

Short article / Article court

Testing for alcohol use in hair: is ethyl glucuronide (EtG) stable in hair?

Dépistage de la consommation d'alcool par l'analyse des cheveux : l'éthyl-glucuronide (EtG) est-il stable dans les cheveux ?

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Abstract – Purpose: Ethyl glucuronide (EtG) is a biological marker in hair used to indicate abstinence or excessive alcohol consumption. The main aim of this paper is to evaluate the stability of EtG in hair samples as it can potentially be affected by normal hygiene, and affect interpretation of the results. **Methods:** EtG was measured by GC-MS/MS in 102 hair samples, which were sectioned in three monthly sections when available to produce 291 sections of hair (sectioned set) and 468 hair samples (not sectioned set), where the most recent centimetre was analysed. **Results:** The 95% percentiles of the EtG levels detected in the not sectioned set and in the first section of the sectioned set were 0.23 ng/mg ($N = 468$) and 0.22 ng/mg ($N = 102$), respectively. The 95% percentiles of the levels detected in the second sections and in the third sections were 0.15 ng/mg ($N = 102$) and 0.10 ng/mg ($N = 87$), respectively. Levels were below cut-off (0.01 ng/mg) in 61% in the not sectioned set and 67% in the sectioned set. Of the samples where EtG was detected, the second section samples showed mean EtG levels 74% lower the levels detected in the first section. The mean levels detected of EtG in third section were 62% the levels of the second section and 47% the levels detected in the first section. Analysis of variance showed the levels of the third section significantly lower ($p < 0.05$) than the first section. **Conclusion:** The results of this study suggest that normal hair hygiene might wash out EtG from the hair. The recommendation is therefore that only the most recent month must reliably monitor abstinence or chronic alcohol abuse using head hair and that data should be evaluated in conjunction with other biochemical tests and clinical evaluation.

Key words: Hair analysis, ethyl glucuronide, alcoholism

Résumé – Objectif : L'éthyl-glucuronide (EtG) est un marqueur biologique dont le dosage dans les cheveux permet d'évaluer la non-consommation ou la consommation excessive d'alcool. L'objectif principal de cet article est d'évaluer la persistance de l'EtG dans les échantillons de cheveux sans traitement capillaire particulier, à prendre en compte dans l'interprétation des résultats. **Méthodes :** La concentration d'EtG a été quantifiée par GC-MS/MS dans 102 échantillons de cheveux coupés en trois segments correspondant chacun à un mois de croissance, lorsque cela s'est avéré possible. Nous avons pu étudier 291 segments (échantillons segmentés) et 468 échantillons de cheveux entiers (échantillons non segmentés) où le centimètre le plus récent a été analysé. **Résultats :** Les 95^{ème} percentiles des concentrations d'EtG dans les cheveux étaient de 0,23 ng/mg ($N = 468$) pour les cheveux non segmentés et respectivement de 0,22 ng/mg ($N = 102$), 0,15 ng/mg ($N = 102$) et 0,10 ng/mg ($N = 87$) pour les premier, second et troisième segment de cheveux analysés. Les concentrations étaient en-deçà du cut-off (0,01 ng/mg) dans 61 % des échantillons non segmentés. Dans les échantillons où l'EtG a été détecté, ceux du deuxième segment ont révélé des niveaux moyens d'EtG 74 % inférieurs à ceux du premier segment. La concentration moyenne d'EtG détectée dans le troisième segment ne s'élevait qu'à 62 % de la concentration dans le deuxième segment, et à 47 % de la concentration dans le premier. Une analyse de variance a montré des niveaux dans la troisième section significativement inférieurs ($P < 0,05$) à ceux de la première section. **Conclusions :** Les résultats de cette étude suggèrent que les soins capillaires courants peuvent éliminer l'EtG des cheveux. Seuls les cheveux correspondant au mois de croissance le plus récent permettraient alors d'évaluer de façon fiable la non-consommation ou la consommation excessive d'alcool ; cette notion serait à confirmer par d'autres analyses biochimiques et examens cliniques.

Mots clés : Cheveux, éthyl-glucuronide, alcoolisme

Travail présenté au 46^{ème} congrès TIAFT, juin 2008, La Martinique

Received 25 September 2008, accepted after revision 26 March 2009

Published online 12 June 2009

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1 Introduction

Due to its ability to detect drugs retrospectively, the usefulness of the detection of drugs in hair is now widely accepted. In the last years an increasing number of papers and reviews have been published, enabling the scientific understanding of issues concerning the detection of drugs in hair [1–7]. Hair analysis differs significantly from other biological samples such as urine or oral fluids in that it provides a longer window of detection. This is particularly relevant for use for drug detection in the workplace, in medico-legal cases and to monitor the success of treatment in a clinical setting as it can prove drug use or abstinence historically.

