Rapid LC-MS/MS Analysis of EtG/EtS and Barbiturates in Normal and Disease State Urine

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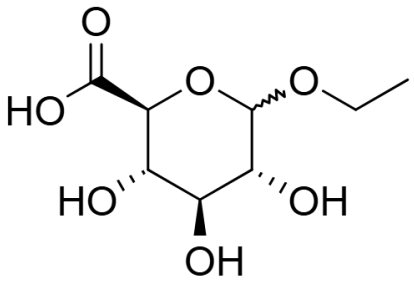
Presentation Objectives

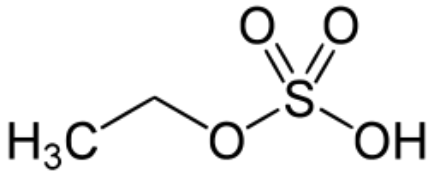
- Background and current challenges
- Benefits of reversed phase chromatography and particle choice
- Method Development
- Inclusion of negative mode drugs- barbiturates
- Conclusions

Analytical Challenges

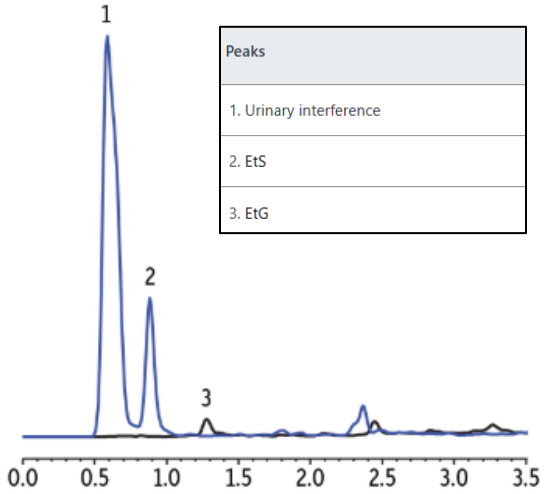
- Small, polar compounds, difficult to retain by reversed phase
- Urinary matrix interference isobaric to EtS
- Needs chromatographic resolution
- Adequate retention from matrix suppression/enhancement zones
- Biological matrix variability sample-to-sample
- IEX methods tend to need extensive pre-conditioning for proper performance



Ethyl Glucuronide



Ethyl Sulfate

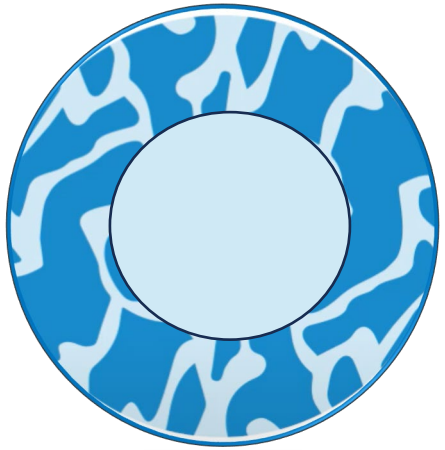


Reversed Phase Method

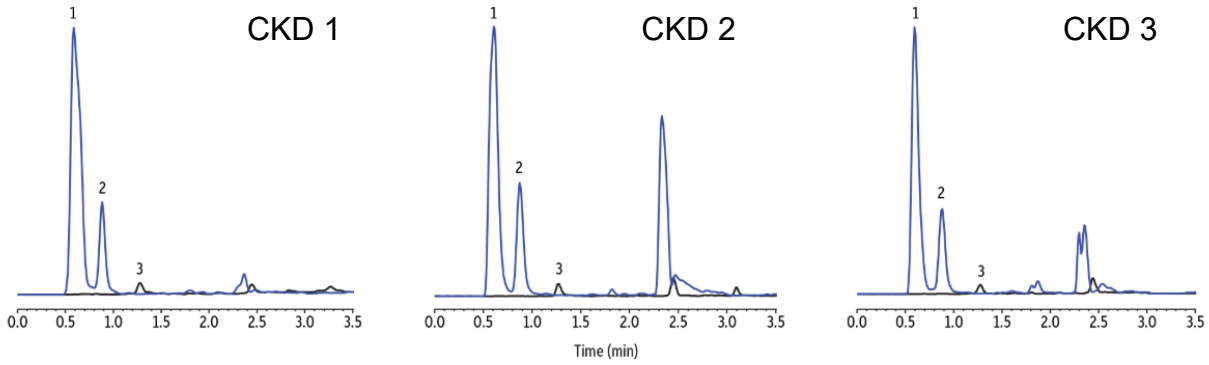
- Fully porous particle greater surface area
- Greater retention of analytes
- Allows for other panels to be ran on the same column
- Additional compounds can be analyzed in the same method…

