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Ethyl glucuronide as a long-term alcohol biomarker in fingernail and hair. Matrix comparison and evaluation of gender bias.

Running head:

EtG as alcohol biomarker in fingernail and hair.

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Key words

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Abstract

Aims: This work aimed to assess the performance of hair and fingernail ethyl glucuronide (EtG) measurement for use as a biomarker of alcohol consumption in persons with known drinking history across a range of drinking behaviours.

Methods: EtG concentrations were assessed from the hair and fingernails of 50 study participants. Alcohol consumption of the previous 90 days was assessed by participant interview using the alcohol time-line follow-back (TLFB) method. EtG concentration was determined using LC/MS-MS using a method which was validated and accredited to ISO/IEC 17025 standards.

Results: There was significant correlation between alcohol consumption and EtG concentrations found in hair and fingernail samples across the study group (n=50). From participants testing positive for EtG (male n=14, female n=13) no significant difference was found between male and female EtG levels in either hair or fingernails. Across all participants there was no significant difference in hair or fingernail EtG concentration between male (n=23) and females (n=27).

Conclusions: Our results support the use of EtG to indicate alcohol consumption over the previous 90 days, or approximately 3 months as is the normal practice in hair analysis. The results confirm that fingernails can be a useful alternative matrix where hair samples are not available.

Short Summary: Hair and fingernail samples were collected from 50 participants who provided details of their previous 90 days alcohol consumption. EtG concentration was measured in the samples using LC/MS-MS. Results showed significant correlation between alcohol consumption and EtG concentration in hair and fingernails. No gender bias was observed.

Introduction

The sensitivity and efficacy of the detection of ethanol biomarkers remains of paramount importance to the fields of legal medicine and forensic toxicology. In recent year keratinous matrices have gained reputation in the forensic sector as reliable mediums to detect such biomarkers of ethanol consumption, in particular ethyl glucuronide (EtG).

EtG, a direct and long-term biomarker of ethanol consumption, has been a valuable asset in clinical and forensic toxicological analysis (Cappelle *et al*. 2017). Upon glucuronidation in the liver, EtG diffuses into and can subsequently accumulate in keratinous tissue via the bloodstream (Keten *et al*. 2013) and through sweat. The latter of which being applicable to EtG accumulation in hair.

Following detection in keratinous specimens, EtG presents the potential to reflect and monitor patterns of ethanol consumption. This is inclusive of periods of abuse or abstinence (Pragst and Yegles 2008; Hastedt *et al*. 2013;), which can be highly significant.

Scalp hair analysis is a reliable medium over more traditional screens for alcoholic metabolites (Stewart *et al*. 2013). As such, its applicability in the detection of EtG has been widely studied (Pragst *et al*. 2010; Lees *et al*. 2012, Suesse *et al*. 2012, Wetterling *et al*. 2014).

However, scalp hair as a medium for the detection of EtG has some limitation. This is with particular regard to the self-reported condition of the hair. This includes, but is not limited to, bleaching and colouring (Sporkert *et al*. 2012; Crunelle *et al*. 2015; Petzel-Witt *et al*. 2018), but the use of hair care products has been shown only to affect fatty acid ethyl esters levels, whilst EtG was unaffected (Gareri *et al* 2011). Conditions such as alopecia, male pattern baldness can also affect the sample collection of hair and the results of any subsequent examination (Cappelle *et al*. 2017). Pre-existing kidney and liver pathologies (Stewart *et al*. 2013) and seasonal changes (Salomone *et al.* 2015) also have been demonstrated to affect EtG concentrations in hair.

Nail specimens have been demonstrated to be a useful alternative to the examination of hair. However, and as noted by Cappelle *et al* (2017), studies into the efficacy and ability of fingernails to demonstrate alcohol consumption are limited in their numbers. It must also be noted that like hair, nail specimens are also subject to external influences which may alter the concentrations of EtG within the nail. Nail biting, diseases of the nail and the cosmetic treatment of nails (Cappelle *et al* 2017) may all contribute to insufficient sample collection or, indeed, the quality of the analysis of EtG.

