A fully validated method for the quantification of ethyl glucuronide and ethyl sulphate in urine by UPLC-ESI-MS/MS applied in a prospective alcohol self-monitoring study

Kummer Natalie[a]✉
Wille Sarah[a]
Di Fazio Vincent[a]
Lambert Willy[b]
Samyn Nele[a]

✉nataliekummer@bluewin.ch

[a] National Institute of Criminalistics and Criminology, Brussels, Belgium
[b] Ghent University, Faculty of Pharmaceutical Sciences, Ghent, Belgium

Postprint version of the article published in *Journal of Chromatography B* ***2013****, 929, 149–154. https://doi.org/10.1016/j.jchromb.2013.04.011.*

Abstract A method for the quantification of ethyl glucuronide (EtG) and ethyl sulphate (EtS) in human urine is developed and fully validated according to the international guidelines. Protein precipitation is used as sample preparation. During the development of the method on an UPLC-ESI-MS/MS using a CSH C18 column, special attention was paid to reduce matrix effects and to improve sensitivity of the second transition for EtS. The method is linear from 0.1 to 10 µg/mL for both analytes. Ion suppression less than 24% was observed for EtG and no significant matrix effect was measured for EtS. This method was used to analyse urine samples obtained from twenty seven volunteers whose alcohol consumption was monitored during the 5 days before sampling.

Keywords Ethyl glucuronide; Ethyl sulphate; Urine; Validation; UPLC-ESI-MS/MS; Alcohol marker; Prospective study

Contents

1 Introduction

Ethyl glucuronide (EtG) and ethyl sulphate (EtS) are two specific metabolites of ethanol, created by conjugation with UDP-glucuronic acid for EtG [1] and with 3'-phosphoadenosine 5'-phosphosulfate for EtS [2].

Quantification of EtG and EtS in urine is used to detect recent alcohol consumption. These biomarkers extend the detection window relative to blood ethanol measurement and, compared to long term biomarkers, allow the detection of drinking of small quantities.

EtG and EtS are detectable in urine up to 24 h after intake of 0.25 g/kg ethanol and up to 48 h after intake of 0.50 g/kg ethanol [2-8]. After alcohol intoxication, they can be detected in urine during a few days. EtG is eliminated according to a half-time of 2.5 h [1, 3]. After consumption of alcohol and depending on the amount of consumed alcohol, urinary concentrations for EtG and EtS can vary from some µg/mL [8-12] to hundreds of µg/mL [3, 5, 7, 11, 13, 14]. Urine samples from alcohol-dependent patients during detoxification can reach EtG concentrations up to 1240 µg/mL [1, 2, 15, 16] and EtS concentrations up to 264 µg/mL [2].

Due to the possibility of finding EtG and EtS in urine even without consumption of alcoholic beverages [8, 9, 17-20], a cut-off limit is used to avoid false positive results. However, cut-offs are not fixed yet in international guidelines. The ones currently used vary between 0.1 and 1.1 µg/ml [21, 22]. A cut-off at 0.1 µg/mL for EtG and at 0.05 µg/mL for EtS has been proposed to exclude repeated intake of alcohol [21]. Urine analysis of teetotallers shows no EtG [1, 23] and no EtS [24] above 0.1 µg/mL

The most commonly applied technique for quantification of EtG and EtS in urine is liquid chromatography coupled to mass spectrometry (LC-MS) [3, 15] or coupled with tandem mass spectrometry (LC-MS/MS) [1, 7, 12, 18, 22, 24-28] in combination with simple dilution or protein precipitation as sample preparation. A few methods have been published using GC-MS [23, 29-31] or capillary zone electrophoresis (CZE) [32-35] for the analysis of EtG and EtS in urine or serum.

To decrease matrix effects, especially for EtS, sample preparation should be adapted. Dilution of urine is the easiest 'sample preparation' method, however high matrix effects and higher instrument maintenance can be problematic in routine analysis. Even with a 1/20 dilution, relevant matrix effect was observed at low concentrations [24]. Liquid-liquid extraction (LLE) and solid phase extraction

(SPE) are conventional sample preparation techniques for non-volatile compounds. Due to the high polar and acidic character of EtG and EtS in combination with a different acidic strength, the development of LLE and SPE is, however, not straightforward. Protein precipitation can be an alternative clean-up method for this type of analytes [7-9, 25] if the matrix effects are carefully monitored.

