TN-1227



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APPLICATIONS

A Single Column and Mobile Phase Workflow for LC-MS/MS Analysis of a Comprehensive Drug **Research Panel and Ethanol Metabolites**

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Overview

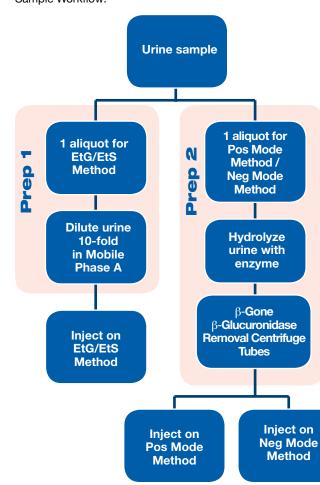
- Simplified workflow for toxicology research
- Positive and negative mode drug research panel and EtG/EtS
- Same mobile phases; same column

Introduction

In this work, we present a simple workflow using one column and one set of mobile phases for ethanol metabolites (EtG/EtS), positive ionizing pain management compounds, and negative ionizing pain management compounds. All three methods were run on a single system without the need to switch columns or mobile phase between methods. This allows labs to utilize their current instrumentation for a variety of applications.

EtG/EtS follows a simple dilute-and-shoot sample preparation. Comprehensive drug research pain management samples are hydrolyzed and cleaned up using β-Gone[™] β-Glucuronidase Removal Tubes that eliminate enzyme proteins without the need for dilution of the sample prior to injection.

Sample Workflow:





Laura Snow

Outside of the lab, Laura enjoys spoiling her dog Maggie and subjecting her husband to novel methods of torture, such as endless playlists of sad songs and long walks on the beach to catch Pokémon.

Materials and Methods Reagents and Chemicals

Analytical reference standards were purchased from Cerilliant® (Round Rock, TX). HPLC-grade solvents were purchased from Honeywell[™] (Muskegon, MI). All other chemicals were obtained from the Sigma-Aldrich® (St. Louis, MO). Water purification via Sartorius arium[®] Comfort II, courtesy of Sartorius Corporation (Bohemia, NY). Campbell enzyme was purchased from Campbell Science (Rockford, IL). IMCSzyme® was purchased from Integrated Micro-Chromatography Systems (Irmo, SC). Kura enzyme was purchased from Kura Biotec® (Puerto Varas, Chile).

Sample Preparation

Prep 1: EtG/EtS samples were prepared as follows:

- 1. Centrifuge urine for 10 minutes at max rpm to remove any particulates
- 2. Transfer 100 µL urine from the top layer to a vial
- 3. Dilute with 900 µL mobile phase A (0.1 % Formic acid in Water)
- 4. Inject sample

Prep 2: Pain management samples were prepared as follows: Hydrolysis of urine with one of the following enzymes Campbell Enzyme:

- - 1. Aliquot 200 µL urine
 - 2. Add 100µL 0.1 M Ammonium acetate buffer (pH 4, adjusted)
 - 3. Add 40 μL Campbell Science β-Glucuronidase Enzyme (Campbell Part No. DR2102)
 - 4. Incubate 1 hour at 50 °C
 - 5. Proceed to β-Gone Centrifuge Tube Protocol

IMCSzyme:

- 1. Aliquot 140 µL urine
- 2. Add 80 µL IMCS buffer (IMCS Part No. 04-EZ-RHB-20)
- 3. Add 30 µL IMCS Enzyme (IMCS Part No. 04-E1F-010)
- 4. Incubate 30 min at 60 °C
- 5. Proceed to β-Gone Centrifuge Tube Protocol

Kura Enzyme:

- 1. Aliquot 200 µL urine
- 2. 100 µL 0.1 M Ammonium acetate buffer (pH 5, adjusted)
- 3. 40 µL Kura Enzyme Solution (Kura Part No. BG100-10ML)
- 4. Incubate 1 hr at 50 °C
- 5. Proceed to β-Gone Centrifuge Tube Protocol

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β -Glucuronidase Removal Protocol

Centrifuge Tube: β-Gone[™] β-Glucuronidase Removal 2 mL Centrifuge Tubes Part No.: 8N-S323-TUK

- Pre-treatment:
 Combine 200 μL of urine hydrolysate with 133 μL of 0.1 %

 Formic acid in Methanol
 Load:

 Load:
 Tap tube prior to use to consolidate sorbent. Load the pre
 - treated sample onto the centrifuge tube.

