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A simplified approach for determination of urinary ethyl glucuronide by gas chromatography–mass spectrometry

Shayani Ghosh, Raka Jain*, Satpal Singh, Ravindra Rao, Ashwani Kumar Mishra and Sonali Jhanjee

Abstract

Urinary ethyl glucuronide (EtG), an alcohol biomarker, plays an essential role in monitoring alcohol abstinence and relapse during treatment for alcohol dependence. Detection of this biomarker has become a routine in many clinical and forensic laboratories over the last few years. Most previously published methods commonly use hyphenated chromatographic techniques along with extensive extraction procedure before analysis. This work aimed to develop and validate an electron impact ionization mode gas chromatography–mass spectrometry method to measure ethyl glucuronide levels in human urine. For its determination, urine samples were dried under a gentle stream of nitrogen, derivatized with N,O-bis(trimethylsilyl) trifluoroacetamide, incubated, and injected into the instrument. The analysis was performed using single quadrupole gas chromatography–mass spectrometry (GC-MS) technology and validation was performed according to the guidelines of the German Society of Toxicology and Forensic Chemistry (GTFCh). The linearity of urinary EtG was obtained in the range of 30–5000 ng/ml with a correlation coefficient (*r*) above 0.999. The extraction recoveries exceeded 80%, and the obtained inter-day and intra-day precisions were below 15%. The achieved limit of detection was 10 ng/ml and limit of quantification achieved was 30 ng/ml. The electron ionization gas chromatography–mass spectrometry technique proves to be a feasible option for determining EtG in human urine when other sophisticated techniques are unapproachable. This method provides a good sensitivity and proves to be cost-effective, robust, and advantageous for both clinical as well as forensic settings.

Keywords: Ethyl glucuronide, Gas chromatography–mass spectrometry, Urine, Ethanol metabolite, Validation, Precision, Accuracy

Introduction

Ethyl glucuronide (EtG) is a non-volatile, water-soluble, and stable direct metabolite of ethanol (Wurst et al. 2003). EtG has been used as a routine biomarker for monitoring alcohol abuse and abstinence. In humans, only a small fraction (0.02–0.06%) of the total ethanol eliminated is bio transformed to EtG which can be detected in body fluids as well as hair (Crunelle et al. 2014; Washlam et al. 2012). Ethyl glucuronide was first detected in human urine in 1967 by Jaakonmaki et al (Jaakonmaki et al. 1967). Urinary EtG is a useful diagnostic parameter for monitoring abstinence as well as relapse in patients with alcohol-dependence syndrome (Ghosh et al. 2019). It can be detected in urine for up to several days after elimination of alcohol; in some cases, it was found 80 h after heavy ethanol consumption (McDonell et al. 2015). Since 1967, a good number of studies were conducted to determine EtG, and its analysis has become routine in many clinical and forensic laboratories (Bicker et al. 2006; Rohrig et al. 2006; Weinmann et al. 2004; Wurst et al. 2003;). The most commonly used technique for detecting EtG in human urine

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is liquid chromatography (LC) coupled with electrospray tandem mass spectrometry (MS) (Bicker et al. 2006; Broucki et al. 2005). Although such analytical techniques are powerful and sensitive, not all laboratories have access to them. Also, various extraction procedures such as liquid–liquid extraction, solid-phase extraction (SPE), or solid-phase microextraction (Acikkol et al. 2015; Favretto et al. 2010; Freire et al. 2008; Janda and Alt 2001) have been employed for the detection of EtG to reduce matrix interference and improve detection. However, such extraction methods are cumbersome, time-consuming, and expensive; they also have marginal extraction efficiency and require large amounts of organic solvents (Sharma et al. 2015). The lengthy extraction protocols and expensive high-resolution instruments demonstrate a need to explore an alternative and less labor-intensive approach. Gas chromatography–mass spectrometry (GC-MS) is a versatile technique that can measure a broad spectrum of primary water-soluble metabolites (Halket et al. 2005). Its strength is in measuring low-molecular-weight and volatile analytes, including small species that are typically not retained well in LC (Zimmermann et al. 2007). With simple derivatization, single quadrupole GC increases the stability of the metabolites and is cost-effective and robust (Halket et al. 2005).

