Simultaneous Analysis of Alcohol Metabolites and Barbiturates by LC-MS/MS

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Abstract

Analysis of alcohol metabolites and barbiturates in urine typically is done using separate methods due to polarity differences between the compound classes and the incompatibility of EtG with enzymatic hydrolysis. In this method, barbiturates were incorporated into an existing EtG/EtS method, providing testing labs with a potential avenue for a more efficient approach through simultaneous analysis.

Related Products

- *Raptor EtG/EtS LC column*
- *UltraShield UHPLC precolumn filter*
- *Reference Standards*
  - *Ethyl-β-D-glucuronide*
  - *Ethyl-β-D-glucuronide-d5*
  - *Ethyl sulfate sodium salt*
  - *Ethyl sulfate-d5 sodium salt*
  - *Pentobarbital*
  - *Butalbital*
  - *Secobarbital*

Introduction

Ethanol metabolites ethyl-β-D-glucuronide (EtG) and ethyl sulfate (EtS) are unique biomarkers of alcohol use. They are frequently monitored to ensure compliance with zero tolerance treatment programs and for abstinence enforcement, situations in which information regarding recent alcohol consumption is required. The analysis of these compounds is especially beneficial in these circumstances because they have a three-day detection window, are relatively stable when samples are stored correctly, and can be detected with high specificity. However, EtG and EtS are both polar, which makes them difficult to retain via reversed-phase chromatography.

Barbiturates are also commonly monitored in urine as part of drug screening programs and, while they are not polar compounds, they are similar to EtG and EtS in that they are both analyzed in negative electrospray ionization (ESI) mode by LC-MS/MS. But, due to polarity and sample preparation differences, the analysis of alcohol metabolites and barbiturates in the same urine sample usually is done using separate methods and LC columns with different stationary phases. Although, in both cases, the methods used must be able to reliably separate the target analytes from coeluting matrix components.

Recently, interest has grown in developing a single negative ESI LC-MS/MS method to analyze both barbiturates and alcohol metabolites in urine, but this presents a significant challenge because the enzymatic hydrolysis sample preparation that often is used for barbiturates would be detrimental to EtG analysis. In this hydrolysis reaction, β-glucuronidase is used to cleave the glucuronide-conjugated barbiturates and convert them into their parent compounds. But, this enzyme will also cleave the glucuronide moiety from EtG, causing it to break down into ethanol which ultimately reduces levels of EtG in urine and leads to inconclusive or inaccurate results.

In this study, a simple dilute-and-shoot LC-MS/MS method was developed for the simultaneous analysis of alcohol metabolites and barbiturates in human urine without enzymatic hydrolysis. This method was developed on a Raptor EtG/EtS column because it has previously been demonstrated to be highly effective at retaining EtG and EtS and separating them from matrix interferences [1]. Using the selectivity of a Raptor EtG/EtS column, analysis of these two classes of compounds was combined into a single, hydrolysis-free method, which ultimately has the potential to increase productivity and reduce costs for labs currently running the same sample through separate methods.

Experimental

Calibration Standards and Quality Control Samples

Human urine (alcohol and barbiturates free) was fortified with EtG, EtS, phenobarbital, butalbital, amobarbital, pentobarbital, and secobarbital in order to prepare the calibration standards and QC samples. The calibration standards concentration range was 50–5,000 ng/mL for all the analytes. Four QC levels were prepared at 50, 125, 750, and 4,000 ng/mL.

Sample Preparation

50 µL of urine was diluted with 950 µL of the working internal standard (100 ng/mL EtG-d5, EtS-d5, and barbiturates-d5 in 0.1% formic acid in water). Samples were vortexed at 3500 rpm for ten seconds to mix, followed by centrifugation at 3000 rpm for five minutes at 10 °C and then injected for LC-MS/MS analysis. Additional urine double blanks were prepared and injected for column equilibration.

Chromatographic Method:

The chromatographic conditions used on a Shimadzu Prominence HPLC connected to a Sciex 4000 for this LC-MS/MS analysis of alcohol metabolites and barbiturates in urine are detailed below. The ion transitions used for each analyte are provided in Table I. Running 30 matrix injections through the full gradient program is recommended for equilibrating the column [2].

