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A Comprehensive Screening of Illicit and Pain Management Drugs from Whole Blood Using SPE and LC/MS/MS

Introduction

Drug analysis from whole blood is gaining popularity due to a more complete measurement of analytes in the biological system. Historically, most drug screens have been performed using immunoassay and other similar technologies. Positive samples are then processed accordingly and analyzed using LC/MS/MS or GC/MS/MS for confirmation and further quantification. Immunoassay methods often lack the specificity and sensitivity necessary for today's complex legal requirements. As a result, LC/MS/MS is a more proficient screening method when an effective pretreatment and sample cleanup method is utilized.

The pretreatment of whole blood samples is an important step in efficiently extracting analytes of interest and maintaining a simplified matrix to analyze. This involves hemolysis to release drugs of interest taken up by the cells as well as precipitation of proteins. However, determining a single pretreatment option for all classes of drugs can be difficult due to varying chemical properties of the target analytes.

Here we demonstrate a fast, effective technique for the targeted analysis of a broad range of drugs from whole blood utilizing a core-shell HPLC/UHPLC column, SPE, and tandem mass spectrometry. We attempted analysis of thirty nine compounds, comprising opiates (both natural and synthetic), amphetamines, benzodiazepines, phenylpiperidines, a muscle relaxant, and some illicit drugs (PCP and cocaine metabolite). Utilizing advanced sample preparation techniques we simplify a complicated matrix to allow for a fast and successful multi-component analysis by LC/MS/MS.

Table 1. List of Pain Panel Drugs

Class	Analyte	Class	Analyte
Benzodiazepines	Alprazolam	Synthetic Opioids	Methadone
	Clonazepam		EDDP
	Diazepam		Fentanyl
	Flunitrazepam		Norfentanyl
	Lorazepam		Meperidine
	Midazolam		Normeperidine
	Nordiazepam		Naloxone
	Oxazepam		Norpropoxyphene
	Temazepam		Propoxyphene
	α-Hydroxyalprazolam		Sufentanil
	Alprazolam		Naltrexone
Opiates	Codeine	Amphetamines	Amphetamine
	Hydrocodone		Methamphetamine
	Hydromorphone		MDMA
	Morphine		MDA
	6-Acetylmorphine (6-MAM)		MDA
	Oxymorphone	Analgesics	Tramadol
Illicit Drugs	Phencyclidine		Carisoprodol
	Benzoylecgonine		Buprenorphine
			Norbuprenorphine

Experimental Conditions

Table 2. List of Pretreatment Conditions Tested (Not Ranked)

Option #	Pretreatment Conditions
1	10% Trichloroacetic Acid
2	6% Perchloric Acid
3	Zinc Sulfate + Acetonitrile
4	90:10 Acetonitrile:Methanol
5	50:50 Acetonitrile:Methanol
6	10:90 Acetonitrile:Methanol
7	Zinc sulfate + 90:10 Acetonitrile:Methanol (Most successful. Used for method.)

SPE Method

Cartridge:	Strata™-X-C 30 mg/3 mL
Part No.	8B-S029-TBJ
Pretreatment:	Add 100 µL 5 % $ZnSO_4.7H_2O$ and lightly vortex. Add 1.5 mL of 90:10 Acetonitrile:Methanol while vortexing. Centrifuge samples at 6000 rpm for 10 min. Add 4 mL of water to the samples to dilute the amount of organic prior to SPE
Condition:	1 mL Methanol
Equilibrate:	1 mL Water
Wash 1:	1 mL 0.1 % Formic Acid
Wash 2:	1 mL 30 % Methanol
Dry:	3 to 4 min at high vacuum (~10" of Hg)
Elute:	2 x 500 µL (2 aliquots of 500 µL) Ethyl acetate:Isopropanol:Ammonium Hydroxide (70:20:10)
Blow Down:	To dryness under Nitrogen at 40-45 °C
Reconstitute:	With 500 µL of 85:15 (A:B) of LC mobile phase

LC Conditions

Column:	Kinetex® 2.6 µm Biphenyl
Dimensions:	50 x 3 mm
Part No.:	00B-4622-Y0
Mobile Phase:	0.1% Formic Acid in Water / 0.1% Formic Acid in Methanol
Gradient:	

Time	A	B
0.0	90	10
2.5	0	100
3.5	0	100
3.51	90	10
5	90	10

Flow Rate:	0.7 mL/min
Injection Volume	10 µL
Temperature:	Ambient

MS/MS Parameters

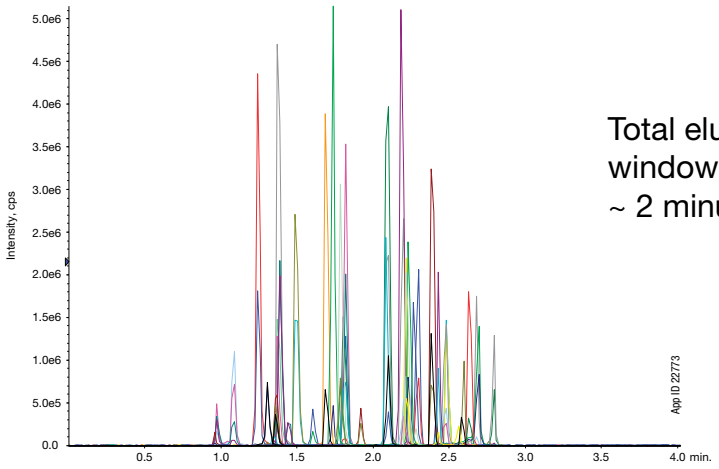
Detection: AB Sciex 4000 QTRAP®, positive polarity

ESI CAD Gas = High; GS1=65, GS2 = 50; curtain gas = 10

ESI spray voltage = 4000 V; Temp = 550°C

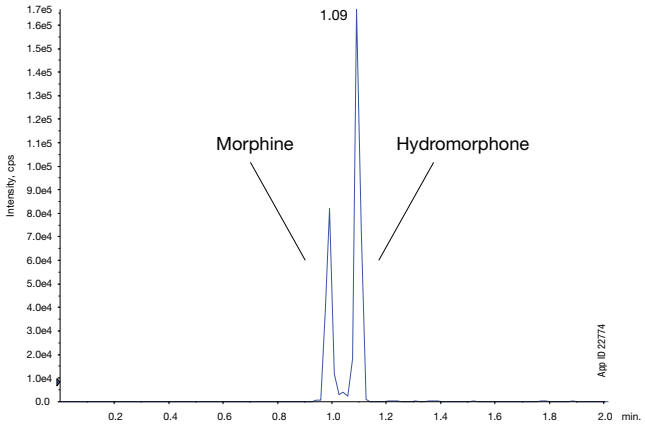
Results

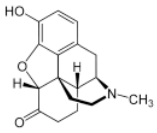
Figure 1. Representative Chromatogram of Drug Panel



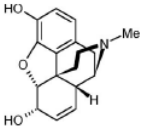
Total elution window of ~ 2 minutes

Figure 2. Selectivity of Isobaric Pair Morphine and Hydromorphone



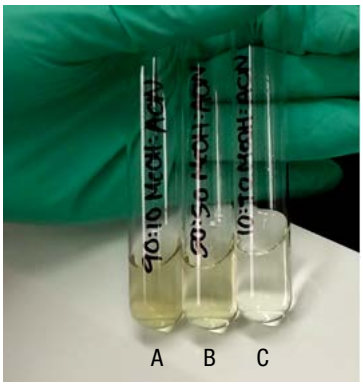


Hydromorphone
$C_{17}H_{19}NO_3$
MW: 285.34



Morphine
$C_{17}H_{19}NO_3$
MW: 285.34

Figure 3. Comparison of Select Pretreatment Extracts Prior to SPE



A higher percent of acetonitrile results in a clearer extract

A= 10:90 Acetonitrile:Methanol
B= 50:50 Acetonitrile:Methanol
C= 90:10 Acetonitrile:Methanol

Figure 4. Comparison of the effects of various pretreatment options on amphetamine

Chromatograms are overlaid with time shift to provide clarity.

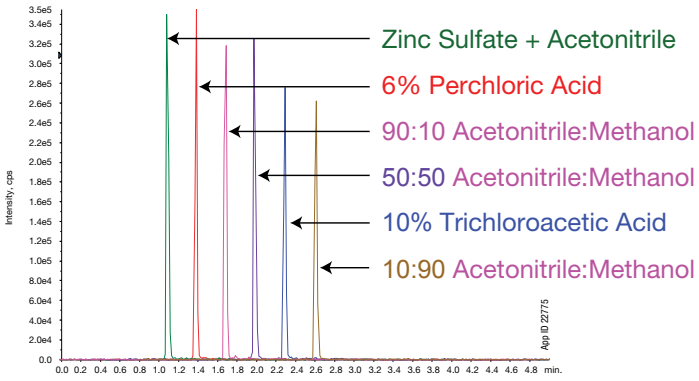
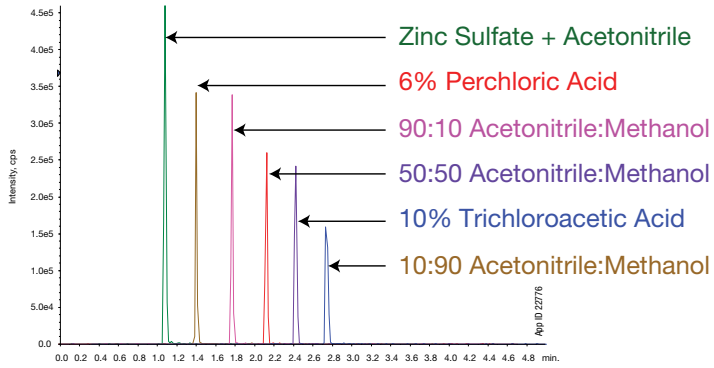


Figure 5. Comparison of the effects of various pretreatment options on benzoylecgonine

Chromatograms are overlaid with time shift to provide clarity.



Summary of Sample Pretreatment Procedure

Different classes of compounds responded better with different pretreatment options

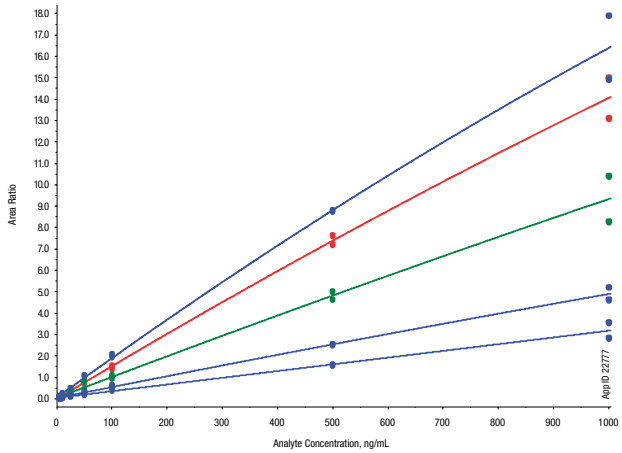
- Zinc Sulfate + Acetonitrile combination was most effective at extracting the benzodiazepines, synthetic opioids, amphetamines, analgesics, and other illicit drugs
- 90:10 Acetonitrile:Methanol produced the best recoveries for the natural opiates

Based on these findings, a pretreatment combination of Zinc Sulfate and 90:10 Acetonitrile:Methanol was determined to be the best option to extract the majority of analytes of interest

Table 3. Calibration Curve Parameters

Class	Analytes	Corr. Coeff.	Class	Analytes	Corr. Coeff.
Benzodiazepines	Alprazolam	0.9968	Opiates	Codeine	0.9919
	Clonazepam	0.9967		Oxycodone	0.9932
	Diazepam	0.9960		Hydromorphone	0.9942
	Flunitrazepam	0.9959		Morphine	0.9949
	Lorazepam	0.9948		6-Acetylmorphine	0.9948
	Midazolam	0.9974		Hydrocodone	0.9979
	Nordiazepam	0.9968	Synthetic Opioids	Methadone	0.9911
	Oxazepam	0.9966		EDDP	0.9960
	Temazepam	0.9957		Fentanyl	0.9934
	α-Hydroxyalprazolam	0.9953		Norfentanyl	0.9935
Amphetamines	Amphetamine	0.9981		Meperidine	0.9906
	Methamphetamine	0.9912		Normeperidine	0.9954
	MDMA	0.9912		Naloxone	0.9923
	MDA	0.9963		Norpropoxyphene	0.9944
	MDEA	0.9923		Propoxyphene	0.9933
Analgesics	Tramadol	0.9930		Sufentanil	0.9972
	Carisoprodol	0.9931		Naltrexone	0.9987
	Buprenorphine	0.9910	Others	Phencyclidine	0.9906
	Norbuprenorphine	0.9943		Benzoylecgonine	0.9902

Figure 6. Calibration Curve for Various Analytes Over 200 Fold Concentration (5-1000 ng/mL)



Top to Bottom:

Alprazolam R^2= 0.9968

Hydrocodone R^2 = 0.9979

Amphetamine R^2 = 0.9981

Naloxone R^2 = 0.9923

Buprenorphine R^2 = 0.9910

Curve Fitting = Quadratic

Regression= 1/x

Table 4. Method Precision and Accuracy Data Based on Replicate Quality Control Samples

Analyte	Class	Expected Conc, ng/mL (Low)	%RSD (Low)	% Accuracy (Low)	Expected Conc, ng/mL (High)	%RSD (High)	% Accuracy (High)
Alprazolam	Benzodiazepines	20	10	108	200	12	104
Clonazepam		20	9	114	200	11	107
Diazepam		20	10	97	200	12	103
Flunitrazepam		20	7	112	200	7	105
Lorazepam		20	15	108	200	10	111
Midazolam		20	7	115	200	4	88
Nordiazepam		20	11	101	200	13	103
Oxazepam		20	6	108	200	12	105
Temazepam		20	7	105	200	9	99
α-Hydroxyalprazolam		20	6	88	200	11	91
Codeine	Opiates	20	10	92	200	9	87
Oxycodone		20	4	95	200	2	93
Hydromorphone		20	6	85	200	14	97
Hydrocodone		20	7	105	200	9	99
Morphine		20	8	91	200	10	86
Methadone	Synthetic Opioids	20	10	110	200	5	105
EDDP		20	10	98	200	2	94
6-MAM		20	7	100	200	7	100
Fentanyl		20	9	115	200	5	90
Norfentanyl		20	12	95	200	4	100
Meperidine		20	7	105	200	7	103
Normeperidine		20	9	103	200	10	102
Naloxone		20	7	118	200	3	111
Norpropoxyphene		20	9	100	200	14	90
Propoxyphene		20	12	111	200	5	101
Sufentanil		20	8	98	200	7	89
Naltrexone		20	4	113	200	11	108
Amphetamine	Amphetamines	20	9	107	200	11	107
Methamphetamine		20	10	115	200	3	96
MDMA		20	13	111	200	8	92
MDA		20	8	102	200	7	101
MDEA		20	16	107	200	3	105
Tramadol	Analgesics	20	4	105	200	3	96
Carisoprodol		20	8	106	200	9	100
Buprenorphine		20	12	104	200	11	101
Norbuprenorphine		20	6	105	200	13	106
Phencyclidine	Others	20	7	110	200	4	92
Benzoylecgonine	Others	20	10	104	200	5	101

Discussion

A combination of zinc sulfate with acetonitrile and methanol worked for the majority of analytes including opiates and synthetic opioids, benzodiazepines, and amphetamines.

The Kinetex 2.6 µm Biphenyl 50 x 3 mm column provided quick separation of all drugs of abuse analytes in a 5 minute cycle time with a total elution window of ~2 minutes (**Figure 1**).

- Excellent resolution and good selectivity of the opiate isobaric species was obtained, such as morphine and hydromorphone (**Figure 2**).
- Fast elution time as a result of a ballistic gradient led to the potential of even greater throughput with the use of multiplex or dual-stream systems.

Strata-X-C is effective for all the classes of pain panel drugs that that are typically monitored in whole blood testing (amphetamines, benzodiazepines, opiates, and other drugs of abuse).

- Successfully removes interfering endogenous compounds such as proteins, which improves sensitivity and also extends column lifetime.
- Allows for sample concentration which greatly increases the sensitivity of assays and allows for older, less sensitive mass spectrometry systems to be effectively used for drugs of abuse testing.

Good linearity ($R^2 \geq 0.990$) was demonstrated for all drugs of abuse compounds over a wide concentration range (**Table 3** and **Figure 6**).

Precision and accuracy were within suitable limits (± 15%) (**Table 4**).

Conclusion

We have developed an effective pretreatment and SPE cleanup method for whole blood followed by targeted LC/MS/MS analysis. Zinc sulfate with an acetonitrile and methanol combination provided the best response for the majority of analytes tested, and the Strata-X-C SPE sorbent chemistry worked well at removing endogenous matrix interferences while retaining a wide range of compounds. The pretreatment/SPE method showed robustness over a dynamic range of concentrations, demonstrating good precision and accuracy. The sample cleanup procedure described here can greatly extend column lifetime and reduce maintenance of the MS system. The use of the Kinetex 2.6 µm Biphenyl provides the necessary selectivity and resolving power to analyze all target drug molecules in a total cycle time of about 5 minutes, including baseline separation of critical isobaric ions.

References

1. S Huq, S Sadjadi, and S Countryman, “Quantitative Bio Analysis of the Most Commonly Used Pain Medications in Urine Using a Reliable Sample Preparation Technique in Combination With an API 5000 LC-MS-MS”, Mass Spec Application for Clinical Laboratory Conference, 2013

2. Dalsgaard et al,Quantitative Analysis of 30 Drugs in Whole Blood by SPE and UHPLC-TOF-MS. (2013) *J of Forensic Science and Criminology* 1(1):101