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Article

A High-Performance Liquid Chromatographic–Tandem Mass Spectrometric Method for the Determination of Ethyl Glucuronide and Ethyl Sulfate in Urine Validated According to Forensic Guidelines

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Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are powerful markers for alcohol intake and abuse. Several analytical procedures for the quantification of EtG and EtG in serum and urine have been developed so far. Many of the published methods show limits of detections (LODs) or limits of quantifications (LOQs) for EtG in urine within the range of 0.1 mg/L or higher. Since this is the actual cutoff value for proving abstinence in Germany, problems may occur if urine samples are highly diluted. In this paper, the validation of a highly sensitive, fast and simple LC–MS–MS for the determination of EtG and EtS in urine is described. The calibration curves for EtG and EtS is linear over the whole range (0.025–2.0 mg/L). Very low detection limits can be achieved (LOD: EtG 0.005 mg/L, EtS 0.005 mg/L; and LOQ: EtG 0.019 mg/L, EtS 0.015 mg/L). All data for selectivity, precision and accuracy, recovery, as well as for the processed sample and the freeze/thaw stability, comply with the guidelines of the German Society of Toxicological and Forensic Chemistry. Strong matrix-related effects can be compensated for by using an internal standard. Finally, the applicability of the procedure is proven by analysis of 87 human urine samples and by successful participation in interlaboratory comparison tests.

Introduction

Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are stable, nonvolatile, and water-soluble metabolites of ethanol. Ethanol is mainly oxidized to acetaldehyde in the liver (90–95%). Only small proportions (<0.1%) are converted into EtG and EtS by conjugation of ethanol with UDP-glucuronic acid or activated sulphate via the action of UDP-glucurosyl transferase (UDP-GT) or sulfotransferase, respectively (1, 2). Due to the specificity of EtG and EtS for the presence of ethanol, they are effective markers of alcohol intake and alcohol abuse. EtG and EtS can be determined in various body fluids for an extended time period after complete elimination of ethanol itself (3–10). After a single moderate intake of alcohol (0.5 g/kg body weight), EtG and EtS are detectable in serum for 10–14 h and in urine for 25–44 h (11, 12). Thus, EtG and EtS close the gap between short-term markers (e.g., ethanol) and long-term markers (e.g., carbohydrate-deficient transferrin in serum). This permits a wide range of applications, such as controlling patients in withdrawal treatment or monitoring alcohol abstinence.

Several methods exist to determine EtG and EtS in urine, blood, hair, and other species. For the analyses of urine and serum, the following analytical methods are used: gas chromatography–mass spectrometry (GC–MS) (13, 14, 16, 31), liquid chromatography–mass spectrometry (LC–MS) (15, 18, 24, 35, 36), LC–multiple mass spectrometry (MS^n) (14, 19–21, 25, 27, 30), LC with pulsed electrochemical detection (22, 26), capillary zone electrophoresis (23, 29, 33, 34), and immunochemical tests (17, 28, 32).

Major differences exist with regard to the applied extraction procedures. Besides simple dilutions or precipitations with methanol or acetonitrile for protein precipitation (13–15, 17–21, 23–25, 27, 29, 30, 32, 34), solid-phase extraction procedures (16, 22, 26, 33, 35, 36) and extractions by microwaves (31) are used for the analysis of urine samples. Basic data for methods for the determination of EtG/EtS in urine and serum are summarized in Table I (13–36).

Many of the published methods showed limits of detections (LODs) or limits of quantifications (LOQs) within the range of 0.1 mg/L or higher. According to the current state of research, this concentration is consistent with the cutoff value for distinguishing between teetotalers and social drinkers. The cutoff value of 0.1 mg/L for EtG in urine has recently been entered into the new guidelines for driving ability diagnostics in Germany (37). However, problems may occur if heavily diluted urine samples are analyzed.

Studies have revealed that EtG and EtS concentrations in urine are heavily influenced by internal dilution of the urine sample (38–40). Therefore, it is recommended to factor the degree of dilution into the evaluation of the results. One possibility is to measure the creatinine content.

Additionally, a highly sensitive method for the determination of EtG and EtS concentrations far below the cutoff level (0.1 mg/L for EtG) is needed for the calculation of the normalized EtG/EtS results ($c_{creatinine} = 100$ mg/dL), designated as EtG_{100}/EtS_{100} (7, 41).

