Uncovering Tracks – Robust, Reproducible Screening Assay for Fentanyls in Urine with LC-HRAM(MS) for clinical research or forensic toxicology

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ABSTRACT

Purpose: To develop and analytically validate a method for screening of fentanyl and fentanyl analogs in urine for clinical research or forensic toxicology.

Methods: The method is based on direct injection of urine into a UHPLC system coupled to a Thermo Scientific™ Q Exactive™ Focus hybrid quadrupole-Orbitrap High Resolution Accurate Mass (HRAM) mass spectrometer.

Results: Results were evaluated using Thermo Scientific™ TraceFinder™ software. The method performance was evaluated by testing selectivity, accuracy and precision at the proposed cutoff level, linearity and matrix effects.

INTRODUCTION

Fentanyl is an opioid used as a pain medication together with other medications for anesthesia. Fentanyl and fentanyl analogues made illegally are also used as recreational drugs. Fentanyl and its analogues are significantly stronger than morphine, with some analogues (carfentanil) exhibiting ~10,000 times higher strength than regular pain medications. The use and abuse of fentanyl and its analogues are also known to cause serious side effects, ranging from respiratory depression to deaths. Problems related to fentanyl and its analogues are still prevalent in many countries according to reports from European Monitoring Centre for Drugs and Drug addiction (EMCDDA). In 2017, deaths due to overdoses of fentanyl and fentanyl analogues were more common than deaths owing to heroin overdose in Sweden1.This study reports the development and analytical validation of a method for detection of fentanyl and some analogues in urine.

MATERIALS AND METHODS

The Screening method was based on reversed phase liquid chromatography (LC) coupled to High Resolution Accurate Mass (HRAM) spectrometry for 14 different Fentanyl analogues and metabolites in urine has been developed using a Thermo Scientific™ Ultimate RS3000 UHPLC system and a Thermo Scientific Q Exactive Focus Orbitrap High Resolution Accurate Mass spectrometer operated in data dependent MS/MS (ddMS2) mode. Identification was based on m/z and retention time. Confirmation of the compounds was performed by matching of the MS2 spectrum of the target compound to the recorded library MS2 spectrum. Ion chromatograms were extracted at ± 5 ppm, and quantitation was based on a two-point calibration curve using internal standard calibration. Four different fentanyls labeled with stable isotopes were used as internal standards. The total analysis time was 5 minutes. Sample preparation was performed by dilution and direct injection of urine into LC-HRAM(MS)

Sample Preparation

Urine was centrifuged at 10000xg for 5 minutes.10 µL urine was diluted with 100 µL Milli-Q® (MilliporeSigma) water containing internal standards. 10 µL was injected into the LC MS System.

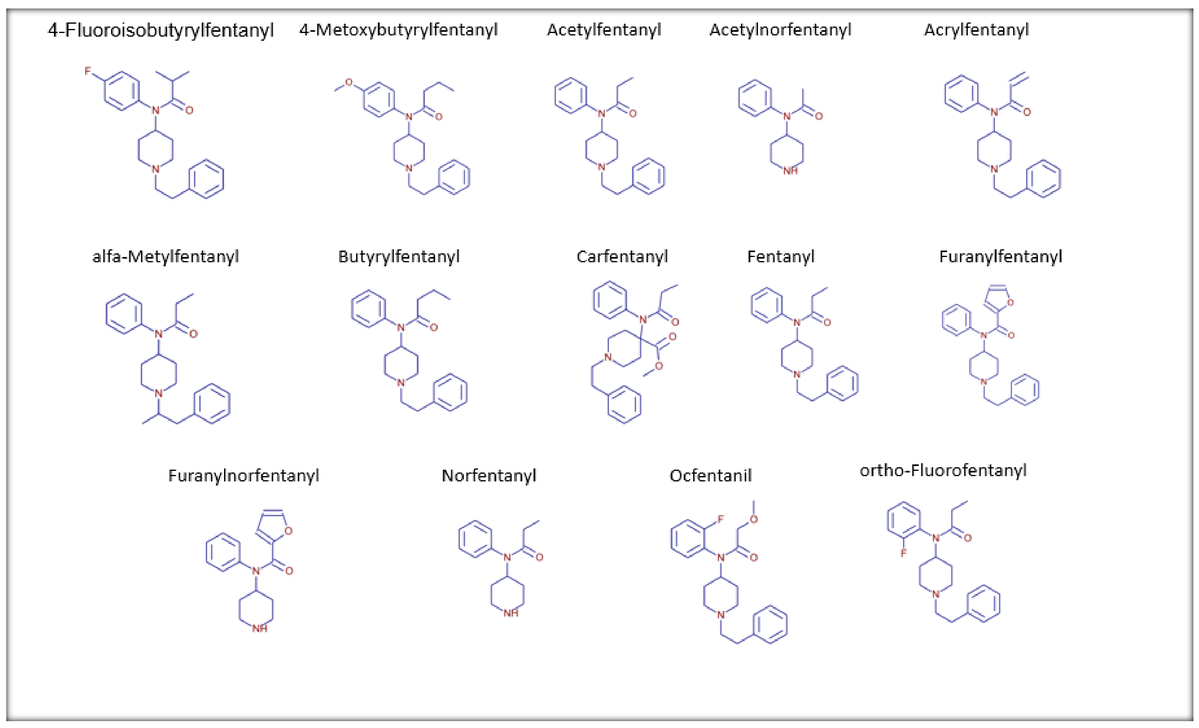
Test Method(s)

14 different Fentanyls were included in the method. The compounds were chosen from previously reported intoxications. Four stable isotope-labelled internal standards were used for quantitation. Details are given in Table 1.

