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Reference:

Cappelle Delphine, Neels Hugo, De Keukeleire Steven, Fransen Erik, Dom Geert, Vermassen Annemie, Covaci Adrian, Crunelle Cleo, van Nuijs Alexander.- Ethyl glucuronide in keratinous matrices as biomarker of alcohol use : a correlation study between hair and nails
Forensic science international - ISSN 0379-0738 - 279(2017), p. 187-191
Full text (Publisher's DOI): <https://doi.org/10.1016/J.FORSCIINT.2017.08.022>
To cite this reference: <http://hdl.handle.net/10067/1467150151162165141>

Accepted Manuscript

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PII: S0379-0738(17)30321-3
DOI: <http://dx.doi.org/10.1016/j.forsciint.2017.08.022>
Reference: FSI 8965

To appear in: *FSI*

Received date: 14-4-2017
Revised date: 29-7-2017
Accepted date: 21-8-2017

Please cite this article as: Delphine Cappelle, Hugo Neels, Steven De Keukeleire, Erik Fransen, Geert Dom, Annemie Vermassen, Adrian Covaci, Cleo L. Crunelle, Alexander L.N. van Nuijs, Ethyl glucuronide in keratinous matrices as biomarker of alcohol use: a correlation study between hair and nails, *Forensic Science International* <http://dx.doi.org/10.1016/j.forsciint.2017.08.022>

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Ethyl glucuronide in keratinous matrices as biomarker of alcohol use: a correlation study between hair and nails

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Highlights

- Evaluation of EtG concentrations in hair, finger- and toenails
- EtG levels in hair and nails significantly and positively correlated
- Higher concentrations present in finger- and toenails compared to hair
- Nails as useful alternative to hair for monitoring of alcohol consumption
- Preliminary cut-off values for EtG concentrations in fingernails proposed

Abstract

To quantify alcohol use, objective, specific and sensitive long-term alcohol markers are necessary. Ethyl glucuronide (EtG), a direct metabolite of alcohol, accumulates in keratinous matrices such as hair and nails, and is a specific and sensitive long-term biomarker for the detection of chronic alcohol consumption. So far, research has primarily focused on the detection of EtG in hair, and studies on its measurement in nails are scarce. In this article, we assessed EtG concentrations in hair, finger- and toenails from the same individuals in order to evaluate the direct correlation between the matrices. To this end, a total amount of 45 hair, 41 fingernail, and 13 toenail samples were collected from patients treated for alcohol use disorders at two psychiatric centers in Belgium. Samples were analyzed by gas chromatography-tandem mass spectrometry. Hair EtG concentrations ranged from < LLOQ to 1149 pg/mg (median = 164 pg/mg, IQR [42; 283]). Fingernail EtG concentrations ranged from < LLOQ to 4090 pg/mg (median = 250 pg/mg, IQR [74; 645]). Toenail EtG concentrations ranged from 127 to 3792 pg/mg (median = 687 pg/mg, IQR [379; 1370]). EtG levels in hair and nails were significantly and positively correlated (p-values < 0.001, $r = 0.70$ and $r = 0.62$, respectively). Higher concentrations were present in finger- and toenails compared to hair, which might be attributed to the slower growth rate of nails, resulting in increased accumulation of EtG. Hence, nail analysis may be interesting when low concentrations of EtG are expected, e.g. to discriminate between teetotalers and social drinkers. In addition, the current study proposes preliminary cut-off values for EtG concentration in fingernails: > 123 pg/mg for chronic excessive alcohol consumption, 59-123 pg/mg for moderate alcohol consumption, and < 59 pg/mg for alcohol abstinence. In light of these results, nails may be a useful alternative to hair samples for monitoring of long-term alcohol consumption, e.g., in cases where hair is not available. Further studies are needed to establish cut-off values for EtG levels in nails.

Keywords

Ethyl glucuronide; Alcohol; Keratinous matrices; Nails; Hair; Correlation

Introduction

The harmful use of alcohol is a worldwide problem and causes a large disease, social and economic burden in societies. Alcohol abuse is directly and indirectly responsible for more than 40 diseases most notably alcohol use disorders, liver cirrhosis, cancers and injuries. Overall, 5.1% of global mortality and 4.1% of disability-adjusted life years (DALYs) lost are directly attributable to alcohol [1]. Moreover, both heavy drinking and alcohol use disorders are associated with a range of negative socio-economic effects, e.g. diminished work productivity, traffic accidents, and criminality [2].

There is a growing need for objective, specific and sensitive long-term alcohol markers for both detection and monitoring of harmful and/or chronic alcohol consumption. Clinical and forensic applications for such alcohol markers are multiple: from the assessment of excessive alcohol use (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).

