A study of distribution of ethyl glucuronide in different keratin matrices

Abstract

Ethyl glucuronide (EtG) is a direct metabolite of ethanol, frequently used as a biomarker of alcohol abuse. To this purpose, EtG is preferentially determined in hair samples, using a cut-off value of 30 pg/mg to discriminate between social and heavy drinkers, as recently fixed by an international consensus conference. Although this cut-off value is assumed for head hair, alternative matrices, such as pubic, axillary and chest hair, are often analyzed when head hair is not available. Previous studies suggested that determination of EtG in various keratin matrices may lead to different results; growth cycle and rate, urine contamination, distribution of sebum glands and other environmental factors are likely to contribute to these differences.

We analyzed more than 2700 samples (head, pubic, chest and axillary hair) to evaluate the inter- and intra-individual distribution of the EtG concentration in the different keratin matrices. The data were interpreted on a statistical basis, on the assumption that large population data-sets will level off the average alcohol consumption of each group. From both inter- and intra-individual distribution data, significant differences were observed in EtG concentrations recorded in head, axillary and pubic hair samples. It is concluded that pubic hair cannot be utilized alternatively to head hair to prove chronic alcohol abuse, nor is axillary hair, since positive and negative biases respectively affect these determinations. In contrast, for chest hair, EtG distributions similar to head hair were found, although the large discrepancy between the examined population dimensions presently prevents any definitive conclusion. Thus, chest hair represents a promising alternative to head hair for EtG determinations, deserving further investigation on samples collected from the same individuals, in order to establish a clear correlation between their respective EtG concentrations.

Keywords

- Ethyl glucuronide;
- Alcohol abuse;
- Cut-off;
- Statistical elaboration;
- Keratin

1. Introduction

Alcohol is the most widely used legal drug of the western world. Therefore, alcoholism is one of the most frequent addictions encountered in many western countries. An important goal of forensic toxicology and clinical medicine is to identify biological markers of ethanol consumption appropriate to diagnose chronic alcohol abuse and to ascertain alcohol abstinence. This objective is particularly relevant when offenders convicted of driving or workplace impairment are ordered to refrain from consuming ethanol [1].

The analysis in various biological matrices of ethanol direct metabolites, such as ethyl glucuronide (EtG), ethyl sulfate (EtS) or fatty acid ethyl esters (FAEEs), presents many advantages with respect to determining the corresponding ethanol concentrations. In contrast with indirect alcohol markers, such as carbohydrate-deficient transferring (CDT) and γ-glutamil transferase (γ-GT), these direct metabolites are more sensitive and specific markers than ethanol itself, because their concentrations in all biological matrices appear to be directly correlated with alcohol intake [2], with minor chance of positive interference.

Lately, EtG has become the most accreditated marker to prove chronic alcohol abuse, and the determination of EtG concentration in head hair is currently used to diagnose this addiction, although some toxicologists have recently raised some warnings about the general applicability of this determination [3] and [4].

EtG is a non-oxidative metabolite of ethanol, arising from its conjugation with glucuronic acid [5]. The phase II conjugation reaction is catalyzed by the endoplasmatic reticulum UDP-glucuronosyltransferase. This non-volatile, stable, polar and hydrophilic conjugate represents about 0.5% of the total ethanol elimination and is detectable for up to 18 h in blood and up to 80 h in urine. A small fraction of EtG is also incorporated into the hair, which is quantified to determine previous alcohol intake for longer periods of time [6] and [7]. The mechanism of EtG incorporation in hair is not completely understood yet, but uptake from sweat and sebum appears to be involved [8]. EtG incorporation proved not to be influenced by the melanin content [9], therefore this excluding any bias due to natural hair color or pigmentation degree. Also, there is no evidence in the literature that belonging to specific ethnic groups may determine another source of bias [10].

EtG concentration in scalp hair is used to discriminate between social and heavy drinkers: recently, an international consensus conference fixed the cut-off value at 30 pg/mg, as measured in the 0–3 cm proximal hair segment [11]. This cut-off value was basically obtained from (i) receiver operating characteristic (ROC) curve analyses, (ii) studies of the correlation between the EtG concentration and the alcohol daily intake (the cut-off value corresponding to 60 g/day of ethanol), and (iii) comparison with other alcohol markers [12]. Other studies suggested to apply either a more conservative 50 pg/mg cut-off value [1] or even a stricter 20 pg/mg concentration [13].

