A Comparison of Extraction Techniques for Fentanyl Analogues in Whole Blood

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Introduction

Fentanyl and fentanyl-related analogues have been identified as the root cause of several notable mass-overdose events in recent years. The demand for testing of these drugs has rapidly increased due to the opioid epidemic affecting many cities across the United States and Canada. Whole blood is a common sample matrix for forensic laboratories as this can be easy to collect and may provide relevant information regarding recent or active use of illicit materials. Obtaining optimal analytical results from whole blood requires adequate sample preparation to remove endogenous interferences and to isolate compounds of interest. Several options exist for effective preparation of whole blood, each with their own merits. Some may involve minimal effort, such as the load-wait-elute technique using supported liquid extraction (SLE+), while others may require more complex methodologies, such as solid phase extraction (SPE) with mixed-mode ion exchange sorbents. Each method of sample preparation will yield extracts of different levels of cleanliness. The results of different extraction techniques for whole blood spiked with 16 fentanyl analogues was collected via LC-MS/MS and compared to identify practical considerations for optimal workflows.

Methods

Reagents and Materials

Standards, Chemicals, Extraction Hardware

All standards were purchased from Cerilliant (Round Rock, TX). LC/MS grade water and methanol (MeOH) were purchased from Honeywell Chemicals (Charlotte, NC). HPLC Plus grade ethyl acetate (EA) and *tert*-Butyl methyl ether (MTBE) was purchased from Sigma-Aldrich (St. Louis, MO). LC/MS Optima grade dichloromethane (DCM), 2-propanol (IPA), and formic acid (FA) were purchased from Fisher Scientific (Waltham, MA), as well as HPLC grade acetonitrile (ACN) and ammonium hydroxide (NH_4OH). Raptor Biphenyl 2.7µm 100 x 2.1mm analytical column was provided by Restek (Bellefonte, PA). Drug-free human whole blood was provided by UTAK (Valencia, CA). EVOLUTE® EXPRESS CX (30 mg bed) extraction plate (601-0030-PX01), ISOLUTE® HCX (25 mg bed) extraction plate (902-0025-PO1), ISOLUTE® SLE+ (400 µL) extraction plate (820-0400-P01), Biotage® PRESSURE+ 96 position positive pressure manifold (PPM-96), and Biotage® SPE Dry 96 (SD-9600-DHS-NA) were supplied by Biotage.

Sample Preparation

Whole Blood Sample Preparation

Each sample was spiked at two known concentrations with all 16 target analytes resulting in stocks of 5 ng/mL and 0.1 ng/mL.

Compounds Included in the Panel

U-47700, sufentanil, valeryl fentanyl, isobutyryl fentanyl, methoxyacetylfentanyl, 4-fluoro isobutyryl fentanyl, carfentanil, fentanyl, alfentanil, norfentanyl, U-51754, butyryl fentanyl, furanyl fentanyl, o-fluorofentanyl, acrylfentanyl, 4-ANPP

Sample Pretreatment

Each extraction protocol utilized a different pretreatment for the whole blood workflows while maintaining a 1:1 dilution of raw sample. For whole blood on ISOLUTE® SLE+, a 1% ammonium hydroxide buffer was used for pretreatment. ISOLUTE® HCX and EVOLUTE® EXPRESS CX workflows utilized a 0.1% formic acid solution for pretreatment. It is reported that the fentanyl analogues do not exhibit significant protein binding during normal metabolism, so aggressive disruption or cell lysis steps were not necessary for these samples. Samples were prepared in triplicate sets along with no matrix controls, extraction blanks, and unextracted standards for use in the calculation of analyte recoveries and matrix effects.

Extraction Procedures

Following pretreatment, sample extraction was performed. Data was obtained for the extraction of whole blood using ISOLUTE® SLE+, ISOLUTE® HCX, and EVOLUTE® EXPRESS CX products. In all experiments, the initial whole blood sample volume was 100 µL, along with 100 µL of the given pretreatment solution. The full workflows for each plate and procedure are detailed in tables and figures 1 and 2.