Our aim was to develop and validate such a sensitive LC–MS–MS procedure for the determination of EtG and EtS in urine. In contrast to previous methods, the procedure presented here combines high sensitivity with relatively strong dilution of the urine. By using a very sensitive tandem mass spectrometer (4000 Q-Trap, Applied Biosystems), EtG and EtS can be determined in very low concentrations. Even in heavily diluted urine samples, EtG and EtS can be determined with

Table I
Basic Data of Published Methods for the Determination of EtG/EtS in Urine and Serum

Year	Analyte	Matrix	Sample preparation	Instrumentation	LOD (mg/L)	LOQ (mg/L)	Lit.
1995	EtG	urine	precipitation	GC–MS	–	–	(13)
1999	EtG	urine/serum	dilution/precipitation	GC–MS and LC–MS–MS	0.1 (both)	–	(14)
1999	EtG	serum	precipitation	LC–MS	0.03	–	(15)
2001	EtG	urine/serum	SPE	GC–MS	0,168/0.037	0,560/0.173	(16)
2002	EtG	urine/serum	dilution	immunochemical (ELISA)	–	–	(17)
2002	EtG	urine	dilution	LC–MS	0.05	0.1	(18)
2004	EtS	urine	dilution	LC–MS–MS	0.05	0.11	(19)
2004	EtG	urine	dilution/precipitation	LC–MS–MS	0.052	0.125	(20)
2004	EtG	urine	dilution/precipitation	LC–MS–MS	0.1	0.3	(21)
2005	EtG	urine	SPE	pulsed electrochemical detection	0.03–0.1	0.1–0.8	(22)
2005	EtG	serum	dilution	capillary zone electrophoresis	0.1	–	(23)
2005	EtS	urine	dilution	LC–MS	0.05	–	(24)
2005	EtG, EtS	urine	dilution	LC–MS–MS	0.025	0.05	(25)
2006	EtG	urine	SPE	pulsed electrochemical detection	0.08	0.3	(26)
2006	EtG, EtS, EtP	urine	dilution/precipitation	LC–MS–MS	–	0.1	(27)
2006	EtG	urine	no	enzyme immunoassay	0.35	–	(28)
2006	EtS	urine	dilution	capillary electrophoresis/UV detection	–	5	(29)
2007	EtG/EtS	serum	dilution/precipitation	LC–MS–MS	0.01	0.05	(30)
2008	EtG	urine	microwaves	GC–MS	0.005	0.1	(31)
2008	EtG	urine	no or dilution	enzyme immunoassay	–	<0.1	(32)
2008	EtS	urine/serum	SPE	capillary zone electrophoresis	0.4–1.0/0.1	0.6–2.0/0.2	(33)
2008	EtG	serum	dilution	capillary zone electrophoresis	0.01	–	(34)
2008	EtG	urine	SPE	LC–MS	–	–	(35)
2010	EtG, EtS	urine	SPE	LC–MS	0.05	0.1	(36)

absolute certainty. As a consequence of the relatively strong dilution of the urine, concentrated urine samples can also be analyzed without problems because matrix effects are minimized. Thereby it is guaranteed that urine samples with a wide range of properties can be analyzed using only one procedure. At the same time, the presented procedure is fast and simple. In contrast to many other procedures, which include time-consuming solid-phase extraction, sample preparation was limited to precipitation with methanol without losing the required sensitivity. The method was fully validated according to the guidelines of the German speaking Society of Forensic Toxicology (GTFCh), and its applicability was proven by analyzing 87 authentic urine samples.

Materials and Methods

Chemical and reagents

EtG/EtS and d_5-EtG/d_5-EtS were purchased from Lipomed (Arlesheim, Switzerland). Methanol, acetonitrile, and formic acid (98%) were obtained from Merck (Darmstadt, Germany). All chemicals were of the highest analytical grade. Water was purified with a NANOpure Diamond Analytic Water Purification System D11901 (Barnstead, Dubuque, IA).

Stock solutions of EtG and EtS (both 1 g/L) as well as of d5-EtG and d5-EtS (both 5 g/L) were prepared in methanol by weighing separately. All solutions were stored at −20°C. Working standard solutions used for calibration were prepared by spiking blank urine at 0.025, 0.05, 0.1, 0.25, 0.5, 1, and 2 mg/L. Quality control samples were prepared at 0.1, 0.35, 0.5, and 1.5 mg/L. All working solutions were stored in a refrigerator (2–8°C).

Sample preparation

For protein precipitation, 20 μL of the internal standard (methanolic solution of d_5-EtG and d_5-EtS (both 2.5 μg/mL) and 280 μL methanol were added to a 100 μL urine sample. The samples were vortexed for a short time and centrifuged (10 min, 3000 g). Then 300 μL of the supernatant were separated and evaporated to dryness under a stream of nitrogen at 40°C. The dried extracts were reconstituted with 600 μL of 0.1% aqueous formic acid and 10 μL aliquots were injected directly into the LC–MS–MS system.