With the consensus of 2009, the Society of Hair Testing (SOHT) accomplished its mission of regulating and standardizing the EtG determination use in the forensic context, but not all uncertainties were solved. For example, an unadulterated 3 cm long scalp hair sample is frequently unavailable for several reasons, including baldness, short cuts, hard cosmetic treatments, alopecia. In these cases, sampling of pubic, axillary, chest or other body hair has alternatively been proposed [14] without any change of the cut-off value for EtG concentration. However, several studies involving both drugs and EtG determinations showed that different keratinic matrices may contain uneven concentrations of these analytes, since there are substantial inter-site differences in hair growth rate and growth cycle, and in the nature and activity of sweat and sebum glands [15]. Further variations could be ascribed to environmental exposure, washing habits and the use of special cosmetic products [16] and [17]. It was also shown that the contact with positive biological matrices, such as urine, can raise the EtG incorporation into hair samples by a factor of up to 400. These external contaminations proved to be sufficient to turn a non-drinker into a drinker [18]. Lastly, it is important to stress that quite large inter-individual variability is expected in the EtG synthesis and metabolism, possibly affecting the conversion fraction of ethanol into EtG, and consequently its concentrations in all biological matrices [3].

In the present study, we utilized the data collected from more than 2700 hair samples to evaluate the inter-individual distribution of EtG, not on the basis of individual alcohol consumption, but from the point of view of the different keratinic matrices (head, pubic, axillary and chest hair) that were analyzed. Samples were obtained from drivers convicted for DUI (driving under the influence of alcohol) going through re-licensing examination.

Our statistical investigation was based on the assumption that large population data-sets will level off the influence of the average alcohol consumption between the groups. It was also considered that alcohol dependence is not a precondition for DUI, but rather the population of DUI offenders is heterogeneous and approximately overlaps a population of social drinkers [19].

Finally, we verified the existence of differences of EtG levels in head, pubic and axillary hair through an intra-individual evaluation. Samples collected from 32 volunteers were analyzed and EtG concentrations were statistically compared.

2. Material and methods

2.1. Chemical, reagents and standard solutions

Ethyl glucuronide (EtG) and ethyl glucuronide–d_5 (EtG–d_5), used as internal standard (IS), were acquired from Medichem® (Germany). Standard solutions of EtG and EtG–d_5 were prepared in methanol at 10 μg/mL concentration and stored at −20 °C. Working methanol solutions of EtG and

its internal standard were prepared by progressive dilution of the standard solutions. Testing and calibration samples were obtained by spiking the blank head hair samples with the working solutions. Blank head hair samples were obtained from non-drinker laboratory personnel (2 subjects), stored at room temperature and used as surrogate matrix.

Acetonitrile CHROMASOLV®, methylene chloride CHROMASOLV®, methanol CHROMASOLV® and formic acid were obtained from Sigma–Aldrich (St. Louis, MO, USA). All chemicals and reagents were of analytical purity grade.

Water was produced by a Milli-Q System (Millipore Corporate Headquarters, Billerica, USA).

2.2. Hair samples pretreatment

The initial pretreatment of hair samples was performed using a standard procedure for EtG determination[20]. Briefly, all hair samples were washed twice using methylene chloride and methanol (3 mL, 3 min) in sequence and then dried. Each sample was cut into small pieces (1–2 mm length) and weighted. About 50 mg of hair was added with 25 ng of EtG–d_5 and 720 µL of a 35:1, v:v water and methanol mixture. Then, samples were centrifuged (4000 rpm, 5 min) and incubated overnight at room temperature. 15 h later, samples were ultra-sonicated with the UCI-150 Ultrasonic Cleaning Bath (Raypa©, Ankara, Turkey) for 90 min and then 100 µL of the liquid phase was transferred into a vial for LC–MS/MS analysis.

