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Analysis of 17 fentanyls in plasma and blood by UPLC-MS/MS with interpretation of findings in surgical and postmortem casework

Jonathan P. Danaceau [a], Michelle Wood [b], Melissa Ehlers [c], Thomas G. Rosano [c,d]

[a] Waters Corporation, Milford, MA, USA
[b] Waters Corporation, Wilmslow, UK
[c] Albany Medical Center (AMC), Albany, NY, USA
[d] National Toxicology Center, Albany NY, USA

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ABSTRACT

The opioid crisis is linked to an increased misuse of fentanyl as well as fentanyl analogs that originate from the illicit drug market. Much of our current understanding of fentanyl and fentanyl analog use in our communities comes from postmortem toxicology findings. In the clinical settings of addiction medicine and pain management, where the opioid abuse potential is high, the use of fentanyl, as well as specific fentanyl analogs, may be underestimated due to limited plasma testing and limited availability of assays with suitable analytical sensitivity and selectivity to detect misuse of fentanyls. We report plasma and blood assays for 17 fentanyls (these include fentanyl, fentanyl analogs, fentanyl metabolites and synthetic precursors) in clinical, and medical examiner, casework. A mixed-mode solid phase extraction of diluted plasma or precipitated blood was optimized for maximum recovery of the fentanyls with minimized matrix effects. Analysis was performed using a Waters ACQUITY UPLC I-Class interfaced with a Waters Xevo TQ-S micro tandem quadrupole mass spectrometer. Method parameters were optimized and validated for precision, accuracy, carryover, linearity and matrix effects. Application studies were performed in postmortem blood obtained in 44 fentanyl-related fatalities and in serial plasma samples from 18 surgical patients receiving intravenous fentanyl therapy while undergoing parathyroidectomy. Fentanyls found in postmortem cases included fentanyl, norfentanyl, despropionyl-fentanyl (4-ANPP), beta-hydroxy fentanyl (β-OH fentanyl), acetyl fentanyl, acetyl norfentanyl, methoxyacetyl fentanyl, furanyl fentanyl, cyclopropyl fentanyl, and para-fluorobutyryl fentanyl, with fentanyl, norfentanyl, 4-ANPP and β-OH fentanyl predominating in frequency. Fentanyl concentrations ranged from 0.2 to 56 ng/mL and fentanyl was nearly always found with 4-ANPP, norfentanyl and β-OH fentanyl. Concentrations of other fentalogs ranged from <1 to 84 ng/mL (extrapolated). In the surgical cases, fentanyl was detected and quantified along with norfentanyl and β-OH fentanyl, but without detection of 4-ANPP in any of the samples. The association and relative concentrations of β-OH fentanyl, fentanyl and norfentanyl in the postmortem and clinical studies indicated a metabolic, rather than an illicit, source of β-OH fentanyl.

1. Introduction

On a global level, overdose deaths due to opioids have dramatically increased in the past several years [1–3]. In North America, the situation remains particularly challenging; while deaths due to heroin and other natural and semi-synthetic opioids have increased substantially, deaths due to synthetic opioids such as fentanyl and fentanyl analogs (fentanyls) increased three-fold from 2015 to 2017 and 10-fold from 2013 to 2017 [3]. The increased confiscations of fentanyl and fentanyl analogs by law enforcement agencies during this period points to illicit production as a major factor in fueling the opioid crisis [4,5]. These data also point to the growing need for identification and quantitation of the fentanyls in forensic as well as clinical toxicology.

Abbreviations: ACN, Acetonitrile; 4-ANPP, 4-Anilino-N-phenethylpiperidine (despropionyl-fentanyl); β-OH fentanyl, beta-hydroxy fentanyl; LC-MS, liquid chromatography-mass spectrometry; LLOQ, lower limit of quantitation; MRM, multiple reaction monitoring; PFBF, para-fluorobutyryl fentanyl; UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry.

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Sensitive and selective identification and quantitation of a growing number of structurally similar fentanyls in biological matrices is analytically challenging. In medical examiner casework and clinical toxicology, where urine and postmortem blood are often the primary drug test specimens, significant progress has been made in the development and application of definitive methods for the analysis of the fentanyls [6–9]. As a result, many fentanyl-related, as well as fentanyl analog-related fatalities, have been reported along with methods of analysis of these agents in forensic casework [10–17]. Methods for alternative matrices, such as oral fluid, have also been reported recently [18–19]. In addition to the analytical challenge is a lack of certainty in interpretation regarding the metabolic versus synthetic-byproduct origin of a number of fentanyls found in postmortem cases. It is known for example, that 4-ANPP is a byproduct of fentanyl synthesis and may be present as an impurity in preparations. However, it has also been proposed as a minor inactive metabolite of fentanyl and some other fentanyl analogs [20–24], consequently the contribution of production or metabolism to the 4-ANPP levels measured in postmortem blood remains a question. In addition, while it is well established that norfentanyl is a major metabolite of fentanyl, hepatic microsomal studies suggest that hydroxy fentanyl may also originate from metabolism of fentanyl [21,25]. Clinical measurement of 4-ANPP and β-OH fentanyl in fentanyl treated patients is limited but could add to our knowledge of a metabolic versus illicit source of these agents. Therefore, in addition to postmortem blood testing is the need for plasma determination of the fentanyls by sensitive and selective methods that will allow clinical monitoring of the high opioid risk population and may further improve our understanding of the fentanyls.

We describe methods for the analysis of fentanyls in plasma and blood, with subsequent application to 44 fatalities involving the fentanyls and to sequential plasma samples from 18 surgical patients receiving intravenous fentanyl therapy while undergoing parathyroidectomy.