 - Mix: Cap and invert the tube 10 times. Vortex for 30 seconds. Centrifuge: Centrifuge tube at 14,000 rpm for 10 minutes (16,000 x g RCF). Collect: Supernatant for analysis and inject sample

Experimental Conditions EtG/EtS HPLC Conditions

S HPLC Conditions			
Column:	Luna® Omega 5 µm Polar C18		
Dimension:	50 x 4.6 mm		
Part No.:	00B-4754-E0		
Guard:	SecurityGuard™	Polar C18	
	Cartridge: AJO-	7601	
Mobile Phase:	A: 0.1 % Formic	acid in Water	
	B: Methanol		
Gradient:	Time (min)	%В	
	0	2	
	2	95	
	2.6	95	
	2.7	2	
	4	2	
Injection Volume:	10µL		
Flow Rate:	0.7 mL/min		
Temperature:	Ambient		

Instrument: Agilent[®] 1260 LC Detector: MS/MS (SCIEX 4500 Triple Quad)

Negative Mode Comprehensive Drug **Research Panel HPLC Conditions**

Dimension: Part No.: Guard:	Luna Omega 5 µm Polar C18 50 x 4.6 mm 00B-4754-E0 SecurityGuard Polar C18 Cartridge: AJ0-7601 A: 0.1 % Formic acid in Water	
MODIIC I Hase.	B: Methanol	aciu in water
Gradient:	Time (min)	%В
	0	50
	1.5	95
	2.5	95
	2.6	50
	3.8	50
Injection Volume:	10µL	
Flow Rate:	: 0.7 mL/min	
	: Ambient : Agilent 1260 LC : MS/MS (SCIEX 4500 Triple Quad)	
201001011		

Positive Mode Comprehensive Drug Research Panel HPLC Conditions

Dimension: Part No.: Guard:	Luna Omega 5 µm Polar C18 50 x 4.6 mm 00B-4754-E0 SecurityGuard Polar C18 Cartridge: AJ0-7601 A: 0.1 % Formic acid in Water B: Methanol	
Gradient:	Time (min)	%B
	0	5
	3	95
	5	95
	5.01	5
	6.5	5
Flow Rate:	0.6 mL/min	
Temperature:	Ambient	
Injection Volume:	10 µL	
Instrument:	Agilent 1260 LC	
Detector:	MS/MS (SCIEX 4	1500 Triple Quad)

Table 1.

EtG/EtS MRM Transitions

ID	Q1	Q3 (quant)	Q3 (qual)
EtG	221.2	75.0	85.1
EtS	124.9	80.1	97.0

Table 2.

Negative Mode Comprehensive Drug Research Panel MRM Transitions

ID	Q1	Q3 (quant)	Q3 (qual)
Butalbital	223.1	42.1	180.0
Secobarbital	237.0	42.1	194.0
Phenobarbital	231.1	42.1	188.0
Amo/pentobarbital	237.0	42.1	182.0
THC-COOH	343.1	299.0	191.2



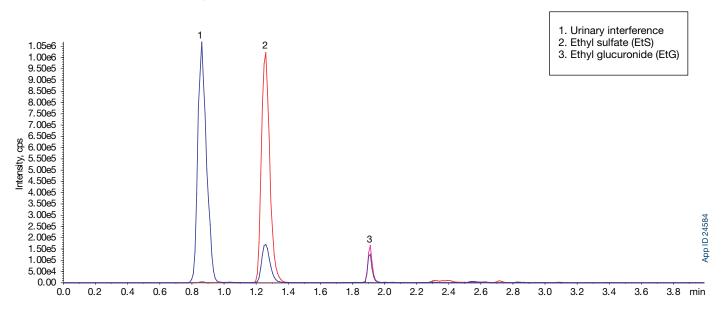
Table 3.

