



TN-0157

Quantitative Analysis of Illicit Drugs of Abuse in Whole Blood by LC-MS/MS

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Introduction

The escalation of many forms of illicit drugs of abuse in forensic toxicology casework necessitates development of a comprehensive analytical method that requires minimum sample preparation. Whole blood has been the biological specimen of choice for many forensic toxicologists, especially in those cases where drug use is presumed with the cause of death. Due to many constraints like budget and resource limitation, there is a high demand for a cost effective, efficient method of analysis for forensic toxicology laboratories.

In this technical note, we demonstrate a fast and effective sample preparation method that targets a broad range of pain management and illicit drugs in whole blood, utilizing Phree™ Phospholipid Removal (PLR) products. Phree allows for a simplified sample cleanup that is efficient and reliable for analysis of thirty-nine compounds, comprising Opiates (both natural and synthetic), Amphetamines, Benzodiazepines, and other illicit drugs (Fentanyl analogs, PCP, and Cocaine metabolites). This effective cleanup technique lends itself useful for detection by LC-MS/MS.

Sample Preparation

Pre-treatment: Aliquot 200 µL of whole blood and add 50 µL of 5 % Zinc Sulfate (w/v) solution spiked with internal standard. Mix the tube for 5-10 sec. Add 600 µL chilled (0 to -20 °C) Acetonitrile / Methanol (95:5, v/v) and mix vigorously for 5-10 sec. Centrifuge the tubes at 3000 rpm for 10 min.

Load: Decant supernatant into a Phree PLR 96-well plate (Part No.: [8E-S133-TGB](#)) followed by the addition of 25 µL 1 % Formic Acid. Mix for 10-15 sec.

Apply: Vacuum at 4-5 psi to collect supernatant.

Evaporate: Sample under a gentle stream of Nitrogen at 40-45 °C.

Reconstitute: Dried samples in 200 µL initial mobile phase.

Note: For phospholipid analysis, make direct injection (bypass evaporation and reconstitution) of eluted samples.

LC Conditions – Quantitative Analysis of Drugs of Abuse Analytes

Column: Kinetex 2.6 µm Biphenyl
Dimension: 50 x 3.0 mm
Part No.: [00B-4622-YO](#)
Mobile Phase: A: 0.1 % Formic Acid in Water
B: 0.1 % Formic Acid in Methanol
Gradient:

Time (min)	%B
0	15
3.5	95
5	95
5.01	15
7	15

Flow Rate: 0.5 mL/min
Injection Volume: 5 µL
Temperature: Ambient
LC System: Agilent 1260 Infinity
Detection: MS/MS
Detector: SCIEX 4500 Triple Quad

LC Conditions – Qualitative Analysis of Phospholipids

Column: Kinetex™ 2.6 µm C18
Dimension: 50 x 2.1 mm
Part No.: [00B-4462-AN](#)
Mobile Phase: A: 0.1 % Formic acid in water
B: 0.1 % Formic acid in Methanol
Gradient:

Time (min)	%B
0	40
0.5	95
11.5	95
11.51	40
13.5	40

Flow Rate: 0.4 mL/min
Injection Volume: 5 µL
Temperature: 40 °C
LC System: Agilent® 1260 Infinity
Detection: MS/MS
Detector: SCIEX® 4500 Triple Quad™

MS/MS Conditions

Ion Source: ESI
Polarity: Positive
Source Temperature: 550 °C
GS1: 40
GS2: 60
CUR: 20
CAD: 6
IS: 4500 V

Table 1. Drugs of Abuse Panel.

Class	Analyte	Class	Analyte
Benzodiazepines	Alprazolam	Synthetic Opioids	Metadone
	Clonazepam		EDDP
	Diazepam		Fentanyl
	Flunitrazepam		Norfentanyl
	Flurazepam		Meperidine
	Lorazepam		Normeperidine
	Midazolam		Naloxone
	Nordiazepam		Norpropoxyphene
	Oxazepam		Propoxyphene
	Temazepam		Sufentanil
Opiates	Codeine	Amphetamines	Amphetamine
	Hydrocodone		Methamphetamine
	Hydromorphone		MDMA
	Morphine		MDA
	6-Monoacetylmorphine (6-MAM)		MDEA
	Oxycodone		Tramadol
Illicit Drugs	Phencyclidine	Analgesics	Carisoprodol
	Benzoylcegonine		Buprenorphine
			Norbuprenorphine



Table 2. MRM Transitions for 39 Drugs of Abuse Analytes.