Reversed-phase (RP) chromatography used with negative electrospray ionization mode (ESI^-) is the most common approach used [1, 3, 7, 12, 14, 15, 18, 26, 28, 36]. The retention of very polar acidic compounds, such as EtG (pKa ~ 2.84) and EtS (pKa ~ -3.14) [12], is achieved in RP only under highly aqueous conditions. As highly aqueous conditions might not be optimal for ESI ionization, post-column addition of an organic modifier is required to enhance the ionization of compounds and so to improve sensitivity. A chromatographic possibility to improve the ionization is to use a normal phase column [25] or another specific column with particular retention behaviour [12]. Nevertheless, normal phase chromatography is known to provide variable retention times [37]. The use of no discharge atmospheric pressure chemical ionization (ND-APCI) [22] or atmospheric pressure chemical ionization APCI [24] is another solution to increase the ionization and so to improve the limit of quantification.

According to international guidelines, forensic analysis by MS/MS requires the detection of minimum two transitions for each compound; one for identification and one for quantification [28, 38]. When LC-MS is used, three characteristic ions are required. Sometimes it is difficult to find a second transition for EtS using LC-ESI-MS/MS [25, 26], because of the low intensity of the second transition and the presence of interfering compounds in urine.

Our aim was to develop a simple and robust method for the quantification of EtG and EtS in urine using an UPLC-ESI-MS/MS system equipped with a triple quadrupole (QQQ) tandem mass spectrometer. The method was validated according to international guidelines. A prospective study, based on 27 volunteers, is used to study the chosen cut-off value (0.1 µg/mL) for EtG and EtS and to estimate the sensitivity and specificity of the method. This method could be used for the surveillance of abstinence in the context of driving licence regranting [22, 36] and for the detection of alcohol uptake prior death in post-mortem cases [39, 40].

2 Experimental

2.1 Chemicals

Ethyl glucuronide (EtG), ethyl sulphate (EtS) and their pentadeuterated analogues (EtG-d_5 and EtS-d_5) were obtained from Sigma-Aldrich (Steinheim, Germany) as a methanolic 1 mg/mL solution. ULC/MS grade acetonitrile, methanol and 0.1% formic acid in water were purchased from Biosolve (Valkenswaard, The Netherlands). Blank urine was purchased from Bio-Rad Laboratories (Nazareth Eke, Belgium). External quality controls Medidrug ETG 1/10-B and Medidrug ETG 2/09-B were both purchased from Medichem (Steinenbronn, Germany). Proficiency tests for EtG and EtS in urine and serum were organised by GTFCh.

2.2 Preparation of Standard Solutions, Calibrators and Quality Control Samples (QC)

Two stock solutions, one for calibration (Cal-Stock) and one for internal quality controls (QC-Stock), with EtG and EtS at a concentration of 20 µg/mL were prepared in methanol. The stock solution with internal standards (IS) at a concentration of 4 µg/mL was prepared in methanol. All solutions were stored at -18°C.

Daily calibration working solutions (Cal-WS) with concentrations of 0.1, 0.5 and 10 µg/mL were prepared diluting the Cal-Stock solution. Calibrators (0.1, 0. 25, 0.5, 2.5, 5.0 and 10 µg/mL) were prepared by spiking 30 µL of the IS solution, 50 µL of commercial blank urine, an adequate amount of Cal-WS, and methanol until a total volume of 280 µL was reached.

Daily quality controls working solutions (QC-WS) with concentration of 0.5 and µg/mL were prepared diluting the QC-Stock solution. Internal quality controls (0.3, 4 and 7.5 µg/mL) were prepared spiking 30 µL of IS, 50 µL of commercial blank urine, an adequate amount of QC-WS and methanol until a total volume of 280 µL.

2.3 Population study

A prospective alcohol self-monitoring study was performed asking 27 volunteers to declare their exact alcohol consumption per day during the 5 days preceding the sampling. Urine samples were collected in 100 mL urine containers from Sarstedt (Nümbrecht, Germany), transferred to 4 mL Greiner bio-one tubes (Frickengrasen, Germany) and stored at 2-8°C until analysis.

EtG_{100} and EtS_{100} concentration were calculated by normalizing the measured EtG and EtS to a creatinine concentration of 100 mg/dL [2].

2.4 Sample preparation

Methanol (250 µL) and 30 µl of the IS solution (4 µg/mL) were added to 50 µL of urine. After precipitation, the sample was centrifuged at 14'000 rpm during 10 min at 4°C. 250 µL of the supernatant was transferred to a total recovery glass vial (Waters, Zellik, Belgium) and evaporated to dryness under a stream of nitrogen using a heated metal block at 38°C. The residue was reconstituted in µL of 0.1 % formic acid in water.

