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TECHNICAL NOTE

Detection of ethyl glucuronide in dried human blood using LC-MS/MS

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Abstract Ethyl glucuronide, as a direct metabolite of ethanol degradation, has proven useful as a long-term marker in many forensic applications. The inability to determine ethyl glucuronide in dried blood left a missing link in many investigations. Here, we describe a new method based on mass spectrometry in a Pauli-type ion trap in order to determine this substance in dried blood samples.

Keywords Ethanol · Ethyl glucuronide · LC/MS · Ion trap

Introduction

Alcoholism is one of the most frequent addictions and, therefore, of crucial interest in forensic investigations [4]. In order to prove evidence for the potential influence of ethanol in criminal investigations, the collection of blood samples from suspects can be directed by the legal authorities. The determination of ethanol is performed either by enzymatic methods or by gas chromatography. In many cases, already dried blood samples can be included in enquires at the crime scene. However, the fairly short duration of ethanol in dried blood samples because of its volatility is a frequently encountered problem in legal investigations. In such cases, it is difficult to make any statements on a potential alcoholisation. β-D-Ethyl glucuronide (EtG) [5, 10, 14, 17] is a direct metabolite of ethanol in urine and blood, and it has been shown that EtG is suitable to follow up previous alcohol consumption [4, 16] even if the uptake has taken place several days (urine) or weeks (hair) ago [1, 6, 7, 9, 11]. EtG measurements have been of particular interest in cases of alcoholics who were hospitalised in the course of alcohol withdrawal [16, 18]. In the past, several methods have been described to quantify EtG in ante-mortem and post-mortem samples [9, 12, 15]. These experimental approaches are highly sensitive but are not suitable to detect EtG in dried blood. Because of the particular circumstances at the crime scene, it is not always possible to collect liquid blood. Therefore, this inability to derive ethanol data from dried blood leaves an important missing link in criminological investigations. Here, we report a new method for the determination of EtG in dried blood by means of a simple, mass spectrometry (MS)-based approach [8]. This set-up could possibly supplement present procedures.

Materials

All solvents for high-performance liquid chromatography (HPLC) and MS were delivered in high purity grade by Merck (Darmstadt, Germany), and penta-deutero-EtG (EtG-D_5) are from Medichem (Steinenbronn, Germany). The microfilter devices are from Amicon (Danvers, MA, USA).

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Methods

In order to obtain ethanol-enriched blood samples, five volunteers (three ♀, two ♂) agreed to consume alcoholic beverages within a period of 1.5–2 h. The amount and specific kind of beverage was left up to the discretion of the subjects. The blood alcohol concentration aimed for should be between 0.5‰ and 1.5‰. All persons gave their informed consent before their inclusion in this study. One hundred millilitre blood was collected 1.5 h after the last consumption of alcohol and supplemented with EDTA to prevent coagulation. The alcohol concentration was determined by Head-Space-GC-FID. The water content was measured gravimetrically by lyophilozation (see Table 1). Before subjecting the samples to the LC/MS method as described below, the samples were spiked with EtG-D_5 as an internal standard (12.5 μg/mL). For the detection of EtG in dried blood, 10 g of blood was collected from the volunteers and left to complete dryness in a large glass beaker for approximately 2 days at ambient room temperature. Two hundred milligrams of the residue was collected and resuspended in 1 mL water, the sample was spiked with EtG-D_5 (12.5 μg/mL) and shaken for 1 h before the LC/MS measurement.

Further, a cotton patch (blue jeans, 15×15 cm) was coated with 10 g of blood donated by the volunteers and left at room temperature until to entire dryness. The patch was cut into small strips and treated in a glass flask with water and supersonic (three extractions with 100 mL water each, treatment for 10 min with supersonic). The collected supernatants were freeze dried; the extraction efficacy was determined gravimetrically. Again, we collected 200 mg of the remainder and resuspended it in 1 mL water, spiked the sample with EtG-D_5 (12.5 μg/mL) and left it shaking for 1 h before LC/MS measurement (see below). In order to compare the results from the various extraction samples, we normalised the data to nanogram EtG/mg dried blood. All samples for EtG measurement by LC/MS were centrifuged for 5 min at 10,000×g and subsequently filtered with a Amicon-Centricon®-centrifugal filter devices (cut off 10,000 kDa). Fifteen microlitre of this filtrate was subjected to LC/MS analysis.