Positive Mode Comprehensive Drug Research Panel MRM Transitions

Analyte	Q1	Q3 (quant)	Q3 (qual)
α -Hydroxyalprazolam	325.1	297.1	216.1
6-MAM	328.1	165.1	211.1
7-Aminoclonazepam	286.0	222.1	121.1
Amitriptyline	278.1	191.0	202.0
Amphetamine	136.1	91.1	119.0
Benzoylecgonine	290.1	168.1	105.0
Buprenorphine	468.3	55.2	396.1
Carisoprodol	261.1	176.2	97.2
Codeine	300.2	152.1	115.1
Cyclobenzaprine	276.1	231.0	215.1
EDDP	278.2	234.2	186.2
Fentanyl	337.3	105.1	188.2
Fluoxetine	310.1	44.1	148.2
Gabapentin	172.1	137.1	95.1
Hydrocodone	300.2	199.0	128.0
Hydromorphone	286.1	185.1	128.0
Imipramine	281.1	86.0	58.1
Lorazepam	321.0	275.0	229.0
MDMA	194.1	105.1	163.1
MDPV	276.2	126.1	175.1
Meperidine	248.2	220.2	174.2
Meprobamate	219.2	158.1	97.0
Methadone	310.0	265.0	105.0
Methamphetamine	150.1	9.0	119.2
Methylphenidate	234.1	84.1	56.0
Morphine	286.1	152.1	165.1
Naloxone	328.2	212.0	253.0
Norbuprenorphine	414.3	55.0	83.0
Nordiazepam	271.0	140.0	165.1
Norfentanyl	233.2	84.1	150.1
Norhydrocodone	286.1	199.1	241.1
Noroxycodone	302.2	284.0	187.2
Nortriptyline	264.2	233.2	117.2
O-Desmethyl-cis-tramadol	250.1	58.1	42.2
Oxazepam	287.0	241.0	269.1
Oxycodone	316.1	241.2	256.2
Oxymorphone	302.1	227.0	198.1
Paroxetine	330.2	192.1	123.0
Phencyclidine	244.1	86.1	159.1
Phentermine	150.1	65.1	133.0
Pregabalin	160.1	55.1	97.1
Ritalinic Acid	219.9	83.9	56.0
Tapentadol	222.1	107.0	121.0
Temazepam	301.1	255.1	177.1
Tramadol	264.1	58.1	42.1
Zolpidem Phenyl-4-COOH	338.1	265.0	292.9

Figure 1.

EtG/EtS Method Representative Chromatogram of a Urine Sample



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Figure 2. Negative mode method representative chromatogram for hydrolyzed urine sample. One transition per compound is shown for clarity.

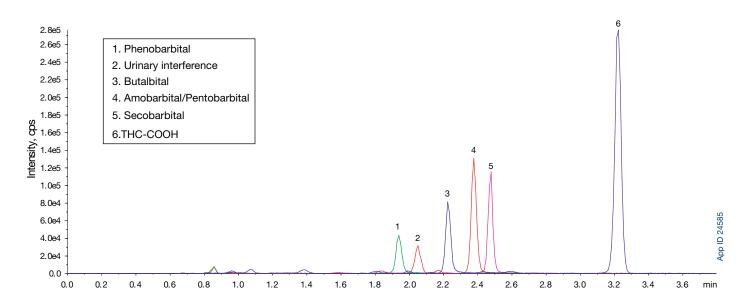




Figure 3.

