RESTEK Pure Chromatography

Rapid EtG & EtS Analysis in Urine and Chronic Kidney Disease State Urine by LC-MS/MS

Jamie York, Samantha Herbick; Restek Corporation

Introduction

Ethyl Glucuronide (EtG) and Ethyl Sulfate (EtS) are established biomarkers for alcohol consumption. The analysis of these compounds poses challenges due to their polar nature, making them challenging to retain by reversed phase chromatography. In addition to this, there are isobaric matrix interferences in urine that require full resolution from the analytes to obtain accurate data. In this work, a rapid LC method was developed to demonstrate excellent resolution between the matrix interferences and analytes of interest without the need for column conditioning.

Methods

Table 1: Method parameters

Column:	Force Biphenyl 100 x 3 mm, 3 µm
Guard Column:	Force Biphenyl EXP Guard Cartridge 5 x 3 mm, Ultra Shield UHPLC PreColumn Filter, 0.2 µm Frit
Column Temperature:	30 °C
Injection Volume:	10 µL
Mobile Phase A:	Water, 0.1% formic acid
Mobile Phase B:	Methanol, 0.1% formic acid
Detection:	ESI (-) MS/MS

Valve Position	Time (min)	Flow Rate (mL/min)	%B
Waste	0.00	0.8	0
MS	*0.50*	0.8	-
MS	*1.74*	0.8	100
Waste	*1.75*	1.0	-
Waste	*3.50*	1.0	100
Waste	*3.51*	0.8	0
Waste	*5.00*	0.8	0

Sample Preparation

***Protein Precipitation*:**

- 50 µL urine
- 10 µL isotopically labelled internal standards
- 150 µL cold acetonitrile
- Vortexed ~ 30 seconds
- Centrifuged 10 minutes 4,200 rpm

Dilution:

- 100 µL of supernatant aliquoted into vial
- 900 µL of water (40x total dilution)
- Vortexed ~ 30 seconds

Column Choice

Three different lots of chronic kidney disease (CKD) urine were used to develop the chromatographic method. These were selected due to pronounced matrix effects. A 100 x 3 mm, 3 µm Force Biphenyl column was used to adequately baseline resolve the isobaric matrix interference, even in the challenging CKD samples (Figure 1).

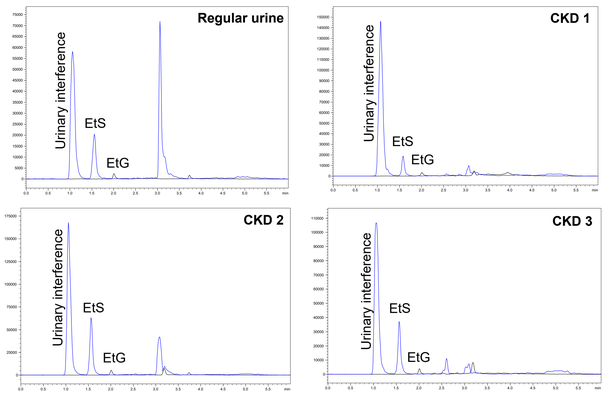


Figure 1: 50 ppb fortified urine analyzed using 100 x 3 mm, 3 µm Force Biphenyl column. A) regular urine; B) CKD urine 1; C) CKD urine 2; D) CKD urine 3. This column dimension adequately resolves matrix interference from urine.

Ion Suppression Study

An ion suppression study was conducted to assess whether the analytes eluted within regions affected by enhanced matrix effects. This was achieved by post-column infusion of 1 ppm EtG and EtS while a blank CKD urine sample was injected. The resulting chromatograms from these experiments are shown in Figure 2.

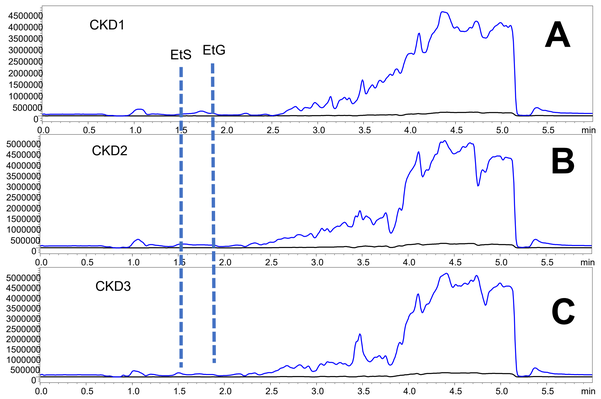


Figure 2: Post infusion of 1 ppm EtG/EtS while simultaneous injection of 1)CKD urine 1; 2)CKD urine 2; 3)CKD urine 3. Dotted line shows where EtG/EtS elute on chromatogram and shows no significant ion suppressions are present during analyte elution.

Recovery & Precision

Calibration standards were constructed using synthetic urine (UTAK, Valencia, CA) fortified with EtG and EtS from 30-2,000 ng/mL. Quality control (QC) samples were prepared at LLQ (100 ng/mL), LQC (160 ng/mL), MQC (400 ng/mL), and HQC (1,600 ng/mL) using six lots of single donor human urine, including two from CKD patients, analyzed in triplicate over three days.

Table 2: Precision and accuracy results for interday experiments at LLQ.

