Solid-Phase Extraction Procedure for Ethyl Glucuronide in Urine

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Abstract

Measurement of the conjugated ethanol metabolite ethyl glucuronide (EtG) in urine is increasingly being used as a biomarker for recent alcohol consumption. Prior to quantification of EtG by mass spectrometric (MS) methods [liquid chromatography (LC–MS or gas chromatography–MS), there is sometimes need for sample cleanup to remove interfering matrix constituents. A solid-phase extraction (SPE) procedure using a HyperSep SAX strong anion exchanger was developed for sample cleanup of urinary EtG prior to LC–MS analysis. The EtG content in a 50–100-µL urine sample was finally reconstituted in the same volume as the original aliquot. The cleaner SPE extracts, without sample dilution, allowed for improved quantification of urinary EtG in the low concentration range. The detection limit of the SPE procedure when combined with LC–MS analysis was < 0.1 mg/L EtG, and the assay imprecision < 5.5% (total CV) in the 0.5–5.0 mg/L concentration range. The absolute recovery of urinary EtG was ~80%, which was compensated for by using a deuterated analogue (EtG-d5) as internal standard. The urinary EtG results with SPE followed by LC–MS were highly correlated ($r^2 = 0.959$) with those obtained using a sensitive and selective ultra-performance LC-tandem MS method.

Introduction

After consumption of alcohol, the minor ethanol metabolite ethyl glucuronide (EtG) can be detected in body fluids, tissues, and hair (1–3). EtG is formed by a phase-II conjugation reaction with glucuronic acid catalyzed by UDP-glucurono-syltransferase (UGT) (4) and makes up only < 0.05% of the ingested ethanol dose (5). The EtG peak concentration shows a time delay compared with the parent compound, and EtG remains detectable in the urine for hours to days after ethanol has been eliminated (1,6,7). The detection window depends primarily on the amount of alcohol ingested (8). This time-lag between ethanol and EtG elimination is the basis for the increasing popularity of urinary EtG testing as a sensitive biomarker for recent drinking with clinical and forensic applications (9). A clinical problem with EtG testing if applying a very low analytical cutoff is the risk for obtaining positive results due to inadvertent exposure to ethanol from use of ethanol-based mouthwash and hand sanitizers (10,11). Another problem that needs to be considered is the risk for false-positive (12) and false-negative (13) results in case specimens are infected with E. coli and stored improperly.

Measurement of EtG and ethyl sulfate (EtS) (14), another conjugated minor ethanol metabolite, is often carried out by liquid chromatography–mass spectrometry (LC–MS) using either single or tandem MS detection (15,16). An immunoassay (DRI EtG, Microgenics) for routine screening of urinary EtG is also available (17). Depending on the amount of alcohol consumed, the time between drinking and urine sampling, and diuresis (5), the EtG concentration may vary considerably between and within individuals (18,19). Prior to analysis by LC–MS, it is sometimes necessary to remove interfering matrix constituents or, in cases of very dilute urine, to pre-concentrate the analyte. A published (20) sample cleanup method for urinary EtG based on solid-phase extraction (SPE) employed a relatively large sample volume and was developed for use in connection with gas chromatography–MS.

The present study aimed to develop an SPE sample cleanup method for urinary EtG that is suitable for use on a small sample volume and in combination with a direct injection LC–MS method. An initial SPE procedure can also improve analytical strategies to comply with proposed forensic standards (21,22), by improving the selectivity and reproducibility of confirmatory MS analysis.

Experimental

Samples

The urine specimens used for this study (approved by the local Ethics committee) were leftover volumes from the routine samples pool sent to the Alcohol Laboratory at the
Karolinska University Hospital (Stockholm, Sweden) for measurement of EtG and EtS. Samples were stored frozen at −20°C, where EtG concentration is known to be stable for months (15). Standard solutions of EtG (Medichem, Steinbronn, Germany) to cover the concentration range 0.1–100 mg/L (0.45–450 µmol/L) were prepared in water. Control urine samples containing ~0.5 mg/L (~2.25 µmol/L) and ~1.0 mg/L (~4.5 µmol/L) EtG were included in each run.

SPE

In the initial phase of this study, several different SPE sorbents were evaluated for the intended purpose (results not shown). The HyperSep SAX strong anion exchanger (Thermo Scientific, Waltham, MA) showed the best extraction yield and lowest chromatographic interference for EtG in urine and was chosen for this work. Prior to use, the HyperSep SAX SPE cartridge (100 mg/1 mL) was conditioned with 1.0 mL each of methanol, deionized water and acetonitrile. The deionized water and all chemicals used for this study were of analytical or high-performance liquid chromatography (HPLC) grade.

