ELSEVIER

Journal of Chromatography B, 758 (2001) 229–234

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Improvement of ethyl glucuronide determination in human urine and serum samples by solid-phase extraction

Ines Janda*, Andreas Alt

Department of Forensic Medicine, University of Ulm, Prittwitzstr. 6, D-89075 Ulm, Germany

Received 12 December 2000; received in revised form 15 March 2001; accepted 19 March 2001

Abstract

An improved method for the determination of ethyl glucuronide (EtG) in human serum and urine was developed using solid-phase extraction (SPE) and gas chromatography (GC) with mass spectrometric detection (MS). EtG was isolated from serum and urine using aminopropyl SPE columns after deproteination with perchloric acid and hydrochloric acid, respectively. The chromatographic separation was performed on a DB 1701 fused-silica column. At a signal-to-noise ratio of 3:1, a quantification limit of 173 and 560 ng/ml and a detection limit of 37 and 168 ng/ml could be determined for serum and urine, respectively. This indicates high specificity and sensitivity of the described method. The mean absolute recovery was ~85%, while intra- and inter-day precision of the assay were all less than 7.5%. The linearity of the calibration curves was satisfying as indicated by correlation coefficients of >0.993. The presented method provides the basis for determination and identification of EtG in human serum and urine samples in a low-concentration range for monitoring alcohol consumption during treatment for alcohol dependence and comorbid alcohol abuse of psychotherapy patients.

Keywords: Ethyl glucuronide

1. Introduction

In the human organism, ~90–95% of ethyl alcohol is eliminated by oxidation, mainly in the liver via alcohol- and aldehyde-dehydrogenase, catalase and the microsomal ethanol-oxidizing system. Minor amounts are excreted by the kidneys (0.5–2%), the lungs (1.6–6%) and the skin (max. 0.5%) [1]. The detoxifying pathway of alcohol elimination via ethyl glucuronide (EtG) is reported to represent ~0.5–1.5% of total ethanol elimination [2–4]. Nevertheless, until recently, only limited attention has been payed to this pathway in comparison to hepatic and extra-hepatic oxidative metabolism.

EtG is a non-volatile, water soluble compound, which can be detected in body fluids, hair and tissue [5–11]. In comparison to ethanol concentration, the maximum EtG concentration in blood shows a time delay of 2–3.5 h [10]. EtG is still detectable in urine up to 80 h after alcohol consumption [10,11]. The detection of alcohol consumption that has taken place several days, weeks or months earlier, via the determination of EtG, represents a useful forensic tool as well as a possible method for monitoring alcohol consumption of patients being treated for alcoholism.

*Corresponding author. Tel.: +49-731-50-26873; fax: +49-731-50-26875.

E-mail address: ines.janda@medizin.uni-ulm.de (I. Janda).

0378-4347/01/$ – see front matter
PII: S0378-4347(01)00186-4

Gas chromatography–mass spectrometry methods without sample pretreatment using acetyl- [12] or trimethylsilyl (TMS)-derivatives [5] have been recently described. However, due to the complex nature of urine and blood, most procedures yield samples with varying degrees of purity. As a result, these methods are frequently hindered by matrix compounds co-eluting with EtG. A liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) method has been published by Nishikawa et al. [13], which requires equipment not available in many laboratories.

To overcome these problems, a method was developed using a solid-phase extraction (SPE) procedure followed by modified GC–MS detection.

2. Experimental

2.1. Materials

Ethyl glucuronide (EtG) and d_5-ethyl glucuronide (d_5-EtG) standards were obtained from Medichem (Stuttgart, Germany). *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane used for derivatization was purchased from Merck (Darmstadt, Germany). SPE-columns (ISOLUTE NH_2 (aminopropyl) 1 g/6 ml) from Separtis (Grenzach-Wyhlen, Germany) were employed for solid-phase extractions. The GC column (DB-1701 fused-silica capillary column, 30 m×0.25 mm I.D., 0.25-μm film thickness) was obtained from J&W Scientific (Folsom, CA, USA). All chemicals (Merck, Darmstadt, Germany) were analytical chemical grade.

2.2. Standard solutions

Stock standard solutions of EtG and d_5-EtG were prepared by dissolution of each compound in methanol to obtain a concentration of 1 mg/ml. These were stored at −20°C and remained stable for several months.

Biological standards were prepared at concentrations of 30, 70, 135, 200, 330, 500 and 700 ng/ml for serum and at concentrations of 70, 135, 330, 700, 1400, 2000, 3300 and 7000 ng/ml for urine by adding appropriate aliquots of the stock solutions to EtG-free samples.

