FORENSICS AND TOXICOLOGY ANALYSIS

ETHYL GLUCURONIDE DETERMINATION IN HAIR AS AN INDICATOR OF CHRONIC ALCOHOL ABUSE: A FULLY VALIDATED METHOD BY GC-EI-MS/MS

Solutions for Your Analytical Business
Markets and Applications Programs

Agilent Technologies
Authorized Partner Laboratory

Market
Forensics and Toxicology

Author(s)
Jennifer P. Pascali[1], Franco Tagliaro[2]

[1] DTOLabs, Analytical Excellence Centre, authorized Agilent Technologies Partner Lab, Resana (TV), Italy.

[2] Department of Public Health and Community Medicine, Unit of Forensic Medicine, University of Verona, Italy.

Abstract

Hair analysis is a powerful tool for the detection of chronic or past drug ingestion and in recent years it has also been exploited for chronic alcohol abuse. Ethyl glucuronide (EtG) determination in hair has been increasingly employed for diagnosing chronic excessive drinking in both clinical and forensic areas.

This work describes a simple fully validated method for the determination of EtG in hair using an Agilent 7890B gas chromatograph coupled to an Agilent 7000C triple quadrupole mass spectrometer detector with an electron impact ion source (GC-EI-MS/MS). N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was used as the derivatizing agent and dimethylformamide (DMF) as the solvent enhancer. Sample decontamination and treatment were performed according to Hastedt et al. (2012). Linearity of the assay was tested between 2.5 – 100 pg/mg. The sensitivity of the method, expressed as limit of quantitation (LOQ), was 2.5 pg/mg. The method has been applied to a number of real samples, with analytical results in the range of < LOQ to 39 pg/mg.

Introduction

Alcohol is the most widely consumed psychoactive substance and is becoming a problematic addiction issue in millions of people worldwide. In fact, unhealthy alcohol use can be either a primary or a secondary cause of liver disease and besides medical complications its abuse can be responsible for severe social problems. From a forensic point of view, it is of extreme importance to monitor alcohol abstinence in patients undergoing alcohol withdrawal treatment when a legal case is proceeding. Laboratory testing traditionally based diagnosis on the assessment of biomarkers such as mean corpuscular volume (MCV), carbohydrate deficient transferrin (CDT) and liver enzymes (gamma-glutamyl tranferase, aspartate aminotransferase and alanine aminotransferase). In the last few years, increased attention has been paid to ethyl glucuronide (EtG), a new specific alcohol intake marker. It is a direct metabolite in the non-oxidative breakdown of ethanol, accounting for less than 0.1 % of total ethanol elimination. In literature, several methods have been published for the determination of EtG in hair for the detection of chronic or past alcohol ingestion, exploiting both liquid chromatography and gas chromatography coupled to mass detection. Due to its high polarity, EtG is incorporated into hair only in very small amounts. Accordingly to the new guidelines of the Society of Hair Testing, EtG cut off levels in hair are recommended at 30 pg/mg to distinguish between moderate and heavy alcohol consumption, whereas a 7 pg/mg cut off level is sufficient to pass the medical assessment in the context of driving ability when alcohol abstinence is required [1,2,3,7]. In view of this, among the published methods, necessary sensitivity has only been achieved when triple quadrupole systems have been used or when complex matrix pre-treatments have been employed. Furthermore, some of them lack the requirements for method validation, necessary for endorsement in the field of forensic science. This paper describes a simple fully validated method for the determination of EtG in hair using a gas chromatograph coupled to a triple quadrupole mass spectrometer detector with an electron impact ion source (GC-EI-MS/MS). N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was used as the derivatizing agent and dimethylformamide (DMF) as the solvent enhancer. Sample decontamination and treatment were performed according to Hastedt et al.[4]. The sensitivity achieved by this method, expressed as Limit of Quantitation (LOQ), was 2.5 pg/mg. This is comparable to LC-MS/MS published procedures, allowing this method to also be applied to social drinking monitoring.

Experimental

Materials

EtG and the internal standard EtG-D5 were purchased from Medichem (Stuttgard, Gemany). Methanol, dichloromethane and DMF were from Carlo Erba, Milan, Italy. MSTFA was purchased from Sigma-Aldrich (Milan, Italy).