For each authentic sample, an additional 1/1000 dilution was systematically performed.

2.5 Liquid chromatography and mass spectrometry conditions

Analyses were performed on an Aquity UPLC coupled to a Xevo TQ MS tandem mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ionization source operated in negative mode. Gradient elution was performed on an Acquity UPLC CSH C_{18} (2.1 x 100 mm, 1.8 µm) column (Waters, Milford, MA, USA) with 0.1 % formic acid in water (A) and acetonitrile (B) at a flow rate of 300 µL/min. The gradient elution started with 99.3 % of solution A for 2.4 min, decreasing to 40% of solution A at 3.0 min, to 20% of solution A at 4.4 min. The washing step contains only 2 % of solution A and holds from 4.41 to 5.40 min. The initial condition is applied from 5.41 min to 7 min. The column temperature was set at 55°C. The injection volume is 5 µL using full-loop mode.

For the MS/MS detection, following parameters were used: temperature of source gas (nitrogen) was 150°C, desolvatation gas (nitrogen) flow was 900 L/h at 650°C, capillary voltage was 1 KV, cone voltage was 26 V with a cone gas flow at 40 l/h, multiplier voltage was 508.42 V, extractor voltage was 3 V, MS mode detection energy was 2, MS/MS mode detection energy was 10, ion energy I was 0.5 V, ion energy II was 0.5 V, entrance and exit potential was 0.5 V and collision gas (argon) flow was 0.35 ml/min. Detection was performed in the multiple reaction monitoring mode (MRM) using the appropriate parameters for each compounds (Table 1).

Table 1: MRM transitions and conditions for EtG, EtS and for their deuterated analogues.

	Precursor ion (m/z)	Product ion (m/z)	Dwell (s)	Cone voltage (V)	Collision energy (eV)
EtG (Quantifier)	220.96	74.95	0.110	30	22
EtG (Qualifier)	220.96	84.96	0.110	30	24
EtG-d5	225.97	84.90	0.190	28	30
EtS (Quantifier)	124.90	80.10	0.110	26	26
EtS (Qualifier)	124.90	96.88	0.110	26	18
EtS-d5	129.97	97.85	0.110	28	18

2.6 Method validation

Selectivity, sensitivity, matrix effects, extraction efficiency, limits, linearity, accuracy and stability were evaluated according to international guidelines [41].

To study endogenous interferences, six blank urine samples from different individuals were analysed. To verify that there were no isotope exchange reactions with non-labelled compounds, two zero samples (blank urine spiked with internal standard) were analysed. According to the EMEA guideline, interferences are acceptable in our type of method as long as the signal was lower than 20% of the response at the LOQ.

Matrix effects are quantified and evaluated using the post-extraction addition technique [42]. For the matrix effect, six blank urine samples from different persons were spiked after the sample preparation and compared with compounds spiked at the same theoretical concentration in the mobile phase. Extraction efficiency is evaluated comparing responses of six blank urine samples spiked before sample preparation with responses of six blank urine samples spiked after sample preparation. These experiments were done at low (0.3 µg/mL), medium (4 µg/mL) and high (7.5 µg/mL) concentration.

The limit of detection (LOD) is determined by analysing decreasing concentrations of the analytes. The LOD is the lowest concentration of the analyte for which the signal-to-noise ratio of both transitions is at least 3/1.

The limit of quantification (LOQ) is the lowest concentration of analyte with a signal-to-noise ratio greater than 10/1 for both transitions and for which the bias and precision deviation is less than 20%. Other identification criteria, such as the ion ratio between the quantifier and the qualifier also had to be reached.

The calibration model (n=6) was tested over the range 0.1, 0.25, 0.5, 2.5, 5 and 10 µg/mL. Calibration model and weighting factor were evaluated for each compound. The goodness of fit was established as the difference between the calculated calibrator value and its nominal value. The variation coefficient should be lower than 15% except for the LOQ (< 20%).

Accuracy is measured through the determination of trueness (bias) and precision (repeatability and intermediate precision). Three internal quality controls at low (0.3 µg/mL), medium (4 µg/mL) and high (7.5 µg/mL) concentration and two external quality controls (ETG 1/10-B and EtG 2/09-B) were analysed in replicates on 8 different days. A single factor ANOVA test with significance level (α) of 0.05 allows calculating bias, repeatability and intermediate precision with these data. The results are acceptable when they are less than 15% (20% for the LOQ). The reproducibility is evaluated by participation in proficiency tests organized by GTFCh.