The objective of this work was to develop a fast, sensitive, and reliable method to detect EtG in human urine using a standard benchtop GC-MS technology that is commonly available in laboratories. The method was validated and applied for the detection of EtG in urine of participants with alcohol dependence syndrome. The study protocol was reviewed and approved by the ethical committee of All India Institute of Medical Science, India.

Methods

Chemicals and reagents

EtG standard was obtained from Cerilliant (TX, USA), and the deuterated internal standard (IS) EtG-D_5 was purchased from TRC (Toronto, Canada). The derivatizing agent, N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA), was procured from Sigma–Aldrich (Missouri, USA). Methanol, acetone, and pyridine were obtained from Merck (Darmstadt, Germany). All chemicals were of highest analytical grade.

Preparation of stock solution and urine specimens

A stock solution of EtG (10,000 ng/ml) was made in methanol and stored at −80 °C. Standard working solutions was freshly prepared each day by dilution with methanol.

Urine sample (30 ml) was collected from six adult volunteers who had no alcohol consumption history in the last 3 months. Each control urine sample was centrifuged at 5000 rpm for 3 min and supernatant was decanted. After pooling the supernatant, a total of 100 ml urine was separated. Ten milliliters of urine sample aliquots was prepared to avoid freeze and thaw cycle and was stored in clean and dry plastic containers. No preservative was used during urine sampling. All urine samples were stored at −80°C until analysis.

Standard preparation and derivatization

After thawing, the aliquot control urine sample was centrifuged at 5000 rpm. One hundred microlitres of urine sample was taken and appropriate volumes of standard EtG solution were added to the blank urine, resulting in final concentration of 5000, 1000, 500, 250, 100, 50 and 30 ng/ml. Then 50 μl of EtG-D_5 (250 ng/ml) was added as an IS before adding methanol to a total volume of 1 ml. Prepared samples were vortexed for 2 min and evaporated to dryness for 20 min under a gentle stream of nitrogen. The dried residue was derivatized with 50 μl of BSTFA and 50 μl of pyridine. The tubes were tightly closed, vortexed (10 s), and incubated in a hot air oven at 100°C for 30 min. After cooling the solution at room temperature, 2 μl of extract was injected into the GC-MS instrument.

Gas chromatography–mass spectrometry conditions

Analysis of EtG was performed in an EI-GC-MS system comprising a gas chromatograph (7890B series, Agilent) equipped with an automatic injector and coupled with a mass spectrometer (5977B). Chromatographic separation was achieved on an Agilent DB-5 fused silica capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness). The carrier gas was helium at a constant flow rate of 1 ml/min. Samples (2 μl) were injected in splitless mode at an injection temperature of 250°C. The oven temperature was maintained at 100°C for 2 min, increased at 10°C/min to 250°C, maintained constant for 2 min, increased again at 15°C/min to 300°C, and held for 1 min. The total run time was 22 min. The mass detector was operated at 70 eV in the EI ionization mode. The ion source was maintained at 230°C. The data was acquired in the selected-ion monitoring (SIM) mode. Quantifier and qualifier ions for each analyte were selected based on their abundance and *m/z* values. The observed trimethylsilyl (TMS) derivative ions were identified for EtG at *m/z:* 160, 261(quantifier), and 405, and for EtG-D_5 at *m/z:* 165, 266 (quantifier), and 410.

Method validation

The analytical method was validated in accordance to guidelines of the German Society of Toxicology and Forensic Chemistry (GTFCh), keeping in view selectivity of the method, linearity, bias, precision, accuracy, recovery, limit of detection (LOD), and limit of quantitation (LOQ) (Peters et al. 2009).

The calibration curve was made by adding defined volumes of methanolic solution of the reference standards of EtG to control urine sample. Spiked quality-control samples were prepared in five replicates at seven concentration levels and 250 ng/mL of internal standard (EtG-D_5) was added. The concentrations of calibration standard were 5000, 1,000, 500, 250, 100, 50, and 30 ng/ml.

