TECHNICAL NOTE 73391

Screening and semi-quantification of fentanyls in human urine using high-resolution Orbitrap mass spectrometry for clinical research or forensic toxicology

Authors: Magnus Olin, Thermo Fisher Scientific, Hägersten, Sweden

Yufang Zheng, Unilabs, Eskilstuna, Sweden

Keywords: Fentanyls, urine, screening, Orbitrap, high-resolution mass spectrometry, NPS

Application benefits

- Fast identification and semi-quantitation of fourteen different fentanyl analogs in human urine
- Minimal offline sample preparation with direct injection of diluted urine

Goal

Implementation of an analytical method for the screening and semi-quantification of fourteen different fentanyl analogs in human urine using a Thermo Scientific™ Q Exactive™ Focus hybrid quadrupole-Orbitrap™ mass spectrometer.

Introduction

Fentanyl is an opioid used as a pain medication together with other medications for anesthesia. Fentanyl and fentanyl analogs made illegally are also used as recreational drugs. Fentanyl and its analogs are significantly stronger than morphine, with some analogs (carfentanil) exhibiting ~10,000 times higher strength than regular pain medications. The use and abuse of fentanyl and its analogs are also known to cause serious side effects, ranging from respiratory depression to death. Problems related to fentanyl and its analogs are still prevalent in many countries according to reports from the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA).

Sample preparation was performed by dilution of urine and addition of internal standard, followed by injection onto a Thermo Scientific™ UltiMate™ 3000 RSLC system. A Q Exactive Orbitrap Focus hybrid quadrupole-Orbitrap mass spectrometer with heated electrospray ionization (HESI) was used for detection by full scan data-dependent MS/MS (FSdd-MS^2). Identification was performed by

matching of the MS/MS spectrum of the target compound to the recorded library MS/MS spectrum. Semi-quantification was performed around the proposed cutoff level and was based on a two-point calibration curve using internal standard calibration. Four stable isotope labeled fentanyl analogs were used as internal standards.

Experimental

Target analytes

Chemical structures of the fourteen fentanyl analogs are presented in Figure 1. The internal standards used for each compound are indicated in Table 1.