2. Materials and methods

2.1. Reference material and reagents

The fentanyls (acetyl norfentanyl, norfentanyl, norcarfentanil, remifentanil acid, remifentanil, acetyl fentanyl, 4-ANPP, fentanyl, furanyl fentanyl, 3-methylfentanyl, carfentanil, methoxyacetyl fentanyl, butyryl fentanyl, para-fluorobutyryl fentanyl, sufentanil) and the internal standards (acetyl norfentanyl-$^{13}C_6$, norfentanyl-d5, acetyl fentanyl-$^{13}C_6$, fentanyl-d5, sufentanil-d5) were obtained from Cerilliant (Round Rock, TX, USA). β-OH fentanyl and cyclopropyl fentanyl were obtained from Cayman Chemical (Ann Arbor, Michigan, USA). Ammonium acetate (trace metal grade), strong ammonia solution (28–30%), ammonium formate (trace metal grade), zinc sulfate heptahydrate, formic acid (Optima grade, 99%), and 85% phosphoric acid solution were obtained from Fisher Scientific (Waltham, MA). LC-MS grade acetonitrile (ACN) and methanol were also from Fisher Scientific. A stock standard of the fentanyls was prepared at analyte concentrations of 10 μg/mL in methanol. Internal standard stock solutions were also prepared in methanol at a concentration of 2 μg/mL. A combined working internal standard solution (1 ng/mL) was prepared daily in an aqueous solution containing 0.1 M zinc sulfate and 0.1 M ammonium acetate for the whole blood analysis. For the plasma analysis, a working internal standard solution (0.2 μg/mL) was prepared in 4% aqueous phosphoric acid. Human whole blood and plasma for quality control pool preparations were obtained from Lampire Biological Supply (Pipersville, PA, USA).

2.2. Postmortem and clinical samples

Discarded postmortem whole blood samples from fentanyl and fentanyl analog-related fatalities were initially collected under medical examiner orders and tested by the Forensic Toxicology Laboratory at the Albany Medical Center (Albany, NY) between December 2018 and August 2019. After all medical examiner testing was completed, in accordance with the laboratory's forensic protocol, the samples were de-identified and tested in the study.

Discarded, de-identified plasma samples used in the study were initially collected intra-operatively under physician orders for the measurement of parathyroid hormone levels from patents undergoing minimally invasive parathyroidectomy at the Albany Medical Center Hospital and Albany Medical Center South Clinical Campus. After the measurement of parathyroid hormone was complete, the samples were used for this current study. The study was performed with review and approval by the Albany Medical College Institutional Review Board.

Subjects received 50–150 μg of intravenous fentanyl upon induction of anesthesia, with further administration as needed for the care of the patients. A baseline specimen was collected prior to the initial administration of fentanyl in all but case 17 where administration occurred before the baseline sample was drawn. Following the requested clinical testing and after sample discard, the samples were de-identified and stored frozen until analysis. Each patient had between 2 and 6 specimens drawn after the initial fentanyl administration; samples were collected intra-operatively (approximately 1–2 h duration) and therefore represent acute administration conditions.

2.3. Blood assay

Analyte negative blood bank blood (300 μL) was added to 300 μL of internal standard reagent containing 0.1 M zinc sulfate and ammonium acetate. Samples were vortex-mixed and allowed to sit for 5 min. Six-hundred microliters of ice-cold ACN:methanol (50:50 v/v) were then added and the samples were briefly vortex-mixed and allowed to sit for a further 5 min before centrifugation for 10 min at 7000×g. Four-hundred microliters of the supernatant were diluted with 1 mL of 4% phosphoric acid and then added, in two aliquots, to a Waters Oasis PRiME MCX μElution plate (Waters Corp, Milford, MA, USA). After loading the plate, the wells were washed with 200 μL of 0.1 M ammonium formate containing 2% formic acid, followed by 200 μL of methanol. Samples were eluted with two 25 μL volumes of ACN:methanol (50:50 v/v) containing 5% strong ammonia solution. All samples were then diluted with 50 μL of water:ACN:formic acid (97:2:1 v/v/v). Five microliters of the diluted eluates were injected onto the UPLC-MS/MS system. Calibration curves for whole blood samples ranged from 50 to 20000 pg/mL. Low, medium, and high quality control pools were prepared at combined analyte concentrations of 75, 2500, and 15000 pg/mL, respectively.

Blood extracts were analyzed on a Waters TQ-S micro tandem mass spectrometer (Waters Corp) equipped with a Waters ACQUITY I-Class UPLC system. Analytical separation was performed using a Waters BEH C_{18} column (1.7 μm, 2.1 × 100 mm) together with a solvent gradient of mobile phase A (MPA) comprising 0.1% formic acid in water, and mobile phase B (MPB), comprising 0.1% formic acid in ACN. The chromatographic flow rate was 0.6 mL/minute. The gradient started at 15% MPB, increased over 2 min to 55% MPB and then to 90% MPB over the next 0.5 min prior to returning to initial conditions. Electrospray analysis was performed in positive ionization mode with a capillary voltage at 1.5 kV. Nitrogen was used as the desolvation gas at 500 °C with a flow of 1000 L/hr and as a cone gas at a flow of 150 L/hr. Mass spectrometer parameters and retention times of the fentanyls and internal standards are listed in Table 1. Phospholipid content was profiled by performing a precursor ion scan of m/z 184.2, over the range m/z 400–900 at a cone voltage of 30 V and a collision energy of 30 eV. Compound identification was based upon reten-

tion time tolerances of ± 2% and product ion ratios were based on the draft OSAC guidelines for mass spectral data acceptance [26], as defined below:

Relative abundance	Acceptance criteria
Greater than 50%	20%
20–50%	25%
Less than 20%	30%

2.4. Plasma assay

Plasma samples (250 μL) were diluted with 250 μL plasma internal standard solution (4% formic acid containing internal standards at 200 pg/mL) and loaded onto a Waters Oasis PRiME MCX μElution plate. As with the postmortem blood assay, wells were then washed with 200 μL of 100 mM ammonium formate containing 2% formic acid, followed by 200 μL of methanol. Samples were eluted with two 25 μL volumes of ACN:methanol (50:50 v/v) containing 5% strong ammonia solution. All samples were then diluted with 50 μL of water:ACN:formic acid (97:2:1 v/v/v). Five microliters of diluted extract were injected onto the UPLC-MS/MS system. Plasma analysis was also performed on a Waters TQ-S micro mass spectrometer. Separation was achieved on a Waters CSH C_{18} analytical column (1.7 μm, 2.1 × 100 mm) and the mobile phase gradient was the same as that used for whole blood analysis.

