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Reference:

Crunelle Cleo, Cappelle Delphine, Flamand Eva, Cox Jeroen, Covaci Adrian, De Doncker Mireille, van Nuijs Alexander, Michielsen Peter, Yegles Michel, Neels Hugo.- Ethyl glucuronide in hair of non-excessive alcohol consumers: correlations and gender influence

Forensic Toxicology - ISSN 1860-8965 - 34:1(2016), p. 186-190

Full text (Publishers DOI): <http://dx.doi.org/doi:10.1007/S11419-015-0291-8>

To cite this reference: <http://hdl.handle.net/10067/1324510151162165141>

Ethyl glucuronide in nails: method validation, influence of decontamination and pulverization, and particle size evaluation

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Abstract

Ethyl glucuronide (EtG), a minor metabolite of alcohol, can be determined in hair and nails to retrospectively detect alcohol consumption. Due to the low EtG concentrations in keratinized matrices (pg/mg range), sensitive analytical methods and optimized sample preparation procedures are essential. While several methods for the detection of EtG in hair have been published, its detection in nails has only been scarcely investigated. In this study, an analytical method for the determination of EtG in nails using gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) was developed and validated, and the influence of sample preparation on EtG concentrations was evaluated. The calibration curve was linear from 2 to 100 pg/mg and the lower limit of quantification (LLOQ) was 2 pg/mg. The method was applied to finger- and toenails of five regular alcohol consumers, with concentrations ranging from < LLOQ to 123 pg/mg. EtG concentrations in the washing solutions did not differ significantly with the use of different decontamination procedures, including hexane, dichloromethane, methanol, acetone and water as solvents. More intense pulverization of the nail samples increased EtG concentrations, parallel with a decrease in particle size, but only until a certain limit. In conclusion, we present a sensitive validated GC-MS/MS technique for the determination of EtG in nails which were pulverized to obtain a high extraction surface area.

Keywords

Ethyl glucuronide, nails, GC-MS/MS, decontamination, pulverization, particle size

Introduction

In forensic and clinical toxicology, the monitoring of substances through the detection of reliable biomarkers in adequate matrices is critical. Beside the more 'traditional' matrices including blood and urine, keratinous matrices such as hair and nails, are particularly interesting owing to their long detection window and their ease of sample collection, transport and storage [1-4]. In 1965 the first applications of nails mainly included the detection of arsenic intoxications and the exposure to metals (e.g.; cadmium, lead and zinc) [5-9]. Gradually, the use of nails expanded towards the detection of pharmaceuticals, drugs of abuse and their metabolites (e.g., antimycotics, amphetamines, opioids and cocaine)[2,3]. Also, nails have been used for the monitoring of alcohol consumption through the detection of ethyl glucuronide (EtG), which is a minor phase II direct metabolite of alcohol. EtG accumulates in keratinous matrices, where it remains detectable for several months [1,3], and provides a specific and sensitive marker for alcohol consumption [10]. Research has mainly focused on the detection of EtG in hair [1], whereas studies on its measurement in nails are sparse [11-15]. However, nail analysis offers advantages compared to hair analysis. First, nails grow slower than hair (3 mm/month for fingernails and 1 mm/month for toenails compared to 1 cm/month for head hair [16]), and would thus allow a larger accumulation of EtG. This can be relevant in situations where low EtG concentrations need to be determined, e.g., for the differentiation between teetotalers and moderate alcohol consumers,. Second, nails provide an alternative in cases where hair is not (sufficiently) available (e.g., alopecia, newborns). Third, cosmetic hair treatments has been proven to reduce EtG content in hair [9], which would be avoided through nail analysis.

Currently available methods for the detection of EtG in nails include liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) operated in electrospray ionization (ESI) mode [11-15]. While no gas chromatography coupled to mass

spectrometry (GC-MS) methods are available for EtG in nails, derivatization followed by a GC-MS determination may improve the analytical sensitivity as has been demonstrated for hair [1].

