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A comparison of the performance of quality controls prepared from spiked, fortified and authentic hair for ethyl glucuronide analysis

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ABSTRACT

Ethyl glucuronide (EtG) quantification in hair was assessed using quality controls prepared by three methods: (a) spiking hair samples with known concentrations of EtG, (b) fortifying hair by incubation of blank hair with EtG for several days or (c) use of authentic hair samples positive for EtG. A liquid chromatography tandem mass spectrometry (LC–MS/MS) method was developed on a Shimadzu model 8030 instrument and validated for the quantification of EtG. For two concentration levels, approximately 50 and 500 pg/mg QCs, EtG concentrations were measured in duplicate ($N = 2$) on 8 days ($N = 16$) and intra-assay precision (repeatability) and inter-assay precision determined using one-way analysis of variance. EtG concentrations measured in authentic hair exhibited poor intra-assay precision, with coefficients of variation of 25.1 and 20.9%, compared with 17.7 and 18.5% for fortified hair and 17.4 and 11.3% for spiked hair, for the lower and higher concentrations respectively. The inter-assay precision for authentic hair was also poorer, 35.7 and 22.5%, compared with fortified (28.2 and 19.8%) and spiked (18.4 and 13.2%) hair for the lower and higher concentrations. Although spiked QCs resulted in a better repeatability and inter-assay precision, the values obtained for QCs prepared from fortified and authentic hair are likely to be more representative of case specimens. These results have implications on the interpretation of EtG concentrations when spiked QCs are used to validate methods.

1. Introduction

There have been a number of papers published on the detection and quantification of ethyl glucuronide (EtG), a minor non-oxidative metabolite of ethanol, in hair. Analysis of EtG in hair is used to corroborate drinking behavior of individuals particularly to assess whether the person is a current alcoholic. The presence of EtG in hair has been studied in newborns [1], teetotallers (alcohol-abstaining individuals) [2], social drinkers [2–4], chronic alcoholics [2,5,6], individuals undergoing alcohol detoxification [7], children [3,4,8] and cadavers [8,9]. Although there are some inter-individual differences in the concentrations of EtG in hair [10], there is a correlation between concentration and the dose of ethanol administered [10–12]. This has led to the Society of Hair Testing (SOHT) implementing cutoffs: currently 7 pg/mg, to differentiate between abstinence and social drinking (<60 g ethanol/day) [13,14]; and 30 pg/mg to distinguish between a social drinker and a chronic alcoholic [14]. The use of these cutoffs necessitates stringent monitoring of the performance of the methods employed such that subjects are not wrongly assessed. This involves ensuring that quality controls (QCs) are appropriate for the application.

In hair analysis, there are 3 alternatives for producing specimens that are used for quality control purposes. These are: (1) spiking of analytes onto blank hair, as practiced for liquid matrices; (2) fortification of blank hair with analytes for a period of several days to allow their incorporation into the hair matrix; and (3) homogenization of authentic hair specimens containing the analytes of interest. The first of these alternatives is by far the most common in hair analysis but does not model incorporation of the drug or metabolite into hair [15] (which can be via the blood stream, sweat and/or sebum [16]). The analyte is placed in contact with the surface of the hair for a limited period of time before its extraction from the sample. It is unknown how much of the analyte, if any, is incorporated into the matrix of the hair, and although extraction recovery and accuracy of measurements can be determined because the concentration of drug or metabolite added to the hair is pre-determined, this is unlikely to reflect case

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specimens. Hair is always washed first, *before* being spiked with the analytes, whereas positive case specimens, which are being verified by the QC, are washed after the drug or metabolite has already become incorporated into the hair in vivo. Therefore, a lower-than-anticipated concentration in the case specimen may be obtained as a result of change in concentration by the washing-out effect.

