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A fully validated method for the quantification of ethyl glucuronide and ethyl sulphate in urine by UPLC–ESI-MS/MS applied in a prospective alcohol self-monitoring study

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ABSTRACT

A method for the quantification of ethyl glucuronide (EtG) and ethyl sulphate (EtS) in human urine is developed and fully validated according to international guidelines. Protein precipitation is used as sample preparation. During the development of the method on an UPLC–ESI-MS/MS system using a CSH C_{18} column, special attention was paid to reduce matrix effects to improve assay sensitivity and to improve detection of the second transition for EtS for specificity purposes. The method was linear from 0.1 to 10 μg/mL for both analytes. Ion suppression less than 24% (RSD < 15%) was observed for EtG and no significant matrix effect was measured for EtS. The recovery was around 80% (RSD < 14%) for both compounds. This method provides good precision (RSD_r and RSD_t < 10%) and bias (<15%) for internal and external quality control samples. The reproducibility of the method was demonstrated by the successful participation to proficiency tests (z-score < 0.86). This method was finally used to analyze urine samples obtained from twenty-seven volunteers whose alcohol consumption during the 5 days before sampling was monitored. Concentrations between 0.5 and 101.9 μg/mL (mean 10.9, median 1.4) for EtG and between 0.1 and 37.9 μg/mL (mean 3.6, median 0.3) for EtS were detected in urine samples of volunteers who declared having consumed alcohol the day before the sampling. EtG and EtS concentrations in urine were highly correlated (r = 0.996, p < 0.001). A moderate correlation between the number of drinks the day before sampling and the concentration of EtG (r = 0.448, p < 0.02) or EtS (r = 0.406, p < 0.04) was observed. Using a cut-off value at 0.1 μg/mL for EtG and EtS, this method is able to detect social alcohol consumption approximately 24 h after the intake, without showing any false positive result.

1. Introduction

Ethyl glucuronide (EtG) and ethyl sulphate (EtS) are two specific metabolites of ethanol, created by conjugation with UDP-glucuronic acid for EtG [1] and with 3′-phosphoadenosine 5′-phosphosulphate for EtS [2].

Quantification of EtG and EtS in urine is used to detect recent alcohol consumption. These biomarkers extend the detection window relative to blood ethanol measurement and, compared to long term biomarkers, allow the detection of drinking of small quantities. This permits to monitor alcohol consumption during withdrawal treatment [3,4] or for workplace testing [5,6]. Several countries, such as Italy [7] and Germany [8,9], have integrated the quantification of EtG and EtS in urine into their licence regranting programme to monitor the abstinence period. In post-mortem cases, the detection of EtG and EtS in urine is usefull to distinguish between antemortem alcohol intake and postmortem formation of ethanol [10–12].

EtG and EtS are detectable in urine up to 24 h after intake of 0.25 g/kg ethanol and up to 48 h after intake of 0.50 g/kg ethanol [2,13–18]. After alcohol intoxication, they can be detected in urine during a few days. EtG is eliminated according to a half-time of 2.5 h [1,13]. After consumption of alcohol and depending on the amount of consumed alcohol, urinary concentrations for EtG and EtS can vary from some μg/mL [11,18–21] to hundreds of μg/mL [13,15,17,21–23]. Urine samples from alcohol-dependent patients during detoxification can reach EtG concentrations up to 1240 μg/mL [1,2,4,24] and EtS concentrations up to 264 μg/mL [2].

Due to the possibility of finding EtG and EtS in urine even without consumption of alcoholic beverages [18,19,25–28], a cut-off limit is used to avoid false positive results. However, cut-offs are

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not fixed yet in international guidelines. The ones currently used vary between 0.1 and 1.1 μg/ml [7,9]. A cut-off at 0.1 μg/mL for EtG and at 0.05 μg/mL for EtS has been proposed to exclude repeated intake of alcohol [9]. Urine analysis of teetotallers shows no EtG [1,29] and no EtS [30] above 0.1 μg/mL.

The most commonly applied technique for quantification of EtG and EtS in urine is liquid chromatography coupled to mass spectrometry (LC–MS) [4,13] or coupled with tandem mass spectrometry (LC–MS/MS) [1,7,11,12,17,26,30–33] in combination with simple dilution or protein precipitation as sample preparation. A few methods have been published using GC–MS [3,29,34,35] or capillary zone electrophoresis (CZE) [36–39] for the analysis of EtG and EtS in urine or serum.

To decrease matrix effects, especially for EtS, sample preparation should be adapted. Dilution of urine is the easiest 'sample preparation' method, however, high matrix effects and higher instrument maintenance can be problematic in routine analysis. Even with a 1/20 dilution, relevant matrix effect was observed at low concentrations [30]. Liquid–liquid extraction (LLE) and solid phase extraction (SPE) are conventional sample preparation techniques for non-volatile compounds. Due to the high polar and acidic character of EtG and EtS in combination with a different acidic strength, the development of LLE and SPE is, however, not straightforward. Protein precipitation can be an alternative clean-up method for this type of analytes [12,17–19], if the matrix effects are carefully monitored. No matrix effects were reported after protein precipitation [12] using a LC–MS/MS system coupled with ion trap mass spectrometer.