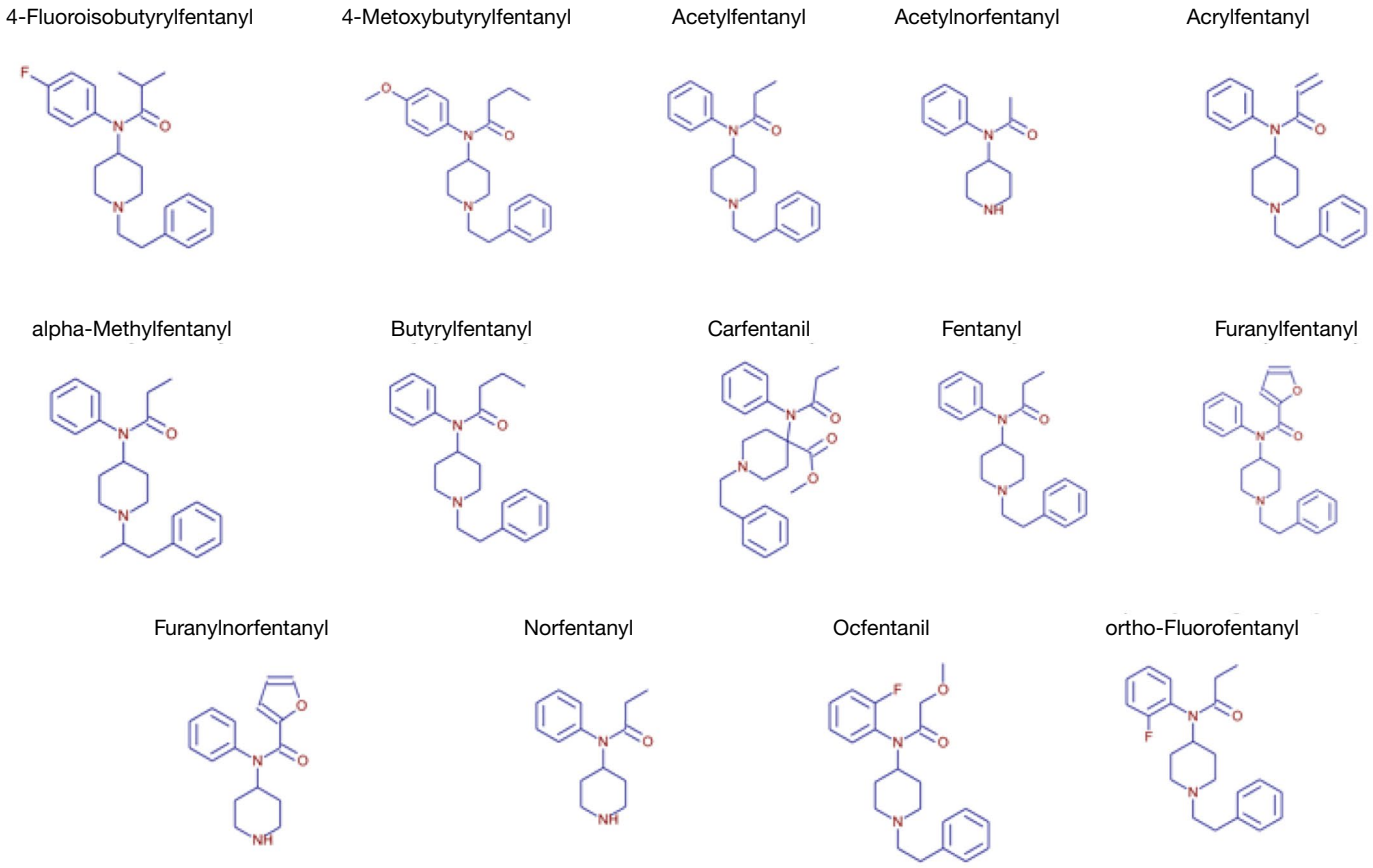

Figure 1. The fourteen fentanyl analogs

Table 1. Compounds, monoisotopic masses and limit of quantitation

Analyte	Chemical formula	Monoisotopic mass (M+H)	Calibrated range (ng/mL)	Internal standard
4-Fluoroisobutyrylfentanyl	$C_{23}H_{29}FN_2O$	369.2337	2–10	Fentanyl-D_5
4-Metoxybutyrylfentanyl	$C_{24}H_{32}N_2O_2$	381.2537	4–20	Fentanyl-D_5
Acetylfentanyl	$C_{21}H_{26}N_2O$	323.2118	1–5	Acetylfentanyl-$^{13}C_6$
Acetylnorfentanyl	$C_{13}H_{18}N_2O$	219.1492	2–10	Noracetylfentanyl-$^{13}C_6$
Acrylfentanyl	$C_{22}H_{26}N_2O$	335.2118	1–5	Fentanyl-D_5
alpha-Methylfentanyl	$C_{23}H_{30}N_2O$	351.2431	4–20	Fentanyl-D_5
Butyrylfentanyl	$C_{23}H_{30}N_2O$	351.2431	2–10	Fentanyl-D_5
Carfentanil	$C_{24}H_{30}N_2O_3$	395.2329	0.5–2.5	Fentanyl-D_5
Fentanyl	$C_{22}H_{28}N_2O$	337.2274	0.5–2.5	Fentanyl-D_5
Furanylfentanyl	$C_{24}H_{26}N_2O_2$	375.2067	1–5	Fentanyl-D_5
Furanylnorfentanyl	$C_{16}H_{18}N_2O_2$	271.1441	1–5	Norfentanyl-D_5
Norfentanyl	$C_{14}H_{20}N_2O$	233.1648	4–20	Norfentanyl-D_5
Ocfentanil	$C_{22}H_{27}FN_2O_2$	371.2129	1–5	Acetylfentanyl-$^{13}C_6$
ortho-Fluorofentanyl	$C_{22}H_{27}FN_2O$	355.2180	4–20	Fentanyl-D_5

Sample preparation

Urine was centrifuged at 10,000 × g for 5 minutes. Calibrators were prepared at the concentrations described in Table 1 by spiking blank human urine with known amounts of pure standard solutions. Certified standard solutions were obtained from Cerilliant, Chiron AS, and Cayman Chemical. First, 10 µL of sample, calibrator, or control sample was diluted with 100 µL of Milli-Q® water (MilliporeSigma) containing internal standards. Then, 10 µL were injected onto the LC-MS system.

Liquid chromatography

Liquid chromatography was performed on an UltiMate 3000 RSLC system using the following mobile phases:

- Mobile phase A: Water + 0.1% NH_4OH
- Mobile phase B: Methanol

Chromatographic separation was achieved on a Waters® Acquity UPLC® BEH 2.1 × 100 mm, 1.7 µm analytical column run at 40 °C. The gradient profile is described in Table 2.

Table 2. Gradient profile

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	0.4	10	90
3.01	0.6	0	100
4.0	0.6	0	100
4.1	0.6	95	5
4.5	0.6	95	5

Mass spectrometry

Detection was performed in full scan – data dependent MS/MS (dd-MS^2) acquisition mode on a Q Exactive Focus hybrid quadrupole-Orbitrap mass spectrometer, equipped with a heated electrospray ionization (HESI) ion source run in positive ion mode. The mass spectrometer was operated in targeted (Confirmation) mode using an inclusion list, which triggers the acquisition of an MS/MS spectrum when the signal for included compounds is above the set threshold. The ion source conditions and mass spectrometry settings are presented in Table 3 and Table 4, respectively. The inclusion list is presented in Table 5.

