TECHNICAL NOTE 73957

Quantitation of 22 fentanyl analog compounds on an Orbitrap Exploris 120 mass spectrometer

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Goal

Demonstrate the sensitivity and benefits of high resolution accurate mass (HRAM) on the Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer for fast, accurate analysis of 22 fentanyl-related compounds, including fentanyl in urine for clinical research and toxicology. Compounds were quantitated using the precursor ion collected by Full MS scan and confirmed by matching experimentally collected MS^2 spectra to an in-house MS^2 library of 213 fentanyl analog compounds (Full MS-ddMS^2 Scan). This experimental mode can be used for retrospective analysis, while also achieving absolute quantitation. The method demonstrates how sensitivity is achieved through HRAM Orbitrap technology.

Application benefits

- Quantitation of 22 known fentanyl-related compounds, including fentanyl, with associated internal standards by HRAM mass spectrometry
- Confident identification by retention time, accurate *m/z*, isotopic pattern, and MS/MS spectral matching
- Targeted quantitation with the capability for retrospective analysis

Introduction

The United States is facing an opioid crisis that includes not only the abuse of prescription drugs but also synthetic opioids. According to the Centers for Disease Control and Prevention (CDC), rates of overdose deaths involving synthetic opioids other than methadone, but including fentanyl, increased by 10% from 2017 to 2018. Over 31,000[1] people died from overdoses involving these compounds in 2018 alone. The CDC, in collaboration with Cerilliant Corporation™, released a Traceable Opioid Material™ Kit (TOM Kit™) consisting of 22 fentanyl analog compounds[2] with matched carbon-13 and nitrogen-15 isotopically labeled internal standards for quantitation and confident identification. Here we present a method for quantitation of the TOM Kit compounds in urine that includes sample preparation by supported liquid extraction (SLE), quantitation by full scan HRAM data, and confident confirmation by retention time, accurate *m/z*, isotopic pattern matching and experimental matching of collected MS^2 spectra to an in-house MS^2 library of 213 fentanyl analog compounds.