Positive mode method representative chromatogram for hydrolyzed urine sample. One transition per compound is shown for clarity.

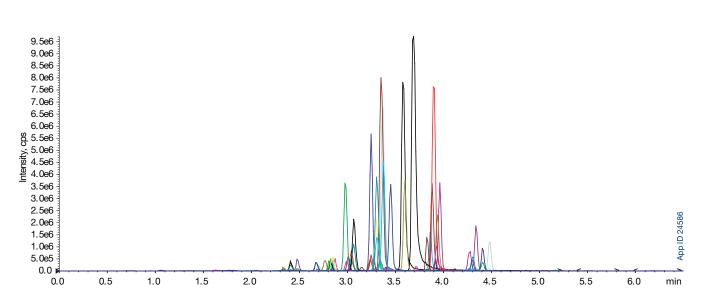


Figure 4. Extracted chromatogram for methamphetamine and phentermine (m/z 150).

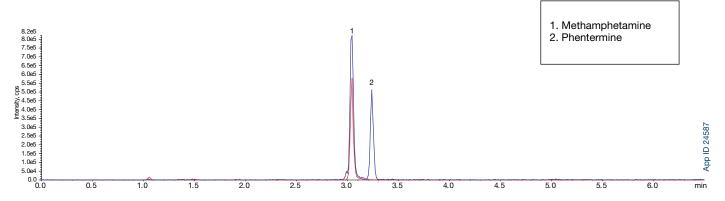




Figure 5.

Extracted chromatogram for tramadol and nortriptyline (m/z 264.1).

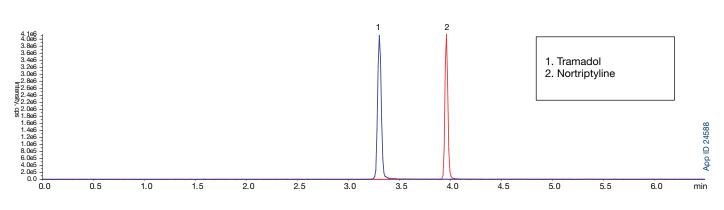


Figure 6. Extracted chromatogram for EDDP and amitriptyline (m/z 278.1).

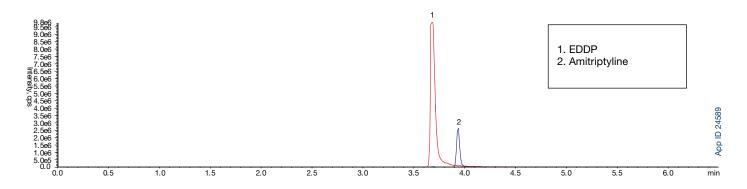




Figure 7.

Extracted chromatogram for morphine, hydromorphone, and norhydrocodone (m/z 286.1).

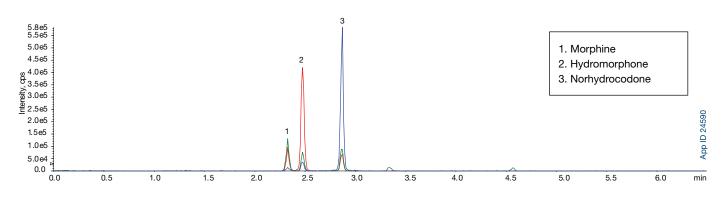


Figure 8. Extracted chromatogram for codeine and hydrocodone (m/z 300.1).

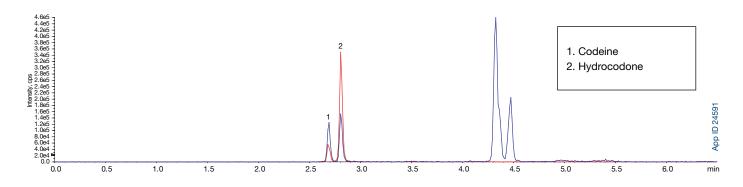




Figure 9.

Extracted chromatogram for oxymorphone and noroxycodone (m/z 302.1).