The LC/MS system consisted of an Agilent 1100 Series binary pump HPLC system coupled by an atmospheric pressure interface electrospray unit to an Agilent VL Paul-type ion trap. EtG separation was performed at constant flow on a polar-endcapped phenylpropyl reversed phase column (Synergy Polar-RP 250×2 mm, 4 μm) with a guard column (4 mm×2 mm, same packing material; Phenomenex, Aschaffenburg, Germany). The mobile phase consisted of aqueous 0.1% formic acid (vol/vol) at a flow-rate of 0.2 mL/min. EtG eluted under these conditions at about 3 min. The interface to the ion trap was operated at 350°C. Analysis was performed in a negative ion mode with −3.6-kV capillary voltage. Nitrogen was used as a nebulising gas for particle evaporation and helium as collision gas to induce directed fragmentation within the ion trap. The maximal accumulation time was 400 ms with ion charge control. Trap and lens parameters were optimised by an infusion of 100 μg/mL EtG in 0.1% formic acid by a syringe pump at constant flow. The analysis is based on fragment ions *m/z* 203, 157, 129, 113, 85, 75 and derived from precursor EtG (*m/z* 221) and *m/z* 208, 157, 129, 113, 85, 75 from precursor EtG-D_5 (*m/z* 226), recorded in the multireaction monitoring mode of the ion trap. The isolation width for fragmentation was *m/z* 1, and the fragmentation amplitude was 0.8 V. Three averages were recorded per data point. Data analysis was performed with the QuantAnalysis® program provided by Agilent.

Results

The aim of the study was to determine the presence of EtG in freshly collected blood and in dried blood as well as in garments containing solidified blood. To obtain blood samples containing ethanol, volunteers agreed to drink alcoholic beverages in order to reach an estimated serum level between 0.5‰ and 1.5‰ after consummation. Blood samples collected from these donators were either directly

Table 1 Determination of EtG content in whole blood and dried blood samples by LC/MS

	Subject 1 (♀)	Subject 2 (♀)	Subject 3 (♀)	Subject 4 (♂)	Subject 5 (♂)
Ethanol content whole blood (‰)[a]	1.68	1.56	0.97	0.49	0.82
Water content (% by weight)	80.0	80.7	80.1	78.7	77.3
EtG content whole blood by LC/MS (μg/mL)	1.2	1.1	0.6	0.3	0.5
EtG content whole blood by LC/MS (ng/mg)	9.1	8.9	4.3	2.1	3.6
EtG content dried blood by LC/MS (ng/mg)	7.1	5.6	3.2	1.3	2.8
EtG content dried blood (cotton) by LC/MS (ng/mg)	7.2	6.7	3.4	1.9	2.8