Calibration ranges for fentanyl and norfentanyl were 4–1000 pg/mL with low, medium and high control pools prepared at concentrations of 30, 150 and 600 pg/mL. Calibration ranges for the remaining fentanyls were 2–500 pg/mL with the three quality control pools prepared at 15, 75 and 300 pg/mL.

2.5. Method validation

Both methods were validated according to SWGTOX guidelines for recovery, matrix effects, accuracy, precision, linearity, specificity, selectivity, sensitivity [27]. Recovery and matrix effects were determined quantitatively by analyte addition experiments [28,29]. Whole blood matrix effects and recoveries were evaluated at three different concentrations, 250, 1000, and 10000 pg/mL. Plasma recoveries and matrix effects were also determined by analyte addition experiments. Due to the more limited range of the plasma assay, recoveries and matrix effects were determined at a single concentration of 1000 pg/mL.

Quantitative validation was achieved by analyzing 6 replicates for inter-day accuracy and precision and 5 replicates between days at 4 QC levels. Lower limits of quantitation were defined as those concentrations at which accuracy was within 20% of target concen-

Table 1
Individual MS parameters of the fentanyls and internal standards analyzed in postmortem whole blood samples. Retention times (R.T.) correspond to the BEH C_{18} column. The first product ion is the quantitative transition.

R.T.	Name	M + H$^+$	Product Ions	Cone voltage (V)	Collision energy (eV)	Internal standard
0.68	Acetyl Norfentanyl	219.1	84.1	4	18	Acetyl Norfentanyl-$^{13}C_6$
			136.1	4	20	
0.67	Acetyl Norfentanyl-$^{13}C_6$	225.1	84.1	4	18	
0.91	Norfentanyl	233.1	84.1	5	15	Norfentanyl-d5
			150.1	5	15	
0.90	Norfentanyl-d5	238.2	84.1	5	15	
1.03	Norcarfentanil	291.1	231.2	5	14	Norfentanyl-d5
			259.2	5	10	
1.06	Remifentanil acid	363.2	214.1	5	30	Acetyl Fentanyl-$^{13}C_6$
			113.0	5	18	
1.18	Remifentanil	377.2	113.0	5	30	Acetyl Fentanyl-$^{13}C_6$
			228.1	5	20	
1.24	Methoxyacetyl Fentanyl	353.2	188.1	10	22	Acetyl Fentanyl-$^{13}C_6$
			105.1	10	37	
1.29	Acetyl Fentanyl	323.1	188.1	5	34	Acetyl Fentanyl-$^{13}C_6$
			105.0	5	22	
1.28	Acetyl Fentanyl-$^{13}C_6$	329.2	188.1	5	22	
1.33	β-OH Fentanyl	353.2	186.1	10	26	Acetyl Fentanyl-$^{13}C_6$
			204.2	10	25	
1.48	4-ANPP	281.2	188.1	8	16	Fentanyl-d5
			105.0	8	30	
1.50	Fentanyl	337.2	188.1	5	22	Fentanyl-d5
			105.0	5	35	
1.49	Fentanyl-d5	342.2	188.1	5	22	
1.55	Furanyl Fentanyl	375.2	188.1	5	22	Fentanyl-d5
			105.0	5	38	
1.59	Cyclopropyl Fentanyl	249.2	188.1	10	25	Fentanyl-d5
			105.1	10	35	
1.65	(±)-cis-3-Methylfentanyl	351.2	202.2	5	22	Sufentanil-d5
			105.0	5	35	
1.66	Carfentanil	395.2	355.2	10	16	Sufentanil-d5
			113.1	10	32	
1.69	Butyryl Fentanyl	351.2	188.1	5	22	Sufentanil-d5
			105.0	5	38	
1.76	para-Fluorobutyryl Fentanyl	369.2	188.1	5	24	Sufentanil-d5
			105.0	5	40	
1.80	Sufentanil	387.2	238.1	10	18	Sufentanil-d5
			355.2	10	18	
1.79	Sufentanil-d5	392.2	238.1	10	18	

trations and precision was less than 20%. Accuracy and precision requirements for all other concentrations were within 15% of target and less than 15% RSD.

3. Results and discussion

3.1. Whole blood assay: Method development and validation studies

Assay optimization and validation was conducted for all pre-analytical and analytical procedures. Pre-analytical preparation of postmortem blood employed zinc sulfate and ammonium acetate treatment with protein precipitation prior to solid phase extraction. Precipitation solvent conditions were optimized for analyte recovery and a methanol:ACN (50:50 v/v) precipitation was selected because it resulted in visibly clearer supernatants, tightly compact precipitate pellets and consistent recoveries. The addition of phosphoric acid to the sample, prior to the solid phase extraction loading step, was also found to improve recoveries, especially among the more polar fentanyls that included the nor-metabolites and remifentanil acid. Fig. 1 shows the mean recoveries and standard deviations resulting from analysis with four pools of analyte-negative blood supplemented with the fentanyls at a concentration of 1000 pg/mL. Recoveries ranged from 53.6 to 84.5% with relative standard deviations under 10% for all analytes demonstrating extraction efficiency. Overall recovery was a balance between developing a simplified workflow that permitted direct loading of the precipitated sample onto the μElution plate. The relatively high percentage of organic solvent (20%) that resulted from this pretreatment step led to lower recoveries for some of the early eluting compounds. Stable isotopically labelled internal standards were available for the two most affected analytes and recoveries were found to be consistent and reproducible. No conditioning or equilibration was necessary for either matrix, further streamlining the workflow.