Table 1. Compounds, internal standards included in the method and their monoisotopic masses

Analytes	Chemical Formula	Monoisotopic mass (M+H)	Internal Standards	Monoisotopic mass (M+H)	LOQ (ng/mL)
4-Fluoroisobutyrylfentanyl	C23H29FN2O	369.2337	Fentanyl-D5	342.2588	2
4-Metoxybutyrylfentanyl	C24H32N2O2	381.2537	Fentanyl-D5	342.2588	5
Acetylfentanyl	C21H26N2O	323.2118	Acetylfentanyl-13C6	329.2319	1
Acetylnorfentanyl	C13H18N2O	219.1492	Noracetylfentanyl-13C6	225.1693	2
Acrylfentanyl	C22H26N2O	335.2118	Fentanyl-D5	342.2588	1
alfa-Metylfentanyl	C23H30N2O	351.2431	Fentanyl-D5	342.2588	4
Butyrylfentanyl	C23H30N2O	351.2431	Fentanyl-D5	342.2588	2
Carfentanyl	C24H30N2O3	395.2329	Fentanyl-D5	342.2588	2
Fentanyl	C22H28N2O	337.2274	Fentanyl-D5	342.2588	0.5
Furanylfentanyl	C24H26N2O2	375.2067	Fentanyl-D5	342.2588	1
Furanylnorfentanyl	C16H18N2O2	271.1441	Norfentanyl-D5	238.1962	1
Norfentanyl	C14H20N2O	233.1648	Norfentanyl-D5	238.1962	4
Norfuranylfentanyl	C16H18N2O2	271.1441	Norfentanyl-D5	238.1962	1
Ocfentanil	C22H27FN2O2	371.2129	Acetylfentanyl-13C6	238.1962	1
ortho-Fluorofentanyl	C22H27FN2O	355.2180	Fentanyl-D5	342.2588	4

Figure 1. Chemical structures of the compounds included in the method



LC-Method:

The LC-system consisted of an Ultimate RS3000 UHPLC High Pressure Gradient pump. The LC-method is described in Table 2. Total cycle time for the method is 5 minutes/sample.

Mobile Phase A: Water + 0.1%NH4OH

Mobile Phase B: Methanol

Column: WATERS® ACQUITY BEH 2.1x100 mm (WATERS)

Temperature: 60 ° C

Injection Volume: 10 µL

Table 2. LC method

Time (min)	Flow rate (mL/min)	%A	%B
0	0.6	95	5
1.3	0.6	95	5
1.31	0.4	50	50
2.6	0.4	20	80
2.8	0.4	15	85
2.9	0.4	10	90
3	0.4	10	90
3.01	0.6	0	100
4	0.6	0	100
4.1	0.6	95	5
4.5	0.6	95	5

MS-Method:

A Q Exactive Focus Orbitrap High Resolution Accurate Mass mass spectrometer was operated in Full Scan/data dependent MS/MS (ddMS2) in confirmation mode (i.e. an inclusion list is used). The ion source settings are presented in Table 3, and the method settings are presented in Table 4.

Table 3. Ion Source Parameters

Tune File	
Spray Voltage	3500
Capillary Temperature	300
Sheath Gas	75
Aux Gas	12.5
Sweep Gas	2
Probe Heater Temp.	450
S-Lens RF Level:	90

Identification was based on *m/z* and retention time. Confirmation of the compounds was performed by matching of the MS2 spectrum of the target compound to the recorded library MS2 spectrum. Ion chromatograms were extracted at ± 5 ppm, and quantitation was based on a two-point calibration curve using internal standard calibration. Quantitation was performed to determine if detected concentrations were above the cutoff value of the method.

For validation, test samples were prepared by spiking blank human urine with known amounts of test compounds. Certified test compounds were obtained from Cerrilliant®, Chiron AS and Cayman Chemical.

TraceFinder software was used for data evaluation.