In this work, bias was obtained as the deviation of the average test result and the actual reference value, thereby giving a measure of the systematic errors; accuracy was obtained as the percentage deviation of each test result and the actual reference value. On the other hand, precision was acquired as the degree of scatter within a set of measurements and was a measure of the random errors. Intra-assay or repeatability expressed the precision under the same operating conditions over a short time interval. Inter-assay, on the other hand, showed the precision between runs and from day to day. The inter-batch and intra-batch coefficient variation to determine precision of the method had to be ≤ 15% (20% near the limit of quantification). Repeatability was determined in multiple measurements of the samples under the same analytical conditions.

The recovery was calculated by comparing the peak areas of the urine spiked standards with peak areas of direct neat standards in methanol. Acceptable specificity was defined as the capability of the method to detect and identify the target component (EtG) in the spiked urine samples without any interference.

The LOD was defined as the lowest concentration of the analyte in the urine matrix that can be identified and reported. It was calculated using a single quantifier ion with a signal-to-noise ratio greater than 3. The LOQ of the method was defined as the lowest concentration of the analyte in the urine matrix that can be measured, reported, and determined with an acceptable precision (RSD ≤ 20%). It was calculated using a single quantifier ion with a signal-to-noise ratio greater than 10. The LOD and LOQ were estimated according to DIN 32645 (Peters et al. 2009).

Results

Selectivity

Six blank urine samples and two zero samples (blanks with internal standard, IS) were tested to ensure no interferences. The retention time (RT) of EtG and EtG-D_5 (IS) were 16.80 and 16.75 min, respectively. As it can be seen in Fig. 1 from the chromatograms obtained for a blank urine sample and a sample spiked with 5000 ng/mg EtG, no interfering signals were observed in the analyte's and IS's retention time window. Figure 2 shows the TMS derivative ions for EtG-D_5 were at *m/z:* 165, 266 (quantifier), and 410, and those for EtG were at *m/z:* 160, 261 (quantifier), and 405.

Linearity

Five calibrators were prepared by spiking blank urine matrix with definite concentration of EtG at five different concentration levels (5000–30 ng/ml). Linearity was verified with the Mandel-F-test at the 99% significance level, after ensuring homogeneity of the variances across the calibration range and the absence of straggler and

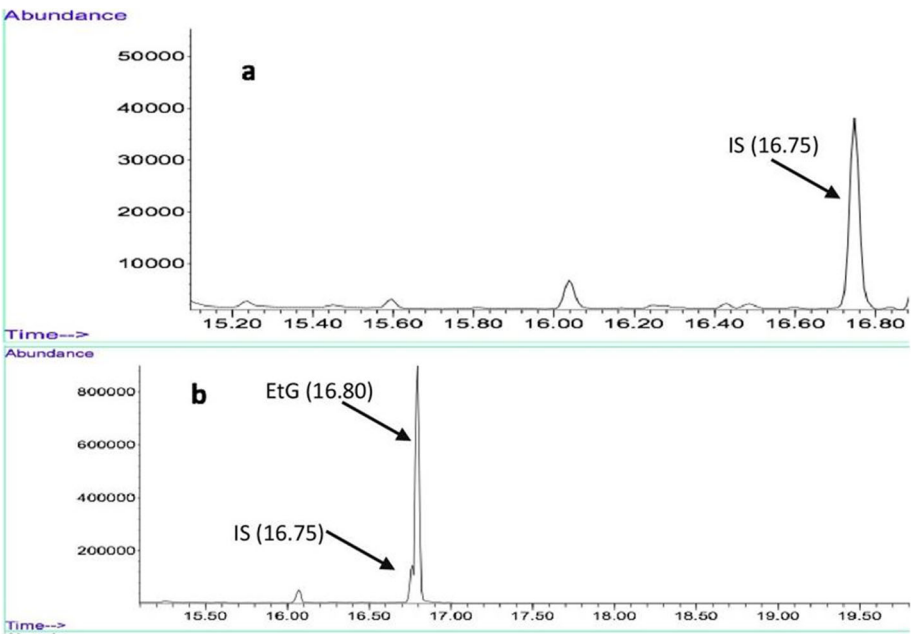

Fig. 1 **a** Chromatogram of blank urine with IS (500 ng/ml). **b** Chromatogram of urine spiked with EtG (5000 ng/ml) and IS (250ng/ml)

