APPLICATIONS

LC/MS/MS Analysis of Ethyl Glucuronide (EtG) and Ethyl Sulfate (EtS) from Urine Using a Luna® Omega 1.6µm UHPLC Column

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Introduction

Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are metabolites of ethanol and are slowly eliminated in the urine. EtG has been measured up to 80 hours after heavy ethanol consumption.

With acceptable limits of quantitation being 500 ng/mL for EtG and 250 ng/mL for EtS[1], an LC column with good polar selectivity is critical since EtG and EtS can elute close to matrix interferences.

In this study, we present a method for fast, accurate and reproducible quantitation and determination of both metabolites using Luna Omega Polar C18, a novel sub-2 µm UHPLC column with polar selectivity and 100 % aqueous stability. The efficiency and selectivity of this novel sub-2 µm phase allows for an accurate and sensitive method while meeting throughput needs.

Materials and Methods

Reagents and Chemicals

Ethyl-β-D-glucuronide, ethyl sulfate, and deuterated standards (EtS-D_5, EtG-D_5) were purchased from Cerilliant (Cerilliant Corporation, Round Rock, TX, USA).

Sample Preparation

Negative human urine was collected and spiked with ethyl-β-D-glucuronide and ethyl sulfate to prepare a stock standard at 20,000 ng/mL. This standard was serially diluted in urine to make a 5 point calibration curve. Two positive samples were also run.

Each standard and sample was diluted 10-fold with mobile phase A (10 mM ammonium formate in 0.1 % formic acid, pH unadjusted) and spiked with internal standard as follows: 100 µL urine + 885 µL mobile phase A + 10 µL EtS-D5 (10 µg/mL) + 5 µL EtG-D5 (100 µg/mL)

Experimental Conditions

LC/MS/MS method was performed using a Luna Omega 1.6 µm Polar C18 column on a Shimadzu® Nexera® X2 with LC30A solvent module (Shimadzu Scientific Instruments, Columbia, MD, USA) and an upper pressure limit of 1000 bar. MS analysis was performed using a SCIEX 4000 QTRAP® LC/MS/MS system (Sciex, Framingham, MA, USA).

LC/MS/MS Conditions

LC Column: Luna Omega 1.6 µm Polar C18
Dimensions: 100 x 2.1 mm
Part No.: 00D-4748-AN
Mobile Phase: A: 10 mM Ammonium Formate in 0.1 % Formic Acid, pH unadjusted (~3.2)
B: 50:50 Methanol/Acetonitrile

Gradient:

Time (min)	% B
0.01	0
1.00	0
2.00	25
2.01	95
3.00	95

Flow Rate: 400 µL/min
Temperature: 40 °C
LC System: Shimadzu Nexera X2
Detection: MS/MS
Detection System: SCIEX 4000 QTRAP

Table 1.
Primary and Secondary EtG/EtS MRM Transitions

ID	Q1	Q3
EtG 1 (Primary)	221.2	75.0
EtS 1 (Primary)	124.9	80.1
EtG_D5	226.1	85.1
EtS_D5	130.0	80.1
EtG 2 (Secondary)	221.2	85.1
EtS 2 (Secondary)	124.9	97.0

Result and Discussion

The use of the Luna® Omega 1.6 µm Polar C18 column allowed for the fast elution of EtG and EtS in less than 2 min (**Fig. 1**), with total run times including column cleaning less than 5 minutes. This fast separation allows for multiplexing techniques to increase throughput. Note that the retention of EtS is ~0.9 minutes, and EtG is ~3 minutes. This ensures that low responding EtG elutes away from major a matrix component that responds in the EtS channel, which is a known cause for signal suppression.

Calibration curves were generated over a concentration of 50 ng/mL to 5000 ng/mL. A quadratic regression was used to determine relative response versus concentration using peak area of EtG and EtS/peak area of IS, with 1/X weighting factor), Correlation coefficient for EtS calibration curves are 0.997 and 0.9994 for primary and secondary MRM's, respectively. Correlation coefficient for EtG calibration curves are 0.9998 and 0.9998 for primary and secondary MRM's, respectively.

Quantitative data for EtS and EtG standards are summarized in **Table 2** and **Table 3**. Sample concentrations were calculated using the primary MRM channels, and results are summarized in **Table 4**.

Table 2.
EtS Quantitation

Sample ID	Analyte Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy (%)
Primary Standard 1	50	46	91.2
Primary Standard 2	100	109	109.0
Primary Standard 3	500	506	101.0
Primary Standard 4	1000	987	98.7
Primary Standard 5	5000	5000	100.0
Secondary Standard 1	50	54	108.0
Secondary Standard 2	100	91	91.4
Secondary Standard 3	500	502	100.0
Secondary Standard 4	1000	1000	100.0
Secondary Standard 5	5000	5000	100.0

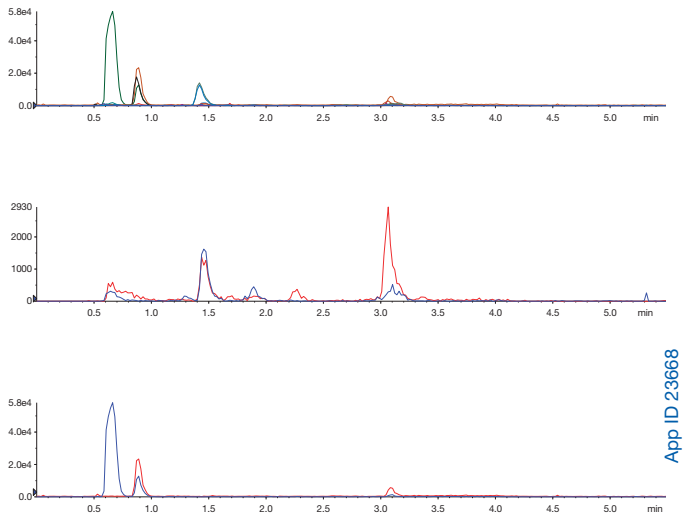
Table 3.
EtG Quantitation

Sample	Analyte Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy (%)
Primary Standard 1	50	54	107.0
Primary Standard 2	100	94	94.0
Primary Standard 3	500	482	96.4
Primary Standard 4	1000	1030	103.0
Primary Standard 5	5000	4990	99.8
Secondary Standard 1	50	54	108.0
Secondary Standard 2	100	91	91.4
Secondary Standard 3	500	502	100.0
Secondary Standard 4	1000	1000	100.0
Secondary Standard 5	5000	5000	100.0

