A No-Drydown SPE Method for Biomarkers of Alcohol Consumption in Human Urine Using ISOLUTE® NH2 SPE Columns Prior to LC-MS/MS

Tested on Patient Samples

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This application note describes the extraction of ethyl glucuronide and ethyl sulfate from human urine using ISOLUTE® NH2 solid phase extraction columns.

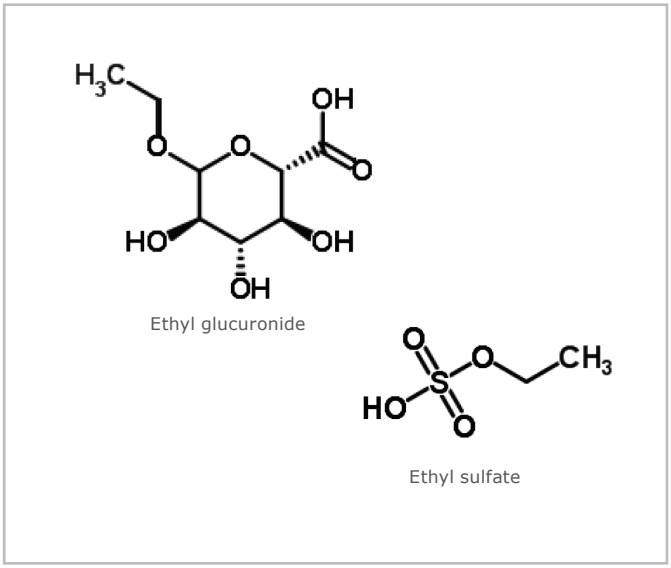

Figure 1. Structures of ethyl glucuronide and ethyl sulfate

Introduction

A recent publication by Ferrara et al[1] illustrated the utility of ISOLUTE NH2 solid phase extraction cartridges as a sample preparation method for alcohol biomarkers in human urine. The method reported a successful throughput of 6,000 samples per year. Method repeatability with %RSDs of <20% was reported for urine concentrations at 500 ng/mL. The LOQ for this method was determined to be 100 ng/mL. The key advantage to this method is the elimination of the evaporation step prior to analysis by LC-MS/MS. This study investigates the parameters and offers some suggestions for method optimization. Initial development studies were performed at the Biotage US Applications Laboratory. The method was then transferred to ARUP for further investigation of method performance with real patient samples that had been previously analyzed with a validated referee method. The results of the orthogonal measurements agreed, to provide similar diagnostic values.

Analytes

Ethyl glucuronide, ethyl sulfate

Sample Preparation Procedure

Format: ISOLUTE NH2 100 mg/1 mL columns, p/n 470-0010-A

The SPE sorbent chemistry is an aminopropyl bonded sorbent (**Figure 2**).

Samples were extracted using ISOLUTE NH2 SPE columns using a method modified from the published method in reference 1.

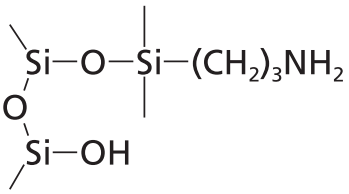

Figure 2. ISOLUTE® NH2 sorbent chemistry

Sample Pre-treatment:	To urine (100 µL), add HCl (6 M, 50 µL) and acetonitrile (1 mL). Centrifuge.
Conditioning:	Condition each column with methanol (2 mL).
Equilibration:	Equilibrate each column with water (2 mL) followed by acetonitrile containing 0.2% (v/v) acetic acid (2 mL).
Sample Loading:	Load sample (2.15 mL total volume) at a flow rate of 1 mL /min.
Interference Wash 1:	Elute interferences with hexane (1 mL). Dry columns for 10 mins under positive pressure.
Analyte Elution:	Elute analytes with 10 mM ammonium formate/formic acid (pH 3, 2 x 750 µL). No dry down is required.

HPLC Conditions

Instrument: Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA)

Column: Phenomenex Synergi 2.5 Hydro-RP (100Å 100 x 2 mm)

Injection volume: 5 µL

Mobile Phase: Solvent A: 0.1% Formic Acid in 18MΩ water
Solvent B: Acetonitrile with 0.1% formic acid

Gradient:

Table 1. Gradient parameters for the sepration ethyl glucuronide and ethyl sulfate

Step	Time (min)	Flow Rate (µL/min)	%A	%B
0	0	300	100	0
1	1.0	300	100	0
2	2.5	300	85	15
3	3.25	300	55	45
4	3.3	300	10	90
5	3.8	350	10	90
6	4.0	350	100	0
7	5.0	300	100	0

Mass Spectrometry Conditions

Instrument: API4000 triple quadrupole equipped with a Turbo Ionspray® interface operated in negative ion mode (Applied Biosystems, Foster City, CA.)