Column: Raptor EtG/EtS 2.7 µm, 100 mm x 2.1 mm (cat.# 9325A12)
Guard column: UltraShield UHPLC precolumn filter 0.2 µm (cat.# 25809)
Sample temp.: 10 °C
Column temp.: 30 °C
Injection volume: 10 µL
Mobile phase A: 0.1% Formic acid in water
Mobile phase B: 0.1% Formic acid in acetonitrile

Time (min)	%B
0.00	5
1.20	20
1.21	35
3.00	45
4.00	5
5.00	stop

Flow rate: 0.5 mL/min
Ion mode: Negative ESI

Table I: Ion Transitions for LC-MS/MS Analysis of Alcohol Metabolites and Barbiturates

Peak Identification	Precursor Ion	Quantitative Ion	Qualitative Ion
Ethyl-β-D-glucuronide-d5 (EtG-d5)	225.9	84.7	-
Ethyl-β-D-glucuronide (EtG)	220.9	74.9	85.0
Ethyl sulfate-d5 (EtS-d5)	129.7	97.7	-
Ethyl sulfate (EtS)	124.8	96.8	79.7
Phenobarbital-d5	236.0	42.0	-
Phenobarbital	231.2	188.0	42.0
Butalbital-d5	228.0	42.0	-
Butalbital	223.0	180.0	42.0
Amobarbital-d5	230.0	42.0	-
Pentobarbital-d5	230.0	42.0	-
Amobarbital-	225.0	182.0	42.0
Pentobarbital	225.0	182.0	42.0
Secobarbital-d5	242.0	42.0	-
Secobarbital	237.0	194.0	42.0

Results and Discussion

Chromatographic Performance

For this work, a previously published, dilute-and-shoot EtG and EtS method [1] was expanded to include the barbiturates by making simple changes to the mobile phase gradient in the original method. As shown in Figure 1, a fast 5-minute analysis of alcohol metabolites and barbiturates was successfully obtained from the direct injection of urine sample supernatant without using enzymatic hydrolysis. All target compounds eluted within three minutes, and the total cycle time was extended to five minutes to allow for column re-equilibration.

The selectivity of the Raptor EtG/EtS column provided ample chromatographic separation of the target analytes from isobaric matrix interferences, making peak identification easy for most compounds. However, amobarbital and pentobarbital could not be separated from each other under these method conditions. These isobaric barbiturates had good sensitivity and peak shape, but because they coelute, accurate quantitation using this method is only possible if just one of the two compounds is present in the sample. Therefore, if individual quantitative results are required for amobarbital and pentobarbital, a separate confirmatory method, such as Restek's barbiturate drug panel [3], will be needed. While a confirmatory method would be required for definitive identification and individual quantitation, the current method is suitable for screening purposes.

Column Robustness

Following 100 sample injections, all chromatographic peaks maintained their initial peak shape, retention time, and intensity (Figure 2). The maximum system pressure also remained at the same level indicating no column clogging had occurred.

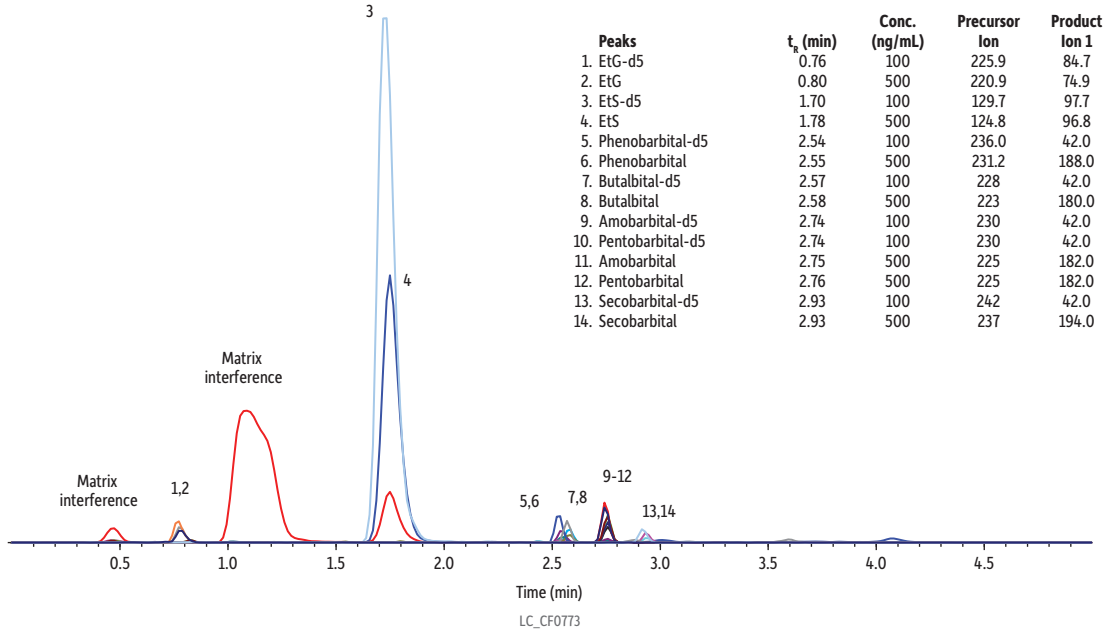
Linearity

Using linear 1/x weighted regression, good linearity results were obtained with r^2 values of 0.992 or greater (Figure 3).