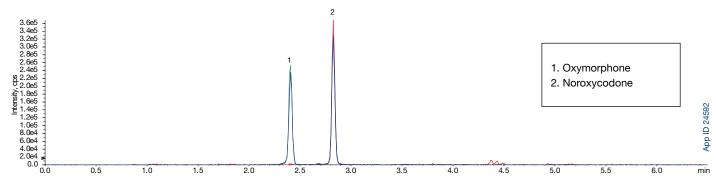


Figure 10. Extracted chromatogram for naloxone and 6-MAM (m/z 328.2).

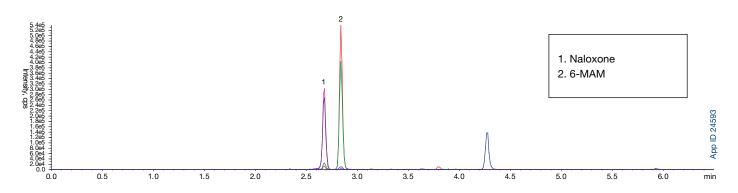




Figure 11.

Extracted chromatogram for fluoxetine and methadone (m/z 310.1 and 310.2).

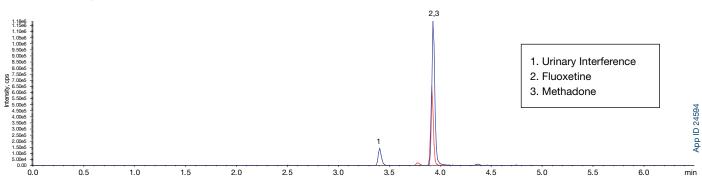
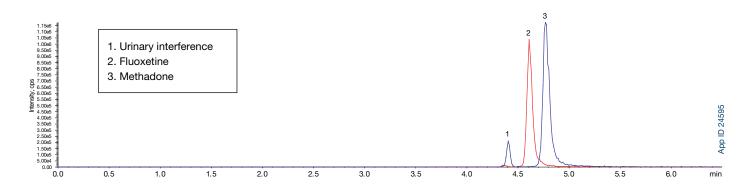


Figure 12. Extracted chromatogram for fluoxetine and methadone (m/z 310.1 and 310.2) with MP A 5 mM ammonium acetate in water.



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Discussion

In previous work, we have demonstrated running positive and negative ionization methods on the same column. However, HPLC methods for ethanol metabolites have always required a separate column and mobile phases. This is primarily because ethyl sulfate is an ionic species and is very difficult to retain using reversed phase mechanisms. The mixed mode selectivity of the Luna® Omega Polar C18 column makes it an excellent choice for analysis of ethyl sulfate and ethyl glucuronide. The polar modified surface provides enhanced polar retention for ethyl sulfate. Ethyl sulfate is retained so that it elutes away from the void volume where salts typically elute. Ethyl sulfate is also sufficiently resolved from the interference peak present in urine samples. Acidifying the aqueous mobile phase with 0.1 % formic acid neutralizes ethyl glucuronide, providing additional retention and improved peak shape for this compound as it is retained by the aliphatic C18 part of the phase. By utilizing this mixed mode selectivity, we are able to achieve excellent separation and peak shape for both compounds (Figure 1).

Acidic compounds, barbiturates and THC-COOH ionize well by negative electrospray ionization and are grouped together in our negative mode ionization panel. These compounds are chromatographically resolved with the exception of structural isomers, amobarbital and pentobarbital which differ only by the position of a methyl group (**Figure 2**).

For the main panel containing the majority of analytes, we were able to analyze 46 compounds in 6.5 minutes using positive electrospray ionization (**Figure 3**). We demonstrated resolution of all common isobaric/isomeric species (**Figures 4-10**), with the exception of fluoxetine and methadone (**Figure 11**). Resolution of fluoxetine and methadone can be improved by switching mobile phase A to aqueous 5 mM ammonium acetate (**Figure 12**), suggesting that a separate method could easily be developed for identification of these compounds, if needed.