Freeze/thaw stability, processed sample stability and long term storage stability are evaluated at low (0.3 µg/mL) and high (7.5 µg/mL) concentration. The mean of the stability should be within 90 – 110% of the mean of the control samples and the 90% confidence interval of the stability sample results should be within ± 20% of the control samples.

3 Results and discussion

3.1 Method Validation

The method was validated for selectivity, matrix effects, extraction efficiency, sensitivity, linearity, accuracy and stability.

Identification of compounds was based on retention time and on the presence of a stable ratio between the two MRM transitions (<20%). It is well known that EtG and EtS can be present in small amounts in urine even without voluntary consumption of alcohol [8, 9, 17-19]. Some blank urine contained EtG and EtS, but the calculated concentrations were lower than the LOQ.

Results of matrix effects and extraction efficiency are presented in Table 2 and Table 3.

Table 2: Matrix effect and extraction efficiency for EtG.

EtG	Low	Medium	High
Matrix effect calculated as % recovery (RSD %)	84 (12)	80 (15)	76 (8)
Matrix effect (% recovery) compensated by IS (RSD %)	110 (11)	102 (4)	102 (3)
Extraction efficiency % (RSD %)	81 (14)	80 (3)	79 (3)

Table 3: Matrix effect and extraction efficiency for EtS.

EtS	Low	Medium	High
Matrix effect calculated as % recovery (RSD %)	106 (9)	95 (8)	88 (3)
Matrix effect (% recovery) compensated by IS (RSD %)	108 (7)	96 (7)	113 (6)
Extraction efficiency % (RSD %)	76 (5)	81 (7)	80 (6)

A reproducible ion suppression of less than 24% is observed for EtG. The use of EtG-d_5 as IS compensated for the matrix effect. No significant matrix effects were observed for EtS. The extraction efficiency of EtG and EtS is reproducible, concentration independent and about 80%.

Using an LC-ESI system equipped with a Q-Trap tandem mass spectrometer, a matrix effect (% recovery) of up to 69 % was calculated for EtG and around 94% for EtS [36]. Using an LC-ESI system equipped with a triple quadrupole mass spectrometer, an ion suppression of up to 20% was calculated for EtS and an ion enhancement of up to 10% was observed for EtG after a 1:20 dilution of urine sample as sample preparation [12]. Using LC-MS/MS with ND-APCI a matrix effect (% recovery) of up to 170% for EtG and EtS was calculated [24].

The LOQ was 0.1 µg/mL for EtG and for EtS (Figure 1). The LOD was 0.06 µg/mL for EtG and 0.08 µg/mL for EtS.

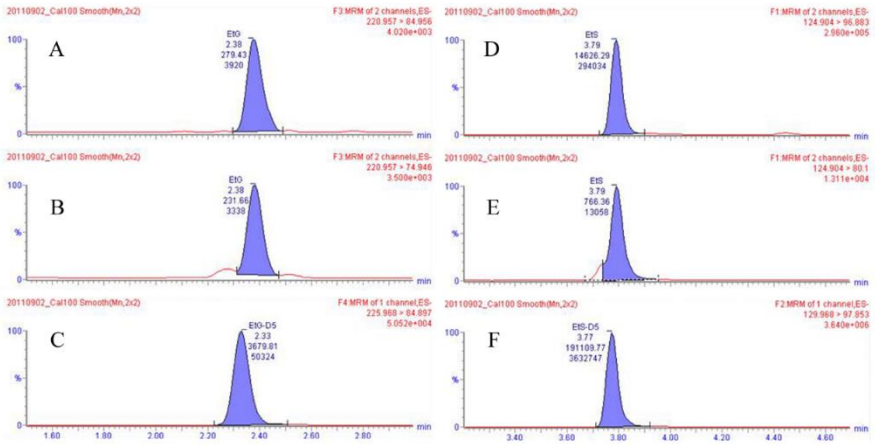

Figure 1: MRM Chromatogram for EtG (m/z 221→75 (A), m/z 221→85 (B)) and EtG-D5 (m/z 226→85 (C)) and EtS (m/z 125→80 (D), m/z 125→97 (E)) and EtS-D5 (m/z 130→98 (F)) at the LOQ (0.1 µg/mL).

For EtG, a weighted (1/x) linear regression line was applied, for EtS a $1/x^2$ weighting was necessary.

The bias of the method is lower than 15%. The repeatability (RSD_r) and intermediate precision (RSD_t) are acceptable, with RSD (%) lower than 10% (Table 4).