Screening for alcohol abuse through questionnaires can be problematic due to participant's denial or recall bias. Indirect alcohol biomarkers, such as aspartate amino transferase (AST-GOT), gamma-glutamyl transferase (GGT) and mean corpuscular volume (MCV) are mainly correlated to liver damage, and are influenced by age, gender and somatic pathologies. As carbohydrate-deficient transferrin (CDT) has a short half-life (8-12 days), its use to detect chronic alcohol consumption is limited. Direct alcohol markers, such as ethyl glucuronide (EtG), a minor phase II metabolite of alcohol, have the advantage of reflecting direct alcohol presence without the biases that influence indirect measures [3].

EtG accumulates in keratinized matrices, such as hair and nails, where it remains detectable for several weeks to months depending on the length of the hair or nail [4]. In comparison to more 'traditional' matrices, such as blood and urine, hair and nails are particularly interesting owing to their long detection window and their ease of sample collection, transport and storage. Over the past years, the monitoring of alcohol consumption through the detection of EtG in hair has been widespread, since EtG in hair has proved to be a reliable specific and sensitive long-term biomarker for the detection of chronic and excessive alcohol consumption [5, 6]. The Society of Hair Testing (SoHT) has stated its confidence in EtG as a marker for alcohol and has established guidelines and cut-off values for EtG concentrations in hair [7]. An EtG concentration in hair of > 30 pg/mg suggests chronic excessive alcohol consumption, a concentration between 7 and 30 pg/mg corresponds to moderate alcohol consumption, and an EtG concentration of < 7 pg/mg does not contradict self-reported abstinence [8].

Research has primarily focused on the detection of EtG in hair, whereas studies on its measurement in nails are sparse [9, 10]. However, nail analysis offers multiple advantages over hair analysis [11, 12]. 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), and would thus allow a more significant accumulation of EtG [13]. 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 can provide an alternative option in cases where hair is not (sufficiently) available (e.g., alopecia, newborns). Third, cosmetic hair treatments have been proven to reduce EtG content in hair, which would be avoided through nail analysis [11, 14]. Possible disadvantages of nails can be insufficient sample amount in cases of (finger)nail biting, presence of nail diseases (e.g. bacterial or fungal infections), and effects of nail polishing or cleaning.

To the best of our knowledge, only two studies compared the presence of EtG in hair and nails [15, 16]. The studies included hair and fingernails collected from a group of college-students with a limited alcohol consumption. Only Berger et al. 2014 considered cosmetic treatment of hair and nails. In the current study, we evaluated EtG concentrations in paired hair and nail samples (both finger- and toenails), from real-life alcohol dependent patients, to investigate the differences and correlations between both matrices. In this way, we evaluated the applicability of nail samples as an alternative to hair for the monitoring of long-term alcohol consumption.

Materials and methods

Reagents

Ethyl glucuronide (EtG) and the internal standard ethyl glucuronide-D₅ (EtG-D₅) in methanol were purchased from Medichem (Stuttgart, Germany). Pentafluoropropionic anhydride (PFPA) 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. 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.

Sample collection

Hair and nail samples were collected from patients engaged in treatment for alcohol use disorders at the psychiatric centers of ZNA Stuivenberg and Multiversum together with an informed consent. The Ethical Committee of the University Hospital of Antwerp (UZA) and the local Ethical Committees of ZNA Stuivenberg and Multiversum approved the study (Belgian registration number B30020169233). Hair samples were collected from the vertex posterior region of the head and cut as closely to the scalp as possible. The first 3 cm segment from the proximal end was used for further analysis. Nail samples were obtained by clipping of the distal edges of all ten finger- and toenails. Finger- and toenail samples were collected and analyzed separately. Samples were stored in aluminum foil at room temperature until analysis.

Data on alcohol consumption

For all patients, the diagnosis of alcohol use disorder was made by the treating psychiatrist. Together with the hair and nail samples, a detailed anamnesis of past alcohol consumption was taken. Patients were asked about their alcohol consumption in the past 12 weeks using either the Timeline Follow-back (TLFB) method or an in-house questionnaire. The total amount of alcohol (in grams) consumed in the past 12 weeks, and per week, was calculated for each subject.

Data on cosmetic treatment

Patients were asked whether or not they had bleached, dyed, permed or thermally straightened their hair in the past year, as cosmetic hair treatment may lead to a degradation or removal of EtG in hair [8, 17]. Nail polishing, the use of acetone and other nail treatment were also recorded. Patients reporting any cosmetic treatment of the hair or nails were excluded from the study.