Table 3. Ion source settings

Parameter	Value
Sheath gas flow rate	75 AU
Aux gas flow rate	12.5 AU
Sweep gas flow rate	2 AU
Spray voltage	3500 AU
Capillary temperature	300 °C
Aux gas heater temperature	450 °C
S-lens RF level	90

Table 4. Mass spectrometer settings

Parameter	Value
General	
Polarity	Positive
dd-MS^2	Confirmation
In-source CID	-
Full MS	
Scan range	215–400 *m/z*
Resolution	70,000
# Scan ranges	1
AGC target	1E+06
Maximum IT	auto
Microscans	1
Spectrum data type	Profile
dd-MS^2 confirmation	
Apex trigger	-
Resolution	17,500
Isolation window	1.5 *m/z*
Isolation offset	-
(N)CE / Stepped (N)CE	ce: 30
Default charge state	1
AGC target	5E+04
Maximum IT	Auto
Loop count	2
Minimum AGC target	1
Intensity threshold	1
Dynamic exclusion	1.0 s
Spectrum data type	Profile

Table 5. Inclusion list

Mass [*m/z*]	Start [min]	End [min]	(N)CE	(N)CE type	MSX ID	Comment
219.1492	1.8	2.6	17	CE		Acetylnorfentanyl
233.1648	2	2.8	17	CE		Norfentanyl
271.1441	2	2.8	17	CE		Furanylnorfentanyl
323.2118	3	3.4	25	CE		Acetylfentanyl
335.2118	2.9	3.7	30	CE		Acrylfentanyl
337.2274	3	3.8	30	CE		Fentanyl
351.2431	3	3.8	30	CE		alpha-Methylfentanyl, Butyrfentanyl
355.2180	3	3.8	30	CE		ortho-Fluorofentanyl
369.2337	3	3.8	30	CE		4-Fluoroisobutyrfentanyl
371.2129	2.8	3.6	30	CE		Ocfentanil
375.2067	2.9	3.7	30	CE		Furanylfentanyl
381.2537	3	3.8	30	CE		4-Methoxybutyrfentanyl
395.2329	3.1	3.5	20	CE		Carfentanil

Identification

The identification of the analytes at the lower limit of quantitation (LLOQ) or higher, was based on the exact mass (extracted using a mass accuracy of ±5 ppm), the retention time, and by matching the acquired MS/MS spectrum against a spectral library. The library was in mzVault™ format and was prepared by recording high-resolution MS/MS spectra of pure standards. The library search was performed in reversed search mode.

Method evaluation

The method developed in this study was tested and analytically validated in terms of accuracy and precision at the lower limit of quantitation (LLOQ), matrix effects, and linearity.

Analytical performance was evaluated using test samples prepared by spiking blank human urine with known amounts of the test compounds. Accuracy was evaluated from the concentrations determined in the test samples as percent of the nominal concentrations. Intra-assay precision was evaluated as the coefficient of variation (%CV) using the same set of test samples. The evaluation of accuracy and precision was performed at LLOQ and analyzed in replicates of five.

Matrix effects were evaluated around the LLOQ level by analyzing urine from 10 different sources, all spiked at the LLOQ concentration. The %CV and the maximum deviation from the mean value were calculated.

The possibility to obtain semi-quantitative results for samples with higher concentrations was investigated by evaluating the linearity of the response for all compounds. Linearity was evaluated by analyzing spiked samples in two different concentration ranges, 0.5–10 ng/mL and 50–100 ng/mL, and evaluating the coefficient of determination (R^2) of the calibration curve.

The capability of the method to unambiguously identify all compounds at the LLOQ was also investigated.

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 4.1 software.

Results and discussion

The data demonstrated good accuracy for the method, with values always between 80.9% and 107.6%. The %CV for inter-assay precision was always below 13.4%. Results for accuracy and precision are reported in Table 6.

The results from the investigation of matrix effects are presented in Table 7. Despite the fact that all compounds did not have a stable isotope labeled analog as an internal standard, the matrix effects were moderate. The %CV of all 10 individually spiked matrix samples was below 20% for all compounds, and the maximum deviation from average was below 30% for all compounds.

All compounds were identified and confirmed in a concentration range 0.5–5 ng/mL. An example of identification using the library search is presented in Figure 2, where the identity of carfentanil was confirmed by using the exact mass (extracted with a mass accuracy of 5 ppm), the retention time, and the match against an mzVault library.