Analyte	Retention Time (min)	Q1 (m/z)	Q3 (m/z)	Analyte	Retention Time (min)	Q1 (m/z)	Q3 (m/z)
Alprazolam	4.8	309.1	281.1	Flurazepam	4	388.2	315.2
Amphetamine	2.3	136.1	91.1	Hydrocodone	2.8	300.2	199
Benzoylcegonine	3.3	290.1	168.1	Hydromorphone	2.1	286.1	185.1
Codeine	2.6	300.2	152.1	Lorazepam	4.3	321.0	275
Diazepam	4.9	285.0	193.2	MDA	2.7	180.1	133
MDMA	2.9	194.1	105.1	MDEA	3	208.2	163
Methamphetamine	2.6	150.1	91	Meperidine	3.4	248.2	220.2
Norbuprenorphine	3.6	414.3	83.2	Methadone	4.4	310.0	265
Oxazepam	4.4	287.0	241	Midazolam	4.1	326.1	291.1
Oxymorphone	2	302.1	227	Morphine	1.9	286.1	152.1
PCP	4	244.3	91	Naloxone	2.56	328.2	212
Propoxyphene	4	340.3	266.3	Naltrexone	2.8	342.2	267.1
Sufentanil	4.1	387.2	238.1	Nordiazepam	4.64	271.0	140
6MAM	2.57	328.1	165.1	Norfentanyl	3.2	233.2	84.1
Buprenorphine	3.9	468.3	55.2	Normeperidine	3.4	234.1	160.1
Carisoprodol	3.9	261.1	176.2	Norpropoxyphene	4.1	308.2	100.1
Clonazepam	4.4	316.1	270.1	Oxycodone	2.8	316.1	241.2
EDDP	4.2	278.2	234.2	Temazepam	4.7	301.1	255.1
Fentanyl	3.9	337.3	105.1	Tramadol	3.2	264.1	58.1
Flunitrazepam	4.7	314.1	268.2				

Results and Discussion

The Kinetex™ 2.6 µm Biphenyl column provides fast chromatographic separation of the comprehensive drug panel, resolving 39 analytes in 7 minutes including 2 minutes of re-equilibration time (**Table 1, Figure 1**). The Phree™ PLR employs a quick two step extraction method requiring a protein precipitation, followed by a simple pass through that selectively captures the phospholipids while eluting the analytes of interest. The prescribed Phree method results in a more rapid and accurate analysis of the large drug panel in whole blood by cleaning up >95 % of the phospholipids compared to protein precipitation when compared in parallel (**Figure 2**).

The tested dynamic range of the assay covers 2.5 % cutoff to 3X cutoff ranging from 0.075 ng/mL to 1500 ng/mL concentration level, depending on the unique cutoff requirement values of the analytes of interest per industry standard. The linear regression value (r) ranging ≥ 0.995 for all analytes while a quadratic fit, with a 1/x weighting factor, was applied (**Figure 3**). The absolute recovery yield for the analytes, spiked in whole blood samples at 20 % cutoff for concentration, extracted by Phree PLR ranged from 76 % to 114 % with a %CV value ≤ 15 % (**Table 3**).

Figure 1. Representative Chromatogram of Illicit Drug Panel Analytes Extracted from Whole Blood Utilizing Phree PLR and Analyzed with a Kinetex 2.6 µm Biphenyl Column.