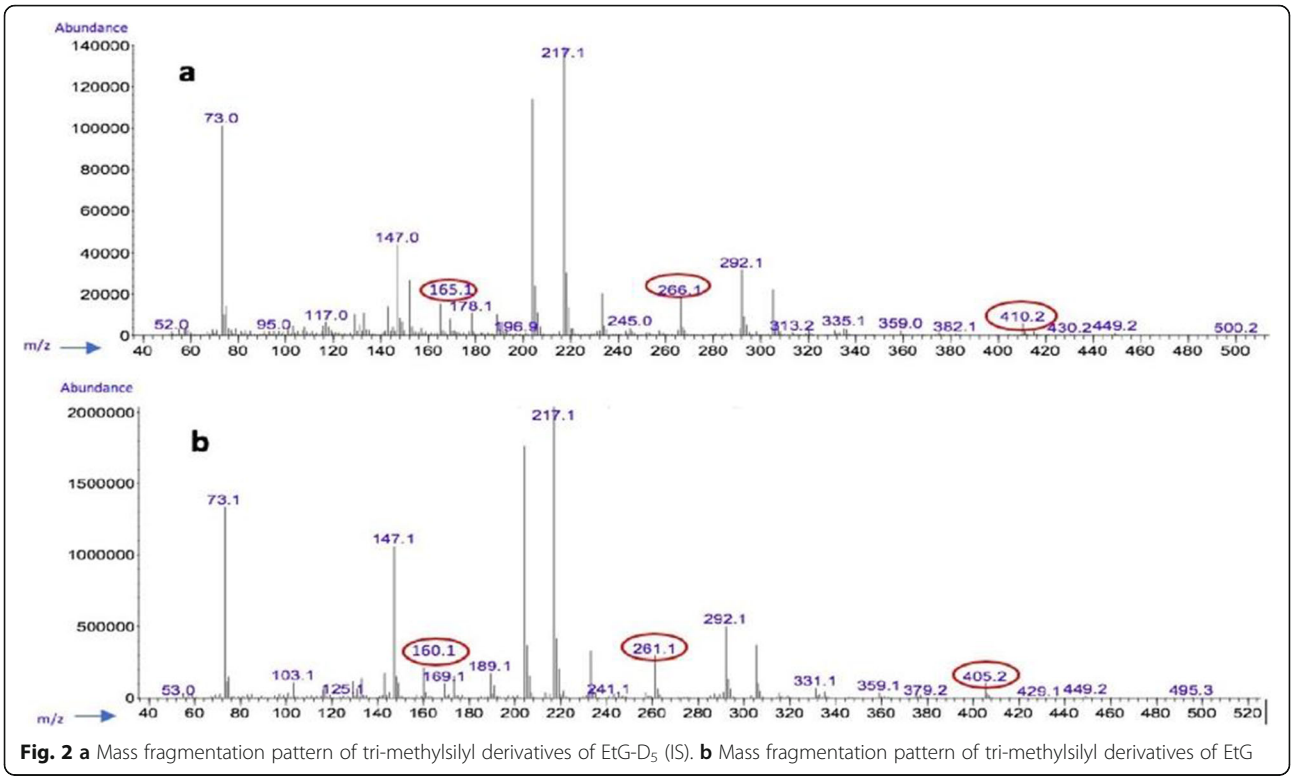

Fig. 2 **a** Mass fragmentation pattern of tri-methylsilyl derivatives of EtG-D_5 (IS). **b** Mass fragmentation pattern of tri-methylsilyl derivatives of EtG

outliers by means of the Grubbs-test at 99% significance level respectively as given in DIN 32645 (Peters et al. 2009).

A total of six replicates for all the calibrators (5000, 1,000, 500, 250, 100, 50, and 30 ng/ml) were analyzed and were found to be homogenous, free of straggler, and outliers. A good linearity was obtained with a slope of 0.96 and a correlation coefficient above 0.999 in the range from 30 to 5000 ng/ml urine as shown in Table 1.