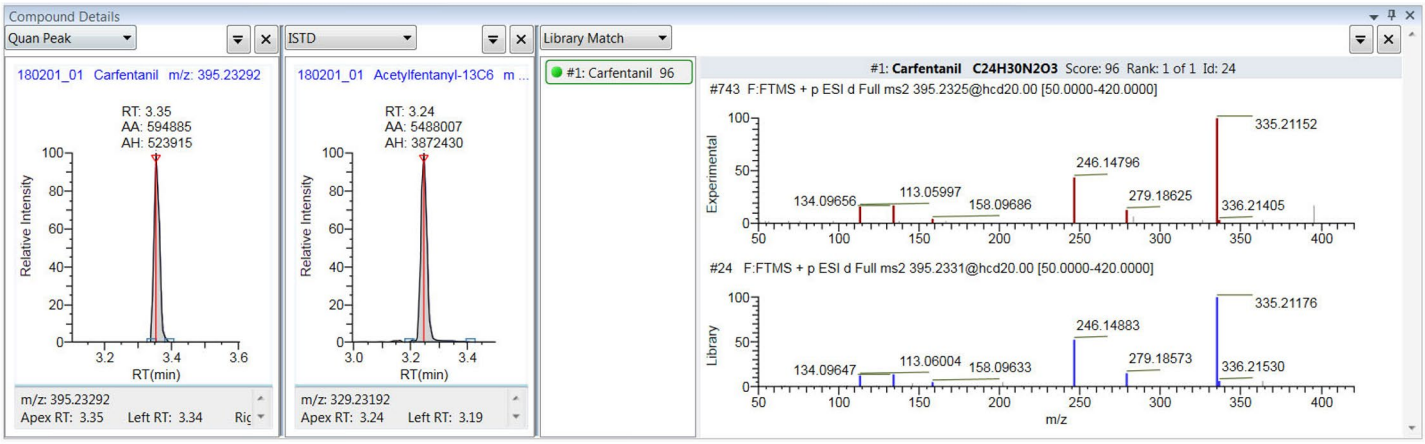

Figure 2. Example of identification of carfentanil at 0.5 ng/mL

Table 6 (part 1). Accuracy and precision results at cutoff level (ng/mL) (n=6)

	4-Fluoroisobutyr-fentanyl	4-Methoxy-butyrfentanyl	Acetyl-fentanyl	Acetylnor-fentanyl	Acryl-fentanyl	alpha-Methyl-fentanyl	Butyryl-fentanyl
Cutoff level	2.00	5.00	1.00	2.00	1.00	4.00	2.00
Mean calc. conc.	2.15	4.93	0.81	2.29	0.96	4.00	2.00
Accuracy (%)	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

Table 6 (part 2). Accuracy and precision results at cutoff level (ng/mL) (n=6)

	Carfentanil	Fentanyl	Furanyl-fentanyl	Furanylnor-fentanyl	Norfentanyl	Ocfentanil	ortho-Fluoro-fentanyl
Cutoff level	0.50	0.50	1.0	1.0	4.0	1.0	4.0
Mean calc. conc.	0.51	0.49	1.00	1.03	3.80	0.96	4.24
Accuracy (%)	102.4	98.4	99.9	103.2	94.9	95.9	106
CV (%)	5.0	4.0	7.7	6.0	3.0	2.5	4.5

Table 7 (part 1). Results from investigation of matrix effects (n=10)

	4-Fluoroisobutyr-fentanyl	4-Methoxy-butyrfentanyl	Acetyl-fentanyl	Acetylnor-fentanyl	Acryl-fentanyl	alpha-Methylfentanyl	Butyryl-fentanyl
Mean (ng/mL)	2.31	4.93	0.89	2.61	1.02	4.63	2.31
CV%	11.1	13.4	10.0	11.0	9.4	11.5	11.6
Max Dev%	19.5	24.2	16.2	16.9	23.0	18.2	18.3

Table 7 (part 2). Results from investigation of matrix effects (n=10)

	Carfentanil	Fentanyl	Furanylfentanyl	Furanylnor-fentanyl	Norfentanyl	Ocfentanil	ortho-Fluoro-fentanyl
Mean (ng/mL)	0.55	0.54	1.11	1.20	4.10	1.06	4.72
CV%	10.6	7.5	12.6	9.5	11.4	6.5	10.7
Max Dev%	21.9	16.9	24.8	15.6	27.8	12.8	22.4

thermo scientific

Conclusions

The results presented in this technical note represent the performance of a method designed for screening of fentanyl and analogs in urine. The method is fast and offers reliable results regarding semi-quantitation and identification around the cutoff level. However, although this screening method provides reliable results, verification of the screening results using a separate analytical method is good practice. Such a procedure will not only confirm findings in the screening method but also rule out human errors like mixing up samples, one of the most common errors in laboratories.

Find out more at **thermofisher.com/clinicalresearch**

ThermoFisher SCIENTIFIC