ISOLUTE® HCX and EVOLUTE® EXPRESS CX Urine and Whole Blood Extraction				
Step	**Volume (µL)**	**Solvent**	**Time (min)**	**Pressure (psi)**
Condition *(HCX only)*	1000	MeOH	1	1-2
Equilibrate *(HCX only)*	1000	0.1% FA	1	1-2
Sample Load	200	Pretreated Sample	1-2	2-4
Wash #1	1000	H_2O	1-2	2-4
Wash #2	1000	0.1% FA	1-2	2-4
Wash #3	1000	MeOH	1-2	3-5
Plate Dry	N/A	N/A	5	20
Elute	2 x 750	DCM/IPA/NH_4OH EA/ACN/NH_4OH [78:20:2]	1-2	2-4

Table 1. *Biotage 96 Positive Pressure Processing Parameters for whole blood samples on ISOLUTE® HCX and EVOLUTE® EXPRESS CX plates. Elution was completed with 2 aliquots of 1 of 2 different complex mixtures.*

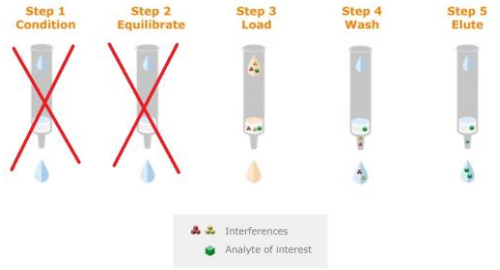

Figure 1. *The water-wettable EVOLUTE® EXPRESS CX polymer does not require conditioning and equilibration, saving time and solvent use.*

ISOLUTE® SLE+ Whole Blood Extraction				
Step	**Volume (µL)**	**Solvent**	**Time (min)**	**Pressure (psi)**
Sample Load	200	Pretreated Sample	1	4-6
Wait	N/A	N/A	5	N/A
Elute	750	DCM/MTBE/EA	N/A	N/A
Wait	N/A	N/A	5	N/A
Elute	750	DCM/MTBE/EA	N/A	N/A
Wait/Elute	N/A	N/A	5	2-4

Table 2. *Biotage 96 Positive Pressure Processing Parameters for whole blood samples on ISOLUTE® SLE+ plate. Each elution step is followed by a 5-minute period before a final push with positive pressure is applied to collect extracts.*

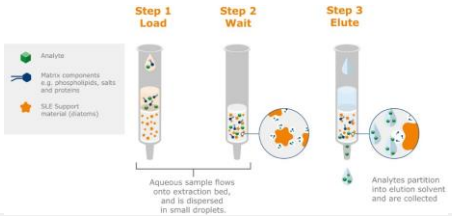

Figure 2. *The simple load-wait-elute workflow for ISOLUTE® SLE+.*

Dry Down and Sample Reconstitution: Eluates were collected into a collection plate. All samples were evaporated to dryness at 40°C with 20 L/min of nitrogen using a Biotage® SPE Dry 96. Extracts were then reconstituted with 50 µL of 50:50 mobile phase A/mobile phase B and analyzed via LC-MS/MS.

Chromatography Parameters

UPLC	Parameter
Column	Restek Raptor Biphenyl 2.7 µm, 100 x 2.1 mm
MPA	0.1% formic acid (aq)
MPB	0.1% formic acid in MeOH
Flow Rate	0.4 mL/min
Column Temp.	40°C
Sample Temp.	15°C
Injection Volume	2 µL

Table 3. *Shimadzu Nexera X2 SIL-30AC UPLC.*

An isocratic gradient was used over a 7.0-minute data window to achieve the chromatographic separation visible in figure 3. Details of chromatography parameters can be found above in table 3.

Mass Spectrometry Parameters

Instrument: SCIEX 5500 triple quadrupole mass spectrometer with Turbo Ionspray® Ion interface (Foster City, CA). Source parameters were optimized and can be found in table 4. Acquisition was conducted by scheduled MRM. Details of monitored transitions are found in Table 5. Data window for each sMRM was set at 60 seconds, with target scan time at 1.0 seconds.

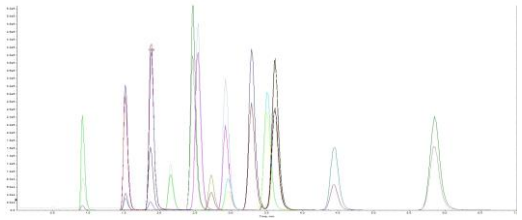

Figure 3. *Chromatograms for each of the 16 analytes at 5 ng/mL*

Ionization Spray Voltage	+4000(V)	**CAD**	8
Source Temp	600 °C	**GS1**	30
Curtain	20	**GS2**	60