2.3. Urine sample preparation

A methanolic solution of d_5-EtG, used as an internal standard, was added to a 1.5-ml aliquot of urine in a 10-ml glass tube to give a final concentration of 5 μg/ml. After addition of 100 μl 3 *M* HCl and 3.5 ml acetonitrile, the sample was briefly vortexed.

2.4. Serum sample preparation

A total of 100 μl of concentrated perchloric acid was added to a 1.5-ml aliquot of serum in a 10-ml glass tube, and d_5-EtG was adjusted to a final concentration of 1 μg/ml as an internal standard. The mixture was briefly vortexed and centrifuged at 3000 rpm for 10 min. Prior to addition of acetonitrile (3.5 ml), the clear supernatant was transferred to a clean tube.

2.5. Extraction

A 5-ml volume of the prepared samples was applied to an aminopropyl (NH_2) cartridge, conditioned with 3 ml methanol, 3 ml demineralized water and 3 ml acetonitrile prior to use. Care was taken to ensure that the columns did not run dry between conditioning steps. The cartridge was then washed with 3 ml *n*-hexane. To remove all residual liquid, a strong vacuum was applied for 15 min. EtG was eluted from the cartridges using 1.8 ml of water containing 2% conc. ammonia. All solvents passed the cartridges at a flow-rate of ~0.5 ml/min. The eluate was evaporated to dryness under a gentle stream of nitrogen using a heated metal block at 30°C.

2.6. Derivatization

Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (100 μl) containing 1% trimethylchlorosilane and pyridine (50 μl) was added to the dry residue. The vial was capped and heated in an oven at 90°C for 30 min. After cooling to room temperature, the vial was uncapped and evaporated to dryness. The residue

was immediately dissolved in 50 μl ethyl acetate and 1 μl was injected into the GC–MS system.

2.7. GC–MS conditions

The analysis was performed using an HP 5973 mass spectrometer equipped with an HP 6890 gas chromatograph and an HP 7683 autosampler (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was achieved on a DB-1701 fused-silica capillary column, 30 m×0.25 mm ID, 0.25-μm film thickness (J&W Scientific, Folsom, CA, USA) with the following temperature program: 60°C for 2 min, then increasing temperature at 10°C/min to 200°C and further at 15°C/min to 250°C, hold for 1 min. Helium was used as the carrier gas. Injections were made in the splitless mode. The injector and transfer line were maintained at 250 and 280°C, respectively.

The mass selective detector was operated in electron impact mode at 70 eV with an ion source temperature of 150°C. Data were acquired in the selected-ion monitoring mode (SIM-mode). The trimethylsilyl (TMS)-derivatives were identified with the masses m/z 160, 261, 405 (target ion) for TMS-EtG (Fig. 1) and m/z 165, 266, and 410 for TMS-d_5-EtG.

2.8. Validation

The calibration curve was determined daily by adding appropriate amounts of EtG standard solution to 1.5 ml serum or urine. A 5-ml volume of prepared solution was applied to the SPE cartridge. The calibration curves consisted of seven (serum) and eight (urine) points ranging from 30 to 700 ng/ml EtG and from 70 to 7000 ng/ml EtG, respectively. Calibration curves were estimated by a weight least-squares regression procedure, using $1/x^2$ as weighting factor.

Quality control (QC) samples at low (50 ng/ml, serum; 100 ng/ml, urine), medium (300 ng/ml, serum; 2000 ng/ml, urine) and high (700 ng/ml, serum; 7000 ng/ml, urine) concentrations were prepared by spiking samples with EtG standard solutions. These samples were stored at −20°C until use. Inter-day precision and accuracy of the method were determined by assaying six replicates of each of the three quality control samples at 5 different days. The results were expressed as the relative standard deviation (RSD) at each level.

Absolute recoveries of EtG from serum and urine were calculated by comparing the peak area of extracted samples spiked with EtG at concentrations of 100 and 2000 ng/ml (serum) and 1000 and 10 000 ng/ml (urine), with those obtained from a standard EtG solution dissolved in 50 μl ethyl acetate. Human serum and urine from 12 people were used to evaluate selectivity. Blank samples were extracted and analyzed for potential interfering peaks coeluting with EtG and d_5-EtG as internal standard.

The sensitivity was determined by spiking serum or urine with increasing concentrations of EtG until response equivalent to three times the background noise was observed. The limit of detection (LOD) and of quantification (LOQ) was calculated according to DIN 32645 using software B.E.N. 2.0 (Institute of Forensic Medicine, Heidelberg, Germany).