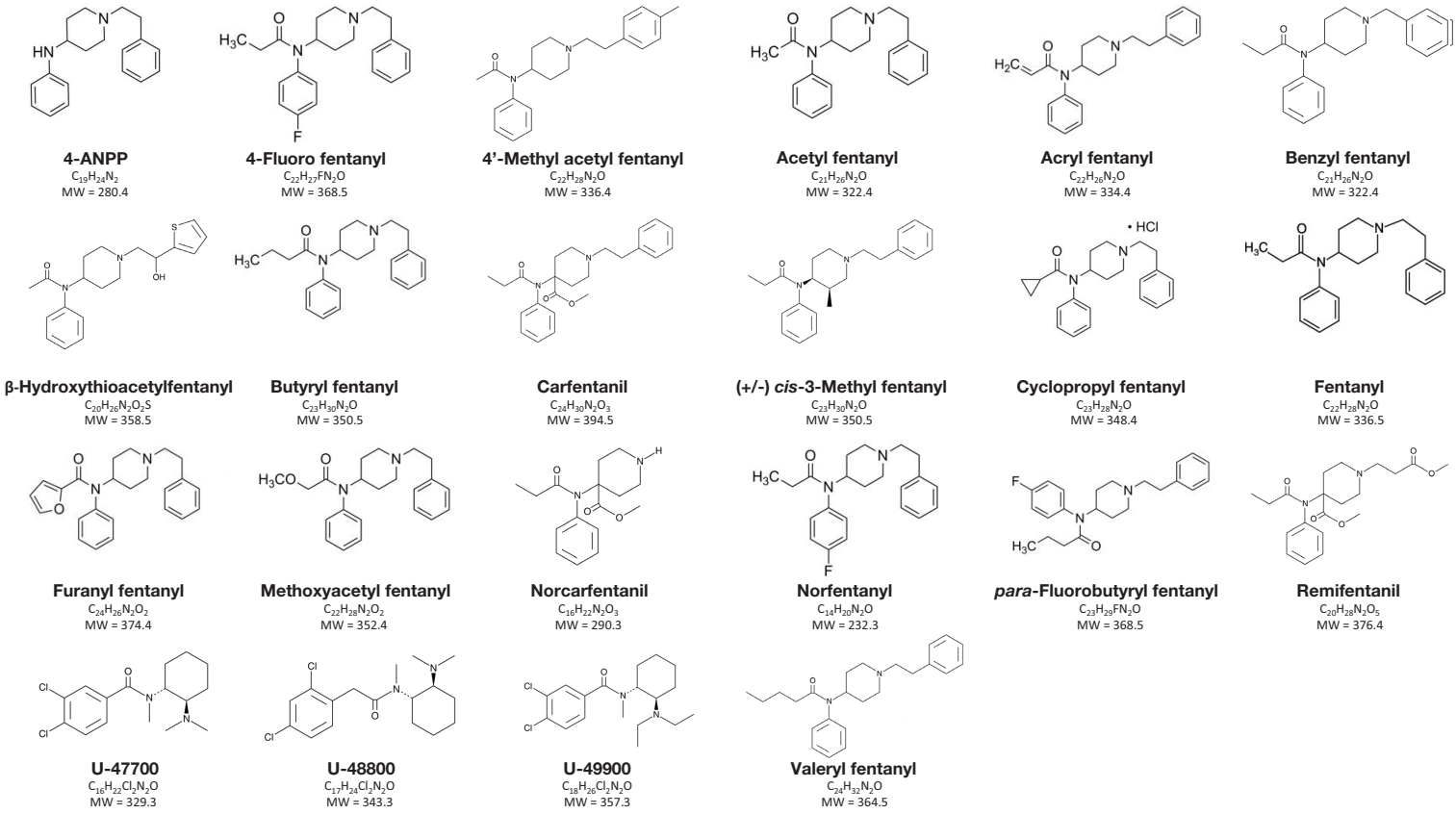

Figure 1. Fentanyl and fentanyl-related compounds analyzed

Experimental

Target analytes

A panel consisting of 22 fentanyl analog compounds was analyzed and quantitated by internal standard calibration. The chemical structures of these compounds are presented in Figure 1. Each fentanyl analog compound had its own isotopically labeled internal standard as provided in the TOM Kit and is listed in Table 4.

Creation of an MS^2 library and targeted inclusion list

An MS^2 library with associated retention times for 213 fentanyl analog compounds was created by analyzing fentanyl compounds contained in two of the CDC TOM Kits (Opioid CRM Kit and Fentanyl Analog Screening Kit with Emergent Panels)[2,3] by the same LC-MS/MS method described here. Fentanyl compounds were prepared in 100 ng/mL mixes containing 10–14 compounds each to avoid co-eluting isomers. Following analysis by LC-MS/MS, the acquired MS^2 spectra were validated for retention time and spectral quality and were used to create an MS^2 library using Thermo Scientific™ mzVault™ software as seen in Figure 2. This library was utilized by Thermo Scientific™ TraceFinder™ software to identify fentanyl analog compounds by matching the experimental MS^2 spectra with the library spectra. An inclusion list was created that contained the name of the fentanyl compound, accurate *m/z*, and retention time to be used as part of the mass spectrometer instrument method.

Figure 2. Library of MS² spectra of 213 fentanyl compounds as viewed in mzVault software

Calibration standards and control samples

All non-labeled standards were combined into a stock solution and diluted in methanol to a concentration of 10 µg/mL. Isotopically labeled standards were combined and diluted in methanol to a concentration of 10 µg/mL as well. A concentrated calibration solution for the non-labeled standard was prepared by diluting the non-labeled standard mix with human urine to a final concentration of 100 ng/mL. A 13-point calibration curve ranging from 0.01 ng/mL to 100 ng/mL was prepared by serial dilution with human urine as the diluent. Quality control (QC) standards were prepared at concentrations of 1.0 ng/mL, 2.5 ng/mL, and 25 ng/mL in urine. A 25 ng/mL internal standard (IS) working solution was created by combining the carbon-13 and nitrogen-15 isotopically labeled standards and diluting in water. The IS working solution was added to the non-labeled standards prior to sample preparation by SLE for absolute quantitation.

Supported liquid extraction preparation

Each of the standards in the 13-point calibration curve and the QC standards were prepared by supported liquid extraction (SLE) with the Biotage™ ISOLUTE™ SLE+400 plates prior to analysis. A 200 µL aliquot was taken from each calibration and QC standard and was combined with 25 µL of the IS working solution and 175 µL of 0.2 % v/v formic acid in water in a microcentrifuge tube and vortexed for 5 seconds. The diluted standard was then pipetted onto the SLE plate with a 2 mL deep-well collection plate attached underneath. The sample was allowed to absorb onto the SLE plate material for five minutes. Then, 900 µL dichloromethane (DCM) was added, without pressure, for five minutes until a short burst of air was applied at 0.7 bar positive to the SLE plate for elution of the entire aliquot of DCM. Next, an additional 900 µL of DCM was added, without pressure, for five minutes, and then a short burst of air was applied at 0.7 bar of pressure to the SLE plate. The collection plate was removed, and the DCM solvent was evaporated under nitrogen gas at a temperature of 50 °C. The dried standards were then resuspended with 100 µL of water:methanol (90:10) (v:v). The 96 deep-well plate was sealed with foil and shaken at 1000 rpm for 5 minutes. The samples were then transferred to autosampler vials with glass inserts for analysis.