Instruments and LC–MS–MS conditions

Analyses were performed on a Shimadzu LC-20A Series system (Shimadzu, Duisburg, Germany) interfaced to a 4000 Q-Trap (Applied Biosystems/Sciex, Darmstadt, Germany) with an electrospray Turbo V Ion source in negative mode. The ESI source settings were: ion-spray voltage, −4500 V; source temperature, 450°C; nebulation and heating gas, (N2), 60 psi and 50 psi, respectively. For chromatographic separation, a polar-endcapped phenylpropyl reversed-phase column (Synergi Polar-RP 250 × 2 mm, 4 μm) with a guard column (ODS Octadecyl 4 mm × 2 mm; Phenomenex, Aschaffenburg, Germany) was used at 40°C. A mobile phase of water containing 0.1% of formic acid (solvent A) and acetonitrile (solvent B) was used with a flow rate of 0.2 mL/min, and the following gradient program was used: 100% A for 6 min; switch to 100% B over 1 min and hold for 2 min; back to 100% A over 1 min and hold for 4 min. Using a tee mixer, acetonitrile was added post-column (0.1 mL/min) to enhance analyte ionization. Detection of the ions was performed in multiple reaction monitoring (MRM) mode, using the following precursor to product ion transitions: EtG 221/75 (target), 221/85 (qualifier 1), 221/113 (qualifier 2); d5-EtG: 226/85 (target), 226/75 (qualifier); EtS 125/97 (target), 125/80 (qualifier 1), 125/64 (qualifier 2); d_5-EtS: 130/98 (target), and 130/80 (qualifier). The expected relative peak areas of the transitions for EtG and EtS are: 221/75:221/85:221/113 × 100:100:60 and 125/97:125/80:125/64 × 100:30:1, respectively. Analysis of the collected data was carried out with Analyst software (Version 1.4.2, Applied Biosystems/Sciex, Darmstadt, Germany).

Results

Validation of the method

The validation was conducted according to the guidelines of the GTFCh (42, 43). The method was validated for selectivity, linearity and sensitivity, precision and accuracy, recovery/matrix effects as well as for processed sample and freeze/thaw stability. All calculations were performed using Valistat software (Version 1.0) (44).

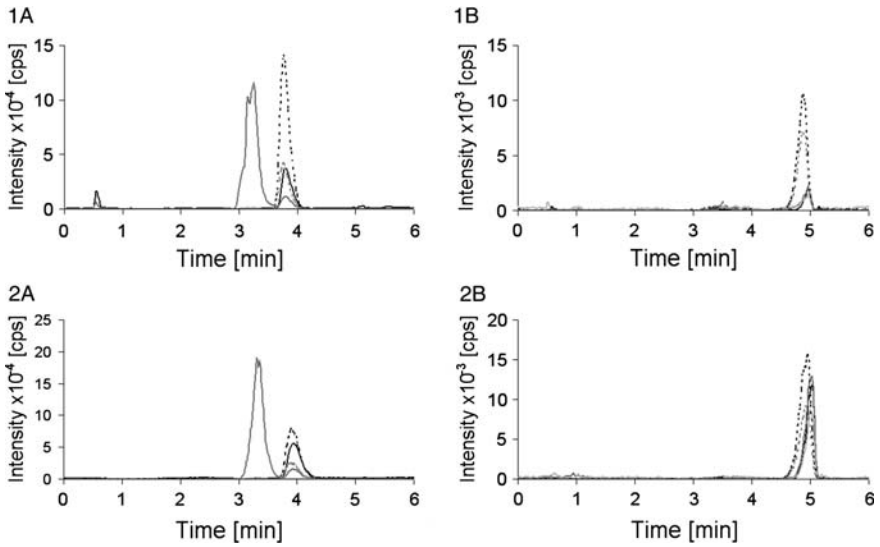

Figure 1. Extracted LC–MS–MS chromatograms of EtS (1A) and EtG (1B), both 0.1 mg/L in urine and of an authentic urine sample, tested positive for EtG (2A) and EtS (2B). The dotted lines correspond to the internal standards. The solid lines in black and grey correspond to the target and qualifiers, respectively.

Selectivity

Five different blank urine samples and five zero samples (all of strict teetotalers) were tested negative for EtG. No interfering peaks appeared at the retention times of the analyte and IS in these samples.

