Ann Toxicol Anal. 2011; 23(4): 183-191
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DOI: 10.1051/ata/2011129

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ANNALES DE TOXICOLOGIE ANALYTIQUE

Original article / Article original

Development and validation of a GC-MS/MS method for the determination of ethylglucuronide in human urine and serum

Développement et validation d'une méthode de dosage de l'éthylglucuronide dans l'urine et le sérum par chromatographie en phase gazeuse couplée à la spectrométrie de masse en tandem

Alaa AlSaabi[1,2], Gilles Tournel[1,3], Benjamin Hennart[1,2], Delphine Notebaert[2] and Delphine Allorge[1,2]*

[1] EA4483, Faculté de Médecine/Pôle Recherche, Université Lille-Nord de France, 1 place de Verdun, 59045 Lille Cedex, France
[2] Laboratoire de Toxicologie, Centre de Biologie-Pathologie, CHRU de Lille, Bd du Pr. J. Leclercq, 59037 Lille Cedex, France
[3] Institut de Médecine Légale, CHRU de Lille, Rue du Pr. A. Verhaeghe, 59037 Lille Cedex, France

Abstract – Objectives: Ethyl-β-D-6-glucuronide (EtG) is a minor phase-II metabolite of ethanol. The aim of this work was to develop and validate a gas chromatography negative chemical ionization tandem mass spectrometry (GC-NCI-MS/MS) method to measure EtG levels in human urine and serum with both high sensitivity and specificity. **Methods:** EtG was extracted and purified from 1 mL urine or 0.5 mL serum by solid-phase extraction (SPE) using Mixed-mode Anion-eXchange (Oasis® MAX) extraction cartridges, followed by derivatization with pentafluoropropionic anhydride (PFPA). The analysis was performed in the multiple reaction monitoring (MRM) mode using the transitions m/z 496→163 (for EtG quantification), m/z 347→163 and m/z 496→119 (for identification), and m/z 501→163 for the internal standard EtG-D5. The validation procedure was performed according to the guidelines of the French Society of Analytical Toxicology (SFTA) and the French Committee of Accreditation (COFRAC; LAB GTA 04). **Results:** Calibration curves were linear in the concentration range of 10 to 10 000 ng/mL and 5 to 1 000 ng/mL in urine and serum, respectively, with a coefficient of correlation (*r*) above 0.996. The LOD and LOQ values were 5 and 10 ng/mL, respectively, for both matrices. The intra- and inter-day precision (relative standard deviation RSD%) and relative bias were less than 20%. **Conclusion:** To our knowledge, this is the first report of the application of a GC-MS/MS method for EtG measurement in urine and serum. The LOQ achieved appears to be better than those reported in the literature using other validated analytical techniques. This method could be used routinely for EtG measurement in various clinical and forensic contexts.

Key words: Ethylglucuronide, gas chromatography-tandem mass spectrometry (GC-MS/MS), ethanol, serum, urine

Résumé – Objectif : L'éthylglucuronide (EtG) est un métabolite mineur de l'éthanol, utilisé comme biomarqueur d'alcoolisation. Ce travail a consisté à développer une méthode simple et rapide de dosage de l'EtG dans l'urine et le sérum par chromatographie en phase gazeuse couplée à la spectrométrie de masse en tandem (GC-MS/MS). **Méthodes :** L'EtG a été extrait et purifié à partir d'1 mL d'urine ou de 0,5 mL de sérum par extraction en phase solide, puis dérivé par l'anhydride pentafluoropropionique (PFPA). L'acquisition a été réalisée en mode MRM (*multiple reaction monitoring*) à l'aide des transitions 501→163 pour l'étalon interne EtG-D5, 347→163 et 496→119 pour l'identification de l'EtG et 496→163 pour la quantification de l'EtG. La validation de la méthode a été réalisée selon les recommandations de la SFTA et du COFRAC. **Résultats :** La linéarité de la méthode a été démontrée pour une gamme de concentrations de 10 à 10 000 ng/mL dans l'urine et de 5 à 1 000 ng/mL dans le sérum, avec un coefficient de corrélation (*r*) supérieur à 0,996. Les limites de détection et de quantification (LDQ) sont respectivement de 5 et 10 ng/mL dans les deux matrices. La répétabilité et la reproductibilité de la méthode ont été démontrées, avec un coefficient de variation inférieur à 20 %. **Conclusion :** Une méthode de dosage par GC-MS/MS de l'EtG dans l'urine et le sérum a été développée et validée. La LDQ de cette méthode apparaît être meilleure que celles précédemment rapportées dans la littérature avec d'autres techniques. Cette méthode est applicable en routine pour le dosage de l'EtG dans un contexte médical ou médico-légal.