	Interday n=9		
	100 ng/mL (LLQ)		
Analyte	**Urine**	**% Recovery**	**%RSD**
EtS	**CKD 1**	111.9	1.5
	CKD 2	111.8	2.2
	3	109.8	2.3
	4	101.3	4.4
	5	105.1	2.6
	6	110.7	3.8
	Synthetic	98.3	3.6
EtG	**CKD 1**	104.6	3.6
	CKD 2	96.5	6.7
	3	107.4	6.8
	4	92.9	6.4
	5	93.3	1.8
	6	103.5	10.1
	Synthetic	103.1	4.1

UTAK ETG Plus Controls

EtG Plus Level 1 Quality Control and EtG Plus Level 2 Quality Control reference material from UTAK were prepared and analyzed utilizing the outlined method. Each level was prepared in six replicates and analyzed over 3 days (n=18). An example chromatogram can be seen in Figure 3 and the results of these experiments can be seen in Table 3.

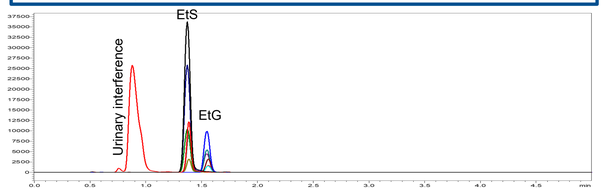


Figure 3: Chromatogram of UTAK EtG Plus Level 1 Urine Control.

Table 3: Precision and accuracy results for UTAK Plus Controls.

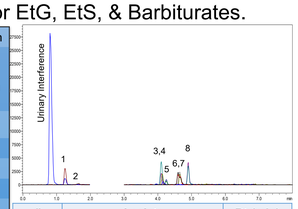
		UTAK EtG Plus Level 1 Urine Control		UTAK EtG Plus Level 2 Urine Control	
	Analyte	**EtG**	**EtS**	**EtG**	**EtS**
	Verified Value (ng/mL)	510	240	1800	800
Day 1 n=6	**% Recovery**	98.33	97.46	106.22	106.59
	%RSD	7.84	3.50	5.68	4.80
	Calculated Concentration (ng/mL)	501.52	233.92	1911.88	852.79
Day 2 n=6	**% Recovery**	103.01	101.70	104.04	105.68
	%RSD	8.63	7.29	4.85	2.82
	Calculated Concentration (ng/mL)	522.80	245.20	1822.78	830.80
Day 3 n=6	**% Recovery**	103.32	99.33	101.90	101.17
	%RSD	10.91	8.50	4.57	3.62
	Calculated Concentration (ng/mL)	524.05	239.74	1784.07	795.07
Interday n=18	**% Recovery**	101.56	99.50	104.05	104.48
	%RSD	2.75	2.13	2.07	2.78
	Calculated Concentration (ng/mL)	516.13	239.62	1839.58	826.22

Additional Compounds: Barbiturates

Table 4: Method parameters for EtG, EtS, & Barbiturates.

Column:	Force Biphenyl 100 x 3 mm, 3 µm
Guard Column:	Force Biphenyl EXP Guard Cartridge 5 x 3 mm, Ultra Shield UHPLC PreColumn Filter, 0.2 µm Frit
Column Temp.:	30 °C
Injection Vol.:	10 µL
Mobile Phase A:	Water, 0.1% formic acid
Mobile Phase B:	Methanol, 0.1% formic acid
Flow Rate:	0.8 mL/min
Detection:	ESI (-) MS/MS

Time (min)	%B
0.00	0
1.75	50
5.50	85
6.00	100
6.01	0
8.00	0



#	Analyte	Rt (min)
1	EtS	1.25
2	EtG	1.67
3	Phenobarbital	4.14
4	Butabarbital	4.15
5	Butalbital	4.25
6	Pentobarbital	4.61
7	Amobarbital	4.67
8	Secobarbital	4.91

Table 5: Precision and accuracy results for interday experiments at LLQ.

		Interday n=9	
		100 ng/mL EtG/EtS	
		200 ng/mL Barbiturates	
Analyte	**Urine**	**% Recovery**	**%RSD**
EtG	**1**	106.3	1.7
	2	107.8	7.0
	3	106.8	1.5
	4	98.1	1.9
	CKD	111.6	2.5
	Synthetic	99.4	3.0
EtS	**1**	108.2	6.1
	2	106.5	2.2
	3	101.1	2.5
	4	104.0	5.4
	CKD	106.6	0.8
	synthetic	102.4	1.9
Amobarbital & Pentobarbital	**1**	100.1	6.8
	2	99.4	4.8
	3	99.8	7.8
	4	101.6	6.9
	CKD	99.3	1.0
	synthetic	97.2	9.4
Butabarbital	**1**	101.7	6.1
	2	100.1	6.5
	3	102.0	8.1
	4	105.1	4.2
	CKD	103.2	7.8
	synthetic	103.2	7.1
Butalbital	**1**	93.8	1.6
	2	97.5	2.3
	3	96.4	9.1
	4	99.1	9.6
	CKD	94.8	5.3
	synthetic	108.0	2.8
Phenobarbital	**1**	100.6	3.5
	2	97.6	6.5
	3	98.3	10.0
	4	101.1	1.7
	CKD	100.2	3.9
	synthetic	97.4	9.4
Secobarbital	**1**	102.1	7.7
	2	100.4	6.4
	3	98.2	4.1
	4	101.0	5.7
	CKD	103.0	8.8
	synthetic	99.2	3.6

Conclusion

Linearity was demonstrated for both analytes with $r^2 \geq 0.99$ and exhibited acceptable intra- and inter-day precision and accuracy. The Force Biphenyl column offered superior robustness over multi-mode columns, which often require extensive pre-conditioning. Also, in contrast to other columns, the Force Biphenyl allowed immediate use without compromising performance. It also provides flexibility for analyzing a broad range of compounds, including other drugs of abuse/novel psychoactive substances, reducing the need for column changes.