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Comparison of Different Whole Blood Sample Pretreatment Methods for Targeted Analysis of Basic Drugs

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Introduction

Drug analysis from whole blood is a challenge due to the complex matrix and the presence of erythrocytes, the concentration of which can vary from sample to sample. Any drug analysis in whole blood generally requires some form of a pretreatment procedure that simplifies the blood matrix before the actual analyte extraction. However, many procedures can efficiently be applied to hemolyze the erythrocytes. Likewise, there are equally many capable methods to precipitate the plasma proteins. Ultimately, a successful pretreatment method should produce a high degree of recovery for all analytes in the sample.

Here, we evaluated several common pretreatment procedures[1,2], that both lyse the cells and precipitate the plasma proteins. These include acidic reagents (10 % TCA and 6 % $HClO_4$), organic solvent mixtures (MeOH and ACN) and a combination of zinc sulfate and an organic solvent (**Table 2**). Subsequently, the clarified supernatant was processed through a polymeric cation exchange SPE (Strata™-X-C) to extract the basic drugs. The list of the class of compounds that were targeted for this work includes amphetamines (amphetamine, methamphetamine, MDA, MDEA, etc), natural and synthetic opiates (morphine, codeine, hydromorphone, hydrocodone, etc) illicit drugs (PCP, benzoylecgonine), benzodiazepines (alprazolam, lorazepam, etc) and analgesics (tramadol).

After the initial evaluation, we constructed a calibration curve using whole blood as a matrix. Replicate analysis of 20 and 200 ng/mL spiked whole blood samples were used for the precision and accuracy study.

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)		MDEA
	Oxymorphone	Analgesics	Tramadol
Illicit Drugs	Phencyclidine		Carisoprodol
	Benzoylecgonine		Buprenorphine
			Norbuprenorphine

Table 2. Evaluated Pretreatment Methods

Acidic Reagents	10 % TCA
	6 % $HClO_4$
Organic Solvents	90:10 ACN:MeOH
	50:50 ACN:MeOH
	10:90 ACN:MeOH
	100 % MeOH
	100 % ACN
Organic Solvent + $ZnSO_4$	100 % ACN
	90:10 ACN:MeOH
	100 % MeOH

Final Sample Preparation Method

Pretreatment:

- Add 0.5 mL whole blood (with EDTA preservative) into a glass tube
- Add 100 µL 5 % (w/v) $ZnSO_4$ and vortex 3-5 sec
- Add 1.5 mL of chilled (~0 °C) 90:10 ACN:MeOH while vortexing
- Centrifuge samples at 6000 rpm for 10 min and transfer supernatant
- To supernatant, add 4 mL of aqueous 0.1 % formic acid to acidify and dilute the mixture

SPE Cartridge:

- Strata-X-C, 30 mg/3 mL (Part no. 8B-S029-TBJ) equipped with an adapter cap (Part no. AH0-7191) and a 12 mL reservoir (Part no. AH0-7003)

Condition: 1 mL Methanol
Equilibrate: 1 mL Water
Wash 1: 1 mL 0.1 % Formic acid in water
Wash 2: 1 mL 30 % Methanol in water
Dry: 3 to 4 mins at high vacuum (~10" of Hg)
Elute: 2x 500 µL (2 aliquots of 500 µL) Ethyl acetate: Isopropanol: Ammonium hydroxide (70:20:10)
Dry down: Evaporate to dryness under nitrogen at 40-45 ºC
Reconstitute: With 500 µL of 85:15 (A:B) of LC mobile phase

Final LC/MS Method

Column: Kinetex® 2.6 µm Biphenyl
Dimensions: 50 x 3.0 mm
Part No.: 00B-4622-Y0
Mobile Phase: A: 0.1 % Formic acid in water
B: 0.1 % Formic acid in methanol
Flow Rate: 0.7 mL/min
Gradient:

Time (min)	% B
0.00	10
2.50	100
3.50	100
3.50	10
5.00	10

Temperature: Ambient
Detection: MS/MS, 4000 QTRAP® (AB SCIEX), ESI+
System: Shimadzu® Nexera® UFLC with LC-30AD pumps
Injection: 10 µL

Figure 1. Acidic supernatant

Addition of Acidic Reagent

- Both 10 % trichloroacetic acid and 6 % perchloric acid produced very clear, colorless supernatants, even after dilution.

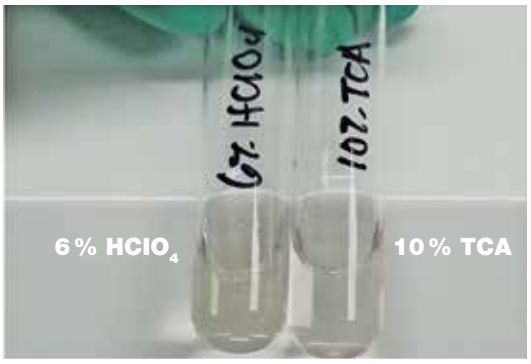
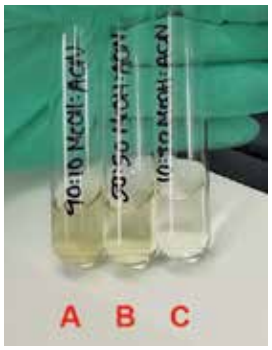


Figure 2. Supernatant from organic solvents

Addition of Organic Solvents

- MeOH and/or mostly methanol solvent produced a supernatant with a slight hazy yellow tint
- Acetonitrile and/or mostly acetonitrile solvent produced a more clear and colorless supernatant. However, the supernatant turned cloudy when diluted.

Supernatant



(A)
90:10 MeOH:ACN

(B)
50:50 MeOH:ACN

(C)
10:90 MeOH:ACN

Supernatant post dilution

Figure 3. Supernatant from $ZnSO_4$ and ACN post dilution

Addition of $ZnSO_4$ and an Organic Solvent

- When added to whole blood, zinc sulfate produced a bright red cloudy solution.
- Upon addition of the organic solvent, a brown precipitate appeared which lead to a clear and colorless supernatant.
- Upon dilution, the solution showed slight turbidity ($ZnSO_4$ and ACN supernatant are shown below).

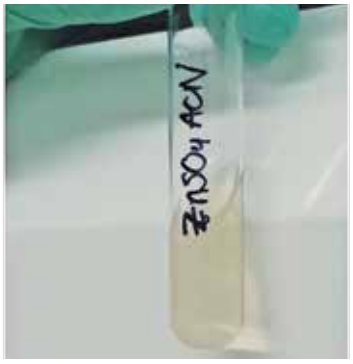


Figure 4. Representative chromatogram of the basic compounds

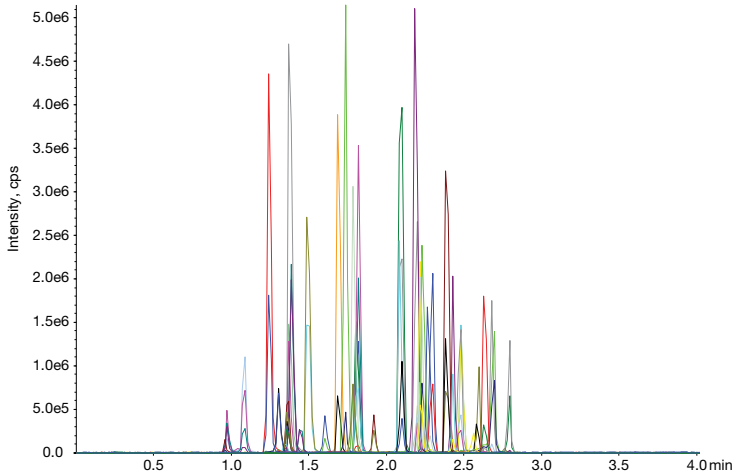


Figure 5. Comparison of the effects of various pretreatment options on amphetamine. Chromatograms are overlaid with time shift to provide clarity.

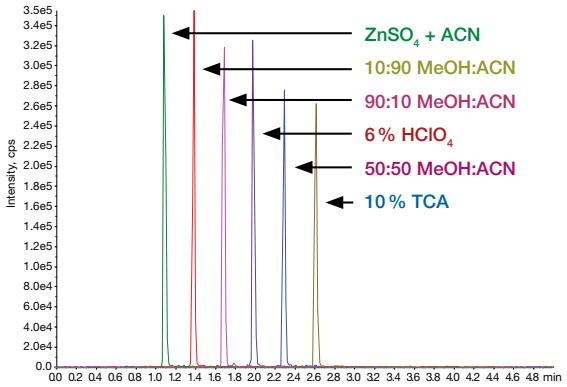


Table 3. Method Precision and Accuracy Data Based on Replicate Quality Control Samples

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	Illicit Drugs	20	7	110	200	4	92
Benzoylecgonine		20	10	104	200	5	101

Figure 6. Comparison of the effects of various pretreatment options on Codeine (peak 1) and Hydrocodone (peak 2). Chromatograms are overlaid with time shift to provide clarity.

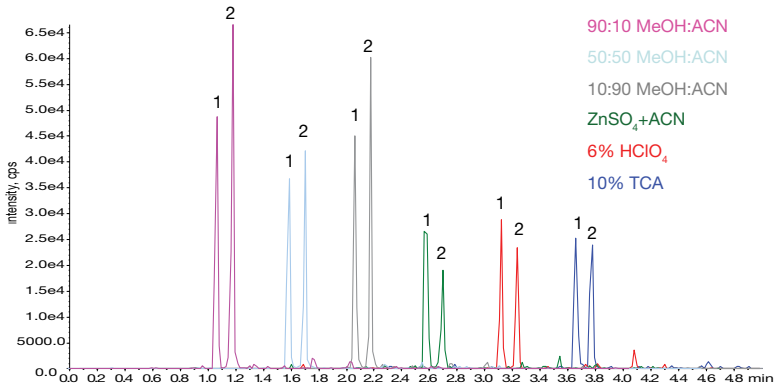
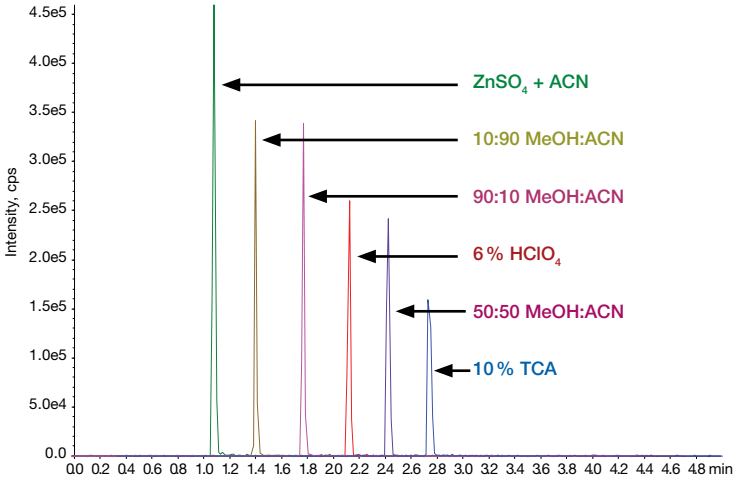


Figure 7. Comparison of the effects of various pretreatment options on Benzoylecgonine. Chromatograms are overlaid with time shift to provide clarity.



Discussion

It appeared that not all pretreatment procedures produced the best results. In general, the acidic pretreatment methods produced the poorest overall responses despite a very clear pretreated sample. (**Figure 1** and **Figure 5**, with some exceptions). This might be due to the hydrophobic nature of many of the compounds used here that are soluble (or stable) in the pretreated acidic solutions (**Figure 6 and 7**).

Use of MeOH alone was not adequate to achieve a clear enough supernatant from whole blood. Acetonitrile with a small amount of MeOH produced better than expected recoveries for some classes of compounds such as opiates (**Figures 2** and **6**).

The pretreatment procedure with $ZnSO_4$ and an organic solvent (acetonitrile or 90:10 ACN:MeOH) produced the most consistent results for many compounds (**Table 3**).

Discussion

We have developed an effective pretreatment and SPE clean-up 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. Further sample cleanup was successfully accomplished by using a cationic exchange SPE, Strata-X-C, sorbent. This combination can greatly improve the column longevity and maintain a clean LC/MS/MS system. The combination of the pretreatment and SPE method can sufficiently be employed for a wide range of basic compounds generally encountered in existing pain panel methods.

Acknowledgement

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