Aside from the analytical method, the sample preparation of complex keratinous matrices is crucial. Sample preparation includes decontamination, homogenization, extraction and clean-up steps. Although these steps influence the obtained results, little attention has been paid to them in regard to nail analyses. In a study on cocaine and opioids by Engelhart et al. over 98% of external contamination could be removed with 2 methanol washes [17]. Lemos and colleagues developed a decontamination procedure consisting of subsequent washes with 0.1% sodium dodecyl sulfate, water and methanol. Efficiency of this procedure was confirmed by negative final methanol washes and it was applied for the detection of cocaine, opioids and cannabinoids[18-20]. Decontamination by washing with water and methanol was proved sufficient to remove external contamination prior to amphetamines and cannabinoids analysis[21]. Regarding the analysis of EtG in nails, two different procedures for decontamination have been described including, dichloromethane methanol (and hexane) as solvents [11,13,15,14]. In neither methods, the efficiency of the decontamination was evaluated. Homogenization of the sample allows optimal extraction of the compound of interest from the matrix. In hair mechanical pulverization increased the extraction efficiency compared to scissor cut samples [22,23]. Studies in nails have shown that cryogenic or frozen pulverization leads to a higher specific surface area and smaller particle sizes compared to cut samples[24,25]. For homogenization prior to EtG analysis in nails, studies employ either no homogenization procedure [13,15], or a mechanical pulverization of the nails [11,12,14], but without any comparison.

Our aim is to develop and validate a specific and sensitive GC-MS/MS method for the determination of EtG in nails. In addition, we compare the efficiency of several

decontamination procedures. Finally, we assess the influence of pulverization on EtG extraction efficiency, subsequently presenting data on nail-particle sizes after mechanical pulverization.

Methodology

Reagents, materials and instruments

EtG and the internal standard EtG-D₅ 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 chemicals and reagents were of analytical purity grade. Stock solutions of EtG (10 ng/μL) and EtG-D₅ (10 ng/μL) 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. Oasis[®] MAX (60 mg, 3 mL) solid-phase extraction (SPE) 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 nail 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[™] SPE Vacuum Manifold (Bellefonte, CA, USA) with 24 ports was employed to load the nail samples and to dry the cartridges. Solvent evaporation was achieved with a Pierce Reacti-Therm III Heating Module (Rockford, IL, USA). Particle size measurements were performed using a Camsizer XT (Retsch Technology GmbH, Haan, Germany) equipped with Camsizer XT software 6.5.1.

The gas chromatographic system consisted 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).

Sample collection

Nail samples were obtained by clipping of the distal edge of finger- and toenails. For method validation, blank nail samples were collected from teetotalers. For decontamination and homogenization experiments, nail clippings were obtained from five volunteers who reported regular alcohol consumption (at least once a week) over the past year without binge drinking events. Nails were collected every 2 weeks over several months, in order to obtain a large quantity of nails per subject (300 – 700 mg). Per subject, finger- and toenails were collected and stored separately, resulting in 5 sets of fingernails and 5 of toenails.

GC-MS/MS validation

The sample preparation steps prior to injection on the GC-MS/MS system are summarized in Table 1.

Chromatographic separation was achieved on a HP-5 MS (5% phenyl methyl siloxane) column (30 m × 0.25 mm × 0.1 µm). The GC-MS/MS procedure was based on a previously validated method for the detection of EtG in hair [26]. The injector temperature was set at 250 °C. The carrier gas was helium with a flow rate of 1.2 mL/min. The oven was initially held at 100 °C for 2.25 min, heated to 170 °C at a rate of 20 °C/min and then programmed at a final temperature of 310 °C at 50 °C/min. The detector was operated in negative ion chemical ionization (NICI) mode at 280 °C. Data acquisition was performed in 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-D₅.

Validation of the analytical method was performed based on the guidelines on Bioanalytical Method Validation provided by the European Medicine Agency (EMA) with modifications [27]. The following performance criteria were evaluated: linearity, calibration range, selectivity, accuracy, precision, lower limit of quantification (LLOQ), and recovery.

Calibration curves were prepared by spiking blank matrix samples with EtG-D₅ and 5 different levels of EtG ranging from 2 to 100 pg/mg (2, 8, 20, 40, 100 pg/mg). In addition, zero blanks (processed matrix sample without EtG and without EtG-D₅), procedural blanks (processed matrix with EtG-D₅) and quality control (QC) samples were included. Calibration curves were constructed by plotting the ratio between the peak area of EtG and EtG-D₅ against the spiked concentration, and were 1/x weighted. Selectivity was assessed through evaluation of blank samples for interfering components.