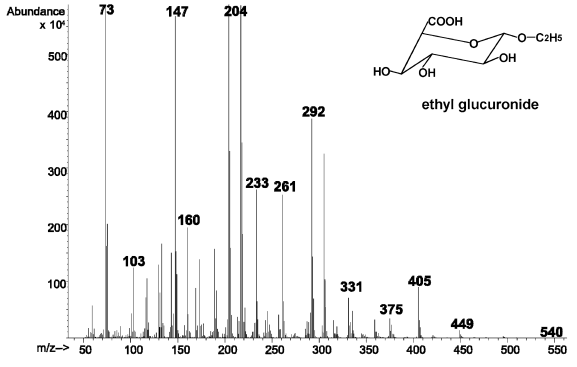

Fig. 1. Chemical structure of EtG and mass fragmentation spectrum of the respective trimethylsilyl (TMS) derivative. Masses for identification of EtG are m/z 160, 261 and 405 (target ion).

3. Results and discussion

3.1. Chromatography

In Figs. 2 and 3 representative chromatograms of a positive urine and serum sample are shown containing 500 and 50 ng/ml EtG, respectively. Both samples were analyzed using the described SPE method (A) and the direct method (B). With the new SPE procedure, the amount of interfering peaks has

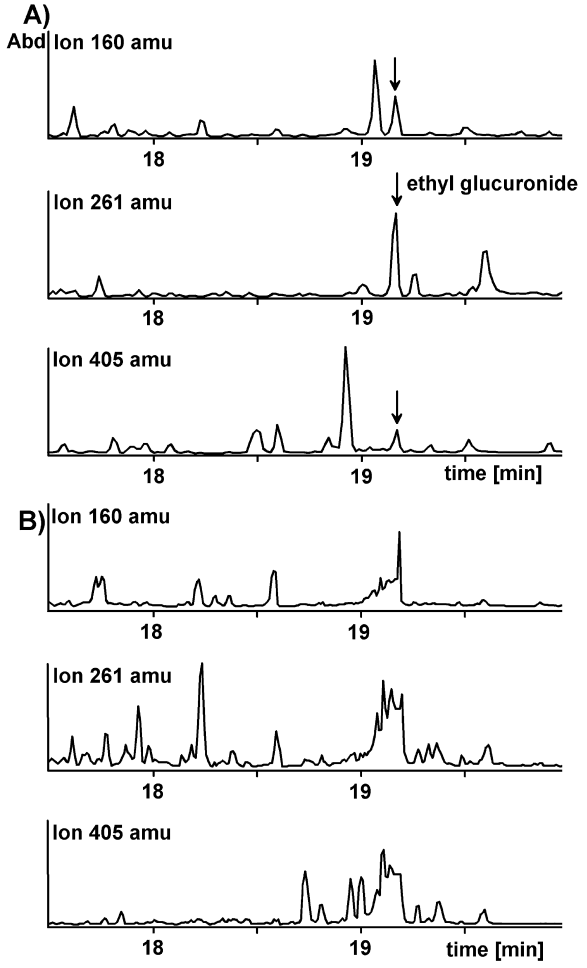

Fig. 2. Typical chromatograms of an EtG-containing urine sample extracted with SPE (A) and without sample preparation (B). Selected ion chromatograms show the trimethylsilyl (TMS)-derivatives identified with the masses m/z 160, 261 and 405 (target ion). EtG concentration was 500 ng/ml.