Table 4. *SCIEX 5500 Triple Quadrupole ESI (+) Turbo Ionspray® Source Parameters.*

Compound	Q1	Q3 1	Q3 2	Retention Time	DP 1	DP 2	EP 1	EP 2	CE 1	CE 2	CXP 1	CXP 2
4-ANPP	281.1	188.2	105.1	1.94	50	50	10	10	20	45	12	8
Acryl Fentanyl	335.3	188.2	105.1	2.54	100	50	10	10	30	55	12	8
Fentanyl	337.2	188.2	105.2	2.62	50	50	10	10	35	50	12	8
o-fluorofentanyl	354.4	188.1	105.2	1.55	100	50	10	10	35	45	10	4
Furanyl fentanyl	375.3	188.2	105.2	3.45	50	50	10	10	35	50	10	8
Alfentanil	417.3	197.1	165.0	2.22	50	50	10	10	40	45	10	10
Isobutyryl fentanyl	351.3	188.2	105.0	3.25	100	100	10	10	35	50	10	8
Butyryl fentanyl	351.3	188.2	105.0	3.59	100	100	10	10	35	50	10	8
Methoxyacetyl fentanyl	353.1	188.1	105.1	1.54	50	50	10	10	35	55	12	8
Valeryl fentanyl	365.3	188.3	105.1	5.84	50	50	10	10	35	50	10	8
4-fluoro-isobutyryl-fentanyl	369.0	188.2	105.1	3.05	50	50	10	10	35	50	10	8
Sufentanil	387.2	111.1	140.2	4.4	100	50	10	10	50	35	8	10
Carfentanil	395.2	113.1	134.0	3.07	150	100	10	10	45	45	8	8
Norfentanyl	233.1	55.1	84.2	0.93	50	50	10	10	52	25	8	8
U-51754	343.1	217.8	112.2	2.83	70	29	10	10	37	38	13	7
U-47700	329.2	172.9	203.9	1.93	50	140	10	10	42	43	11	14

Table 5. *sMRM information for each fentanyl analogue monitored in this workflow.*

Results

Extraction Recoveries

Recoveries varied greatly for some analytes, depending on the extraction technique and elution solvent applied. Figure 4 illustrates the recoveries of all 16 analytes at 0.1 ng/mL from whole blood using each different applicable extraction technique.

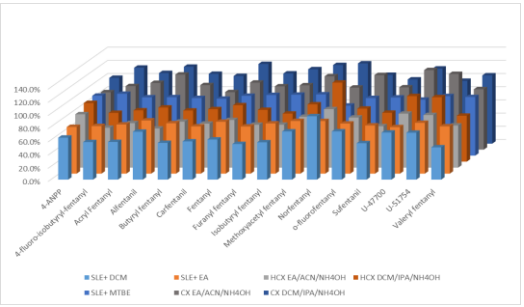

Figure 4. *Variations in recoveries for whole blood extraction of fentanyl analogues using different techniques.*

Extraction Matrix Effects

The measured matrix effects for each extraction did display notable variation. Figure 5 illustrates the matrix effects for each of the analytes at 0.1 ng/mL from the whole blood extractions. Some of the compounds demonstrated either ion suppression or ion enhancement, specifically with respect to the SLE+ extraction. This indicates these extracts were simply not as clean as other approaches, such as the EVOLUTE® EXPRESS CX or ISOLUTE® HCX methods.

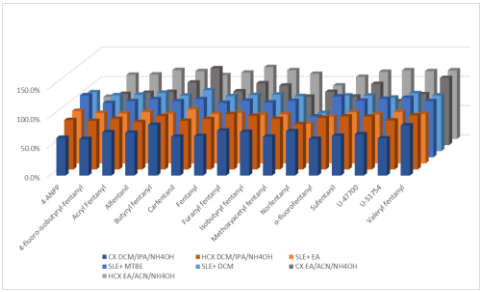

Figure 5. *The measured matrix effects for each whole blood extraction.*

Conclusions

- The whole blood extractions produced qualitatively similar results, although the EVOLUTE® EXPRESS CX samples were relatively cleaner.
- Although each extraction method is suitable, the EVOLUTE® EXPRESS CX sample preparation method with the DCM/IPA/NH_4OH elution solvent provided the best recoveries of our target analytes with the least amount of matrix effects.
- If a simpler approach is desired, using the ISOLUTE® SLE+ can provide ease of use with clean extracts for the fentanyl analogues.

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