Automated Sample Preparation for the Monitoring of Ethanol Metabolites in Urine by LC-MS/MS

Beckman Coulter Biomek 4000 Workstation and SCIEX Triple Quad™ 4500 LC/MS/MS system

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Abstract

Ethylglucuronide (EtG) and Ethylsulphate (EtS) are stable Phase II metabolites of ethanol, which can be detected in urine until several days after alcoholic beverage consumption. In this application note, we describe an LC-MS/MS method for the analysis of EtG and EtS in urine employing a simple, automated sample preparation methodology using a liquid handling-based automation system. Automation of the sample preparation for analysis by LC-MS/MS has the potential to increase throughput, reduce active bench time, and minimize the opportunity for human error. Our method enabled the quantification of EtG and EtS over a large concentration range, spanning from 0.5X to 10X the cut-off value, with accuracies ranging from 97-104%, and CV less than 4% taking into account both sample preparation and LC-MS/MS analysis.

Introduction

LC-MS/MS is a powerful technology that is increasingly used by forensic laboratories for the monitoring of drugs in urine samples. The time and effort to process these samples can be significant and the required resources increase with sample number. In addition, the likelihood of errors increases with sample throughput. These challenges can be minimized by automating the sample preparation prior to analysis by LC-MS/ MS. In addition to minimizing active sample processing time, automation can also increase the consistency of results by reducing user-touser sample processing variability and the opportunity for errors. In this study the Biomek 4000 Workstation (Figure 1) was used to automate the sample processing for the forensic screening of two metabolites of ethanol, EtG and EtS, in human urine on the SCIEX Triple Quad™ 4500 LC/MS/MS system. The cut-off values were 500 ng/mL and 100 ng/mL, for EtG and EtS, respectively. Standard curves were generated across five concentration levels (0.5X, 1X, 3X, 5X, 10X cutoff value) with excellent accuracy, precision, and curve linearity.

Figure 1. Beckman Coulter Biomek 4000 Workstation (left) and SCIEX Triple Quad™ 4500 LC/MS/MS system (right).

Materials and Methods

Automated Sample Preparation

Table 1 describes the steps in the automated sample preparation protocol for the analysis of EtG and EtS. Briefly, pre-cleared urine controls and samples were diluted with mobile phase A in a 96-well plate, a deuterated ethyl glucoronide internal standard was added to each well, and the samples were thoroughly mixed using an orbital shaker. The 96-well plate was centrifuged offline to clear any precipitate and 200 µL of the supernatant were transferred to a new plate for analysis by LC-MS/MS.

This sample preparation was automated on a Biomek 4000 Workstation utilizing single and 8-channel pipetting tools. To initiate sample processing, a user simply places tips, plates, and reservoirs on the deck as directed in the software (Figure 2) and then highlights the wells containing samples and controls. Reagents were added to full plate columns using the multichannel tools while partial columns (if any) were added using the single channel tool.

Upon completion of the method, a text file containing information for each sample well (i.e. original sample barcodes) is generated in a format that allows direct import into the Analyst software for LC-MS/MS analysis. This ensures that sample data is propagated throughout the workflow.

Step 1	Barcoded urine samples are added to a deepwell plate ("Samples 500 µL") and precleared by centrifugation at 4500 rpm in an Allegra X-30R (Beckman Coulter).	Manual
Step 2	100 µL of samples, calibrators, and QC controls are transferred to a deepwell plate ("Assay").	Automated
Step 3	850 µL of Mobile Phase A are added to each well.	Automated
Step 4	50 µL of internal standard (deuterated ethyl glucuronide, EtG-D5) are added to each well and the plate is shaken to mix.	Automated
Step 5	Samples are centrifuged at 4500 rpm in an Allegra X-30R, for 15 minutes.	Offline
Step 6	200 µL of supernatant are transferred to a flat-bottomed plate ("STD") for analysis.	Automated
Step 7	Analysis by LC-MS/MS system.	