Accuracy and Precision

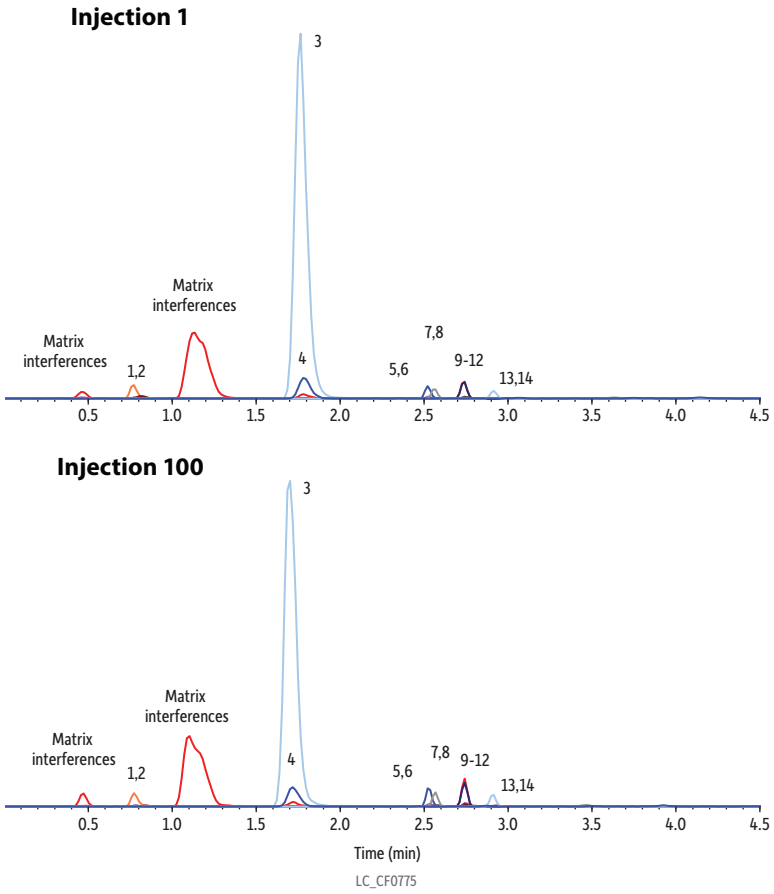
Precision and accuracy analyses were performed on three different days. Method accuracy was demonstrated by percent recovery values being within 15% (20% for the LLOQ level) of the nominal concentration on all three days. The %RSD was 1.10-12.5% and 0.8-13.7% for intra- and interday comparisons, respectively, across the LLOQ, low, mid, and high QC levels indicating good method precision (Table II).

Figure 1: Analysis of Alcohol Metabolites and Barbiturates in Fortified Human Urine (500 ng/mL Calibration Standard)



Peaks	t_R (min)	Conc. (ng/mL)	Precursor Ion	Product Ion 1	Product Ion 2
1. EtG-d5	0.76	100	225.9	84.7	-
2. EtG	0.80	500	220.9	74.9	85
3. EtS-d5	1.70	100	129.7	97.7	-
4. EtS	1.78	500	124.8	96.8	79.7
5. Phenobarbital-d5	2.54	100	236.0	42.0	-
6. Phenobarbital	2.55	500	231.2	188.0	42.0
7. Butalbital-d5	2.57	100	228	42.0	-
8. Butalbital	2.58	500	223	180.0	42.0
9. Amobarbital-d5	2.74	100	230	42.0	-
10. Pentobarbital-d5	2.74	100	230	42.0	-
11. Amobarbital	2.75	500	225	182.0	42.0
12. Pentobarbital	2.76	500	225	182.0	42.0
13. Secobarbital-d5	2.93	100	242	42.0	-
14. Secobarbital	2.93	500	237	194.0	42.0

