Published in final edited form as:

Forensic Sci Int. 2015 December ; 257: 435–441. doi:10.1016/j.forsciint.2015.10.021.

Postmortem Tissue Distribution of Acetyl Fentanyl, Fentanyl and their Respective Nor-Metabolites Analyzed by Ultrahigh Performance Liquid Chromatography with Tandem Mass Spectrometry

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Abstract

In the last two years, an epidemic of fatal narcotic overdose cases has occurred in the Tampa area of Florida. Fourteen of these deaths involved fentanyl and/or the new designer drug, acetyl fentanyl. Victim demographics, case histories, toxicology findings and causes and manners of death, as well as, disposition of fentanyl derivatives and their nor-metabolites in postmortem heart blood, peripheral blood, bile, brain, liver, urine and vitreous humor are presented. In the cases involving only acetyl fentanyl (without fentanyl, n=4), the average peripheral blood acetyl fentanyl concentration was 0.467 mg/L (range 0.31 to .60 mg/L) and average acetyl norfentanyl concentration was 0.053 mg/L (range 0.002 to 0.086 mg/L). In the cases involving fentanyl (without acetyl fentanyl, n=7), the average peripheral blood fentanyl concentration was 0.012 mg/L (range 0.004 to 0.027 mg/L) and average norfentanyl blood concentration was 0.001 mg/L (range 0.0002 to 0.003 mg/L). In the cases involving both acetyl fentanyl and fentanyl (n=3), the average peripheral blood acetyl fentanyl concentration was 0.008 mg/L (range 0.006 to 0.012 mg/L), the average peripheral blood acetyl norfentanyl concentration was 0.001 mg/L (range 0.001 to 0.002 mg/L), the average peripheral blood fentanyl concentration was 0.018 mg/L (range 0.015 to 0.021 mg/L) and the average peripheral blood norfentanyl concentration was 0.002 mg/L (range 0.001 mg/L to 0.003 mg/L). Based on the toxicology results, it is evident that when fentanyl and/or acetyl fentanyl were present, they contributed to the cause of death. A novel ultrahigh performance liquid chromatography (UPLC) tandem mass spectrometry (MS/MS) method to identify and quantify acetyl fentanyl, acetyl norfentanyl, fentanyl and norfentanyl in postmortem fluids and tissues is also presented.

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Keywords

Postmortem Tissue Distribution; Acetyl Fentanyl; Fentanyl; UPLC/MS/MS

Introduction

Acetyl fentanyl is a new “designer fentanyl” with no medical use in the United States. It can be clandestinely manufactured and is also available from internet drug sites (1). Acetyl fentanyl is estimated to be 15 times more potent than morphine and one third as potent as fentanyl in mice (2). In comparison, fentanyl is approximately 50 to 100 times more potent than morphine (3). Since late 2013, there have been alarming reports of heroin laced with clandestinely manufactured fentanyl and acetyl fentanyl which has resulted in numerous fatal narcotic overdose cases (4–6). Most of these cases reports with fentanyl and acetyl fentanyl fatalities lack postmortem drug findings and the cause and manner of death was established by case history and testing of drugs found at the scene. A review of The Center of Disease Control reports of fourteen acetyl fentanyl related deaths in Rhode Island presented only qualitative blood test results indicating the presence of acetyl fentanyl (7). One recently published case report of a single acetyl fentanyl fatality presented quantitative acetyl fentanyl findings of 0.26 mg/L peripheral blood, 0.25 mg/L heart blood, 1 mg/Kg liver and 2.6 mg/L urine as measured by gas chromatography mass spectrometry (8).

We present fourteen cases of acetyl fentanyl and fentanyl fatalities with demographics and the postmortem tissue distribution fentanyl, acetyl fentanyl and their respective nor-metabolites in heart blood, peripheral blood, bile, brain, liver, urine and vitreous humor. Additionally, we present a novel ultrahigh performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method for the analysis of fentanyl, acetyl fentanyl and their respective nor-metabolites in postmortem specimens.