[a] By Headspace GC/FID

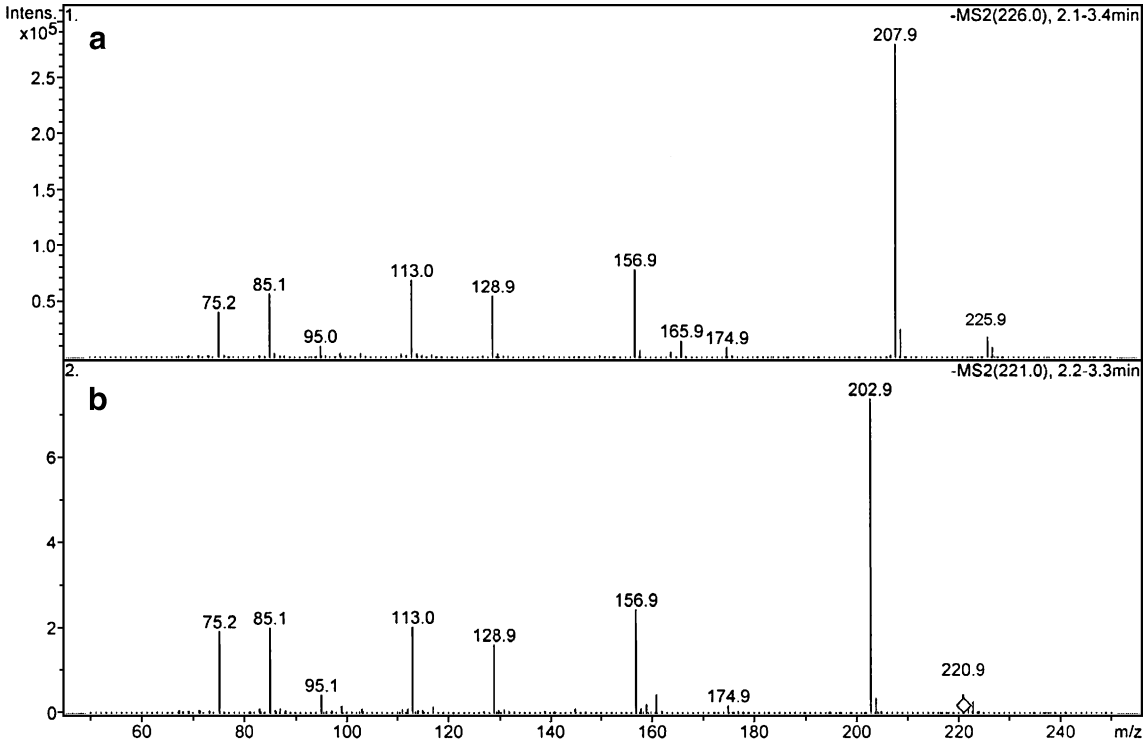

Fig. 1 MS/MS pattern of EtG (**a**) and EtG-D_5 (**b**). The parent molecules 221 (**a**) and 226 (**b**) are extensively cleaved. The intensities of *m/z* fragments 203, 157 (**a**) and 208, 157 (**b**) are used for quantification

admitted to LC-MS/MS analysis or completely dried at room temperature either before or after application to a patch of cotton (blue jeans). EtG from solidified blood samples was recovered by extraction with water as described in "Methods". Purified samples were subjected to a directed fragmentation in a Pauli-type ion trap. In order to compare different samples, EtG values from dried blood extractions were referred as (nanogram EtG/milligram dried blood) and determined in triplicate (Table 1). In the same manner, the EtG concentration in whole blood (microgram EtG/millilitre whole blood) was converted by taking into account the measured water content (nanogram EtG/milligram dried blood) in order to facilitate the comparison to the samples derived from extraction (dried blood and cotton patches).

In the trap, the EtG-$(M)^-$ parent ion *m/z* 221 is cleaved by directed collision with helium into several fragments, *m/z* 203, 157, 129, 113, 85, 75 (Fig. 1a). The HPLC-peak corresponding to these fragments of EtG is eluted at about 3 min (Fig. 2). The intensity of the *m/z* 203, 157 fragments

