TECHNICAL NOTE 21989

Measuring alcohol biomarkers from urine – a splitting headache!

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Goals

- To overcome a significant matrix effect causing peak splitting of ethyl sulfate and the labeled internal standard. This issue was present in six individual sources and varied in severity when using competitor biphenyl column chemistry.
- To demonstrate the use of the Thermo Scientific™ Hypersil GOLD™ VANQUISH™ aQ phase in place of the biphenyl phase for the determination of EtS and EtG.
- To assess the linearity and accuracy and precision of the developed assay on the revised column chemistry.

Introduction

Ethanol metabolites are often monitored for clinical research and forensic purposes. These programs often require a robust, non-invasive, and simple testing protocol to determine abstinence. Monitoring blood alcohol content is not suitable in many cases as it requires an invasive sampling technique and the half-life of ethanol and its major metabolites are short. This means that by the time a sample is taken for testing, the ethanol and major metabolites have already been excreted. Therefore, there has been a growing interest in monitoring of minor metabolites such as ethyl sulfate (EtS) and ethyl glucuronide (EtG) as they are better biomarkers for recent exposure to ethanol, even a few days after the ethanol was consumed.

Experimental

Consumables

Thermo Scientific™ SOLA™ SCX Solid Phase Extraction (SPE) Plate 96-well, 10 mg/2 mL	P/N 60309-002
Thermo Scientific™ Hypersil GOLD™ VANQUISH™ aQ UHPLC column 1.9 µm, 100 x 2.1 mm	P/N 25302-152130-V
Thermo Scientific™ WebSeal™ 96-well non-coated plastic microplates, square well (pack of 5)	P/N 60180-P212
Thermo Scientific™ WebSeal 96-well non-coated plastic microplates, square well (pack of 50)	P/N 60180-P202
Thermo Scientific™ WebSeal™ non-sterile mat, blue silicone (pack of 5)	P/N 60180-M122

Reagents

Thermo Scientific™ UHPLC-MS grade water	P/N W8-1
Thermo Scientific™ Optima™ UHPLC-MS grade methanol	P/N A456-1
Thermo Scientific™ Optima™ UHPLC-MS grade acetonitrile	P/N A956-1
Fisher Chemical™ analytical grade formic acid	P/N F/1900/PB08

Instrumentation

Thermo Scientific™ Vanquish™ Horizon UHPLC system consisting of the following:

• System Base Vanquish™ Horizon	P/N VH-S01-A
• Binary Pump H	P/N VH-P10-A
• Split Sampler HT	P/N VH-A10-A
• Column Compartment H	P/N VH-C10-A
• Active Pre-heater	P/N 6732.0110
Thermo Scientific™ TSQ Quantiva™ Triple Quadrupole Mass Spectrometer	P/N IQLAAEGAAXFAOUMZZZ

UHPLC conditions

Table 1. UHPLC parameters

UHPLC conditions	
Mobile phase A:	0.5% formic acid in water
Flow rate:	0.2 mL/min
Run time:	7.5 min
Column temperature:	20°C, with active pre-heating and still air mode
Injection volume:	10 µL

MS/MS conditions

Table 2. MS/MS source parameters

MS source parameters	Setting
Source	H-ESI
Polarity	Negative
Spray voltage	3000 V
Vaporizer temperature	400 °C
Sheath gas pressure	50 Arb
Aux gas pressure	15 Arb
Ion transfer tube temperature	350 °C
CID gas pressure	1.5 mTorr

Table 3. Compound transition details

Compound	Polarity	Precursor (*m/z*)	Product (*m/z*)	Collision energy (V)
Ethyl glucuronide	Negative	221.0	75.1	15.1
Ethyl sulfate	Negative	125.0	97.0	17.0
Ethyl glucuronide-d5	Negative	226.1	75.0	16.0
Ethyl sulfate-d5	Negative	130.0	98.0	17.1

Sample preparation

1. Dilute 100 µL of urine with 900 µL of 0.5% formic acid in water.
2. Condition the SOLA SCX 96 Well plate with 1 mL of methanol followed by 1 mL of 0.5% formic acid in water.
3. Load the 1 mL of diluted urine onto the plate.
4. Collect the eluent in a fresh 96-well plate.
5. Inject on the UHPLC-MS/MS system.

Data processing

The Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), version 7.2.8, was used for data acquisition and analysis.