Table 4: Trueness (bias) and precision (repeatability (RSDr) and intermediate precision (RSDt)).

	EtG				**EtS**			
	Nominal value (µg/mL)	RSDr (%)	RSDt (%)	Bias (%)	Nominal value (µg/mL)	RSDr (%)	RSDt (%)	Bias (%)
LOQ	0.100	6.64	9.97	3.70	0.100	3.59	3.59	-4.65
1/10-B	0.878	3.63	3.42	-4.18	0.920	2.22	4.10	-12.53
2/09-B	3.020	3.60	3.84	-1.41	1.750	3.10	5.67	-4.45
QC Low	0.300	6.41	7.42	-0.74	0.300	4.08	7.27	6.02
QC Medium	4.000	2.38	5.05	-1.00	4.000	3.32	4.35	-0.01
QC High	7.500	2.29	5.67	0.26	7.500	2.39	5.20	-4.95

No instability was observed for samples staying in the autosampler during 24 and 72 h. Moreover, EtG and EtS were stable after three freeze/thaw cycles, after 2 months at -20°C and after 2 months at 4°C.

The reproducibility of the method was tested passing three proficiency tests organized by GTFCh. The concentrations of EtG and EtS and the z-scores are presented in Table 5.

Table 5: Target value and z-score for EtG and EtS for each proficiency tests.

	EtG		**EtS**	
	Nominal value (µg/mL)	z-score	Nominal value (µg/mL)	z-score
Proficiency test EtG 3/11	1.450	-0.22	0.885	0.57
Proficiency test EtG 1/12	0.800	-0.46	1.100	-0.86
Proficiency test EtG 2/12	0.556	0.10	1.070	0.23

Using an Acquity CSH C_{18} column on an UPLC-ESI-MS/MS system equipped with a triple quadrupole, allows to fulfil forensic analysis requirements and to reach an LOQ for EtG and EtS of 0.1 µg/mL.

Most published methods (see Table 6) using LC-MS(/MS) showed an LOQ at 0.1 µg/mL or higher. By using LC-ESI systems equipped with very sensitive tandem mass spectrometer (4000 Q-Trap) [8, 36, 43], very low detection limits could be achieved (LOQ at 0.05 µg/mL or lower). By using LC-ESI systems equipped with a triple quadrupole as tandem mass spectrometer, no published method excepted one [9], has been reported with an LOQ at 0.1 µg/mL for both EtG and EtS. Unfortunately, for the method with an LOQ lower than 0.1 µg/mL for both EtG and EtS, no details on the validation of the method are given in the publication.

Table 6: Methods used for the quantification of EtG/EtS in urine (Q = quadrupole; QQQ = triple quadrupole).

Method (MS Type)	Mode and transition for the detection		LOQ (µg/mL)		Ref.
Column	EtG	EtS	EtG	EtS	
HPLC-ESI-MS (Q)	SIM		0.1	-	[3]
Hypercarb	221				
HPLC-ESI-MS (Q)	SIM	SIM	0.1	0.1	[15]
Hypercarb	221	125			
HPLC-ESI-MS/MS (Ion Trap)	MRM	MRM	0.13	0.2	[25]
ZIC-HILIC	221→203 (113)	125→98 (125)			
HPLC-ESI-MS/MS (Q-Trap)	MRM	MRM	0.1	0.1	[12]
Mixed-modal RP/WAX	221→75 (85)	125→97 (80)			
HPLC-ESI-MS/MS (Q-Trap)	MRM	MRM	0.019	0.015	[8, 36]
Synegy polar RP	221→75 (85, 113)	125→97 (80,64)			
HPLC-ESI-MS/MS (Q-Trap)	MRM	MRM	0.05	0.05	[43]
Chrompack Inertsil ODS-3	221→75 (85)	125→97 (80)			
HPLC-ND-APCI-MS/MS (Ion Trap)	Product ion scan	SIM	0.1	0.1	[22]
Hypercarb	221→Scan 50-230	125→125			
HPLC-APCI-MS/MS (QQQ)	MRM	MRM	0.1	0.1	[24]
Restek Ultra Aqueous C18	221→75 (85, 57)	125→97 (96, 45)			
HPLC-ESI-MS/MS (QQQ)	MRM	MRM	0.15	0.1	[26]
Synegy polar RP	221→75 (85)	125→97 (80, 125)			
HPLC-ESI-MS/MS (QQQ)			0.1	0.2	[7]
Synegy polar RP	221→75 (203, 113, 85)	125→97 (80)			
UPLC-ESI-MS/MS (QQQ)	MRM	MRM	0.37	0.2	[18]
Acquity UPLC HSS T3	221→85	125→97			
HPLC-ESI-MS/MS	SIM (MRM)		0.1	-	[1]
Hypercarb	221→221 (75)				
HPLC-ESI-MS/MS (QQQ)	MRM		0.15	-	[28]
Synegy polar RP	221→75 (85)				
HPLC-ESI-MS/MS (QQQ)	MRM	MRM	0.05	0.04	[9]
Synegy polar RP	221→75 (85, 203, 113)	125→97 (80)			