Table 4.
Sample Results

Sample Name	Analyte Peak Name	Calculated Concentration (ng/mL)	Analyte Peak Area (counts)	IS Peak Area (counts)
Sample 1	ETS-1	763	6.75E+04	6.76E+04
Sample 2	ETS-1	405	4.14E+04	7.50E+04
Sample 1	ETG-1	5000	4.69E+04	6.35E+04
Sample 2	ETG-1	2230	2.66E+04	5.93E+04

Figure 1.
Extracted Ion Chromatograms for EtG/EtS and their Deuterated Internal Standards (EtG-D_5 and EtS-D_5), 500ng/mL



App ID 23668

Figure 2.
Extracted Ion Chromatograms for Positive Sample 1

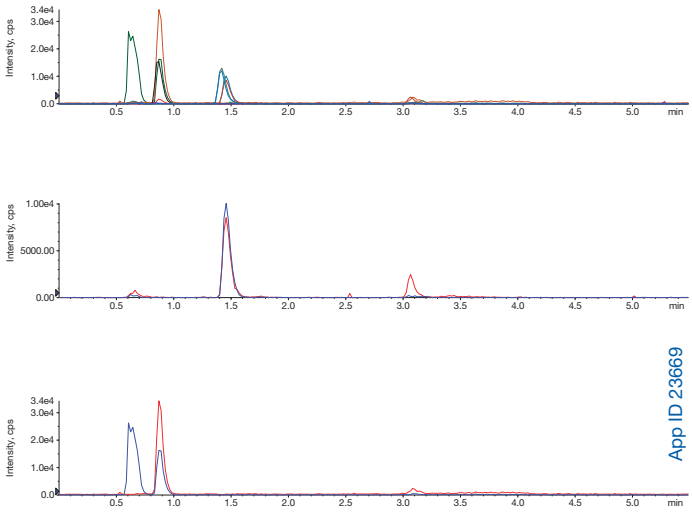
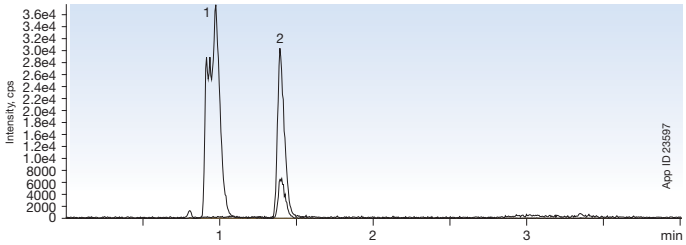


Figure 3.
Extracted Ion Chromatograms for EtG and EtS, 100 ng/mL



Further Method Development

Another method was implemented using different mobile phase conditions, to compensate for differences in instrumentation and method requirements.

LC/MS/MS method was performed on an Agilent® 1260 (Agilent Technologies, Santa Clara, CA, USA) with an upper pressure limit of 600 bar. MS analysis was performed using a SCIEX API 4000™ MS/MS.

LC/MS/MS Conditions

LC Column: Luna Omega 1.6 µm Polar C18
Dimensions: 100 x 2.1 mm
Part No.: 00D-4742-AN
Mobile Phase: A: 10 mM Ammonium Formate pH 3
Formic Acid, pH unadjusted (~3.2)

B: Acetonitrile with 0.1% Formic Acid

Gradient:

Time (min)	% B
0	0
1	50
1.1	0
5	0

Flow Rate: 300 µL/min
Temperature: 30°C
LC System: Agilent 1260
Detection System: SCIEX API 4000

Conclusions

A method using a Luna Omega 1.6 µm Polar C18, a new UHPLC column with excellent polar selectivity, can be used for accurate and quantitative analysis for ethanol metabolites ethyl glucuronide and ethyl sulfate. The method shows good linearity and accuracy from the concentration ranges of 50 to 5000 ng/mL.

Another method was also presented here as proof of concept for mobile phase optimization. This method also showed good separation for both EtG and EtS, with run times of less than 5 minutes, and could be used for further studies for increasing sensitivity.

References:

1. Jatlow, Peter I. et al. “Ethylglucuronide and Ethyl Sulfate Assays in Clinical Trials, Interpretation and Limitations: Results of a Dose Ranging Alcohol Challenge Study and Two Clinical Trials.” Alcoholism, clinical and experimental research 38.7 (2014): 2056–2065. PMC. Web. 4 May 2016.

APPLICATIONS

Ordering Information

Luna® Omega

1.6 µm Minibore Columns (mm)					SecurityGuard™ ULTRA Cartridges‡
Phases	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	3/pk
Polar C18	00A-4748-AN	00B-4748-AN	00D-4748-AN	00F-4748-AN	AJ0-9505
C18	00A-4742-AN	00B-4742-AN	00D-4742-AN	00F-4742-AN	AJ0-9502

‡ SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

If Luna analytical columns do not provide at least an equivalent separation as compared to a competing column of the same particle size, similar phase and dimensions, return the column with comparative data within 45 days for a FULL REFUND.

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