Accuracy and precision were evaluated by analyzing replicated blank matrix samples spiked at concentration levels of 2 pg/mg (LLOQ samples) and 40 pg/mg (QC samples). Within-run accuracy and precision were determined by analyzing 4 samples in a single analytical run. Between-run accuracy and precision were calculated from samples analyzed in different runs on 3 separate days. Acceptance criteria were: 1) accuracy (bias) within 15% of the nominal value for QC samples and within 20% for LLOQ samples, and 2) precision (coefficient of variation (CV)) lower than 15% for QC samples and lower than 20% for LLOQ samples. The extraction recovery was calculated by comparison of responses from standards spiked before extraction to standards spiked after extraction.

Decontamination experiment

Three different procedures were tested on 5 sets of nails, either finger- or toenails, each in triplicate on 30 mg of sample. The first 2 procedures were retrieved from earlier publications, and consisted of washing with dichloromethane and methanol (DCM-MeOH) [13,15] or washing with hexane, dichloromethane and methanol (Hex-DCM-MeOH) [11,14]. The third procedure was based on a method for EtG analysis in hair [26] and included washing with water and acetone (H₂O-Ac). The decontamination protocol was similar between procedures and consisted of subsequent addition of 1 mL of washing solvent, vortex mixing for 30 s and

solvent removal. For each procedure, the washing solvents were collected, combined, evaporated to dryness, derivatized with HFBA, and injected into the GC-MS/MS system.

Homogenization experiment

The homogenization experiment was performed on another 30 mg of nail samples (5 sets of fingernails and 5 sets of toenails) previously washed with water and acetone. Per set of nails, 5 procedures were compared: cut nails without further pulverization ($T_p = 0$ min), 3 min of pulverization ($T_p = 3$ min), 6 min of pulverization ($T_p = 6$ min), 9 min of pulverization ($T_p = 9$ min), and 12 min of pulverization ($T_p = 12$ min). Each procedure was performed in duplicate or triplicate, depending on the amount of sample remaining. After homogenization, samples were further analyzed for EtG concentrations as presented in Table 1.

Particle size measurements

From the samples pulverized for 3 min, 6 min, 9 min and 12 min, approximately 5 mg was collected for particle size measurements. As described in the standard ISO 13322-2, particle size was assessed by dynamic digital image analysis and using wet dispersion. Briefly, a pulverized sample was added to the dispersion area with approximately 300 mL isopropanol as dispersion medium. The dispersion medium was degassed to remove possible air bubbles, and agglomerates were broken down by ultrasound. A centrifugal pump was used to keep the particles in suspension and prevent sedimentation during analysis. A gap width of 4 mm was set as spacing through which the flow was optically assessed and analyzed, and a total number of 20000 image frames per sample were recorded. The areal density was set between 0.4 and 0.6%. Per sample 3 measurements were performed to assess repeatability. After each sample, the liquid was drained, the area rinsed 3 times, and subsequently refilled with medium for the next measurement. From the obtained measurements, Camsizer XT software was used to assess the shape, calculate the mean and median diameter, and the range of the diameter of particles within each sample.

Data analysis

Statistical analysis was performed using R (version 3.1.2., The R Foundation for Statistical Computing). Normality was tested on raw data and log-transformed data. For all statistical tests, a p-value < 0.05 was considered statistically significant. Results are presented as mean $\pm$ standard error (SE). For the decontamination experiment, the EtG concentrations in the washing solutions were analyzed using logistic regression, with the outcome variable being the presence (yes/no) of detectable EtG (i.e., > LLOQ). All other outcome variables (EtG concentrations in the nail samples, influence of pulverization and origin of the nails (finger or toe) on EtG concentrations, and effect of pulverization time on particle sizes) were analyzed using linear mixed models, accounting for the dependence between observations within the same individual. Significance of the fixed effects was tested using an F-test with Kenward-Roger correction for multiple testing. A log transformation was carried out if the outcome variable was not normally distributed. Post-hoc analysis was performed using a Tukey test for multiple testing.

Results

Method validation

As the response in blank samples was less than 20% of the LLOQ (2 pg/mg) for EtG and less than 5% for EtG-D₅, the absence of interfering substances was accepted. The calibration curve was linear ($R^2 > 0.99$) in the range from 2 to 100 pg/mg. The back-calculated concentrations of all the calibrators were within $\pm 15\%$ of the nominal value.