Mots clés : Éthylglucuronide, éthanol, urine, sérum, GC-MS/MS

Received 21 July 2011, accepted after revision 4 November 2011
Published online 12 December 2011

* Delphine Allorge, dallorge@univ-lille2.fr

1 Introduction

Alcohol abuse is one of the most frequent addictions worldwide and causes many serious social problems and pathologies [1]. The World Health Organization (WHO) has estimated that two billion people consume alcoholic beverages worldwide, with 76.3 million of those suffering diagnosable alcohol-use disorders [2]. For these reasons, a significant amount of research has focused on finding a useful and reliable marker of alcohol consumption. Such a marker would potentially allow clinicians, researchers and forensic experts to focus on groups with an increased risk of alcoholism, to monitor more efficiently curative treatment programs, and to determine the extent to which alcohol plays a role in the neurological impairment of drivers involved in accidents [3].

The known enzymatic and hematological alcohol markers (*e.g.* g-glutamyltransferase (GGT), aspartate and alanine aminotransaminases (AST, ALT) and mean corpuscular volume (MCV)) provide some information relating to alcohol use or abuse, but they cannot be considered to be satisfactory with regard to sensitivity and selectivity. Furthermore, these biomarkers can be influenced by age, gender, a variety of substances and non-alcohol-associated diseases, and they do not cover fully the time axis for alcohol intake [4].

Direct biomarkers, such as ethanol itself or some of its metabolites, have better specificity and reflect recent alcohol consumption. Ethanol itself is a good marker of alcohol consumption. However, its detection in body fluids is only possible during a relatively short time after alcohol intake, with a maximum blood concentration obtained 30 min to 1 h after ingestion and an average rate of elimination from blood of 0.10 to 0.2 g/L/h in occasional drinkers and of 0.25 to 0.35 g/L/h in chronic drinkers [5]. Because of its relatively short half-life in the body, its use is limited in practice to the diagnosis of acute alcohol intake and not of chronic alcohol abuse.

Fatty acid ethyl esters (FAEEs) are also direct alcohol markers containing the unchanged ethyl group of ethanol. After ethanol consumption, they are enzymatically formed in a side route of the ethanol metabolism in almost all tissues from free fatty acids or lipids. FAEEs are detectable in blood up to 24 h after the end of drinking and accumulate in fat tissues. They have proved to be interesting biomarkers of alcohol consumption [6].

Ethylglucuronide (ethyl-β-D-6 glucuronic acid, EtG) is formed by the conjugation of ethanol with activated glucuronic acid. It represents only 0.5% of complete alcohol elimination [7, 8], whereas more than 95% of ethanol is eliminated by oxidation, mainly in the liver [8]. EtG is a non-volatile acidic water-soluble and stable metabolite of ethanol that can be detected in various body fluids, tissues and hair. Compared to that of the conventional enzymatic markers, EtG analysis is much more sensitive and specific. Furthermore, it presents a particularly interesting detection window, as EtG can still be detected even after complete elimination of ethanol from the body [9, 10], specifically in urines where it can be detected up to 3 days [9]. All these aspects make EtG one of the most relevant biomarkers of alcohol consumption which can be applied for both clinical and forensic analyses.

Numerous analytical methods have been developed for EtG analysis in biological matrices, such as nuclear magnetic resonance (NMR) [11], capillary zone electrophoresis (CZE) [12, 13], gas chromatography–mass spectrometry (GC-MS) [14–16], liquid chromatography–mass spectrometry (LC-MS) [17–19], liquid chromatography with pulsed electrochemical detection [20] and immunochemical test [21, 22]. Each method has inherent strengths and weaknesses in terms of specificity, sensitivity, assay complexity, cycle-time and instrumentation cost and/or availability.