2.3. LC–MS/MS method

All the analyses were performed using an Agilent Technologies (Milan, Italy) HPLC 1100 liquid chromatograph interfaced to an Applied Biosystem API 4000TM triple-quadrupole mass spectrometer (Applied Biosystem Division Headquarters, Foster City, USA) operating in ESI-negative ion mode. LC separation was performed using a LiChroCART, purospher STAR RP-18E column (150 mm × 4.6 mm i.d.), with particle size of 5 µm. The elution solvents were formic acid 5 mM (component A) and acetonitrile (component B). The mobile phase eluted under the following linear gradient conditions: (A:B, v:v) from 98:2 to 10:90 in 8.30 min, isocratic elution at 90% B for 2 min, final re-equilibration for 6 min. The flow rate was 0.5 mL/min. The ESI source was held at 550 °C. EtG and EtG–d_5 were eluted respectively in about 7.91 and 7.85 min. For each sample, 10 µL of liquid phase was injected and the data were acquired at unit mass resolution in selected-reaction monitoring (SRM) mode, using the mass transitions listed in Table 1. Two MS/MS transitions were utilized for identifying and determining EtG and its internal standard. Identification criteria for the analyte was established according to international [21] and [22] and national guidelines [23]. The EtG transition 221 > 75 was used for quantification. To maximize the fragment

ion signals while maintaining comparable precursor ion abundance, for EtG and EtG–d_5 a different collision energy was optimized. The experimental conditions are shown in Table 1.

Table 1.

SRM transitions and experimental conditions for EtG and EtG–d_5 determination.

Compound	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Declustering potential (V)	Collision cell exit potential (V)	Collision energy (eV)
EtG	221	85	−47	−12	−25
		75	−47	−12	−22
EtG–d_5	226	85	−48	−12	−22
		75	−48	−12	−21

2.4. Method validation

The analytical method was validated in accordance with the recognized International Standard ISO/IEC 17025:2005 and accredited. The following parameters were investigated: linearity range, precision, accuracy and trueness, detection and quantification limits (LOD and LOQ), recovery, sensitivity and specificity. Carry-over effect phenomenon was also evaluated.

The linear calibration model was checked by analyzing three replicates of blank head hair samples spiked with working solutions at the final concentration of 10, 30, 50, 100, 300 pg/mg. The calibration was completed by internal standardization with EtG–d_5. The linear calibration parameters were obtained using the least squares regression method. The correlation coefficient (R^2) was utilized to estimate linearity.

Intra-assay precision (%), accuracy and trueness (both expressed as bias 100%) were evaluated by analyzing, on the same day and in the same conditions, 10 blank head hair samples spiked with EtG at 10, 30 and 50 pg/mg concentrations. Although the acceptance criteria for precision and accuracy are not fixed by internationally standardized rules, according to national guidelines [21], [22], [23] and [24] and literature reports [8], [25], [26] and [27], assay precision was considered satisfactory when CV% values were below 20%, while accuracy was satisfactory when the experimentally determined concentrations lied within ±20% from the expected values. Accuracy was also positively verified by regular participation to the interlaboratory proficiency tests organized by the Centre Universitaire Romand de Médecine Légale (University Hospital of Geneva, Switzerland) for the Society of Hair Testing.

The limit of detection (LOD) was estimated as the concentration of the analyte that gives a signal (peak area) equal to the average background of the blank (S_{blank}) plus three times its standard deviation (LOD = $S_{blank} + 3s_{blank}$), while the LOQ was estimated as LOQ = $S_{blank} + 10s_{blank}$. The noise was measured from −0.05 min before the peak onset till the beginning of the peak for each

analyte. The LOD value estimated from calculation was experimentally confirmed by analyzing 5 blank head hair samples spiked with the analyte at the concentrations corresponding to the estimated LOD value. The LOQ value was confirmed in daily laboratory work, including the analysis of a LOQ control sample at the beginning and end of each session.

Relative extraction recovery was determined by comparing the experimental of two sets of solutions at 30 pg/mg concentration. In the first set, 10 blank head hair samples were spiked with the analyte at the final concentration of 30 pg/mg before the extraction step, while in the second set, the liquid phase extracted from blank head hair samples was spiked with 30 pg/mg.

Selectivity was determined using 10 blank head hair samples spiked at the final EtG concentration of 30 pg/mg. The identification criteria for each analyte were satisfied in each sample. Specificity was determined by analyzing 10 blank head hair samples. For each sample the signal-to-noise ratio (S/N) was measured for the corresponding mass transitions at the expected retention time windows.

Carry-over effect was evaluated by injecting an alternate sequence of 5 blank samples and 5 blank samples spiked with EtG at high concentration (up to 300 ng/mg). To ensure the absence of carry-over, for each transition, the signal-to-noise ratio (S/N) had to be lower than 3.

