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The determination of ethyl glucuronide in hair: Experiences from nine consecutive interlaboratory comparison rounds

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ABSTRACT

The increasing request for hair ethyl glucuronide (HEtG) in alcohol consumption monitoring according to cut-off levels set by the Society of Hair Testing (SoHT) has triggered a proficiency testing program based on interlaboratory comparisons (ILC). Here, the outcome of nine consecutive ILC rounds organised by the SoHT on the determination of HEtG between 2011 and 2017 is summarised regarding interlaboratory reproducibility and the influence of procedural variants. Test samples prepared from cut hair (1 mm) with authentic (in-vivo incorporated) and soaked (in-vitro incorporated) HEtG concentrations up to 80 pg/mg were provided for 27–35 participating laboratories. Laboratory results were evaluated according to ISO 5725-5 and provided robust averages and relative reproducibility standard deviations typically between 20 and 35% in reasonable accordance with the prediction of the *Horwitz* model. Evaluation of results regarding the analytical techniques revealed no significant differences between gas and liquid chromatographic methods In contrast, a detailed evaluation of different sample preparations revealed significantly higher average values in case when pulverised hair is tested compared to cut hair. This observation was reinforced over the different ILC rounds and can be attributed to the increased acceptance and routine of hair pulverisation among laboratories. Further, the reproducibility standard deviations among laboratories performing pulverisation were on average in very good agreement with the prediction of the *Horwitz* model. Use of sonication showed no effect on the HEtG extraction yield.

1. Introduction

Ethyl glucuronide is an effective alcohol consumption marker [1–3] with increasing relevance in the monitoring of heavy chronic drinking [4], workplace surveillance [5] traffic safety [6,7] or forensic investigations [8]. Based on scientific studies, the Society of Hair Testing (SoHT) has fixed cut-off levels for EtG in hair for the assessment of alcohol consumption [9]. This implies that laboratory results on EtG concentrations in hair need to be quantitative and have to fulfill requirements regarding trueness and precision. For that purpose, the SoHT offers interlaboratory proficiency tests for society members on a regular basis.

In addition, the proficiency testing scheme aims at improving quality and conformance of the determination of HEtG among laboratories. In this context, the impact of different analytical procedures, particularly the sample pre-treatment on the HEtG recovery, is of great interest with respect to a harmonisation of the analytical protocol.

In this paper, the outcome of nine consecutive proficiency testing rounds on the determination of HEtG is evaluated with emphasis on the effect of sample preparation and instrumental determination on the interlaboratory comparability of results.

2. Materials and methods

2.1. Preparation and characterisation of the test batches

The preparation of a large amount of a homogeneous hair sample suitable for an ILC is a challenging process. Two main aspects have to be taken into account. Firstly, authentic, soaked or spiked starting material can be used and secondly the solid material has to be homogenised either by cutting into short snippets or by powdering the hair.

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For the first four rounds, authentic hair from Caucasian donors not having undergone cosmetic bleaching or colouring and with preliminarily determined EtG contents was washed and then cut to 1 mm snippets using a procedure developed by MEDICHEM Diagnostica. Fortified hair was obtained by prolonged soaking of EtG-free hair in an aqueous EtG solution. Afterwards, the solution was removed and the hair was repeatedly washed with water until the concentration of EtG in hair remained constant. In contrast, spiked hair was prepared by gravimetric fortification of blank hair without washing afterwards. If necessary, each type (authentic, soaked and spiked) EtG containing and EtG free batches were blended and homogenised to attain EtG levels suitable for testing participants' ability to determine EtG in the cut-offs ranges set by the SoHT [9]. After homogenisation, each test batch was bottled in 100 mg units. At least 10 bottled units were submitted to a homogeneity test using a validated GC/MS [11], GC–MS/MS [12] or HPLC–MS/MS procedure [13]. EtG solutions in water were gravimetrically prepared and bottled in 1 mL ampoules by MEDICHEM. For rounds 5–9, only authentic donor hair was used. After testing a hair sample for EtG, in each case a retained sample is stored for one year. After this time period, the anonymised material is pooled according to its EtG concentration. The hair strands of these hair pools are then washed, cut into snippets of 1 mm length, and homogenised by a multistep sieving procedure. This sieving was done on a vibration screening machine AS 200 (Retsch, Haan, Germany) and four sieves of different mesh sizes. Homogeneity was finally tested using a validated LC–MS/MS method [14] applied for the routine case work of the laboratory. The homogenised hair snippets material was bottled in aliquot samples of approximately 100 mg by Zurich Institute of Forensic Medicine, Zurich, Switzerland.