Table 4. Mass spec method

General	
Polarity	positive
dd-MS²	Confirmation
In-source CID	—
Full MS	
Scan range	215 to 400 m/z
Resolution	70,000
# Scan ranges	1
AGC target	1e6
Maximum IT	auto
Microscans	1
Spectrum data type	Profile
dd-MS² Confirmation	
Apex trigger	—
Resolution	17,500
Isolation window	1.5 m/z
Isolation offset	—
(N)CE / stepped (N)CE	ce: 30
Fixed first mass	—
Default charge state	1
AGC target	5e4
Maximum IT	auto
Loop count	2
Minimum AGC target	1.00
Intensity threshold	auto
Dynamic exclusion	1.0 s
Spectrum data type	Profile

RESULTS

Results from Method Characterization

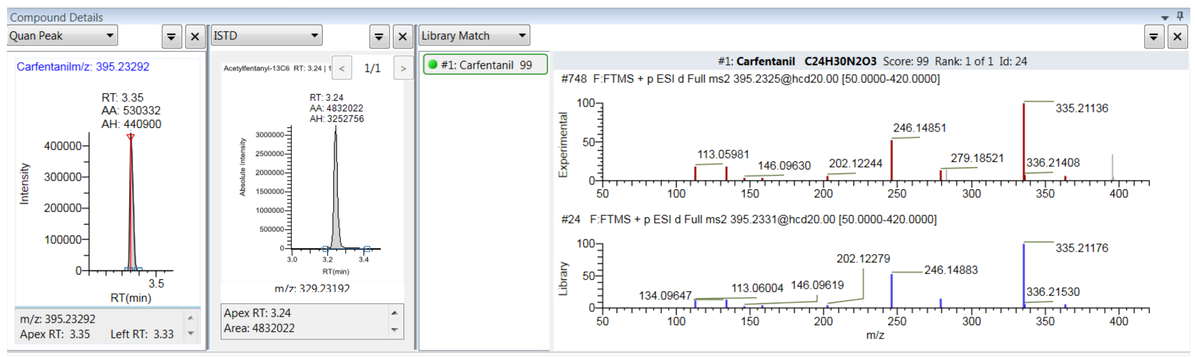
The method developed in this study was tested and analytically validated by monitoring accuracy of identification, linearity, matrix effects, accuracy and Coefficient of Variation (%CV) at the lower limit of quantitation. All compounds present in the spiked matrix were detected and confirmed at 0.5 – 5 ng/mL. The accuracy (bias%) and %CV was within ±15% for all compounds. The accuracy and precision (%CV) at the lower limit of quantitation is presented in table 5.

Table 5. Accuracy and precision at the cutoff level

	4-Fluoroisobutyryl fentanyl	4-Metoxybutyry lfentanyl	Acetylfentanyl	Acetylnorfentanyl	Acrylfentanyl	alfa-Metylfentanyl	Butyrylfentanyl
Nominal conc (ng/mL)	2	5	1	2	1	4	2
Mean	2.15	4.93	0.81	2.29	0.96	4.00	2.00
Accuray%	107.6	98.6	80.9	114.5	95.6	99.9	99.9
CV%	5.5	13.4	4.0	1.8	8.0	7.7	7.7
	Carfentanil	**Fentanyl**	**Furanylfentanyl**	**Furanylnorfentanyl**	**Norfentanyl**	**Ocfentanil**	**ortho-Fluorofentanyl**
Nominal conc (ng/mL)	0.5	0.5	1	1	4	1	4
Mean	0.51	0.49	1.00	1.03	3.80	0.96	4.24
Accuray%	102.4	98.4	99.9	103.2	94.9	95.9	106.0
CV%	5.0	4.0	7.7	6.0	3.0	2.5	4.5

The identity of all compounds was confirmed by the accurate mass (±5 ppm), the retention time (± 0.25 min) and matching against a spectral library (reversed search) recorded using pure standards. An example is presented in figure 2.

Figure 2. Example of confirmation: Carfentanil at 0.5 ng/mL



The identity of Carfentanil was confirmed by the exact mass ±5ppm, the retention time and matching against a library spectrum. Regular

The linearity of the method was good at 50-1000 ng/mL for all compounds. Most of the compounds also showed linearity from 0.5-10 ng/mL. The Lower Limit of quantitation was set with regards to this.

The stability of all compounds in urine was good at room temperature (nominally 22 ° C), in refrigerator (nominally 4° C) and in refrigeration (nominally -18° C).

DISCUSSION

There is a steady increase in use and demand of methods for known/unknown screening and untargeted/targeted quantitation of analytes in complex biological matrices. Methods based on Thermo Scientific Q Exactive are suitable for this task since data can be collected in untargeted mode, which makes gives the possibility for retrospective interrogation of data. The instrument is sensitive enough for quantitation, even at the low levels required for Fentanyls, and it can provide HRAM spectra that together with retention time and accurate mass.

CONCLUSIONS

In this study, we report a robust, reliable, and reproducible method for screening of Fentanyls in Urine for clinical research or forensic toxicology.

REFERENCES

1. Swedish National Threat Assessment on fentanyl analogues and other synthetic opioids, Swedish National Board of Forensics, The Swedish Police Authority, National Operations Department, A503.217/2017, October 2019

TRADEMARKS/LICENSING