Fortification of hair has previously been suggested as an improvement to the practice of analyte spiking [15]. In this situation, the drug or metabolite is typically incubated with the hair for a period of several days. Like case specimens the hair is either cut or pulverized after the analyte has been incorporated into hair, washed and then an extraction performed. This approach has been used to produce QCs for the analysis of methamphetamine and amphetamine [15,17] as well as cocaine, benzoylecgonine, cocaethylene, phencyclidine, codeine, morphine, 6-acetylmorphine and Δ^9-tetrahydrocannabinol [17].

The third approach, use of an authentic analyte-containing hair sample, should be most representative of case specimens as the analyte has been incorporated into this matrix following the consumption of drug. While this would provide an ideal specimen for proficiency testing programs it is very difficult to know the absolute concentration of analyte in the hair specimen. Furthermore, old case samples may not always be available for use as QCs.

Alongside the numerous options for QC preparation, different acceptable practices for hair analysis are promoted by relevant professional societies. While SOHT advocates drug fortification of hair specimens [18], the GTFCh (German society for Toxicological and Forensic Chemistry) endorses authentic hair and spiked hair in its guidelines [19]. With regards to EtG testing in hair, the majority of published papers have reported the spiking of ethyl glucuronide onto hair to produce QCs [2,6,7,10,20–30]. To our knowledge, there have been only three instances where authentic hair has been used as a QC for validation or for regular performance testing of a method for EtG in hair [10,25,31]. In one of these studies, analysis of an authentic hair specimen containing 29 pg/mg demonstrated higher imprecision compared with spiked QCs at a similar concentration [10].

Proficiency testing of EtG in hair is offered by SOHT [32] and the GTFCh [33]. The results, however, show wide concentration ranges and high coefficients of variation (CVs). Hair from alcohol-consuming individuals is pooled and either cut or powdered to produce the quality control samples for proficiency testing. As the samples are pooled, care must be taken to ensure homogeneity of specimens, particularly when cut hair is used as opposed to pulverized hair. Although authentic hair is employed for these samples, the samples are typically analyzed by participating laboratories on isolated occasions for the purpose of involvement in the proficiency test, each laboratory employing its own method of detection. There are many issues proficiency tests do not address such as the reproducibility and inter-assay precision of an individual method and ultimately how the uncertainty of measurement is applied to reporting against the cut-off concentrations [34]. It is very uncommon for methods to be validated based on many replicate analyses of these quality controls over many days. This could be due to the ease of preparing custom-made QCs at concentrations befitting of the intended application. Furthermore, it is costly to use proficiency test samples for method validation. For example, current prices are €100 for a single proficiency test from GTFCh where a single sample of 100 mg is provided [33]. Validation of methods typically requires many replicate samples to be analyzed on many days, and subsequent testing of QCs on a daily basis. Use of a commercial quality control is therefore impractical and expensive for the average laboratory.

There is a need to determine whether the conventional approach of spiking EtG into hair can be justified, and if not, to propose a suitable alternative. To our knowledge, no instance of fortification of hair with ethyl glucuronide has been reported in quality control procedures. The purpose of this study was to develop a liquid chromatography tandem mass spectrometry (LC–MS/MS) method for the quantification of EtG in hair in order to assess the reliability of quantification of quality controls prepared by three procedures: (a) spiking hair samples with known concentration of EtG, (b) fortifying hair by incubation of blank hair with EtG for several days or (c) use of authentic hair samples positive for EtG.

