APPLICATIONS

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

Laura Snow, Danielle Moore
Phenomenex, Inc., 411 Madrid Ave., Torrance, CA 90501 USA

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.

Overview

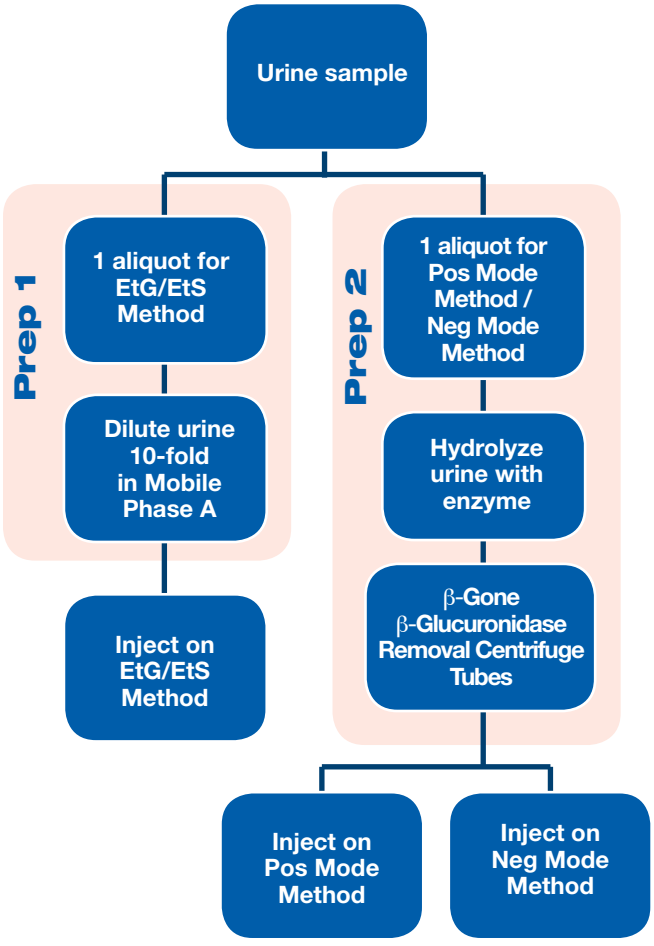
- *Simplified workflow for toxicology research*
- *Positive and negative mode drug research panel and EtG/EtS*
- *Same mobile phases; same column*

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:



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 100 µL 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

APPLICATIONS

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: 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

Column: Luna® Omega 5 µm Polar C18
Dimension: 50 x 4.6 mm
Part No.: 00B-4754-E0
Guard: SecurityGuard™ Polar C18 Cartridge: AJ0-7601
Mobile Phase: A: 0.1 % Formic acid in Water
B: Methanol

Gradient:

Time (min)	%B
0	2
2	95
2.6	95
2.7	2
4	2

Injection Volume: 10 µL
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

Column: Luna Omega 5 µm Polar C18
Dimension: 50 x 4.6 mm
Part No.: 00B-4754-E0
Guard: SecurityGuard Polar C18 Cartridge: AJ0-7601
Mobile Phase: A: 0.1 % Formic acid in Water
B: Methanol

Gradient:

Time (min)	%B
0	50
1.5	95
2.5	95
2.6	50
3.8	50

Injection Volume: 10 µL
Flow Rate: 0.7 mL/min
Temperature: Ambient
Instrument: Agilent 1260 LC
Detector: MS/MS (SCIEX 4500 Triple Quad)

Positive Mode Comprehensive Drug Research Panel 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: AJ0-7601
Mobile Phase: 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 4500 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

