Sample Preparation for Fentanyl Analogs in Whole Blood

Fentanyl and its analogs are part of the growing opioid crisis in the United States. According to the latest data from the National Survey on Drug Use and Health[1] , more than 2.1 million Americans struggle with an opioid use disorder for either prescription pain relievers or heroin. The National Center for Injury Prevention and Control estimated that the total annual economic burden[2] of prescription opioid misuse in the United States is $78.5 billion, which includes increased healthcare costs, substance abuse treatment, lost productivity, and criminal justice. Because of this, both routine clinical and post-mortem toxicology testing demands have rapidly increased. Whole blood is a common choice for sample matrix due to its availability, but the viscosity can make blood a challenge to work with. This difficulty demonstrates a real need for robust sample preparation methods to extract opioids from dirty biological matrices across many industries, including medicine, workplace testing, and forensics.

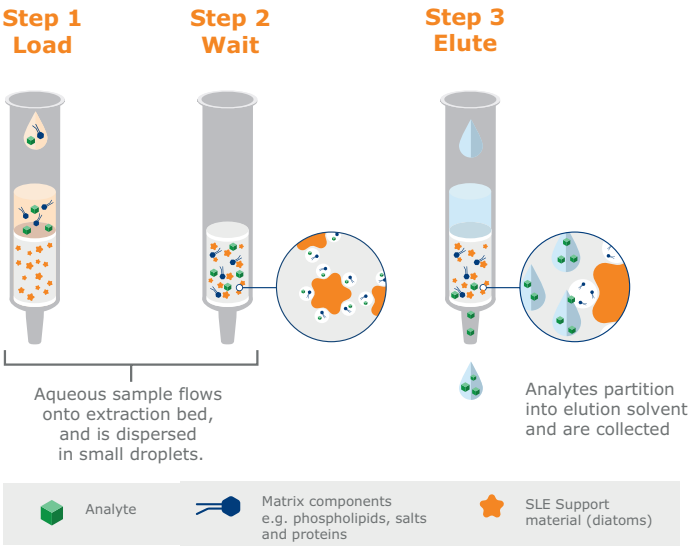

Figure 1. Typical ISOLUTE® SLE+.

Analytes

4-ANPP (4-aminophenyl-1-phenethylpiperidine), 4-fluoro-isobutyryl-fentanyl, acryl fentanyl, alfentanil, butyryl fentanyl, carfentanil, fentanyl, furanyl fentanyl, isobutryl fentanyl, methoxyacetyl fentanyl, norfentanyl, o-fluorofentanyl, sufentanil, U-47700, U-51754, valeryl fentanyl

Methods

There are several different extraction methods that can be used to isolate fentanyl compounds from whole blood samples.

The simplest technique is supported liquid extraction using ISOLUTE® SLE+. This product, available in 96-well plate or cartridge format, employs the mechanism of a liquid-liquid extraction with a diatomaceous earth sorbent, allowing for complete separation of the aqueous and organic layers (see Figure 1).

The first step is loading samples onto the diatomaceous earth material. A five-minute wait time allows the aqueous sample to fully adsorb onto the sorbent. Next, an elution step follows using a water-immiscible organic solvent like dichloromethane (DCM), ethyl acetate (EA), or MTBE (tert-butyl methyl ether). This step targets the compounds of interest allowing them to elute off of the diatomaceous earth sorbent, while leaving behind any aqueous impurities and other unwanted components.

Table 1. Supported Liquid Extraction (SLE) methodology (ISOLUTE® SLE+ 400 µL plate, p/n 820-0400-P01).

Step	Conditions
Blood sample volume	100 µL
Pre-treatment	100 µL 1% NH_4OH (aqueous)
Load sample	
Elution	2 x 750 µL dichloromethane OR
	2 x 750 µL ethyl acetate OR
	2 x 750 µL MTBE

Following elution, evaporate samples to complete dryness using a Biotage® SPE Dry 96 plate evaporator. Reconstitute in 50 µL 50:50 mobile phase A/mobile phase B.