In spite of this, Cingolani *et al* (2004) found nail samples to be more useful than hair specimens in terms of the available concentrations of drugs of abuse and their metabolites. Morini *et al* (2012) seemingly support this, reporting that EtG in one volunteer accumulated at a higher rate in nails than in hair. While it is evident that both keratinous matrices possess unique benefits in the detection of EtG and indeed, of biomarkers of drugs of abuse, the comparability of both mediums to each other has not been widely studied. Cappelle *et al* (2017) demonstrate one such study where a significant and positive correlation was noted between the two mediums.

Fosen *et al* (2017) noted that EtG disappeared from fingernail specimens faster than expected. This is of interest when using nails to determine abstinence. Berger *et al*. (2014) showed that that both keratinous matrices were useful in the identification and detection of EtG. However, it was noted in this study that hair specimens acted as a more qualitative indicator of EtG, an observation in alignment with Morini *et al*. (2012), whilst nails provided a more quantitative insight.

Similarly, Jones *et al*. (2012) suggested that, on comparison, fingernails may be regarded as the more effective medium to detect EtG. This was due to a higher detected concentration of EtG in this specimen type and through a lack of observed gender bias in the investigation of nail specimens. Jones *et al*. (2012) further concluded that nails should be used as an

additional tool in the arsenal of assays that can be used to investigate patterns of ethanol consumption.

While the above studies have given valuable insight into the comparability of hair and fingernail specimens in the detection of EtG, the true extent of this is not fully understood. The present paper represents an effort to further elucidate the comparability of hair and fingernail specimens in their respective abilities to detect EtG. In doing so, it is hoped that the applicability of fingernails as a detection medium, and EtG as a long-term biomarker of ethanol consumption can be further demonstrated.

Materials and methods

Participants

50 participants were recruited from the Dorset area of the United Kingdom either via email or telephone. Participants were interviewed by trained personnel about their past 90 days alcohol use and then completed a self-reported alcohol consumption proforma. Alcohol consumption of the prior 90 days was collected using the Alcohol Timeline Follow Back (TLFB) Interview (Sobell *et al*., 1986). The TLFB is well validated in college students, community residents and in participants in alcohol treatment, to address retrospective alcohol consumption for time frames of ≤1 year (Sobell *et al*., 1986). Our inclusion criteria were (a) participants should have >3 cm length scalp hair, and sufficient fingernail to provide clippings >5 mg; (b) participants provide detailed alcohol intake of the prior 90 days before sample collection using a modification of the Alcohol TLFB. Our exclusion criteria were: Individuals with nail varnish or nail treatments, bleached hair, with cosmetically straightened and/or permed hairs, and with gastrointestinal-, kidney- or liver-pathologies were excluded.

Head hair was cut from participants from the vertex posterior at a thickness approximately of a pencil and stored in aluminium foil indicating the root end. Participants were provided with nail clippers and asked to provide up to 10 of their own fingernail clippings.

EtG measurement

50 hair samples and 50 fingernail samples were analysed for EtG concentration at Cansford Laboratories (www.cansfordlabs.co.uk). All samples were washed before extraction and analysed for EtG by LC/MS-MS utilising an in-house method which was validated and accredited to ISO/IEC 17025 standards for specificity, sensitivity, linearity, accuracy, precision, recovery. The LC/MS-MS analysis was performed on an Agilent 1200

HPLC stack coupled to an Agilent 6495 triple quadrupole mass spectrometer equipped with an electrospray ionization source (ESI) (Agilent Technologies, Santa Clara, CA, USA). The analysis was performed in negative ion mode with a -3500 V charge on the capillary. The gas temperature was set to 210 °C with a flow of 13 L/min. The nebuliser was set to 30 psi and the electron multiplier voltage (EMV) was set at 500 V. Data acquisition was performed in multiple reaction monitoring (MRM) mode with positive ESI using one principal MRM transition (85.2) for quantitation and one additional transition to serve as a qualifier (75.3). Ultra high purity nitrogen gas was used as the collision gas. The HPLC column used was the Acquity UPLC HSS T3 C18, 3x50mm, 1.8 µm column (Waters, UK) with a flow rate of 0.350 mL/min. Gradient separation was achieved using methanol containing 0.1% formic acid and 18 MΩ water containing 0.1% formic acid. 10 µL of the extracted hair sample (or standard) containing deuterated internal standards was injected onto the column.