An additional advantage of the solid phase extraction method was the reduction of matrix effects to less than 13%, also shown in Fig. 1. Matrix effects in blood can be the result of the co-elution of phospholipids that cause ion suppression [29–32] and build up on the analytical column and within the source region of the mass spectrometer. Mixed-mode solid phase extraction significantly reduced the levels and effects of these phospholipids. Fig. 2 shows the phospholipid profiles of whole blood samples prepared by protein precipitation only in comparison with the results after combined use of precipitation and solid phase extraction. The combination eliminated greater than 99% of the residual phospholipids in the final extract. Recovery and matrix effect studies at lower (250 pg/mL) and higher (10000 pg/mL) concentrations of analytes were also carried out; no concentration-dependent effects were seen, indicating consistent analyte recovery and low matrix effects across the analytical range of the assay.

A rapid chromatographic separation method was developed and optimized for analyte selectivity. Representative chromatograms showing separation of the fentanyls in a postmortem blood extract is displayed in Fig. 3. All fentanyls eluted from the column within two minutes; the total cycle time of four minutes included elution and re-equilibration. The functional isomers butyryl fentanyl and cis-3-methyl fentanyl were not fully baseline-separated, but each isomer produced a unique product ion fragment during collision induced dissociation allowing unambiguous identification and quantification for these analytes (Table 1). Chromatographic interferents were evaluated but not found in either analyte-negative blood or the internal standard preparation.

Only 5 stable isotopically labelled internal standards were available at the time of assay development. Analytes were assigned to internal standards based upon similarities in extraction efficiency (recovery) and matrix effects, followed by retention time.

The assay was further validated for linearity, accuracy, precision, sensitivity, specificity, and carryover according to SWGTOX guidelines [27]. Calibration curves for the whole blood samples (50–20000 pg/mL) were linear with r^2 values $\geq$ 0.997. All analytes were validated for lower limits of quantitation (LLOQ) of 50 pg/mL, apart from remifentanil acid and 4-ANPP, which both validated for LLOQs of 100 pg/mL. Mean (range) inter-assay biases (N = 5 batches) for the fentanyls at 750, 7500 and 15000 pg/mL concentrations in blood were −1.0% (−4.5 to 3.0%), −1.8% (−8.3 to 0%) and −0.2% (−4.4 to 1.5%) respectively. Mean inter-assay relative standard deviations (range) for the fentanyls in replicate low, medium and high control pool concentrations were 3.3% (1.1–16.1%), 2.0% (1.0–3.0%) and 4.5% (3.1–8.9%). Mean carryover (N = 6) was 0.05% (0.00–0.65%) for negative control sample analysis following the analysis of the high calibrator concentration at a concentration

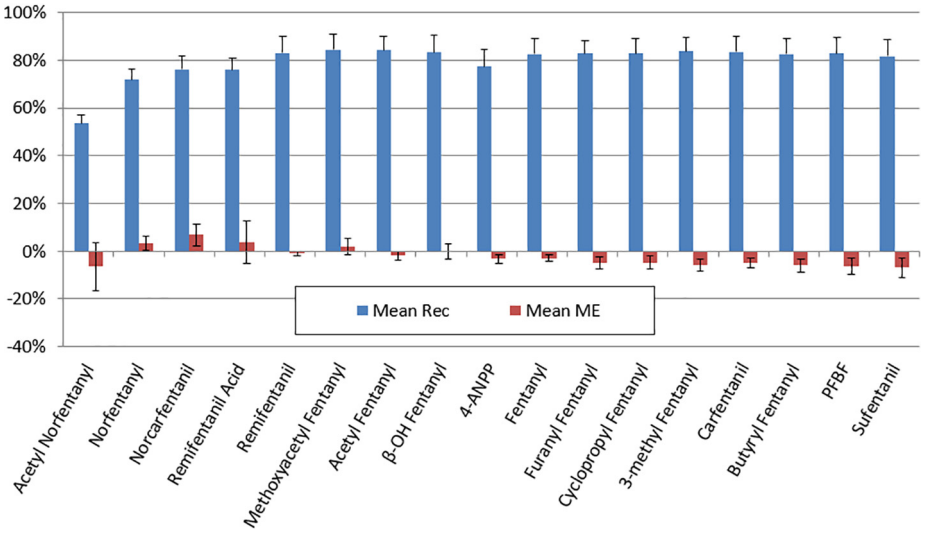

Fig. 1. Mean recovery and matrix effects in the postmortem blood assay. Four lots of blank whole blood were evaluated for recovery and matrix effects as described in the Materials and Methods section. Solid bars represent the mean recovery (blue) or matrix effects (orange). Error bars indicate standard deviations. Recoveries ranged from 53.6 to 84.5%. Matrix effects were all less than 13%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