A 50–100-µL aliquot of urine sample or EtG standard (the recovery of EtG was demonstrated to be linear up to 100-µL sample volume) was mixed with 100 µL of the deuterated internal standard solution (0.55 mg/L EtG-d5 in water, Medichem), 200 µL water, and 700 µL acetonitrile. The specimen was passed through the SPE cartridge by applying a gentle vacuum, and the column was subsequently washed with 1.0 mL water followed by 1.0 mL acetonitrile. The cartridge was dried under vacuum for 2 min and the retained content including EtG eluted with 1.0 mL of a solution containing acetonitrile, water, and formic acid (95:4:1, v/v). The eluted fraction was evaporated to dryness under a stream of nitrogen gas at 30°C using a metal block, and the final extract was reconstituted in the same volume of water as the original urine aliquot. For routine work, the SPE method for urinary EtG used 50-µL sample volume and was automated using an ASPEC XL4 SPE system (Gilson, Middleton, WI).

LC–MS analysis

In the present study, quantification of EtG in the collected SPE fractions and without prior urine sample cleanup was performed using a direct electrospray HPLC–MS method as detailed elsewhere (15). Analysis was performed using an Agilent series 1100 LC system connected to an SL MS detector (Palo Alto, CA) with selected-ion monitoring at m/z 221 for EtG and m/z 226 for the penta-deuterated analogue. The chromatographic column was a porous graphite HyperCarb column (100 × 2.1-mm i.d., Thermo Scientific), and the injection volume was 10 µL. The lower limit of quantification (LOQ) of the LC–MS method was previously reported to be ~0.1 mg/L, and the lower limit of detection (LOD) was ~0.05 mg/L. In clinical use, a cut-off limit for urinary EtG of 0.5 mg/L is routinely applied to retain a high sensitivity of the alcohol biomarker but overcome the risk for obtaining false-positive results due to unintentional ethanol exposure (10).

For comparison, EtG quantification was performed by a very sensitive (LOQ <0.01 mg/L) and fast (retention time for EtG ~1.4 min) ultra-performance liquid chromatography (UPLC)–tandem MS method as detailed elsewhere (23). Analysis was performed using a Quattro Premier XE tandem MS with an ESI source and the MassLynxTM/TargetLynxTM Software version 4.1 (Waters, Milford, MA). Separation was achieved on a high-strength silica trifunctional C18 column (100 × 2.1 mm, 1.8-µm, Waters) (23).

Statistical calculations were performed using MedCalc software.

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**Figure 1.** Chromatograms for urinary EtG by electrospray LC–MS with selected-ion monitoring at m/z 221. The results for four urine samples using the direct injection method (10-fold dilution with internal standard solution) (15) and following sample cleanup by the HyperSep SAX SPE procedure are compared.

**Figure 2.** Distribution of urinary EtG concentrations determined by the SPE and LC–MS procedure in 93 of 173 urine samples that were either classified as EtG negative (i.e., below the LOQ of 0.1 mg/L) by the direct injection LC–MS method (15) or contained interfering peaks in the chromatograms making quantitation of EtG difficult.
Results and Discussion

Four example chromatograms from the LC–MS analyses of EtG in urine samples using the direct injection routine method (10-fold dilution of samples) (15) and following sample preparation by SPE are shown in Figure 1. Following the SPE sample cleanup, much less chromatographic interference was typically observed at the retention time for EtG, although interfering peaks were not always completely eliminated. The cleaner SPE extracts without sample dilution allowed for improved quantification of EtG in the low concentration range. Among 173 urines from the routine samples pool that were either classified as EtG negative by the direct injection LC–MS method (i.e., below the LOQ of ~0.1 mg/L) or for which correct EtG quantification was difficult because of interfering peaks in the chromatograms, 93 (54%) were found to be positive for EtG (range 0.01–0.49 mg/L, mean 0.09) after sample cleanup by the SPE procedure (Figure 2).

Imprecision data for the HyperSep SAX SPE procedure with LC–MS quantification of urinary EtG are given in Table I. In the concentration range 0.5–5.0 mg/L EtG, the coefficient of variation (total CV) was < 5.5%. The absolute recovery of EtG in the SPE sample preparation step was determined by spiking four different EtG-negative urines with 0.5 and 5.0 mg/L EtG and comparing the LC–MS peak areas with those obtained after direct injection. The recoveries ranged between 81 and 86% (mean 83%) for the low EtG concentration and 77 and 81% (mean 78%) for the high concentration.

In routine use, the ~80% absolute recovery of urinary EtG was compensated for by using the penta-deuterated analogue (EtG-d5) as internal standard. Accordingly, the EtG levels in 482 routine urine samples quantified by LC–MS after SPE sample cleanup showed a good and statistically highly significant correlation (r2 = 0.959, p < 0.001) with the corresponding results obtained by the very sensitive and selective UPLC–MS–MS method (23) (Figure 3). The intercept of the regression line was close to the origin, the slope was close to one, and only a few outliers were identified.

To conclude, the HyperSep SAX SPE method for urinary EtG was demonstrated to provide substantial and reproducible sample cleanup of the analyte. The cleaner SPE extracts without sample dilution resulted in an improved analytical sensitivity by LC–MS in the low concentration range. An initial SPE procedure will enhance the selectivity of mass spectrometric quantitation methods for EtG and can thus be applied in connection with confirmatory analysis of immunological screening results, to comply with proposed forensic standards. However, it should be pointed out that if applying a very low clinical cutoff, the risk for positive EtG results due to unintentional ethanol exposure has to be considered.

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References