Instrumentation

GC	7890B
MS	7000C
Autosampler	120 CTC Pal System

Chromatographic Conditions

Column: HP-5 column (12 m x 0.200 mm x 0.33 µm)	
Injection Volume	1 µL, Splitless
Injection Port Temperature	250 °C
Source Temperature	230 °C
Initial Column Temperature	100 °C for 1 minute
Rate 1	30 °C/min to 200 °C
Rate 2	15 °C/min to 290 °C for 5 minutes
Aux Temperature	290 °C

MS/MS MRM Transitions

EtG	261→143 @ 10; 261→73 @ 20
EtG-D5	266→143 @ 20

Sample treatment

Hair samples were divided into 1-3 cm segments depending on the total length of the hair. The hair samples were then washed twice with 5 mL of dichloromethane for 10 minutes and twice with 5 mL of methanol for 10 minutes. The hair samples were then dried at room temperature and cut into small pieces (2-5 mm). Around 45-55 mg of each hair sample was weighted and transferred into a glass tube. 500 µL of deionized water and EtG-D5 (to a final concentration of 10 pg/mg) were added before centrifugation (3000 rpm for 10 minutes). Samples were incubated overnight at 60 °C and ultrasonicated for 10 minutes. The supernatant was taken to dryness under a gentle stream of air at 60 °C for 80 minutes. Samples were reconstituted in 40 µL MSTFA and 10 µL DMF, and incubated for 40 minutes at 60 °C. One microliter was injected onto the GC-EI-MS/MS.

Software

MassHunter acquisition, qualitative, quantitative

Results and Discussion

Validation of the method

The validation was carried out according to the international forensic guidelines [5]. The method was validated for selectivity, linearity and sensitivity, precision and accuracy. All calculations were performed using the MassHunter software.

Selectivity

Seven different hair samples (different colours and hairdresser's treatments) of strict teetotallers were analysed with and without internal standard (IS) in order to determine any endogenous interferent. All tested samples showed no interferent peak at the retention time of the analyte and IS.

Linearity and sensitivity

The calibration was evaluated by analysing five replicates of spiked hair samples at 2.5, 5, 10, 25, 30, 50 and 100 pg/mg. An extracted ion chromatogram of a spiked hair sample at 30 pg/mg is shown in figure 1. The peak area ratio of EtG/EtG-D5 was plotted against the relative concentration of EtG/EtG-D5. The seven point calibration curve, determined by the least squares regression method, was linear over the range with an equation of y= 1.315x + 0.131, R square 0.9979 [figure 2]. The LOQ, defined as the lowest concentration with an accuracy RSD < 20 %, was 2.5 pg/mg. The limit of detection (LOD), defined as signal to noise ratio equal to 5, was 1.3 pg/mg.

