Gas chromatographic determination of ethyl glucuronide in hair : Comparison between tandem mass spectrometry and single quadrupole mass spectrometry

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Cappelle D[1], Neels H[2], Yegles M[3], Paulus J[3], van Nuijs AL[4], Covaci A[4], Crunelle CL[4]

[1]Toxicological University of Antwerp, Universiteitsplein 1, B2610 Antwerp, Belgium. Electronic address: delphine.cappelle@uantwerpen.be.

[2]Toxicological University of Antwerp, Universiteitsplein 1, B2610 Antwerp, Belgium; Toxicology Laboratory, ZNA Stuivenberg Hospital, Lange Beeldekenstraat 267, B2060 Antwerp, Belgium.

[3]Laboratoire National de Santé, Service de Toxicologie, 1, Louis Rech, L3555 Dudelange, Luxembourg.

[4]Toxicological University of Antwerp, Universiteitsplein 1, B2610 Antwerp, Belgium.

Abstract

Ethyl glucuronide (EtG), a minor metabolite of ethanol, accumulates in hair and is currently used as a long-term marker for the detection of chronic and excessive alcohol consumption. Sensitive methods are required to differentiate teetotalers from moderate drinkers according to the established cut-off (i.e., 7 pg/mg hair). The aim of this study was to develop a sensitive method using gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) operated in the negative ion chemical ionization (NICI) mode. The validated method was applied to hair samples from teetotalers, moderate and excessive alcohol consumers, and results were compared to a previously validated GC-NICI-MS method. The developed GC-NICI-MS/MS method showed linearity over a range from 2 to 400 pg/mg hair, with a limit of detection (LOD) of 0.05 pg/mg hair and a lower limit of quantification (LLOQ) of 0.2 pg/mg hair, compared to an LOD of 0.5 pg/mg hair and LLOQ of 1.5 pg/mg hair obtained with GC-

NICI-MS. Furthermore, lower background noise was observed using GC-NICI-MS/MS. Comparison of results of hair samples (n = 58) obtained by GC-NICI-MS and GC-NICI-MS/MS showed no significant difference between both methods (paired-sample t-test, $p > 0.05$; mean CV = 1.0%). The differences between both methods were larger for EtG concentrations < 30 mg/pg hair (mean CV = 1.7%) than for EtG concentrations > 30 mg/pg hair (mean CV = 0.7%). This suggests a higher selectivity of GC-NICI-MS/MS at lower concentrations. In conclusion, by using GC-NICI-MS/MS, a higher analytical selectivity and an improved signal to noise ratio, can be achieved. Although GC-NICI-MS would not change the interpretation of the EtG concentrations, the present GC-NICI-MS/MS method should preferentially be used for the determination of EtG in hair, especially when differentiating between teetotalers and moderate drinkers according to the current cut-off (i.e., 7 pg/mg hair).

Keywords

Ethyl glucuronide; Alcohol; Hair; GC-MS/MS; Negative ion chemical ionization; Validation.

Introduction

The abuse of alcohol is a worldwide problem associated with negative effects on health and society [1]. The detection of alcohol through direct and indirect alcohol markers can be seen as a useful tool to detect harmful and/or chronic alcohol consumption. Clinical and forensic applications for these alcohol markers are widespread: from the detection of alcohol dependence (e.g. in treatment settings and in forensic cases) to the monitoring of alcohol abstinence, (e.g. during pregnancy, in child custody cases and in liver transplant procedures due to alcoholic liver failure).