Liquid chromatography

A 15.5-minute chromatographic method was used for the chromatographic separation of fentanyl and fentanyl analog compounds using a Thermo Scientific™ Vanquish™ Flex UHPLC system consisting of a binary pump, a column compartment, and an autosampler. The separation was performed on a Thermo Scientific™ Accucore™ Phenyl Hexyl column (2.1 mm × 100 mm, 2.6 µm) maintained at 40 °C. Mobile phases consisted of 2 mM ammonium formate in water with 0.1% formic acid for mobile phase A and a mixture of 2 mM ammonium formate in methanol:acetonitrile (50:50 v:v) with 0.1% formic acid for mobile phase B. Chromatographic separation of a 15 µL injected sample was achieved by gradient elution under the conditions described in Table 1.

Table 1. LC gradient

Time (min)	Flow rate (mL/min)	% A	% B
0	0.5	99	1
1	0.5	99	1
10	0.5	1	99
11.5	0.5	1	99
11.51	0.5	99	1
15.5	0.5	99	1

Mass spectrometry

Compounds were detected on an Orbitrap Exploris 120 mass spectrometer equipped with a Thermo Scientific™ OptaMax™ NG ion source with a heated electrospray ionization probe. The mass spectrometer source and scan settings are listed in Table 2 and Table 3, respectively. Full MS scan was used for quantitation, while targeted MS^2 scan by data-dependent analysis was used for confirmation. A targeted mass inclusion list containing all 213 fentanyl compounds with expected retention times was used to give preference to fentanyl compounds for MS^2 data collection.

Table 2. Source parameters for Orbitrap Exploris 120 mass spectrometer

Source parameter	Value
Positive ion	3,500 V
Sheath gas	55 AU
Aux gas	10 AU
Sweep gas	1 AU
Ion transfer tube temp.	325 °C
Vaporizer temp.	350 °C
Probe position	1.5, M

Table 3. MS scan parameters

Source parameter	Value
Global settings	
Scan type	Full MS/ddMSMS
RF %	80
Mass calibration	Thermo Scientific™ Easy-IC™ option
Data type	Profile
Full MS scan	
Resolution	60,000
Max injection time	Auto
Scan range	100–1000
ddMSMS	
Intensity threshold	1.0e5
Targeted mass list tolerance	≤5 ppm, ±0.5 min
Isolation window	1.5 *m/z*
Stepped NCE	18.75, 37.5, 56.25
First mass	40 *m/z*

Data analysis

Data was acquired and processed using Thermo Scientific™ TraceFinder™ 5.1 software. Quantitation was performed by internal standard calibration. The mzVault library of 213 fentanyl MS^2 spectra was referenced. The isotopic pattern score and library match score thresholds were set to 90% and 80%, respectively, for confident compound identification.

