A Novel Solution for EtG/EtS Analysis in Human Urine by LC-MS/MS

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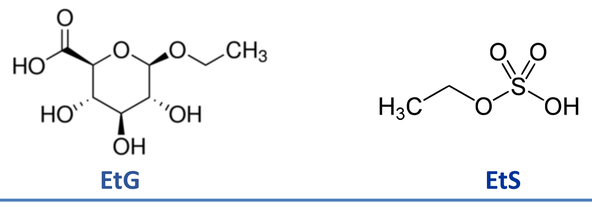
Introduction

Ethyl Glucuronide (EtG) and Ethyl Sulfate (EtS) are unique biomarkers of alcohol use. The detection of these metabolites has proven advantageous for zero tolerance treatment programs and abstinence enforcement where information regarding recent alcohol consumption is required. The analysis of EtG and EtS offers many advantages for abstinence monitoring including the detection window (~3 days), stability in stored specimens (non-volatile), and specificity. EtG and EtS are both polar which makes them difficult to retain via reversed-phase chromatography. Both compounds are also very sensitive to matrix interferences which can result in being unable to achieve low limits of detection. Isobaric interferences can also make quantitation impossible. In this study, a simple dilute and shoot method was developed, validated, and applied to patient samples for the analysis of EtG and EtS in human urine by LC-MS/MS enabled by the use of the novel Raptor EtG/EtS column.

Figure 1: Structures of EtG and EtS



Sample Preparation

Human urine (alcohol free) was fortified with EtG and EtS in order to prepare the calibration standards and QC Samples. The concentration of calibration standards ranged from 50 – 5,000 ng/mL for both analytes. Four QC levels were prepared at 50, 150, 750, and 4,000 ng/mL.

50 µL of urine was diluted with 950 µL of the working internal standard (100 ng/mL EtG-d_5/25 ng/mL EtS-d_5 in 0.1% formic acid in water). Samples were vortexed at 3500 rpm for ten seconds to mix followed by centrifugation at 3000 rpm for five minutes at 10°C. Additional double blanks were extracted for column equilibration.

Methods

Table 1: Analytical Conditions

Column:	Raptor EtG/EtS 2.7 µm, 100 mm x 2.1 mm (cat# 9325A12)		
Guard Column:	UltraShield UHPLC PreColumn Filter, 0.2 µm frit (cat# 25809)		
Mobile phase A:	0.1% Formic acid in water		
Mobile phase B:	0.1% Formic acid in acetonitrile		
Gradient:	Time (min.)	Flow (mL/min)	%B
	0.00	0.5	5
	2.50	0.5	35
	2.51	0.5	5
	4.00	0.5	5
Oven Temp..:	35°C		
Sample Temp.:	10°C		
Inj. Volume:	10 µL		
Ion Mode:	ESI-		

Table 2: Analyte Transitions

Analyte	Precursor Ion	Product Ion	Product Ion
EtG-d_5	225.9	84.9	-
EtG	220.8	84.9	74.8
EtS-d_5	129.7	97.7	-
EtS	124.7	96.8	79.7