3.2 Population study

Twenty seven urines samples from volunteers were analysed. Urine samples from volunteers (n=14) who did not drink alcoholic beverages the day before the sampling were all negative for EtG and EtS using a cut-off at 0.1 µg/mL.

In 10 samples from volunteers who declared having consumed alcohol the day before the sampling (n=13) a concentration between 0.4 and 102 µg/mL for EtG and between 0.145 and 379 µg/mL for EtS was determined (Figure 2).

In one case (Figure 2; A), no EtS (EtS_{100}<0.1 µg/mL and EtG_{100}=1.4 µg/mL) was detected after the consumption of two alcohol units. In another case (Figure 2; B), EtG was not detected 24 hours after the ingestion of 1 glass of alcohol (EtG_{100}<0.1 µg/mL and EtS_{100}=0.1 µg/mL). In one sample (Figure 2; C), neither EtG nor EtS (EtG100 and EtS100<0.1 µg/mL) were detected 24 hours after the consumption of one glass of alcohol. After the consumption of two units of alcohol, EtS concentration in urine is below 0.1 µg/mL and EtG concentration is above 0.1 µg/mL in 6 out of 7 cases [21]. In 2 out of 5 cases, EtG concentration is above 0.1 µg/mL in urine 24 hours after a consumption of 1 glass of alcohol.

One volunteer (Figure 2; D), who has declared a consumption of five alcohol units the day before the sampling, has no EtG and no EtS in urine (EtG_{100} and EtS_{100} <0.1 µg/mL). The creatinine concentration measured in that sample was abnormally low (12 mg/dl). In Germany, a urine creatinine concentration below 20 mg/dl is declared as “not useable” for analysis [44]. This abnormal dilution of urine can explain the absence of EtG and EtS in this sample [45].

The three subjects who have declared a consumption of alcohol (2, 4 and 6 glasses) 2 days before the sampling and no consumption the day before were all negative for EtG and EtS. Kinetic studies show that EtG and EtS are detectable in urine up to 24 h after intake of about 2 units of alcohol and up to 48 h after intake of about 4 units of alcohol [2-8].

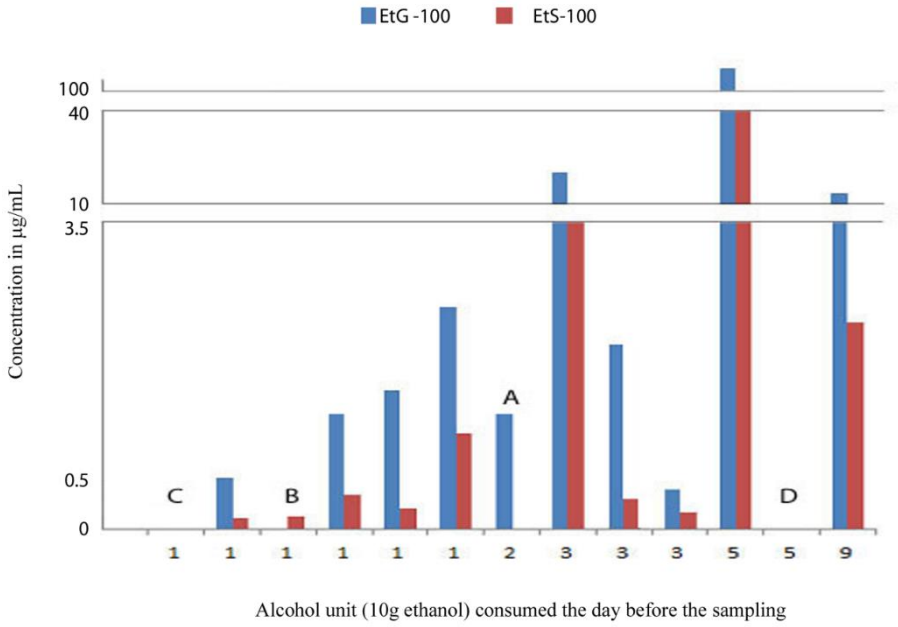

Figure 2: EtG and EtS concentrations normalized to 100 mg/dl creatinine in subjects who declared having been drinking alcohol 24 hours before the sampling.