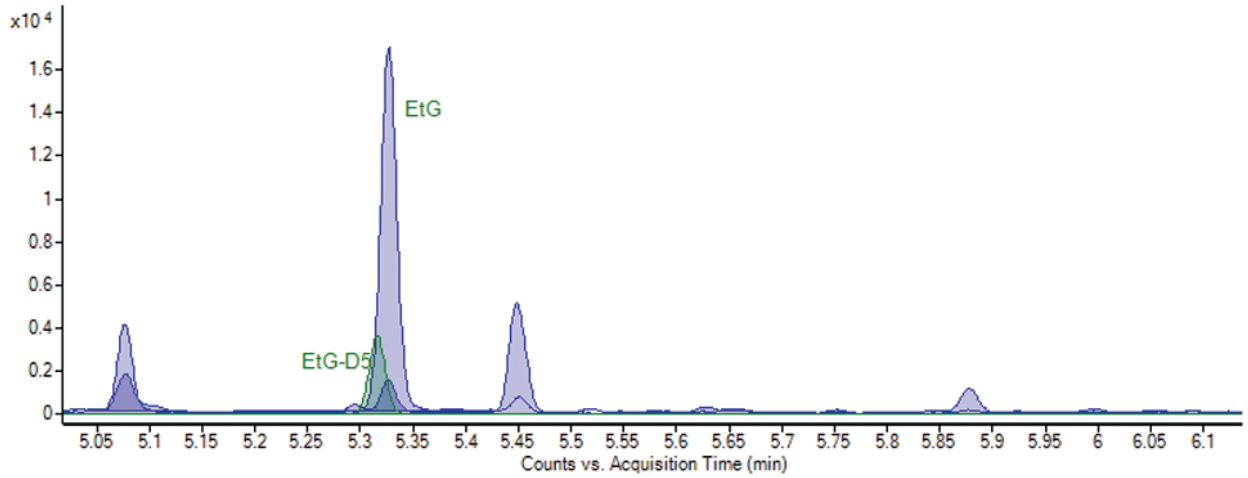

Figure 1. EIC chromatogram of EtG spiked hair sample at 30 pg/mg.

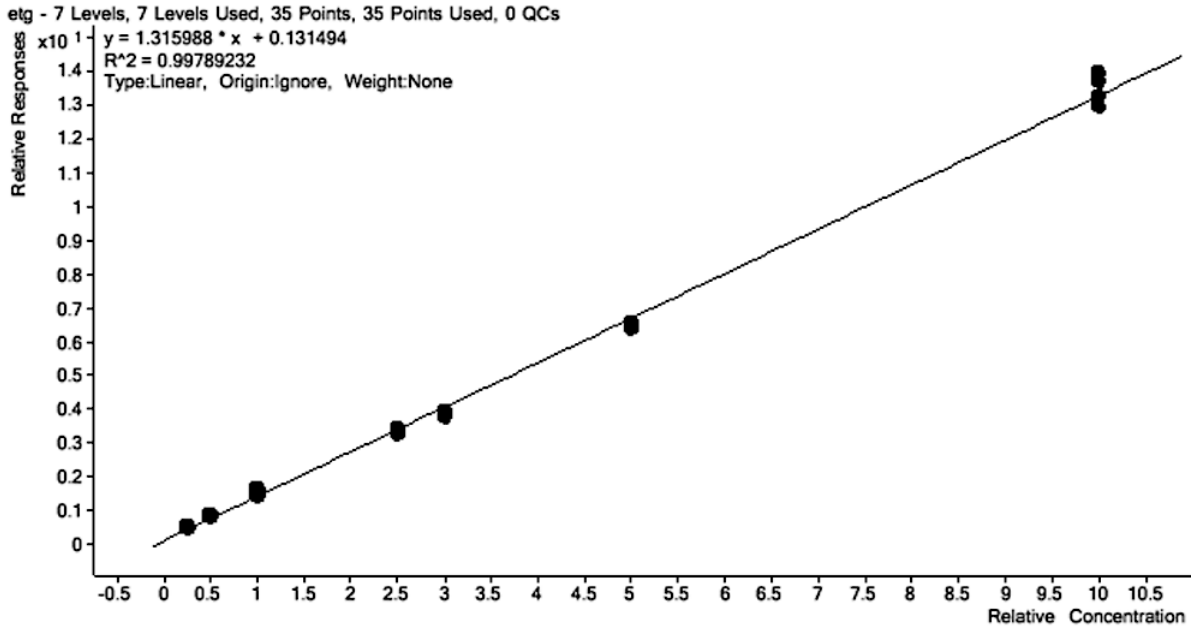

Figure 2. Calibration curve of spiked hair samples, 7 levels, 5 repetitions each [x: concentration EtG/concentration EtG-D5, ng/mL; y: area EtG/area EtG-D5].

Precision and accuracy

Accuracy and precision of the method were assessed by analysing quality control (QC) samples at concentrations of 2.5 (LOQ), 10, 30 and 100 pg/mg. Five replicates of each standard were analysed on five nonconsecutive days. The intra- and inter-day precision fitted the requirements of the forensic guidelines and the RSDs were always below 10 % in QC 10, 30 and 100 pg/mg and below 20 % for the LOQ. Accuracy, calculated as bias in the difference between expected concentration and measured concentration, was below 10 %. Precision and accuracy data are summarised in Table 1.

