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Effects of various sample pretreatment procedures on ethyl glucuronide quantification in hair samples: Comparison of positivity rates and appraisal of cut-off values

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ABSTRACT

The quantification of ethylglucuronide (EtG) in hair is nowadays recognized as the approach with the highest diagnostic performance to evaluate harmful drinking. A widely accepted cut-off of 30 pg/mg has been selected after several accurate compared studies. While most of the studies that were used to establish the appropriate cut-off value prescribed to cut hair into small segments before their extraction, hair milling has subsequently been identified as the most efficient pretreatment procedure and was therefore recommended in the last Consensus document issued by the Society of Hair Testing. In this study, we initially compared the results obtained with the two sample preparations, namely cutting and milling, both being applied to the same specimens ($n = 781$). Among these, 205 samples produced measurable EtG values with both methods, with differences ranging from −41.7% up to +415% (the mean increase in EtG concentration, switching from cutting to milling, was +62.1% and the median was +42.3%). Among the aforementioned 205 samples, 29 specimens (3.7% of the total 781 samples) produced significantly different outcome, being classified as negative (i.e., below 30 pg/mg) if the cutting procedure is used, but largely positive (above 40 pg/mg) when milling is used. Subsequently, the positivity rates obtained on a large population dataset (>27,000 samples) with the two procedures, were retrospectively compared using variable cut-offs values. The percentage of head hair samples with EtG concentration exceeding 30 pg/mg upon application of the milling procedure shows a 45% increase (from 10.9% to 15.8%) with respect to cutting procedure, whereas the fraction of hair samples with EtG exceeding 40 pg/mg (10.5%) overlaps the percentage of positive samples obtained after cutting pretreatment and applying a cut-off of 30 pg/mg. On the basis of these results, it would be worth considering the application of cut-off values linked with the pretreatment procedure, taking into account the results of forthcoming inter-laboratory calibrations.

1. Introduction

An important goal of forensic and clinical toxicology is to identify appropriate biological markers of ethanol consumption to evaluate harmful drinking [1–5] or to ascertain alcohol abstinence [6–8]. With these aims, the detection of ethylglucuronide (EtG) in hair represents nowadays the approach with the highest diagnostic performances [9,10]. The Receiver Operation Characteristics (ROC) curve [11] is an efficient statistical method, used to evaluate the discrimination power of a certain biomarker and select a suitable cutoff with optimized sensitivity and specificity. This technique was consistently applied in hair EtG studies to compare the performance of this molecule with that of other excessive alcohol consumption biomarkers [4,9,12–16]. In all these studies, the diagnosis of chronic alcohol abuse by means of hair EtG determination was based on a cut-off ranging from 25 and 30 pg/mg, which resulted highly accurate (AUC > 0.9). In five of the aforementioned papers [4,9,13–15], the sample treatment

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prescribed to cut the hair specimen in 1–2 mm segments, while only two studies [12,16] utilized a mill to pulverize the samples. In the past, the Society of Hair Testing (SoHT) established the cut-off value that supports a diagnosis of chronic excessive alcohol consumption at the level of 30 pg EtG/mg, measured in the 0–3 cm up to 0–6 cm proximal segment [17], but more recently the SoHT also recommended to "powder hair prior to the extraction of EtG" [18]. With this supplement, the pre-analytical protocol is gaining higher attention.

A meta-analysis on the distribution of EtG concentrations in the hair of teetotalers, social-, and heavy drinkers [19] showed that in none of the examined studies (0 out of 13) EtG concentrations in the hair of teetotalers and social drinkers exceeded 30 pg/mg. Only 2 studies out of 13 used to pulverize hair by milling [12,20]. Furthermore, it was highlighted that only in a minority of the analyzed studies (3 out of 13), heavy drinkers (self-declared EDAI $>$ 60 g) occasionally exhibited EtG concentrations lower than 30 pg/mg, indicating that this cut-off is affected by a limited false negative effect. In two of the latter studies [21,22], the hair samples were cut into 1–2 mm segments and not milled. A similar pooled and meta-analysis was based on 8 studies which reported paired data for 70 subjects [23]. A median EtG concentration of 51.5 pg/mg was found for heavy drinkers, with 95% CI 38.4–86.2 pg/mg, and interquartile range 30–140 pg/mg. Furthermore, sensitivity and specificity indexes were calculated and plotted against several possible EtG decision limits. The 30 pg/mg cut-off led to the best diagnostic efficiency, with 0.97 specificity and 0.85 sensitivity. The diagnostic performance data for the EtG test derived from the meta-analysis (cut-off fixed at 30 pg/mg) proved even better than those calculated from the pooled analysis, yielding a sensitivity of 0.96 and a specificity of 0.99. Among eight considered studies, hair samples were cut in seven cases and milled only in one.