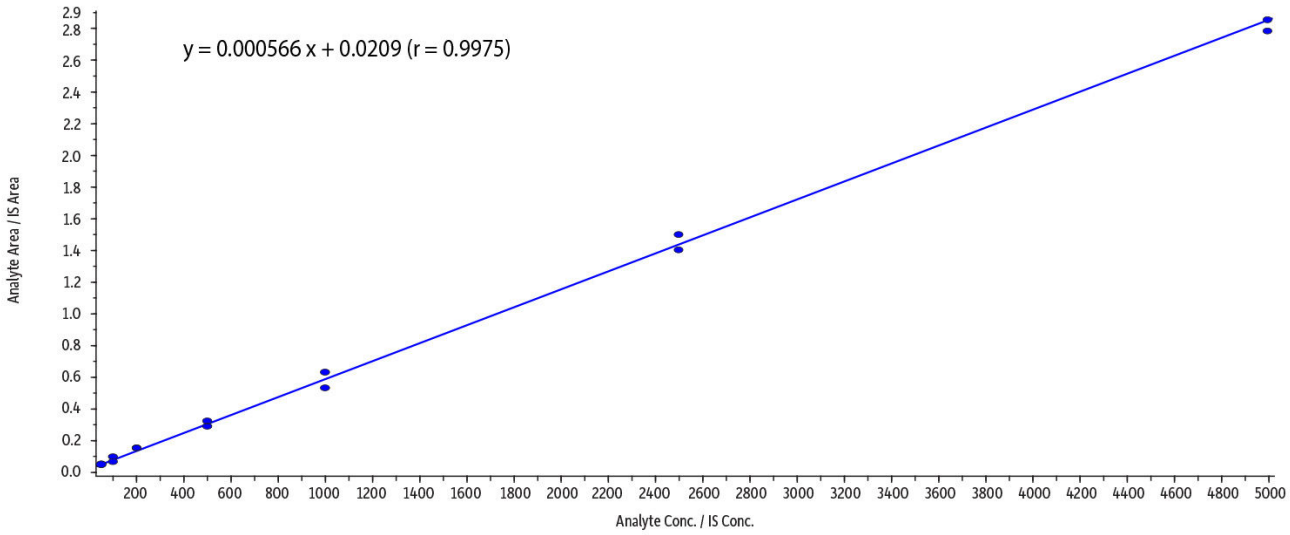
Figure 2: Lifetime Testing: Raptor EtG/EtS Provide Consistent Results Even After 100 Sample Injections



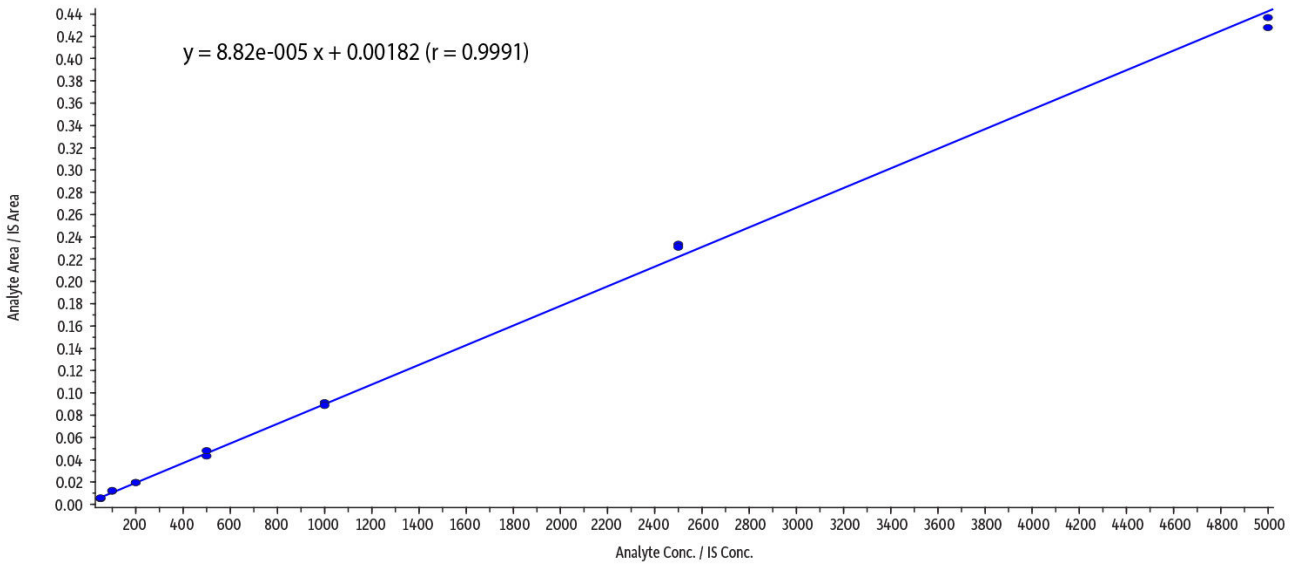
Peaks	t_R (min)	Conc. (ng/mL)	Precursor Ion	Product Ion 1	Product Ion 2
1. EtG-d5	0.76	100	225.9	84.7	-
2. EtG	0.80	500	220.9	74.9	85
3. EtS-d5	1.70	100	129.7	97.7	-
4. EtS	1.78	500	124.8	96.8	79.7
5. Phenobarbital-d5	2.54	100	236.0	42.0	-
6. Phenobarbital	2.55	500	231.2	188.0	42.0
7. Butalbital-d5	2.57	100	228	42.0	-
8. Butalbital	2.58	500	223	180.0	42.0
9. Amobarbital-d5	2.74	100	230	42.0	-
10. Pentobarbital-d5	2.74	100	230	42.0	-
11. Amobarbital	2.75	500	225	182.0	42.0
12. Pentobarbital	2.76	500	225	182.0	42.0
13. Secobarbital-d5	2.93	100	242	42.0	-
14. Secobarbital	2.93	500	237	194.0	42.0