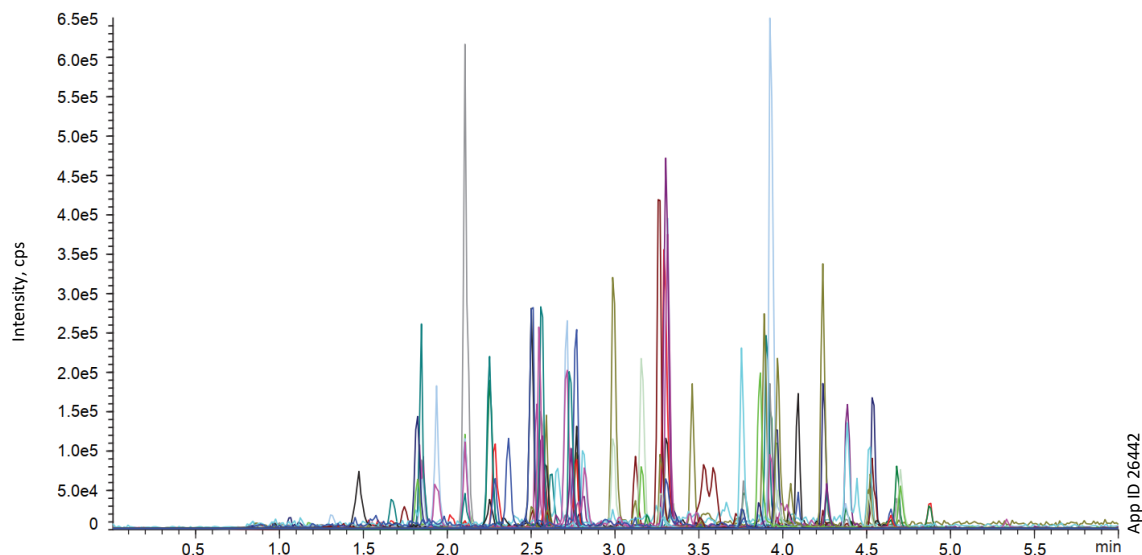


Figure 2. Representative Chromatogram of Phospholipid Analysis in Whole Blood Comparing Protein Precipitation (PPT) and Phree™ PLR and Analyzed with a Kinetex™ 2.6 μm C18 Column.