Initial Toxicological Analysis

Postmortem blood, vitreous and/or urine specimens were screened for volatiles by headspace gas chromatography, drugs of abuse by immunoassay (acetaminophen, barbiturates, benzodiazepines, cannabinoids, carisoprodol/meprobamate, benzoylecgonine, fentanyl, methadone, methamphetamine/MDMA, opiates, oxycodone, and salicylates) and alkaline extractable drugs by full scan gas chromatography mass spectrometry (GC/MS) (9). In the cases containing fentanyl and/or acetyl fentanyl, the immunoassay screens yielded presumptive fentanyl positive results (Immunalysis, Pomona, CA). Fentanyl and acetyl fentanyl immunoassay results were confirmed by full scan GC/MS analysis of alkaline blood extracts using an Rtx-5 (30 m x 0.25 mm x 0.25 μ m) column (Restek, Bellefonte, Pennsylvania). The initial oven temperature of 100°C was held for 1 minute, followed by a 15°C ramp to 230°C, then a 12°C ramp to 300°C followed by a 10 minute hold. A novel UPLC/MS/MS method for the simultaneous identification and quantitation of acetyl fentanyl, acetyl norfentanyl, fentanyl and norfentanyl in postmortem specimens was developed to maximize sensitivity and lower the limit of quantitation.

UPLC/MS/MS Method for Acetyl Fentanyl, Fentanyl, Acetyl Norfentanyl and Norfentanyl

Reagents

The primary reference materials for fentanyl, norfentanyl, acetyl fentanyl, acetyl norfentanyl, and their respective internal standards (ISTD) fentanyl-d₅, norfentanyl-d₅, acetyl fentanyl-¹³C₆, and acetyl norfentanyl-¹³C₆ were purchased from Cerilliant (Round Rock, Texas). Ammonium formate, ammonium hydroxide, glacial acetic acid, isopropanol, methylene chloride, methanol, sodium phosphate monobasic, sodium phosphate dibasic and deionized (DI) water were purchased from Fisher Scientific (Hanover Park, Illinois). All reagents were ACS grade or better. SPEC® MP3 solid phase extraction (SPE) columns were purchased from Agilent Technologies, Inc, (Santa Clara, California). Medical grade nitrogen was purchased from National Welders Supply Company (Richmond, Virginia). Drug-free expired whole blood was obtained from Transfusion Medicine Laboratory at Virginia Commonwealth University Health System. The blood was certified drug free by screening for alkaline extractable drugs by GC/MS and UPLC/MS/MS prior to use.

Sample Preparation

Pre-extraction preparation was unnecessary for the whole blood and vitreous humor specimens. Bile, gastric contents and urine were diluted 1:10 with DI water, mixed using a vortex mixer and allowed to stand for 1 hour. Two-gram aliquots of brain and liver tissue were diluted with 6.0 g of DI water and homogenized using an IKA®-Labortechnik Ultra-Turrax T25 homogenizer (Wilmington, NC). Samples that were determined to have concentrations that exceeded upper limit of the calibration were diluted to bring their concentrations within the linear range of the assay.

Sample Extraction

Five nanograms (5 ng) of fentanyl-d₅, and norfentanyl-d₅ and 10 ng of acetyl fentanyl-¹³C₆, and acetyl norfentanyl-¹³C₆ were added to 1.0 mL or 1.0 g aliquots of calibrators, quality control (QC) specimens or test specimens followed by the addition of 1 mL of pH 6.0 phosphate buffer. Samples were mixed using a vortex mixer for 30 seconds and centrifuged for 5 minutes. SPEC MP3 SPE columns were conditioned with 0.4 mL of methanol followed by 0.4 mL of 100 mM phosphate buffer (pH 6). The samples were added to the columns and aspirated, followed by 0.4 mL of DI water and 0.4 mL of 100 mM acetic acid. Columns were then dried under vacuum. The fentanyl derivatives, their metabolites and ISTDs were eluted with 1 mL of 78:20:2 dichloromethane/isopropanol/ammonia (v:v:v). The eluted extracts were evaporated under nitrogen and reconstituted in 100 µL of mobile phase A. These solutions were transferred to autosampler vials for analysis by UPLC/MS/MS.