Fully Porous Particles (FPP)

Superficially Porous Particles (SPP)



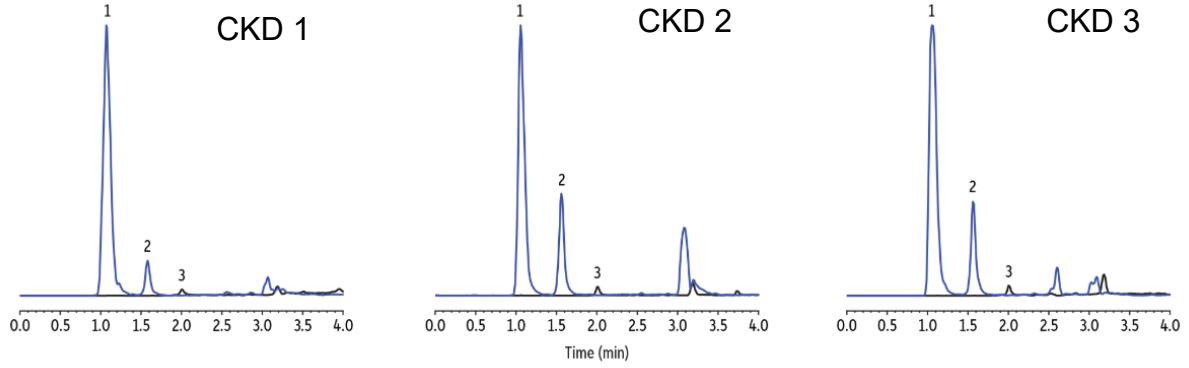
Method Development: Column Choice



LC_CF0844

Peaks	t_R (min)	Precursor	Product 1	Product 2
1. Urinary interference	0.60	125.0	80.1	–
2. EtS	0.88	125.0	96.8	80.1
3. EtG	1.28	221.1	75.1	85.2