2.5. Study protocol

For the inter-individual study, samples of head, pubic, axillary or chest hair were collected, between September 2009 and July 2010, by the Medical Committee for driving licences from the Italian city of Cuneo ($N = 2743$), located in Northern Italy. This Committee examines a broad range of individuals, namely owners of driving licences, requested to undertake medical examination in order to obtain the renewal of their suspended or expired licence.

For each subject only one keratinic sample was collected, with the following distribution: head hair 2286 (83.3%), axillary hair 345 (12.6%), pubic hair 61 (2.2%), and chest hair 51 (1.9%). All these samples were used to evaluate the differences in EtG concentration and the percentage of positive samples within each group, according to the cut-off value of 30 pg/mg.

For the intra-individual study, samples of head, pubic and axillary hair were simultaneously collected from 32 patients (7 women and 25 men, aged 28–60 years, mean = 43.8) from an Alcohol Abuse Treatment Service of the city of Turin (Italy). From the medical records, the patients were classified as heavy drinkers ($N = 12$), social drinkers ($N = 11$) and non-drinkers, i.e., people who abstained from alcohol consumption for at least the last 6 months ($N = 9$). Only the patients with a certain group attribution were included in the study. From the medical records for each patient, general information was collected about the estimated daily intake of ethanol in the past 6

months. Information of relevant diseases and both recent and previous use of other legitimate and illicit drugs was also recorded.

All hair samples were cut as close as possible to the scalp, using freshly disinfected scissors. The samples were stored at room temperature and analyzed within 10 working days. Only the proximal 0–6 cm segment was analyzed whenever a longer head hair sample was collected. Shorter head hair, as well as pubic, axillary and chest hair samples were analyzed in their full length.

The study protocol was approved by the internationally recognized Ethics Committee at San Luigi Gonzaga University Hospital [28] and [29]. All patients gave written informed consent before attending the study and an anonymous code was attributed to each participating subject to adhere to privacy regulation.

2.6. Statistical analysis: inter-individual evaluation

Under the hypothesis of independent samples population, the Yates' chi-square test was selected for conformity assessment. The 2 × 2 contingency tables were constructed by listing the number of positive and negative samples for each couple of keratinic matrices under examination. The total percentage of positive samples was considered as the expected value and used to build the expected cell frequency. Lastly, the chi-square test was performed, corrected by the Yates factor when a large discrepancy between the compared group populations was observed. The statistical model is reported in Table 2.

Table 2.

Statistical model: construction of observed and expected cell frequencies in 2 × 2 contingency tables. Computation of Yates' chi-square test.

Observed cell frequency (o)				Expected cell frequency (e)		
	A	B	Total		A	B
Positive	a	b	$(a + b)$	Positive	$[(a + b) \times (a + c)]/N$	$[(a + b) \times (b + d)]/N$
Negative	c	d	$(c + d)$	Negative	$[(c + d) \times (a + c)]/N$	$[(c + d) \times (b + d)]/N$
Total	$(a + c)$	$(b + d)$	N[a]			
Yates' chi-square value (df; CI%) = $\sum(\lvert o - e\rvert - 0.5)^2/e$						

[a] $N = (a + c) + (b + d)$.

When the critical chi-square value at 95% confidential interval (CI) and 1 degree of freedom (df) proved larger than the calculated Yates' chi-square value, the null hypothesis H_0 (no significative differences between two groups) was retained. At a 95% CI and 1 df the critical chi-square value is

3.84. All statistical analyses were conducted using the software packaging SPSS® (SPSS Inc., Chicago, USA), version 16.0, for Windows.

2.7. Statistical analysis: intra-individual evaluation

Intra-individual samples typically yield dependent data; therefore, the Friedman non-parametric hypotheses test and the post hoc Wilcoxon test were chosen to verify the occurrence of statistically significant differences between pubic, axillary and head hair among samples collected from the same individuals. Due to the different growth rate and quiescence periods of pubic and axillary hair, the correlation with a fixed alcohol consumption period was possible only for head hair.

For the Friedman Test, the null hypothesis H_0 affirms that there are no significant differences among at least two keratinic matrices. For the post hoc Wilcoxon test, the null hypothesis H_0 states that there are no significant differences between the two keratinic matrices under examination. A significant level (a two tailed *P*-value) of 0.05 (CI = 95%) was chosen for all statistical tests. When the experimental value proved smaller than the critical *P*-value, the hypothesis H_0 was rejected.