Results and discussion

Chromatography issues with urine samples

Historically, phenyl or biphenyl phases have been used to separate EtS and EtG due to the ability to use 100% aqueous conditions. Chromatography was initially assessed using a competitor biphenyl column and the application progressed to individual sources of urine known to be free of EtS and EtG. It was at this time a significant chromatographic issue was observed in the form of split peaks for ethyl sulfate (Figure 1). Initially, this was suspected to be a specificity issue caused by other polar isobaric components of the urine when looking at samples without internal standard. Upon the addition of internal standard, the peak splitting phenomenon was also observed for the labeled internal standard, which mirrored the peak trace of the non-labeled compound. This made the likelihood of the issue being a genuine specificity issue to be extremely remote.

As the chromatography was acceptable when no matrix was present, a significant matrix effect was attributed to be the cause of the issue, though no investigative work was performed to identify the components causing the problem. As the issue was present to varying degrees in six individual sources, it was clear that biphenyl column chemistry was not suitable for this analysis in urine samples and alternative selectivity was required. Owing to the requirement for 100% aqueous starting conditions, the Hypersil GOLD VANQUISH aQ phase was used. Excellent peak shape was observed with no interfering peaks and no peak splitting in the same urine samples that were so problematic on the competitor biphenyl column (Figure 2). Linearity, accuracy, and precision were subsequently determined using the newly established chromatographic conditions.

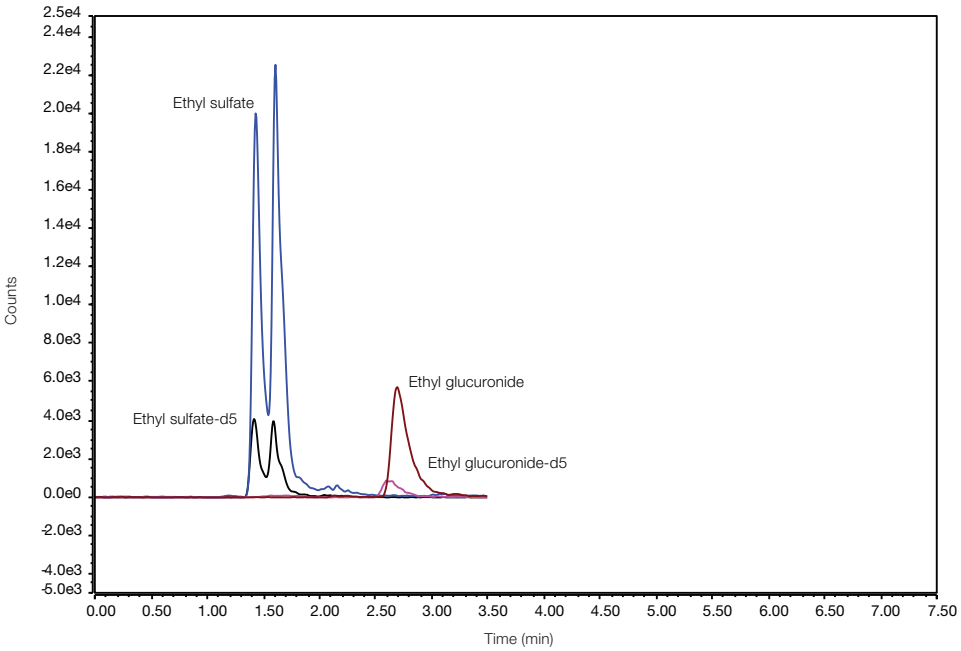

Figure 1. Chromatogram showing ethyl sulfate and ethyl glucuronide at the high QC level in human urine on a competitor biphenyl column R