Ethyl glucuronide (EtG) is a minor metabolite of ethanol that accumulates in hair and has proved to be a specific and sensitive long-term biomarker for the detection of chronic and excessive alcohol consumption [2]. The metabolization of ethanol to EtG represents approximately 0.05% of the total alcohol elimination [3]. Consequently, only small amounts of EtG accumulate in hair, providing EtG concentrations in the lower picogram range: > 30 pg/mg hair in alcohol-dependent individuals [2, 4], between 7 and 30 pg/mg hair for moderate alcohol consumers, and < 7 pg/mg hair for teetotalers [5]. Sensitive analytical methods are thus required for the reliable determination of such low EtG concentrations. The current methods offer lower limits of quantification (LLOQs) varying between 2 and 5000 pg/mg depending on the method applied, with LLOQs generally higher (> 10 pg/mg hair) for liquid chromatography (LC) methods compared to gas chromatography (GC) methods (< 10 pg/mg hair; for a review see Crunelle et al., 2014). The use of negative ion chemical ionization (NICI) instead of electron impact (EI) improves the analytical sensitivity of GC methods, with LLOQs of 5000 [6] and 300 [7] pg/mg hair for EI compared to LLOQs of 6 [8] and 2.3 [9] pg/mg hair for NICI. Tandem mass spectrometry (MS/MS) minimizes background interferences and allows multiple reaction-monitoring (MRM) and should thus result in higher sensitivity and selectivity. Indeed, GC-MS/MS methods operated in EI mode showed an improved sensitivity [10, 11], but this increase could not be observed between GC-NICI-MS and GC-NICI-MS/MS methods [12, 13].

In this article, we validated a GC-NICI-MS/MS method for the determination of EtG in hair after heptafluorobutyric anhydride (HFBA) derivatization. By analyzing hair samples from teetotalers, moderate, and excessive alcohol consumers, the applicability of the method should be demonstrated. In addition, the GC-NICI-MS/MS method was compared to a GC-NICI-MS method, in order to assess the possible advantages of MS/MS and MRM in the determination of EtG in hair from teetotalers, moderate and excessive alcohol consumers.

Materials and methods

Samples

Hair samples together with self-reported data about alcohol consumption were obtained from 58 volunteers: teetotalers (no alcohol consumption; n = 2), moderate alcohol consumers (< 60 g alcohol/day; n = 20) and excessive alcohol consumers (> 60 g alcohol/day; n = 36). Samples were collected from the vertex posterior of the head, and the first 3 cm segment from the proximal end was used for further analysis.

Chemicals

Ethyl glucuronide (EtG) and the internal standard ethylglucuronide-D_5 (EtG-D_5) in methanol were purchased from Medichem (Stuttgart, Germany). Heptafluorobutyric anhydride (HFBA) was obtained from Sigma Aldrich (Bornem, Belgium). Methanol, ammonium hydroxide solution (25%), ethyl acetate, formic acid (98% - 100%), and acetone were supplied by Biosolve (Valkenswaard, the Netherlands). All chemical and reagents were of analytical purity grade.

Standard solutions

Stock solutions of EtG (1 mg/mL) and EtG-D_5 (0.2 mg/mL) were prepared in methanol. The working solutions were prepared in methanol by further dilution of the stock solutions. All solutions were stored at -20 °C.

Instrumentation

Oasis® MAX (60 mg, 3 mL) extraction cartridges were acquired from Waters (New Bedford, MA, USA). A ball mill of type MM2 (Retsch, Haan, Germany) was used for the pulverization of the hair samples. Extraction was performed with an ELMA TI-H-15 ultrasonication bath

(Elma Hans Schmidbauer GmbH & Co. KG, Singen, Germany) and centrifugation with a Sigma centrifuge (Osterode am Harz, Germany). A Supelco Visiprep^{TM} SPE Vacuum Manifold (Bellefonte, CA, USA) with 24 ports was employed to load the hair samples and to dry the cartridges. Solvent evaporation was achieved with a Pierce Reacti-Therm III Heating Module (Rockford, IL, USA).

Both GC-NICI-MS and GC-NICI-MS/MS analyses were carried out on a GC-MS/MS system consisting of a 7890A gas chromatograph, equipped with an automatic injector AS 7693 and coupled to a 7000C triple quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was achieved on a HP-5 MS (5% phenyl methyl siloxane) column (length 30 m × internal diameter 0.25 mm × film thickness 0.25 μm).