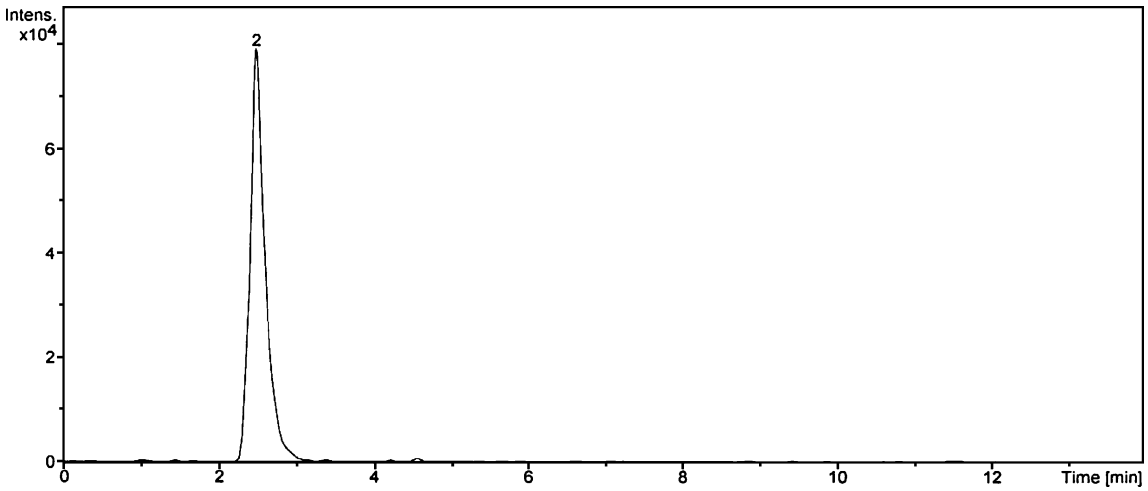

Fig. 2 LC/MS elution profile chromatogram of an EtG-containing blood sample (10 μg/mL). The signal intensity of the ion trap because of the *m/z* fragments 203 and 157 of the EtG fragmentation is recorded. The EtG-derived fragments elute at about 3 min

was used for quantification of EtG within a range between 0.5 and 20 μg/mL. EtG-D_5 was added as an internal standard in order to account for any variations, such as differences in extraction efficiency between various samples (Fig. 3). Analogously, EtG-D_5 gives rise to the corresponding fragments *m/z* 208, 157, 129, 113, 85, 75 (Fig. 1b). The deuterated molecule can be determined in the same manner as the non-deuterated compound. The extraction recovery of samples from air-dried blood was 90% (±3%) and from the blood-soaked jeans patches 85% (±4%; mean value of 5 determinations each), and it was measured gravimetrically. For this purposes, a defined amount of blood was applied to a patch of cotton and left at room temperature until to entire dryness (see "Methods"). After several cycles of extraction and lyophilozation, the extraction efficacy can be calculated by comparing the extraction yield to the weight of the initially applied blood sample. A blank blood sample without any alcohol did not give rise to any significant signals in the LC/MS analysis. The validation of our procedure was performed using a common validation program [13] (VALISTAT. based on Microsoft Excel). This program complies with the guidelines of the Society of Toxicological and Forensic Chemistry (GTFCh). The blood samples for our experiments were donated by volunteers from the department of legal medicine. Serum obtained from these whole blood samples have been used in the same department in routine forensic drug analysis as certified and accredited by the DACH (Deutsche Akkrediterungsstelle Chemie). In all these experiments, no matrix effect was observed. A linear calibration model has been used for the transition *m/z* 221 >203, 157 as quantifier and *m/z* 221 >113 as qualifier. The calibration curve was linear up to 20 μg/mL. Linear regression with various calibration concentrations (0, 0.5, 1, 2, 3, 4, 5, 10, 20 μg/mL EtG) was used for the calculation of the limit of quantification (LOQ) and the limit of detection (LOD) with a significance level of 99% (*k*=3). Limit of quantification is 0.3 μg/mL (α-error 1% for the quantifier); limit of detection is 0.1 μg/mL (α-error 10% for the qualifier). The intraday variability was determined by analysing three EtG concentrations with four assays for each concentration. Similarly, the interday variability was tested by analysing four samples at three EtG concentrations on eight consecutive days [13]. These data are summarised in Table 2.