Linearity and calibration

Calibration was evaluated by analyzing six replicates of spiked urine samples with EtG and EtS at 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, and 2.0 mg/L. The two 7-point calibration curves, fitted by least squares regression, were linear over the whole range. The average equations were:

$$y = 2.98x\,10^{-3}x + 1.14 \times 10^{-2} (R = 0.9989) \text{ and}$$

$$y = 1.44x\,10^{-3}x + 9.71x\,10^{-3} (R = 0.9982)$$

for EtG and EtS, respectively. Based on the EtG/EtS calibrator, concentrations at 0.010, 0.015, 0.020, 0.025, and 0.030 mg/L were used; the limits of detection (LOD) and the limits of quantitation (LOQ) were calculated according to the German Industrial norm DIN 32645 (45).

The results were: EtG 0.005 mg/L (LOD) and 0.019 mg/L (LOQ) and EtS 0.005 mg/L (LOD) and 0.015 mg/L (LOQ). Figure 1 shows an extracted ion LC–MS–MS chromatogram of EtS and EtG both 0.1 mg/L in urine (1) and a chromatogram of an authentic urine sample, tested positive for EtG and EtS (2).

Precision and accuracy

Accuracy and precision of the method were assessed by analyzing QC samples at low, middle, and high concentration levels in relation to the calibration range. The concentration levels (0.1, 0.35, and 1.5 mg/L) were chosen with regard to the current cut-off values for EtG ($c_{EtG} = 0.1$ mg/L). Two replications at each concentration level were analyzed on eight consecutive days. Summarized results for precision and accuracy can be found in Table II.

Table II
Precision and Accuracy Data for the Validated LC–MS–MS Method

	EtG			EtS		
	Low QC 0.1 mg/L	Med QC 0.35 mg/L	High QC 1.5 mg/L	Low QC 0.1 mg/L	Med QC 0.35 mg/L	High QC 1.5 mg/L
Characteristics						
Mean	0.093	0.383	1.567	0.101	0.359	1.552
SD	0.004	0.014	0.058	0.007	0.010	0.054
RSD%	4.61	3.57	3.70	7.14	2.79	3.48
Accuracy						
Variance	−0.007	0.033	0.067	0.001	0.009	0.052
Bias, %	−7.13	9.51	4.44	0.93	2.68	3.44
Intra-day precision						
SD	0.004	0.014	0.035	0.003	0.009	0.029
RSD%	4.5	3.78	2.26	3.15	2.42	1.84
Inter-day precision						
SD	0.004	0.014	0.060	0.007	0.010	0.055
RSD%	4.61	3.78	3.80	7.31	2.81	3.55

Extraction recovery and matrix-related ionisation effects

Extraction recoveries of EtG and EtS from urine were determined by comparing the analyte responses of pre-extraction spiked samples to those of post-extraction spiked samples. Six replicates at low (0.1 mg/L) and high (1.5 mg/L) concentration levels were analyzed.

Matrix-related ionization effects were evaluated by comparing the analyte responses of post-extraction spiked samples to those of non-matrix prepared samples representing 100% recovery. Six replicates at low (0.1 mg/L) and high (1.5 mg/L) concentrations levels were analyzed. Relative peak areas were calculated and compared. All results for extraction recovery and matrix-related ionization effects are summarized in Table III.

Stability of processed samples

For estimating the stability of the processed samples under the conditions of LC–MS–MS analysis, prepared urine samples at two different concentrations (0.1 and 1.5 mg/L) were analyzed three times (immediately after sample preparation, two days

Table III
Summarized Data for Extraction Recovery and Matrix-Related Ionisation Effects

	Extraction recovery		Matrix related effects	
	0.1 mg/L	1.5 mg/L	0.1 mg/L	1.5 mg/L
EtG	95%	93%	79%	69%
d_5-EtG	93%	92%	69%	68%
EtS	98%	92%	104%	94%
d5-EtS	98%	92%	98%	95%

Table IV
Data Concerning the Stability of the Processed Samples

	EtG			EtS		
	Day 0	Day 3	Day 7	Day 0	Day 3	Day 7
0.1 mg/L	100%	101%	91%	100%	97%	91%
0.5 mg/L	100%	101%	87%	100%	94%	86%
1.5 mg/L	100%	101%	88%	100%	95%	87%

later and after one week). During the whole week, samples were stored in a cooled auto sampler at 10°C. Relative peak areas related to day 0 were calculated for each concentration (Table IV). The calculated decrease of EtG/EtS and d_5-EtG/d_5-EtS peak areas was less than 14% at all times.