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Solid phase extraction (SPE) can be also be used to isolate the fentanyl compounds. A silica-based sorbent (ISOLUTE® HCX) or a polymeric sorbent (EVOLUTE® EXPRESS CX) can both be used. Each of these options utilize a mixed-mode extraction mechanism (non-polar interactions and cation exchange) to allow for additional sample purification without loss of analyte. The silica-based sorbent requires traditional SPE conditioning and equilibration steps prior to sample loading. These silica-based plates also require longer drying steps before the compounds can be fully eluted from the sorbent. In contrast, the polymeric sorbent is water-wettable, which permits the exclusion of the condition and equilibration steps, and also promotes less pre-elution plate drying time (see Figure 2).

Tables 1 and 2 detail the methods used for the extraction of fentanyl compounds from whole blood. These methods can be processed manually using a Biotage® VacMaster™ 96 vacuum manifold or a Biotage® PRESSURE+ 96 Positive Pressure Manifold. The extraction can also be automated using the Biotage® Extrahera™ Automated Sample Preparation system.

Table 2. Solid Phase Extraction (SPE) methodologies.

Step	ISOLUTE® HCX 25 mg plate (p/n 902-0025-P01)	EVOLUTE® EXPRESS CX 30 mg plate (p/n 601-0030-PX01)
Blood sample volume	100 µL	100 µL
Pre-treatment	100 µL 0.1% formic acid (aqueous)	100 µL 0.1% formic acid (aqueous)
Extraction		
Condition	1 mL methanol	NONE
Equilibrate	1 mL 0.1% formic acid (aq)	NONE
Load	200 µL of pre-treated sample	
Wash 1	1 mL water	1 mL water
Wash 2	1 mL 0.1% formic acid (aq)	1 mL 0.1% formic acid (aq)
Wash 3	1 mL methanol	1 mL methanol
Plate Dry	10 min at 20 psi	1 min at 20 psi
Elution	2 x 750 µL 78:20:2 DCM/IPA/NH_4OH OR	2 x 750 µL 78:20:2 DCM/IPA/NH_4OH OR
	2 x 750 µL 78:20:2 EA/ACN/NH_4OH	2 x 750 µL 78:20:2 EA/ACN/NH_4OH

After elution, evaporate samples to complete dryness using a Biotage® SPE Dry 96 plate evaporator. Reconstitute in 50 µL 50:50 (v/v) mobile phase A/mobile phase B.

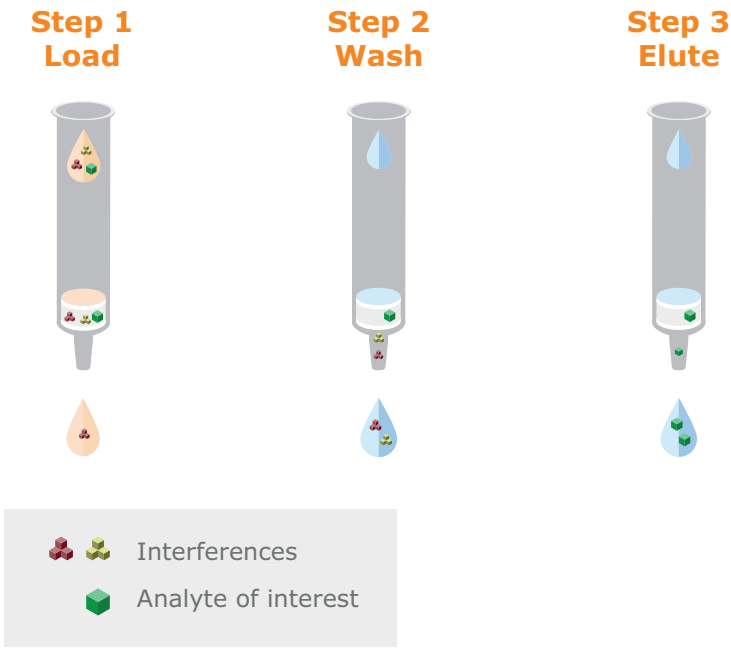

Figure 2. Typical workflow for solid phase extraction using EVOLUTE® EXPRESS.

LC Parameters

Instrument
Shimadzu Nexera X2

LC Column
Restek Raptor Biphenyl 100 x 2.1 mm, 2.7 µm (Cat # 9309A12)

Column Temperature
40 °C

Mobile Phase A
0.1% formic acid in water

Mobile Phase B
0.1% formic acid in methanol

Isocratic Flow
50:50 Mobile Phase A/Mobile Phase B

Flow Rate
0.4 mL/min

Run Time
7.00 minute

Injection
2 µL

MS/MS Parameters

Instrument
SCIEX 5500 Triple Quadrupole

Source Gas
600 °C

Curtain Gas
20

Collision Gas (CAD)
8

Ionspray Voltage
4000

Ion Source Gas 1
30

Ion Source Gas 2
60

Positive Polarity
Table 3 shows the MS parameters for each compound in the panel.