Calibration curves for quantitative analyses of EtG were obtained in the concentration range 6.8 and 487.8 pg/mg of hair with a coefficient of correlation (R^2) better than 0.98 for EtG when spiked to blank hair samples and submitted to the extraction method. Inter-run repeatability (determined by the relative standard deviation) was <20%. Cut-offs used in this study were 7 pg/mg for hair and fingernail.

Alcohol consumption

The accuracy of recorded alcohol consumption from our participants was maximised by our collection personnel through giving guidance on standard drink measures (our guidance was that one standard drink is equivalent to one 330ml can/bottle of 5% alcohol content beer, or 175ml of 12% alcohol content wine), and by giving examples as to how many standard drinks different styles of alcoholic drinks may contain. Participants were encouraged to use personal calendars to aid recall, and to consider their regular drinking patterns. Participants were interviewed to collect data on nail varnish / polish application, hair bleaching or

straightening / perming and presence of gastrointestinal conditions as detailed above to aid application of our inclusion / exclusion criteria.

Analyses

Normality was tested using histograms and QQ-plots of the raw data. The data showed non-normality and as such non-parametric statistics were applied to further data assessment. Correlation between alcohol consumption and EtG levels in hair and fingernails was assessed using Spearman correlation. Difference in median concentration between hair and fingernail EtG was determined using Wilcoxon signed rank test. Difference in hair EtG between male and females, and fingernail EtG between male and females was assessed using Mann-Whitney U test.

For qualitative analysis drinking groups were defined as low (0 -90 standard drinks in 90 days for men, and 0 – 45 standard drinks in 90 days for women), moderate (between 90 and 180 drinks in 90 days for men, and 45 to 90 drinks in 90 days for women) and high (over 180 drinks in 90 days for men, and over 90 drinks in 90 days for women). These levels were decided upon as a result of US National Institute on Alcohol Abuse and Alcoholism guidance on drinking levels which defines moderate drinking as 1 drink per day for women and 2 drinks per day for men. Our levels were extrapolated out to the 90 day time period. Alcohol consumption of study participants is summarised in Table 1.

This study received ethical approval from Bournemouth University ethics committee (reference 20404) and all subjects gave informed consent.

Table 1. Summary of alcohol consumption of participants during the assessed time period.

Male study participants	Number of participants	Average number of standard drinks consumed in 3 months
Low drinker	16	38.8
Moderate drinker	4	136
High drinker	3	293
Female study participants		
Low drinker	17	13.9
Moderate drinker	3	64
High drinker	7	153.6

Results

Table 2 displays hair and fingernail EtG concentrations from all participants (n=50), grouped according to gender and the following drinking categories as defined earlier: low, moderate, high. In brackets are data for participants testing positive for EtG in both hair and fingernails (n=21). 54% of participants in this study were female, and the average age in the group was 37.5 years. 92% of participants reported drinking alcohol, 8% abstained from alcohol, 14% drank moderately and 20% were classed as heavy drinkers. Hair EtG concentration ranged from 0 to 119 pg/mg whilst fingernail EtG ranged from 0 to 102 pg/mg. Across the study group both hair and nail matrices showed good agreement, with only 6 of 50 samples testing positive in one matrix, and negative in the other. These 6 samples displayed low EtG concentrations ranging from 2 – 8 pg/mg.

The Spearman correlation coefficient between alcohol consumed and EtG levels in hair was 0.5182, p = 0.00562 and statistically significant. The Spearman correlation coefficient between alcohol consumed and EtG levels in fingernails was 0.66539, p = 0.00015 and statistically significant.

Median concentration of EtG from female participants was 12 pg/mg in hair, and 16 pg/mg in fingernails. Median concentration of EtG from male participants was 10.5 pg/mg in hair, and

8.5 pg/mg in fingernails. Wilcoxon signed rank was used to explore the difference in median EtG concentrations between paired hair and nail samples and the results were not significant ($p = >0.05$).

From our 50 participants mean hair EtG concentration was 9.1 pg/mg, and mean nail EtG concentration was 9.9 pg/mg. When examined by gender, females (n=27) displayed mean hair EtG concentration of 4.9 pg/mg and mean fingernail EtG concentration of 9.1 pg/mg, whereas male participants (n=23) showed mean hair EtG concentration of 14.1 pg/mg and 10.9 pg/mg in fingernails.