Parameter	QC 2.5 pg/mg	QC 10 pg/mg	QC 30 pg/mg	QC 100 pg/mg
Intra-day				
mean	2.7	10.3	28.0	100.9
SD	0.07	0.05	0.50	2.90
RSD, %	2.5	0.5	1.7	3.0
Absolute bias	+ 0.2	+ 0.3	- 2	+ 1
Bias, %	+ 7	+ 0.1	- 6	+ 1
Inter-day				
mean	2.6	10.2	29.7	102
SD	0.40	0.83	2.00	3.50
RSD, %	15.0	8.0	6.8	3.4
Absolute bias	+ 0.1	+ 0.2	- 0.03	+ 2.0
Bias, %	+ 3.8	+ 2.0	- 0.1	+ 2.0

Table 1. Summary of intra-day and inter-day QC sample data.

Stability

For estimating the stability of the processed sample, three QC levels at 30, 50 and 100 pg/mg were prepared and analysed on day 0 and day 4. Samples prepared on day 0 and analysed on day 4 were compared in terms of absolute peak areas and calculated concentration with samples analysed soon after preparation. During the experiment, the samples were stored on the GC autosampler at room temperature. Instability of the process would have been indicated by a decrease of peak areas of more than 15% according to Albermann et al. [6]. The calculated decrease of measured EtG was around 42 %, well above the fixed 15%, as could be expected when a derivatizing step is involved.

Application of the method

The applicability of this method has been tested on a few authentic hair samples (n=7) from subjects with a well-known drinking behaviour. The EtG concentrations varied between < LOQ and 7.5 pg/mg for moderate drinkers (n=4, CDT concentrations < 1.8 %). No EtG was determined in teetotallers hair samples (n=2, CDT concentrations < 1.0 %), who declared no use of any alcohol containing food or medicaments nor use of any hair cosmetics potentially containing EtG. A single well-known heavy drinker (reported daily alcohol intake: ten units, whose habit was also proved by a CDT value of 6.0 %) showed an EtG concentration of 39 pg/mg, above the cut-off of 30 pg/mg suggested by the Society of Hair Testing. The results are summarized in Table 2.

Sample	Hair (mg)	EtG concentration (pg/mg)
Abstinent 1	76	<LOQ
Abstinent 2	59	<LOQ
Social drinker 1	49	4
Social drinker 2	50	<LOQ
Social drinker 3	53	7.5
Social drinker 4	20	<LOQ
Heavy drinker	38	39.0

Table 2. Summary of EtG concentrations in real hair samples.

Interfering peaks were not observed, but in one case the relative peak area of the transition 261→73 was badly influenced by the matrix, giving some complication in the quantitation process, although the target ion was not affected. The MRM chromatogram of a real sample is shown in Figure 3.