Alcohol is consumed in much higher quantities by a higher number of people in comparison to the consumption patterns of other drugs. Nonetheless the usefulness of biological markers of alcohol misuse, such as the measurement of ethyl glucuronide (EtG) in hair is still under evaluation. Alcohol is a drug very commonly used and misused worldwide, and its use affects directly families, children and the overall economy due to premature death, absenteeism and violent crime. Because of the scale of the problems related to alcohol misuse, the monitoring of use and abstinence is in great demand. Traditionally, biochemical tests, direct and indirect alcohol markers, have been used in the follow up of people misusing alcohol, however, no single test performed in one occasion is sufficient to provide evidence that a person uses alcohol in large quantities and regularly or to disprove this satisfactorily [8].

Because hair grows at a reasonably constant rate of one centimetre per month, with a range of 0.7 to 1.5 centimetre per month [9, 10] analysis of EtG in one-centimetre sections of hair in theory could give a pattern of alcohol use over a monthly time frame. The use of monthly sectional analysis of EtG in hair is therefore a potentially neat tool that could help alcohol treatment efficacy, by allowing treatment compliance to be checked over longer periods and, in cases of more intermittent use, by ascertaining frequency of use. Ideally, levels of EtG in the hair sample should reflect the amount of alcohol taken over the period covered.

In the literature there are many papers reporting different cut-off levels to indicate abstinence, moderate and alcohol abuse [11–16]. However, there is also a consensus that positive result above cut-off should not be regarded as definitive, above certain levels it would imply that the subject has a probable alcohol abuse. Although there is as yet no total agreement on the various cut-off values to enable grading the different degrees of alcohol use, there is a consensus that when no levels of EtG are detected it implies abstinence, although this is not definitive. Higher cut-offs values have been suggested to exclude false positive results [15]. Unlike the detection of illicit drugs in hair where a single positive test can be sufficient to conclude that a person used the drug within certain periods of time over many months, a positive EtG result may not be satisfactorily conclusive, because there is a demand to establish the frequency and quantity of alcohol consumed from levels of EtG detected in hair.

Whilst the correlation between cut-off levels and the degree of alcohol use are being investigated, the interpretation of a negative result has a different aspect. Hair dyes and bleaches can damage hair, causing drugs to leach out; nonetheless the

detection of illicit drugs in hair is likely even though the levels are reduced [6]. The effect of dyes and bleaches on EtG levels in hair will be at least equally detrimental. Due to its polarity, EtG is highly water-soluble, and normal hair hygiene could potentially remove EtG from the hair shaft. If normal hygiene was a factor affecting the levels of ETG in hair, the sectioning hair samples over longer hair samples might not be such a definitive indicator of alcohol abstinence.

The main aim of this paper is to present data from sectioning analysis of head hair samples to aid the interpretation and outline of potential limitations of sectional analysis over longer period of time of EtG results.

2 Materials and methods

2.1 Hair samples

Two sets of samples were included in this study. One set consisted of 102 hair samples from various sources were cut into three sections measuring one centimetre long to represent approximately three months in individual one-month periods. The samples received for testing were from a very heterogeneous group and the information regarding alcohol use by clients was not available. The first section represented the most recent period, followed by the second section representing prior month before collection and the third section the earliest month. Each section was therefore analysed for EtG as multiple sections of one month, covering three months, however, only 87 samples were sufficient long to produce a third section. The second set of samples consisted of 468 hair samples tested for EtG only on the most recent section. No sectional analysis was performed in this set of samples.

2.2 Reagents

Methanol, ammonia 35%, ethyl acetate and Glacial Acetic Acid were HPLC Grade (Fisher Scientific, Loughborough, UK), BSTFA was GC Grade (Sigma, Poole, UK). The cartridges used in the solid phase extraction were anion exchange mixed mode cartridges (MAX) (Waters, Massachusetts, USA)

The standards of Ethylglucuronide (EtG) used in the calibration and quality control samples were purchased from two separate sources, from Lipomed (Arllesheim, Switzerland) and from Medichem (Steinenbronn, Germany). The deuterated internal standard, Ethylglucuronide-D5 (EtG-D5), was from Medichem.