Figure 3: Calibration Curves

EtG



EtS



Phenobarbital

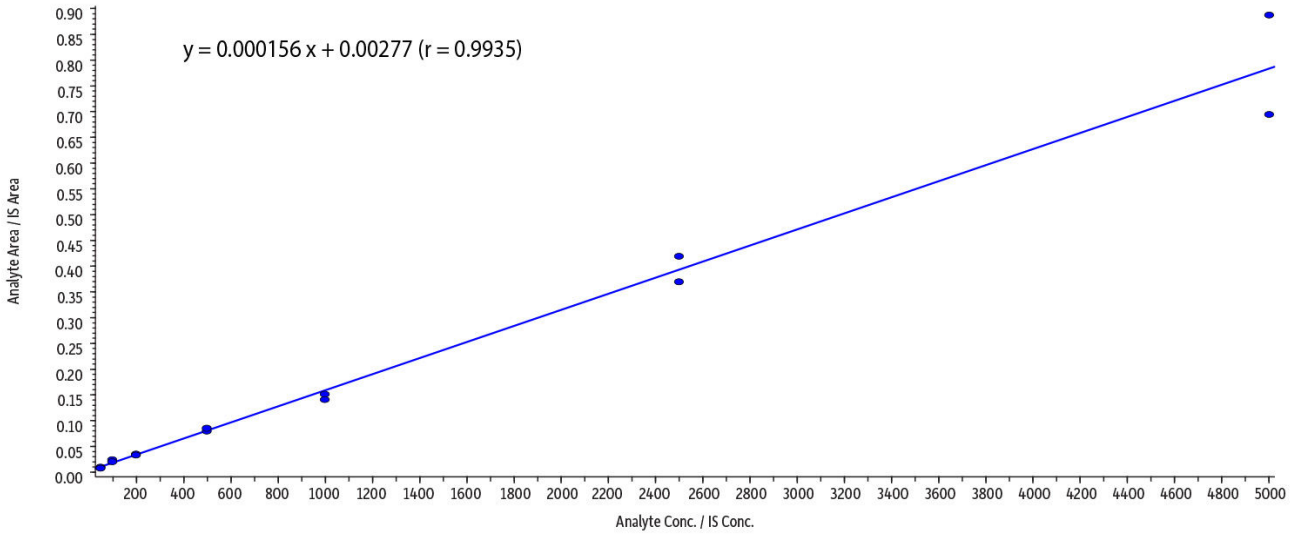
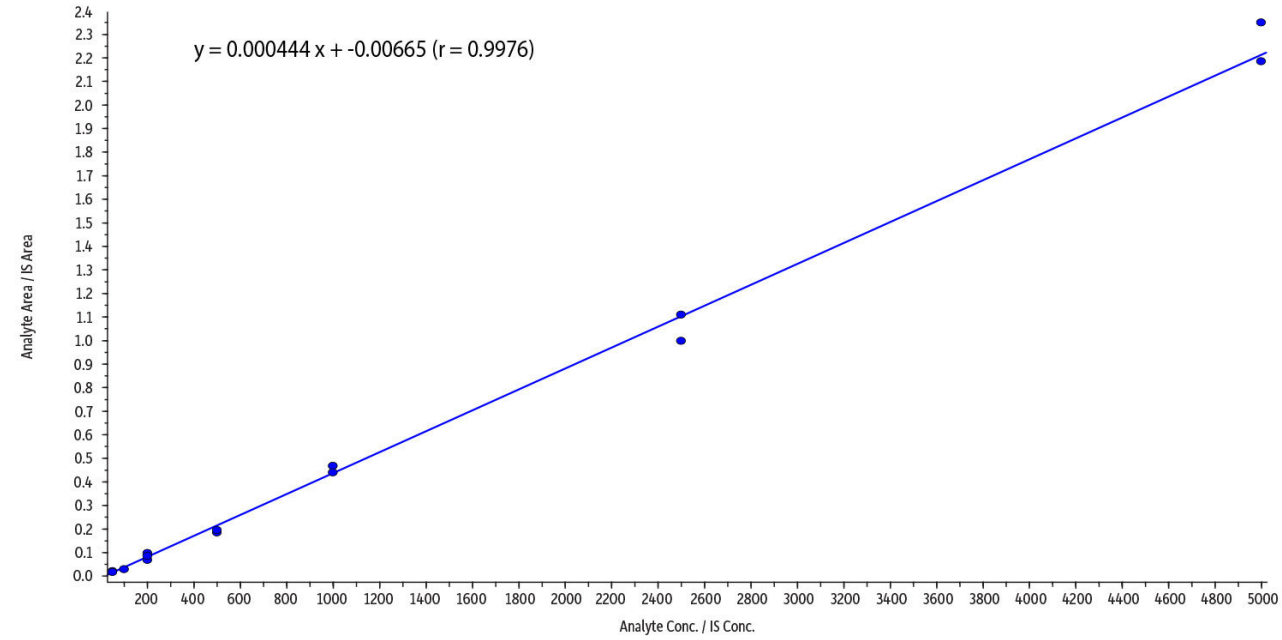