50 x 3 mm, 3 µm



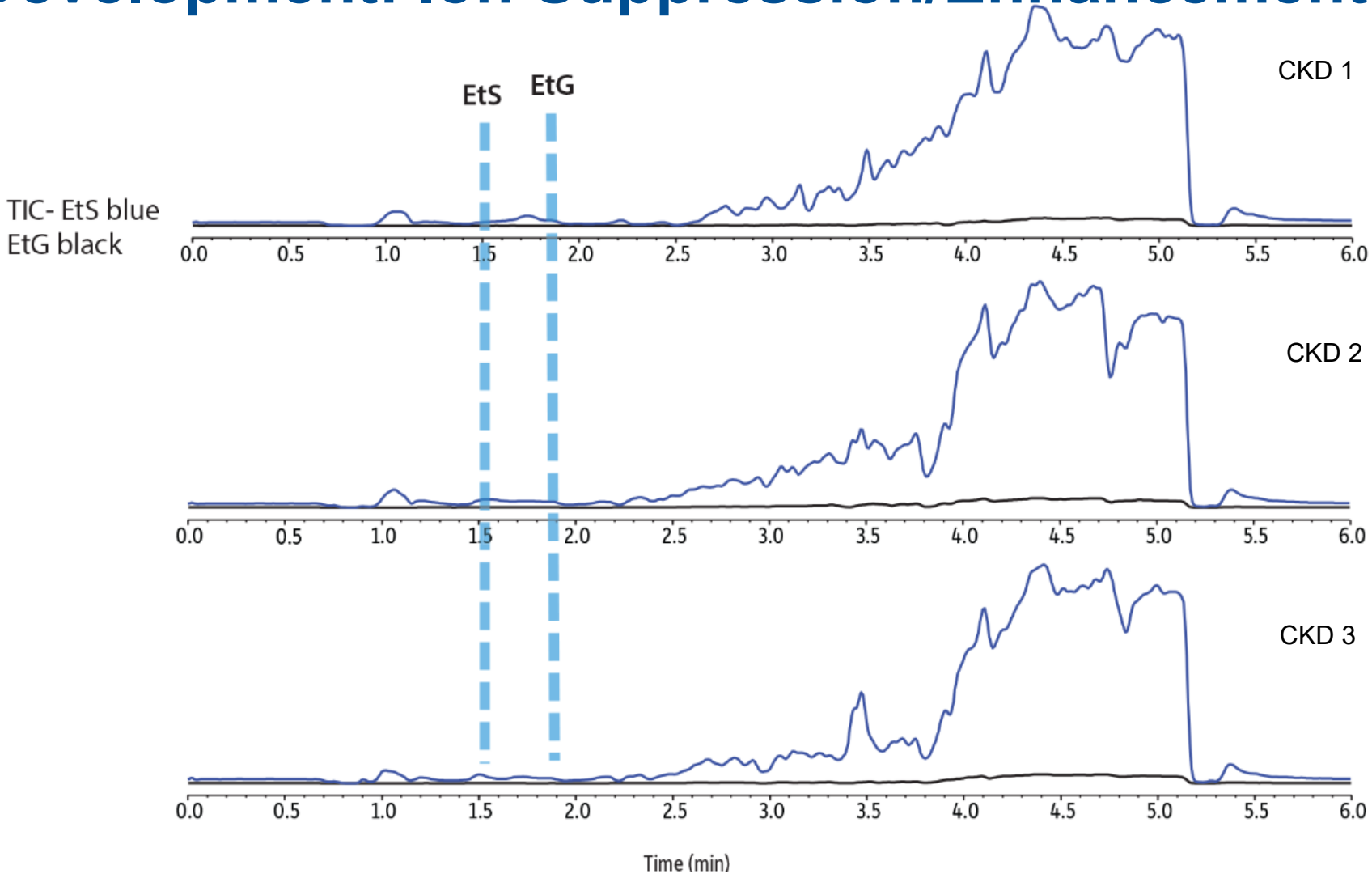
LC_CF0845

Peaks	t_R (min)	Precursor	Product 1	Product 2
1. Urinary interference	1.06	125.0	80.1	–
2. EtS	1.56	125.0	96.8	80.1
3. EtG	2.01	221.1	75.1	85.2

100 x 3 mm, 3 µm

Force Biphenyl 125.0>80.1 urinary interference

Method Development: Ion Suppression/Enhancement



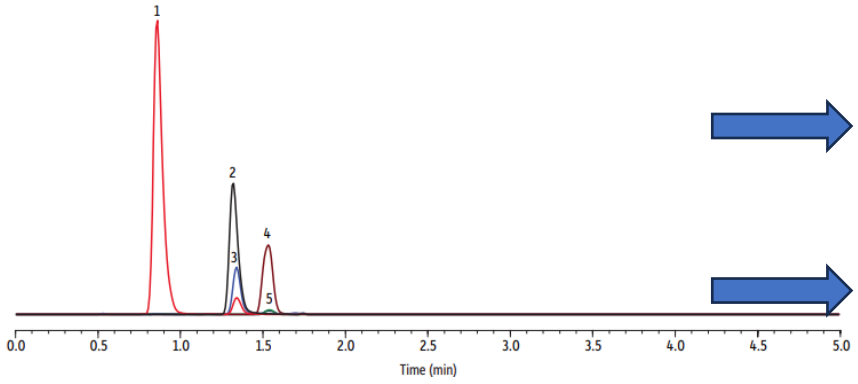
1 ng/mL EtG & EtS in solvent infused post column, simultaneous injection of CKD urine

Sample Prep

- Urine (50 µL) aliquoted to 2 mL centrifuge tube, isotopically labeled IS added
- Cold acetonitrile (150 µL) added to the tube, vortexed 30 seconds and centrifuged 10 mins 4200 rpm
- Supernatant (100 µL) was aliquoted to an autosampler vial and water (900 µL) added
- Sample capped, vortexed 30 seconds and 10 µL injection onto the LC-MS/MS

