APPLICATIONS

Two New Approaches to Analyzing EtS/EtG from Urine

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Overview

- *Three new methods for EtS/EtG Analysis*
- *2 new HPLC/UHPLC stationary phases*
- *Method development tips*

Introduction

There is rarely a "silver bullet" in method development. A method that works great for one lab, may not work for another lab for a variety of reasons. Difference in systems, samples, and the nature of mass spectrometry can negatively affect transferring a method. In this technical note, we offer three options for the analysis of ethyl glucuronide (EtG) and ethyl sulfate (EtS) from urine to help alleviate typical concerns seen with method transfer.

EtS and EtG are direct metabolites of alcohol (ethanol). Both metabolites can be detected up to 3 days after alcohol consumption.[1] Additionally, these analytes are non-volatile and therefore more stable in stored specimens than alcohol.

While the long detection window and sample stability in urine offer strong benefits over blood alcohol level testing, the two compounds themselves pose several challenges. First, both compounds are very polar and therefore don't retain well on common reversed phase LC stationary phases. Secondly, sensitivity is generally low under negative ionization mode (ESI-) due to urinary matrix interferences (isobaric or not) that suppress analyte signal.

Ideally, a forensic toxicologist would want a method that retains these polar compounds, gives excellent efficiency, sensitivity and peak shape, and separates common urine interferences. Here we offer three novel methods on two stationary phases—Luna® Omega 5 µm Polar C18 and Luna Omega 5 µm PS C18—that give increased retention, efficiency, and peak shape. While both of these novel stationary phases can provide stability in 100 % aqueous mobile phases and enhanced retention for highly polar molecules such as EtG and EtS that do not typically retain well on conventional C18 phases, the primary distinction between the two phases is that the Luna Omega 5 µm PS C18 phase contains a positively-charged surface group that can interact via polar and ionic mechanisms with polar acids like EtS and result in dramatic shifts in selectivity and retention.

Materials and Methods

Analytical reference standards and human urine were purchased from Cerilliant Corporation (Round Rock, TX, USA) and BioreclamationIVT (Chestertown, MD, USA). All other chemicals were obtained from the Sigma-Aldrich Company (St. Louis, MO).

Experimental Conditions

All LC/MS/MS analyses were performed using Luna Omega 5 µm Polar C18 and Luna Omega 5 µm PS C18 columns on an Agilent® 1200 LC system (Agilent Technologies, Palo Alto, CA, USA) and SCIEX API 4000 (SCIEX, Framingham, MA, USA).

Sample Preparation

Urine was spiked with EtG and EtS to a final concentration of 500 ng/mL. The spiked urine was then diluted 10-fold using the same aqueous buffer that was used for the LC method on which the samples were run (either water with 0.1 % formic acid or 5 mM ammonium formate pH 3.3). It is critical to match the diluent with the buffer that is used for the LC method in order to optimize chromatographic behavior.

Luna Omega 5 µm Polar C18 Method Development

For the analyses performed using the Luna Omega 5 µm Polar C18 column, we identified two mobile phase options. The first method consisted of a classical LC/MS mobile phase of water with 0.1 % formic acid and methanol with 0.1 % formic acid. Representative XIC for the spiked urine sample are shown in **Figure 1**.

For the second mobile phase option, we used 5 mM ammonium formate (adjusted to pH 3.3 using formic acid) as the aqueous component and 0.1 % formic acid in acetonitrile as the organic solvent. Representative XIC for the spiked urine sample are shown in **Figure 2**.

LC/MS/MS Conditions #1

Column: Luna Omega 5 µm Polar C18
Dimensions: 50 x 4.6 mm
Part No.: 00B-4754-E0
SecurityGuard™ Cartridge: AJ0-7601
Mobile Phase: A: 0.1% Formic acid in Water
B: 0.1% Formic acid in Methanol
Gradient:

Time (min)	B (%)
0	0
3	90
3.1	0

Flow Rate: 0.7 mL/min
Injection Volume: 5 µL
Temperature: 25°C
Detector: SCIEX API 4000™ MS/MS