When considering only the participants that tested positive for EtG in both hair and fingernails (n=21) the results are as follows: mean hair EtG concentration was 16.4 pg/mg, and mean nail EtG concentration was 23 pg/mg. When examined by gender, females (n=11) displayed mean hair EtG concentration of 11.5 pg/mg and mean fingernail EtG concentration of 22.5 pg/mg, whereas male participants (n=10) showed mean hair EtG concentration of 21.9 pg/mg and 23.7 pg/mg in fingernails (Table 2).

From participants testing positive for EtG in either hair or fingernail samples (male n=14, female n=13), analysis was conducted to investigate gender bias. Mann Whitney U test showed no significant difference found between male and female EtG levels in either hair ($p=0.94$, not significant at $p <0.05$) or fingernails ($p = 0.65$, not significant at $p < 0.05$). Across all 50 participants there was also no significant difference in hair EtG concentration between male (n=23) and females (n=27) ($p = 0.60$, not significant at $p <0.05$), or fingernail EtG concentration between male and females ($p = 0.64$, not significant at $p < 0.05$).

Table 2. Hair and fingernail EtG concentrations from all participants (n=50), grouped according to gender and reported drinking category. In brackets are the results from participants testing EtG positive in both hair and fingernails (n=21).

			Mean pg/mg	**Standard Deviation** pg/mg	**N**
Hair EtG	Category	Male Low Drinker	10.2 (26.7)	29.3 (45.3)	16 (6)
		Female Low Drinker	2.3 (12.3)	6.7 (13.1)	17 (3)
		Male Moderate Drinker	4.5 (9)	5.4 (2.8)	4 (2)
		Female Moderate Drinker	8 (10)	5.3 (5.6)	3 (2)
		Male High Drinker	47.7 (20.5)	47.1 (3.5)	3 (2)
		Female High Drinker	9.9 (11.5)	7.8 (7.1)	7 (6)
Fingernail EtG	Category	Male Low Drinker	4.3 (10.5)	9.6 (14.1)	16 (6)
		Female Low Drinker	1.4 (7.7)	3.6 (5.7)	17 (3)
		Male Moderate Drinker	13 (22)	14.6 (17)	4 (2)
		Female Moderate Drinker	10.7 (16)	13.6 (14.1)	3 (2)
		Male High Drinker	43.3 (65)	52.7 (52.3)	3 (2)
		Female High Drinker	27.4 (32.0)	22.4 (20.7)	7 (6)

Discussion

Our results showing the correlation between alcohol intake and levels of EtG in hair, support the use of EtG to indicate alcohol consumption over the previous 90 days, or approximately 3 months as is the normal practice in hair analysis. The correlation between alcohol consumption and levels of EtG in fingernails confirms that fingernails can be a useful alternative matrix where hair samples are not available. At this time the prevalence of research supporting the incorporation and binding mechanisms of EtG in hair make this matrix perhaps a better choice than fingernails while the research is comparatively lacking for fingernail biology. The Society of Hair Testing recommend cut-offs for EtG hair testing to define abstinence (< 7 pg/mg) and chronic excessive alcohol consumption (> 30 pg/mg) (Society of Hair Testing. 2016). Analysis of our study data suggests that these cut-offs are appropriate when all participants reporting low and moderate levels of drinking (n=40) tested below 30 pg/mg, with the exception of 1 male. The male volunteer in question reported consumption of 81 standard drinks in 90 days, and had an EtG concentration of 119 pg/mg and 39 pg/mg in hair and fingernail respectively. This result may have arisen as a result of under reporting of alcohol consumption. An alternative explanation could be that this volunteer may have exposed his hair to an alcohol-based perfume during the study period. Morini *et al* (2018) demonstrated that prolonged exposure of hair to alcohol-based perfumes may increase hair EtG levels, resulting in false positives.

