Application Note

Clinical Research

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Quantitative LC/MS/MS Analysis of Ethyl Glucuronide and Ethyl Sulfate Using a Charged Surface-C18 Column

Authors

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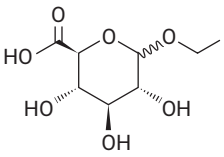
Abstract

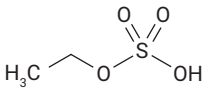
The Agilent InfinityLab Poroshell 120 CS-C18 column is a hybrid end-capped C18 phase on a superficially porous particle modified to have a charged surface. This application note demonstrates a robust, reproducible method for detecting the ethyl alcohol metabolites ethyl glucuronide (EtG) and ethyl sulfate (EtS) in urine samples. The method was developed on an Agilent 1290 Infinity II LC and both the Agilent 6470 triple quadrupole and Ultivo triple quadrupole LC/MS systems. Samples were diluted with mobile phase in vials. The quantitative method had a calibration range of 100 to 10,000 ng/mL. The results showed excellent linearity, with an R^2 >0.99. Retention time stability and reproducibility were evaluated. Multiple dilutions and injection volumes were also tested to determine which gave the best peak shape and area counts.

Introduction

This application note describes the development of an analytical method for the accurate determination of two alcohol consumption markers. The markers, ethyl glucuronide (EtG) and ethyl sulfate (EtS), were analyzed in urine. The samples were analyzed using an Agilent 1290 Infinity II LC and both the Agilent 6470 and Ultivo triple quadrupole LC/MS systems with Agilent Jet Stream technology. Using tandem mass spectrometry (MS/MS) and multiple reaction monitoring (MRM), this method was linear from 100 to 10,000 ng/mL.



Ethyl glucuronide
Log P = –1.4



Ethyl sulfate
Log P = –2.1

Figure 1. Ethyl glucuronide (EtG) and ethyl sulfate (EtS).

Experimental

LC configuration and parameters

Configuration		
1290 Infinity II Binary Pump (G7120A)		
1290 Infinity II Multisampler (G7167B)		
1290 Infinity II Multicolumn Thermostat Column Compartment (G7116B)		
Needle Wash	IPA:MeOH:ACN:Acetone (50:20:20:10)	
Autosampler Temperature	4 °C	
Injection Volume	5 µL	
Analytical Column	Agilent Poroshell CS-C18 (2.1 × 50 mm or 2.1 × 100 mm, 2.7 µm) (p/n 679775-942 or 675775-942)	
Column Temperature	40 °C	
Mobile Phase A	0.01% Formic acid in water	
Mobile Phase B	Methanol	
Flow Rate	0.350 mL/min	
Gradient	2.1 × 50 mm column Time (min) %B 0.00 2 3.00 2 3.01 98 3.99 98 4.00 2	2.1 × 100 mm column Time (min) %B 0.00 2 1.20 2 3.00 50 3.01 98 3.99 98 4.00 2
Stop Time	4.0 min	4.0 min
Post Time	1.0 min	1.4 min

LC/TQ mass spectrometer configuration and parameters

Triple Quadrupole Mass Spectrometer Configuration		
	6470 LC/TQ (G6470B)	Ultivo LC/TQ (G6465B)
Ionization Mode	Negative	Negative
Drying Gas Temperature	200 °C	200 °C
Drying Gas Flow	12 L/min	12 L/min
Nebulizer Pressure	50 psi	50 psi
Sheath Gas Temperature	350 °C	350 °C
Sheath Gas Flow	12 L/min	12 L/min
Nozzle Voltage	2,000 V	2,000 V
Capillary Voltage, Negative	5,000 V	5,000 V
Delta EMV, Negative	300 V	0 V
CAV:	3 V	3 V

MRM transitions

Compound	Precursor	Product	Dwell (msec)	Frag (V)	CE (V)
EtG	221.1	75	50	100	16
EtG	221.1	85	50	100	20
EtG-d5	226.1	75	50	100	16
EtS	125.1	97	50	80	20
EtS	125.1	80	50	80	40
EtS-d5	130.1	98	50	80	20

Software

Data acquisition: MassHunter Acquisition Software (B.09.00).

Data analysis: MassHunter Quantitative Analysis Software (B.09.00) and Qualitative Analysis Software (B.09.00)

Chemicals and reagents

Optima grade methanol and formic acid were from Fisher Scientific (Hampton, NH). Chemical standards were purchased from Cerilliant (Round Rock, TX). Human urine was purchased from Golden West Diagnostics (Temecula, CA).