Instrumental Analysis

The UPLC/MS/MS analysis of fentanyl, acetyl fentanyl and their nor-metabolites was performed on a Waters Xevo TQD LC/MS mass spectrometer attached to an ACQUITY UPLC® System controlled by MassLynx software (Milford, Massachusetts). Chromatographic separation was performed on an Allure Biphenyl 5 µm x 100 mm x 2.5

mm column (Restek, Bellefonte, Pennsylvania). The column was kept at 40°C and 5 µL of sample was injected. The mobile phase consisted of A: 10 mM ammonium formate and 0.1% formic acid in deionized water and B: methanol. The following gradient was used: 0.00 to 1.5 minutes at 95% A and 5% B, 1.5 to 3 minutes at 60% A and 40% B, 3 to 3.5 minutes at 100% B and then return to 95% A and 5% B at 3.6 minutes. The flow rate was 0.6 mL/minute. The source temperature was set at 150°C with a capillary voltage of 3.00 kV. The desolvation temperature was set at 600°C with a gas flow rate of 650 L/hour. The cone flow rate was set at 100 L/hour. The acquisition mode was Multiple Reaction Monitoring (MRM). The retention times (min), cone voltage (V), transition ions (m/z) and corresponding collection energies (eV) for all the compounds can be found in Table 1. The total run time for the analytical method was 4.0 minutes.

Method Validation Procedure

The evaluation of the whole blood assay was conducted over five days. Sample batches were analyzed as recommended for biomedical assay validation for linearity, lower limit of quantitation (LOQ), accuracy/bias, precision, dilution integrity, carryover, selectivity, absolute recovery, ion suppression and stability (10,11). Validation sample batches contained calibrators, drug free controls (negative control) with internal standard (ISTD) added and a double negative control containing neither drugs, drug metabolites, nor ISTDs. Aliquots of whole blood QC specimens were analyzed in triplicate on 5 days. Whole blood QC specimens were prepared with the following target values: limit of quantitation quality control (LOQC), 0.0005 mg/L of fentanyl and norfentanyl and 0.0010 mg/L of acetyl fentanyl and acetyl norfentanyl; low control (LQC), 0.0015 mg/L of fentanyl and norfentanyl and 0.0030 mg/L of acetyl fentanyl and acetyl norfentanyl; medium control (MQC), 0.0075 mg/L of fentanyl and norfentanyl, and 0.0150 mg/L of acetyl fentanyl and acetyl norfentanyl; high control (HQC), 0.0400 mg/L of fentanyl and norfentanyl and 0.0800 mg/L of acetyl fentanyl and acetyl norfentanyl; and dilution control (DQC), 0.5000 mg/L of fentanyl and norfentanyl and 1.000 mg/L of acetyl fentanyl and acetyl norfentanyl. All QC specimens were stored at -20°C until testing.

Linearity, limit of quantitation

Linearity of the assay was verified from seven point calibration curves prepared in certified in-house drug-free whole blood. Calibrators were the following concentrations: 0.0005, 0.001, 0.002, 0.005, 0.010, 0.020 and 0.050 mg/L for fentanyl and norfentanyl; and 0.001, 0.002, 0.005, 0.010, 0.025, 0.050 and 0.100 mg/L for acetyl fentanyl and acetyl norfentanyl. A linear regression of the ratio of the analyte to that of their respective ISTDs versus corresponding concentration was used to construct the calibration curves. The lower limit of quantitation (LOQ) was administratively set at 0.0005 mg/L for fentanyl and norfentanyl and 0.001 mg/L for acetyl fentanyl and acetyl norfentanyl. LOQC samples were used to verify that the LOQ was within $\pm 20\%$ of the target value and had a response at least ten times greater than the signal to noise ratio of drug-free whole blood.

Accuracy/bias and precision

Accuracy/bias and precision were determined from the prepared QC whole blood samples. The accuracy/bias was calculated for each concentration over the five validation runs by

combining all the data from all five days. The within-run and between-run precision were calculated using the one-way analysis of variation approach (ANOVA).