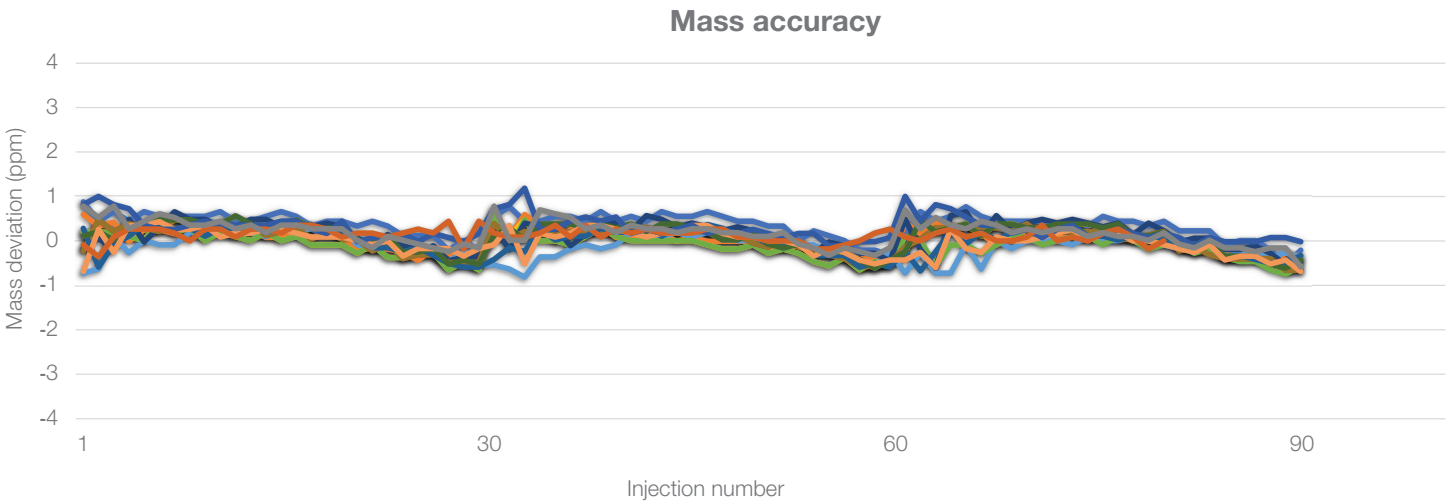

Figure 3. Mass accuracy for 90 calibration and quality control standards injected over three days of analysis. % RSD for all repeated injected standards was < 0.5%.

Results and discussion

All fentanyl compounds eluted chromatographically between 3.8 and 6.2 minutes as shown in Figure 6. Internal calibration was used for all 22 fentanyl compounds with each fentanyl standard having a corresponding stable-isotope-labeled internal standard.

Method evaluation

The limit of quantification (LOQ) for each analyte was determined as the lowest value in the calibration curve giving an average % bias between nominal and back-calculated concentration within ±20%, a % CV below 20% for 3 replicate injections of calibrators, and a correlation coefficient greater than 0.99 for all used calibrators. Acceptance criteria for the quality controls was ±20%. Analysis of calibration curves and quality controls were repeated across three days for intra-day reproducibility and LOQ assessment. The calibration approach was determined using data from three replicate injections of each calibrator across three days with results listed in Table 4. Mass accuracy for 90 injections of calibration and QC standards across three days was <1 ppm and is shown in Figure 3. Chromatograms of LLOQ displayed in "stick mode" depicting Full MS scan and accurate *m/z* for 4-ANPP is shown in Figure 4.

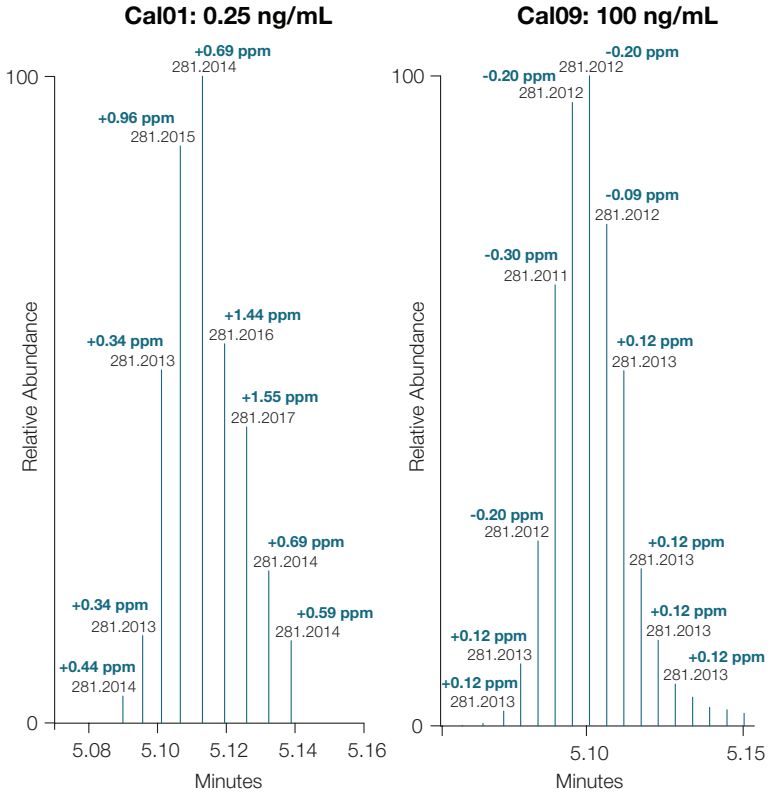

Figure 4. Full MS Scan chromatograms for 4-ANPP at the LLOQ (Cal01, 0.25 ng/mL) and highest calibrator (Cal09, 100 ng/mL). Chromatograms are depicted in "stick" mode where each stick represents a Full MS Scan collected across the chromatographic peak. The accurate mass detected and calculated mass difference from theoretical mass are depicted to show the excellent mass accuracy of the instrument; <2 ppm for all scans collected.

Fentanyl analog compounds results

Data processing results for 4-ANPP can be viewed in Figure 5. Details of the calibration approach and LOQ for each analyte are reported in Table 4. The Orbitrap Exploris 120 mass spectrometer demonstrated LOQs ≤ 0.25 ng/mL for all but six of the 22 fentanyl compounds, and all were quantitated linearly up to 100 ng/mL, having an R^2 above 0.99. Representative chromatograms for the lowest calibrator for 4-ANPP (0.25 ng/mL), norfentanyl (0.1 ng/mL), and norcarfentanil (1.0 ng/mL) are shown in Figure 7.

A method published by the CDC for the detection of fentanyl compounds by LC-MS/MS was followed for sample preparation guidelines with only slight variations made to the formic acid content prior to supported liquid extraction.[4] This was necessary when early eluting compounds, norfentanyl and norcarfentanil, were not recovered post-extraction using 0.1% formic acid. Different pH and solvents were tested for extraction efficiency with ultimate recovery of norfentanyl and norcarfentanil recovered with 0.2% formic acid.

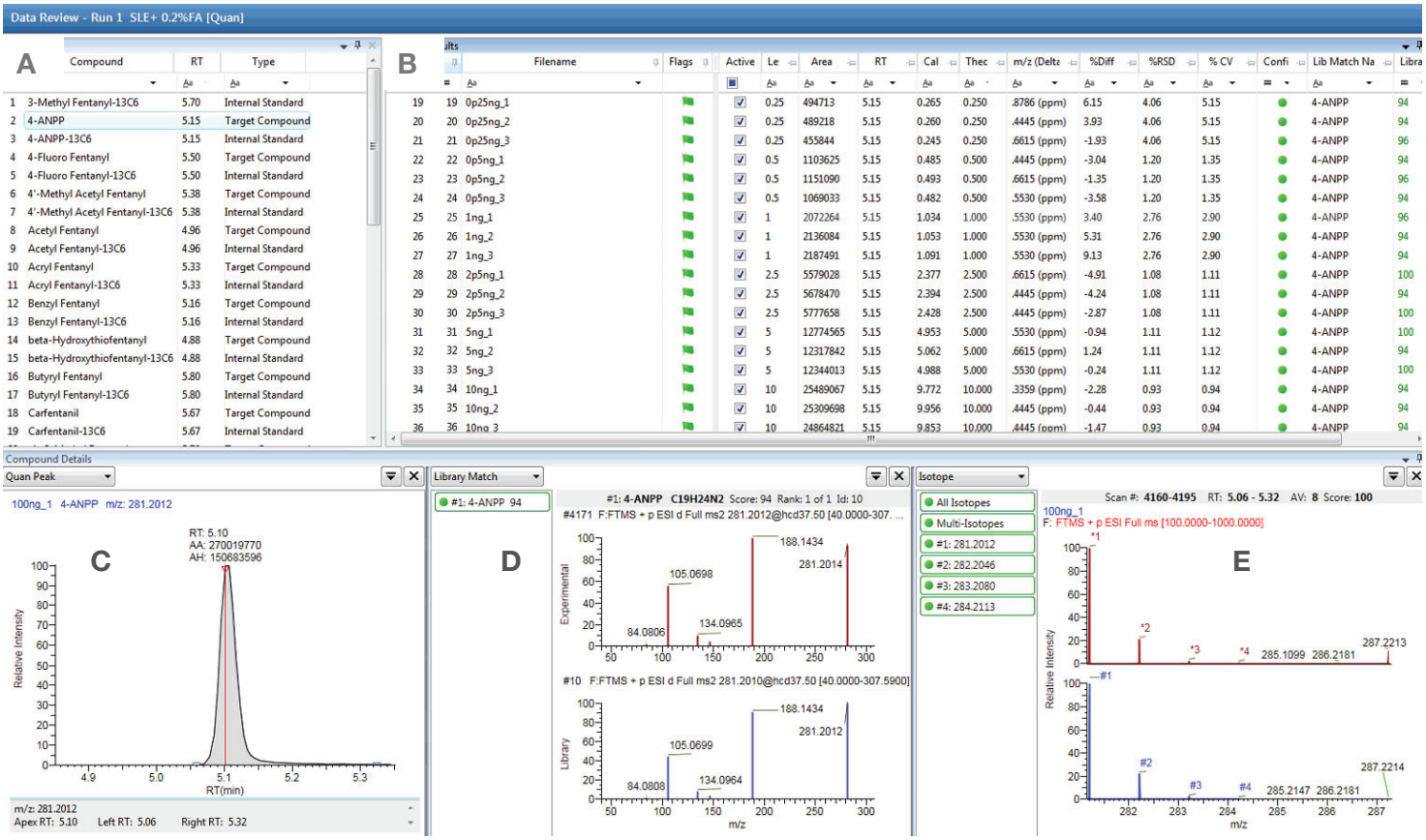

Figure 5. Fentanyl and fentanyl-related compound results as viewed in TraceFinder 5.1 software. The compound of interest, 4-ANPP, is highlighted in panel (A), the quantitative results listed in panel (B), chromatogram of the quantitative peak in panel (C), MS^2 library match with experimental spectra shown in red and library spectra in blue listed in panel (D), and isotope matching of experimental in red and theoretically calculated in blue in panel (E).

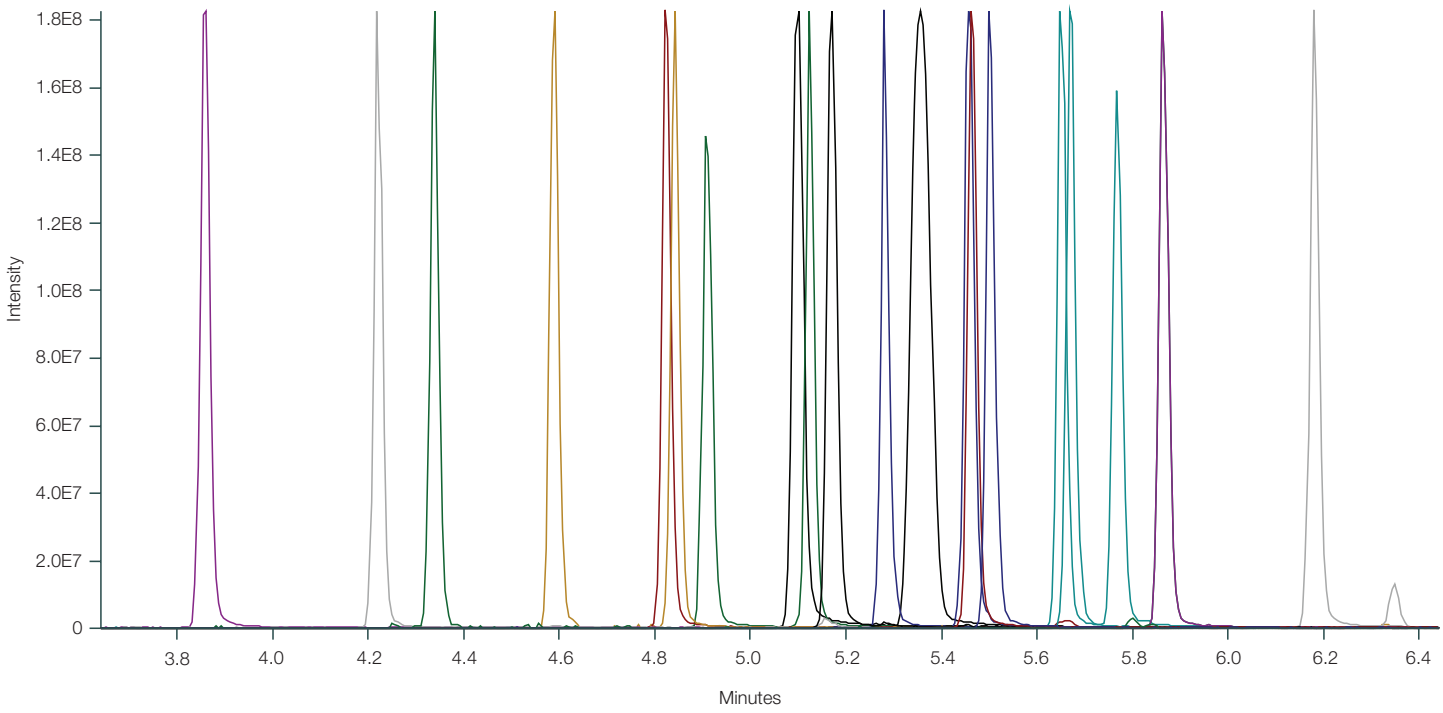

Figure 6. Total ion chromatogram of fentanyl and fentanyl-related compounds at 100 ng/mL

Table 4. Fentanyl standards analyzed. Molecular formula of fentanyl compound with corresponding internal standard, accurate protonated *m/z*, retention time, limit of quantitation, and curve weighting. All compounds were detected linearly to a maximum concentration of 100 ng/mL with the origin ignored.

Compound	Molecular formula	Internal standard	$[M+H]^+$	Retention time (min)	LOQ (ng/mL)	Weighting
4-ANPP	$C_{19}H_{24}N_2$	4-ANPP-$^{13}C_6$	281.20123	5.11	0.25	1/X
4-Fluoro fentanyl	$C_{22}H_{27}FN_2O$	4-Fluoro fentanyl-$^{13}C_6$	355.21802	5.47	0.25	1/X
4'Methyl acetyl fentanyl	$C_{22}H_{28}N_2O$	4'Methyl acetyl fentanyl-$^{13}C_6$	337.22744	5.37	0.25	1/X
Acetyl fentanyl	$C_{21}H_{26}N_2O$	Acetyl fentanyl-$^{13}C_6$	323.21179	4.96	0.25	1/X
Acryl fentanyl	$C_{22}H_{26}N_2O$	Acryl fentanyl-$^{13}C_6$	335.21179	5.28	0.25	1/X
Benzyl fentanyl	$C_{21}H_{26}N_2O$	Benzyl fentanyl-$^{13}C_6$	323.21179	5.13	0.25	1/X
β-Hydroxythiofentanyl	$C_{20}H_{26}N_2O_2S$	β-Hydroxythiofentanyl-$^{13}C_6$	359.17878	4.84	0.25	1/X
Butyryl fentanyl	$C_{23}H_{30}N_2O$	Butyryl fentanyl-$^{13}C_6$	351.24309	5.80	0.25	1/X
Carfentanil	$C_{24}H_{30}N_2O_3$	Carfentanil-$^{13}C_6$	395.23292	5.67	0.25	$1/X^2$
cis-3-Methyl fentanyl	$C_{23}H_{30}N_2O$	3-Methyl fentanyl-$^{13}C_6$	351.24309	5.70	0.25	1/X
Cyclopropyl fentanyl	$C_{22}H_{28}N_2O$	Cyclopropyl fentanyl-$^{13}C_6$	337.22744	5.64	0.25	1/X
Fentanyl	$C_{22}H_{28}N_2O$	Fentanyl-$^{13}C_6$	337.22744	5.42	0.25	1/X
Furanyl fentanyl	$C_{24}H_{26}N_2O_2$	Furanyl fentanyl-$^{13}C_6$	375.20670	5.50	0.50	$1/X^2$
Methoxyacetyl fentanyl	$C_{22}H_{28}N_2O_2$	Methoxyacetyl fentanyl-$^{13}C_6$	353.22235	4.83	0.25	$1/X^2$
Norcarfentanil	$C_{16}H_{22}N_2O_3$	Norcarfentanil-$^{13}C_6$	291.17032	4.26	1.00	1/X
Norfentanyl	$C_{14}H_{20}N_2O$	Norfentanyl-$^{13}C_6$	233.16484	3.86	0.10	1/X
para-Fluorobutyryl fentanyl	$C_{23}H_{29}FN_2O$	*para*-Fluorobutyryl fentanyl-$^{13}C_6$	369.23367	5.87	0.50	1/X
Remifentanil	$C_{20}H_{28}N_2O_5$	Remifentanil-$^{13}C_6$	377.20710	4.59	1.0	1/X
U-47700	$C_{19}H_{26}N_2O_5$	U-47700-$^{13}C_2$, $^{15}N_2$	363.19145	5.17	0.25	$1/X^2$
U-48800	$C_{16}H_{22}Cl_2N_2O$	U-48800-$^{13}C_3$, $^{15}N_2$	329.11820	5.46	0.50	$1/X^2$
U-49900	$C_{18}H_{26}Cl_2N_2O$	U-49900-$^{13}C_5$	357.14950	5.45	0.25	1/X
Valeryl fentanyl	$C_{24}H_{32}N_2O$	Valeryl fentanyl-$^{13}C_6$	365.25874	6.18	2.5	1/X

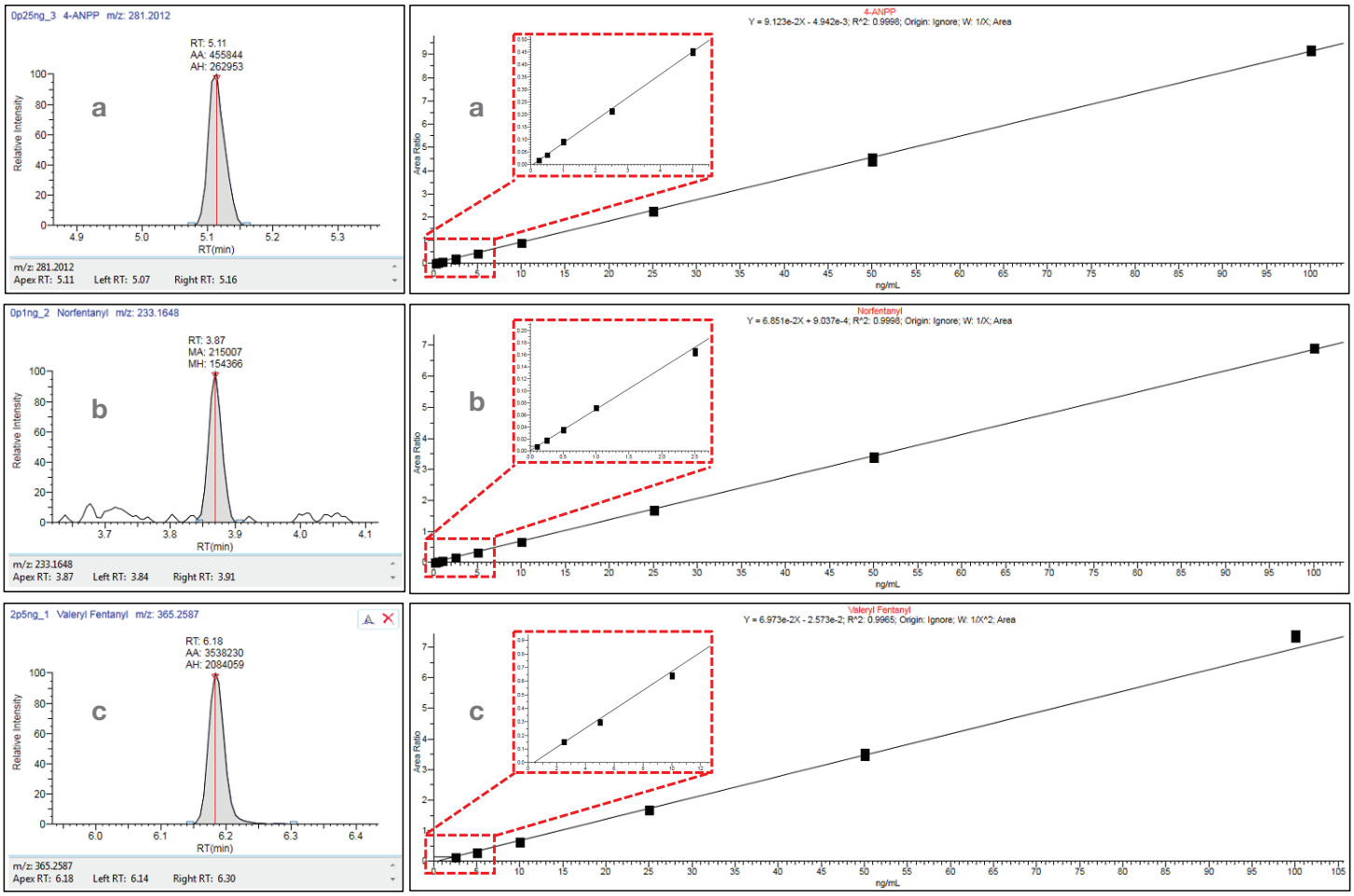

Figure 7. Chromatograms at the LLOQ and calibration curves for (a) 4-ANPP, (b) norfentanyl, and (c) valeryl fentanyl

Conclusion

A sensitive and reproducible method for the quantitative measurement of 22 fentanyl compounds in urine was developed on the Vanquish Flex UHPLC system and Orbitrap Exploris 120 mass spectrometer. Sample preparation involved sample cleanup by SLE followed by a 15-minute UHPLC gradient separation and Orbitrap HRAM detection. The method offers confident compound identification by confirmation of retention time, accurate *m/z*, isotopic pattern matching to calculated theoretical isotopic pattern, and matching experimentally collected MS^2 spectra to an in-house MS^2 library of 213 fentanyl analog compounds. The method enables retrospective data analysis, due to Full MS scan data collection, while also achieving the sensitivity required for routine quantitation.

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