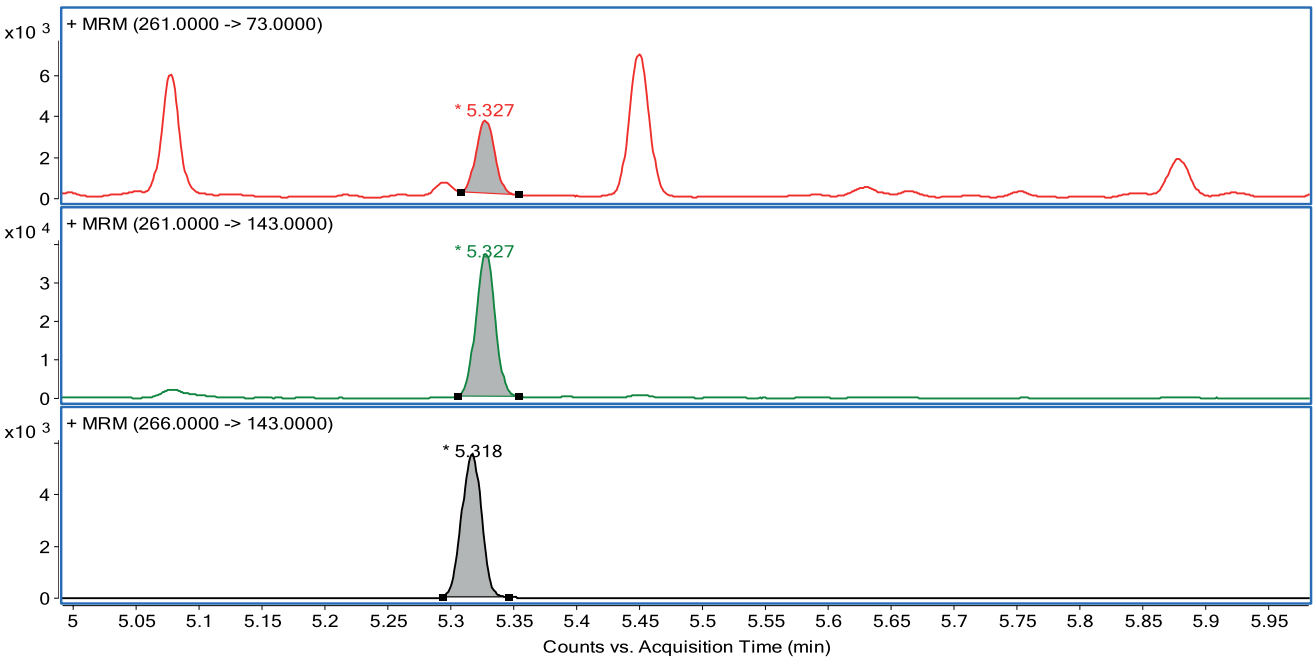

Figure 3. MRM Chromatogram of a real hair sample (Transitions 261→143, 261→73 for EtG, 266→143 for IS); EtG calculated concentration 7.5 pg/mg.

Conclusion

Hair analysis for EtG has been increasingly employed for diagnosing chronic excessive drinking or for monitoring abstinence in both clinical and forensic fields. From an analytical point of view, the literature has traditionally been focused on methods based on liquid chromatography coupled to mass spectrometry mainly because of the fast sample treatment procedure and very low detection limits. However, due to the introduction of more sensitive gas chromatography-triple quadrupole instrumentation, more attention has recently been paid to gas chromatography methods. This paper demonstrates that EtG can be successfully determined in hair by GC-EI-MS/MS. The method has been fully validated and is linear over the range of 2.5 – 100 pg/mg, with a LOQ of 2.5 pg/mg.

References

1. Marques PR. "Levels and types of alcohol biomarkers in DUI and clinic samples for estimating workplace alcohol problems", Drug Test Anal., 2, 4**, 2012**.

2. Boscolo-Berto R, Viel G, Montisci M, Terranova C, Favretto D, Ferrara SD, "Ethyl glucuronide concentration in hair for detecting heavy drinking and/or abstinence: a meta-analysis", Int J Legal Med., 3, 127, **2013**.

3. Morini L, Colucci M, Ruberto MG, Groppi A, "Determination of ethyl glucuronide in nails by liquid chromatography tandem mass spectrometry as a potential new biomarker for chronic alcohol abuse and binge drinking behaviour", Anal Bioanal Chem., 5, 402, **2012**.

4. Hastedt M, Herre S, Pragst F, Rothe M, Hartwig S, "Workplace alcohol testing program by combined use of ethyl glucuronide and fatty acid ethyl esters in hair", Alcohol Alcohol, 2, 47, **2012**.

5. Peters FT, Drummer OH, Musshoff F, "Validation of new methods", Forensic Sci Int, 2-3, 165, **2007**.

6. Albermann ME, Musshoff F, Aengenheister L, Madea B, "Investigations on the influence of different grinding procedures on measured ethyl glucuronide concentrations in hair determined with an optimized and validated LC-MS/MS method", Anal Bioanal Chem, 3, 403, **2012**.

7. Dufaux B, Agius R, Nadulski T, Kahl HG, "Comparison of urine and hair testing for drugs of abuse in the control of abstinence in driver's license regranting", Drug Test Anal, 6, 4, **2012**.

Solutions for your analytical business
Markets & Applications Programs
www.agilent.com/chem/solutions_app

Published in Germany, December 18, 2013
5991-3713ENE

Agilent Technologies