For all rounds, samples containing hair snippets were offered to the participants of the ILC. Additionally, in round 3, five pulverised hair samples were provided. Pulverised hair was prepared with a ball mill operated at room temperature as it is known from the literature that cooling is not necessary to retain the HEtG content [10].

2.2. Organisation of the proficiency testing rounds

The SoHT determined the timeline for each interlaboratory comparison round, the HEtG concentration ranges, and the number of test samples.

Test samples were accompanied by a short description of the analytical tasks and a questionnaire on details regarding the sample treatment and the analytical method. The organisation followed the recommendations given in ISO 17043 and ISO 13528 [15]. These proficiency testing rounds were carried out once per year between 2011 and 2015 and biannually in 2016 and 2017.

2.3. Handling and statistical treatment of laboratory data

Participants' identities were only known to the organiser and distributer and all data were handled strictly anonymous. After scrutiny for obviously erroneous data and feedback with participants in a few cases the laboratory results were processed according to international standard ISO 5725-5 [16] using the ProLab™ Plus software (QuoData GmbH, Dresden, Germany). The evaluation procedure employs a *Huber* estimator as robust consensus mean X of all laboratory means and a robust estimate for the reproducibility standard deviation s_R. This procedure provides reliable estimates for X and s_R regardless of the distribution of analytical values and without identification and assessment of outliers.

3. Results and discussion

3.1. Outline of the interlaboratory comparison rounds

The original focus of the interlaboratory comparisons has always been proficiency testing, enhancement of analytical quality and assessment of interlaboratory variability of HEtG values obtained by different analytical methods. For that purpose, test samples consisting of cut hair were used. However, the meanwhile published observation of higher HEtG averages after hair pulverisation [11,17–21] led to the idea to investigate the effect of reduced particle sizes on the extraction efficiency also on the interlaboratory scale. Furthermore, since authentic hair materials with suitable EtG concentrations are scarcely available in quantities required for reference materials and proficiency test materials the concept of adjusting defined EtG concentrations by artificial incorporation was adopted. Thus, authentic hair with physiologically incorporated EtG was compared with hair presenting soaked and spiked EtG contents, respectively.

3.2. Synopsis of test materials and quantitative HEtG results

Table 1 summarises the test materials and of the results of all interlaboratory comparison rounds. Statistical data X,s_r and s_R were evaluated using all received laboratory data regardless of the respectively employed analytical procedures.

Table 1 contains also the predictions for the reproducibility standard deviations s_R obtained from the *Horwitz* model which has been found to provide a good estimate for the interlaboratory scatter of results in case of established routine analytical methods [22]. Obviously, the s_R obtained in the interlaboratory comparisons are in good accordance with the *Horwitz* model. These PTs provide a standard z score based proficiency assessment where absolute z scores $\leq$2 are considered as satisfactory, those between 2 and 3 as questionable and those above 3 as unsatisfactory. However, the decisive items in this context are the assigned value and the standard deviation for each test material and need to be set by the PT provider. In the following, a detailed analysis of the last 9 rounds and the implication for HEtG proficiency assessment is presented.

3.3. The influence of sample preparation and instrumental determination on the between-laboratory reproducibility standard deviation

Hair analysis requires a complex procedure including sample preparation, extraction, clean-up, and instrumental quantification. To date, there is no standardized protocol and laboratories perform a variety of extraction and clean-up variants and employ either high performance liquid chromatography or different derivatisation procedures followed by gas chromatography connected with a (tandem-) mass spectrometer. Due to the number of participants in the SoHT interlaboratory comparisons (27–35) hardly any given combination of procedural variants is performed in sufficient number to allow mutual comparison. Thus, only those methodological alternatives applied by sufficiently large groups of participants may be compared. Criteria for grouping were the use or disuse of sonication during extraction, the application of either gas chromatography or liquid chromatography, and hair pulverisation prior to extraction vs extraction of hair snippets.

The application of sonication during hair extraction did not lead to a significant tendency towards higher HEtG values. Likewise, no effect was obtained using gas or liquid chromatography (data not shown). In contrast, the pulverisation performed routinely by some