Sample preparation and materials

Nail and hair samples were processed according to a previously described method [18, 19]. Briefly, samples were washed with water and acetone, dried, cut into 1 - 2 mm pieces, and pulverized for 5 min at 30 Hz in a ball mill of type MM400 (Retsch, Haan, Germany). Then, an accurately weighed portion between 15 and 35 mg of each sample was transferred to a tube. To this tube, 2 mL of water was added and the samples were ultrasonicated for 2 h with an ELMA TI-H-15 ultrasonication bath (Elma Hans Schmidbauer GmbH & Co. KG, Singen, Germany). Next, samples were spiked with 2.4 ng of EtG-D₅ as internal standard, vortexed, and centrifuged at 5000 rpm for 10 min using a Sigma centrifuge (Osterode am Harz, Germany). Solid-phase extraction was then performed by transferring the supernatant to Oasis® MAX (60 mg, 3 mL) extraction cartridges which were acquired from Waters (New Bedford, MA, USA). The cartridges were placed on a Supelco Visiprep™ SPE Vacuum Manifold (Bellefonte, CA, USA) with 24 ports, and conditioned with 2 mL of methanol and 2 mL of water. After loading of the samples, the cartridges were washed with 1 mL of water/ammonia solution (95/5, v/v) 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 using a Pierce Reacti-Therm III Heating Module (Rockford, IL, USA). The residues were derivatized with PFPA (30 min, 70 °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 into the gas chromatography-tandem mass spectrometry (GC-MS/MS) system.

GC-MS/MS instrumentation and conditions

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). Chromatographic separation was achieved on a HP-5 MS (5% phenyl methyl siloxane) column (15 m × 0.25 mm × 0.25 µm).

The GC-MS/MS procedure was based on a previously validated method for the detection of EtG in hair and in nails [18, 19]. The method has a lower limit of quantification (LLOQ) of 2 pg/mg. The injector temperature was set at 150 °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 ionization source was operated in negative ion chemical ionization (NICI) mode at 150 °C. Data acquisition was performed in multiple reaction-monitoring (MRM) mode. The monitored ion transitions were m/z 496 → 163 (quantifier) and 347 → 163 (qualifier) for EtG, and m/z 501 → 163 for EtG-D₅.

Data analysis

Statistical analysis was performed using R (version 3.3.1., The R Foundation for Statistical Computing). Normality was tested through histograms and QQ-plots of the raw data. Due to non-normality of the data, further analyses were performed with non-parametric statistics. Differences in median concentrations were assessed using Wilcoxon signed rank test. The associations between hair and nails (finger- and toenails) were expressed using Spearman correlation. For the correlation between hair and fingernails, an 80% prediction interval was calculated. In addition, using the regression coefficients β_0 and β_1 , the existing cut-off concentrations for EtG in hair [8] were translated to preliminary cut-off concentrations in fingernails. For all statistical tests, a p -value < 0.05 was considered statistically significant. Results are presented as mean ± standard deviation (SD) or as median [interquartile range (IQR)].

Results

A total number of 45 patients engaged in treatment for alcohol use disorders were included in the study. Eleven participants were female and 34 participants were male. Patients were between 18 and 77 years old with a mean age of 45.3 ± 13.1 years. Nine patients provided hair, finger- and toenail samples, 32 patients provided hair and fingernail samples, and 4 patients provided hair and toenail samples. To collect information on past alcohol consumption the TLFB method was used in 26 patients, while the in-house questionnaire was used in 19 patients. Median alcohol consumption in the past 12 weeks was 11507 g (IQR [5475; 18927]). Median alcohol consumption per week was 959 g (IQR [456; 1577]).

Comparison of medians

Hair EtG concentrations ranged from < LLOQ to 1149 pg/mg (median = 164 pg/mg, IQR [42; 283]). Fingernail EtG concentrations ranged from < LLOQ to 4090 pg/mg (median = 250 pg/mg, IQR [74; 645]). Toenail EtG concentrations ranged from 127 to 3792 pg/mg (median = 687 pg/mg, IQR [379;

1370] pg/mg). The differences in median EtG concentrations between paired hair and fingernail samples, and between paired hair and toenail samples were significant (p-values < 0.001).

Correlations

Figure 1 displays the scatterplots of the three correlations. Two of these were statistically investigated, and were found to be positive and significant. The Spearman-correlation coefficient between EtG concentrations in hair and in fingernails was 0.70 (p-value < 0.001, $\beta_0 = 3.11$, $\beta_1 = 0.50$, see Figure 2). For this correlation, the 80 % prediction interval was calculated (see Figure 3). This interval around the regression line contains 80% of the individuals' observations, which means that for a given EtG concentration in hair, the prediction interval delineates the range in which the corresponding EtG concentration in fingernails will be situated for 80% of the individuals. Thus, the prediction interval in combination with the regression coefficients β_0 and β_1 enable to make some preliminary predictions of cutoff concentrations for fingernail analysis based on those that exist for hair analysis (see Table 1). The correlation coefficient between EtG concentrations in hair and toenails was 0.62 (p-value = 0.02, $\beta_0 = 4.96$, $\beta_1 = 0.36$).