Long term and freeze/thaw stability

According to the guidelines of the GTFCh, all forensic urine samples are stored in a cool place for six months (46). Often, urine sample are frozen and thawed several times for conducting different analyses. To determine a potential decrease of EtG/EtS concentrations, spiked urine samples at three different concentrations (0.1, 0.5, and 1.5 mg/L) are aliquoted and deep-frozen. Stability samples were analyzed immediately after spiking, two months later, and 6 months later. Freeze/thaw stability samples were analyzed before and after 3 freeze-thaw cycles. Samples were frozen at −20°C and thawed for at least 5 h. The EtG/EtS concentrations were determined and the percentage decreases were calculated. All results are summarized in Table V.

External quality control

Since last year, the institute of legal medicine/Bonn has been taking part in periodical inter-laboratory comparison tests. Aliquots of spiked urine samples are sent to all participating laboratories and the determined concentrations are compared and assessed. Until now all inter-laboratory comparison tests were passed with a maximum deviation of 9% from the desired concentration for EtG and 12% for EtS.

Application of the method

For some time it has been possible to participate in an alcohol abstinence monitoring program at the institute of legal medicine in Bonn. According to the new German guidelines for driving ability diagnostics, the participants have to pass several urine tests with unpredictable summoning. Up to 87 urine samples were analyzed. 79 of them were tested negative ($c_{EtG} < 0.1$ mg/L). The remaining 8 urine samples revealed EtG concentration from 0.17 to 20 mg/L.

Table V
Summarized Data for Long Term and Freeze/Thaw Stability for EtG and EtS

	EtG			EtS		
	0.1 mg/L	0.5 mg/L	1.5 mg/L	0.1 mg/L	0.5 mg/L	1.5 mg/L
2 month	91%	88%	85%	90%	91%	92%
6 month	85%	93%	95%	92%	91%	98%
Frozen/thaw	85%	88%	87%	79%	91%	93%

Discussion

EtG and EtS are minor metabolites of ethanol, which are sensitive markers for determining recent alcohol intake in clinical and forensic investigations. In addition, EtG and EtS analyses in urine and hair provide valid parameters for monitoring alcohol abstinence.

A sensitive, simple, and rapid LC–MS–MS method for the quantification of EtG and EtS in urine has been developed and fully validated according to the German guidelines. Despite very simple sample preparation including only protein precipitation, very low detection limits could be achieved (LOD 0.005 mg/L and 0.004 mg/L, LOQ 0.019 mg/L, and 0.015 mg/L for EtG and EtS, respectively). According to the guidelines for driving ability diagnostics, urine samples with creatinine levels of 20 mg/dL or higher are accepted for analysis (37). Owing to the very low LOQ, it is possible to detect an excess of the cutoff level ($c_{EtG} = 0.1$ mg/dL) by creating the EtG_{100}, even if the creatinine level is very low (20–40 mg/dL).

Very good results could be achieved for precision and accuracy. The method proved accurate within 10%. The intra- and inter-day precision (RSD) values for the QC samples were less than 5% at all times. Besides satisfactory long term and freeze/thaw stability, the method showed an acceptable drop regarding the stability of the processed samples. The calculated decrease of EtG/EtS and d_5-EtG/d_5-EtS peak areas was less than 15% and the extraction recovery of the procedure was better than 90% at all times. The disadvantages of the LC–MS–MS procedure that was used become obvious when looking at the high matrix related effects (up to 31%). In order to counteract these, the urine samples are strongly dissolved (1:8) during sample preparation. However, complete elimination of the matrix effects could not be achieved. Many of the polar urine contents have similar retention times to EtG and EtS and are not separated from the analyte peaks by dilution of the sample. Thus, by using an internal standard that is equally affected, it is possible to compensate the impact of the matrix related effects. The applicability of the validated procedure could be approved by analysis of 87 urine samples, which were analyzed to prove alcohol abstinence. Additionally, 4 comparison tests were passed within the last year.

Conclusion

A specific, sensitive, and robust method for quantitation of EtG and EtS in human urine using LC–MS–MS has been developed and fully validated. Validation data for selectivity, linearity precision and accuracy, recovery as well as for processed sample and freeze/thaw stability was satisfactory and corresponded to the guidelines of the GTFCh. The applicability was proven by analyzing 87 authentic urine samples. As a result of the very

low sensitivity, EtG and EtS can be determined in very low concentrations. Abstinence tests, for example, can be performed by analyzing heavily diluted as well as highly concentrated urine samples without any problems. The validated procedure will provide a powerful tool for monitoring alcohol abstinence and distinguishing between "social" and heavy drinkers.

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