2.3 Analysis of EtG in hair samples and uncertainty of the measurements

Each section of hair was weighed and washed with methanol and the samples are dried to remove all traces of methanol for approximately 45 min at 45 °C. To the dried hair, 1 mL of water and 200 µL of internal standard solution, ethylglucuronide-D5, at 40 ng/mL were added and the

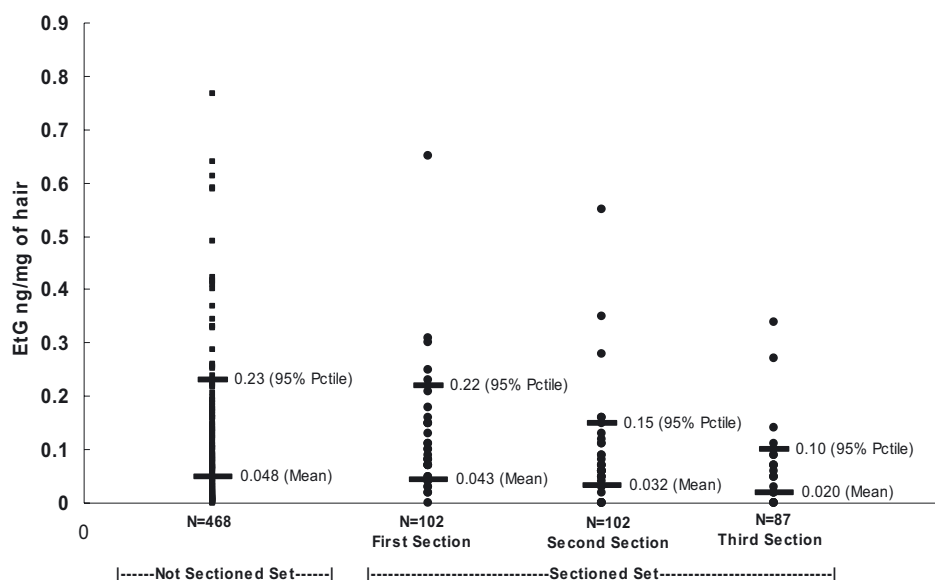


Fig. 1. Levels of EtG detected in the not sectioned set and in the sectioned set showing the 95% percentiles and mean for the results. Levels of EtG were detected 230 hair samples in the not sectioned set and in 34 of the first and the second sections and in 23 of the third sections. The first section represented the most recent period, followed by the second section representing prior month before collection and the third section the earliest month.

mixture was sonicated for at least three hours. An aliquot of 0.5 mL of the aqueous extract was applied to a conditioned solid phase anion exchange mixed mode cartridges - MAX (Waters, Massachusetts, USA). Interference elution was achieved by using 1 mL of 5% ammonia in water, followed by 1 mL of water and finally by 1 mL of methanol. The column was then dried under full vacuum for 5 min and eluted with 1 mL of 5% acetic acid in methanol under gravity. The final extracts were evaporated to dryness and reconstituted in 10 µL of ethyl acetate and 10 µL of BSTFA for the derivatisation. The derivatised extracts were injected onto a gas-chromatograph Varian Inc. 1200 triple quadrupole for GC-MS/MS (Walton-on-Thames, UK) equipped with capillary column 15 m × 0.25 mm Varian, Factor Four). Injector was at 280 °C, splitless, helium at 2.0 mL/min, column temperature 75 °C, 1 min hold, 20 °C/min to 120 °C, hold 0 min then 75 °C/min to 300 °C, hold 0 min then 50 °C/min to 320 °C and hold for 1 min (total time 7.05 min). Mass spectrometer temperatures: source 200 °C, interface 280 °C. Ion transitions monitored: EtG = 261 > 143, and EtG-D5 = 266 > 143.

The method was fully validated in-house in accordance ISO 17025 requirements and meet the minimum requirements to accurately identify EtG. The limit of detection (LOD) was 0.005 ng/mg and the limit of quantification (LOQ) was 0.01 ng/mg assuming a 20 mg hair sample. Uncertainty of measurement and bias associated with the variability of the analytical tests estimates the measurement, taking into account the analytical variables. The uncertainty associated with the extraction efficiency is not possible to accurately determine, because of the drug's intrinsic nature within the hair matrix. The uncertainty of measurement was thus estimated based on a standard uncertainty multiplied by a coverage factor of $k = 2$ to provide a level of confidence of approximately 95% [17]. The estimated uncertainty and bias of EtG measurements in

hair were ±36% and -2%, respectively. The assay was linear over the calibration range 0.01 ng/mg–1.0 ng/mg.