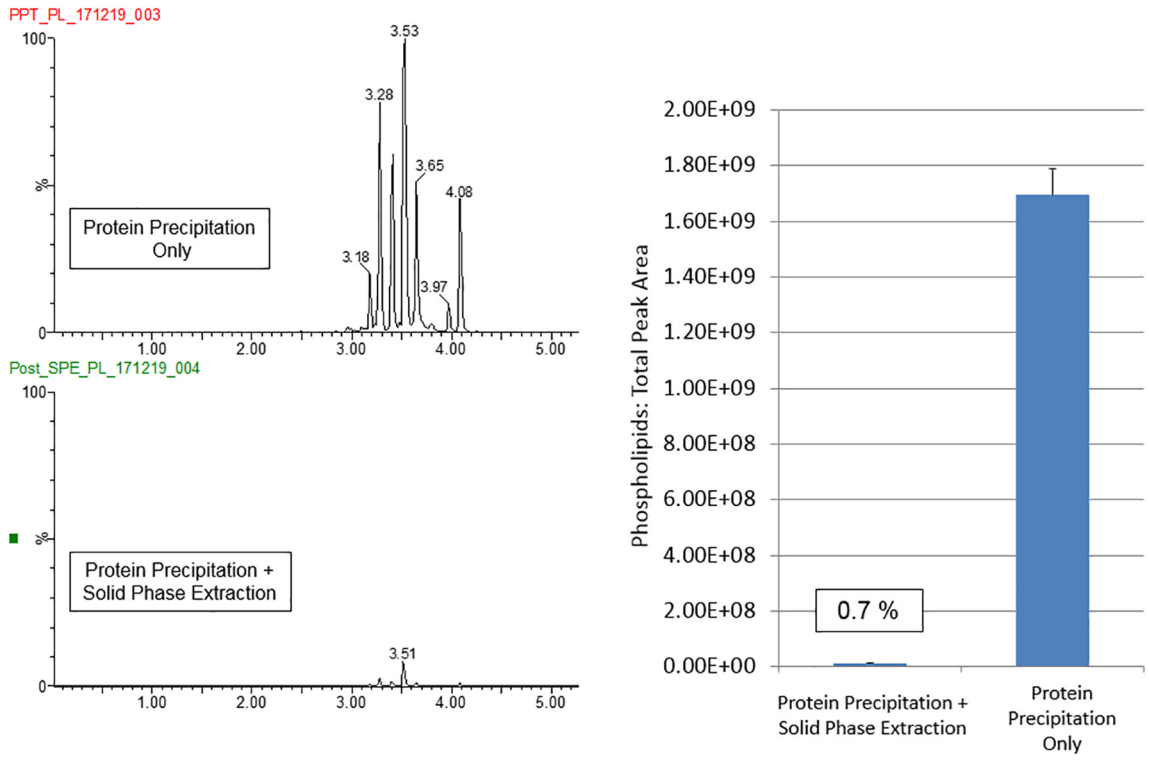

Fig. 2. Comparison of phospholipid content with, and without, solid phase extraction (SPE). On the left are total ion chromatograms of residual phospholipids in blood samples subjected to protein precipitation only (upper) and protein precipitation followed by solid phase extraction (lower). The mean area from each set of samples is shown on the right. SPE removed greater than 99% of residual phospholipids from whole blood samples compared to protein precipitation only.

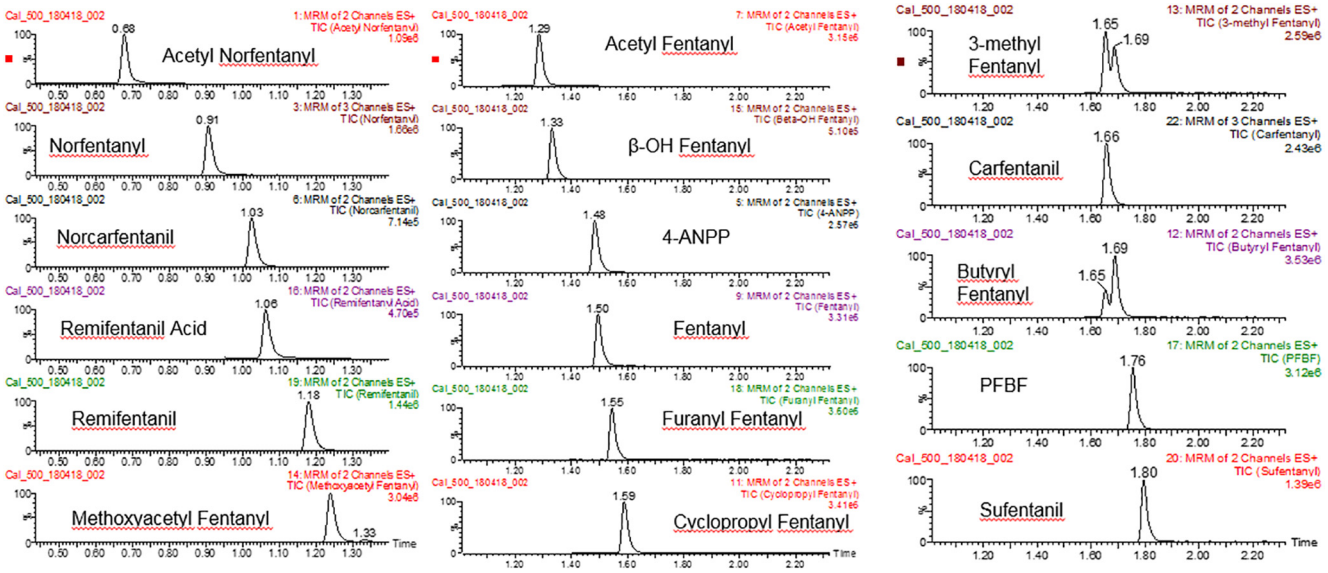

Fig. 3. Chromatography of fentanyls extracted from blank whole blood samples. Separation was achieved on a Waters BEH C_{18} column, 1.7 μm; 2.1 × 100 mm as described in Materials and Methods. All analytes eluted in under 2 min.

of 20000 pg/mL Proficiency of the blood assay was further validated by correlation with an external reference laboratory using 30 postmortem blood samples assayed in the reference lab for 4-ANPP, para-fluorobutyryl fentanyl, and furanyl fentanyl. Fig. 4 shows composite comparison data for the three fentanyl analogs with a regression equation of y = 0.87x – 0.004 and a correlation coefficient of 0.96.

3.2. Plasma assay: Method development and validation studies

The plasma assay was initially validated for acetyl norfentanyl, norfentanyl, methoxyacetyl fentanyl, acetyl fentanyl, β-OH fentanyl, 4-ANPP, and fentanyl only. A low, but consistent level carryover of 4-ANPP in the whole blood assay (mean 0.65%, range 0.55–0.73%) was observed but met the validation criteria for the concen-

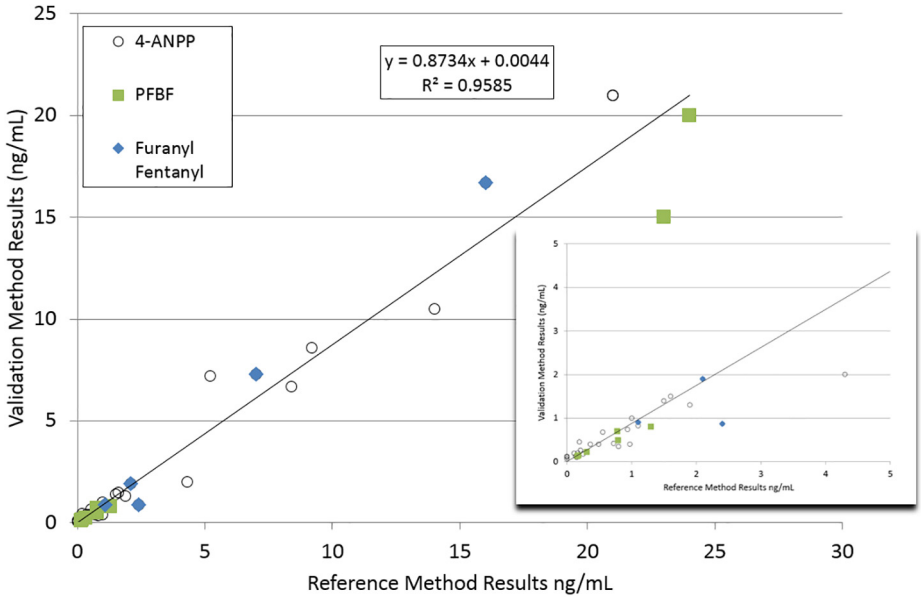

Fig. 4. Blood concentrations of 4-ANPP, para-fluorobutyryl fentanyl, and furanyl fentanyl in postmortem cases compared with reference laboratory results. The equation represents the linear regression of the results of all positive samples combined.

tration range used in the postmortem casework. In the subsequent development of a plasma assay, however, a lower limit of quantitation was required and was achieved by use of an alternate analytical column. Studies with a CSH C_{18} analytical column revealed a mean ($N = 6$) 4-ANPP carryover of only 0.15% (range 0.11–0.18%) in analyte negative plasma analyzed after analysis of plasma containing a high 4-ANPP concentration of 2500 pg/mL. The CSH C_{18} column was therefore adopted for use in the plasma assay and performed with comparable selectivity and slightly shortened retention time for the individual fentanyls compared to the BEH column.

Analytical performance studies with the plasma assay resulted in extraction recoveries ranging between 88 and 95% and standard deviations under 9.5% across all the fentanyls. Matrix effects ranged from −8% to −22% with standard deviations of less than 14%. All calibrations were linear with r^2 values ≥ 0.997. The lower limits of quantitation (LLOQ) were 2 pg/mL for acetyl norfentanyl, 4 pg/mL for norfentanyl and fentanyl, and 5 pg/mL for the remaining compounds. Average inter-assay biases at low, medium and high quality control pool concentrations were 0.3% (range −4.3–6.7%), 1.6% (−12.0–13.40%) and −2.3% (-5.5–0.7%), respectively. Precision studies showed inter-assay relative standard deviations for corresponding control pool concentrations of 8.0% (3.0–17.1%), 5.3% (2.8–9.3%) and 3.3% (2.1–5.2%).

3.3. Application of assays in postmortem blood and clinical plasma

Availability of validated blood and plasma assays allowed a comparative study of postmortem blood and clinical samples. Analysis of fentanyls was conducted in a cohort of postmortem blood samples from 44 fatalities involving fentanyl and fentanyl analogs. Table 2 shows the diversity of fentanyls detected in postmortem blood along with their concentration. The frequency of positive findings were as follows: 4-ANPP (38), fentanyl (36), norfentanyl (35), β-OH fentanyl (28), acetyl fentanyl (13), para-fluorobutyryl fentanyl (9), methoxyacetyl fentanyl (6), furanyl fentanyl (5), cyclopropyl fentanyl (3) and acetyl norfentanyl (2), and resulted from the multiple fentanyl findings in each case. Fentanyl was found in over 80% of the cases at a mean concentration of 14710 pg/mL (median 11000 pg/mL) and in close association with norfentanyl, its major metabolite. A steep progression in mean concentration (pg/mL) for the other fentanyls including acetyl norfentanyl (284), β-OH fentanyl (444), acetyl fentanyl (1096), norfentanyl (2609), 4-ANPP (3583), para-fluorobutyryl fentanyl (4284), cyclopropyl fentanyl (4753). furanyl fentanyl (5522), fentanyl (14705) and methoxyacetyl fentanyl (>20000 pg/mL) was found along with a significant variation in blood concentration between cases. The diversity of fentanyls seen in opioid misuse, together with the most adverse of drug use outcomes, reinforces the requirement for definitive methods, of appropriate selectivity and specificity, to be used in testing for synthetic opioid use, not only in fatalities, but in the clinical setting as well.

In total, 175 identifications of fentanyls were made in the 44 postmortem cases. While fentanyl was present in many of the cases, the fentanyl analogs were significant among these findings. The analogs para-fluorobutyryl fentanyl, methoxyacetyl fentanyl, furanyl fentanyl, and cyclopropyl fentanyl were present in the blood from 20 of the fatalities and appeared consistent with fentanyl analog administration and overdose. These four analogs were found along with fentanyl use in 12 cases but were also found independent of fentanyl use in eight additional cases where analogs were the only opioids detected in blood. The four analogs also predominated in concentration relative to other detected fentanyls in over 70% of the fatalities in which they were involved. Methoxyacetyl fentanyl was found in cases collected early in the study at concentrations that exceeded the calibration range of the assay. Concentration ranges for cyclopropyl fentanyl (3420–6130 pg/mL), furanyl fentanyl (867–16700 pg/mL) and para-fluorobutyryl fentanyl (141–20000 pg/mL) also varied considerably but remained within the limits of quantitation of the blood assay. It should also be noted that the acetyl fentanyl analog was also found in 13 postmortem cases, two with concentrations exceeding 4000 pg/mL and the rest with levels less than 500 pg/mL.

The detection patterns of β-OH fentanyl and 4-ANPP raise further questions regarding potential metabolic or precursor sources in these cases. β-OH fentanyl has been referenced as an illicitly produced fentanyl analog associated with toxicity [33], but fentanyl metabolism also has been suggested by *in vitro* microsomal and hepatocyte studies [20,21]. *In vivo* studies in support of fentanyl metabolism to β-OH fentanyl have not been previously

Table 2
Fentanyls and their concentration (pg/mL) in de-identified postmortem blood collected from fatalities involving fentanyl and fentanyl analogs.

Case	4-ANPP	Fentanyl	Norfentanyl	β–OH fentanyl	Acetyl fentanyl	Acetyl norfentanyl	Furanyl fentanyl	Methoxyacetyl fentanyl	Cyclopropyl fentanyl	para-Fluorobutyryl fentanyl
1	21,500						16,700			
2	1970						885			751
3	1100	282	71				867			
4	7190						1860			697
5	15,700							84,500		
6	118	5330	1120	134						15,100
7		5700	1010	426						
8	147	2860	4350	313						490
9	6750	579	319					30,000		
10	1350	2340	287	118						
11	394	9130	1860	169						225
12	3500							36,500		
13	8620							24,700		
14	390	10,400	5030	483					4710	
15	116								6130	
16	124	6620	1810	188						141
17	117	18,800	3640	297	74					
18	1470	36,700	12,500	954						
19	403	24,600	1410	265	69					
20	294	13,000								20,100
21	182	14,400	1550	272						
22	742	51,000	10,200	2560						
23	212	4100	1050	116				121	3420	
24	1390	24,800	4030	247						
25	45,300				142			69,900		
26	677	20,900	3550	352						143
27	263	6480	1280	277						
28	416	28,500	5260	1200	364					
29	1860	56,400	3270	738	445					
30	185	12,300	3240	436						
31	828	31,600	1440	338						
32	226	17,800	13,600	905	299	87				
33	10,500	212	53				7300			
34	447	15,100	2420	519						
35	451	41,300	311							
36		2050	146		52					909
37	347	12,100	1840	208	8130	481				
38	528	11,600	357		109					
39	194	9360	564	139	192					
40		7790	910		4060					
41		12,500	308	125						
42		2420	413	156	80					
43	139	7020	1240	447	231					
44		3290	892	53						

reported to our knowledge. A metabolic source of 4-ANPP has been suggested by an early report of three patients with qualitative identification of 4-ANPP following intravenous administration of fentanyl [20] and by a hepatic microsomal study [21]. It is known, however, that 4-ANPP is a precursor intermediary in the synthesis of fentanyl. A metabolic versus illicit production source of β-OH fentanyl and 4-ANPP in our cases is not clear from the postmortem data alone but several observations can be made. First, β-OH fentanyl was present in 28 of the fatality cases but did not predominate in opioid concentration in any of the cases. Importantly, β-OH fentanyl showed a striking 100% association with the presence of fentanyl and also was not detected in the eight cases where fentanyl was not detected. On average, β-OH fentanyl concentrations were 3.4% of the fentanyl concentrations and ranged up to 11%. The close association of fentanyl with β-OH fentanyl and the lower levels of β-OH fentanyl relative to fentanyl in these overdoses are consistent with a metabolic source of the β-OH fentanyl but the possibility of illicit exogenous use cannot be ruled out. Second, 4-ANPP was present in blood from 38 cases and was detected in cases with, and without, evidence of fentanyl use. The concentration of 4-ANPP ranged widely and did not appear to show any correlation with fentanyl or any of the analogs. The furanyl fentanyl and methoxyacetyl fentanyl positive samples had substantial concentrations of 4-ANPP compared with the cyclopropyl fentanyl cases suggesting a possible variation in synthesis or clean up efficiency in illicit laboratory production. These findings suggest a synthesis byproduct origin of 4-ANPP rather than metabolism, but again, the data is only suggestive.

The plasma assay was also applied in a study of clinical samples from patients receiving pharmaceutical grade fentanyl and these results may shed additional light on the questions raised in the postmortem casework, particularly with respect to the source of acetyl fentanyl, β-OH fentanyl and 4-ANPP. We analyzed 70 baseline and post-dose plasma samples from 18 individuals receiving intravenous fentanyl during a surgical procedure. Methoxyacetyl fentanyl, acetyl fentanyl, noracetyl fentanyl and 4-ANPP were not detectable. Fentanyl, norfentanyl and β-OH fentanyl, however, were present as shown in Table 3. As anticipated, the level of fentanyls determined with clinical use is much lower than observed in the fatal overdose cases and the lower limit of quantitation used in the plasma assay was clearly needed. Consistent with the dose and recency of fentanyl administration in these clinical cases, fentanyl was found in highest concentration, with a mean of 397 pg/mL and ranging from 7 to 1500 pg/mL. Even with acute administration of fentanyl, metabolism to norfentanyl was quantifiable in 52 of the 53 samples containing fentanyl. The mean concentration of norfen-

Table 3
Fentanyls and their concentration in plasma from de-identified serial blood samples collected for parathyroid hormone measurement in 18 parathyroidectomy cases at the Albany Medical Center (AMC).

	Sample	Norfentanyl (pg/mL)	β-OH Fentanyl (pg/mL)	Fentanyl (pg/mL)
Case 1 Baseline	AMC Sample 001B	nd	nd	nd
	AMC Sample 002	20.7	5	203.2
	AMC Sample 003	28.4	7.5	172
Case 2 Baseline	AMC Sample 004B	nd	nd	nd
	AMC Sample 005	17.6	nd	526.3
	AMC Sample 006	27.2	8.2	369
	AMC Sample 007	32.6	12.9	366.2
Case 3 Baseline	AMC Sample 008B	nd	nd	nd
	AMC Sample 009	6.8	nd	341.9
	AMC Sample 010	10.3	nd	327.1
	AMC Sample 011	11.4	nd	289.1
Case 4 Baseline	AMC Sample 012B	nd	nd	nd
	AMC Sample 013	20.7	6.7	480
	AMC Sample 014	23.5	5	322
	AMC Sample 015	24.4	nd	283.7
Case 5 Baseline	AMC Sample 016B	nd	nd	nd
	AMC Sample 017	11.1	nd	467
	AMC Sample 018	15.3	nd	330.5
	AMC Sample 019	16.9	nd	448.4
Case 6 Baseline	AMC Sample 020B	nd	nd	nd
	AMC Sample 021	5.8		134.6
	AMC Sample 022	19.7	5.8	307.7
Case 7 Baseline	AMC Sample 023B	nd	nd	nd
	AMC Sample 024	17.5	nd	221.7
	AMC Sample 025	26.1	6.5	322.8
Case 8 Baseline	AMC Sample 026B	nd	nd	nd
	AMC Sample 027	25.3	5.5	561.9
	AMC Sample 028	36.2	19.6	1250.3
	AMC Sample 029	35.2	13.2	963.2
Case 9 Baseline	AMC Sample 030B	nd	nd	nd
	AMC Sample 031	5.3	nd	465.1
	AMC Sample 032	14.8	nd	347.6
	AMC Sample 033	12.6	nd	406.9
Case 10 baseline	AMC Sample 034B	nd	nd	nd
	AMC Sample 035	19.6	nd	616.5
	AMC Sample 036	22.3	9.8	592.6
	AMC Sample 037	26.5	5.1	512.3
Case 11 Baseline	AMC Sample 038B	nd	nd	nd
	AMC Sample 039	44.9	nd	310.9
	AMC Sample 040	36.7	nd	349.3
Case 12 Baseline	AMC Sample 041B	nd	nd	nd
	AMC Sample 042	3.4	nd	194.1
Case 13 Baseline	AMC Sample 043B	nd	nd	nd
	AMC Sample 044	12.6	nd	99.8
	AMC Sample 045	13	nd	92.4
	AMC Sample 046	22.4	nd	175.7
Case 14 Baseline	AMC Sample 047B	nd	nd	nd
	AMC Sample 048	10.5	nd	404.2
	AMC Sample 049	13.3	nd	311.6
	AMC Sample 050	15.1	nd	282
Case 15 Baseline	AMC Sample 051B	nd	nd	nd
	AMC Sample 052	11.5	nd	644.9
	AMC Sample 053	10.4	nd	816.5
Case 16 Baseline	AMC Sample 054B	nd	nd	7.3
	AMC Sample 055	40.9	14.8	352.1
	AMC Sample 056	37.9	13	294.4
Case 17 *	AMC Sample 057	7.6	5.6	1494.1
	AMC Sample 058	13.1	10.8	621.4
	AMC Sample 059	12.1	7.4	500.3
	AMC Sample 060	13	9.1	551.2
	AMC Sample 061	13.6	11.7	411.7
	AMC Sample 062	15.5	12	392.2
	AMC Sample 063	19.8	11.3	295
Case 18 Baseline	AMC Sample 064B	nd	nd	nd
	AMC Sample 065	12.8	5.1	196
	AMC Sample 066	13.4	nd	163.2
	AMC Sample 067	14.9	nd	159.7
	AMC Sample 068	16.6	5.2	151.9
	AMC Sample 069	20.1	nd	136.9

nd = none detected. LLOQs were 5 pg/mL for 4-ANPP and β-OH Fentanyl and 4 pg/mL for norfentanyl and fentanyl.
*Baseline specimen not available for testing.

tanyl was 14 pg/mL with a range of 4–45 pg/mL. Norfentanyl represented 6% of the fentanyl concentration on average, with a range up to 17%. β-OH fentanyl was also measurable in 24 of the fentanyl positive samples but at lower concentration than norfentanyl with an average β-OH fentanyl concentration of 9 pg/mL and a range of 5 to 20 pg/mL. β-OH fentanyl quantitation averaged 44% (19 to 86%) of norfentanyl and 2% (range 0.5–4%) of fentanyl. For comparison, postmortem blood β-OH fentanyl levels averaged 19% (5 to 42%) of the corresponding norfentanyl concentration and 3% (1–11%) of the fentanyls concentration. These data would support the conclusion that β-OH fentanyl is a significant metabolite of fentanyl that is present in the circulation following acute fentanyl administration in either therapeutic or fatal doses.

The complete absence of detectable 4-ANPP in the clinical study may be a result of a lower fentanyl concentration achieved in therapeutic use independent of a metabolic or synthesis byproduct origin. Therapeutic concentration of fentanyl averages 3% of the mean as well as median concentration in fentanyl-related fatalities. A proportionate reduction in 4-ANPP levels would still result in concentrations measurable with the greater sensitivity of the plasma assay and should be detectable if 4-ANPP were metabolic in origin. The data is not conclusive and may be explained by a lower 4-ANPP concentration in pharmaceutical fentanyl due to manufacturing practices and governmental oversight. If further studies substantiate this explanation, then 4-ANPP may be considered a concentration-based marker of illicitly sourced fentanyl.

4. Conclusions

Sensitive and selective methods for the analysis of a panel of fentanyls have been developed and validated for use in blood or plasma testing. The assays have been validated based on current standards of practice and are applicable to both forensic and clinical testing. Application in postmortem blood analysis shows concomitant use of fentanyl and fentanyl analogs along with a high frequency of 4-ANPP associated with the cases. Clinical studies in plasma from patients receiving pharmaceutical grade fentanyl revealed an association of fentanyl with norfentanyl and β-OH fentanyl. The postmortem and clinical findings indicate a metabolic source for the β-OH fentanyl determined in these cases.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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