Standards and curve preparation

Standard and internal standard (IS) stock solutions were diluted in methanol and spiked into urine matrix. The calibration curve was prepared by serial dilution with the high calibrator at 10,000 ng/mL EtG and 1,000 ng/mL EtS. Calibrators were prepared as shown in Table 1.

MS time segments for 2.1 × 50 mm and 2.1 × 100 mm column

Segment No.	Time (2.1 × 50)	Time (2.1 × 100)	Scan Type	Diverter Valve	Data Store
1	0.0 min	0.0 min	MRM	Waste	No
2	0.8 min	1.0 min	MRM	MS	Yes
3	3.2 min	3.4 min	MRM	Waste	No

The IS solutions were prepared at concentrations of 50 µg/mL EtG and 5 µg/mL EtS, for a final concentration in samples of 500 ng/mL and 50 ng/mL, respectively. Quality control solutions were prepared independently of the calibrators at concentrations of 200 and 3,000 ng/mL for EtG and 20 and 300 ng/mL for EtS.

Table 1. Calibration curve concentrations.

Calibrator	EtG (ng/mL)	EtS (ng/mL)
1	100	10
2	250	25
3	500	50
4	1,000	100
5	2,500	250
6	5,000	500
7	10,000	1,000

Sample preparation

All calibrators, controls, and biological samples were prepared using a 1:50 dilution. A volume of 10 µL of urine was added to max recovery vials and 490 µL of 0.01% formic acid in water was added. Each vial was spiked with 10 µL of IS solution. Samples were capped and vortexed.

Data analysis

Data were acquired and analyzed using the MassHunter software suite. Data acquisition was performed using MassHunter Acquisition Software (B.09.00). Data were analyzed using MassHunter Quantitative Analysis Software (B.09.00) and Qualitative Analysis Software (B.09.00). All analytes were normalized to internal standards.

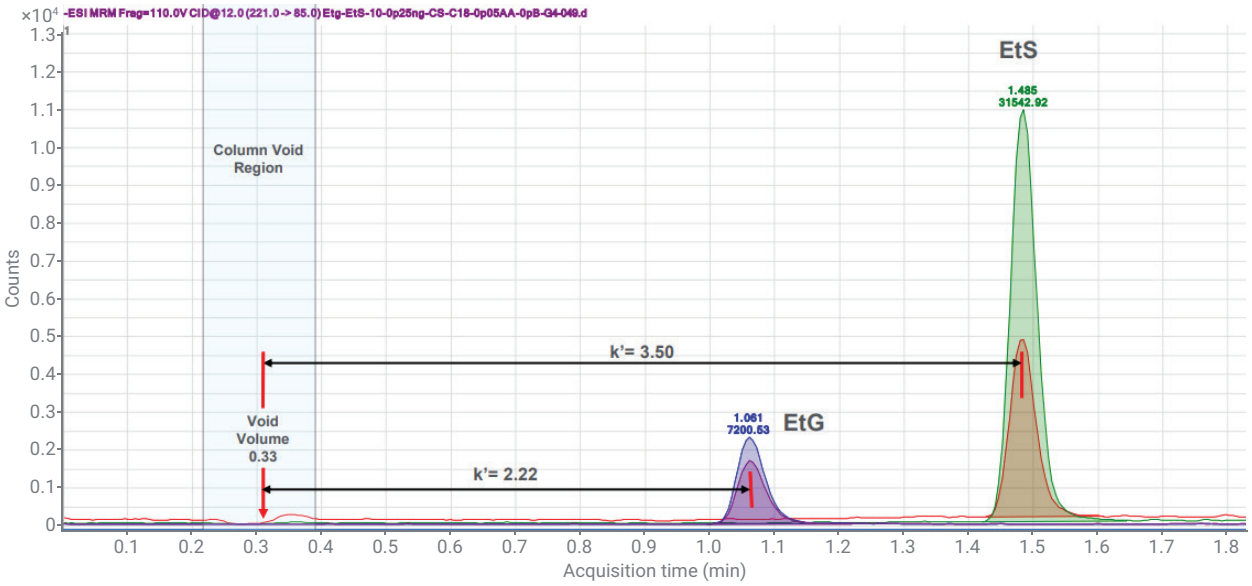


Figure 2. Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are well-retained and separated using Agilent InfinityLab Poroshell 120 CS-C18 column.