Bias, accuracy, and precision

Bias was expressed in percentage and calculated from the average of all measurements and the accepted reference value at three different concentrations (30, 250, 5000 ng/ml) along the linearity range. The obtained values were within ± 15% (±20% near the limit of quantification) and were in accordance with the acceptance criteria of the German Society of Toxicological and Forensic Chemistry (GTFCh) as shown in Table 2 (Peters et al. 2009). Accuracy was determined by spiking blank samples with EtG (n = 6) at three different concentrations on three consecutive days, and the accuracy was expressed as the percentage of the obtained concentration divided by the expected spiked concentration. The obtained accuracy of the method at the three concentrations, 30, 250, and 5,000 ng/ml, was 91.11%, 96.60%, and 92.86%, respectively. Intra and inter-day assay precisions were expressed as the relative standard deviation (RSD) and was determined at three controlled concentrations: 30, 250, and 5000 ng/ml. The intra-day precision was determined by assaying six replicates of the urine matrix at three concentrations on the same day. Similarly, the inter-day precision was calculated for

Table 1 Response function for EtG in urine at 99% confidence intervals

Concentration range (ng/ml)	30–5000
Slope	0.96± 0.01
Intercept	0.5± 0.1
R^2	0.9998
F-Test for outlier's (99%)	Passed
Mandel-Test for linearity (99%)	Passed

Table 2 Validation data for bias, precision, and accuracy

Concentrations (ng/ml)		30	250	5000
Intra-day Precision	Average	26.8	244.7	4790
	SD	1.2	3.9	190.4
	RSD (%)	4.4	1.6	4
Inter-day Precision	Average	27.3	241.5	4643
	SD	1.5	4.9	316.4
	RSD (%)	5.5	2.0	6.8
Accuracy (%)		91	97	93
Bias (%)		13	2	10

Fig. 3 Reconstructed ion chromatogram of 10 ng/ml (LOD)

six replicates at three different concentrations for three consecutive days. The obtained values for both intra-day and inter-day precision are shown in Table 2.

Recovery

The recovery was determined by comparing the peak areas of the extracted standards with those of neat standards (n = 6) at three different concentrations: 30, 250, and 5,000 ng/ml. The percentage EtG recoveries at these concentrations were 85 ± 1.8, 83 ± 2.3, and 80 ± 3.5, respectively.

Limit of detection and limit of quantification

For LOD, three replicates of urine matrix spiked with lowest concentration (10 ng/ml) of EtG were examined for three consecutive days. A signal-to-noise ratio of 3.9 was obtained at 10 ng/ml and the reconstructed ion chromatogram (RIC) of the same is shown in Fig. 3. Simultaneously, for LOQ, three replicates of urine matrix spiked with the expected concentration (30ng/ml) of EtG were examined for three consecutive days. A signal-to-noise ratio of 10.6 was obtained at 30 ng/ml and the RIC of the same is shown in Fig. 4.

Application of the method

The applicability of the developed method was examined on urine specimen of ten patients with alcohol dependence syndrome seeking treatment from a tertiary de-addiction center in northern India. The recruited patients were diagnosed with alcohol dependence syndrome as per the International Classification of Diseases, Version-10 with alcohol consumption within the last 24 h. For every urine sample obtained, a breath alcohol measurement was performed in parallel and the time since the last alcohol consumed was recorded. Breath ethanol measurements were performed using AlcoMate