Dilution integrity

Dilution integrity was determined from the prepared DQC that was diluted 1:10 with drug free whole blood before analysis. The accuracy/bias was calculated for each analyte over the five validation batches by combining the data from all five days. The within-run and between run precision were calculated using ANOVA.

Carryover

Sample carryover of the fentanyl derivatives was evaluated in each of the five validation batches using two different procedures. First, immediately following the injection of the highest calibrator (0.050 mg/L for fentanyl and norfentanyl; 0.100 mg/L for acetyl fentanyl and acetyl norfentanyl) a negative control (drug-free) was injected. Second, an injection of HQC (0.040 mg/L of fentanyl and norfentanyl and 0.080 mg/L of acetyl fentanyl and acetyl norfentanyl) was immediately followed by injection of the LQC (0.0015 mg/L of fentanyl and norfentanyl and 0.003 mg/L of acetyl fentanyl and acetyl norfentanyl). This procedure was routinely applied each time the high calibrator, HQC and LQC samples were analyzed.

Selectivity

The selectivity of the assay was determined using six different lots of certified drug free whole blood. Each individual lot was analyzed with and without internal standard. Drug free whole blood specimens were also fortified with common therapeutic drugs, drugs of abuse and metabolites including acetaminophen, amitriptyline, amphetamine, benzoylecgonine, buprenorphine, caffeine, cocaine, codeine, desipramine, diazepam, imipramine, lorazepam, methamphetamine, morphine 6-monoacetylmorphine, norbuprenorphine, nortriptyline, oxazepam, oxycodone, oxymorphone, oxazepam, phencyclidine, temazepam, tetrahydrocannabinol and tetrahydrocannabinol carboxylic acid.

Absolute recovery

The absolute percent recovery of the assay was assessed at 0.005 mg/L fentanyl, norfentanyl and their ISTDs and 0.010 mg/L for acetyl fentanyl and acetyl norfentanyl and their ISTDs (n=3). The absolute percent recovery was determined by comparing extracted matrix with the analytes added post-extraction versus extracted samples in which the analytes were added pre-extraction. The matrix samples were prepared by first extracting drug free whole blood by the presented method and then adding fentanyl, acetyl fentanyl, norfentanyl and acetyl norfentanyl and ISTD at the appropriate concentrations. The extracted samples were prepared by adding the drugs, drug metabolites and ISTDs to blank matrix at the appropriate concentrations and then extracting the samples using the presented method. The absolute recovery of the assay was determined by comparing the absolute area of the extracted samples to the absolute peak area of matrix samples.

Ion suppression/enhancement

Ion suppression or enhancement percentages of the assay were determined at the high and low QC concentrations using ten different lots of whole blood matrix samples extracted and run in duplicate and compared to six un-extracted samples prepared at the high and low QC concentrations. Matrix samples were prepared by first extracting drug free whole blood by the presented method and then adding fentanyl, acetyl fentanyl, norfentanyl and acetyl norfentanyl and ISTDs at the appropriate concentrations. The ion suppression of the assay was determined by comparing the absolute area of the matrix samples with the absolute area of un-extracted samples at the both the high and low QC concentrations.

Stability

Stability of the fentanyl, acetyl fentanyl, norfentanyl and acetyl norfentanyl in whole blood was determined under several specific conditions and time intervals. The experiments were performed using three of the control specimens: LQC, MQC and HQC. All studies included three replicate analyses of each QC specimen. Blood specimens were stored frozen and thawed for re-analysis. QC specimens stored at -20°C were put through three freeze/thaw cycles with the last freeze cycle lasting 24 hours. They were then extracted and quantitated against freshly prepared calibrators. The “bench-top” stability of the fentanyl derivatives in whole blood was assessed to evaluate the possible effects of specimen transportation and processing in the laboratory by having the QC specimens sit at room temperature for 72 hours. They were then extracted and quantitated against freshly prepared calibrators. The “post-preparative” stability of the fentanyl derivatives in whole blood was evaluated by having extracts sit in the UPLC/MS/MS’s auto-sampler. A batch of the extracted LQC, MQC and HQC were quantitated against freshly prepared calibrators. The extracted controls were then allowed to sit in the auto-sampler for 72 hours at 5°C after which they were re-injected and quantitated from the initial calibration. The results of the initial analysis were compared to those of the re-injected samples.