Chromatographic Performance

Peaks	t_R (min)	Conc. (ng/mL)	Precursor	Product 1	Product 2
1. Urinary interference	0.89	-	125.0	80.1	-
2. EtS-d5	1.34	1000	130.0	97.9	-
3. EtS	1.34	100	125.0	96.8	80.1
4. EtG-d5	1.54	2000	226.0	75.0	-
5. EtG	1.54	100	221.1	75.1	85.2



Column	Force Biphenyl 100 mm, 3.0 mm ID, 3 µm (cat.# 962931E)		
Guard Column	Force Biphenyl EXP guard cartridge 5 x 3.0 mm ID (cat.# 962950253); an UltraShield UHPLC precolumn filter 0.2 µm frit (cat.# 25811) was installed before the guard cartridge.		
Column Temperature	30 °C		
Injection Volume	10 µL		
Mobile Phase A	Water, 0.1% formic acid		
Mobile Phase B	Methanol, 0.1% formic acid		
Detector	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

Accuracy and Precision

- Calibration standards prepared in synthetic urine (UTAK)
- Linearity 30-2,000 ng/mL
- QC samples prepared over 3 days, analyzed in triplicate
- Intraday recovery 85.1-114.6%, %RSD<9.6
- Interday recovery 91.9-111.9%, %RSD<10.1

Interday							
		LQC (100 ng/mL)		MQC (400 ng/mL)		HQC (1600 ng/mL)	
Analyte	Urine Sample#	% Recovery	%RSD	% Recovery	%RSD	% Recovery	%RSD
EtS	**CKD 1**	111.9	1.5	100.1	6.7	106.9	4.0
	CKD 2	111.8	2.2	102.0	3.8	104.4	5.4
	3	109.8	2.3	106.2	8.2	107.6	5.3
	4	101.3	4.4	95.9	5.2	101.0	9.4
	5	105.1	2.6	103.6	7.5	106.8	4.9
	6	110.7	3.8	106.4	4.3	106.7	3.7
	synthetic	98.3	3.6	91.9	2.0	95.3	5.2
EtG	**CKD 1**	104.6	3.6	93.8	9.5	94.4	3.6
	CKD 2	96.5	6.7	94.1	0.9	100.2	2.5
	3	107.4	6.8	100.1	6.8	104.1	5.7
	4	92.9	6.4	93.5	7.5	100.1	3.2
	5	93.3	1.8	97.4	5.1	95.3	2.1
	6	103.5	10.1	96.6	7.5	95.1	3.2
	synthetic	103.1	4.1	100.2	4.2	100.0	9.3