To our knowledge, a quantification of EtG by a GC-MS/MS method has not been published for analysis in urine and serum. The objective of the present work was to develop and validate an accurate and precise analytical method for EtG dosage in urine and serum with both high sensitivity and specificity. For this reason, a solid-phase extraction (SPE) step followed by a gas chromatography tandem mass spectrometry (GC-MS/MS) analysis in a negative chemical ionization (NCI) mode was chosen. Following validation, the method was tested for the analysis of EtG in anonymous serum samples available in our laboratory.

2 Materials and methods

2.1 Chemicals and reagents

EtG (ref: EGL-332-10) and its deuterated analogue EtG-D5 (ref: EGL-780-10), used as an internal standard (IS), were obtained from Lipomed (Souffelweyersheim, France). Methanol (MeOH, ref: 20837.320) and formic acid (99–100%, ref: 20318-297) were obtained from VWR Prolabo (Fontenay-sous-Bois, France). Ammonium hydroxide solution (25%, ref: 1.05432.1000) and hexane (ref: HEO2212500) were purchased from Merck (Chibret, France) and Scharlau (France), respectively. The derivatization agent, pentafluoropropionic anhydride (PFPA 99%, ref: 206-604-2), was obtained from Sigma Aldrich (Saint-Quentin Fallavier, France). All chemicals were of the highest analytical grade. Solid phase extraction (SPE) Oasis® MAX cartridges (3 mL, 60 mg, ref: 86000368) and a SPE tank system working under vacuum were supplied by Waters (Saint-Quentin-en-Yvelines, France).

2.2 Samples preparation

Blank urine and serum samples were collected from five adult volunteers (who are not alcohol consumers or had stopped drinking alcohol for at least one week), here referred to as alcohol abstainers, and were analysed for the presence of EtG before the validation phase. Citrated tubes were used for blood sampling, whereas clean and dry containers were used for urine samples. Blood samples were centrifuged immediately to separate the serum. No additional preservative was used during the sampling. All samples were stored at –20°C in order to maintain a good stability along the validation time [23].

2.2.1 Urine sample preparation

Appropriate volumes of standard EtG solutions were added to 1 mL of blank urine, resulting in final concentrations of 10, 100, 1 000, 5 000, 8 000 and 10 000 ng of EtG per mL of urine. Then, 25 µL of each urine sample was diluted by the addition of 975 µL of distilled water in the presence of 25 µL of EtG-D5 solution (1 000 ng/mL in MeOH). The final mixture was vortexed and transferred onto an Oasis® MAX SPE cartridge.

2.2.2 Serum sample preparation

Calibration samples were prepared by adding suitable amounts of the EtG standard solutions to 0.5 mL of blank serum, resulting in final concentrations of 5, 10, 50, 100, 500 and 1 000 ng/mL. 50 µL of EtG-D5 solution (1 000 ng/mL in MeOH) was added to each sample. Then, these samples were applied for clean-up and extraction to an Oasis® MAX cartridge.

2.3 Extraction procedure and derivatization

The prepared samples were applied to an Oasis® MAX cartridge conditioned with 1 mL of MeOH and 1 mL of deionized water. Special care was taken to ensure that the columns did not dry out between the conditioning steps. To prevent the column from drying-out, which could reduce the extraction yield, once the conditioning has started, we maintained water in the SPE column by replacing water that drained through the column. The cartridge was then washed with 1 mL of ammonium hydroxide (NH_4OH, 2%) and, secondly, with 1 mL of MeOH. A strong vacuum was applied for 5 min to remove all residual liquid. EtG was eluted from the cartridge using 1 mL of a methanol/formic acid (98:2, v/v) solution. The eluate was evaporated to dryness under a stream of nitrogen using a heated metal block at 70°C. The residue was derivatized with 100 µL of pentafluoropropionic anhydride (PFPA) which had been previously shown to be the best agent for EtG derivatization with good stability up to 1 h of incubation at room temperature, as well as at 60°C [16]. The tubes were tightly closed, mixed by vortexing (10 s), heated for 30 min at 70°C, then dried under N_2 and, finally, the residue was reconstituted in 50 µL of hexane. One µL of extract was injected into the GC-MS/MS system.