Sample preparation

Hair samples were processed according to a previously described method [14]. Briefly, the samples were washed with water and acetone, dried, cut into 1 - 2 mm pieces, and pulverized in a ball mill. Then, 2 mL of water was added to approximately 30 mg of the pulverized samples, and the samples were ultrasonicated for 1.5 h. Next, the samples were spiked with 2 ng of EtG-D_5 as internal standard, vortexed, and centrifuged at 5000 rpm for 10 min. Solid-phase extraction was then performed by transferring the supernatant to the cartridges, previously conditioned with 2 mL of methanol and 2 mL of water. The columns were washed with 1 mL of water/ammonia (5%) solution and 2 mL of methanol, and subsequently dried for 5 min. Elution was performed using 2 mL of 2% formic acid in methanol and the eluate was evaporated to dryness under a nitrogen gas stream at 37 °C. The residues were derivatized with HFBA (30 min, 60 °C), dried under a nitrogen gas stream, and then reconstituted in 50

μL of ethyl acetate. Finally, the extracts were transferred to vials and 1 μl was injected twice into the GC-MS/MS system (for GC-NICI-MS analysis and for GC-NICI-MS/MS analysis).

GC-MS/MS conditions

The injector temperature was set at 250 °C. The carrier gas was helium with a flow rate of 1 mL/min. The oven was initially held at 100 °C for 2 min, heated to 170 °C at a rate of 10 °C/min and then programmed at a final temperature of 300 °C at 40 °C/min. The detector was operated in the negative ion chemical ionization (NICI) mode and the detector temperature was 280 °C. The GC-NICI-MS analyses were done as published elsewhere [14]. For the GC-NICI-MS/MS analyses data acquisition was performed in the multiple reaction-monitoring (MRM) mode. The monitored ion transitions were m/z 596 → 213 (quantifier) and 397 → 213 (qualifier) for EtG, and m/z 601 → 213 for EtG-D_5.

Validation

Validation of the analytical method was based on the international guidelines of the European Medicine Agency (EMA) and the Food and Drug Administration (FDA) [15, 16]. The following quality criteria were taken into account: recovery, linearity, accuracy, precision and sensitivity limits (LLOQ, LOD).

The extraction recovery was calculated as the percentage response in extracted samples relative to samples where the standard was added after extraction. A calibration curve, consisting of blank samples spiked at 7 different concentrations (2, 4, 10, 50, 100, 200, 400 pg/mg), was constructed to evaluate linearity. Accuracy and precision were assessed for replicated quality control (QC) samples at a concentration of 25 pg/mg. Intra-day precision and accuracy were assessed by analyzing 10 QC samples in the same analytical run. Inter-day precision and accuracy were calculated on QC samples analyzed in 6 different runs, on

149 separate days. Acceptance criteria were 1) a bias less than 15% for accuracy, and 2) a
150 coefficient of variation (CV) lower than 15% for precision [15, 16].
151 The LLOQ was defined as the lowest concentration producing peaks with a signal to
152 noise ratio of at least 10, and with an accuracy and precision within ± 20% of the reference
153 concentration. The LOD was calculated as the lowest concentration producing peaks with a
154 signal to noise ratio of at least 3 [15, 16].
155
156 ***Data analysis***
157 Statistical analysis was performed using IBM® SPSS® Statistics version 22. The normality of
158 the data was evaluated, followed by a parametric paired-sample t-test on the normally
159 distributed data to assess the differences in concentrations obtained with GC-NICI-MS and
160 GC-NICI-MS/MS. Regression analysis was performed to evaluate the correlation between
161 both methods. A p-value < 0.05 was considered as statistically significant.
162 **Results and discussion**
163 ***Method validation***
164 Mean overall extraction recovery was 98.8% (n = 3). The calibration curve (7-point model)
165 was linear in the range from 2 to 400 pg/mg hair. As presented in Table 1, intra- and inter-day
166 accuracy and precision for replicated QC samples at 25 pg/mg were within the acceptance
167 criteria of 15%. Additional quality control was achieved through successful participation in
168 the EtG proficiency testing in hair organized by the Society of Hair Testing.
169 The LOD and LLOQ obtained for the GC-NICI-MS/MS method were 0.05 pg/mg and
170 0.2 pg/mg hair, respectively. Thus, when compared to GC-NICI-MS (LOD = 0.5 pg/mg;
171 LLOQ = 1.5 pg/mg), the use GC-NICI-MS/MS enables the achievement of a lower LOD and
172 LLOQ [14]. Furthermore, the LOD and LLOQ obtained with the presented GC-NICI-MS/MS
173 method are lower than those reported by previously published GC-NICI-MS/MS methods