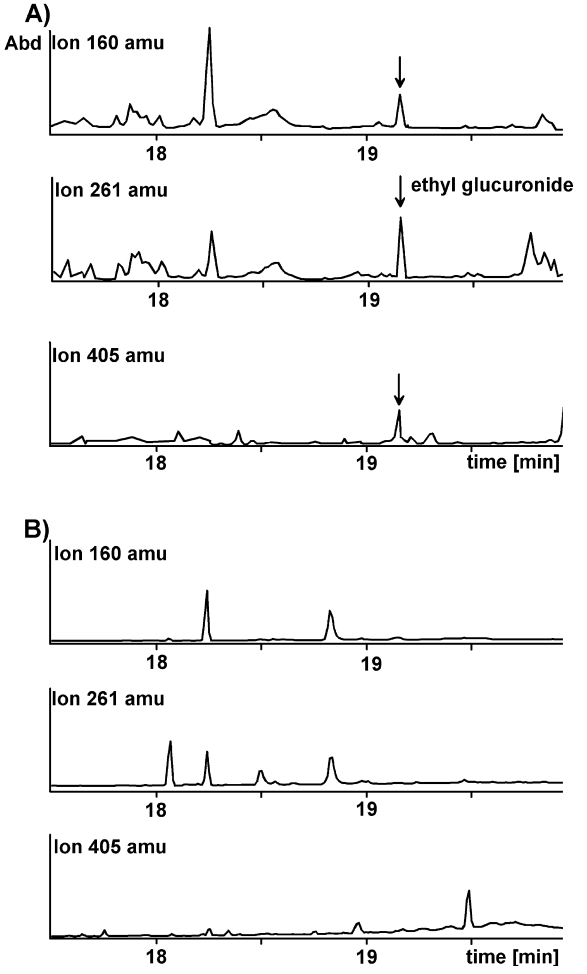

Fig. 3. Typical chromatograms of an EtG-containing serum sample extracted with SPE (A) and without sample preparation (B). Selected ion chromatograms show the trimethylsilyl (TMS)-derivatives identified with the masses m/z 160, 261 and 405 (target ion). EtG concentration was 50 ng/ml.

been remarkably lowered and the signal-to-noise ratio has been slightly enhanced compared to methods published up to now. This is especially important in the low-concentration range, where matrix effects normally lead to a lower signal-to-noise ratio. Due to the initial sample purification, which is not performed in the direct method, clear detection, identification and quantification of EtG in concentrations as small as 50 ng/ml serum is now possible.

In previous studies, hydrophobic separation columns, e.g. DB-5, were applied for the GC–MS analysis of EtG [9,10,12]. However, for EtG, which is an extremely polar compound, the use of a hydrophilic column was thought to be preferable. In this study, the application of a polar capillary column (DB-1701) led to an enhancement of chromatography including better separation of matrix components and avoidance of peak-tailing.

3.2. Concentration range, linearity and selectivity

For the analysis of serum and urine from alcoholics and social drinkers, concentrations may vary from a few nanograms per milliliter to several micrograms per milliliter [6,10,12,14]. To achieve the goal of developing a method able to analyze specimens collected from a variety of sources, it was necessary to establish linearity for a wide range of concentrations. Therefore, linearity was studied from 30 to 700 ng/ml for EtG in serum and from 70 to 7000 ng/ml in urine using a series of spiked samples. Within these ranges, good correlation of nominal concentration and detector response was observed. The corresponding calibration data are presented in Table 1. The tested ranges represent EtG concentrations commonly measured in serum and urine after the consumption of 44–90 g alcohol [10]. Moreover, low EtG concentrations are also relevant as an abstinence control in treatment for alcohol dependence.

To estimate the influence of endogenous alcohol as a possible source of EtG, serum and urine samples collected from 12 different abstinent persons were analyzed. In these samples neither interference with EtG nor EtG itself could be detected, and therefore, the amount of endogenous EtG can be ignored. Most probably, this is due to a very low physiological ethanol concentration in blood, which ranges from 0.1 to 0.2 mg/l [15–18].

Table 2
Absolute recoveries of EtG extracted from human serum and urine

Matrix	EtG concentration (ng/ml)	Recovery (%±SD)
Serum	100	85.0±1.8
	2000	93.5±2.0
Urine	1000	90.1±2.2
	10 000	97.0±1.1

Standard deviation (SD) was determined by repeat experiments ($n=4$).

3.3. Recovery

Recovery was evaluated by comparing the peak area of ethyl glucuronide resulting from spiked samples with the peak area obtain after injection of a methanolic standard prepared at the same concentration. The absolute values were determined for serum and urine using two different concentrations each (Section 2.8). These concentrations were adapted to commonly expected EtG amounts, which are higher for urine than for serum. The recoveries of EtG from human serum and urine are presented in Table 2. Good recoveries were obtained at all

Table 1
Calibration data and linearity of determination of EtG in human serum and urine

Nominal concentration (ng/ml)	Serum			Nominal concentration (ng/ml)	Urine		
	Concentration found (ng/ml), mean±SD	RSD (%)	Accuracy (%)		Concentration found (ng/ml), mean±SD	RSD (%)	Accuracy (%)
30	29±2	6.6	96.6	70	75±2	2.8	107.1
70	73±1	1.4	104.2	135	140±5	3.7	103.7
135	130±8	5.9	96.3	330	318±10	3.0	96.4
200	189±15	7.5	94.5	700	701±3	0.4	100.1
330	346±14	4.2	104.8	1400	1469±23	1.6	104.9
500	509±37	7.4	101.8	2000	2154±67	3.3	107.7
700	704±26	3.7	100.6	3300	3285±22	0.7	99.5
				7000	6981±34	0.5	99.7
R^2	0.993±0.003	0.3			0.998±0.002	0.2	

R^2 depicts the respective correlation coefficient. Standard deviation (SD) was determined by repeat experiments ($n=5$). Intra-day precision is given as relative standard deviation (RSD).