2.4 Instruments and GC-MS/MS conditions

Identification and quantification of EtG were performed in a GC-MS/MS system, which consists of a gas chromatograph (7890A series, Agilent, Massy, France) equipped with an automatic injector (7683B series, Agilent), coupled with a tandem mass spectrometer (Quattro MicroTM GC MICROMASS®, Waters). Chromatographic separation was achieved with a fused silica capillary column AT5-ms (ref: 15807, Alltech, Templemars, France) (30 m × 0.25 mm × 0.25 µm).

The carrier gas was helium with a constant flow of 1 mL/min. One µL was injected in splitless mode at an injection temperature of 250°C. The initial oven temperature of 60°C was kept for 2 min, increased first at 35°C/min to 250°C, and then kept at this temperature for 8.43 min. The transfer line was held at 270°C. Retention times were of 6.53 min for EtG and 6.52 min for EtG-D5. Samples were ionized by NCI with methane, as the reagent gas, at a pressure of 0.2 mTorr. The ion source temperature was kept at 100°C.

Data acquisition and MS control were performed using the software Mass-Lynx version 4.1 (Waters). The GC-MS/MS was performed in multiple reaction monitoring (MRM) mode. The precursor ions m/z 496 and 347 for EtG and m/z 501 for EtG-D5 were selected in the first quadrupole. These precursor ions were chosen for further fragmentation according to their selectivity and abundance in the mass spectra. The resulting product ions m/z 119 and 163 for EtG and m/z 163 for EtG-D5 were selected in the second quadrupole after collision (in the collision cell) with argon, as the collision gas, at a pressure of 5 mTorr. The collision energy was maintained at 30, 10 and 8 eV for the transitions m/z 496→119, 347→163 and 496→163, respectively, and 10 eV for the transition m/z 501→163. Transition m/z 496→163 was retained for the EtG quantification, whereas transitions m/z 347→163 and m/z 496→119 were used for the EtG identification. The electron multiplier was set up at 550 V.

2.5 Validation procedure

The analytical method was validated according to the guidelines of the French Society of Analytical Toxicology (SFTA) [24] and of the COFRAC (LAB GTA 04) [25]. The validation parameters were based on the following criteria: specificity, linearity, precision (repeatability and intermediate precision), reproducibility, limit of detection (LOD), and limit of quantification (LOQ).

2.5.1 Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the studied analyte in the presence of other possible interferences or endogenous components in the sample. In order to evaluate the selectivity of our method five blank samples of urine and serum (no analyte or IS added), obtained from alcohol abstainers, and five “zero” samples (blanks with IS) were extracted by SPE, then derivatized and analysed by GC-MS/MS.

2.5.2 Linearity/calibration curves

The linearity of an analytical method corresponds to its ability, within a given range, to obtain results directly proportional to the analyte concentration in the sample. Calibration samples were prepared by adequately spiked blank samples with appropriate volumes of standard EtG solution. Five replicate measurements at six different spiked concentrations were analysed (10, 100,

Tableau I. Relative standard deviation (RSD%) values and IS response for blank serum samples.

Assay	EtG peak area	IS peak area	Area ratio	Average ($n = 5$)	Standard deviation ($n = 5$)	RSD%
1	54	2 959	0.0182	0.01846	0.001073	17.2
2	72	3 853	0.0194			
3	53	2 870	0.0184			
4	44	2 434	0.0181			
5	60	3 283	0.0182			

1 000, 5 000, 8 000 and 10 000 ng/mL for urine and 5, 10, 50, 100, 500 and 1 000 ng/mL for serum). Furthermore, the internal standard (EtG-D5) was used to eliminate injection error while maintaining a constant area ratio for concentration quantification. Calibration curve was established using the Internal Standard method by plotting peak area ratios of EtG to IS against concentration of EtG. Six points, for both urine and serum, were calculated and fitted by a linear regression.

2.5.3 Lowest limit of detection and quantification (LOD and LOQ)

The limit of detection (LOD) of a compound analysed in a matrix is the lowest concentration of this compound that produces a detectable signal with a defined reliability, statistically different from that produced by a "blank" sample in the same conditions. It was also defined as the lowest concentration that gives a response of at least three times the average of the baseline noise ($S/N \geq 3$, as determined by the Analyst software). The limit of quantification (LOQ) was defined as the lowest amount of analyte that can be quantitatively determined with an acceptable intra-day precision (RSD% less than 20%), corresponding to a signal/noise ratio (S/N) ≥ 10, as determined by the Mass Lynx software.