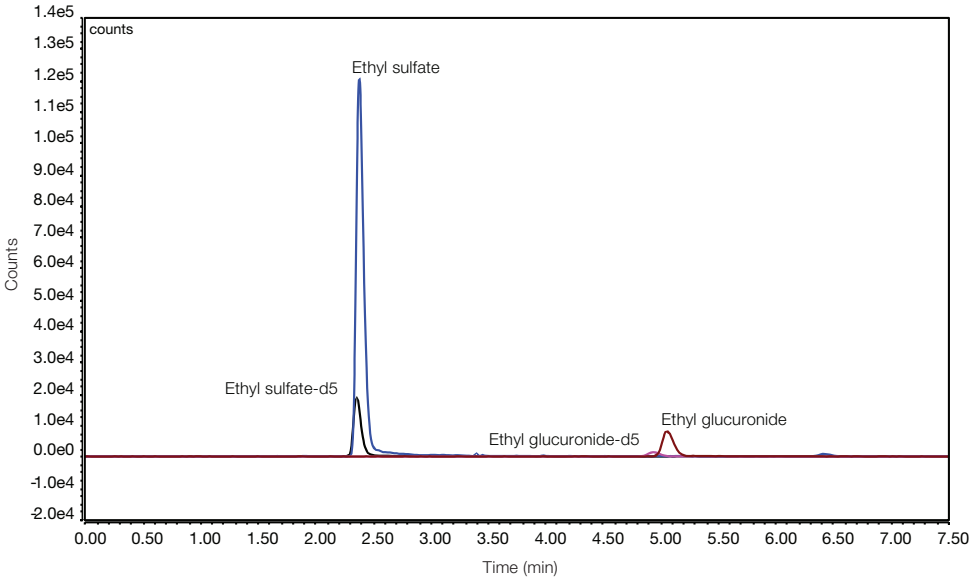

Figure 2. Chromatogram showing ethyl sulfate and ethyl glucuronide at the high QC level in human urine on the Hypersil GOLD VANQUISH aQ UHPLC column

SOLA is a fritless SPE product specifically designed for improved robustness and reproducibility with biological samples such as urine. The mixed mode SOLA SCX phase was chosen for sample cleanup and used in a 'non-retentive' mode. The polar, acidic ethanol metabolites passed through the sorbent while any basic or sufficiently lipophilic compounds were retained. This procedure appeared extremely efficient at removing urobilin from the samples, giving a clear extract for injection regardless of the color of the urine initially loaded.

Calibration model and range

The calibration line for both ethyl sulfate and ethyl glucuronide used a linear regression with $1/x^2$ weighting, with an R^2 value of 0.9963 and 0.9992, respectively. The calibration range for ethyl sulfate was 20–2000 ng/mL and 100–10000 ng/mL for ethyl glucuronide.

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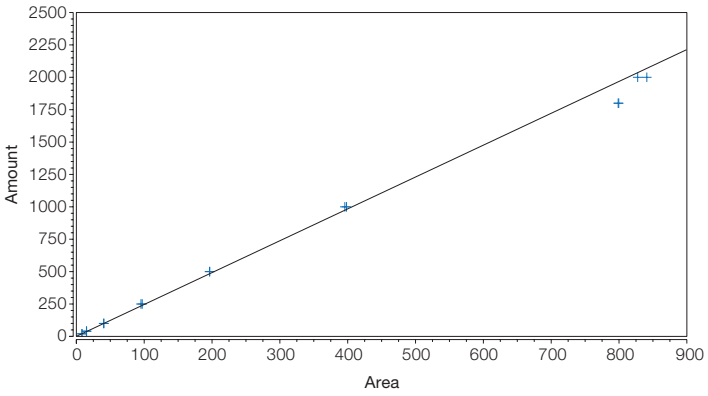

Figure 3. Example calibration curve for ethyl sulfate using the Hypersil GOLD VANQUISH aQ UHPLC column

Table 4. Accuracy and precision of EtS in human urine

	Ethyl sulfate (ng/mL)			
	LLOQ	Low	Medium	High
	20.0	**50.0**	**320**	**1600**
	18.3	45.0	316	1606
	18.2	49.4	308	1614
	18.2	43.5	333	1602
	19.1	47.5	289	1691
	18.9	46.5	325	1644
	18.4	48.8	295	1798
Mean	**18.5**	**46.8**	**311**	**1660**
Bias %	**-7.4**	**-6.4**	**-2.9**	**3.8**
CV %	**2.1**	**4.8**	**5.5**	**4.5**

Table 5. Accuracy and precision of EtG in human urine

	Ethyl glucuronide (ng/mL)			
	LLOQ	Low	Medium	High
	100	**250**	**1600**	**8000**
	103	240	1673	7476
	99.5	271	1556	7653
	90.5	242	1706	7479
	108	265	1506	7609
	98.1	270	1740	8304
	97.5	265	1578	8453
Mean	**99.4**	**259**	**1630**	**7830**
Bias %	**-0.6**	**3.6**	**1.9**	**-2.1**
CV %	**5.8**	**5.4**	**5.7**	**5.5**

Accuracy and precision data

The accuracy and precision of the assay using the revised chromatographic conditions on the Hypersil GOLD VANQUISH aQ UHPLC column were tested and shown to be acceptable for both ethyl sulfate and ethyl glucuronide (Tables 4 and 5).

Conclusion

- Simple 'pass-through' SPE procedure for rapid sample cleanup and removal of matrix components
- Isocratic elution using 100% aqueous mobile phase
- Alternative selectivity on Hypersil GOLD VANQUISH aQ phase solves the peak splitting issue for ethyl sulfate in human urine samples

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