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Determination of ethyl glucuronide in human hair samples: A multivariate analysis of the impact of extraction conditions on quantitative results

Alexander Mueller[a,*], Hilke Jungen[a], Stefanie Iwersen-Bergmann[a], Lars Raduenz[a], Susanne Lezius[b], Hilke Andresen-Streichert[a]

[a] University Medical Center Hamburg-Eppendorf, Department of Legal Medicine, Toxicology, Hamburg, Germany
[b] University Medical Center Hamburg-Eppendorf, Department of Medical Biometry and Epidemiology, Hamburg, Germany

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ABSTRACT

Background: Ethyl glucuronide (EtG), a minor metabolite of ethanol, is used as a direct alcohol biomarker for the prolonged detection of ethanol consumption. Hair testing for EtG offers retrospective, long-term detection of ethanol exposition for several months and has gained practical importance in forensic and clinical toxicology. Since quantitative results of EtG hair testings are included in interpretations, a rugged quantitation of EtG in hair matrix is important. As generally known, sample preparation is critical in hair testing, and the scope of this study was on extraction of EtG from hair matrix.
Methods: The influence of extraction solvent, ultrasonication, incubation temperature, incubation time, solvent amount and hair particle size on quantitative results was investigated by a multifactorial experimental design using a validated analytical method and twelve different batches of authentic human hair material. Eight series of extraction experiments in a Plackett-Burman setup were carried out on each hair material with the studied factors at high or low levels. The effect of pulverization was further studied by two additional experimental series. Five independent samplings were performed for each run, resulting in a total number of 600 determinations.
Results: Considerable differences in quantitative EtG results were observed, concentrations above and below interpretative cut-offs were obtained from the same hair materials using different extraction conditions. Statistical analysis revealed extraction solvent and temperature as the most important experimental factors with significant influence on quantitative results. The impact of pulverization depended on other experimental factors and the different hair matrices themselves proved to be important predictors of extraction efficiency.
Conclusions: A standardization of extraction procedures should be discussed, since it will probably reduce interlaboratory variabilities and improve the quality and acceptance of hair EtG analysis.

1. Introduction

Since its introduction more than 15 years ago, hair ethyl glucuronide (hEtG) has become an applied state marker of alcohol consumption. Interpretative cut-offs for both abstinence control (7 pg/mg) and the detection of excessive chronic alcohol consumption (30 pg/mg) are established [1] and numerous analytical methods including validation data are described in literature (for a comprehensive review refer to Crunelle et al. [2]). Due to the low concentrations of hEtG, mass spectrometry based techniques (LC/MS/MS, GC/MS, GC/MS/MS) are common in hEtG testing.

It is well known that the release and extraction of analytes from hair matrix is one of the most critical issues in hair analysis [3,4]. Polar solvent extraction is almost exclusively used for hEtG analysis. There are obvious differences in the described extraction conditions: hair samples are grounded to fine powders [5,6] or cut in small pieces [7,8] in stead of, water [5,6,8,9], methanol [10] or mixtures of solvents [7,11] are used to extract hEtG. Extraction is carried out for different time periods from 15 min [12] to two days [13] and at different temperatures (room temperature [3,14], 37 °C [15], 50 °C [16]) using different amounts of solvent (from 15 mg [6] to 146 mg [9] hair per mL solvent). Most of the authors

* Corresponding author at: University Medical Center Hamburg-Eppendorf, Department of Legal Medicine, Toxicology, Butenfeld 34, 22529 Hamburg, Germany. Fax: +49 40741058660.
E-mail address: al.mueller@uke.de (A. Mueller).

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use ultrasonication [3,5–9,11,12,16], while some others do not [13,15,17]. Considerable inter-laboratory variabilities are observed in proficiency testings for hEtG [18]. There are several previous studies, which investigated the impact of sample preparation on quantitative hEtG results but they all focused on a single factor, namely the applied disintegration techniques of milling or cutting hair samples prior to extraction. Consistently, an increasing recovery of hEtG by milling hair samples was reported, but this effect was found to be highly variable and virtually unpredictable, as recently presented by Salomone et al. [19] on a large dataset. To evaluate the impact of further methodological factors, which have not yet been considered in previous studies, a multifactorial approach was used to evaluate the influence of six experimental variables on quantitative hEtG recoveries from authentic human hair material and a multifactorial experimental design. There were general considerations in study design: since the number of required measurements increases exponentially with the number of the investigated factors, the number of methodological variables was limited and a reduced factorial design was used to avoid an unacceptable large number of runs. Replicate samplings were necessary to control sample homogenity and analytical reproducibilty and the experiments had to include a representative number of authentic hair materials. The study was carried out in a Plackett-Burman-Design [20] and investigated the impact of ultrasonication, sample solvent, sample to solvent ratio, incubation time, incubation temperature and hair particle size.

2. Materials and methods

2.1. Chemicals and reagents

All solvents and reagents for sample preparation were of quality for analysis (p.a.) and were obtained from Merck Schuchard (Hohenbrunn, Germany). Solvents and chemicals for LC/MS/MS analysis (water, acetonitrile, formic acid) were of specified LC/MS grade (Chromasolv®) and purchased from Fluka (Munich, Germany). Stock solutions (1.0 mg/mL in methanol) of ethyl glucuronide (EtG) and d5-ethyl glucuronide (d5-EtG) were obtained from Lipomed (Weil am Rhein, Germany).