Results and discussion

Effect of dilution and injection volume on chromatography

To ensure the best results, a dilution study was performed testing dilutions of 1:10, 1:20, and 1:50. Samples were diluted with mobile phase A: 0.01% formic acid in water. In addition to assessing the dilution, the injection volume was also tested at 0.5, 1, 2, 5, 10, and 20 µL injections. For this study, a urine sample was spiked at 2,000 ng/mL EtG and 200 ng/mL EtS to ensure adequate response. This study used a Poroshell 120 CS-C18, 2.1 × 50 mm column. By comparing area count and peak shape, it was determined that a 1:50 dilution with a 2 µL injection volume yielded the best results for both ethyl glucuronide and ethyl sulfate (Figure 3).

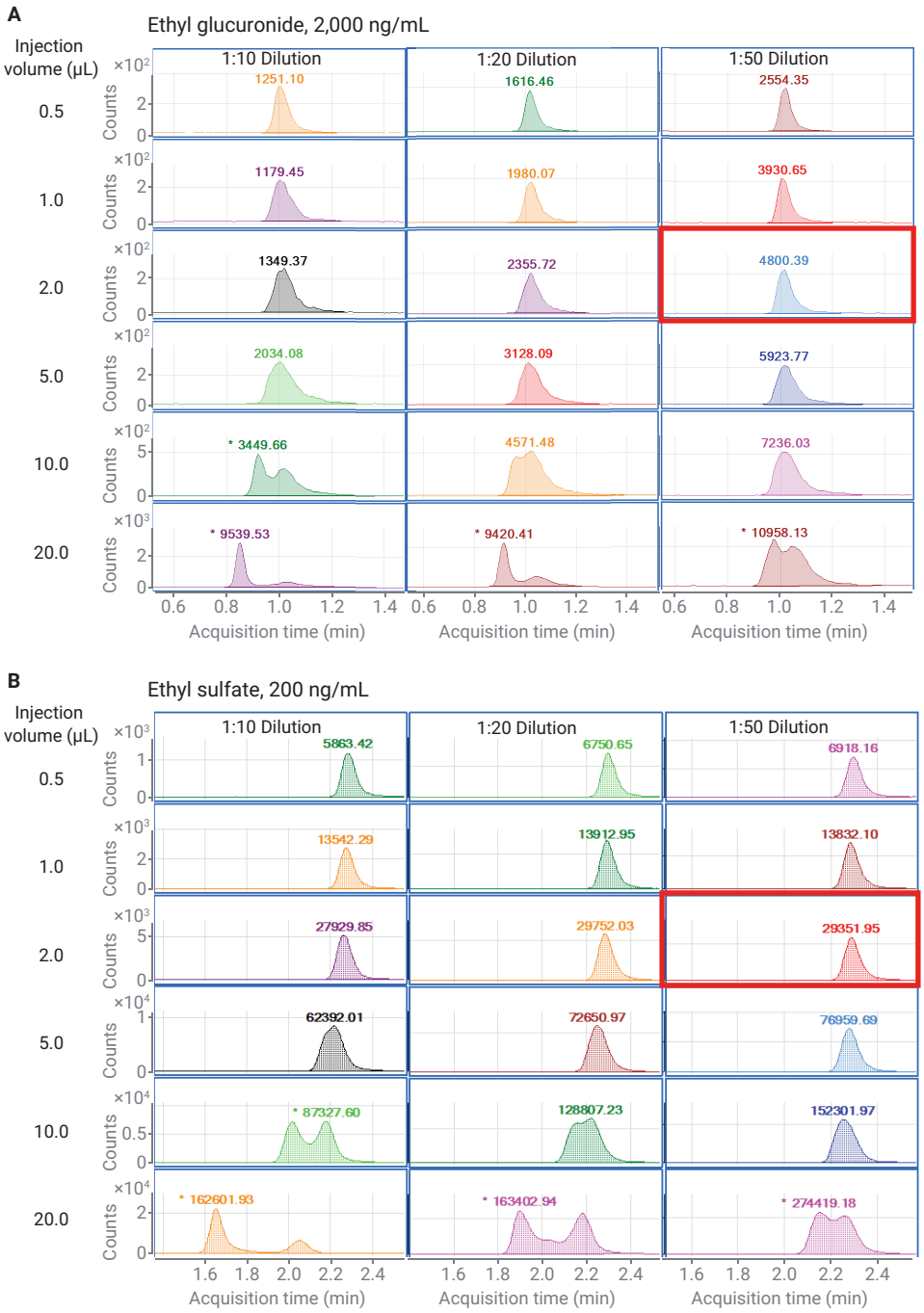


Figure 3. Both sample dilution and injection volume were assessed. A 1:50 sample dilution with a 2 µL injection was determined to provide the best results, using an Agilent InfinityLab Poroshell 120 CS-C18, 2.1 × 50 mm, 2.7 µm column.