APPLICATIONS

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

APPLICATIONS

Figure 1.
EtG/EtS Method Representative Chromatogram of a Urine Sample

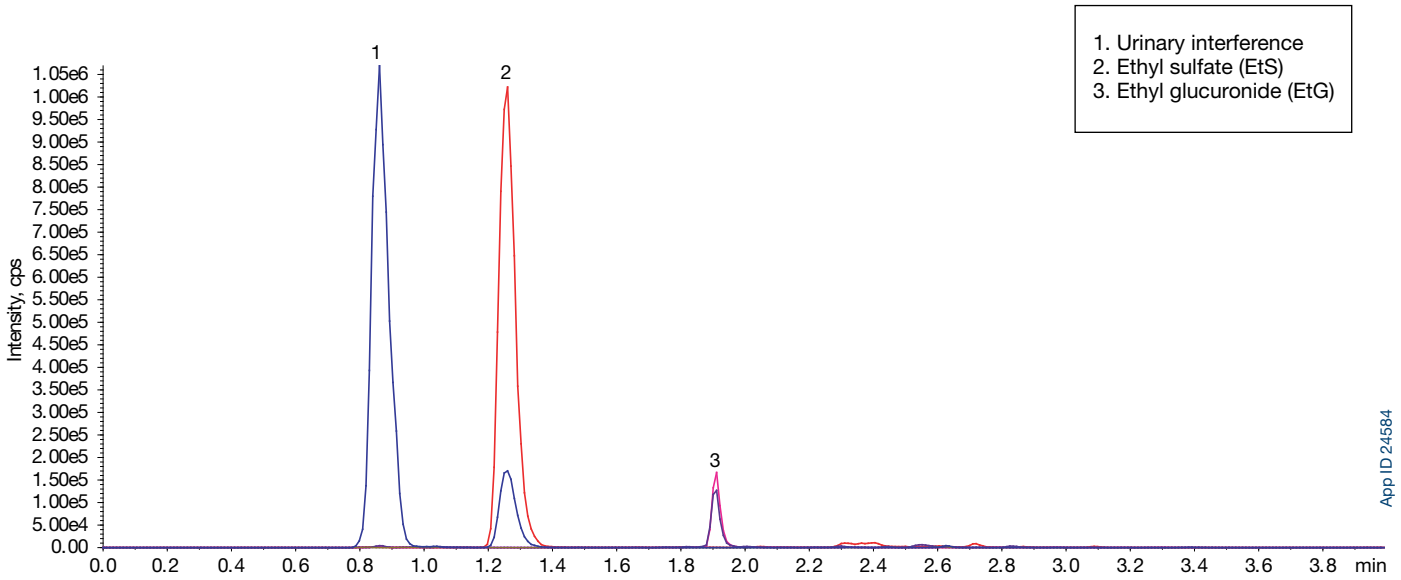
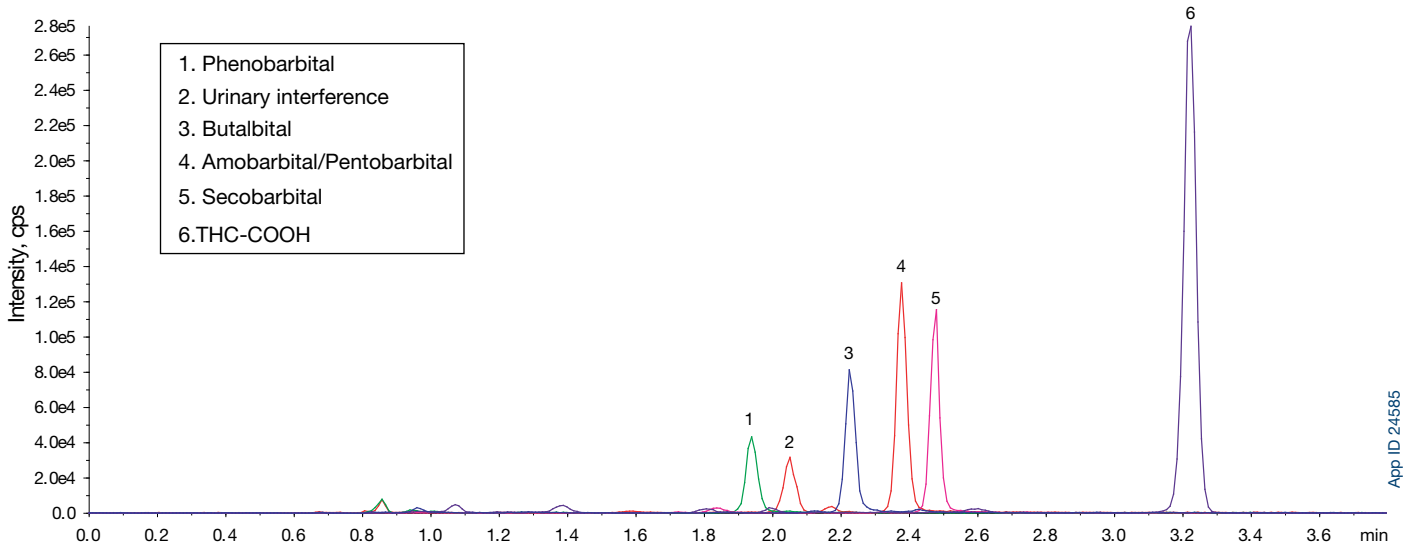