Results

The method validation yielded linear regression correlation coefficients (r^2) for all calibration curves in the five batches with a range of 0.9981 to 1.000 for all analytes. The LOQC samples were within $\pm 20\%$ of the target value and had responses at least ten times greater than the signal to noise ratio of drug-free whole blood. The accuracy of the QC specimens were determined to range from 92% for the fentanyl MQC, to 115% for the acetyl fentanyl DQC with coefficients of variation (CV) 11% for all the fentanyl derivatives. The within-run and between-run precision of the QC specimens were determined to range from (CV) 4% to 11% (Table 2). Lack of carryover was verified for the fentanyl derivatives in the negative control as well as the analyses of the LQC samples immediately following the HQC which did not demonstrate a significant quantitated bias of more than 20%. No matrix peaks or drugs co-eluted or interfered with fentanyl, acetyl fentanyl, norfentanyl and acetyl norfentanyl and their respective ISTDs.

The absolute percent recovery for fentanyl derivatives and ISTDs ranged from 94% to 118% (Table 3). Ion suppression or enhancement for the fentanyl derivatives ranged from -25% to

15% (Table 4). The results of these stability tests demonstrated that fentanyl, acetyl fentanyl, norfentanyl and acetyl norfentanyl spiked into whole blood were stable under the conditions of the freeze-thaw, bench-top and post-preparative studies. All fentanyl derivatives and their nor-metabolite QC concentrations were within $\pm 20\%$ of the target values.

Demographics, case histories, toxicology findings, cause and manner of death for the fourteen fentanyl and acetyl fentanyl cases are presented in Table 5. The demographics of the victims in the presented deaths were as follows: 86% were male and 14% were female, average age was 41 years (range 26 to 55 years) and 86% were Caucasian and 14% were African American. All but one had a history of heroin abuse and/or evidence of drug use at the death scene such as powders, needles, syringes, tourniquets and fresh track marks. Heroin use was evidenced by toxicology findings, 6-acetylmorphine (6-AM) and/or morphine, in 79% (11/14) of the deaths. Fentanyl was present in 71% (10/14) and acetyl fentanyl present in 50% (7/14) of these cases. All the deaths involved multiple drugs with seven deaths involving drugs of abuse other than opiates or fentanyl derivatives; alprazolam (6 cases), cocaine (1 case), oxycodone (1 case), methadone (2 cases), hydromorphone (1 case) and methamphetamine (1 case), see Table 5. The deaths appeared to occur rapidly after drug administration as evidenced by powders, spoons and syringes nearby (sometimes the syringe was extending from the decedent's injection site). Two of the decedents were found dead in the same house with two unused lines of powdered drugs on the table, along with straws, presumably for insufflation. All but one of the decedents was pronounced dead on scene. This decedent was transported to the emergency department and expired the following day.

In the reported fentanyl and/or acetyl fentanyl cases, morphine was either not detected or present at extremely low free and total peripheral blood concentrations, averaging 0.010 mg/L and 0.030 mg/L, respectively (Table 6). The average fentanyl and acetyl fentanyl peripheral blood concentrations was 0.018 mg/L and 0.008 mg/L, respectively, (Table 6). These data are consistent with reported lethal fentanyl intoxications and demonstrates that the fentanyl derivatives were the major intoxicants in these deaths (12–18). In the cases involving acetyl fentanyl where fentanyl was not detected nor was evidence of heroin use present, the average acetyl fentanyl peripheral blood concentration was 0.47 mg/L (range 0.31 to 0.60 mg/L) and average noracetyl fentanyl concentration was 0.053 mg/L (range 0.02 to 0.086 mg/L), Table 6. The higher blood concentrations of acetyl fentanyl compared to fentanyl may be due to victims administering larger doses of the acetyl analogue and/or be reflective of its lower potency compared to fentanyl; therefore, greater blood and/or brain concentrations of acetyl fentanyl may be required to cause death than those cases with fentanyl.