Quantitation results

Results were based on a 7-point calibration curve ranging from 100 to 10,000 ng/mL for EtG and 10 to 1,000 ng/mL EtS. Figure 4 demonstrates calibration curve linearity in triplicate. Both compounds were linear within their analysis range.

Accuracy and reproducibility

Excellent reproducibility was observed for both analytes over 1,000 injections as shown in Figure 5. Five samples with different EtG and EtS concentrations were injected 200 times each. Samples 1 to 4 had reportable EtG and EtS, while sample 5 was a negative with no alcohol consumption within 72 hours. Retention time stability was also assessed over 800 separate injections; results are shown in Figure 5.

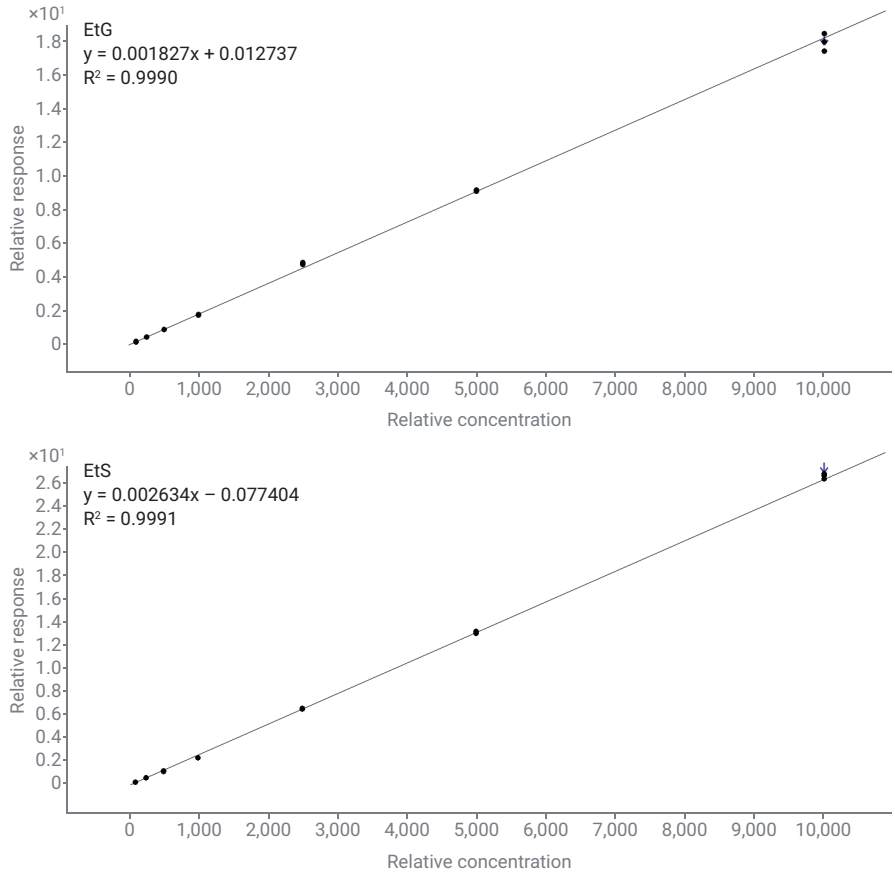


Figure 4. Calibration curves, in triplicate, for ethyl glucuronide and ethyl sulfate.