3 Results

3.1 Sectioned hair samples set

The 95% percentile of the EtG levels detected in the first section was 0.22 ng/mg ($N = 102$); in the second sections and in the third sections were 0.15 ng/mg ($N = 102$) and 0.10 ng/mg ($N = 87$), respectively (Fig. 1). The mean (\pm standard deviation) values of the EtG levels for each of the section groups were 0.043 (± 0.092) ng/mg, 0.032 (± 0.077) ng/mg and 0.020 (± 0.054) ng/mg of hair, respectively (Fig. 1).

Table I shows the levels detected in the sections of the samples in the group where the hair was sectioned and EtG levels were above analytical cut-off ($N = 34$). EtG was not detected or the levels detected were below the analytical cut-off in 67% of first and second sections ($N = 68$); and 74% of the third sections ($N = 64$).

Of the samples where EtG was detected, the second section samples showed mean EtG levels 74% lower the levels detected in the first section. The mean levels detected of EtG in third section were 62% the levels of the second section and 47% the levels detected in the first section. Analysis of variance showed the levels of the third section significantly lower ($p < 0.05$) than the first section.

3.2 Not sectioned hair samples set

The 95% percentile of the EtG levels in the set of samples, which were not sectioned, was 0.23 ng/mg ($N = 468$) and

Table I. Levels of EtG detected in sections of hair samples from 34 different individuals which were sectioned. first section is the most recent month, near to the scalp, the second section representing two months before sample collection and the third section the earliest period, three month before sampling.

Case	First section ng/mg	Second section ng/mg	Third section ng/mg	Decreasing levels
1	0.02	0.00	0.00	yes
2	0.02	0.00	0.00	yes
3	0.03	0.00	IS	yes
4	0.04	0.02	IS	yes
5	0.07	0.00	IS	yes
6	0.08	0.05	0.00	yes
7	0.08	0.03	0.00	yes
8	0.08	0.06	0.02	yes
9	0.11	0.06	0.02	yes
10	0.11	0.08	IS	yes
11	0.13	0.07	0.07	yes
12	0.15	0.06	0.03	yes
13	0.15	0.07	IS	yes
14	0.15	0.07	0.05	yes
15	0.18	0.09	0.10	yes
16	0.21	0.11	IS	yes
17	0.22	0.13	0.07	yes
18	0.23	0.16	IS	yes
19	0.25	0.15	0.14	yes
20	0.10	0.09	0.06	yes
21	0.31	0.28	0.10	yes
22	0.65	0.55	0.34	yes
23	0.00	0.00	0.02	No
24	0.03	0.05	0.05	No
25	0.04	0.04	IS	No
26	0.05	0.05	IS	No
27	0.05	0.06	IS	No
28	0.07	0.06	0.09	No
29	0.07	0.06	0.07	No
30	0.07	0.06	0.05	No
31	0.09	0.11	0.09	No
32	0.10	0.12	0.11	No
33	0.16	0.16	IS	No
34	0.30	0.35	0.27	No

IS = insufficient sample.

the mean (\pm standard deviation) was 0.048 (\pm 0.010) ng/mg. EtG was not detected or the levels detected were below the analytical cut-off in 61% of the samples ($N = 287$). Analysis of variance did not show a statistically significant difference between the levels detected in the set where the samples were not sectioned with the first section in the group of sectioned hair samples ($p > 0.1$).

4 Discussion and conclusions

The results presented in this study showed a trend of EtG results where older (distal) hair segments have overall lower levels in comparison to the most recent sections (proximal), corresponding to more recent periods. The pattern of this occurrence suggests that it is improbable that the trend seen in