Table 1. Automated sample preparation protocol for LC-MS/MS analysis of alcohol in human urine samples

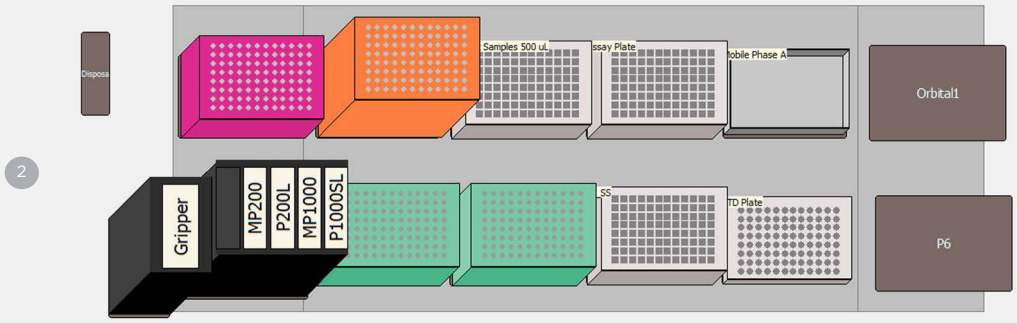

Figure 2. Software representation of the Biomek 4000 deck illustrating the tips, plates, and reservoirs utilized for the automated alcohol analysis method.

Analyses by LC/MS/MS

HPLC separation was performed using a Shimadzu Prominence LC-20XR system and mass spectrometric detection was performed using the SCIEX Triple Quad™ 4500 LC/MS/MS system (Figure 1), equipped with Turbo V™ ionization source (Temperature = 600°C; Gas1 = 60; Gas2 = 50; Curtain Gas = 25). The temperature of the autosampler was set at 15°C.

The prepared samples were injected onto the system, and the chromatographic separation was achieved using a Phenomenex Synergi 2.5 µm Hydro-RP 100A column (50 x 3.0 mm), at 40°C. The separation employed a binary gradient of mobile phases A (HPLC-grade water with 0.1% formic acid) and B (methanol with 0.1% formic acid). The LC-MS/MS data acquisition was done using the Analyst 1.6.2 software. MultiQuant™ 3.0.1 software was used for data processing, and reporting. Two MRM transitions were used to monitor each analyte, and a single MRM transition was used to monitor the internal standard EtG-D5 (see Table 2). The Scheduled MRM™ algorithm was employed, to maximize the acquisition dwell time for each analyte and thereby improve data quality.

The LC-MS/MS analysis was performed in negative ionization mode (Ion Spray voltage = -4500). 10 µl of sample were injected and run at a flow rate of 0.7 mL/min for a 5 min run time with the following time profile for mobile phase B: 0-1.5 minute hold at 0% B; 1.5-2.5 minute ramp from 0-80% B; 2.5-3.0 minute hold at 80% B; 3.0-4.0 minute decrease to 0% B, 4.0-5.0 minute hold (re-equilibrate) at 0% B (Figure 3). Each analyte was monitored during a 60-second detection window centered on the expected Retention Time.

	Q1	Q3	CE (V)
Ethyl Glucuronide 1	220.9	85.1	-20
Ethyl Glucuronide 2	220.9	74.9	-18
Ethyl Sulphate 1	124.8	97	-18
Ethyl Sulphate 2	124.8	79.7	-40
Ethyl Glucuronide-D5	225.9	85.1	-24

Table 2. MS/MS Conditions for the analysis of EtG and EtS.

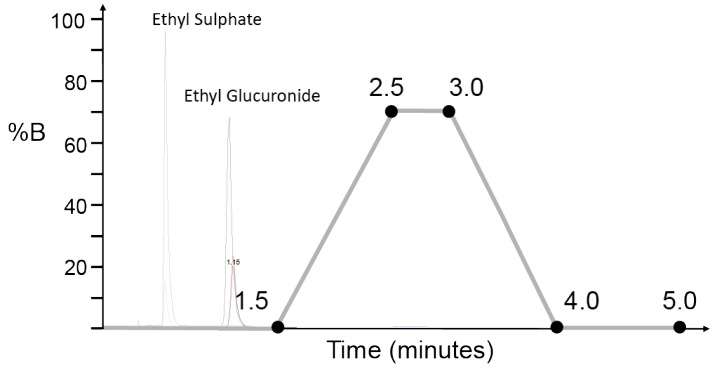

Figure 3. HPLC gradient (% Mobile Phase B) for the analysis of EtG and EtS on the SCIEX Triple Quad™ 4500 LC/MS/MS system, using negative electrospray ionization (ESI), with a run-time of 5 minutes. Overlaid is a representative chromatogram displaying the analytes at their respective cut-off concentration levels.