EtG and EtS were in agreement in 25 out of 27 cases. EtG and EtS concentration in urine were highly correlated ($r=0.996$, $p<0.001$). A moderate correlation between the number of drinks the day before the sampling and the concentration of EtG ($r=0.448$, $p<0.02$) and EtS ($r=0.406$, $p<0.04$) in urine was observed. This result can be explained by the high inter-individual variation of EtG and EtS concentration in urine after the consumption of equal amounts of ethanol [7].

Using a cut-off at 0.1 µg/mL, this method is able to detect alcohol consumption approximately 24 hours after the intake, without showing any false positive results. A cut-off at 0.1 µg/mL for EtG and at 0.05 µg/mL for EtS has been proposed to exclude the repeated intake of alcohol [21]. However, knowing that some blank urines samples contain a small amount of EtG and EtS, a cut-off value of 0.1 µg/mL for EtG and EtS was chosen to distinguish between intentional and unintentional alcohol consumption.

4 Conclusion

This report describes a validated method for the quantification of EtG and EtS in urine by UPLC-ESI-MS/MS using protein precipitation as clean-up step. The chromatographic run time for one analysis is 7 minutes. The recovery was around 80% for both compounds and the matrix effect calculated as % recovery was on average 80% for EtG and on average 96% for EtS. This method provides good precision (RSD_r and RSD_t < 10%) and bias (< 15%). To avoid false positive results, a cut-off value at 0.1 µg/mL was chosen.

A good reproducibility of the method was demonstrated passing three proficiency tests organized by GTFCh with z-scores of less than 1.

Acknowledgment

We thank all volunteers for their collaboration.

References

[1] N. Stephanson, H. Dahl, A. Helander, O. Beck, Therapeutic Drug Monitoring, 24 (2002) 645-651.

[2] A. Helander, O. Beck, Journal of Analytical Toxicology, 29 (2005) 270-274.

[3] H. Dahl, N. Stephanson, O. Beck, A. Helander, Journal of Analytical Toxicology, 26 (2002) 201-204.

[4] M.H. Wojcik, J.S. Hawthorne, Alcohol and Alcoholism, 42 (2007) 317-320.

[5] G. Høiseth, J.P. Bernard, N. Stephanson, P.T. Normann, A.S. Christophersen, J. Mørland, A. Helander, Alcohol and Alcoholism, 43 (2008) 187-191.

[6] G. Høiseth, J.P. Bernard, R. Karinen, L. Johnsen, A. Helander, A.S. Christophersen, J. Mørland, Forensic Science International, 172 (2007) 119-124.

[7] C. Halter, S. Dresen, V. Auwaerter, F. Wurst, W. Weinmann, International Journal of Legal Medicine, 122 (2008) 123-128.

[8] F. Musshoff, E. Albermann, B. Madea, International Journal of Legal Medicine, 124 (2010) 623-630.

[9] A. Thierauf, H. Gnann, A. Wohlfarth, V. Auwärter, M.G.e. Perdekamp, K.-J. Buttler, F.M. Wurst, W. Weinmann, Forensic Science International, 202 (2010) 82-85.

[10] A. Thierauf, C.C. Halter, S. Rana, V. Auwaerter, A. Wohlfarth, F.M. Wurst, W. Weinmann, Addiction, 104 (2009) 2007-2012.

[11] F.M. Wurst, S. Dresen, J.P. Allen, G. Wiesbeck, M. Graf, W. Weinmann, Addiction, 101 (2006) 204-211.

[12] W. Bicker, M. Lämmerhofer, T. Keller, R. Schuhmacher, R. Krska, W. Lindner, Analytical Chemistry, 78 (2006) 5884-5892.

[13] G. Høiseth, R. Karinen, A.S. Christophersen, L. Olsen, P.T. Normann, J. Mørland, Forensic Science International, 165 (2007) 41-45.

[14] K. Borucki, R. Schreiner, J. Dierkes, K. Jachau, D. Krause, S. Westphal, F.M. Wurst, C. Luley, H. Schmidt-Gayk, Alcoholism: Clinical and Experimental Research, 29 (2005) 781-787.

[15] A. Helander, M. Böttcher, C. Fehr, N. Dahmen, O. Beck, Alcohol and Alcoholism, 44 (2009) 55-61.