Figure 2.
Negative mode method representative chromatogram for hydrolyzed urine sample. One transition per compound is shown for clarity.



APPLICATIONS

Figure 3.
Positive mode method representative chromatogram for hydrolyzed urine sample. One transition per compound is shown for clarity.

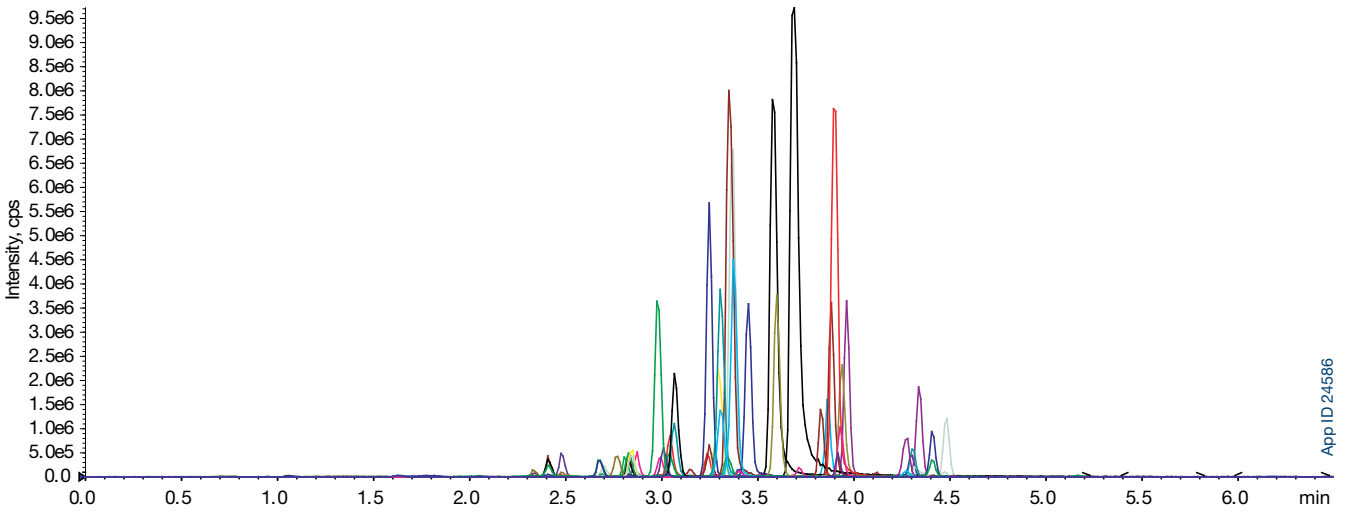
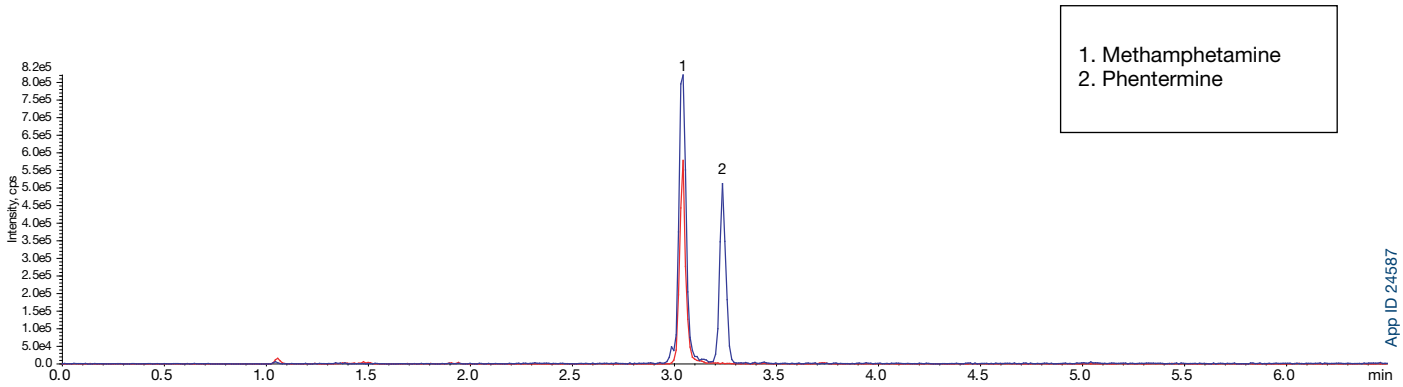