The within- and between-run accuracy and precision results are presented in Table 2 and are within the acceptance criteria of 15% for the QC samples and 20% for the LLOQ samples. For QC samples (n = 12) within- and between-run accuracy (% bias) ranged between 0.8 and 1.4, and within- and between-run precision (% CV) ranged between 3.1 and 6.7. For

LLOQ samples (n = 12) within- and between-run accuracy (% bias) ranged between 6.5 and 10.5, and within- and between-run precision (% CV) ranged between 3.3 and 8.2. Mean overall SPE recovery was 88.5%.

Decontamination experiment

EtG concentrations in the washing solutions and the respective nail samples are presented in Table 3 as mean of the experiments in triplicate. The raw (untransformed) data showed a non-normal distribution. The data on EtG concentrations in the nails were normalized by a logarithmic transformation. However, the data on EtG concentrations in the washing solutions remained non-normal due to the presence of multiple samples < LLOQ. The outcome variable was therefore recoded into a binary outcome variable indicating whether or not the EtG concentrations reached the LLOQ, and analyzed using logistic regression. The effect of decontamination procedure on the outcome was not significant ($p = 0.13$), which means that the probability to reach the LLOQ did not differ significantly between the 3 procedures. The data on EtG concentrations in the nails were analyzed using a linear mixed model with the log-transformed EtG concentrations as outcome variable, and the decontamination procedure as fixed effect. The effect of the procedure on the EtG concentrations in nails was significant ($p = 0.01$). Post-hoc analysis showed that the Hex-DCM-MeOH procedure led to significantly higher EtG concentrations compared to the DCM-MeOH procedure ($p = 0.007$). Ratios between EtG concentrations in the nails compared to EtG concentrations in the washing solutions were stable and ranged between 0.13 and 0.21 (mean 0.17 ± 0.03), with one exception for set 4 which had a higher nail/washing EtG ratio of 0.63. The low number of samples with EtG concentrations > LLOQ did not allow a statistical analysis of the ratios.

Homogenization experiments

Results of the homogenization experiment are presented in Table 4 as the mean of the experiments performed in duplicate or triplicate. The influence of pulverization and origin of

the nails (finger or toe) on EtG concentrations, was modeled using linear mixed models. Fixed effects included origin, pulverization time and their interaction. The interaction between the origin of the nails and pulverization time was not significant. This means that the EtG concentrations between the 5 homogenization procedures are not significantly different for finger- and toenails. This non-significance is not surprising, due to the large variances between the individuals. Although one model could theoretically fit for both finger- and toenails, the effect of pulverization time was studied separately. For toenails, the effect of pulverization time on EtG concentration was not significant ($p = 0.35$). In fingernails, the effect of pulverization time on EtG concentration was significant ($p=0.045$). Post hoc testing with Tukey correction showed that the no pulverization lead to a lower EtG concentration compared to the four other times. The difference in EtG concentration with 3 and 6 minutes is marginally significant ($p=0.07$ and 0.06 , respectively), and the difference with 9 and 12 minutes is significant ($p=0.03$ and 0.02). -No significant differences are observed between the other homogenization times. EtG concentration versus the time of pulverization is visualized in Figure 1. Compared to no pulverization, EtG concentration increase with pulverization. Although there is a lot of variance between subjects, in general pulverization for longer than 3 min do not lead to strong increases in EtG concentrations ~~increased with pulverization time in a more or less linear way. A similar pattern was observed in finger and toenails.~~

Results from particle size measurements after 3 min, 6 min, 9 min and 12 min of pulverization are presented in Table 5. The effect of pulverization time on particle size was analyzed using a linear mixed model with the fraction of particles $< 600 \mu\text{m}$ as outcome variable. There was no significant interaction between the origin of the nails (finger or toe) and the pulverization time ($p = 0.39$). The particle sizes did not differ significantly between the pulverization times, but this non-significance is probably due to the low number of observations. Therefore finger-

and toenails were analyzed in separate models. These models showed that neither in finger-, nor in toenails, there was a significant effect of pulverization time on particle size (toenails: $p=0.68$, fingernails: $p=0.13$) Figure 2 shows the fraction of particles $< 600 \mu\text{m}$ as a function of pulverization time. For most subjects, the highest fraction of particles $< 600 \mu\text{m}$ (smallest particle sizes) are reached after 9 min of pulverization. However, additional pulverization to 12 min leads to decrease in fraction of particles $< 600 \mu\text{m}$ in a more or less pronounced way depending on the subject.