Figure 3: Calibration Curves *(continued)*

Butalbital



Secobarbital

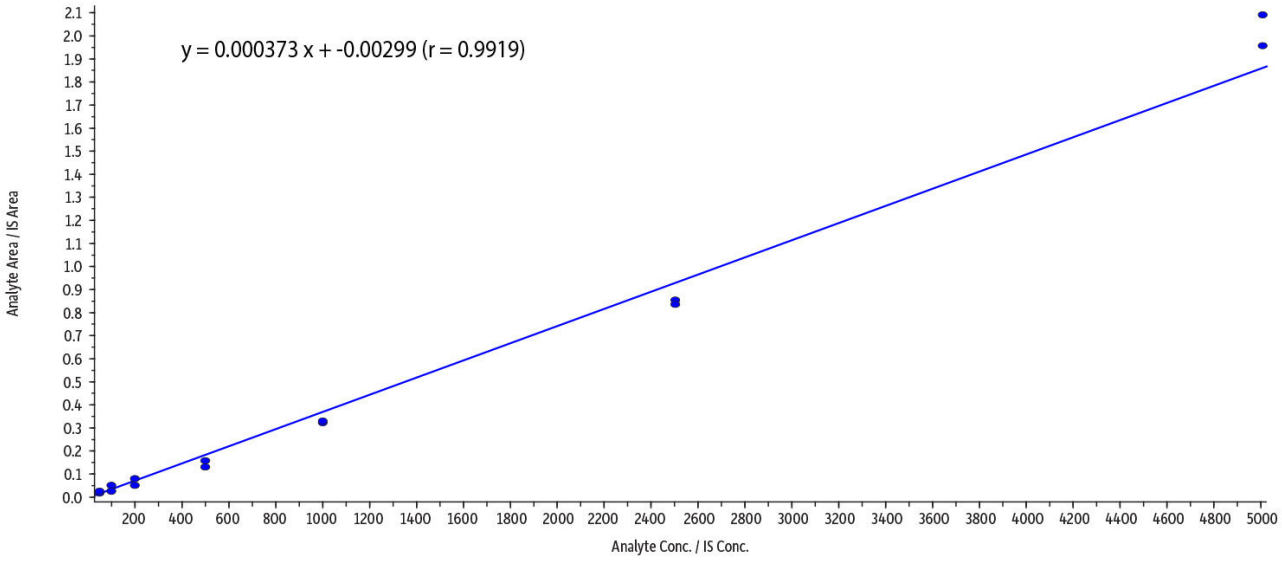


Table II: Interday Accuracy and Precision of QC Samples*

Analyte	Avg. Conc. (ng/mL)	Avg. Accuracy (%)	%RSD
QC LLOQ (50 ng/mL)			
Ethyl-β-D-glucuronide (EtG)	55.0	110	7.70
Ethyl sulfate (EtS)	54.3	109	13.7
Phenobarbital	59.7	119	9.60
Butalbital	58.3	117	13.2
Secobarbital	43.0	86.0	6.25
QC Low (125 ng/mL)			
Ethyl-β-D-glucuronide (EtG)	123	98.4	4.10
Ethyl sulfate (EtS)	125	100	0.800
Phenobarbital	136	109	5.80
Butalbital	128	102	5.50
Secobarbital	131	104	4.20
QC Mid (700 ng/mL)			
Ethyl-β-D-glucuronide (EtG)	729	104	11.3
Ethyl sulfate (EtS)	669	95.6	6.10
Phenobarbital	723	103	8.40
Butalbital	659	94.1	3.50
Secobarbital	676	96.5	7.20
QC High (4000 ng/mL)			
Ethyl-β-D-glucuronide (EtG)	4262	107	5.90
Ethyl sulfate (EtS)	4090	102	3.60
Phenobarbital	4003	101	8.50
Butalbital	3987	99.7	6.50
Secobarbital	3720	93.0	8.40

*While good peak shape and sensitivity were obtained, individual results for amobarbital and pentobarbital could not be reported because these isobaric compounds were not chromatographically separated.