Reversed-phase (RP) chromatography used with negative electrospray ionization mode (ESI^-) is the most common approach used [1,4,11,13,17,23,26,31,33,40]. The retention of very polar acidic compounds, such as EtG (pKa ~2.84) and EtS (p*K*a ~−3.14) [11], is achieved in RP only under highly aqueous conditions. As highly aqueous conditions might not be optimal for ESI ionization, post-column addition of an organic modifier is required to enhance the ionization of compounds and so to improve sensitivity. A chromatographic possibility to improve the ionization is to use a normal phase column [12] or another specific column with particular retention behaviour [11]. Nevertheless, normal phase chromatography is known to provide variable retention times [41]. The use of no discharge atmospheric pressure chemical ionization (ND-APCI) [7] or atmospheric pressure chemical ionization APCI [30] is another solution to increase the ionization and so to improve the limit of quantification.

According to international guidelines, forensic analysis by MS/MS in MRM mode requires the detection of minimum two transitions for each compound; one for identification and one for quantification [33,42]. When LC–MS is used, three characteristic ions are required. Sometimes it is difficult to find a second transition for EtS using LC–ESI-MS/MS [12,31], because of the low intensity of the second transition and the presence of interfering compounds in urine.

Low limits of quantification have been reported using a LC–MS/MS system coupled with ion trap mass spectrometer [7,11,12,43]. Using LC–ESI-MS/MS systems equipped with a triple quadrupole, only one published method [19] has reported an LOQ of 0.1 μg/mL for EtG and EtS. Unfortunately no details of the method validation are given in that publication.

As seen before, several LC–MS (/MS) methods have been described for the quantification of EtG and EtS in urine, but most of them are not fully validated (following all criteria for chromatographic assays), especially regarding the measurement of bias and precision with external and certified quality controls. Moreover, to our knowledge, to date, only two published reports [43,44] have evaluated the reproducibility of the method by participation to interlaboratory tests.

We describe the development of a simple and robust method for the quantification of EtG and EtS in urine using an UPLC–ESI-MS/MS system equipped with a triple quadrupole (QQQ) tandem mass spectrometer, with an LOQ at 0.1 μg/mL both for EtG and EtS. The method was fully validated according to international guidelines. A prospective study, based on 27 volunteers, is applied to evaluate the selected cut-off value (0.1 μg/mL) for EtG and EtS and to estimate the sensitivity and specificity of the method. This method could be used for future surveillance of abstinence in the context of driving licence regranting [7,8] and for the detection of alcohol intake prior death in selected post-mortem cases [10–12].

2. Experimental

2.1. Chemicals

Ethyl glucuronide (EtG), ethyl sulphate (EtS) and their pentadeuterated analogues (EtG-d_5 and EtS-d_5) were obtained from Sigma–Aldrich (Steinheim, Germany) as a methanolic 1 mg/mL solution. ULC/MS grade acetonitrile, methanol and 0.1% formic acid in water were purchased from Biosolve (Valkenswaard, The Netherlands). Blank urine was purchased from Bio-Rad Laboratories (Nazareth Eke, Belgium).

2.2. Standard solutions, calibrators and quality control samples (QC)

Two stock solutions, one for calibration (Cal-Stock) and one for internal quality controls (QC-Stock), with EtG and EtS at a concentration of 20 μg/mL were prepared in methanol. The stock solution with internal standards (IS) at a concentration of 4 μg/mL was prepared in methanol. All solutions were stored at −18 °C.

Daily calibration working solutions (Cal-WS) with concentrations of 0.1, 0.5 and 10 μg/mL were prepared diluting the Cal-Stock solution. Calibrators (0.1, 0.25, 0.5, 2.5, 5.0 and 10 μg/mL) were prepared by spiking 30 μL of the IS solution to 50 μL of commercial blank urine, an adequate amount of Cal-WS, and methanol until a total volume of 280 μL was reached.

Daily quality control working solutions (QC-WS) with concentration of 0.5 and 5 μg/mL were prepared diluting the QC-Stock solution. Internal quality controls (0.3, 4 and 7.5 μg/mL) were prepared spiking 30 μL of IS to 50 μL of commercial blank urine, an adequate amount of QC-WS and methanol until a total volume of 280 μL.

External quality controls Medidrug ETG 1/10-B and Medidrug ETG 2/09-B were both purchased from Medichem (Steinenbronn, Germany). Proficiency tests for EtG and EtS in urine organized by the German Society of Toxicological and Forensic Chemistry (GTFCh) were also performed for quality control purposes.

2.3. Population study

A prospective alcohol self-monitoring study was performed asking 27 volunteers to declare their exact alcohol consumption per day during the 5 days preceding the sampling. Urine samples were collected in 100 mL urine containers from Sarstedt (Nümbrecht, Germany), transferred into 4 mL Greiner bio-one tubes (Frickengrasen, Germany) and stored at 2–8 °C until analysis. Samples were analyzed within 5 days after collection.

EtG_{100} and EtS_{100} concentration were calculated by normalizing the measured EtG and EtS to a creatinine concentration of 100 mg/dL [2].