To elaborate the statistical tests, a randomly generated numerical value (Excel Microsoft Office 2007) comprised between 0 and the LOD was assigned to the samples with non-measurable (below the LOD) EtG concentration.

3. Results and discussion

3.1. Validation

Linearity was observed in the range from 10 pg/mg to 500 pg/mg with a correlation coefficient (R^2) of 0.995. Intra-assay precision was satisfied, as the variation coefficient (CV%) was below 10.0% at 10 pg/mg EtG concentration, and below 5.0% at 30 and 50 pg/mg. Both accuracy and trueness, expressed as percent bias, proved lower than 15% at 10, 30 and 50 pg/mg.

LOD and LOQ values, calculated from the analyses of multiple blank head hair samples, were respectively 3 and 10 pg/mg. The detection of 3 pg/mg was experimentally confirmed from five spiked blank hair samples. The accurate quantification of 10 pg/mg was verified within the daily work using control samples.

An extraction recovery of 97.1 ± 1.4% (mean ± SD%) was estimated for samples spiked with EtG at 30 pg/mg concentration. No carry-over effect was observed under the conditions described in the experimental section.

Selectivity and specificity proved successful. SRM chromatograms from both positive and negative head hair samples showed no interfering signals at the retention time where the analyte was expected to elute.

3.2. Statistical evaluation of data: inter-individual distribution

The percentage of results classified as positive from overall head hair samples reached 16.1%, when a standard 30 pg/mg cut-off value was applied (EtG ranging from 30 up to about 1200 pg/mg, median = 63.5, IQR = 62.6). A significantly lower percentage (10.9%) of positive samples was obtained if a more conservative 50 pg/mg cut-off value was utilized (median = 87.3, IQR = 78.8). Among axillary hair samples, only 3.5% yielded EtG concentrations above 30 pg/mg, with values for positive samples ranging from 30 pg/mg to 185 pg/mg (median = 60.5, IQR = 59.3). In contrast, positive pubic and chest hair samples exceeded the overall percentage: in particular, more than half (60.7%) pubic hair samples were classified as positive, with EtG concentration ranging from 30 pg/mg to about 30,000 pg/mg (median = 199.0, IQR = 599.3), while chest hair samples with EtG values above the cut-off were 21.6%, ranging from 30 to 174 pg/mg (median = 62.0, IQR = 126.0). If a cut-off value of 50 pg/mg was applied, the percentage of non-complying samples in axillary, pubic and chest hair dropped respectively to 2.3%, 49.2% and 5.9% (Fig. 1).

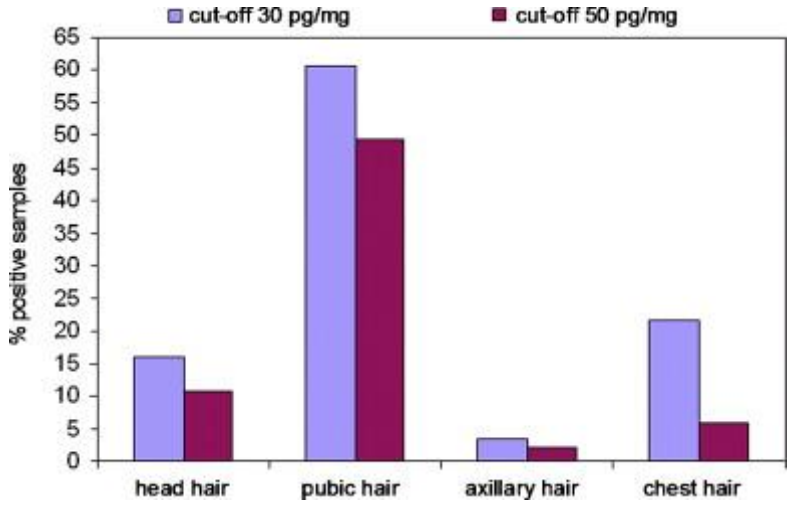

Fig. 1.

Percentage of positive samples for head, pubic, axillary and chest hair at 30 pg/mg and 50 pg/mg for the inter-individual data.

Although big discrepancies were observed among the percentages of non-complying hair samples collected from different body areas, the data were obviously subjected to large random variability, as no preliminary classification of samples on average alcohol daily intake was possible. Therefore an unconditional statistical inference of data was needed in order to draw any ultimate conclusion.