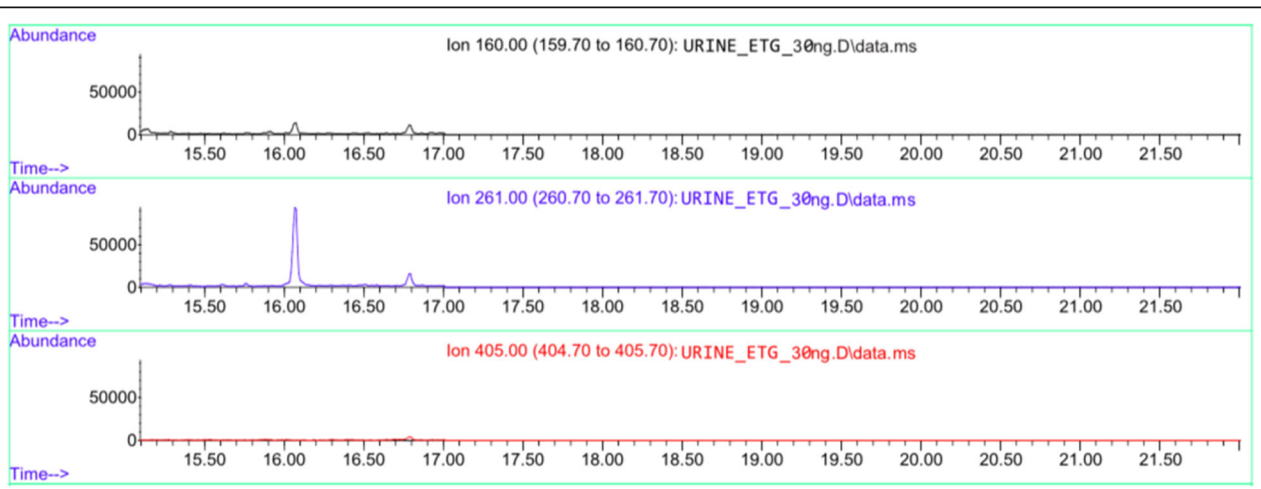

Fig. 4 Reconstructed ion chromatogram of 30 ng/ml (LOQ)

Table 3 Analyzed urine samples and their breath test values

Sample number	Hours since last alcohol consumed	BAC (%)	Urine EtG (ng/ml)
A1	07	0.40	7061.59
A2	06	0.35	59,432.56
A3	06	0.40	83,329.86
A4	06	0.40	6322.72
A5	08	0.40	547.67
A6	09	0.40	44.88
A7	12	0.10	8764.54
A8	09	0.40	78,943.44
A9	06	0.23	79,177.76
A10	04	0.40	56,990.33

Premium (Model AL7000) alcohol tester, and the results were expressed as the corresponding blood alcohol concentration (BAC; range 0.000–0.400%). All samples were analyzed as real routine samples and the results are shown in Table 3. Figure 5 (83,329 ng/ml) and Fig. 6 (45ng/ml) shows the chromatogram of two patient's urine sample showing presence of EtG.

Discussion

Ethyl glucuronide has proven to be a reliable biomarker for monitoring alcohol consumption and relapse (Washlam et al. 2014). It has been successfully used in both clinical and medicolegal toxicology (Santunione et al. 2018). Although several studies have been published on determining EtG levels in urine, most of them employed highly sophisticated and expensive techniques like LC-MS or LC-MS-MS (Helander et al. 2010). Even with such hyphenated techniques becoming popular, some laboratories still have access only to EI-GC-MS instrumentation. This study discusses a fast and reliable GC-MS method with optimized chromatographic separation for the quantitation of EtG in urine using a DB-5