Figure 4.
Extracted chromatogram for methamphetamine and phentermine (m/z 150).



APPLICATIONS

Figure 5.
Extracted chromatogram for tramadol and nortriptyline (m/z 264.1).

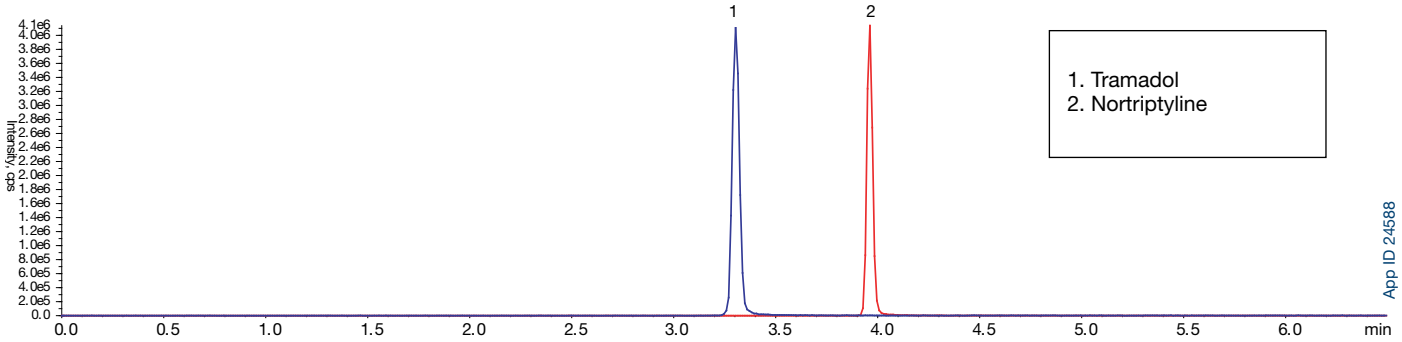
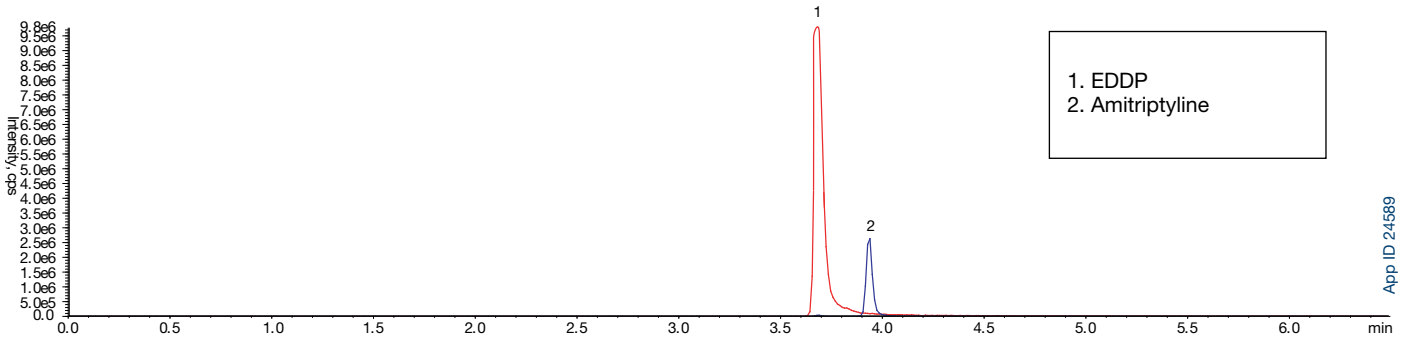