Table 3 shows for the three alternative keratinic matrices under examination the 2 × 2 contingency tables versus head hair. The values obtained from the Yates' chi-square test indicate that the null-hypothesis has to be rejected for axillary (χ^2 = 32.04) and pubic hair (χ^2 = 66.10), while it is accepted for chest hair (χ^2 = 0.59).

Table 3.

Observed and expected cell frequency for head, pubic, axillary and chest hair.

	Observed cell frequency			Expected cell frequency	
	Head	Pubic	Total	Head	Pubic
Positive	367	37	404	393.5	10.5
Negative	1919	24	1943	1892.5	50.5
Total	2286	61	2347	2286	61
	Observed cell frequency			Expected cell frequency	
	Head	Axillary	Total	Head	Axillary
Positive	367	12	379	329.3	49.7
Negative	1919	333	2252	1956.7	295.3
Total	2286	345	2631	2286	345
	Observed cell frequency			Expected cell frequency	
	Head	Chest	Total	Head	Chest
Positive	367	11	378	369.8	8.2
Negative	1919	40	1959	1916.2	42.8
Total	2286	51	2337	2286	51

The first contingency table shows the observed and the expected cell frequencies for pubic and head hair. In this case, the expected cell frequency of positive samples (10.5) is significantly smaller than the observed cell frequency (37). After Yates' chi-square test, the null hypothesis H_0 can be rejected, indicating that the difference between the percentage of positive pubic and head hair samples is statistically significant, i.e., an overestimation of the average alcohol intake from EtG concentration in pubic hair is most likely to occur.

The second table shows the observed and the expected cell frequencies for axillary and head hair. In this case, the expected value for axillary hair (49.7) is greater than the observed one (12),

yielding again a Yates' chi-square value high enough to reject the null hypothesis H_0. Once more, it is concluded that there is a significant difference between head and axillary hair results and that an underestimation of the average alcohol consumption is likely to occur when axillary hair is analyzed in place of head hair.

The last contingency table considers the observed and the expected cell frequencies for chest and head hair. The 2 frequencies appear quite close to each other. Indeed, the Yates' chi-square test proves that these small frequency differences are not statistically significant and the null hypothesis H_0 is accepted. The present result suggests that chest hair has to be seriously evaluated as a potential alternative keratinic matrix to scalp hair, although the large discrepancy between the dimensions of populations examined in the present study, still prevents any definitive conclusion.

3.3. Statistical evaluation of data: intra-individual distribution

In order to further verify the origin of the discrepancies observed in EtG concentrations in the different keratinic matrices, an intra-individual study under strictly controlled conditions was conducted on a relatively small population (32 individuals), from which pubic, axillary and head hair samples were collected at the same time.

Table 4 shows all the relevant information for the examined population. For each patient, it is reported the classification (non-drinker, social or heavy drinker), the length of the analyzed head hair segment and the EtG results for axillary, pubic and head hair. In Fig. 2, the three EtG concentrations are graphically compared for each patient (indicated with a case number). The data on *y*-axis are reported in logarithmic scale.

Table 4.

Intra-individual results of EtG concentration in head, pubic, axillary hair for 32 patients.

Case	Category[a]	Analyzed head hair segment (cm)	EtG concentration[b] (pg/mg)		
			Head hair	Pubic hair	Axillary hair
1	S	3.0	22	4777	22
2	A (1 year)	6.0	<LOD	<LOD	<LOD
3	A (1 year)	6.0	<LOD	<LOD	–
4	S	6.0	<LOD	<LOD	<LOD
5	A (8 months)	2.5	<LOD	–	<LOD
6	A (1 year)	1.0	<LOD	<LOD	<LOD
7	S	2.0	<LOD	215	<LOD
8	S	6.0	12	738	<LOD
9	H	6.0	37	647	62
10	H	2.5	206	39315	374
11	S	3.0	30	344	<LOD
12	S	6.0	20	5108	–
13	S	1.0	<LOD	47	<LOD
14	H	3.0	94	13992	64
15	H	6.0	35	8230	95
16	S	6.0	22	41	<LOD
17	H	3.0	614	78400	204
18	A (1 year)	6.0	<LOD	49	<LOD
19	A (1 year)	6.0	<LOD	76	<LOD
20	A (26 months)	4.0	<LOD	<LOD	<LOD
21	H	2.0	382	6819	81
22	H	6.0	229	308	–
23	H	6.0	243	5204	58
24	H	4.5	220	5614	91
25	S	6.0	<LOD	13	<LOD
26	A (6 months)	6.0	<LOD	<LOD	–
27	H	4.0	218	20382	162
28	S	2.5	30	381	–
29	H	4.0	58	581	<LOD
30	S	2.5	12	1188	<LOD
31	A (6 months)	–	–	379	<LOD
32	H	4.0	89	772	27

[a]A = non-drinker (abstinence period); S = social drinker; H = heavy drinker.