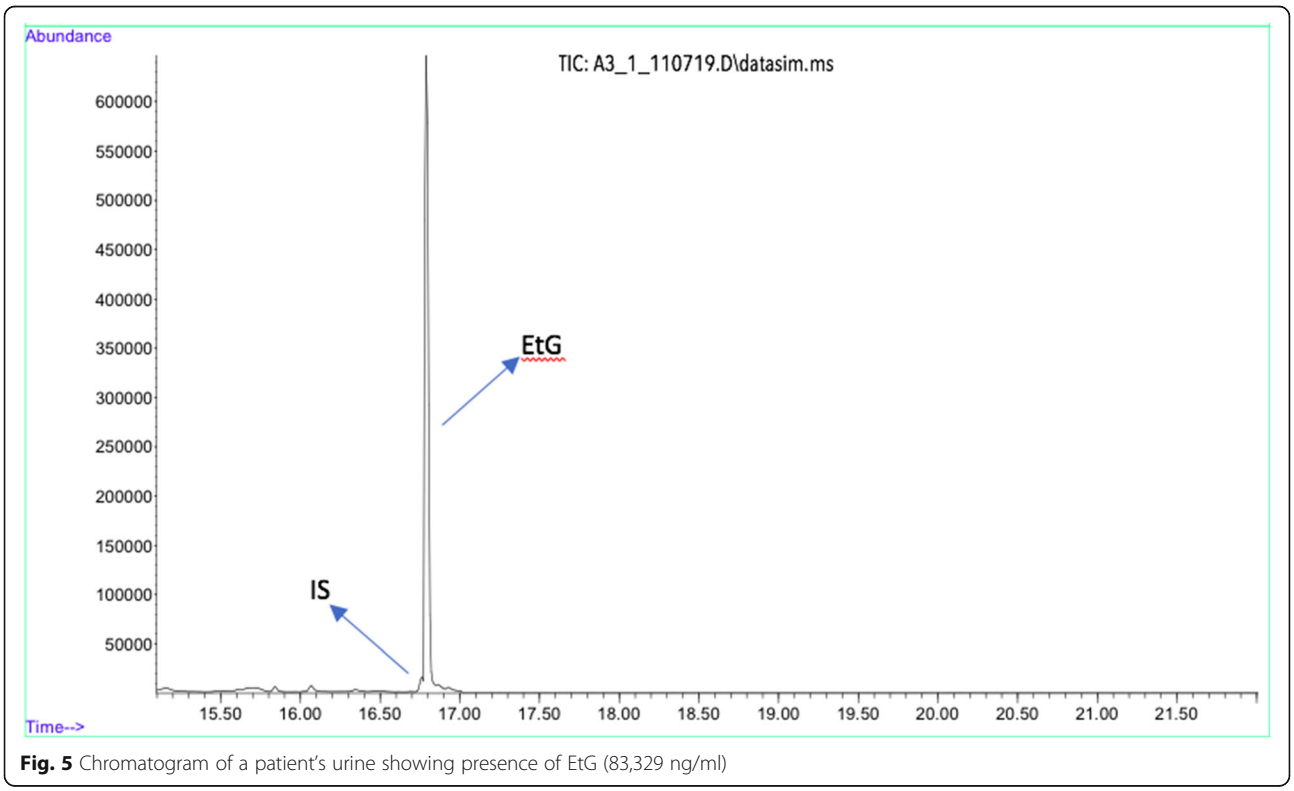

Fig. 5 Chromatogram of a patient's urine showing presence of EtG (83,329 ng/ml)

