replicate, was:

$$\sigma_{WR} = \frac{SD_{(lnR2-lnR1)}}{\sqrt{2}} \tag{1.2}$$

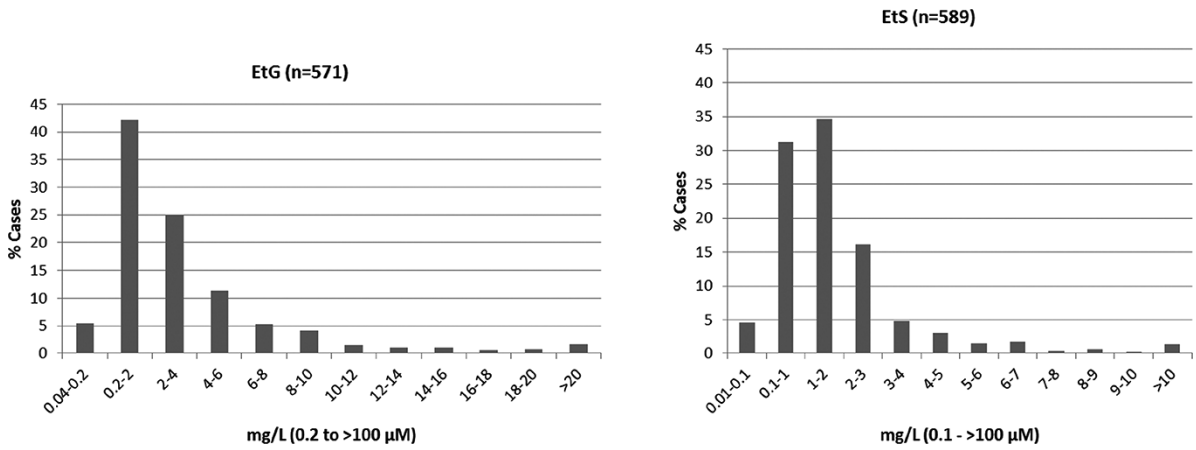

Figure 7. EtG and EtS concentration range and concentration distribution in authentic postmortem whole blood samples during a period of 19 months (n = number of forensic cases).

The assay precision was estimated based on routine analyses during a period of 17 months; about 580 postmortem and 220 antemortem pairwise samples were included. Satisfactory assay precision was achieved in authentic forensic toxicology samples, with RSD $\leq$ 6.7% in postmortem and $\leq$ 5.1% in antemortem whole blood samples (Figure 6).

The performance of the method has also been evaluated by participation in nine external proficiency testing rounds since 2015. The z-scores were calculated as the difference between our result and the consensus mean of the results of the participating laboratories and divided by the standard deviation for proficiency assessment. A z-score $\leq \pm 2$ is acceptable. The z-scores were in the range –0.38 to 0.35 for both compounds, thus indicating good accuracy.

Concentration Distribution in Authentic Samples

All of the received postmortem whole blood samples were screened and quantified for ethanol by a headspace gas chromatography–flame ionization detection method (40). All ethanol-positive ($\geq$ 0.1 g/kg) postmortem whole blood samples were analyzed for EtG and EtS. The concentration range and concentration distribution found in our postmortem whole blood samples (n = 747) were investigated over a period of 19 months (Figure 7). EtG was found in 571 cases and EtS in 589 cases. The median concentration of EtG was 2.2 mg/L (10 µM) and EtS was 1.4 mg/L (11 µM) in postmortem whole blood. These results were in accordance with previously reported EtG and EtS concentration levels found in postmortem samples (3). In about 17% of the samples EtG and/or EtS were not found above half the cut-off (Table II), which may indicate postmortem formation of ethanol. Routine measurement of EtG and EtS should therefore be recommended in order to distinguish antemortem ingestion and postmortem formation of ethanol.

Conclusion

In the present study, a simple, sensitive and high-throughput UHPLC–MS-MS method based on PPT followed by filtration on phospholipid removal 96-well plate, using only 100 µL postmortem blood and acidic mobile phase has been developed and fully validated. Deuterated analogs were used as IS for both compounds. This improves the robustness of the quantitative determination with respect to variation in experimental conditions and reduces possible ME. The procedure was found to be sufficiently sensitive and specific to be applicable in monitoring recent alcohol use and cover well the concentrations found in authentic samples. The assay was successfully applied on authentic postmortem cases, demonstrating high specificity and satisfactory analytical quality of the chromatographic performance for both analytes irrespective of the degree of putrefaction. Good correlation and good agreement of quantified results were found by the presented method when compared to the former method and external proficiency testing samples. In addition, the reproducibility of the method was documented with a large number of authentic samples with RSD $\leq$ 5.1% in antemortem and $\leq$ 6.7% in postmortem whole blood samples. The method has proven to be robust, reliable and has been applied in routine analysis of forensic samples for more than 4 years, analyzing about 6,000 samples.

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