[b]LOD is 3 pg/mg. N dash indicates absence of sample.

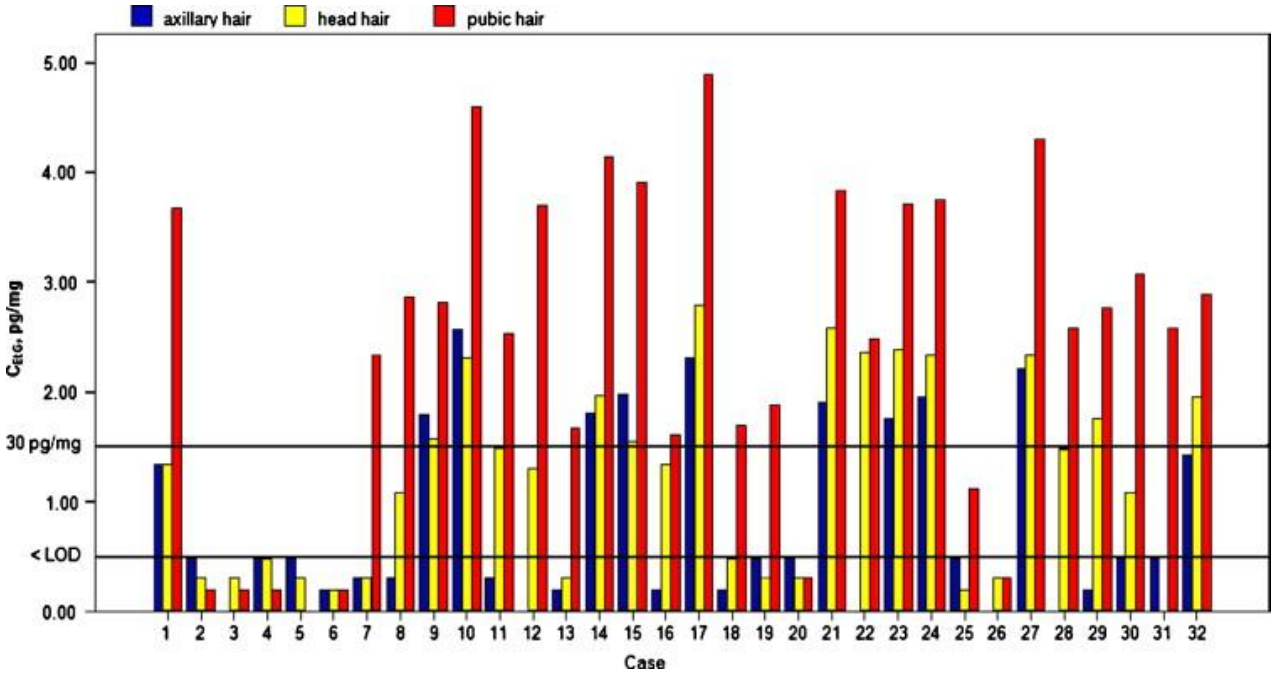

Fig. 2.

Intra-individual distribution of EtG results in head, pubic and axillary hair samples for 32 patients. The EtG results are expressed in logarithmic scale (base 10).

From Table 4, it is clear that all subjects who abstained from alcohol consumption for at least 6 months (cases 2, 3, 5, 6, 18, 19, 20, 26 and 31) had EtG levels in head and axillary (when available) hair samples below the LOD, while the EtG concentrations in pubic hair were below the LOD value for only 5 patients out of 8 (cases 2, 3, 6, 20 and 26). Three individuals (cases 18, 19 and 31) exhibited EtG concentration in pubic hair clearly above the cut-off value. Thus, these subjects would have been incorrectly classified as alcohol abusers had the pubic hair been analyzed instead of the head hair.