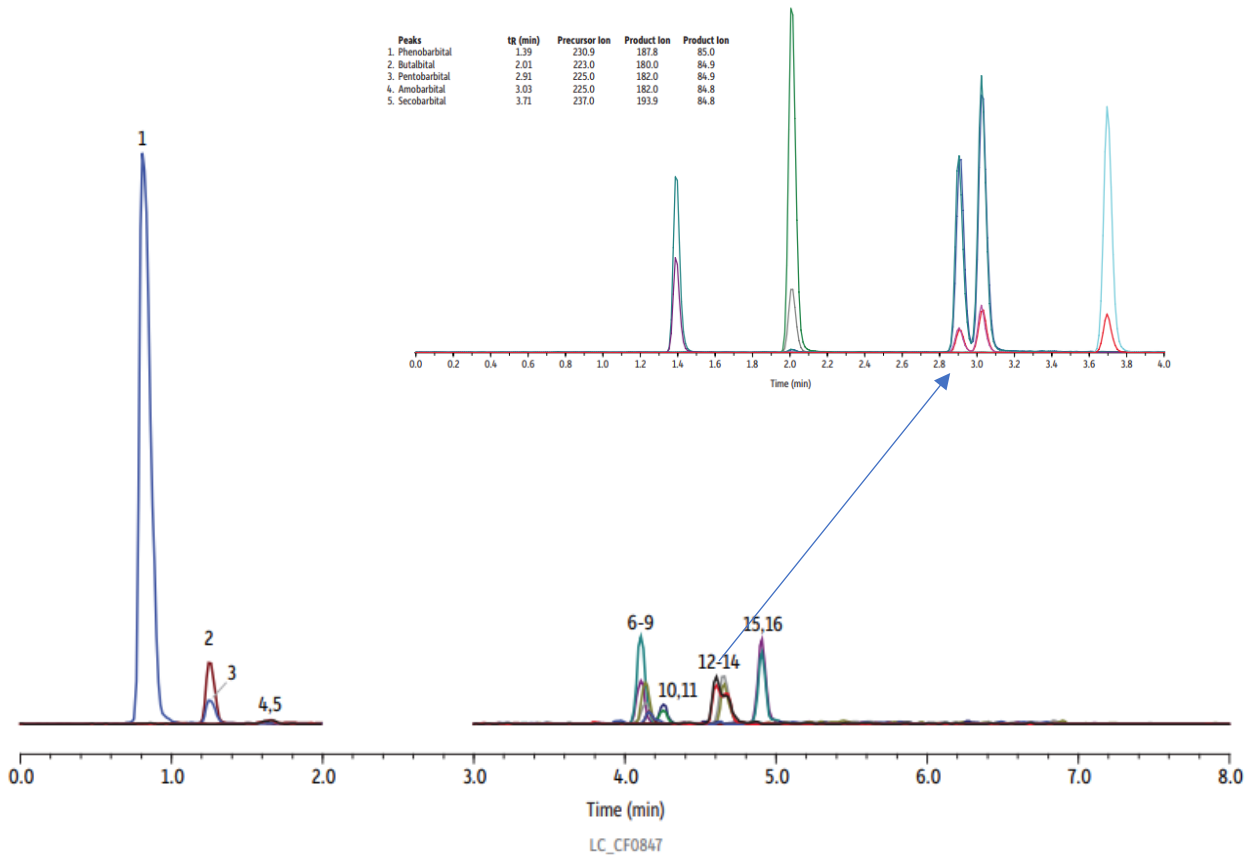
Accuracy and Precision

		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	98.3	97.5	106.2	106.6
	%RSD	7.84	3.50	5.68	4.80
	Calculated Concentration (ng/mL)	502	234	1912	853
Day 2 (n=6)	% Recovery	103.0	101.7	104.0	105.7
	%RSD	8.63	7.29	4.85	2.82
	Calculated Concentration (ng/mL)	523	245	1823	831
Day 3 (n=6)	% Recovery	103.3	100.0	101.9	101.2
	%RSD	10.91	8.50	4.57	3.62
	Calculated Concentration (ng/mL)	524	240	1784	795
Interday (n=18)	% Recovery	***101.6***	***100.0***	***104.1***	***104.5***
	%RSD	***2.75***	***2.13***	***2.07***	***2.78***
	Calculated Concentration (ng/mL)	516	240	1840	826

EtG Plus Level 1 & Level 2 (UTAK) prepared 6x

Combined Method

- Barbiturates can often require separate panel, negative mode
- Consolidate barbiturates with alcohol metabolites, negative mode panel
- Amobarbital and pentobarbital can be identified using secondary method when needed