2.5.4 Accuracy and precision

Precision is the degree of scatter within a set of measurements and a measurement of the random errors. It was evaluated by calculating repeatability (intra-day precision) and intermediate precision (inter-day precision). Accordingly, intra-day assay or repeatability expresses the precision under the same operating conditions over a short-time interval and inter-day assay refers to the precision between runs and from day to day. Intra and inter-day assay precisions were expressed by the relative standard deviation (RSD). The acceptance criteria according to the SFTA guidelines are RSD% less than 20% over the calibration range. The RSD% was calculated using the following equation:

RSD% = (standard deviation/average) × 100

Five replicates for each calibration level were analysed on the first day of the validation procedure. Intra-day precision was calculated by analyzing all concentrations points. Inter-assay precision was assessed for each matrix at two different concentrations (100 and 5 000 ng/mL for urine and 10 and 100 ng/mL for serum), following a typical 6-day validation procedure.

3 Results

3.1 Selectivity

For each monitored transition, no chromatographic interference peak was observed around the retention times of EtG and IS in both urine and serum-free specimens, indicating that the method provides satisfactory selectivity for EtG analysis. In addition, no signal loss or increase (ion suppression) was observed at the retention time windows of EtG or EtG-D5. The RSD% values and the IS response for blank serum samples are shown in Table I. The retention times of EtG and IS were 6.53 and 6.52 min, respectively. Representative mass chromatograms of 3 blank urine samples and a spiked sample with the IS at 50 ng/mL are shown in Figure 1.

3.2 Linearity, LOD and LOQ

Good linearity was observed for peak intensity within the specified EtG concentration range (10 to 10 000 ng/mL for urine and 5 to 1000 ng/mL for serum). The correlation coefficients of the calibration curves were systematically >0.996. A signal-to-noise ratio of 5 (LOD) at 5 ng/mL was reached for both urine and serum. The LOQ was estimated to be at least 10 ng/mL for both matrices. Figure 2 shows MRM chromatograms of serum and urine samples at 10 ng/mL of EtG with a $S/N = 10$ and 12.87, respectively, which give a LOQ = 10 ng/mL for serum and a LOQ < 10 ng/mL for urine. The concentration of 10 ng/mL was defined as the LOQ, since the relative standard deviation of 5 assays at this concentration was lower than 20% for both matrices and the calibration curve was still linear.

3.3 Precision

Good intra- and inter-day precision were observed with a RSD% lower than 20% over the calibration range. This data confirmed the stability of the calibration curve along time