Discussion

Previous research has indicated that there is a medium to strong correlation between alcohol consumption and the concentration of EtG in hair [20]. EtG concentrations in the proximal 3 cm of head hair reflect the alcohol consumption over the past 3 months. Therefore, EtG in hair is used as a long-term marker for alcohol consumption and cut-off values have been proposed by the SoHT to discriminate between abstinence, social, and excessive drinking. This study investigates the correlation of EtG concentrations in two keratinous matrices: hair and nails. Our results confirm that there is a significant and positive correlation between EtG concentrations in hair and in nails (both finger- and toenails). In addition, median concentrations are significantly different between hair and nails (both finger- and toenails). These findings are in agreement with those reported in earlier studies by Jones et al. and Berger et al. [15, 16] and suggest that nails can be used as an additional and/or alternative matrix for long-term alcohol consumption monitoring.

Concentrations in nails were higher in nails compared to hair, which might be attributed to the slower growth rate of nails (1 cm/month for head hair compared to 3 mm/month and 1.1 mm/month for fingernails and toenails, respectively [11, 21]), resulting in higher accumulation of EtG in nails. This observation has two important implications: (i) analysis of nails can be interesting when low concentrations of EtG are expected, i.e. in the case of discrimination between teetotalers and social drinkers; and (ii) cut-off values as established for hair analysis by the SoHT are not valid for nail analysis and future studies should focus on establishing cut-off values for EtG which are relevant for nails. Based on the calculated 80% prediction interval, we already propose preliminary cutoff values for EtG concentrations in fingernails. An EtG concentration of > 123 pg/mg fingernail would suggest chronic

excessive alcohol consumption, a concentration between 59 and 123 pg/mg fingernail would point towards moderate alcohol consumption, and an EtG concentration of < 59 pg/mg fingernail would indicate alcohol abstinence. Due to the fact that some participants were already engaged in treatment for alcohol use disorders for a longer period of time, we could include some participants that had been abstinent for several days to weeks. This is reflected in the lower concentrations of EtG detected in the hair of some participants (see Table S1 in supplementary material). Therefore, although the participants were collected in a group of alcohol abusers, we had a relatively good spreading of the amounts of alcohol consumed. The proposed cut-offs are based on a limited amount of samples and need to be confirmed in future studies.

Due to the limited number of paired finger- and toenail samples ($N = 9$), the difference in median concentrations and the correlation were not statistically evaluated. From the presence of a correlation between hair and nails, and the similarity between finger- and toenails, one can expect a correlation between finger- and toenails will be present. Additionally, due to the slower growth rate of toenails compared to fingernails, higher concentrations can be expected in toenails. The average growth rate of fingernails is 3.0 mm per month, while toenails grow at an average rate of 1.1 mm per month [11]. To the best of our knowledge, this article is the first to report EtG concentrations in both finger- and toenails. However, the number of toenail samples was rather small ($N = 13$), and therefore more research is needed to investigate the proposed hypotheses.

In comparison to the two articles that previously investigated EtG concentrations in hair and fingernail samples from the same subject [15, 16], the current article encompasses a wider range of EtG concentrations (not only low to moderate alcohol consumption), a wider range of ages (not only college-aged students) and the collection of toenails. This study also includes the registration and exclusion of cosmetic treatments.

Conclusion

Today, the detection of EtG in hair samples is used in routine laboratories to evaluate long-term alcohol consumption. This study shows that EtG concentrations in hair and nails (both finger- and toenails) are significantly and positively correlated. In addition, higher concentrations were observed in nails compared to hair. The results of this study indicate that nails can be a suitable alternative for hair. In view of this, we propose some preliminary cut-off values for fingernail analysis, but future studies are needed confirm these.

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 postdoctoral fellowship. We dedicate this paper to Prof. Dr. Sandra Apers of the NatuRA research

group of the Department of Pharmaceutical Sciences of the University of Antwerp who passed away too early.

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Captions to figures

Figure 1: Matrix of scatterplots showing the different correlations in EtG concentrations investigated: hair versus fingernails, hair versus toenails, and fingernails versus toenails. The values for the variables were log-transformed.

Figure 2: Correlation between EtG concentrations in hair and fingernail samples. The values for both variables were log-transformed.

Figure 3: Plot of the log-transformed hair concentrations versus log-transformed fingernail concentrations. The solid line shows the linear regression line. The dashed lines show the 80% prediction interval, which is the interval around the regression line containing 80% of the individuals' observations.

Table 1: Preliminary predictions of cutoff values for fingernail analysis based on those that exist for hair analysis.

	Cutoff value in hair	Predicted cutoff value in fingernails
Excessive alcohol consumption	> 30 pg/mg	> 123 pg/mg
Moderate alcohol consumption	7-30 pg/mg	59-123 pg/mg
Alcohol abstinence	< 7 pg/mg	< 59 pg/mg