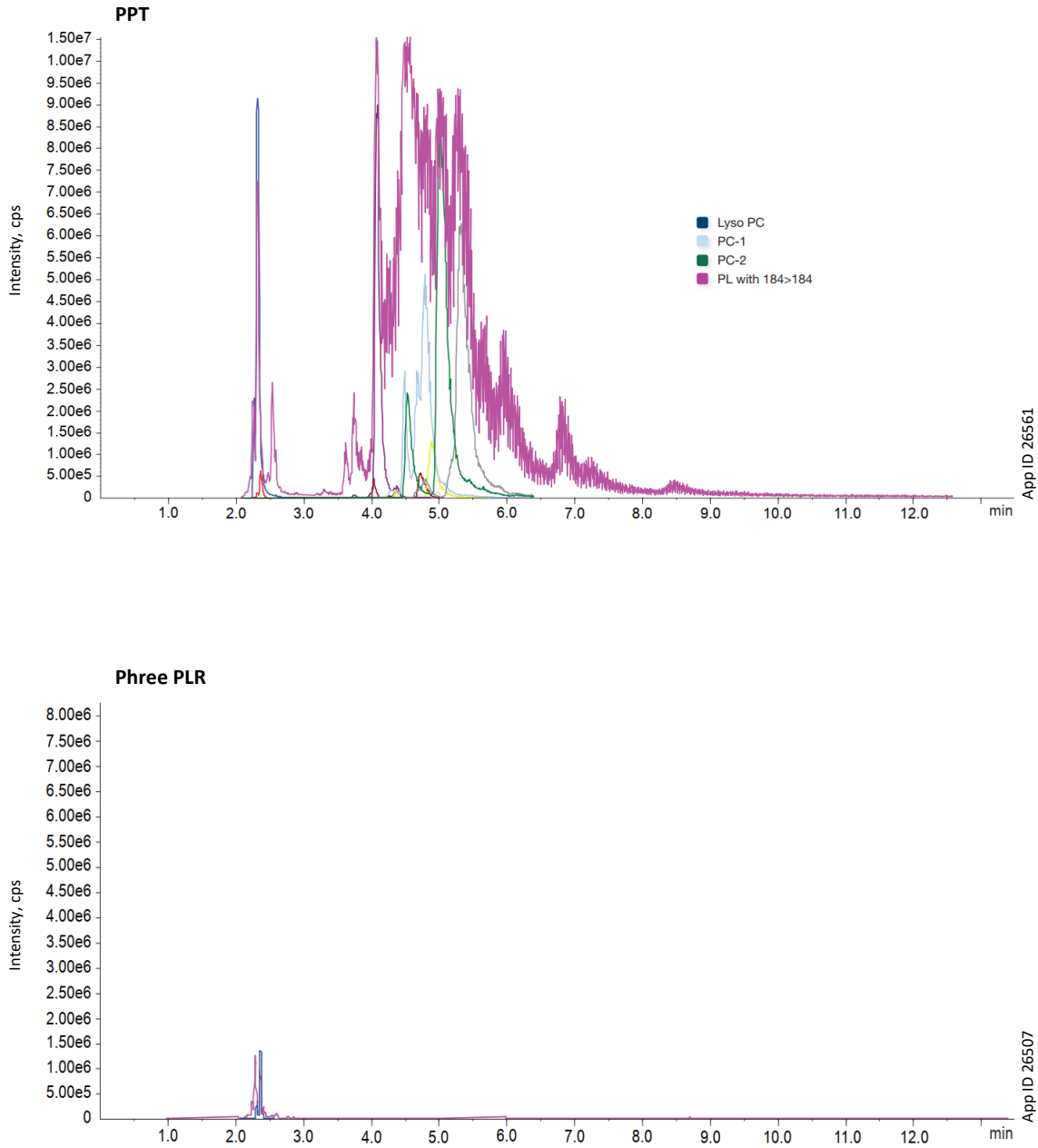


Figure 3. Calibration Curves for Selected Analytes in Whole Blood Sample Extracted Using a Phree™ PLR 96-well Plate.

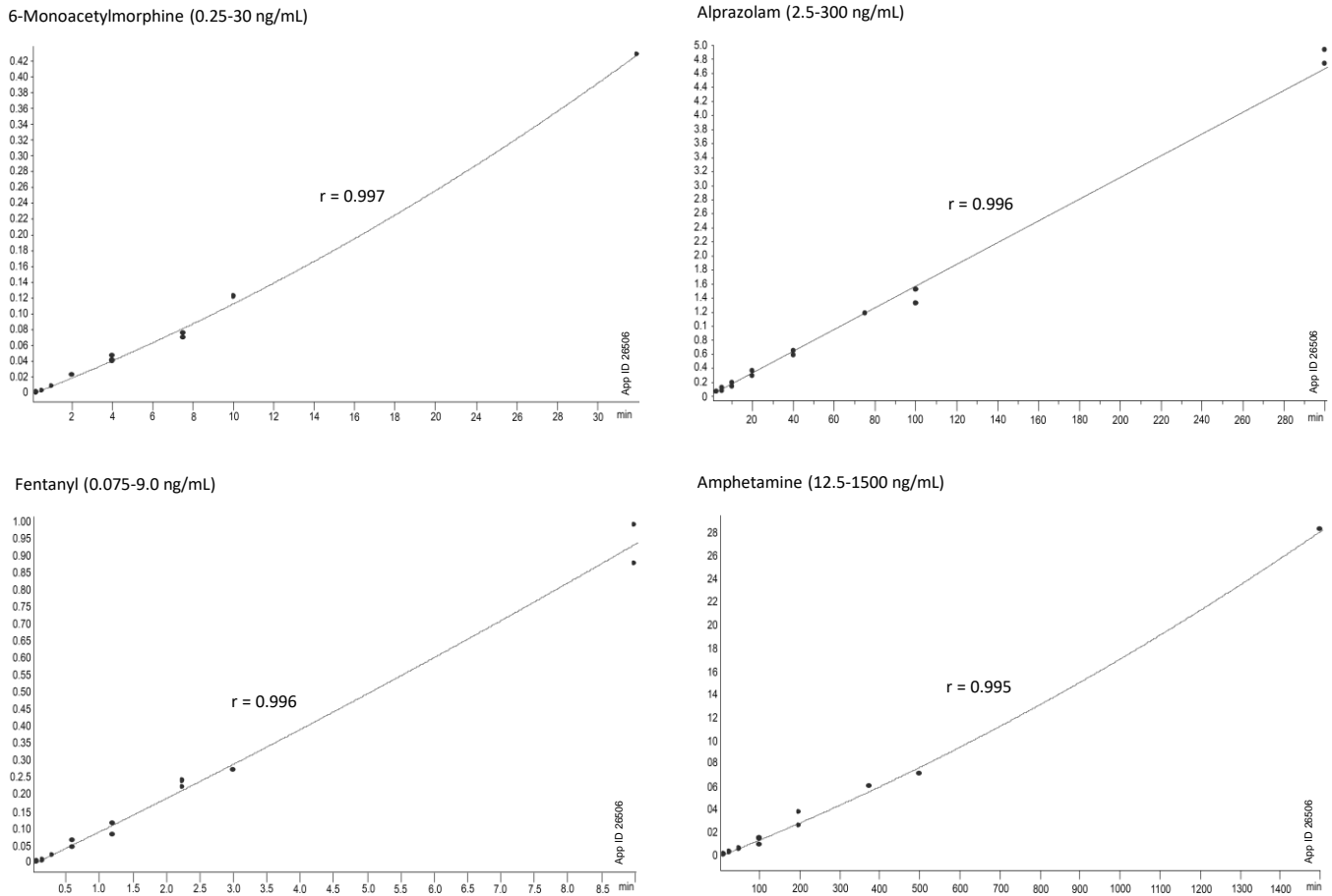


Table 3. Recovery Comparison Between Phree PLR and PPT.

Analyte	Phree PLR		PPT		Analyte	Phree PLR		PPT	
	% Recovery	%CV (N=4)	% Recovery	%CV (N=4)		% Recovery	%CV (N=4)	% Recovery	%CV (N=4)
Alprazolam	108	5.5	93	4.3	Flurazepam	109	3.9	112	13.3
Amphetamine	107	4.9	88	9.6	Hydrocodone	104	11.3	85	8.6
Benzoylcegonine	102	3.1	87	3.9	Hydromorphone	103	8.7	78	7.2
Codeine	76	13.9	84	7.5	Lorazepam	86	15.3	100	4.1
Diazepam	98	5.7	85	9.9	MDA	90	4.4	78	7
MDMA	104	13.7	75	0.9	MDEA	106	13.8	81	9.7
Methamphetamine	103	2.8	78	13.5	Meperidine	108	2.5	98	4.9
Norbuprenorphine	85	12.4	95	10.3	Methadone	105	3.1	99	14.9
Oxazepam	106	7.8	93	4.1	Midazolam	108	5.5	107	11.1
Oxymorphone	81	8.9	72	11.7	Morphine	88	5.2	92	15.7
PCP	111	3.3	80	7.4	Naloxone	88	8.2	76	15.1
Propoxyphene	89	12	93	12.5	Naltrexone	84	15	94	14.1
Sufentanil	114	3.1	92	14.3	Nordiazepam	105	1.6	98	5.3
6-MAM	83	3.3	71	2.9	Norfentanyl	97	4.3	99	6.5
Buprenorphine	113	10.5	93	12.5	Normeperidine	106	4.9	93	8.5
Carisoprodol	86	5.3	103	11.9	Norpropoxyphene	98	11.7	99	15
Clonazepam	99	5.7	94	2	Oxycodone	86	8.2	95	2.4
EDDP	82	11.6	84	4.1	Temazepam	110	3.1	90	4.6
Fentanyl	112	1.6	103	9.3	Tramadol	104	9	87	10.2
Flunitrazepam	83	9.1	86	1.9					

Conclusion

The prescribed sample prep method utilizing a Phree™ PLR extraction resulted in a rapid quantitation of a wide range of analytes while meeting the demand of a high throughput environment.

Phree Ordering Information

Phree Phospholipid Removal Products

Part No.	Description	Unit
8B-S133-TAK	Phree Phospholipid Removal Tabbed 1 mL Tubes	100/pk
8E-S133-TGB	Phree Phospholipid Removal 96-Well Plates	2/pk

Kinetex™ Ordering Information

Phases	2.6 µm Midbore™ Columns (mm)			SecurityGuard™ ULTRA Cartridges (mm)‡		
	30 x 3.0	50 x 3.0	75 x 3.0	100 x 3.0	150 x 3.0	3/pk
EVO C18	00A-4725-Y0	00B-4725-Y0	—	00D-4725-Y0	00F-4725-Y0	AJ0-9297
PS C18	00A-4780-Y0	00B-4780-Y0	—	00D-4780-Y0	00F-4780-Y0	AJ0-8950
Polar C18	—	00B-4759-Y0	—	00D-4759-Y0	00F-4759-Y0	AJ0-9531
Biphenyl	—	00B-4622-Y0	—	00D-4622-Y0	00F-4622-Y0	AJ0-9208
XB-C18	00A-4496-Y0	00B-4496-Y0	00C-4496-Y0	00D-4496-Y0	00F-4496-Y0	AJ0-8775
C18	00A-4462-Y0	00B-4462-Y0	00C-4462-Y0	00D-4462-Y0	00F-4462-Y0	AJ0-8775
C8	00A-4497-Y0	00B-4497-Y0	00C-4497-Y0	00D-4497-Y0	00F-4497-Y0	AJ0-8777
HILIC	00A-4461-Y0	—	—	00D-4461-Y0	00F-4461-Y0	AJ0-8779
Phenyl-Hexyl	—	00B-4495-Y0	—	00D-4495-Y0	00F-4495-Y0	AJ0-8781
F5	—	00B-4723-Y0	—	00D-4723-Y0	00F-4723-Y0	AJ0-9321

for 3.0 mm ID

Phases	2.6 µm Minibore Columns (mm)			SecurityGuard ULTRA Cartridges (mm)‡		
	30 x 2.1	50 x 2.1	75 x 2.1	100 x 2.1	150 x 2.1	3/pk
EVO C18	00A-4725-AN	00B-4725-AN	—	00D-4725-AN	00F-4725-AN	AJ0-9298
PS C18	00A-4780-AN	00B-4780-AN	—	00D-4780-AN	00F-4780-AN	AJ0-8951
Polar C18	00A-4759-AN	00B-4759-AN	—	00D-4759-AN	00F-4759-AN	AJ0-9532
Biphenyl	00A-4622-AN	00B-4622-AN	—	00D-4622-AN	00F-4622-AN	AJ0-9209
XB-C18	00A-4496-AN	00B-4496-AN	00C-4496-AN	00D-4496-AN	00F-4496-AN	AJ0-8782
C18	00A-4462-AN	00B-4462-AN	00C-4462-AN	00D-4462-AN	00F-4462-AN	AJ0-8782
C8	00A-4497-AN	00B-4497-AN	00C-4497-AN	00D-4497-AN	00F-4497-AN	AJ0-8784
HILIC	00A-4461-AN	00B-4461-AN	00C-4461-AN	00D-4461-AN	00F-4461-AN	AJ0-8786
Phenyl-Hexyl	00A-4495-AN	00B-4495-AN	00C-4495-AN	00D-4495-AN	00F-4495-AN	AJ0-8788
F5	00A-4723-AN	00B-4723-AN	—	00D-4723-AN	00F-4723-AN	AJ0-9322

for 2.1 mm ID

‡ SecurityGuard ULTRA Cartridges require holder, Part No.: [AJ0-9000](#)

Have questions or want more details on implementing this method? We would love to help!
Visit www.phenomenex.com/Chat to get in touch with one of our Technical Specialists



Need a different column size or sample preparation format?

No problem! We have a majority of our available dimensions up on www.phenomenex.com, but if you can't find what you need right away, our super helpful Technical Specialists can guide you to the solution via our online chat portal www.phenomenex.com/Chat.

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