Table 3. MS Parameters for all Fentanyl Compounds.

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
Isobutryl 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

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Results

For every method, LOQs were established down to 0.1 ng/mL for all compounds in the panel. The recoveries and matrix effects shown are using a 0.1 ng/mL sample in whole blood. Overall, recoveries using EVOLUTE® EXPRESS CX and a DCM/IPA/NH_4OH elution solvent were higher than the other methods assessed (Figure 3). However, increased signal suppression was evident in the DCM/IPA/NH4OH elution solvent with the EVOLUTE EXPRESS CX data set. The lowest recoveries were seen using ISOLUTE® SLE+ with a DCM elution solvent, but were still above 50% for all compounds in the panel. Using ISOLUTE HCX with the EA/ACN/NH_4OH elution solvent demonstrated the least amount of matrix effects (Figure 4). The most suppression was found when either the ISOLUTE HCX or EVOLUTE EXPRESS CX methods were paired with the DCM/IPA/NH4OH elution solvent. Suppression with these methods ranged from 10–40%.

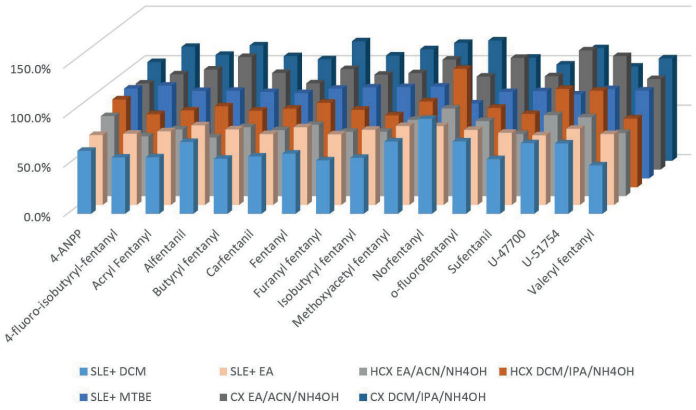

Figure 3. Recoveries of Fentanyl compounds using various extraction techniques and elution solvents.

Conclusion

There are several different extraction techniques that can be used to extract fentanyl compounds from whole blood samples. It is important to consider the compounds in the panel, the desired extract cleanliness, compound recoveries, and extraction time to determine which method best fits the application. If using ISOLUTE SLE+, an elution with MTBE has the highest recoveries and fewest matrix effects. If using ISOLUTE HCX or EVOLUTE EXPRESS CX, an elution solvent of DCM/IPA/NH_4OH has the highest recoveries, but slightly increased matrix effects.

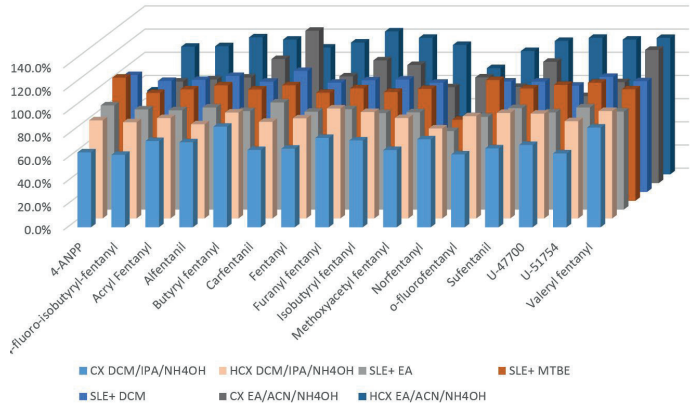

Figure 4. Matrix effect for Fentanyl compounds using various extraction techniques and elution solvents.

References

1. https://nsduhweb.rti.org/respweb/homepage.cfm
2. Med Care. 2016 Oct;54(10):901-6. The Economic Burden of Prescription Opioid Overdose, Abuse, and Dependence in the United States, 2013

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Literature Number: PPS546

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