Conclusion

It was demonstrated that the Raptor EtG/EtS column provides excellent performance for the simultaneous analysis of alcohol metabolites and barbiturates in human urine. Isobaric matrix interferences were easily resolved, preventing issues with peak identification and quantitation, even though only minimal sample preparation (dilute-and-shoot) was used. With a simple sample preparation procedure (no enzymatic hydrolysis) and 5-minute analysis time, this procedure can provide accurate, high-throughput monitoring of alcohol and barbiturates in urine. The single negative ESI LC-MS/MS method demonstrated here combines panels for higher sample throughput; however, it is important to note that EtG samples cannot undergo enzymatic hydrolysis, so laboratories considering adopting this method must independently determine the viability of analyzing their barbiturate samples without enzymatic hydrolysis.

References

1. J. Steimling, R. Alagandula, and F. Carroll, Successful strategies for the analysis of EtG and EtS in urine: rugged sample preparation and analysis conditions for high-throughput labs, Restek Corporation, 2020 https://www.restek.com/technical-literature-library/articles/successful-strategies-for-the-analysis-of-ETG-and-ETS-in-urine

2. Tech tip: column conditioning ensures consistent EtG/EtS results, Restek Corporation, 2017 https://www.restek.com/technical-literature-library/articles/tech-tip-column-conditioning-ensures-consistent-ETGETS-results/

3. Barbiturate drug panel on Raptor C18 by LC-MS/MS, Restek Corporation, https://www.restek.com/en/pages/chromatogram-view/LC_CF0619

ordering notes

Certificates of analysis for new Restek LC columns are now provided electronically. To view and download, visit www.restek.com/documentation then enter your cat.# and serial #.

Raptor LC Columns

Restek chemists became the first to combine the speed of superficially porous particles (also known as SPP or "core-shell" particles) with the resolution of highly selective USLC technology. This new breed of chromatographic column allows you to more easily achieve peak separation and faster analysis times with existing HPLC and UHPLC instrumentation. Learn more about Raptor LC columns at www.restek.com/raptor

Raptor EtG/EtS LC Column

- Proven performance for accurate, reliable ethyl glucuronide (EtG) and ethyl sulfate (EtS) analysis.
- Strong retention consistently resolves analytes from matrix interferences.
- Long column lifetime ensures consistent performance injection after injection.
- Fast, 4-minute, dilute-and-shoot LC-MS/MS analysis supports high sample throughput.
- Save time and increase certainty with Restek's definitive EtG/EtS method and quality reference standards.

Did you know consistent EtG/EtS results depend on proper column conditioning? Learn how to ensure stable performance at www.restek.com/EtGtip

Stationary Phase Category: Proprietary
Ligand Type: Proprietary
Particle: 2.7 µm superficially porous particle (SPP or "core-shell" particle) silica
Pore Size: 90 Å
Carbon Load: Proprietary
End-Cap: Proprietary
Surface Area: 130 m^2/g
Recommended Usage:
pH Range: 2.0–8.0
Maximum Temperature: 40 °C
Maximum Pressure: 600 bar/8700 psi

Properties:
• Resolution of EtG and EtS from matrix interferences.
• Increased retention of EtG and EtS compared to traditional phases.

Switch to the Raptor EtG/EtS column when:
• Other columns can't resolve EtG and EtS from matrix components.
• You need high-throughput EtG/EtS analysis.
• Low-level detection limits are desired.

ID	Length	qty.	cat.#
2.1 mm	100 mm	ea.	9325A12

24995

UltraShield UHPLC PreColumn Filter

- Cost-effective protection for UHPLC systems.
- Reliable way to filter out particulates and extend column lifetime.
- Minimize extra column volume and maximize UHPLC sample throughput vs. guard cartridges.
- Connects easily to any column with Parker-style ports; not compatible with Waters columns.
- Leak tight to 15,000 psi (1034 bar).
- 0.5 µm or 0.2 µm stainless-steel frit in a stainless-steel body with PEEK ferrule.

Specifications
Inlet/Outlet: Female/Male 10-32
Port Geometry: Parker (1/16 CPI)
Material: stainless steel, PEEK ferrule
Filter: 0.5 µm or 0.2 µm stainless steel
Pressure Rating: 15,000 psig (1034 bar)
Wrench Flat: 5/16"

Description	Porosity	qty.	cat.#
UltraShield UHPLC PreColumn Filter	0.5 µm frit	ea.	24995
	0.5 µm frit	5-pk.	24996
	0.5 µm frit	10-pk.	24997
	0.2 µm frit	ea.	25809
	0.2 µm frit	5-pk.	25810
	0.2 µm frit	10-pk.	25811

Restek Reference Standards

Ethyl-β-D-glucuronide (EtG)

Alcohol metabolite biomarkers for monitoring alcohol consumption in urine samples.