this study sample was due to any change in the amount of alcohol use. The consistency of the results obtained in the not sectioned hair samples set and in the first section in the sectioned hair samples set indicates that there was no sampling bias. The most appropriate explanation for the trend in the EtG results is likely to be attributed to normal hair hygiene. To further investigate the proposition that normal hygiene has an effect on EtG levels in hair, cocaine results were examined in 216 sectioned hair samples that were received for analysis over the same period of the present study, and were segmented in three month sections. These showed an opposite pattern of results, in that the mean of the most recent period was almost half the earliest period (Fig. 2), thus corroborating the fact that EtG is affected by normal hair hygiene. The types of clients TrichoTech receive samples from are mostly medico-legal cases related to family law, and in these cases clients generally tend try to stop taking drugs to have accesses to children, for example. It is to be expected therefore that there will be a pattern of less cocaine consumption in the most recent period. The same type of clients routinely request EtG analysis and such pattern would also be expected in hair samples tested for EtG. In theory, normal hygiene of the hair would affect older hair to a greater extent, as older hair has been submitted to normal washes more times than the newer hair growth, causing EtG to be washed out from the hair. This effect was not observed with cocaine analysis. The relevance of the normal hygiene effect is when the retrospective estimation of alcohol consumption over a period of many months is sought. If normal hygiene is a factor affecting the levels of ETG in hair, the interpretation of a negative result over a longer period of time will be less likely to be indicative of abstinence because it is not possible to rule out the effects of cosmetics in hair, including normal shampooing.

Although it is highly likely that EtG detected above a certain level might indicate excessive alcohol use, the conclusion based solely on the EtG levels may not be definitive to establish the quantity of alcohol consumed. EtG data from hair should be evaluated in conjunction with other biochemical tests and clinical evaluation and should be regarded as corroborative evidence in a number of cases. No single biochemical test performed in one occasion is sufficient to provide evidence that a person uses alcohol in large quantities and regularly. EtG test may be a useful diagnostic instrument for alcohol dependency and it should not be relied upon when used in isolation as this could be misleading with regards to the limitations of such tests. Unlike drug testing in hair of illicit drugs, where a single positive test can be sufficient to conclude that a person used drugs, a positive EtG result can only be 'suggestive' of alcohol misuse and a negative result 'indicative' that there is no evidence of heavy drinking and generally neither should be regarded as definitive.

However, the potential effects of normal hygiene may not be significant in some specific situations. An example was described by Wurst *et al.* where in a case of drink drive, the most recent section of hair was negative in contrast with a positive EtG found in distal parts of the hair when the individual was convicted of drink drive [14]. The negative EtG result in this particular case was obviously conclusive and any loss of EtG due to normal hygiene was less relevant. In contrast,

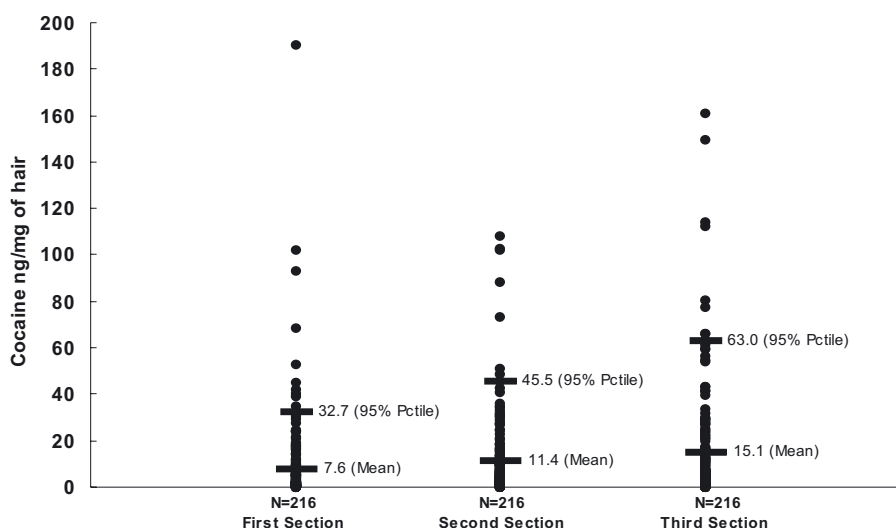


Fig. 2. Levels of cocaine detected in 216 hair samples which were segmented into three one-centimetre sections showing the 95% percentiles and mean for the results for each section group. The first section represented the most recent period, followed by the second section representing prior month before collection and the third section the earliest month.

more recent studies highlighted the usefulness of the measurement of EtG in segmental hair to provide an overview of the drinking history of patients but strongly recommended a careful interpretation of the results [15, 16]. The implication is that we still need to be cautious regarding the interpretation of the results for individual cases, specially using sectional analysis to establish pattern of alcohol use until further scientific evidence regarding the stability of EtG in hair becomes available. Retrospective estimation of alcohol consumption over a period of many months is therefore less useful on routine management of alcohol use. In addition, when sectional analysis is performed, careful interpretation of the EtG results is mandatory. The results presented in this paper imply therefore that only the most recent month must reliably monitor abstinence or chronic alcohol abuse.

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