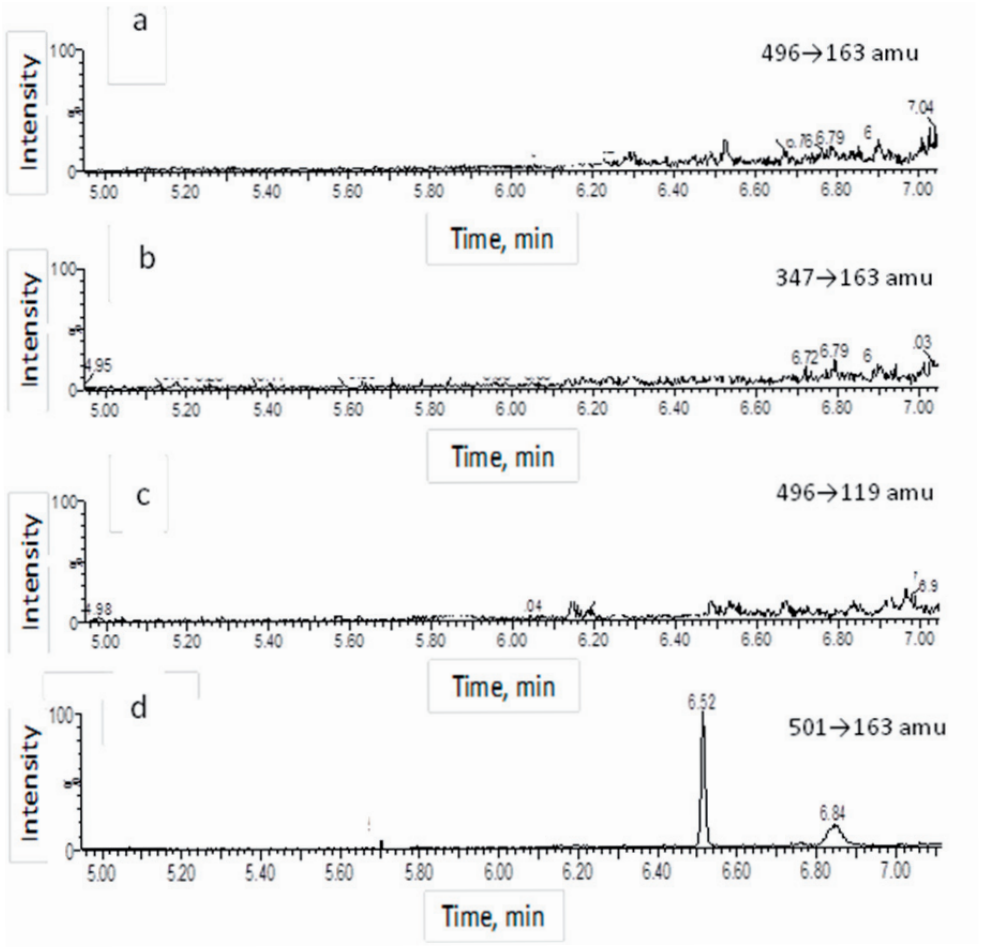

Fig. 1. MRM chromatograms of blank urine samples (a, b, c) and a spiked sample with the internal standard at 50 ng/mL (d).

Tableau II. Relative standard deviation (RSD%) values of the intra- and inter-day precision for urine and serum matrices.

	Urine						Serum					
Concentration	**C_1**	**C_2**	**C_3**	**C_4**	**C_5**	**C_6**	**C_1**	**C_2**	**C_3**	**C_4**	**C_5**	**C_6**
Average	0.03	0.117	1.139	5.931	10.137	12.637	0.078	0.128	0.675	1.357	9.364	19.695
Standard deviation	0.002	0.003	0.093	0.367	1.001	0.772	0.006	0.011	0.082	0.13	0.607	1.052
RSD%[a]	6.66	2.56	8.16	6.18	9.87	6.1	7.69	8.59	12.14	9.57	6.48	5.34

	Urine		Serum	
Concentration	$C_{2 = 100\ ng/mL}$	$C_{4 = 5\ 000\ ng/mL}$	$C_{2 = 10\ ng/mL}$	$C_{4 = 100\ ng/mL}$
Average	0.109	6.195	0.185	0.889
Standard deviation	0.006	0.596	0.016	0.04
RSD%[b]	5.5	9.62	8.64	4.49

[a]RSD% values of the intra-day precision; [b]RSD% values of the inter-day precision

(at least for 6 days, the period of the validation protocol). The RSD% values that were used to estimate intra-day and inter-day precision are shown in Table II.

3.4 Application of the method

The developed and validated method of EtG in serum was used to analyse 22 anonymous and unrelated serum samples