Chromatograms

Figure 2: EtG & EtS in Human Urine on Raptor EtG/EtS

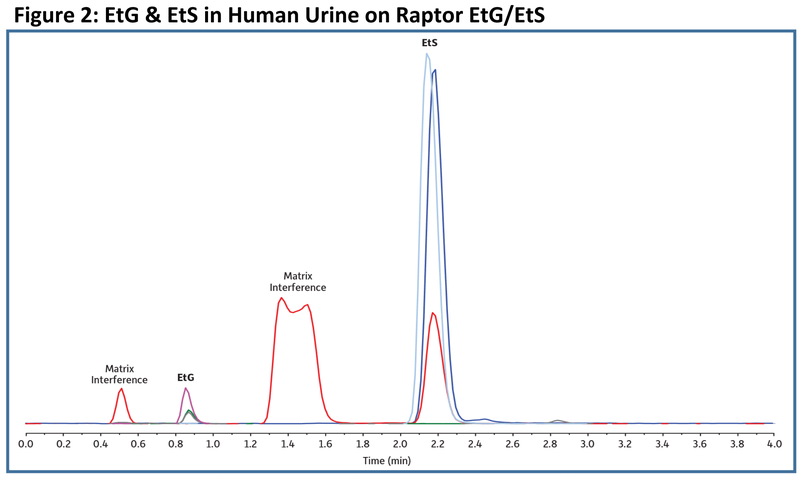


Figure 3: 1000 Sample Injection Lifetime on Raptor EtG/EtS

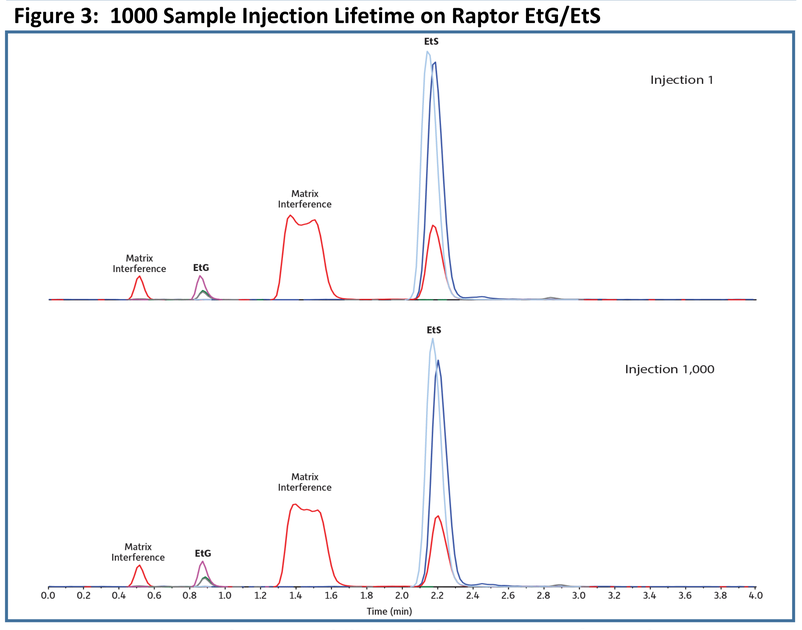


Figure 4: Matrix Resolving Capabilities of the Raptor EtG/EtS Column

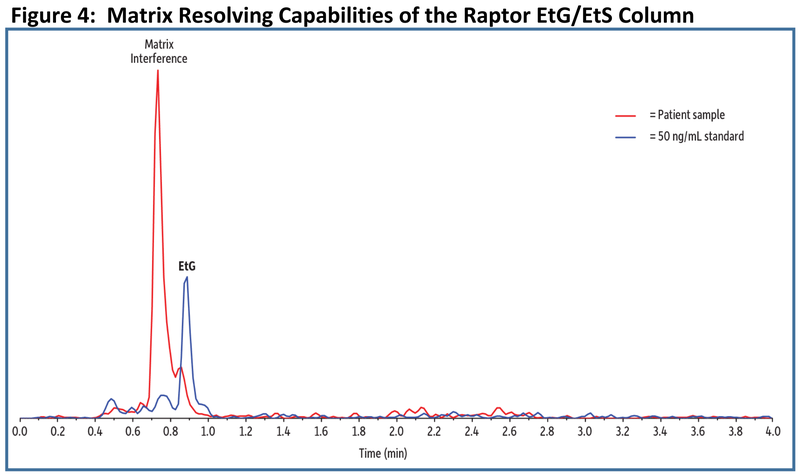
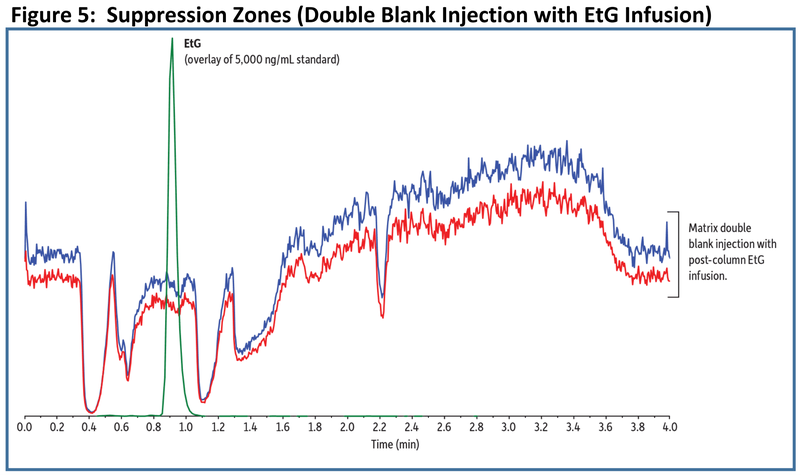