Our results show a significant and positive correlation between alcohol consumption and EtG values in both hair and nails, whilst median concentrations were not statistically different between the two matrices. Very few studies to date have directly compared EtG levels in hair and fingernails. These studies are shown in Table 3. Many of the reported studies, including ours, have not been absolutely controlled in terms of participant alcohol consumption, as it was based on people's declaration of their alcohol consumption. Whilst efforts have been made to ensure reliable self-reporting of alcohol consumption this is often an area where error may arise. In addition, the analysis of EtG levels in hair as compared to fingernails will

be affected by hair bleaching and dyeing. The process of bleaching and / or dyeing may lower EtG concentration in hair, therefore artificially increasing the difference in EtG levels when comparing hair to fingernails. Petzel-Witt *et al* suggested a possible gender bias for EtG determination in hair, likely caused by the influence of bleaching and colouring on EtG concentration. They showed a decrease in EtG content of at least 10% in a controlled study where hair samples analysed for EtG were re-analysed following various treatment protocols. In their study, permanent bleaching and dyeing showed the most severe reduction in EtG levels. Our results, when examined by gender, showed that females (n=27) had mean hair EtG concentration of 4.9 pg/mg and mean fingernail EtG concentration of 9.1 pg/mg, whereas male participants (n=23) showed mean hair EtG concentration of 14.1 pg/mg and 10.9 pg/mg in fingernails. However these results were not statistically different. Our results in hair confirm those of Crunelle *et al* (2014) who demonstrated that no gender bias exists for EtG in hair.

Table 3. Published research on the evaluation of EtG in hair and nails, sorted by number of cases studied.

Source of EtG	Matrix displaying highest EtG levels	Number of study participants	Analytical methodology	Age of participants	Gender of participants *	Monitoring duration	Reference	Summary of conclusions
Hair and Nails	Nails	606	LC/MS-MS	18-25	Male and **Female**	3 months	(Berger *et al*., 2014)	In both hair and fingernails at three measured EtG thresholds, sensitivity was greatest for high risk drinking group. Proposed threshold for EtG in nails of 56 pg/mg for high risk drinking.
Hair and Nails	Nails	606	LC/MS-MS	18-26	Male and **Female**	NA	(Jones *et al*., 2012)	EtG levels in fingernail were higher than in hair, and correlation of matched pairs was observed.
Hair, Nails and Meconium	Hair and nails were all negative	151	LC/MS-MS	32 (mean)	**Female**	9 months	(Morini *et al*., 2013)	EtG in hair and nails lack sensitivity to detect lower that daily alcohol consumption and lower than 1-2 units per day.
Hair and Nails	No significant difference	50	LC/MS-MS	37.5 (mean)	Male and **Female**	3 months	This work	Correlation observed between alcohol consumption and EtG concentrations in hair and fingernail samples. No significant difference in hair or fingernail EtG concentration between male and female participants.
Hair and Nails (finger and toe)	Nails	45	GC/MS-MS	18-77	**Male** and Female	3 months	(Cappelle *et al*., 2017)	EtG concentration in hair and nails were correlated. EtG was higher in finger and toe nails than in hair. Proposed EtG cut-offs for fingernails of >123 pg/mg for chronic excessive alcohol consumption and <59 pg/mg for abstinence.
Hair and Nails	Nails	40	LC/MS-MS	47 (mean)	**Male** and Female	3 months	(Fosen *et al*., 2017)	EtG was higher in nails than in hair, and correlated in the two matrices. EtG disappeared from nails after approx. 2 months abstinence. Proposed higher cut-off for EtG in nails, than in hair.

***** The gender with the most subjects is shown with **bold** font.

Conclusion

Our findings support the use of EtG as a biomarker of alcohol consumption over the previous 90 days using either hair or fingernail samples where results showed a significant correlation between alcohol consumption and EtG concentration between both matrices. Our results showed no significant difference between paired hair and nail samples, and no gender bias in either matrix, suggesting that whilst hair may remain the medium of choice to demonstrate alcohol consumption over longer periods of time, fingernails are also a viable matrix for this purpose. Both hair and fingernail samples are easy to collect, and serve as a reliable alcohol use monitoring strategy. Public health practitioners as well as reporting scientists must be aware that hair and fingernails both display advantages and limitations when monitoring EtG concentration. With proper consideration of factors such as matrix growth rate and effect of cosmetic treatment, EtG testing in hair and fingernails can provide long-term quantitative data on drinking behaviours. Such data though must not be used in isolation. Blood alcohol tests, clinical assessments and medical and social history can be usefully combined with hair and nail alcohol biomarker testing to provide a comprehensive overview of an individual's drinking behaviour.

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