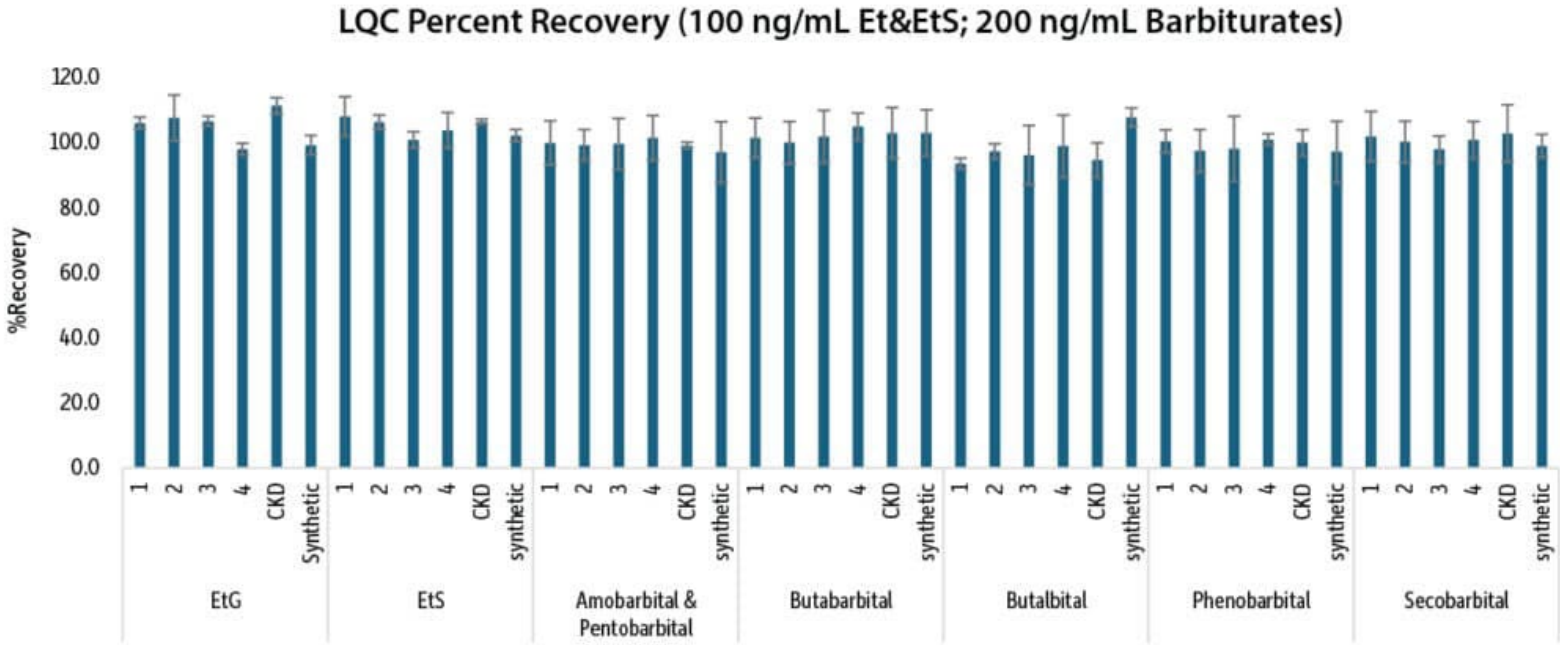
Peaks	tR (min)	Conc. (ng/mL)	Precursor	Product 1	Product 2
1. Urinary interference	0.89	-	125.0	80.1	-
2. EtS	1.22	100	125.0	96.8	80.1
3. EtS-d5	1.21	1000	130.0	97.9	-
4. EtG	1.65	100	221.1	75.1	85.2
5. EtG-d5	1.64	2000	226.0	75.0	-
6. Phenobarbital-d5	4.15	4000	236.0	42.0	-
7. Phenobarbital	4.18	200	231.2	42.1	188.1
8. Butabarbital-d5	4.18	4000	216	41.9	-

Peaks	tR (min)	Conc. (ng/mL)	Precursor	Product 1	Product 2
9. Butabarbital	4.19	200	211.0	168.3	42.0
10. Butalbital-d5	4.23	4000	228.0	185.3	-
11. Butalbital	4.27	200	223.0	42.1	180.3
12. Amobarbital	4.63	200	225.0	182.3	42.1
13. Amobarbital-d5	4.69	4000	230.0	42.0	-
14. Pentobarbital	4.71	200	225.0	182.3	42.1
15. Secobarbital-d5	4.92	4000	242.0	199.3	-
16. Secobarbital	4.94	200	237.0	42.1	194.2

Column	Force Biphenyl (cat.# 962931E)
Dimensions:	100 mm x 3 mm ID
Particle Size:	3 µm
Pore Size:	100 Å
Guard Column:	Force Biphenyl EXP guard cartridge 5 mm, 3 mm ID, 3 µm (cat.# 962950253)
Temp.:	30 °C

Combined Method Accuracy and Precision

- Calibration standards prepared in synthetic urine (UTAK)
- Linearity 30-2,000 ng/mL alcohol metabolites, 60-4,000
- QC samples prepared over 3 days, analyzed in triplicate
- Intraday recovery 87.3-114.8%, RSD<10.7%
- Interday recovery 92.7-112.9%, RSD<11.9%



Conclusions

- Reversed phase chromatography was implemented for the analysis of EtG & EtS
- Matrix interferences resolved, no pre-conditioning
- Developed using various urine types, including CKD, and calibration performed using synthetic urine
- Dedicated method targeting alcohol metabolites and consolidated method for both alcohol metabolites & barbiturates
- Additional flexibility with Force Biphenyl to utilize the column for other panels (DOA), reducing the need for column switching