[16] F.M. Wurst, S. Seidl, D. Ladewig, F. Müller-Spahn, A. Alt, Addiction Biology, 7 (2002) 427-434.

[17] T.P. Rohrig, Journal of Analytical Toxicology, 30 (2006) 703-704.

[18] G. Høiseth, B. Yttredal, R. Karinen, H. Gjerde, A. Christophersen, Journal of Analytical Toxicology, 34 (2010) 84-88.

[19] A. Costantino, E.J. DiGregorio, W. Korn, S. Spayd, F. Rieders, Journal of Analytical Toxicology, 30 (2006) 659-662.

[20] A. Thierauf, A. Wohlfarth, V. Auwärter, M.G.e. Perdekamp, F.M. Wurst, W. Weinmann, Forensic Science International, 202 (2009) e45-e47.

[21] M. Albermann, F. Musshoff, E. Doberentz, P. Heese, M. Banger, B. Madea, International Journal of Legal Medicine, (2012) 1-8.

[22] D. Favretto, A. Nalesso, G. Frison, G. Viel, P. Traldi, S. Ferrara, International Journal of Legal Medicine, 124 (2010) 161-164.
[23] I. Janda, A. Alt, Journal of Chromatography B: Biomedical Sciences and Applications, 758 (2001) 229-234.
[24] J. Beyer, T. Vo, D. Gerostamoulos, O. Drummer, Analytical and Bioanalytical Chemistry, (2011) 1-8.
[25] Al-Asmari, Journal of Analytical Toxicology, 34 (2010) 261-272.
[26] S. Dresen, W. Weinmann, F.M. Wurst, Journal of the American Society for Mass Spectrometry, 15 (2004) 1644-1648.
[27] F. Wurst, M. Yegles, C. Alling, S. Aradottir, J. Dierkes, G. Wiesbeck, C. Halter, F. Pragst, V. Auwaerter, International Journal of Legal Medicine, 122 (2008) 235-239.
[28] W. Weinmann, P. Schaefer, A. Thierauf, A. Schreiber, F.M. Wurst, Journal of the American Society for Mass Spectrometry, 15 (2004) 188-193.
[29] G. Schmitt, R. Aderjan, T. Keller, M. Wu, Journal of Analytical Toxicology, 19 (1995) 91-94.
[30] F.M. Wurst, C. Kempter, J. Metzger, S. Seidl, A. Alt, Alcohol, 20 (2000) 111-116.
[31] I.Á. Freire, A.M.B. Barrera, P.C. Silva, M.J.T. Duque, P.F. Gómez, P.L. Eijo, Journal of Applied Toxicology, 28 (2008) 773-778.
[32] F.A. Esteve-Turrillas, W. Bicker, M. Lämmerhofer, T. Keller, W. Lindner, Electrophoresis, 27 (2006) 4763-4771.
[33] B. Jung, J. Caslavska, W. Thormann, Journal of Chromatography A, 1206 (2008) 26-32.
[34] L. Křivánková, J. Caslavska, H. Malášková, P. Gebauer, W. Thormann, Journal of Chromatography A, 1081 (2005) 2-8.
[35] M. Nováková, L. Křivánková, ELECTROPHORESIS, 29 (2008) 1694-1700.
[36] M. Albermann, F. Musshoff, B. Madea, Analytical and Bioanalytical Chemistry, 396 (2012) 2441-2447.
[37] P. Cabarcos, H.M. Hassan, M.J. Tabernero, K.S. Scott, Journal of Applied Toxicology, (2012).
[38] L. Rivier, Analytica Chimica Acta, 492 (2003) 69-82.
[39] G. Høiseth, R. Karinen, L. Johnsen, P.T. Normann, A.S. Christophersen, J. Mørland, Forensic Science International, 176 (2008) 147-151.
[40] C.C. Halter, A. Laengin, A. Al-Ahmad, F.M. Wurst, W. Weinmann, K. Kuemmerer, Forensic Science International, 186 (2009) 52-55.
[41] S. Wille, F. Peters, V. Di Fazio, N. Samyn, Accreditation and Quality Assurance, 16 (2011) 279-292.
[42] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Analytical Chemistry, 75 (2003) 3019-3030.
[43] L. Politi, L. Morini, A. Groppi, V. Poloni, F. Pozzi, A. Polettini, Rapid Communications in Mass Spectrometry, 19 (2005) 1321-1331.
[44] T. Arndt, Forensic Science International, 186 (2009) 48-51.
[45] M.E. Albermann, F. Musshoff, B. Madea, Journal of Chromatographic Science, 50 (2012) 51-56.