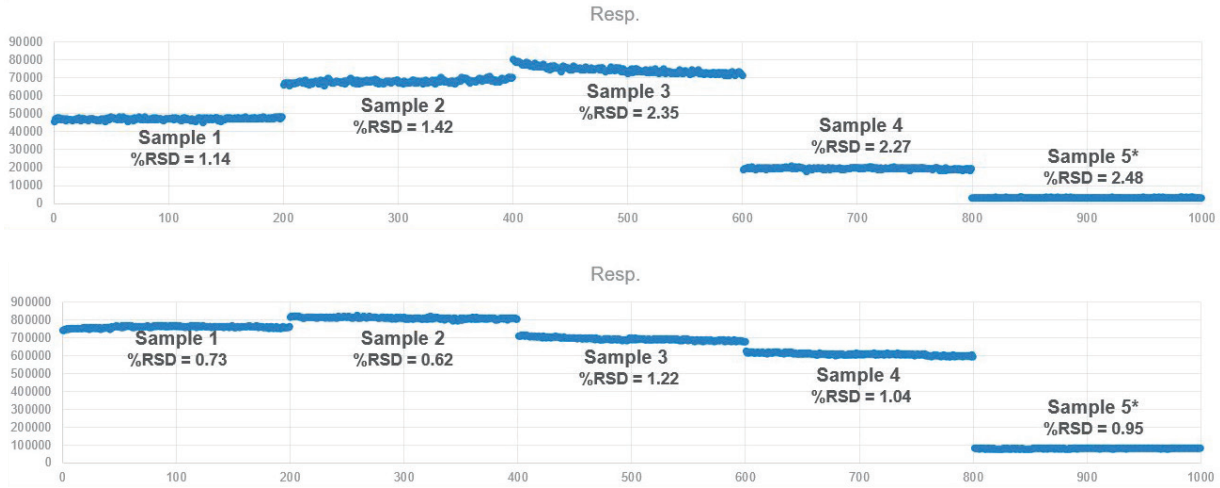


Figure 5. One thousand injections, 200 per sample, showed reproducible response across the run. **Note:** Sample 5 had not consumed alcohol within 72 hours of collection.

Conclusion

An Agilent InfinityLab Poroshell 120 CS-C18 column retained both analytes with acceptable k' values. A simple "dilute and shoot" sample preparation and analysis using an Agilent triple quadrupole LC/MS system showed accurate, reproducible results.

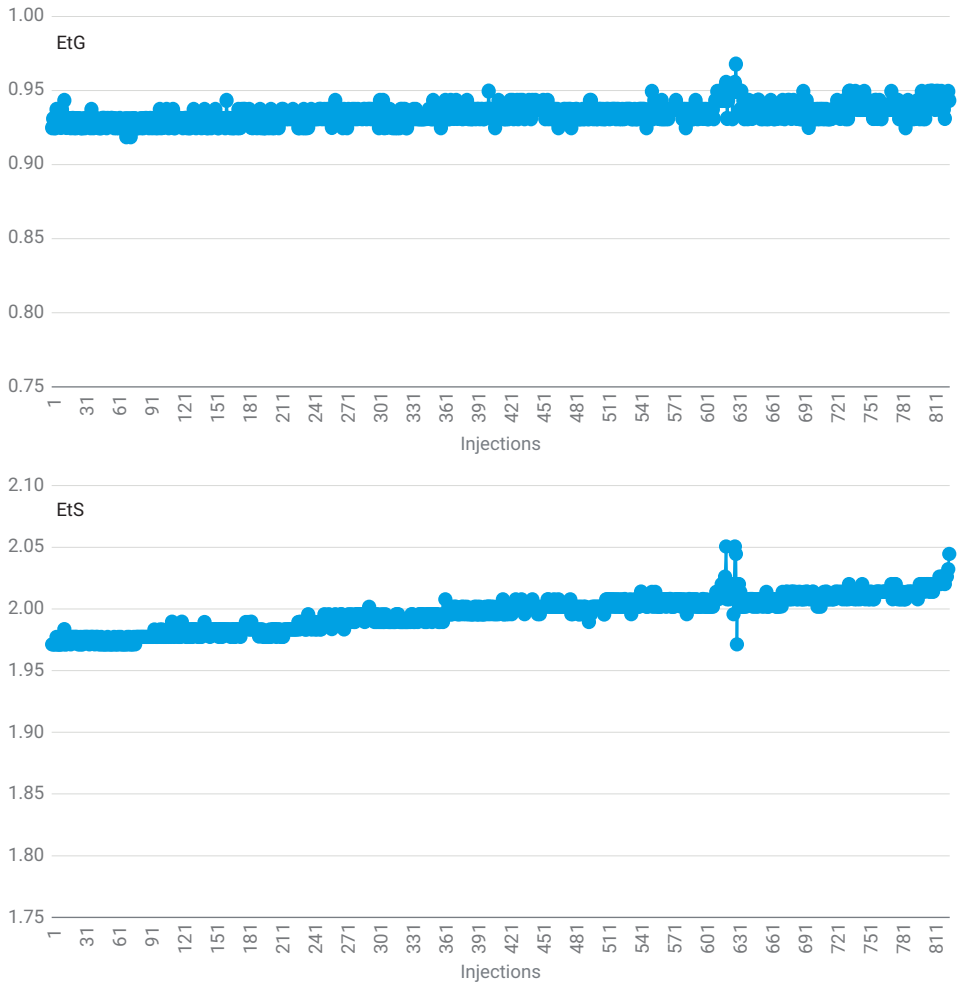


Figure 6. Retention times were stable over 800 injections.

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