The disposition of fentanyl, acetyl fentanyl and their nor-metabolites are presented in Table 7. In case 1, the relatively low concentrations of fentanyl, acetyl fentanyl and metabolites in blood, brain and liver as compared to bile and urine values indicate a delayed death. Conversely in cases 2 and 3, the relatively high concentrations of both fentanyl and acetyl fentanyl in brain and liver as compared to their concentrations in the fluids of elimination, bile and urine, indicate rapid deaths. In cases 5, 6 and 8, the relatively high concentrations of both fentanyl in brain and liver as compared to fentanyl concentrations in bile and urine,

would indicate rapid deaths. This interpretation of rapid death is further supported by the parent fentanyl urine concentrations being two to four times greater than those of norfentanyl in these three cases. In case 11, the decedent was transported to the emergency department and expired the following day. At autopsy his acetyl fentanyl and acetyl norfentanyl peripheral blood concentrations were relatively low compared to bile and urine values. These drug disposition findings are consistent with the case history of delayed death. A similar acetyl fentanyl disposition finding indicative of delayed death is seen in case 14 which also involved alprazolam. Cases 12 and 13 with huge acetyl fentanyl brain and liver concentrations compared to blood values were very likely rapid deaths.

Discussion

The presented case demographics are remarkably similar to the fentanyl and fentanyl analog fatalities reported in California in the 1980's (12–14). California reported an average age of 32 years (range, 19 to 57 years) with victims being 78% male and 79% Caucasian. In addition, other similarities included evidence of drug use or drug paraphernalia at the death scenes with a suggestion of rapid demise after drug administration. California had five victims surviving long enough to be transported to a hospital, whereas this study had one such victim. Cocaine and alcohol were other commonly detected drugs. In addition, many of the decedents were found dead in bathrooms, bedrooms and hotel rooms, in isolation, perhaps in an attempt to conceal drug use. However, the fatal fentanyl blood concentrations in the California fatalities were much lower with an average value of 0.03 mg/L compared to the presented study with an average fentanyl value was 0.018 mg/L in the cases containing both fentanyl and acetyl fentanyl and an average fentanyl value of 0.012 mg/L in the cases containing only fentanyl. This could be due to a difference in analytical technique since the California fentanyl concentrations were based on radioimmunoassay testing and not UPLC-MS/MS.

In the initial cases in which fentanyl and/or acetyl fentanyl were detected, the deaths were unusual in that the decedent had a history of heroin use with drug paraphernalia found at the scene, yet the blood morphine concentrations were remarkably low compared to other fatal heroin overdose deaths. It was evident some additional intoxicant was involved in the case. Once fentanyl was identified and quantitated, the presence of the earlier eluting compound with m/z 146 ion raised the suspicion of another fentanyl derivative. The identification of m/z 231, m/z 14 less than fentanyl, led to the identification of acetyl fentanyl. Many of the fentanyl analogues have common ions, therefore, it is prudent to obtain a full scan mass spectra to compare against certified reference material for definitive identification.

At the time the autopsy and toxicology testing were completed on these cases, there were no published reports of acetyl fentanyl concentrations in biological specimens, including human postmortem tissues or fluids. Therefore, the assertions in this report that acetyl fentanyl caused and/or contributed to the death in these cases were based on the likely pharmacological similarity of acetyl fentanyl to fentanyl, autopsy findings and individual case histories. In the cases with acetyl fentanyl only, the acetyl fentanyl concentrations were incredibly high, requiring dilution for analysis. Three of the four acetyl fentanyl deaths occurred within 2 days of each other. In addition, the acetyl fentanyl concentrations were a

lot higher than what had been seen in the previously reported fentanyl fatalities. This raised the suspicion that these more recent acetyl fentanyl cases appeared to be related to a new supply of drug in the area. Law enforcement was able to link the three cases to the same drug dealer. Since the time these cases were analyzed, there is a single case report of an acetyl fentanyl fatality with an acetyl fentanyl concentration of 0.26 mg/L peripheral blood, 0.25 mg/L heart blood, 1 mg/Kg liver and 2.6 mg/L urine as measured by gas chromatography mass spectrometry (8).