Table 3
Inter-day precision (RSD) and accuracy of determination of EtG in human serum and urine

Matrix	EtG concentration (ng/ml)	RSD (%)	Accuracy (%)
Serum	50	2.8	102.5
	300	4.6	98.9
	700	3.1	99.2
Urine	100	3.8	98.4
	2000	3.4	99.5
	7000	2.2	99.7

Measurement was done independently at 5 days.

concentration levels examined, demonstrating the excellent efficiency of the SPE method. In previous studies, recovery of 78.1% in serum has been reported at a concentration of 2000 ng/ml using LC–ESI-MS analysis [13]. Using GC–MS analysis, ~70% and 80% have been determined for serum and urine, respectively [12,10]. Therefore, due to initial SPE, recovery could significantly be enhanced in comparison to methods without sample extraction. In addition, SPE allows the use of higher sample volumes due to selective extraction of the analyte prior to analysis.

3.4. Sensitivity, precision and accuracy

For serum, an LOD of 37 ng/ml and an LOQ of 173 ng/ml could be observed. For urine, 168 and 560 ng/ml were found, respectively. Recently, Schmitt et al. [12] found an LOD of 100 ng/ml for serum and urine.

The relative standard deviation (RSD) of individual calibration points (Table 1, intra-day-precision) and quality control samples (Table 3, inter-day-precision) was 7.5% for serum and 3.8% for urine, indicating good reproducibility. Accordingly, the deviation between found and added concentration (accuracy) is presented in Tables 1 and 3. These results demonstrate good precision and analytical recovery emphasizing that the described method is well suited for an improved determination of EtG in human serum and urine.

4. Conclusion

A new SPE procedure for the improved determination of EtG in human serum and urine has been developed and validated. The method proved to be sensitive, specific and accurate for the determination of EtG in a higher sample volume (1.5 ml). In addition, the method allows the identification of positive samples in the lower concentration range. In comparison to previous published procedures the signal-to-noise ratio was significantly enhanced and possibly interfering matrix components could be removed.

References

[1] H.J. Mallach, in: H.J. Mallach, H. Hartmann, V. Schmidt (Eds.), Alkoholwirkung beim Menschen, Thieme, Stuttgart, 1987, p. 13.
[2] O. Neubauer, Arch. Exp. Pathol. Pharmakol. 46 (1901) 133.
[3] I.A. Kamil, J.N. Smith, R.T. Williams, Biochem. J. 51 (1952) 32.
[4] P.I. Jaakonmaki, K.L. Knox, E.C. Horning, M.G. Horning, Eur. J. Pharmacol. 1 (1967) 63.
[5] A. Alt, F.M. Wurst, S. Seidl, Blutalkohol 34 (1997) 360.
[6] S. Seidl, F.M. Wurst, A. Alt, Blutalkohol 35 (1998) 174.
[7] F.M. Wurst, Ch. Kempter, J. Metzger, S. Seidl, A. Alt, Alcohol 20 (2000) 111.
[8] A. Alt, I. Janda, S. Seidl, F.M. Wurst, Alcohol Alcohol. 35 (2000) 313.
[9] F.M. Wurst, R. Schüttler, C. Kempter, S. Seidl, T. Gilg, K. Jachau, A. Alt, Alcohol Alcohol. 34 (1999) 262.
[10] G. Schmitt, P. Droenner, G. Skopp, R. Aderjan, J. Forensic Sci. 42 (1997) 1099.
[11] F.M. Wurst, C. Kempter, S. Seidl, A. Alt, Alcohol Alcohol. 34 (1999) 71.
[12] G. Schmitt, R. Aderjan, T. Keller, M. Wu, J. Anal. Toxicol. 19 (1995) 91.
[13] M. Nishikawa, H. Tsuchihashi, A. Miki, M. Katagi, G. Schmitt, H. Zimmer, Th. Keller, R. Aderjan, J. Chromatogr. 726 (1999) 105.
[14] G. Schmitt, R. Aderjan, M. Kirchner, H. Zimmer, Blutalkohol 35 (1998) 428.
[15] R. Sprung, W. Bonte, E. Rüdell, M. Domke, C. Frauenrath, Blutalkohol 18 (1981) 67.
[16] A.W. Jones, Forensic Sci. Prog. 5 (1991) 31.
[17] A.W. Jones, G. Mardh, E. Änggard, Pharmacol. Biochem. Behav. 18 (1983) 267.
[18] Y.M. Ostrovsky, P. Pronko, S.N. Shishkin, V.B. Kolesnikov, S.I. Volynets, Alcohol 6 (1989) 97.