with LLOQs of 8.4 pg/mg [13] and 2.8 pg/mg [12]. As a consequence, and in contrast to previous publications [12, 13], the current method demonstrates the higher sensitivity of MS/MS.

Application to real hair samples

Hair samples, collected from teetotalers (n = 2), moderate (n = 20) and excessive (n = 36) alcohol consumers, were analyzed with the developed GC-NICI-MS/MS method and the results were compared with those obtained using the GC-NICI-MS method published by Kerekes et al. [14] (see Table 2).

Hair EtG concentrations obtained by GC-NICI-MS and GC-NICI-MS/MS did not differ significantly from each other (paired-sample t-test, $p > 0.05$). Calculation of the coefficient of variation (CV) of the obtained EtG concentrations for each sample showed that the results were similar for both methods (mean CV = 1.0%). The concentrations of EtG obtained by GC-NICI-MS/MS were highly correlated to those obtained by GC-NICI-MS ($R^2 = 0.999$, $p < 0.01$, see Figure 1). Thus, using one method instead of the other would not change the interpretation of the EtG concentrations regarding the differentiation between teetotalers, moderate and excessive alcohol consumers.

The differences between both methods were larger for EtG concentrations < 30 pg/mg hair (mean CV = 1.7%; see Figure 2a) than for EtG concentrations > 30 pg/mg hair (mean CV = 0.7%; see Figure 2b). In contrast to GC-NICI-MS, using GC-NICI-MS/MS no decrease of sensitivity was observed at lower concentrations. These findings suggest a better selectivity of GC-NICI-MS/MS at lower concentrations.

Furthermore, a low background noise was observed using GC-NICI-MS/MS through the use of specific MRM transitions (see Figure 3).

This higher selectivity of GC-NICI-MS/MS at lower concentrations in combination with its high sensitivity, underlines the usefulness of GC-NICI-MS/MS instead of GC-NICI-MS for the differentiation between teetotalers and moderate alcohol consumers.

Conclusions

A GC-NICI-MS/MS method for the determination of EtG in hair was validated, applied to hair samples, and compared to a previously published GC-NICI-MS method. Using the presented GC-NICI-MS/MS method, a higher analytical selectivity and an improved signal to noise ratio were achieved, compared to earlier published methods. Therefore, this GC-NICI-MS/MS method provides sensitive and reliable results, and should preferentially be used for the determination of EtG in hair, especially when differentiating between teetotalers and moderate alcohol consumers according to the current cut-off (i.e., 7 pg/mg hair).

Captions to figures

Table 1:

Accuracy and precision measured at a concentration of 25 pg/mg.

Table 2:

Hair EtG concentrations for different drinking behaviors obtained with GC-NICI-MS and GC-NICI-MS/MS.

Figure 1:

Comparison of hair EtG concentrations of teetotalers, moderate and excessive alcohol consumers obtained by GC-NICI-MS and GC-NICI-MS/MS.

Figure 2:

Comparison of hair EtG concentrations obtained by GC-NICI-MS and GC-NICI-MS/MS. (a) Hair EtG concentrations of teetotalers and moderate alcohol consumers (concentrations < 30 pg/mg hair). (b) Hair EtG concentrations of excessive alcohol consumers (concentrations > 30 pg/mg hair).

Figure 3:

Chromatogram obtained by GC-NICI-MS/MS at an EtG concentration of 4 pg/mg hair (m/z 596 → 213 (quantifier); 397 → 213 (qualifier)) and an EtG-D5 concentration of 20 pg/mg hair (m/z 601 → 213).

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