For most social and heavy drinkers (cases 8, 11, 14, 16, 17, 21, 23, 24, 27, 29, 30 and 32), the measured EtG concentration in axillary hair was lower than in head hair and even below the LOD in five cases, while in a few other cases (9, 10 and 15), all relative to homeless people with poor hygiene, the opposite trend was observed.

Nine social drinkers (cases 1, 7, 8, 11, 12, 13, 16, 28 and 30) with compliant head hair EtG concentrations (i.e., ≤30 pg/mg) exhibited pubic hair EtG concentrations largely exceeding the cut-off value (mean = 1427 pg/mg; range = 41–5108 pg/mg). Had pubic hair been analyzed instead of head hair for official purposes, all nine subjects would have been incorrectly classified as alcohol abusers. Overall, the results obtained by analyzing pubic hair samples indicate that 38.7% of the subjects under study (12/31) would have been incorrectly classified as ethanol abusers.

In some cases, EtG concentrations in pubic hair were 2 or 3 order-of-magnitude higher than in head hair, producing a largely overestimated alcohol intake.

It is relevant to note that the EtG concentration in head hair was above the cut-off value (30 pg/mg) for all the alcohol abusers and below it for all other subjects, non-drinkers and social drinkers, confirming that EtG determination in scalp hair is an efficient tool to differentiate the subjects according to their ethanol consumption, using a cut-off value of 30 pg/mg.

By applying the Friedman test, the null hypothesis H_0 was rejected (experimental P-value = 0.000), confirming that significant differences exist at least in one among the three keratinic matrices under examination. To further evaluate if the statistically significant differences were also present for each pair of matrices, the post hoc Wilcoxon test was applied. The test demonstrated that the null hypothesis H_0 had to be rejected in all cases. The experimental P-value between pubic and head hair samples was equal to 0.000, while between axillary and head hair samples was equal to 0.038.

4. Conclusions

Ethyl glucuronide is a sensitive, specific and reasonably accurate biomarker for alcohol consumption; in particular, its concentration in head hair is known to be correlated with the average alcohol intake for extended periods of time. On the other hand, alternative matrices and/or methods appear to be necessary when head hair is not available. EtG concentration can be conveniently determined in other keratinic matrices, including pubic, axillary and chest hair, but the practical worth of such determinations for establishing the average alcohol intake has been repeatedly questioned. Other warnings about EtG determination in head hair have been recently raised on the general applicability of a 30 pg/mg cut-off value in the forensic context, as a consequence of the alleged inter-individual variability in ethanol metabolism, EtG secretion and physical characteristics.

In the present extended epidemiological evaluation of EtG determination in various keratinic matrices, these issues are primarily considered, so that a few ultimate conclusions can be drawn and some further preliminary suggestions are advanced.

First of all, neither pubic nor axillary hair can be suitably collected for the diagnose of alcohol consumption from individuals whose head hair is absent. Indeed, a large overestimation is generally observed when pubic hair is analyzed, reasonably due to EtG contamination from urine and consequent inclusion into the keratinic matrix. On the other hand, the analysis of EtG in axillary hair frequently yields a large underestimation of alcohol consumption, as part of the secreted EtG is likely to be washed down by sweating or detergents, before it is incorporated into the keratinic matrix.

Secondly, the extremely large variability of positive and negative bias in pubic and axillary hair, respectively, excludes any opportunity of fixing different cut-off values for these alternative keratinic matrices.

Our inter-individual epidemiological evaluation indicates that chest hair may represent an appropriate alternative to head hair for EtG determination, although the large difference between the examined population dimensions presently prevents any definite conclusion. An intra-individual investigation, similar to the one undertaken for pubic and axillary hair in the present study, appears to be necessary before any unquestionable deduction is determined.

Another issue emerging from the present work is that quite a large percentage (5.2%) of positive head hair EtG results was within 30–50 pg/mg, opening the question of inter-individual variability and the resulting risk of false positive classification. Further studies are undoubtedly necessary to investigate possible biological discriminating factors, such as gender, age, body-mass index and ethnic or geographical origin, which will be undertaken in our future work.

In conclusion, we firmly believe that EtG is the most objective biomarker for alcohol abuse when determined in head hair. Nonetheless, both random and class-related inter-individual variability is likely to influence the correlation between EtG concentration and alcohol consumption. Accordingly, we suggest to responsibly consider a careful surveillance policy for the evaluation of subjects whose EtG result is close to the cut-off value (i.e., 20–50 pg/mg) before any sanction is assigned.

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