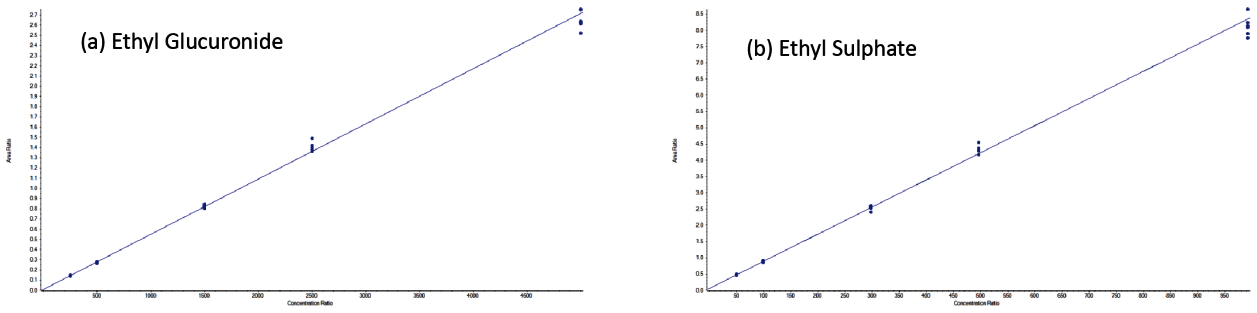

Figure 4. Calibration curves plotting Area Ratio (Y-axis) vs. Concentration Ratio (X-axis) for (a) EtG, and (b) EtS.

FOLD CUTOFF VALUE	MEAN CONC. (ng/mL)	ACCURACY (%)	CV (%)
0.5	252.3	100.9	3.8
1	487.9	97.6	2.4
3	1507.8	100.5	1.8
5	2582.8	103.3	3.2
10	4883.9	97.7	3.4

Table 3. Statistical summary (n=6) for the quantitation of EtG by LC-MS/MS, using automated sample preparation on the Biomek 4000 workstation.

FOLD CUTOFF VALUE	MEAN CONC. (ng/mL)	ACCURACY (%)	CV (%)
0.5	49.7	100.3	3.6
1	98.1	99	2.3
3	297.0	99.8	2.8
5	513.3	103.5	2.9
10	966.1	97.4	3.8

Table 4. Statistical summary (n=6) for the quantitation of EtS by LC-MS/MS, using automated sample preparation on the Biomek 4000 workstation.

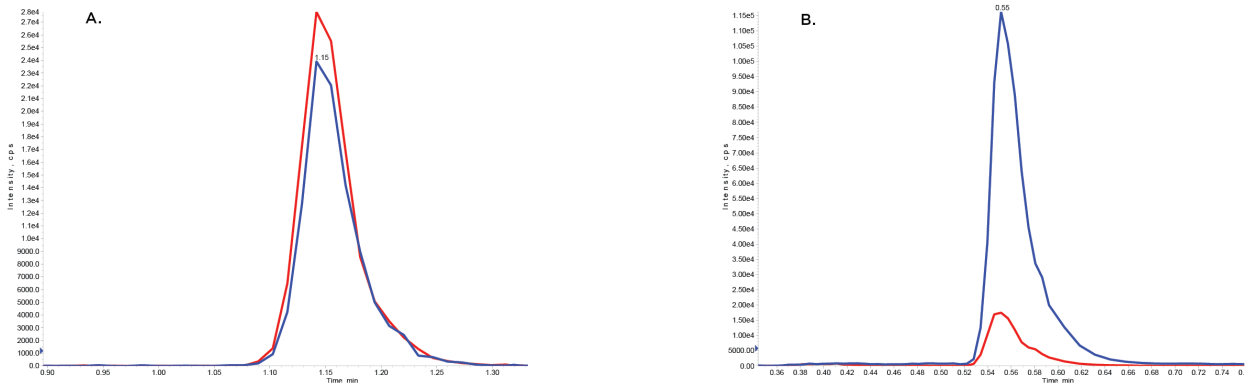

Figure 5. Representative chromatograms showing two MRMs per analyte at the cut-off level for (a) EtG (500 ng/mL), and (b) EtS (100 ng/mL), demonstrating the excellent sensitivity of this

Conclusions

This study describes a successful application of an automated sample preparation protocol for analysis of metabolites of ethanol, EtG and EtS, in urine samples by LC-MS/MS. The Beckman Coulter Biomek 4000 Workstation was used toprepare the calibration curves and urine samples prior to end-point analysis. The SCIEX Triple Quad™ 4500 LC/MS MS system was used for the identification and quantification of EtG and EtS in the processed samples. In summary, this automated method offers a simple, rapid, accurate and reproducible solution for the quantitation of ethanol metabolites that is suitable for implementation in routine testing laboratories.

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