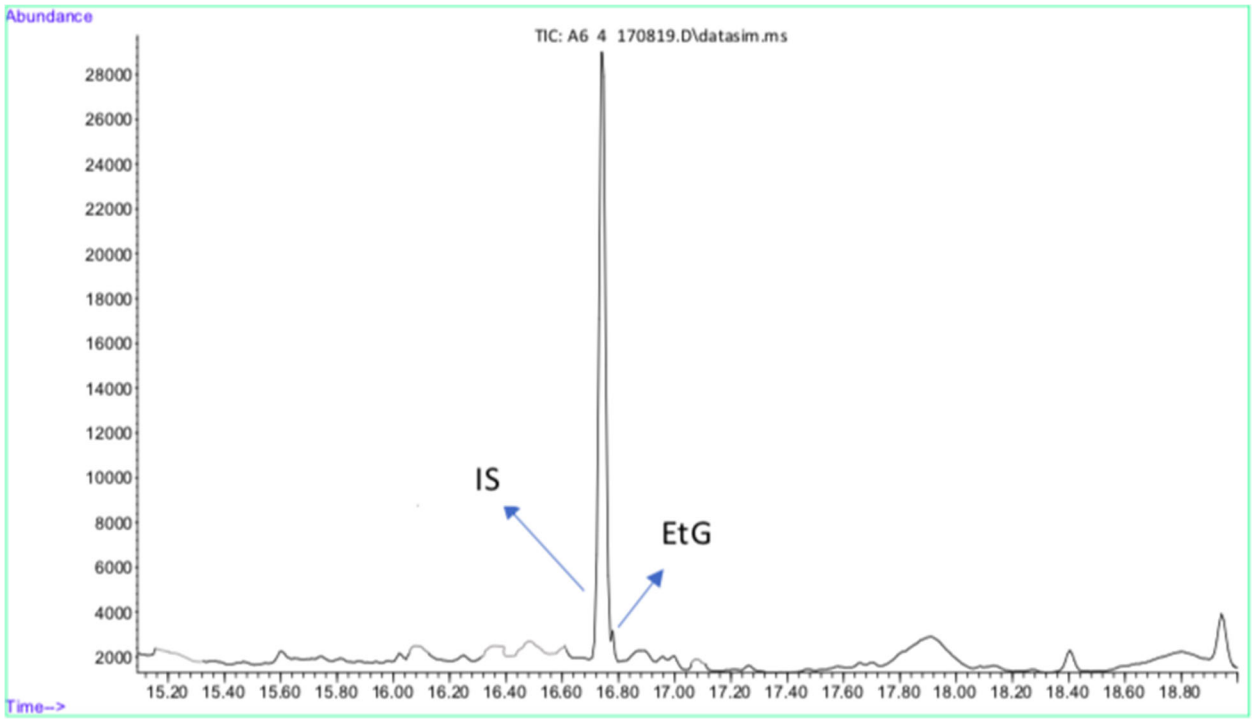

Fig. 6 Chromatogram of a patient's urine showing presence of EtG (45 ng/ml)

fused silica capillary column. The calibration curve ranged between 30 and 5000 ng/ml. Additionally, analysis of metabolites in biological matrices often involves certain complex extraction methods (Zheng et al. 2008) However, the present method did not employ any sample pre-treatment technique and obtained a good LOQ (30 ng/ml) and LOD (10 ng/ml). The LOQ achieved by this method indicated a higher sensitivity than in other studies, where conventional extraction techniques were used with sensitive analytical instruments; comparisons are briefly shown in Table 4. Bias, precision, and accuracy obtained fulfill the validation requirements. This method has been successfully used in our laboratory to analyze EtG in the urine of patients with alcohol dependence syndrome. The developed procedure will be advantageous in analyzing EtG in de-addiction or medico-legal cases for the detection and monitoring of alcohol consumption.

Conclusion

A validated simple and reproducible GC-MS method which fulfills the requirements for confirmatory and quantitative analysis of EtG in urine has been discussed in this study. The step by step method validation has been performed according to guidelines of the GTFCh and general rules for bioanalytical method validation (Peters et al. 2009). The method was observed to be advantageous and sensitive as compared

Table 4 A comparison of the LOD, LOQ, extraction techniques, and instruments used in our study and other published methods

Urine matrix	Our results	Freire et al. 2008	Janda and Alt 2001	Sharma et al. 2015	Favretto et al. 2010	Shah and Lacourse 2006	Beyer et al. 2011	Broucki et al. 2005
Extraction technique	None	Microwave-assisted extraction	Solid-phase extraction	Solid-phase extraction	Solid-phase extraction	Solid-phase extraction	None	Cation-exchange resin
Method	GC-MS	GC-MS	GC-MS	GC-MS	LC-MS	LC with pulsed electrochemical detection	LC-MS/MS	LC-MS/MS
LOD (ng/ml)	10	5	168	50	5	80	100	Unknown
LOQ (ng/ml)	30	100	560	150	100	370	100	100

to other discussed methods for analyses of EtG in urine. One of the advantages of the developed method is the achieved LOQ, which yet has not been achieved by any other hyphenated chromatographic technique. Furthermore, it is cost-effective and requires no cumbersome extraction protocols. It is a viable option for analyzing EtG in laboratories that have access to standard benchtop EI-GC-MS technology.

Abbreviations

BAC: Blood alcohol concentration; BSTFA: N, O-Bis(trimethylsilyl) Trifluoroacetamide; EI-GC-MS: Electron ionization-gas chromatogrpahy–mass spectrometry; EtG: Ethyl glucuronide; GTFCh: German Society of Toxicology and Forensic Chemistry; IS: Internal standard; LOD: Limit of detection; LOQ: Limit of quantitation; LC: Liquid chromatography; LC-MS-MS: Liquid chromatography tandem mass spectrometry; RIC: Reconstructed ion chromatogram; RSD: Relative standard deviation; RT: Retention time; SIM: Single ion monitoring; SPE: Solid phase extraction; TMS: Trimethylsilyl derivative ions

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Authors' contributions

RJ and SG contributed to the design of the study. SG performed the experiments. SS assisted in analyzing the data. RJ, RR, SJ, and AKM gave their valuable suggestions during the course of validation. SG wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable

Declarations

Ethics approval and consent to participate

This study was approved by the institute ethics committee. Informed consent has been obtained from the participating individuals.

Consent for publication

The authors have no objection regarding publication of the article

Competing interests

The authors declare that they have no competing interests.

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