

Extraction and Analysis Methods for Fentanyl and Fentanyl Analogues in Whole Blood and Urine

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Introduction

Fentanyl and fentanyl-related analogues have been identified as the root cause of several notable drug overdoses 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. Urine and whole blood are common matrices of choice for forensic laboratories as they can be easy to collect, and they provide relevant information regarding recent or active use of illicit materials. Obtaining optimal analytical results from either matrix will require adequate sample preparation to remove interferences and isolate compounds of interest. Several options exist for effective preparation of whole blood and urine. Some may involve minimal effort, such as the dual-mode extraction (DME+), while others may require more complex methodologies, such as solid phase extraction (SPE) with mixed-mode polymeric ion exchange sorbents. Each method of sample preparation will yield extracts of different levels of cleanliness. The results of different extraction procedures for both whole blood and urine 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 (NH₄OH). Raptor Biphenyl 2.7 µm 100 x 2.1 mm analytical column was provided by Restek (Bellefonte, PA). Drug-free human whole blood was provided by UTAH (Valencia, CA). Drug-free human urine was collected from a willing donor. EVOLUTE® EXPRESS CX (30 mg bed) extraction plate (601-0030-PX01), ISOLUTE® HCX (25 mg bed) extraction plate (902-0025-PO1), ISOLUTE® HYDRO DME+ (400 mg bed) extraction plate (970-0400-PZ01), ISOLUTE® SLE+ (400 µL) extraction plate (820-0400-PO1), 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 and Urine Sample Preparation

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

Compounds Included in the Panel

U-47700, sufentanil, valeryl fentanyl, isobutyryl fentanyl, methoxyacetyl fentanyl, 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 both whole blood and urine 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, ISOLUTE® HYDRO DME+, and EVOLUTE® EXPRESS CX workflows utilized a 0.1% formic acid solution for pretreatment. It is reported that the fentanyl analogues do not undergo any significant conjugation during normal metabolism, so hydrolysis steps were not necessary for the urine 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 the urine samples with ISOLUTE® HYDRO DME+, ISOLUTE® HCX, and EVOLUTE® EXPRESS CX, whereas whole blood extraction data was collected using ISOLUTE® SLE+, ISOLUTE® HCX, and EVOLUTE® EXPRESS CX products. In all experiments, the initial urine or whole blood sample volume was 100 µL. For ISOLUTE® HYDRO DME+, each urine sample was pretreated with 100 µL of 0.1% formic acid. The resulting 200 µL was then added to the ISOLUTE® HYDRO DME+ plate, followed by 600 µL of ACN to each sample well. The contents of each well were mixed via aspiration and dispense 5 times before positive pressure was applied to push the samples through the ISOLUTE® HYDRO DME+ media. Whole blood samples on ISOLUTE® SLE+ were first pretreated with 100 µL of 1% NH₄OH. Each pretreated sample was loaded onto the ISOLUTE® SLE+ plate with positive pressure and permitted to absorb for 5 minutes. After this waiting period, samples were eluted with 2 aliquots of 750 µL of one of three elution solvents: DCM, MTBE, or EA. The more complex workflows on ISOLUTE® HCX and EVOLUTE® EXPRESS CX are detailed in table 1.

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 ₂ O	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 ₄ OH EA/ACN/NH ₄ OH [78:20:2]	1-2	2-4

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

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
MFA	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 2. 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 1.

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 6. Acquisition was conducted by scheduled MRM (transition information not presented here but is available upon request). Data window for each SMRM was set at 60 seconds, with target scan time at 1.0 seconds.

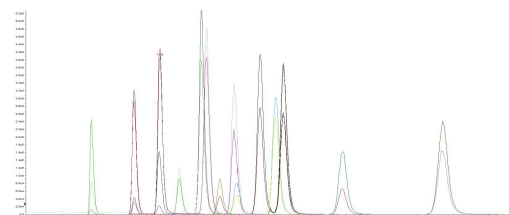


Figure 1. Chromatograms for each of the 16 analytes at 5 ng/mL

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

Table 3. SCIEX 5500 Triple Quadrupole ESI (+) Turbo Ionspray® Source Parameters.

Results

Extraction Recoveries

Recoveries varied greatly for some analytes, depending on the sample matrix, as well as the extraction technique and elution solvent applied. Figure 1 illustrates the recoveries of all 16 analytes from whole blood using each different applicable extraction technique.

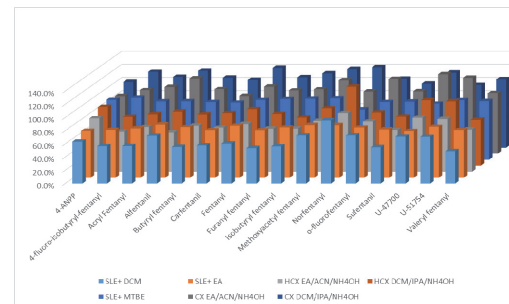


Figure 2. Variations in recoveries for whole blood extraction of fentanyl analogues using different techniques.

Similarly, the extraction of the fentanyl analogues from urine demonstrated variation in recovery with respect to the choice of technique and elution solvent applied. The urine extraction results are found in figure 3.

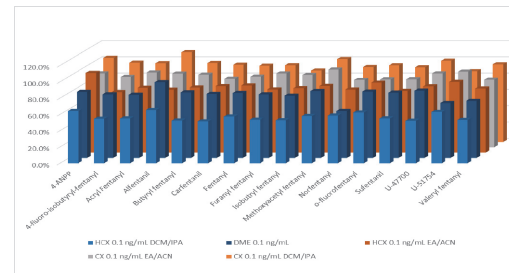


Figure 3. Variations in recoveries for urine extraction of fentanyl analogues using different techniques.

Extraction Matrix Effects

The measured matrix effects for each extraction and sample matrix did display notable variation. Figure 4 illustrates the matrix effects for each of the analytes and the whole blood extractions, while figure 5 contains the results of the urine protocols. Some of the compounds demonstrated either ion suppression or ion enhancement, specifically with the flow-through technique of ISOLUTE® HYDRO DME+. This indicates these extracts were simply not as clean as other approaches, such as the EVOLUTE® EXPRESS CX or ISOLUTE® SLE+ methods.

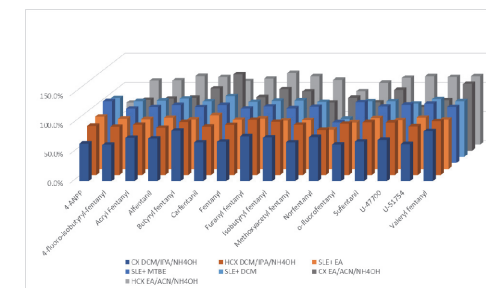


Figure 4. The measured matrix effects for each whole blood extraction.

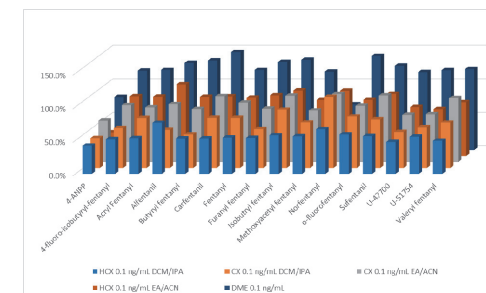


Figure 5. The measured matrix effects for each urine extraction.

Conclusions

- For the urine extractions, ISOLUTE® HYDRO DME+ is a fast and cheap option for sample preparation, although the resulting extracts are not as clean as other methods and does yield increased matrix effects.
- 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/NH₄OH 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.