Discussion

So far, only three studies investigated the detection of EtG in nails as a marker for monitoring alcohol consumption over extended periods of time [11-15]. These studies provided the first evidence that EtG could be detected in fingernails using LC-MS/MS. Moreover, EtG concentrations in fingernails were found to be correlated with those in hair, and suggested a higher sensitivity of fingernails compared to hair [13]. To the best of our knowledge, the current study is the first to report on the detection of EtG in toenails. Differences between finger- and toenails were not significant but this may be due to low number of samples. More research is required to investigate whether finger- and toenails are substitutable. Furthermore, comparison of concentrations in finger- and toenails could be interesting in the evaluation of the extent of incorporation through sweat and external contamination, and deserves further investigation. In contrast to the published procedures, the current study comprises the development and validation of a GC-MS/MS based method. Advantages of GC-MS/MS compared to LC-MS/MS are a less expensive apparatus, less pronounced matrix effects and enhanced sensitivity through derivatization. Indeed, the method presented here in with HFBA derivatization has a lower LLOQ compared to the other studies on EtG in nails that encompassed a full validation using similar criteria (2 pg/mg compared to 10 and 8 pg/mg).

As in hair, the cut-off to discriminate between teetotalers and moderate drinkers has been established at 7 pg/mg [28], this drop in LLOQ below 7 pg/mg may be of significant importance to discriminate between both categories.

Procedures of decontamination and homogenization of nails were evaluated. Experiments on decontamination compared 3 procedures. The choice of decontamination solvents was based on previous publications of EtG detection in nails [11,13-15] and on a recently published method for EtG detection in hair [26]. The EtG concentrations in the washing solutions did not differ significantly between the 3 procedures, indicating that none of the procedures removed significantly more EtG from the nails. The effect of the decontamination procedure on EtG concentrations in nails was significant: decontamination using the Hex-DCM-MeOH procedure resulted in significantly higher EtG concentrations compared to the DCM-MeOH procedure. Ratios between EtG concentrations in the nails compared to EtG concentrations in the washing solutions were stable among the decontamination procedures, which suggest that there is no overall difference between procedures. These experiments underline the need for an adequate decontamination procedure. Decontamination should remove only external contamination and not incorporated EtG, thus higher EtG concentrations in washing solutions are not always desired. As a result, the choice of an optimal decontamination procedure is not straightforward, and as the current study does not provide enough data to draw definitive conclusions, further research is required. To this end, concentrations in nails should be compared to alcohol consumption and reference material for nails should be developed. Pulverization (in comparison to no pulverization) allows the achievement of small particle sizes and a homogeneous powder, and thereby enhances the extraction efficiency, reduces the extraction time from the matrix and improves determination of the EtG concentrations in nails. As such, cutting of nail samples without further pulverization may underestimate the actual EtG concentrations and

consequently influence the interpretation of results. Due to the large variances between subjects, drawing definitive conclusions on optimal pulverization time is rather difficult. In theory, longer pulverization times would result in higher EtG concentrations and smaller particle sizes. Experimental data, however show that this theory is only true until a certain point after which longer pulverization times results in decreased EtG concentrations and bigger particles. Possible explanations are the formation of agglomerates and EtG degradation during extensive pulverization.

To minimize the measuring variation, all experiments were performed in duplicate or triplicate. Limitations of the study include the small number of subjects. Also further research is needed to confirm these findings and to establish optimal decontamination and homogenization procedures.

Conclusions

This study presents the development and validation of a sensitive GC-MS/MS method for the determination of EtG concentration in both finger- and toenails. The results from decontamination and homogenization experiments stress the importance of washing and pulverization steps in nail samples to achieve accurate and reliable nail EtG results that can be used to evaluate alcohol consumption over extended periods of time.

Acknowledgements

Delphine Cappelle is grateful to the Toxicological Centre, University of Antwerp for her PhD scholarship. Dr. Alexander van Nuijs acknowledges the Flanders Scientific Funds for Research (FWO) for his fellowship.

338 **Conflict of Interest**

339 Kai Dueffels is employed by Retsch Technology GmbH. The remaining authors declare that
340 they have no financial or other relations that could lead to a conflict of interest.

341 **Figures**

342 **Fig 1** Scatterplot of the EtG concentrations (pg/mg) in finger- and toenails as a function of
343 pulverization time (min). Each line represents data from one subject

344

345 **Fig 2** Scatterplot of the fraction of particles < 600 μm in finger- and toenails as a function of
346 pulverization time (min). Each line represents data from one subject

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