Ion Source Temperature: 700 °C

Spray Voltage: -4500V

The MRM transitions used are detailed in **Table 2**.

Table 2. MS/MS transitions for the detection of ethyl glucuronide and ethyl sulfate

Analyte	g/mole	MRM Transition (m/z)	Declustering Potential (DP)	Collision Energy	Dwell Time (ms)
Ethyl Sulfate (quantifier)	126.3	124.8 → 96.8	-38	-22	100
Ethyl Sulfate (qualifier)	126.3	124.8 → 79.8	-38	-36	100
Ethyl Sulfate-D_5	131.3	130.1 → 98.1	-38	-22	100
Ethyl Glucuronide (quantifier)	222.2	220.6 → 84.9	-44	-22	100
Ethyl Glucuronide(qualifier)	222.2	220.6 → 74.8	-44	-22	100
Ethyl Glucuronide-D_5	227.2	226.6 → 85.1	-44	-22	100

Reagents

18 MΩ water was collected in-house from a Barnstead NanoPure filter system. Optima/LC-MS grade methanol and acetonitrile were obtained from Fisher Scientific. Ethyl glucuronide and ethyl sulfate (and deuterated analogs) were purchased from Cerilliant Corp. The biological fluids were prepared and collected in-house.

Results and Discussion

The method was evaluated at the Biotage US Applications Lab (Charlotte, NC) prior to method transfer to ARUP Laboratories (Salt Lake City, UT). The LC column was a Restek Allure Organic Acids column (4.6mm x 150mm x 5µm). Since the sorbent bed size of the SPE column was not reported in the original publication, a development study was performed comparing 50 mg and 100 mg columns under various conditions. In general, the 100 mg columns outperformed the 50 mg columns yielding improved analyte response.

Choice of the second equilibration step of the published method was evaluated as there seemed to be more than one viable option (formic acid, hydrochloric acid or acetic acid in acetonitrile). The adoption of acetic acid in this modified method favored the peak area response of EtS in a screening experiment. The effect of acetic acid concentration in acetonitrile was then evaluated and the final concentration optimized.

An investigation of the interference wash was also undertaken. In urine samples, it was suspected that interferences from the endogenous composition on the matrix would be polar. For this reason, a development study was performed considering H_2O, EtAc, MTBE and THF as alternative wash solvents. It was determined that all of these solvents failed to secure reasonable recovery for the selected analytes. This was interesting as the primary interaction of this sorbent follows a weak ion exchange binding mechanism.

The pH of the elution step was also considered for optimization (pHs =3,4,5). It was determined that varying the elution pH offered no significant effect in the peak area response of the extracted analytes. This data set may suggest that the buffer is displacing the retained analyte by mass action (negating the analyte-sorbent interaction). The elution volume described in the published method was also reduced to allow for improved analyte sensitivity.

This modified method was transferred to ARUP for further evaluation with real patient samples. The LC column was changed to a Phenomenex Synergi 2.5 Hydro-RP (100Å 100x2 mm) in order to fit the existing workflow. Typical chromatograms for this method are detailed in **Figure 3**. Towards quantitative measurements, analyte suppression studies were evaluated via post column infusion experiments (syringe pump operated at 10µL/min). It was determined that ISOLUTE® NH2 extracts measured cleaner versus a dilute and shoot prep over a designated chromatographic time period. Typical chromatograms obtained by this method are given in **Figure 4**.

Towards measurements in real patient samples, this ISOLUTE NH2 method was able to differentiate between patient positive and patient negative samples over a range of clinical interest (n=6: 4 positive, 2 negative). Calibration curves are shown in **Figure 5**, with examples of real patient data also shown.

The ISOLUTE NH2 cartridge format demonstrated as a viable option for urine measurements over a relevant concentration range in clinical reference laboratories.

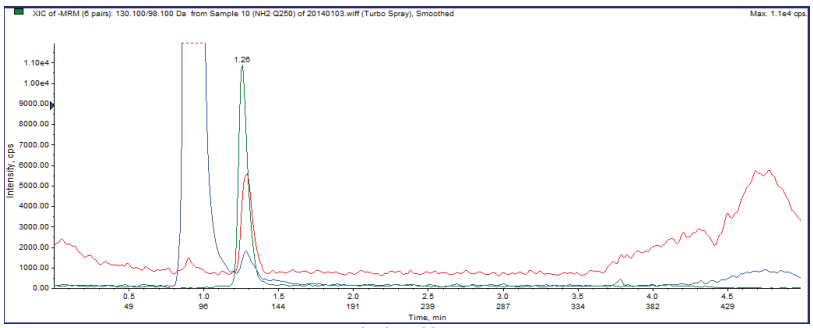

Figure 3. A typical chromatogram obtained from the extraction of a 250 ng/mL fortified specimen of Urine. The upper chromatogram represents ethyl sulfate, the lower chromatogram represents ethyl glucuronide. In each, the green trace represents the pentadeuterated internal standard. Analytes elute at 1.28 (EtS) and 2.38 (EtG) minutes respectively. Note the endogenous peak in the ethyl sulfate trace. Care must be taken when optimizing chromatography to ensure separation from ethyl sulfate.