2.2. Hair material and sample preparation

Authentic hair was collected from volunteers in a barber's shop. The only precondition was a maximum hair length of 6 cm and no additional data was collected. Randomized hair sampling was carried out on 12 days. Cut hair specimens from individuals in hair dressing settings were daily collected in a bucket resulting in 12 batches of different hair material, which were designated by letters A–L. Hair colors were mixed and varied from white to black. Because the hair samples were collected completely anonymously, information about the ethnic of the volunteers was not available. Hair was decontaminated by a four step decontamination procedure. Ethanol, water, acetone and methylene chloride were subsequently suspended to the hair material (50 mL solvent/g hair) and ultrasonication was applied for 5 min. After drying, each hair batch was extensively homogenized to fine snippets (1–2 mm) using a pair of scissors. Aliquots (2 g) of each batch material were further pulverized using a ball mill (MM2000, Fa. Retsch, Haan, Germany).

50 mg of samples were weighed in 10 mL conical glass vials, the extraction solvent and 5 ng of internal standard d5-EtG (using a working solution of 1 ng/μL in methanol) were added. To ensure a complete covering of the hair material with the extraction solvent and to remove air bubbles inside the sample, a short centrifugation (5 min, 4000 g) was performed after.

Extraction at 60 °C was carried out in an incubator (Kelvitron®, Heraeus, Hanau, Germany), whereas the samples at room temperature were placed in an orbital shaker (Promax® 1020, Heidolph, Schwabach, Germany). An ultrasonic bath (Sonorex® RK102H, Bandelin, Berlin, Germany) was used for ultrasonication. Extracts were separated by centrifugation (10 min, 4000 g) and additional filtration (Chromafil® filtration cartridges, 6 mL PET, 0,20 μm, Macherey-Nagel, Düren, Germany). Evaporation of the extraction solvent was carried out at 70 °C using a gentle stream of nitrogen. After reconstitution in 100 μL of mobile phase A, the samples were analyzed by liquid chromatography/mass spectrometry.

Calibrators and quality control samples were prepared by addition of EtG to blank hair samples of children that were tested hEtG negative before.

2.3. Liquid chromatography/tandem mass spectrometry

Chromatography was performed by Waters Acquity® UPLC with a C18 Acquity® HSS T3, 1.7 μm, 2.1 × 150 mm column at 40 °C. The injection volume of the samples was 5 μL, the flow rate at 0.3 mL/min and the binary gradient as follows: 0–3.8 min: 2%–30% B, 4.0–6.9 min: 2%B. Mobile phase A consisted of water with 0.1% formic acid and mobile phase B was acetonitril with 0.1% formic acid. A Waters XEVO® TQ-MS triple quadrupole mass analyzer operating in multiple reaction monitoring was used for detection, five transitions were monitored: EtG: m/z 221 > 75 (quantifier), m/z 221 > 85, m/z 221 > 57, m/z 221 > 113 (qualifiers), d5-EtG (internal standard I.S.) m/z 226 > 85. Six spiked calibration levels (5, 20, 40, 60, 80, 100 pg/mg) and two quality control samples (7 and 35 pg/mg) were included in each experimental series.

2.4. Method validation

Validation parameters including accuracy, interferences, linearity of calibration, matrix effects and recovery complied with international standards [21] and the method is in the accredited area of our laboratory. The limit of detection of the analytical method was 1.7 pg/mg and the lower limit of quantification was 4.7 pg/mg. Intraday CV was 6% (QC1) and 5% (QC2) and interday CV 10% (QC1) and 6% (QC2) respectively. Special attention was paid to in-process-stability of the analyte, since different temperatures, incubation times and solvents were used in this study. Therefore, ten solutions (five aqueous, five methanolic) of EtG and d5-EtG were incubated for 24 h at 4 °C, 20 °C and 60 °C. The solutions at 60 °C were additionally treated by one hour of ultrasonication. Stability was studied by the absolute signal areas (average and CV) of EtG and d5-EtG.

2.5. Experimental design

Six experimental factors, which were supposed to influence hEtG recovery were selected for this study and two different levels (each a "high" and a "low" one) were defined for these factors: ultrasonication ("high": 1 h, "low": 0 h), incubation temperature ("high": 60 °C, "low": 20 °C), incubation time ("high": 24 h, "low": 2 h), hair particle size ("high": powder "low": snippets), solvent type ("high": water, "low": methanol) and sample/solvent ratio ("high": 25 mg/mL, "low": 12.5 mg/mL). As shown by Table 1, eight series of extraction experiments (V1–V8) were arranged according to Plackett-Burman. [20] with the studied factors at high or low level. Since hair is different, the experiment was carried out on 12 different batches of hair materials. Because of the inhomogeneous nature of hair and because batches contained hair materials from different individuals, replicate measurements from five independent samplings of each 50 mg hair were performed.