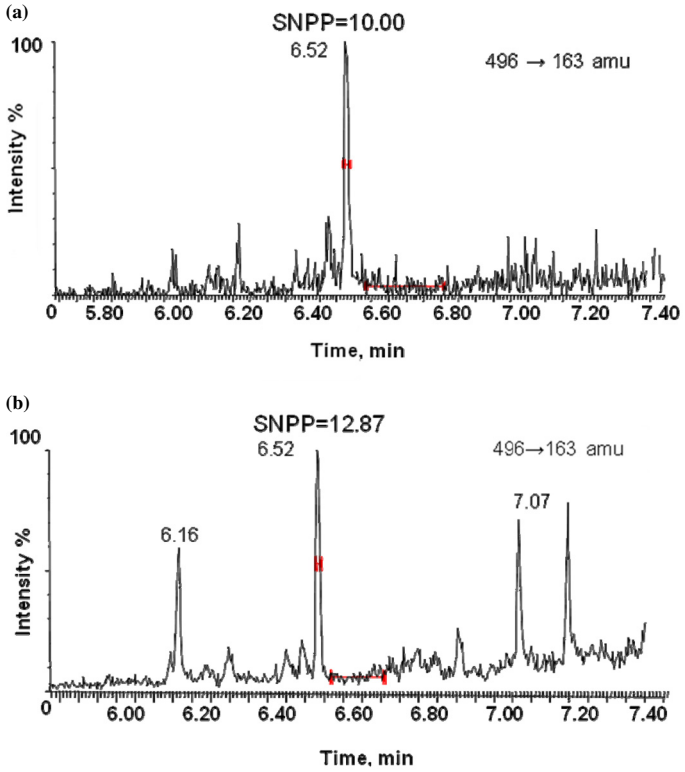

Fig. 2. MRM chromatograms of serum (a) and urine (b) samples at 10 ng/mL of EtG with a *S/N* = 10 and 12.87, respectively, which give a LOQ = 10 ng/mL for serum and a LOQ = 7.8 ng/mL for urine.

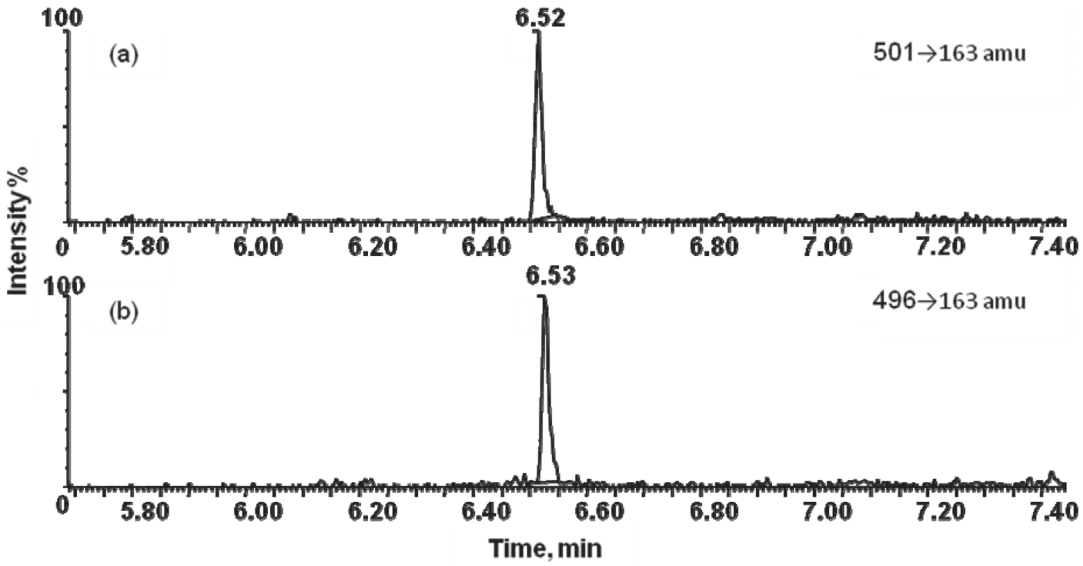

Fig. 3. MRM chromatograms of an authentic serum specimen with both transitions for (a) IS at 50 ng/mL and (b) EtG at 55.7 ng/mL.

obtained from the Toxicology laboratory of the CHRU of Lille and compared with the serum ethanol concentrations determined by headspace GC. Table III lists the results together with blood alcohol levels. As depicted, even though some samples were negative for ethanol detection, it was possible to detect EtG. Some samples had to be diluted because of high concentration values. The mean concentration value for serum EtG was 1 975 ng/mL (range 10–3941 ng/mL), with some values under the LOQ. An authentic serum specimen at 55.7 ng/mL is shown in Figure 3. This result shows the usefulness of EtG detection and dosage compared to that of ethanol to assess ethanol consumption.

Tableau III. Comparison of ethanol and EtG concentrations in 22 serum samples.