Ethyl-β-D-glucuronide (EtG) (17685-04-0)

CAS #	Conc. in Solvent	CRM?	Min Shelf Life on Ship Date	Max Shelf Life on Ship Date	Shipping Conditions	Storage Temp.	qty.	cat.#
17685-04-0	1000 µg/mL in methanol, 1 mL/ ampul	Yes	6 months	36 months	Ambient	0 °C or colder	ea.	34101

Ethyl-β-D-glucuronide-d5 (EtG-d5)

Alcohol metabolite biomarkers for monitoring alcohol consumption in urine samples.

Ethyl-β-D-glucuronide-d5 (EtG-d5) (1135070-98-2)

CAS #	Conc. in Solvent	CRM?	Min Shelf Life on Ship Date	Max Shelf Life on Ship Date	Shipping Conditions	Storage Temp.	qty.	cat.#
1135070-98-2	1000 µg/mL in methanol, 1 mL/ ampul	Yes	6 months	36 months	Ambient	0 °C or colder	ea.	34102

Ethyl sulfate sodium salt (EtS)

Alcohol metabolite biomarkers for monitoring alcohol consumption in urine samples.

Ethyl sulfate sodium salt (EtS) (546-74-7)

CAS #	Conc. in Solvent	CRM?	Min Shelf Life on Ship Date	Max Shelf Life on Ship Date	Shipping Conditions	Storage Temp.	qty.	cat.#
546-74-7	1000 µg/mL in methanol, 1 mL/ ampul	Yes	6 months	36 months	Ambient	0 °C or colder	ea.	34103

Ethyl sulfate-d5 sodium salt (EtS-d5)

Alcohol metabolite biomarkers for monitoring alcohol consumption in urine samples.

Ethyl sulfate-d5 sodium salt (EtS-d5) (1329611-05-3)

CAS #	Conc. in Solvent	CRM?	Min Shelf Life on Ship Date	Max Shelf Life on Ship Date	Shipping Conditions	Storage Temp.	qty.	cat.#
1329611-05-3	1000 µg/mL in methanol, 1 mL/ ampul	Yes	6 months	36 months	Ambient	0 °C or colder	ea.	34104

Butalbital

Note: Stressed shipping studies indicate that this product remains stable after being continuously exposed to an elevated temperature of 40 °C for 7 days. Ground freight is the default shipping method for this product; expedited methods are available upon request.

U.S. DEA-exempted formulation—no additional customer permits or licensing are required to purchase within the U.S.

Butalbital (77-26-9)

CAS #	Conc. in Solvent	CRM?	DEA Status	Min Shelf Life on Ship Date	Max Shelf Life on Ship Date	Shipping Conditions	Storage Temp.	qty.	cat.#
77-26-9	1000 µg/mL in P&T methanol, 1 mL/ampul	Yes	Exempt	6 months	24 months	Ambient	10 °C or colder	ea.	34032

Pentobarbital

Note: Stressed shipping studies indicate that this product remains stable after being continuously exposed to an elevated temperature of 40 °C for 7 days. Ground freight is the default shipping method for this product; expedited methods are available upon request.

U.S. DEA-exempted formulation—no additional customer permits or licensing are required to purchase within the U.S.

Pentobarbital (76-74-4)

CAS #	Conc. in Solvent	CRM?	DEA Status	Min Shelf Life on Ship Date	Max Shelf Life on Ship Date	Shipping Conditions	Storage Temp.	qty.	cat.#
76-74-4	1000 µg/mL in P&T methanol, 1 mL/ampul	Yes	Exempt	6 months	24 months	Ambient	10 °C or colder	ea.	34036

Secobarbital

Note: Stressed shipping studies indicate that this product remains stable after being continuously exposed to an elevated temperature of 40 °C for 7 days. Ground freight is the default shipping method for this product; expedited methods are available upon request.

U.S. DEA-exempted formulation—no additional customer permits or licensing are required to purchase within the U.S.

Secobarbital (76-73-3)

CAS #	Conc. in Solvent	CRM?	DEA Status	Min Shelf Life on Ship Date	Max Shelf Life on Ship Date	Shipping Conditions	Storage Temp.	qty.	cat.#
76-73-3	1000 µg/mL in P&T methanol, 1 mL/ampul	Yes	Exempt	6 months	24 months	Ambient	10 °C or colder	ea.	34038

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