Figure 5: Suppression Zones (Double Blank Injection with EtG Infusion)



Results and Discussion

Chromatographic Performance: A fast four minute chromatographic analysis (Figure 2) was obtained from the direct injection of supernatant. EtG and EtS are clearly resolved from isobaric matrix interference making peak identification easy.

Column Robustness: Following 1,000 sample injections, all chromatographic peaks maintained the initial peak shape, retention time, and intensity (Figure 3). The maximum system pressure also remained at the same level indicating no column clogging had occurred.

Linearity: Using linear 1/x weighted regression for EtG and EtS, both compounds showed good linearity with r^2 values of 0.999 or greater.

Accuracy & Precision: Precision and accuracy analyses were performed on three different days. The method accuracy was demonstrated to be within 6.3% of the nominal concentration for all QC levels for both EtG and EtS. The %RSD was 1.28-9.19% and 4.01-6.82% for intra- and inter-run, respectively, at the QC LLOQ. The %RSD was 0.675-7.78% and 1.00-4.99% for intra- and inter-run, respectively, at the QC Low, Mid, and High levels indicating good method precision (Table 3).

Table 3: Inter-run Accuracy and Precision of QC Samples

Analyte	QC LLOQ 50 ng/mL			QC Low 150 ng/mL			QC Mid 750 ng/mL			QC High 4,000 ng/mL		
	Avg. Conc. (ng/mL)	Avg. Accuracy (%)	%RSD	Avg. Conc. (ng/mL)	Avg. Accuracy (%)	%RSD	Avg. Conc. (ng/mL)	Avg. Accuracy (%)	%RSD	Avg. Conc. (ng/mL)	Avg. Accuracy (%)	%RSD
EtG	51.2	102	6.82	143	95.2	4.99	749	99.8	3.68	3,949	98.7	2.04
EtS	46.9	93.7	4.01	143	95.6	1.42	762	102	1.00	3,958	98.9	1.59

Selectivity: The Raptor EtG/EtS column not only offers superior resolution from EtS isobaric matrix interference (Figure 2), but also the ability to resolve less common EtG isobaric interferences that could otherwise result in false positives (Figure 4).

Matrix Effect: Samples prepared in matrix show approximately 80% and 100% of the signal compared to sample prepared in solution for EtG and EtS, respectively, across all QC levels. The sensitivity and consistency of the EtG response at the LLOQ is made possible since the elution time is outside of a zone of matrix suppression (Figure 5).

Sample Analysis: The robustness of the method was further tested by monitoring five patient samples with positive results for both EtG and EtS in the lower end of the linear range across multiple instrument platforms and nine column lots of Raptor EtG/EtS. The precision of the results (n=9) was found to range from 3.24-11.2% for both analytes over multiple days and sample preparations indicating excellent robustness and ease of method transfer (Table 4).

Table 4: Inter-run Precision of Patient Samples Across Multiple Instrument Platforms and Nine Column Lots

Patient Sample	AVG EtG Concentration (ng/mL)	%RSD	AVG EtS Concentration (ng/mL)	%RSD
37	216	6.13	78.0	3.56
44	1167	4.81	300	3.24
50	98.2	9.76	82.4	4.01
51	319	8.33	233	3.63
53	247	11.2	163	8.70

Conclusions

It was demonstrated that the Raptor EtG/EtS column is excellent for the rapid and accurate analysis of EtG and EtS in human urine. Isobaric matrix interferences are easily resolved preventing issues with peak identification and quantitation. The elution time of EtG outside of a zone of matrix suppression typically observed with dilute-and-shoot assays allows for superior sensitivity. With a fast and simple sample preparation procedure and four minutes of chromatographic analysis time, the established method provides accurate, high-throughput monitoring of alcohol consumption.