2. Materials and methods

2.1. Chemicals and reagents

Ethyl glucuronide and ethyl glucuronide-d_5 (both 1 mg/mL in methanol and with a purity of >98.5%) were purchased from Lipomed (PM separations, Capalaba, Australia). HPLC-grade acetonitrile (99.9%), methanol (99.8%) and dichloromethane (99.9%) were sourced from Lichrosolv (Merck Pty Ltd, Kilsyth, Australia) and formic acid (98–100%) was sourced from Emsure (Merck Pty Ltd, Kilsyth, Australia). Distilled deionized water was filtered on Millipore Milli-RO (reverse osmosis) and Milli-Q systems (North Ryde, Australia) connected in tandem, resulting in a water purity of >18 mΩ. Authentic hair (hair which has not been manipulated by the addition of EtG), along with blank hair from 10 alcohol-abstaining individuals, was obtained from volunteers within the laboratory, or members of their families, and stored at room temperature. The EtG-free hair was sourced from either children, or teetotallers and the hair was confirmed to be below the limit of quantification (5 pg/mg) for EtG prior to use. The pooled blank hair was prepared from brown and black hair. The authentic hair used in this study was brown (lower concentration QC) and black (higher concentration QC). Although detailed information on hair treatment was not collected from donors, in all cases the hair was of natural color. No hair dyes or bleach had been used by any of the individuals to limit the variables as far as possible to method of QC preparation.

2.2. Preparation of working solutions

Stock solutions of ethyl glucuronide were prepared in water, at concentrations of 1, 5, 10, 50, 100, 150 and 200 ng/mL (corresponding to the calibrants, 5, 25, 50, 250, 500, 750 and 1000 pg/mg, respectively) and stored at 4 °C for a maximum of 4 weeks. These aqueous calibrants were spiked into hair (see below). Although stability of EtG was not systematically investigated, the calibrants were also analyzed directly to monitor the stability of EtG and there was no noticeable decrease in peak intensity over a 4-week timeframe. Furthermore, EtG is known to be stable in aqueous matrices over similar timeframes [35]. Internal standard solution consisted of 1 μg/mL ethyl glucuronide-d_5 in water. Spiked QC solutions, at concentrations of 10 and 100 ng/mL, were prepared in water independently of standard solutions. Methanolic solutions of EtG at concentrations of 5–3000 ng/mL were prepared once for the purpose of fortifying blank hair.

2.3. Preparation of calibrants, QCs and blank hair samples

Calibrants and spiked QCs were prepared on a daily basis by cutting EtG-free hair into lengths of approximately 10 cm and placing aliquots (approximately 2 g) into 20-mL glass vials (Perkin Elmer Pty Ltd, Rowville, Australia) which were filled with dichloromethane. The samples were mixed on a vortex (Ratek Instruments Pty Ltd, Boronia, Australia) for 30 s and sonicated for 10 min in a Bandelin Sonorex sonicator (LKB Instruments, Mt Waverley, Australia). The solvent was removed and replaced with 20 mL methanol and the samples were further vortexed for 30 s and the methanol decanted. Any residual methanol was removed with a glass Pasteur pipette and the hair was dried under nitrogen on a Dri-Block® DB-3D Techne Sample Concentrator (DKSH, Hallam, Australia). The hair was then cut with scissors into pieces of less than 2 mm in length. Aliquots of 100 (±1) mg of cut hair were then accurately weighed out, on pieces of paper, on a XS105DU Excellence balance (Mettler Toledo, Port Melbourne, Australia) for each standard and spiked QC before transferring to 16 mm × 100 mm glass culture tubes (Rowe Scientific, Hallam, Australia). Standard solution or spiked QC solution (500 μL) was added to 20 μL internal standard solution in an autosampler vial which was vortex-mixed, and these solutions were transferred to the prepared aliquots of blank hair. The resultant spiked QCs contained concentrations of 50 pg/mg (QCL) and 500 pg/mg (QCH) of EtG. The method of preparation of spiked QCs is outlined in Fig. 1 and is compared with the preparation of QCs from fortified and authentic hair, as described below.

Fortified QCs were prepared by cutting EtG-free hair into lengths of approximately 10 cm. Two aliquots of hair (approximately 2 g) were inserted into glass vials to which 20 mL methanolic fortifying solutions were added. Fortification of hair with 20 mL of an initial concentration of 5 ng/mL (for QCL) and 50 ng/mL (for QCH) of methanolic EtG was necessary to achieve final concentrations in hair that were comparable to spiked QCs and the available authentic hair samples. The hair samples were incubated at room temperature for 5 days and were placed on a Ratek suspension mixer (Adelab Scientific, Thebarton, Australia) during the day to