Conclusion

Postmortem toxicology laboratories should be cognizant of their cases in which the case history and circumstances suggest an opiate overdose-related death, yet the toxicology findings are negative or have only trace amounts of drugs detected. These cases could potentially contain fentanyl, acetyl fentanyl, other fentanyl analogs and/or other designer drugs. The presented cases, with associated drug concentrations, case histories, demographics and causes and manners of death may help provide assistance with the interpretation of the postmortem findings. The disposition data for fentanyl and acetyl fentanyl and their nor-metabolites demonstrate the utility and importance of disposition in interpreting toxicology findings. In addition, the rapid identification of new or unusual drugs of abuse or illicit drug mixtures in fatal poisoning may aid law enforcement in the timely identification of new illicit drug sources, hopefully saving lives in the process.

Acknowledgments

This project was supported in part by the National Institute on Health (NIH) grant P30DA033934.

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Highlights

- Novel UPLC/MS/MS method to quantify acetyl fentanyl, fentanyl and their metabolites
- Postmortem tissue disposition of acetyl fentanyl, fentanyl and their metabolites
- Case histories and toxicology findings in 14 acetyl fentanyl and fentanyl deaths

Table 1Fentanyl Analogues; retention times (RT), cone voltages (V), transition ions (m/z) and collision energies (CE)

Designer Drug	RT (min)	Cone (V)	Trans Ions (m/z)	CE (eV)
Fentanyl	2.8	50	337>105 337>188	36 24
Fentanyl-d ₅	2.8	50	342>105 342>188	36 24
Norfentanyl	2.2	40	233>84 233>177	16 14
Norfentanyl-d ₅	2.2	40	238>85 238>182	16 14
Acetyl fentanyl	2.1	48	323>105 323>188	35 23
Acetyl fentanyl- ¹³ C ₆	2.1	48	329>105 329>188	35 23
Acetyl norfentanyl	2.8	32	219>85 219>56	17 25
Acetyl norfentanyl- ¹³ C ₆	2.8	32	225>84 225>56	17 25

One-Way Analysis of Variation (ANOVA) Approach to Calculate Combined Within-Run and Between-Run Precision (% CV)

Table 2

Fentanyl (% CV)	LOQC (0.0005 mg/L)	LQC (0.0015 mg/L)	MQC (0.0075 mg/L)	HQC (0.040 mg/L)	DQC (0.50 mg/L)
Within-Run	6	11	6	8	5
Between-Run	11	10	7	9	5
Norfentanyl (% CV)	LOQC (0.0005 mg/L)	LQC (0.0015 mg/L)	MQC (0.0075 mg/L)	HQC (0.040 mg/L)	DQC (0.50 mg/L)
Within-Run	11	4	7	7	5
Between-Run	10	6	7	7	5
Acetyl Fentanyl (% CV)	LOQC (0.0010 mg/L)	LQC (0.0030 mg/L)	MQC (0.0150 mg/L)	HQC (0.080 mg/L)	DQC (1.00 mg/L)
Within-Run	9	4	7	4	4
Between-Run	9	5	6	4	4
Acetyl Norfentanyl (% CV)	LOQC (0.0010 mg/L)	LQC (0.0030 mg/L)	MQC (0.0150 mg/L)	HQC (0.080 mg/L)	DQC (1.00 mg/L)
Within-Run	5	5	5	4	9
Between-Run	8	7	7	5	9

Table 3

Assay Recovery

Drug	% Abs Recovery (% Mean $\pm$ SD, n=3)
Acetyl Fentanyl	102 $\pm$ 1
Acetyl Fentanyl- ¹³ C ