APPLICATION

A Unified Sample Preparation Procedure for General Unknown Screening (GUS) of Compounds in Whole Blood Samples

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Introduction

The identification of drugs from whole blood by high resolution mass spectrometer serves as an effective screening tool in both clinical and forensic labs. General Unknown Screening (GUS) is particularly important in cases where the affected individual is incapacitated, unaware of what was taken (or ingested), or deceased. In addition, whole blood presents a challenging matrix that often requires an elaborate sample preparation procedure.

Several parameters were considered for a sample preparation protocol. The sample cleanup should not favor one class of compounds at the expense of other classes and should generally yield a high degree of recovery for all analytes. The sample preparation method should also produce an adequately clean sample to present to MS for detection. Lastly, the final extracted sample should present a good short-term stability to prevent premature compound loss while these samples are waiting to be analyzed.

The objectives of these efforts were:

- Develop a universal sample preparation procedure for whole blood samples to: lyse the erythrocytes, precipitate the majority of plasma proteins, and remove high levels of phopholipids present in the sample
- Neutralize the undesirable effect of strong solvent

Experimental Conditions

Sample Preparation

1. Aliquot 200 µL EDTA whole blood into individually labeled glass tubes.
2. Add 50 µL 7 % Zinc acetate (w/v) (or 5 % Zinc sulfate (w/v)) to the whole blood and vortex the tubes for 3-5 sec.
3. Add 650 µL chilled (0 to -20 °C) 95:5 Acetonitrile/Methanol.
4. Vortex vigorously for 10-15 sec.
5. Centrifuge the tubes at 3000 rpm for 7-10 min.
6. Carefully remove the supernatant and combine with 25 µL 1 % Formic acid (v/v).
7. Transfer the acidified supernatant into Phree™ Phospholipid Removal 1 mL tubes.
8. Apply a vacuum (1-2 in Hg) or positive pressure (3-4 psi) to collect the final extract.
9. Transfer the final extract to an autosampler vial and proceed to analysis. No dry down is needed!

HPLC Conditions

Column: Kinetex® 2.6 µm Biphenyl
Dimensions: 100 x 3.0 mm
Part No.: 00D-4622-Y0
Mobile Phase:: A: 5 mM Ammonium Formate with 0.05 % Formic Acid
B: 0.05 % Formic Acid in Acetonitrile
Temperature: 50 °C
Static Mixer: 25 µL (Analytical Scientific Instruments US, part no. 40X-0025HP)
Gradient:

Time (min)	% B	Flow Rate (µL/min)
0.00	0	800
0.50	0	800
2.00	30	700
6.50	80	700
8.00	80	700
8.01	0	700
9.00	0	700

MS/MS Conditions

MS System: Agilent® 6550 Q-TOF

Acquisition Method

Ion Source	Dual AJS ESI
Polarity	Pos/Neg
Gas Temp	225 °C
Drying Gas	14 L/min
Nebulizer	40 psig
Sheath Gas Temp	350 °C
Sheath Gas Flow	11 L/min
VCap	3000 V
Nozzle Voltage	500 V
Fragmentor	380 V
Min Range	75 m/z
Max Range	1000 m/z
Rate	10 spectra/s
Time	100 ms/spectrum
Collision Energy	0 V
Reference Mass 1	121.0509 m/z
Reference Mass 2	922.0098 m/z

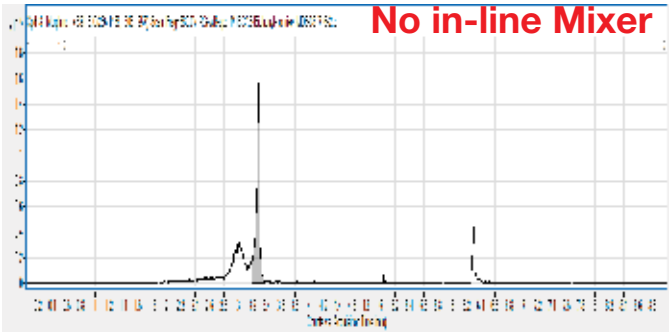
Data Analysis Method

	Values to match	Mass
Formula Matching	Mass tolerance	+/- 10ppm
Positive Ions	Charge carrier	+H
Negative Ions	Charge carrier	-H
Scoring	Mass score contribution	100
	Isotope abundance score contribution	60
	Isotope spacing score contribution	50
	Expected MS mass variation	2.0mDa + 5.6ppm
	Expected MS isotope abundance variation	7.5%
Result Filters	Only generate compounds for matched formula	Yes
	Warn if the unobserved 2nd ion's abundance is expected to be	>50
	Do not match if the unobserved 2nd ion's abundance is expected to be	>200

Figure 1.
Trapped residue from post-mortem blood samples on Phree™ Phospholipid Removal media

Figure 2.
Final whole blood extract

Figure 3.
Ameliorating effect of 25 µL mixer on Morphine peak



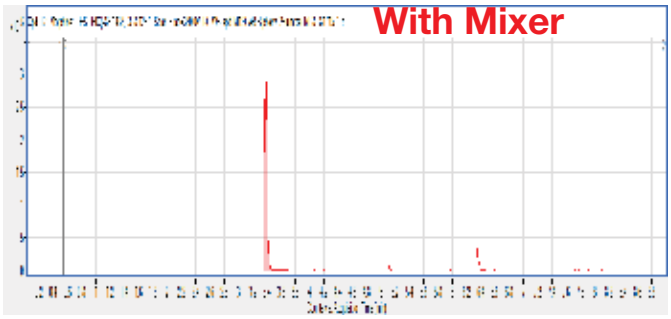
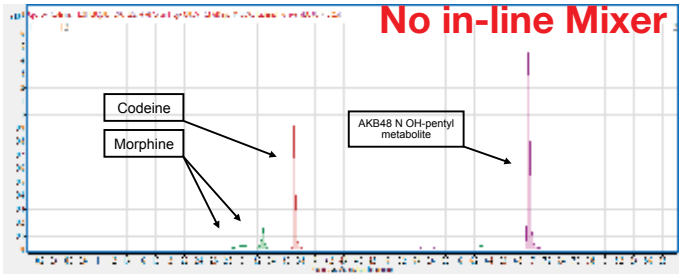
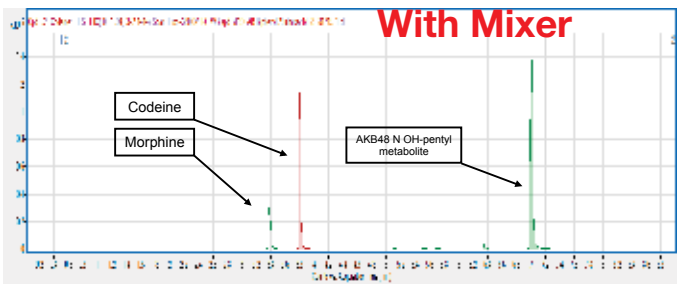


Figure 4.
Ameliorating effect of a mixer on early eluting peak (Morphine) and minor effect on mid and late eluting peaks





Results

Sample Preparation/Extraction

- The post-mortem samples produced a light blue/grey color in the supernatant which was retained by the Phree™ Phospholipid Removal media (**Figure 1**).
- Combination of Zinc acetate with chilled 95:5 Acetonitrile/ Methanol produced a very clear and color-free supernatant (**Figure 2**).

Liquid Chromatography

- With the addition of the 25 µL mixer in-line with the LC column and adjusting the LC gradient to better focus the analytes on column, we overcame the undesirable strong solvent effect on chromatography (**Figures 3** and **4**).
- The addition of the mixer increased the system volume and increased the retention time.

Library Search and Identification

- An extensive library of 7,000 compounds was searched for peak identification. The search criteria included mass error ≤10ppm of the ionic species and isotope fitting. Retention time matching (≤0.15 min) is also imposed on 560 compounds in the database for which reference standards are available. An overall score of 70 % or greater was considered a good library match to minimize false positives. Further confirmation of ambiguous peaks can be achieved by conducting dynamic MS/MS of the suspect peaks.
- A training set of 70 reference standards spiked into whole blood was used to evaluate the performance of method. All 70 compounds were identified at 100 ng/mL with target scores of 84-99 %. No false positives were identified using our database of 560 drugs of abuse and designer drugs with available retention times. Recovery rates for the compounds in the training set range between 74 % and 105 %.

Conclusion

- We have successfully demonstrated a simple and fast sample preparation procedure that is suitable for screening many compounds from a whole blood matrix.
- The final sample extracts contain approximately 65-70 % acetonitrile and provide a suitable solution for the stability of many compounds.
- The addition of a static mixer aids in negating the detrimental strong solvent effect on the chromatography thus allowing for the direct injection of the sample with no need for dry-down or reconstitution of dried residue.
- Careful library search parameters has lead to very successful unknown analyte identification with no false-positive IDs.

References

1. Sadjadi, S; Huq, S; Orlowicz, S; Snow, L; Comparison of Different Whole Blood Sample Pretreatment Methods for Targeted Analysis of Basic Drugs; MSACL US, 2015
2. Sadjadi, S; Anspach, J; Preston, J; Aslan, L; Farkas, T; Simple Yet Effective Method for Overcoming Strong Injection Solvent Effects, Proceedings of 42nd Symposium of HPLC Separation and Related Techniques, 2015

Ordering Information

Kinetex® HPLC Columns

5 µm Minibore Columns (mm)				SecurityGuard™ ULTRA Cartridges‡
Phases	30 x 2.1	50 x 2.1	100 x 2.1	3/pk
Biphenyl	00A-4627-AN	00B-4627-AN	00D-4627-AN	AJ0-9209 for 2.1 mm ID

5 µm MidBore™ Columns (mm)				SecurityGuard ULTRA Cartridges‡
Phases	50 x 3.0	100 x 3.0	150 x 3.0	3/pk
Biphenyl	00B-4627-Y0	00D-4627-Y0	00F-4627-Y0	AJ0-9208 for 3.0 mm ID

5 µm Analytical Columns (mm)					SecurityGuard ULTRA Cartridges‡
Phases	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	3/pk
Biphenyl	00B-4627-E0	00D-4627-E0	00F-4627-E0	00G-4627-E0	AJ0-9207 for 4.6 mm ID

2.6 µm Minibore Columns (mm)					SecurityGuard ULTRA Cartridges‡
Phases	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	3/pk
Biphenyl	00A-4622-AN	00B-4622-AN	00D-4622-AN	00F-4622-AN	AJ0-9209 for 2.1 mm ID

2.6 µm MidBore Columns (mm)				SecurityGuard ULTRA Cartridges‡
Phases	50 x 3.0	100 x 3.0	150 x 3.0	3/pk
Biphenyl	00B-4622-Y0	00D-4622-Y0	00F-4622-Y0	AJ0-9208 for 3.0 mm ID

2.6 µm Analytical Columns (mm)				SecurityGuard ULTRA Cartridges‡
Phases	50 x 4.6	100 x 4.6	150 x 4.6	3/pk
Biphenyl	00B-4622-E0	00D-4622-E0	00F-4622-E0	AJ0-9207 for 4.6 mm ID

1.7 µm Minibore Columns (mm)				SecurityGuard ULTRA Cartridges‡
Phases	50 x 2.1	100 x 2.1	150 x 2.1	3/pk
Biphenyl	00B-4628-AN	00D-4628-AN	00F-4628-AN	AJ0-9209 for 2.1 mm ID

‡SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

Phree™ Phospholipid Removal Products

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

Accessories

Collection Plates (deep well, polypropylene)		
AH0-7192	96-Well Collection Plate 350 µL/well	50/pk
AH0-7193	96-Well Collection Plate 1 mL/well	50/pk
AH0-7194	96-Well Collection Plate 2 mL/well	50/pk
AH0-8635	96-Well Collection Plate, 2 mL Square/Round-Conical	50/pk
AH0-8636	96-Well Collection Plate, 2 mL Round/Round, 8 mm	50/pk
AH0-7279	96-Well Collection Plate, 1 mL/well Round, 7 mm	50/pk
Sealing Mats		
AH0-8597	Sealing Mats, Pierceable, 96-Square Well, Silicone	50/pk
AH0-8598	Sealing Mats, Pre-Slit, 96-Square Well, Silicone	50/pk
AH0-8631	Sealing Mats, Pierceable, 96-Round Well 7 mm, Silicone	50/pk
AH0-8632	Sealing Mats, Pre-Slit, 96-Round Well 7 mm, Silicone	50/pk
AH0-8633	Sealing Mats, Pierceable, 96-Round Well 8 mm, Silicone	50/pk
AH0-8634	Sealing Mats, Pre-Slit, 96-Round Well 8 mm, Silicone	50/pk
AH0-7362	Sealing Tape Pad	10/pk
Vacuum Manifolds		
AH0-6023*	SPE 12-Position Vacuum Manifold Set, for tubes	ea
AH0-6024*	SPE 24-Position Vacuum Manifold Set, for tubes	ea
AH0-8950	Strata 96-Well Plate Manifold, Universal with Vacuum Gauge	ea

*Manifolds include: Vacuum-tight glass chamber, vacuum gauge assembly, polypropylene lid with gasket, male and female luers and yellow end plugs, stopcock valves, collection rack assemblies, polypropylene needles, lid support legs. Waste container included with 12-position manifold.

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.

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