In the recent SoHT Consensus document [18], it is said that laboratories using hair preparation procedures other than powdering (i.e. cutting) should demonstrate comparable recovery of EtG. Principally, this approach is leading the way as only similar efficiency of extraction protocol and analytic allows the use of a common cut-off value. However, it was demonstrated by different authors that grinding the hair sample leads to higher extraction yields rather than cutting hair into short pieces. In particular, it was shown that grinding or milling hair provides 0.95- to 1.8-fold higher extraction after overnight incubation, and to 1.4- to 2.3-times higher extraction yields after 2 days incubation with water [24,25]. A more recent study on two authentic positive hair samples from two alcohol consumers also showed that an extensive pulverization of hair samples leads to a significantly higher amount of detectable EtG [26].

In the study presented hereby, we initially compared the results obtained with the two sample preparations, namely cutting and milling, both being applied to the same specimens. Our aim was to evaluate the difference in the extraction yield between the two procedures on a large number of samples. Subsequently, the positivity rates obtained on different groups of samples with the two procedures, namely cutting and milling, were retrospectively compared using variable cut-offs values.

2. Materials and methods

2.1. Reproducibility of the analytical results after the cutting and milling procedures

A pooled hair sample from 15 donors (approximately 2 g) was accurately homogenized and analyzed 10 times after extraction with the cutting preparation procedure, and 10 times after extraction with the milling preparation procedure. Each aliquot weighted approximately 40 mg. The variability was expressed by means of CV%.

2.2. Study protocol for the direct comparison of the two crumbling treatments executed on the same hair samples

The direct comparison of the two hair crumbling procedures was executed on 781 samples (either head or chest hair) randomly selected. All samples were collected during year 2015 from subjects who underwent medical examination either within alcohol abuser's rehabilitation programs or for driving relicensing protocols. Several medical committee located in Piedmont, northern Italy, commissioned these analyses. All hair samples were cut as close as possible to the scalp or the skin surface, using freshly disinfected scissors. The samples were stored at room temperature and analyzed within 10 working days. Only the proximal 0–3 cm segment was analyzed whenever longer head hair samples were collected. When more than one lock of hair from one subject was available, the material was pooled and homogenized. These hair samples were washed twice using methylene chloride and methanol in sequence [27]. Dried hair was separated into two aliquots of similar weight. One aliquot was cut into small snippets (about 1 mm) with freshly cleaned scissors, whereas the second aliquot was pulverized using a metal beads mill. A Precellys 24 Tubes Homogenizer (Bertin Pharma, France) equipped with six 2.8 mm metal beads was used for hair milling. EtG extraction was carried out overnight at room temperature with a 35:1 water:-methanol mixture. Then, the samples were sonicated and an aliquot of liquid phase was transferred into a vial for UHPLC–MS/MS analysis. The two aliquots were extracted simultaneously and analyzed in the same batch (single injection). The comparison of the procedures was made on the EtG concentrations found in the two hair aliquots.

2.3. Determination of EtG

Analyses were performed using a Shimadzu Nexera UHPLC-system (Shimadzu, Duisburg, Germany) interfaced to an AB Sciex API 5500 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) with an electrospray (ESI) source operating in the negative ion mode. The limits of detection (LOD) and quantification (LOQ) were 3 and 10 pg/mg respectively. The method was internally validated and accredited in accordance with ISO/IEC 17025:2005 rules. The laboratory performances in hair EtG analysis were constantly monitored through regular participation to inter-laboratory proficiency tests organized by SoHT and the Gesellschaft für Toxikologische und Forensische Chemie (GTFCh).

2.4. Study protocol for comparative evaluation of the percentage of positive samples according to different cut-offs

The comparison of EtG positive samples distributions according to the hair crumbling procedure (cutting or milling) was executed using both prior results and more recent (year 2015) data. These include (i) 24,771 samples (22,825 head hair and 1946 chest hair) analyzed after cutting and presented in a previously published study [27], (ii) 5118 samples (4632 head hair and 486 chest hair) analyzed after cutting, and (iii) 2730 samples (2461 head hair and 269 chest hair) analyzed after milling. Groups i and ii were united in order to obtain a single group of hair prepared by cutting, for a total of 27,457 head hair samples and 2432 chest hair samples. For the large statistical population investigated, the different cohorts of subjects can be viewed as global distributions with specific properties, rather than collections of independent individuals [27,28].