Table 2 Validation of EtG extraction from whole blood

Variation	2 (μg/mL)	5 (μg/mL)	10 (μg/mL)
Intraday			
Spiked EtG concentration			
N	4	4	4
Standard deviation	0.127	0.181	0.217
% Coefficient of variation	4.83	3.54	3.67
% Accuracy (bias)	−3.63	−2.95	0.19
Interday			
Spiked EtG concentration			
N	32	32	32
Standard deviation	0.151	0.168	0.377
% Coefficient of variation	5.97	3.53	3.93
% Accuracy (bias)	−4.04	−3.68	−3.03

Discussion

Here, we described for the first time the determination of EtG in dried human blood by applying a LC-MS method employing a Pauli-type ion trap. In contrast to MS/MS ionisation on a quadrupole instrument, which is used in many GC/MS and LC/MS applications, the fragmentation on an ion trap is mass selective. The different mode of ionisation assures that for ion-trap MS/MS, the spectra are generally the result of a single stage of ionisation, which

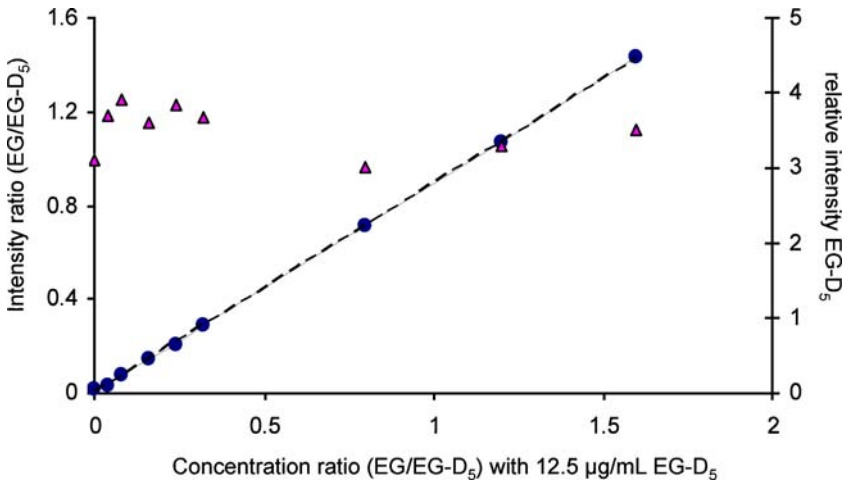

Fig. 3 LC/MS analysis of whole blood EtG samples. A whole blood sample was spiked with EtG and a constant amount of EtG-D_5 (final concentration 12.5 μg/mL) and processed before MS as described in "Methods". EtG: *filled dots*, EtG-D_5: *filled triangles*. The ordinate on the *right* shows the intensity of the EtG-D_5 signal

explains why ion-trap MS/MS spectra are often clearer [8]. The procedure works reliably up to a LOQ of 0.3 μg/mL and the LOD is 0.1 μg/mL. The blood samples can be stored up to several weeks at 4°C without any significant change in the EtG concentration. Therefore, EtG seems to be a molecule whose stability is not affected by enzymes or other degrading substances present in blood [12]. Further, the extraction/air-drying procedure does not substantially affect EtG stability. However, the slightly lower recoveries of EtG from dried blood and blood-soaked cotton (Table 1) might be attributed to the combined effects of lower extraction yield and minor degradation of EtG during air drying [12]. Even if we found differences in absolute EtG concentrations between varying samples (blood, dried blood, cotton-sample), we could show that changes in EtG concentration within one set of samples relate to the ethanol content. However, the kinetics of degradation for ethanol and EtG are distinct. Recent studies on the kinetics of ethanol and EtG clearly showed that the elimination of the metabolite is delayed by approximately 3 h compared to ethanol [2, 3]. Therefore, if this kinetic framework is not taken into account, it is not feasible to derive directly the amount of alcohol at the crime scene from EtG measurements performed later on in the laboratory. Our data indicated that an EtG content of more than 3 ng/mg dried blood relates to a forensic relevant degree of alcoholisation. To substantiate our findings, we intend to continue these experiments on an extended data base taking into account the aforementioned differences in elimination between ethanol and EtG. The determination of EtG in dried blood by a certified protocol could be an interesting supplement to the panel of existing methods.

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