Analyte	Retention time (min)
EtS	1.36
EtG	2.05

LC/MS/MS Conditions #2

Column: Luna Omega 5 µm Polar C18
Dimensions: 50 x 4.6 mm
Part No.: 00B-4754-E0
SecurityGuard™ Cartridge: AJ0-7601
Mobile Phase: A: 5 mM Ammonium formate (pH 3.3)
B: 0.1% Formic acid in Acetonitrile
Gradient:

Time (min)	B (%)
0	5
3	90
3.1	5

Flow Rate: 0.7 mL/min
Injection Volume: 5 µL
Temperature: 25°C
Detector: SCIEX API 4000 MS/MS

Analyte	Retention time (min)
EtS	1.21
EtG	1.86

[1]The Role of Biomarkers in the Treatment of Alcohol Use Disorders (2012). SAMHSA Advisory. Volume 11 Issue 2. Available at: http://store.samhsa.gov/shin/content/SMA12-4686/SMA12-4686.pdf

Figure 1. XIC for EtS and EtG Using LC/MS/MS Conditions #1

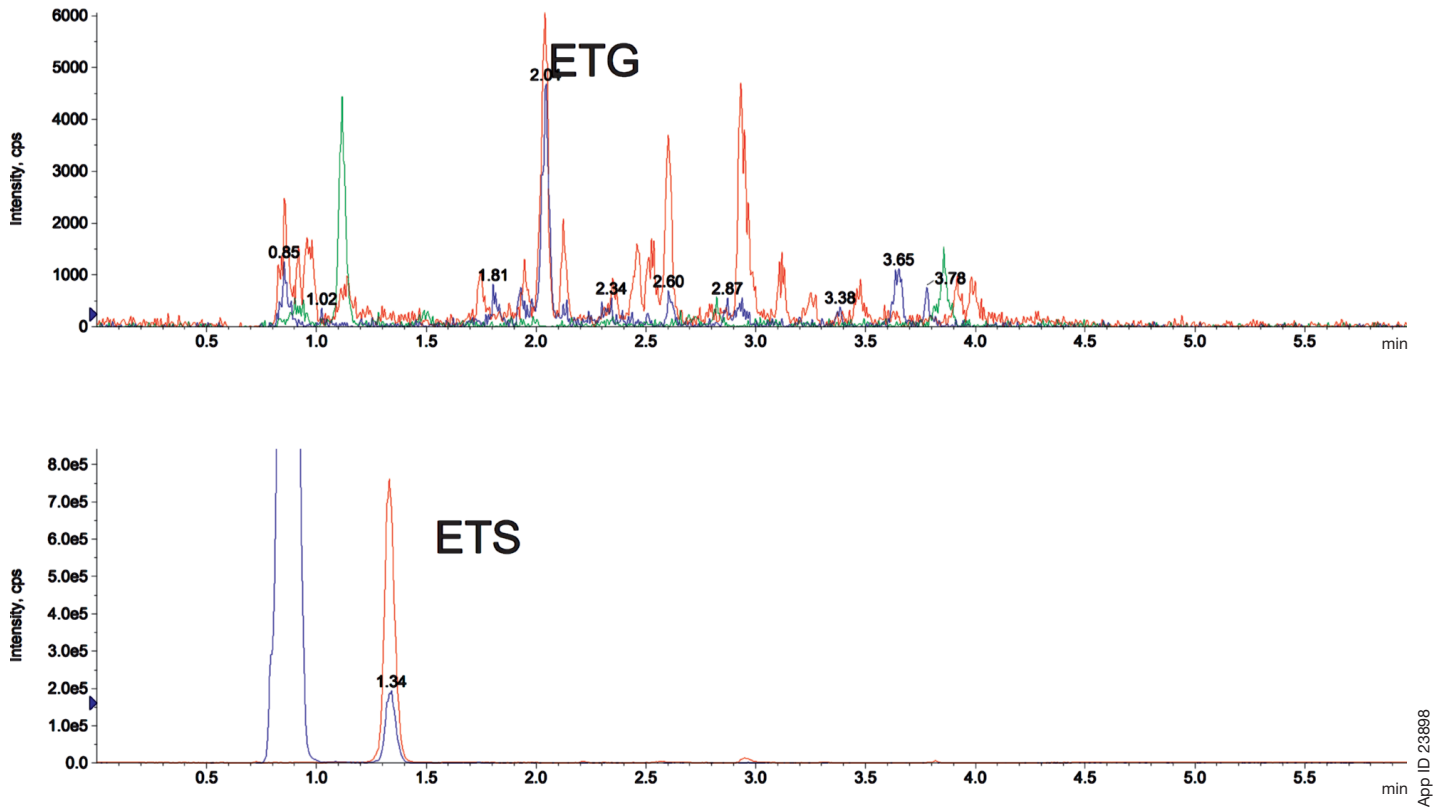
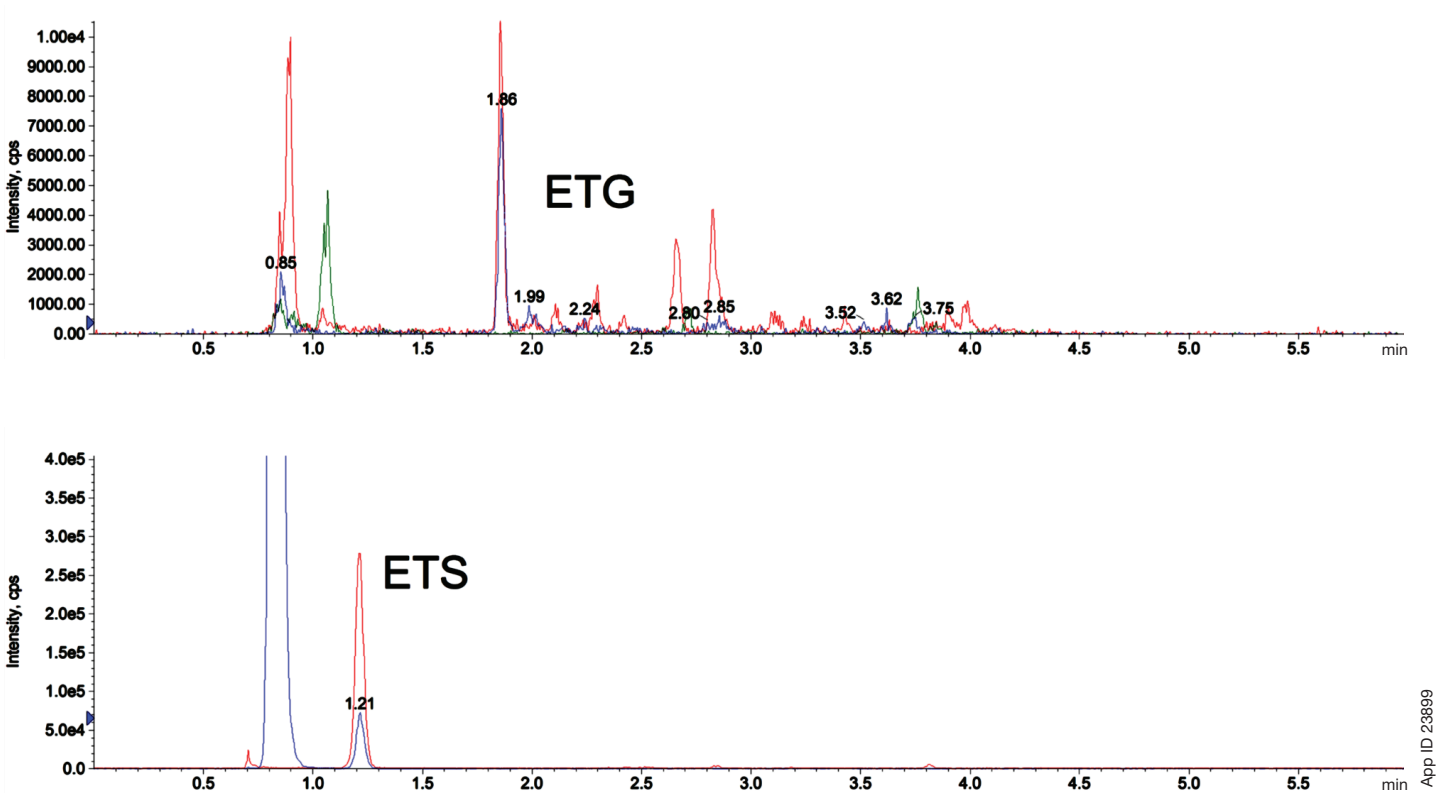