In order to quantitate several drugs that may be present in a glucuronide form in urine, the sample must first be hydrolyzed. A common method for achieving this is enzymatic hydrolysis using β -glucuronidase. Proteins collecting on the front of the column are a concern as they can clog the frit at the head of the column if not adequately removed from the sample. Traditional dilute-andshoot methods typically dilute the sample anywhere from 5-fold to 20-fold and rely on centrifugation to pull the protein out of the sample into a pellet. Protein precipitation methods use organic solvent in a ratio of 2-4x the sample volume to crash the protein out of solution, however this technique requires that the sample be subsequently diluted prior to injection on-column. The dilution step is needed to avoid poor chromatography due to strong solvent effects.

Using β -Gone[™] β -glucuronidase Removal Centrifuge Tubes, the media targets β -glucuronidase in the sample. The sample is then spun down to remove the media and proteins leaving a clean sample. By combining, the simple β -Gone sample preparation with our Luna Omega Polar C18 column, which is highly retentive for polar compounds, we eliminate the need to dilute the urine sample prior to injection. This allows for greater sensitivity compared to dilute-and-shoot and protein crash with organic solvent. β -Gone sample preparation has been shown to increase column lifetime and provide greater sensitivity compared to dilute-and-shoot sample preparation. ^{1,2}

Conclusion

Using the unique mixed-mode selectivity of the Luna Omega Polar C18 column, we were able to use one set of mobile phase conditions and a single column to run pain panel drugs as well as ethanol metabolites.

The additional retention provided by the Polar C18 chemistry allows for direct injection of the sample after β -Gone sample prep, no dilution prior to injection required.

References

- 1. Analyzing β -Gone β -Glucuronidase Removal Centrifuge Tube Recovery and Clean Up; TN-0107. Matthew Brusius, Jessica Detsch, and Eric Chapa. Phenomenex, Inc.
- Improved Sensitivity of Hydrolyzed Urine Samples Using β-Gone β-Glucuronidase Removal Products; TN-0101. Matthew Brusius. Phenomenex, Inc



Order Information

β-Gone [™] β-Glucuronidase Removal Products		and a second sec	
Part No.	Description	Unit	(Gone
8B-S139-TAK	1 mL Tubes, Recombinant Enzyme	100/Box	
8B-S322-DAK	1 mL Tubes, Non-Recombinant Enzyme	100/Box	GGone
8E-S139-TGA	96-Well Plate, Recombinant Enzyme	1/Box	
8E-S322-DGA	96-Well Plate, Non-Recombinant Enzyme	1/Box	
8N-S323-TUK	2 mL Centrifuge Tubes, Recombinant and Non-Recombinant Enzyme	100/Box	

Contraction of the

Luna[®] Omega HPLC Columns

5 µm Analytic	al Columns (mm)				SecurityGuard Cartridges (mm)
Phases	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	4 x 2.0*
Polar C18	00B-4754-E0	00D-4754-E0	00F-4754-E0	00G-4754-E0	AJ0-7601
				for ID:	3.1-8.0 mm

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

Presston[™] 100 Positive Pressure Manifold

Part No.	Description
AH0-9334	Presston 100 Positive Pressure Manifold, 96-Well Plate
AH0-9342	Presston 100 Positive Pressure Manifold, 1 mL Tube Complete Assembly
AH0-9347	Presston 100 Positive Pressure Manifold, 3 mL Tube Complete Assembly
AH0-9343	Presston 100 Positive Pressure Manifold, 6 mL Tube Complete Assembly

Presston 100 Tube Adapter Kits (for AH0-9334)

Part No.	Description	and the second s
AH0-9344	1 mL Tube Adapter Kit	0 00
AH0-9345	3 mL Tube Adapter Kit	
AH0-9346	6 mL Tube Adapter Kit	



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