Figure 6.
Extracted chromatogram for EDDP and amitriptyline (m/z 278.1).



APPLICATIONS

Figure 7.
Extracted chromatogram for morphine, hydromorphone, and norhydrocodone (m/z 286.1).

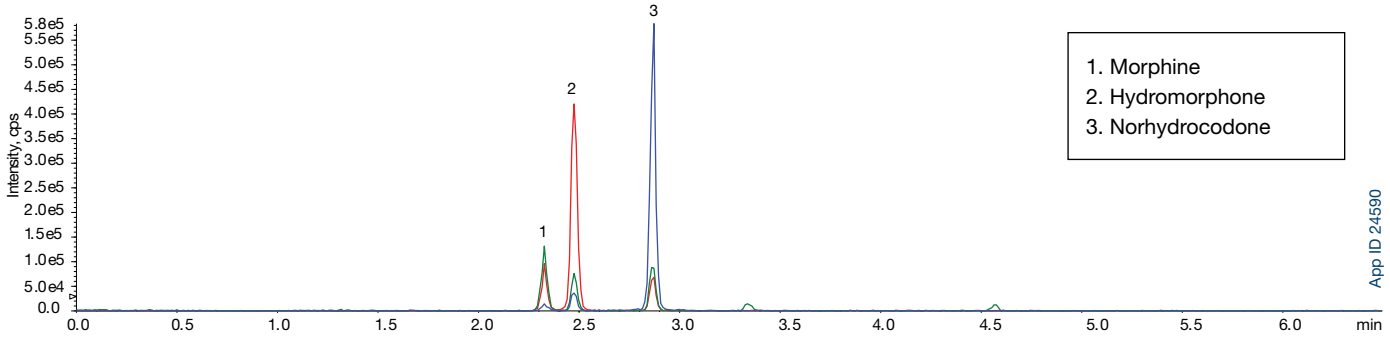
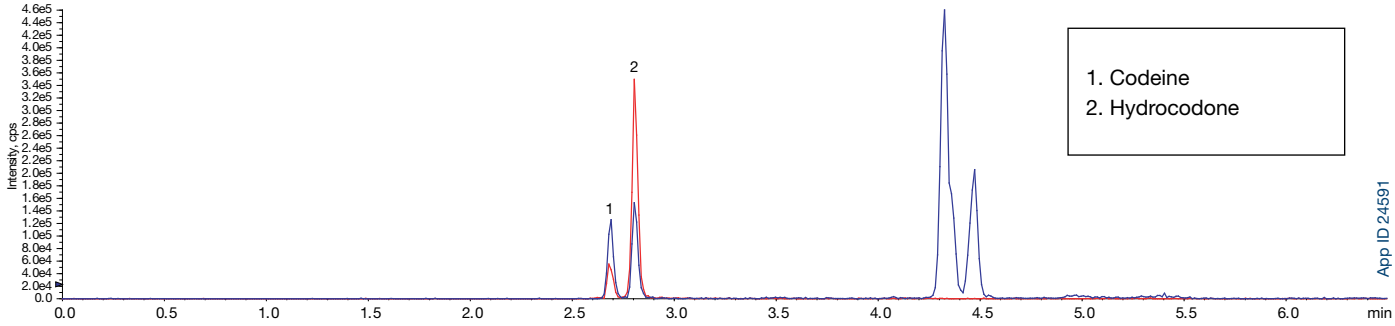


Figure 8.
Extracted chromatogram for codeine and hydrocodone (m/z 300.1).