Sample number	EtG (ng/mL)	Ethanol (g/L)
1	< LOQ[a]	0
2	< LOQ	0
3	< LOQ	0
4	< LOQ	0
5	< LOQ	0
6	< LOQ	0
7	10	0
8	14.7	0
9	16	0
10	18.3	0
11	19.3	0
12	19.8	0
13	28.8	0
14	55.7	0
15	402	0.2
16	1215	0
17	1150	0.15
18	1068	1.3
19	1888	2.9
20	2478	2.5
21	3628	2.7
22	3941	1.9

[a]<LOQ means lower than 10 ng/mL

4 Discussion

EtG is a biomarker of alcohol used in clinical and medico-legal toxicology [26]. A sensitive, simple and reliable GC-MS/MS method has been developed and validated to determine EtG in human urine and serum according to the guidelines of the French Society of Analytical Toxicology (SFTA). GC-MS/MS could be a method of choice for EtG analyses as it combines the advantages of GC (high separation power) and tandem-mass spectrometry (high selectivity), minimizing or eliminating background interferences and, therefore, improving selectivity and sensibility. For the pre-analytical phase, a solid-phase extraction (SPE) was applied using Oasis® MAX cartridges. The principle of these cartridges combines anion exchange and reverse phase interactions. SPE could thus be a method of choice for EtG extraction because of its acidic properties which make it mainly retained by ionic interactions. This characteristic allows a washing step with methanol that removes interference compounds. Methanol elution of EtG in acidic conditions (formic acid/HCOOH/2%) enables fast evaporation of the solvent in comparison to other published methods where aqueous solutions have been used [27]. The calibration curve for EtG (10–10 000 ng/mL for urine; 5–1 000 ng/mL for serum) were obtained with a good linearity and with the correlation coefficient of $r > 0.996$.

To our knowledge, this is the first report of the application of a GC-MS/MS method for EtG dosage in urine and serum. Furthermore, it is noteworthy that our achieved LOQ of EtG (10 ng/mL) in the studied biological matrices appeared to be better than those reported in the literature with different analytical methods (Table IV). In the case of urine, several publications list a LOQ between 50 and 560 ng/mL [14, 18, 20, 28–31]. For serum samples, the published LOQ range between 45 and 500 ng/mL [15, 29, 32–35].

Tableau IV. Comparison of the LOD and LOQ of EtG in urine and serum between our GC-MS/MS method and other published methods.

Urine	**Our results**	Favretto (2010) [30]	Shah (2006) [20]	Janda (2001) [29]	Freire (2008) [14]	Thierauf (2009) [28]	Beyer (2011) [31]
Method	GC-MS/MS	LC-MS	LC-PED	GC-MS	GC-MS	LC-MS/MS	LC-MS/MS
LOD (ng/mL)	**5**	50	80	168	5	5	100
LOQ (ng/mL)	**10**	100	370	560	100	50	100
Serum	**Our results**	Schmitt (1997) [15]	Nishikawa (1999) [33]	Janda (2001) [29]	Morini (2007) [34]	Halter (2008) [32]	Jung (2009) [35]
Method	GC-MS/MS	GC-MS	LC-MS	GC-MS	LC-MS/MS	LC-MS/MS	CZE
LOD (ng/mL)	**5**	100	30	37	9	–	–
LOQ (ng/mL)	**10**	–	100	173	45	100	500

GC, Gas Chromatography; LC, Liquid Chromatography; PED, Pulsed Electrochemical Detection; CZE, Capillary Zone Electrophoresis

In addition to the high sensitivity of our method, the calculated precision values fulfill the requirements of validation. The method has been successfully applied to the analysis of anonymous serum samples obtained from our Toxicology Laboratory, and compared with the serum ethanol concentrations determined by headspace GC. The present GC-MS/MS procedure for EtG could be usefully applied for documentation of alcohol abstinence and for investigation of transportation accidents, monitoring of motorists with alcohol abuse histories who have abstinence as a condition for continued driving privileges.

5 Conclusion

In this study, a sensitive and reliable analytical GC-NCI-MS/MS method for the determination of EtG in urine and serum was developed and validated. The performance of this method is sufficient to measure low concentrations of EtG in both matrices, with a LOQ better than those obtained with other techniques. The method has been shown to be precise, and it has been successfully used in our laboratory. Therefore, this assay could be routinely used in the detection of EtG in various clinical and forensic contexts.

Acknowledgements

This work was supported by the Université Lille-Nord de France and the Centre Hospitalier Régional et Universitaire de Lille. We are grateful to Dr. S. W. Ellis for the english revision of the manuscript.

Conflicts of interest. The authors declare that there are no conflicts of interest.

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