Matrix Suppression

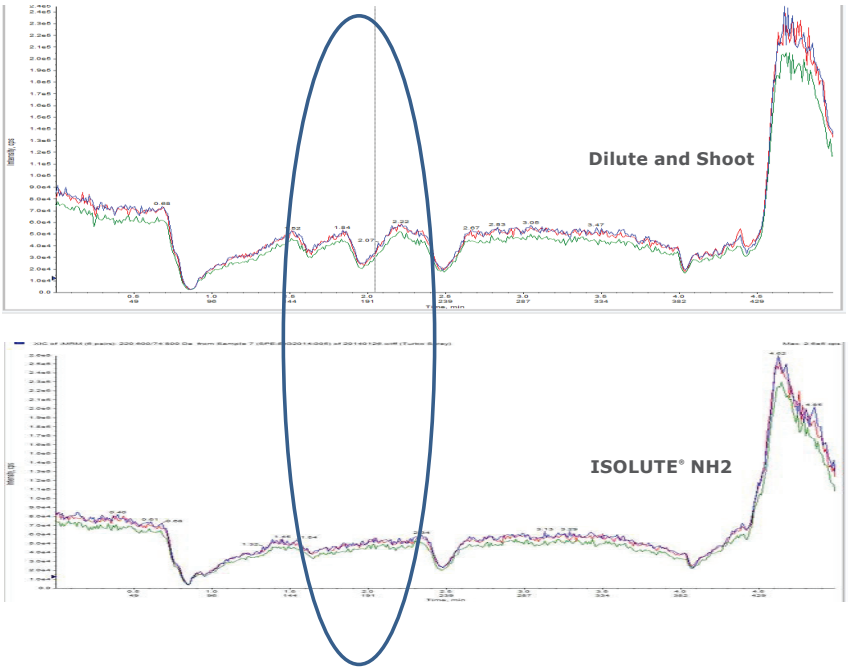

Figure 4. Post-column infusion comparison of a real patient urine sample treated by a) dilute and shoot and b) ISOLUTE® NH2 SPE.

Calibration Curve Examples

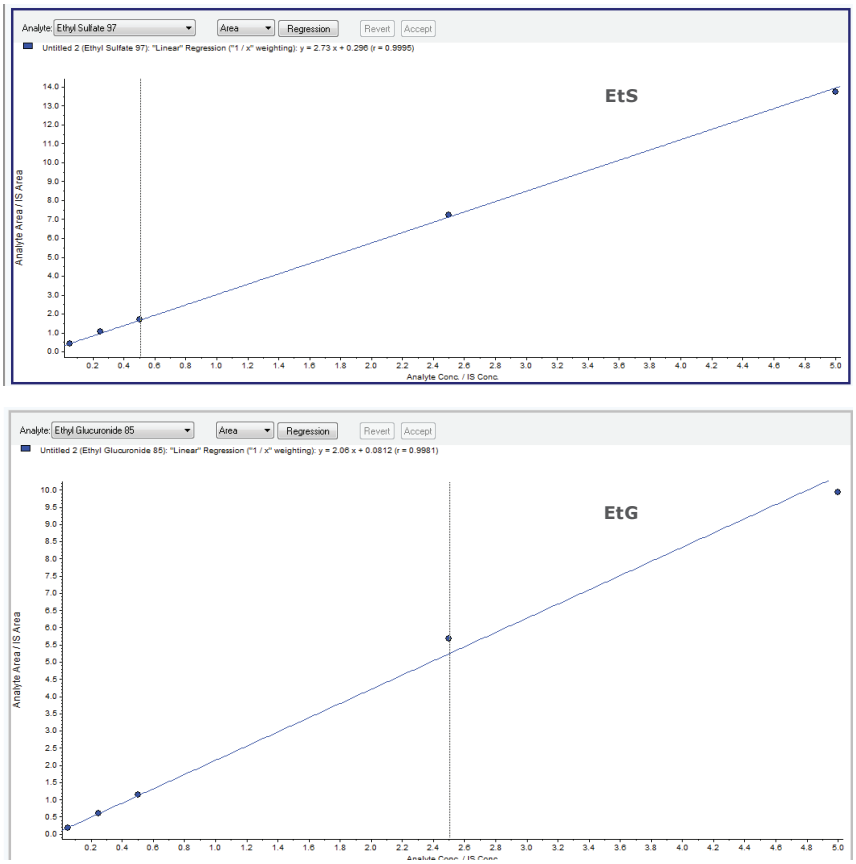

Figure 5. Calibration plots for fortified urine samples

Real Patient Data

De-identified donor specimens that had previously screened positive for EtG by immunoassay were analyzed by the described method. Below represent the tabulated concentrations of EtG and EtS:

Sample	EtG ng/mL	EtS ng/mL
A	<LOQ	<LOQ
B	<LOQ	<LOQ
C	12000	4740
D	34900	11100
E	1260	571
F	1490	567

Below are chromatograms from Sample A (**Figure 6**) and Sample E (**Figure 7**) as examples of extracted donor specimen:

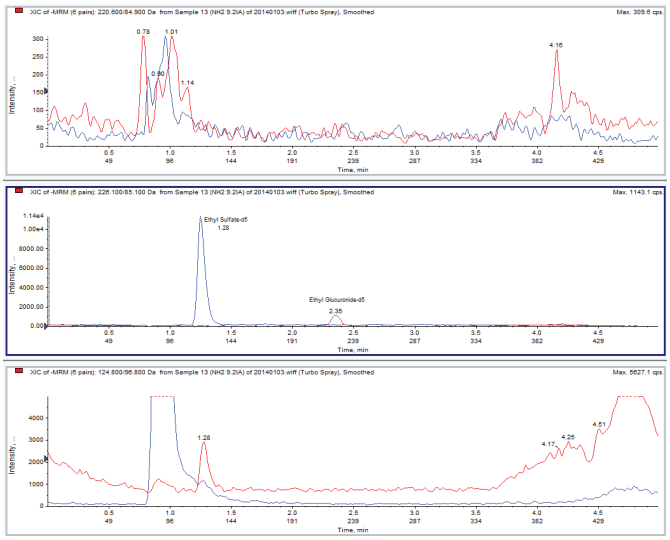

Figure 6. *Sample A.* (<LOQ). This is a donor specimen that previously screened negative for EtG by immunoassay. The upper chromatogram represents ethyl glucuronide. The middle chromatogram represents the pentadeuterated internal standard traces for both EtS and EtG. The lower trace represents ethyl sulfate. Each internal standard is fortified at the same concentration. The difference in response for each analyte is typical for this analysis.

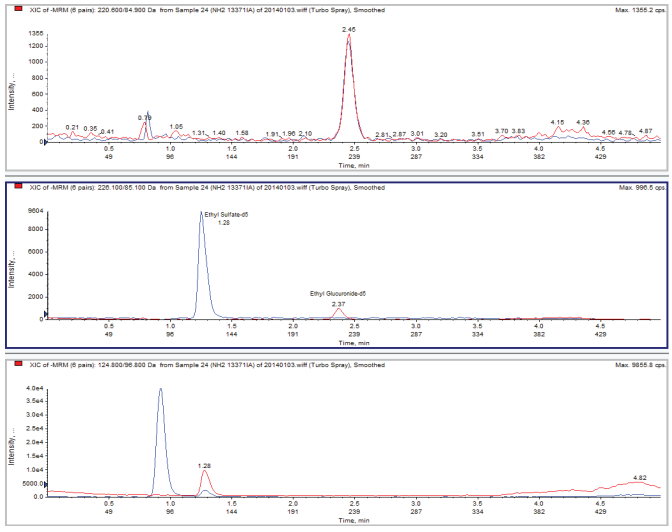

Figure 7. *Sample E.* (EtG = 1260 ng/mL; EtS = 571 ng/mL). This is a donor specimen that previously screened positive for EtG by immunoassay. The upper chromatogram represents ethyl glucuronide. The middle chromatogram represents the pentadeuterated internal standard traces for both EtS and EtG. The lower trace represents ethyl sulfate. Each internal standard is fortified at the same concentration. The difference in response for each analyte is typical for this analysis.

Acknowledgment:

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Reference:

1. Donata Favretto & Alessandro Nalesso & Giampietro Frison & Guido Viel & Pietro Traldi & Santo Davide Ferrara, Int. J. Legal Med. (2010) 124:161–164

Ordering Information

Part Number	Description	Quantity
470-0010-A	ISOLUTE® NH2 100 mg/1 mL	100
PPM-48	Biotage® Positive Pressure Manifold 48 Position	1
C103198	TurboVap® LV 100/120 V	1
C103199	TurboVap® LV 220/240 V	1

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