APPLICATIONS

Figure 9.
Extracted chromatogram for oxymorphone and noroxycodone (m/z 302.1).

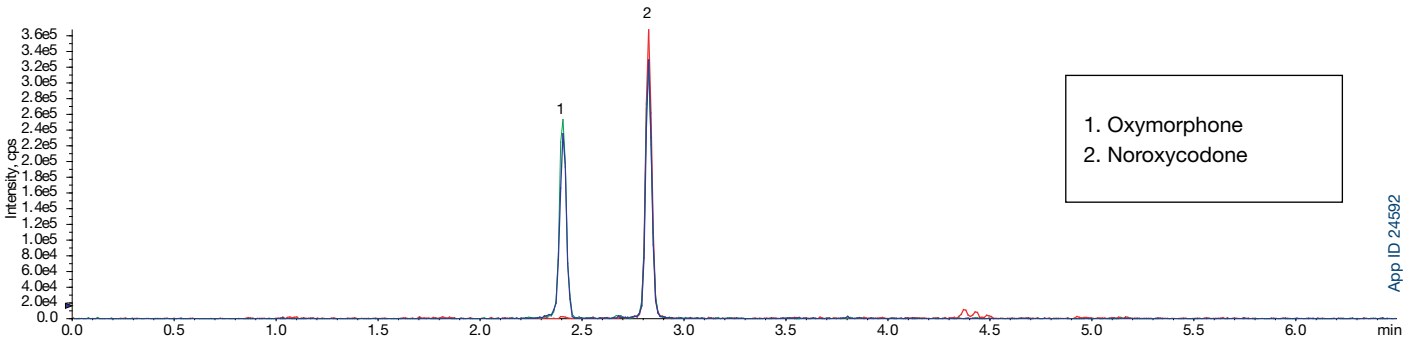
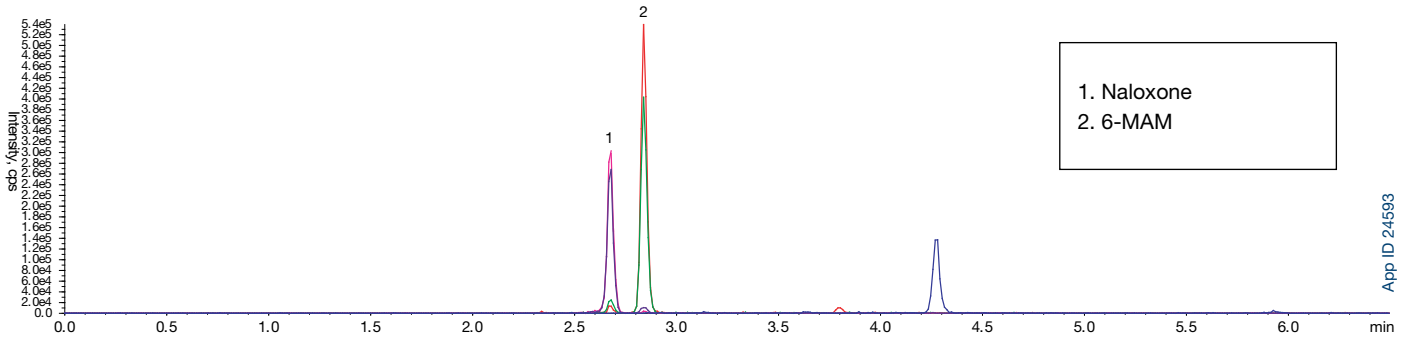


Figure 10.
Extracted chromatogram for naloxone and 6-MAM (m/z 328.2).