Figure 2. XIC for EtS and EtG Using LC/MS/MS Conditions #2



Using the Luna® Omega 5 µm Polar C18, both mobile phase options yield similar retention behavior for the EtG and EtS peaks. In addition, in both cases, the EtS peak is well-resolved from the large isobaric urinary interference that elutes early in the run. The principle difference between the two methods is that, in our hands, the method using 5 mM ammonium formate pH 3.3 appears to result in fewer potential interferences in the EtG transitions (**Figure 2**). Customer should evaluate both options and determine which works best in the context of their sample preparation procedures, samples, and instrumentation.

Luna Omega 5 µm PS C18 Method Development

Using the second LC column option, Luna Omega 5 µm PS C18, which contains the positively charged functional groups on the surface of the silica, we found that the ideal selectivity was achieved when using the 5 mM ammonium formate buffer system. Representative XIC are shown in **Figure 3**.

LC/MS/MS Conditions

Column: Luna Omega 5 µm PS C18
Dimensions: 50 x 4.6 mm
Part No.: 00B-4753-E0
SecurityGuard™ Cartridge: AJ0-7606
Mobile Phase: A: 5 mM Ammonium formate (pH 3.3)
B: 0.1 % Formic acid in Acetonitrile

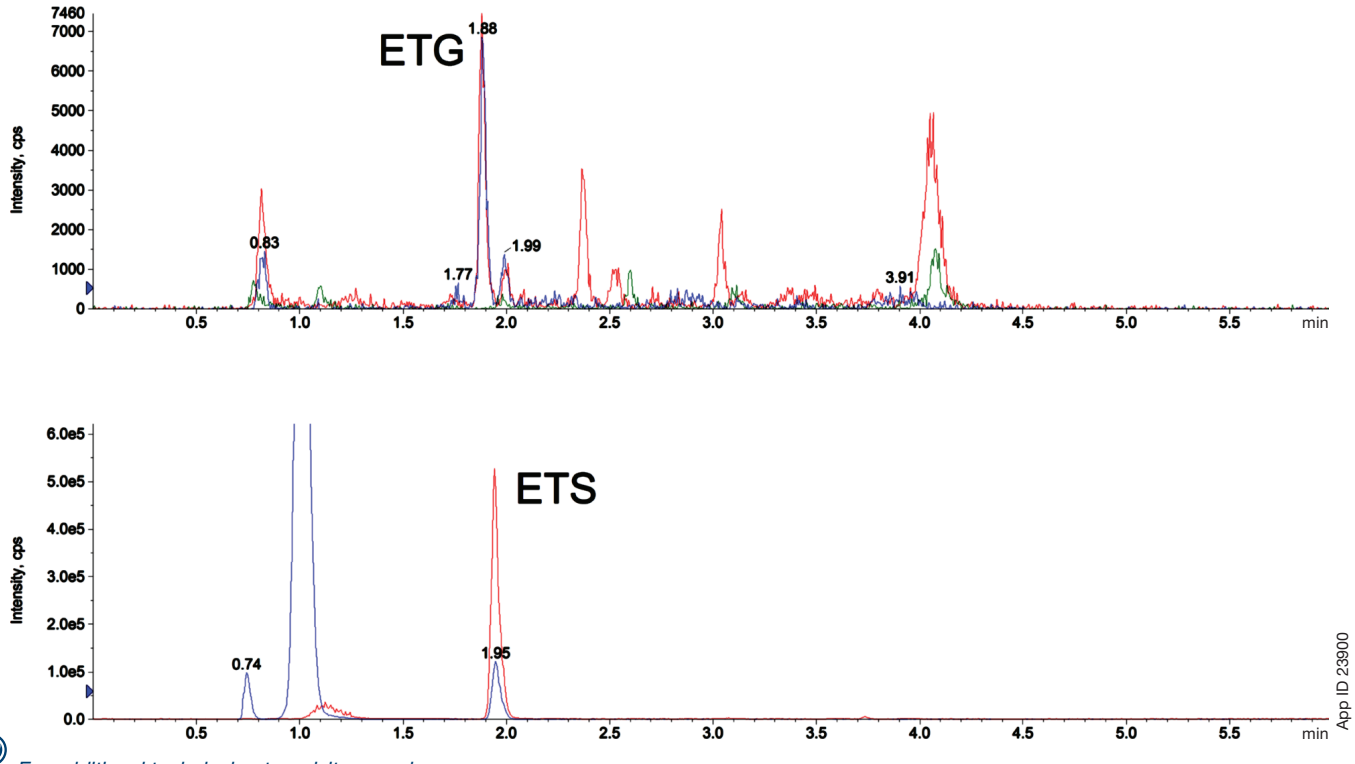
Gradient:

Time (min)	B (%)
0	0
3	90
3.1	0

Flow Rate: 0.7 mL/min
Injection Volume: 5 µL
Temperature: 25°C
Detector: SCIEX API 4000™ MS/MS

Analyte	Retention time (min)
EtS	1.95
EtG	1.88

Figure 3. XIC of EtS and EtG from Urine using Luna Omega 5 µm PS C18



PS C18 Results and Discussion

While EtG elutes at approximately the same time as the previously described methods, EtS retention is dramatically increased using the Omega PS C18 column—and actually eluted at about the same time or slightly later than the EtG peak. This dramatic shift in retention moves the EtS analyte further from the large urinary isobaric interference. It should be noted that, as a method development tip, adjusting the pH of the mobile phase can be used to modify the retention of that EtS peak. Increasing the mobile phase pH will result in further increasing the retention of the EtS, while decreasing the mobile phase pH will reduce the retention of the EtS peak. This can be a valuable tool in fine-tuning your method.

This enhanced retention of EtS is due to polar and ionic interactions with the unique positive surface charge of the Luna Omega PS C18 phase chemistry. This unique mixed-mode retention mechanism can be sensitive to pH fluctuations caused by the plug of urine injected onto the column, so it was necessary to use the ammonium formate buffer as the aqueous component of the mobile phase because of its improved buffering capacity (as compared to simple water/formic acid). If we ran the same separation using just water with 0.1 % formic acid as the aqueous buffer, we found that the retention of EtS was greatly increased, but it was subject to peak distortion and not suitable for accurate quantitation at injection volumes greater than ~5 µL.

Conclusion

In this technical note, we offer three possible solutions for the separation of EtS and EtG in urine. Evaluating different methods on different columns is the best way to identify the solution that works best for your lab. In our method development, on our systems, we have determined that using Luna Omega PS C18 with an ammonium formate buffer provided the best retention of EtS and the least amount of interference, but your results may vary depending upon the specifics of your laboratory. However, we are confident that one of these solutions, if not all, will work well for you.

APPLICATIONS

Luna Omega Ordering Information

5 µm Minibore, MidBore™, and Analytical Columns (mm)

Phases	50 x 2.1	100 x 2.1	50 x 3.0	100 x 3.0	50 x 4.6	100 x 4.6	SecurityGuard™ Cartridge 4 x 2.0*/10pk	SecurityGuard™ Cartridge 4 x 3.0*/10pk
Polar C18	00B-4754-AN	00D-4754-AN	00B-4754-Y0	00D-4754-Y0	00B-4754-E0	00D-4754-E0	AJ0-7600	AJ0-7601
PS C18	00B-4753-AN	00D-4753-AN	00B-4753-Y0	00D-4753-Y0	00B-4753-E0	00D-4753-E0	AJ0-7605	AJ0-7606
						for ID:	2.0 - 3.0 mm	3.2 - 8.0 mm

* SecurityGuard Analytical Cartridges require holder, Part No.: KJ0-4282

1.6 µm Minibore Columns (mm)

Phases	50 x 2.1	100 x 2.1	SecurityGuard™ ULTRA Cartridges‡ 3/pk
Polar C18	00B-4748-AN	00D-4748-AN	AJ0-9505
PS C18	00B-4752-AN	00D-4752-AN	AJ0-9508
			for 2.1 mm ID

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

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guarantee

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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