APPLICATIONS

Figure 11.
Extracted chromatogram for fluoxetine and methadone (m/z 310.1 and 310.2).

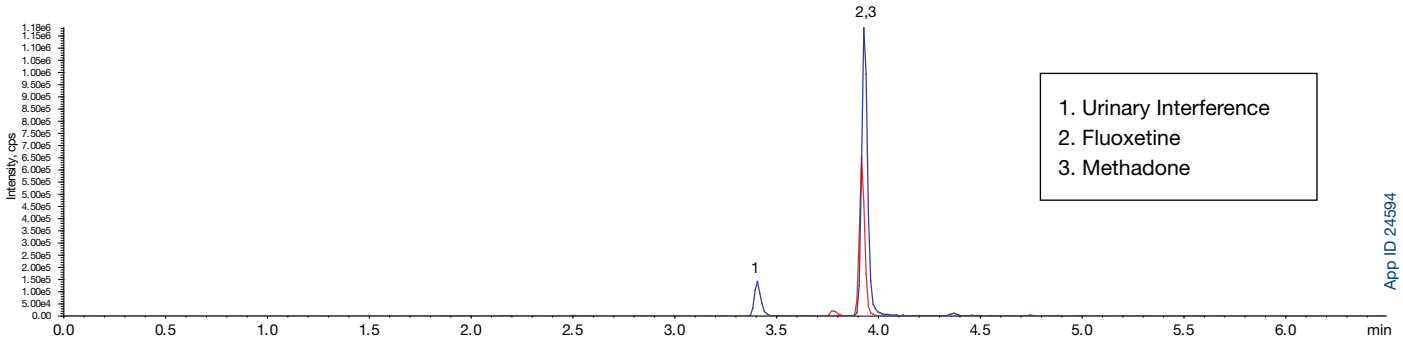
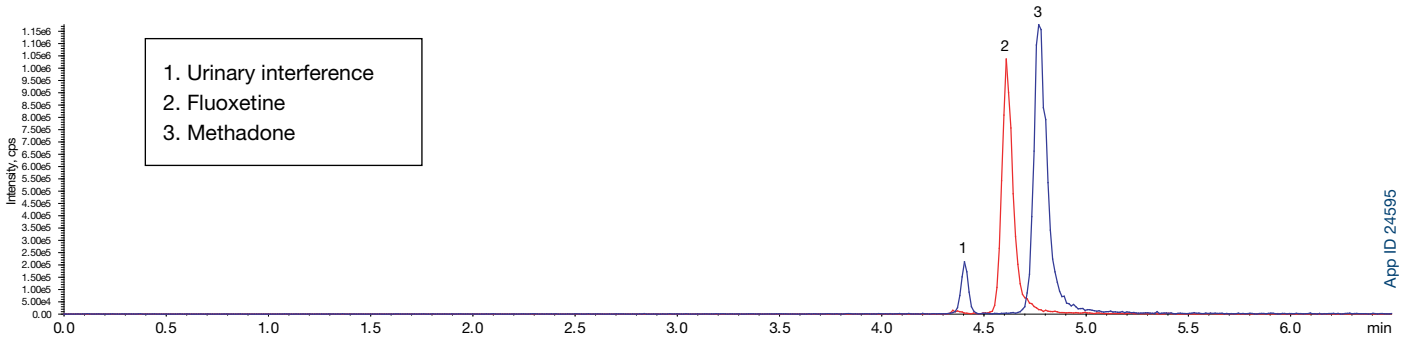


Figure 12.
Extracted chromatogram for fluoxetine and methadone (m/z 310.1 and 310.2) with MP A 5 mM ammonium acetate in water.



APPLICATIONS

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-and-shoot 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.
2. Improved Sensitivity of Hydrolyzed Urine Samples Using β-Gone β-Glucuronidase Removal Products; TN-0101. Matthew Brusius. Phenomenex, Inc

APPLICATIONS

Order Information

β-Gone™ β-Glucuronidase Removal Products

Part No.	Description	Unit
8B-S139-TAK	1 mL Tubes, Recombinant Enzyme	100/Box
8B-S322-DAK	1 mL Tubes, Non-Recombinant Enzyme	100/Box
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

Luna® Omega HPLC Columns

5 µm Analytical 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
AH0-9344	1 mL Tube Adapter Kit
AH0-9345	3 mL Tube Adapter Kit
AH0-9346	6 mL Tube Adapter Kit

WARRANTY Phenomenex warrants that for a period of 12 months following delivery, the Presston 100 Positive Pressure Manifold you have purchased will perform in accordance with the published specifications and will be free from defects in materials or workmanship. In the event that the Presston 100 Positive Pressure Manifold does not meet this warranty, Phenomenex will repair or replace defective parts. Please visit www.phenomenex.com/Presston for complete warranty information.

guarantee

If Phenomenex products in this technical note do not provide at least an equivalent separation as compared to other products of the same phase and dimensions, return the product with comparative data within 45 days for a FULL REFUND.

APPLICATIONS

Australia
t: +61 (0)2-9428-6444
f: +61 (0)2-9428-6445
auinfo@phenomenex.com

Austria
t: +43 (0)1-319-1301
f: +43 (0)1-319-1300
anfrage@phenomenex.com

Belgium
t: +32 (0)2 503 4015 (French)
t: +32 (0)2 511 8666 (Dutch)
f: +31 (0)30-2383749
beinfo@phenomenex.com

Canada
t: +1 (800) 543-3681
f: +1 (310) 328-7768
info@phenomenex.com

China
t: +86 400-606-8099
f: +86 (0)22 2532-1033
phen@agela.com

Denmark
t: +45 4824 8048
f: +45 4810 6265
nordicinfo@phenomenex.com

Finland
t: +358 (0)9 4789 0063
f: +45 4810 6265
nordicinfo@phenomenex.com

France
t: +33 (0)1 30 09 21 10
f: +33 (0)1 30 09 21 11
franceinfo@phenomenex.com

Germany
t: +49 (0)6021-58830-0
f: +49 (0)6021-58830-11
anfrage@phenomenex.com

India
t: +91 (0)40-3012 2400
f: +91 (0)40-3012 2411
indiainfo@phenomenex.com

Ireland
t: +353 (0)1 247 5405
f: +44 1625-501796
eireinfo@phenomenex.com

Italy
t: +39 051 6327511
f: +39 051 6327555
italiainfo@phenomenex.com

Luxembourg
t: +31 (0)30-2418700
f: +31 (0)30-2383749
nlinfo@phenomenex.com

Mexico
t: 01-800-844-5226
f: 001-310-328-7768
tecnicomx@phenomenex.com

The Netherlands
t: +31 (0)30-2418700
f: +31 (0)30-2383749
nlinfo@phenomenex.com

New Zealand
t: +64 (0)9-4780951
f: +64 (0)9-4780952
nzinfo@phenomenex.com

Norway
t: +47 810 02 005
f: +45 4810 6265
nordicinfo@phenomenex.com

Puerto Rico
t: +1 (800) 541-HPLC
f: +1 (310) 328-7768
info@phenomenex.com

Spain
t: +34 91-413-8613
f: +34 91-413-2290
espinfo@phenomenex.com

Sweden
t: +46 (0)8 611 6950
f: +45 4810 6265
nordicinfo@phenomenex.com

Switzerland
t: +41 61 692 20 20
f: +41 61 692 20 22
swissinfo@phenomenex.com

United Kingdom
t: +44 (0)1625-501367
f: +44 (0)1625-501796
ukinfo@phenomenex.com

USA
t: +1 (310) 212-0555
f: +1 (310) 328-7768
info@phenomenex.com

All other countries
Corporate Office USA
t: +1 (310) 212-0555
f: +1 (310) 328-7768
info@phenomenex.com

www.phenomenex.com

Phenomenex products are available worldwide. For the distributor in your country, contact Phenomenex USA, International Department at international@phenomenex.com

TN56120917_W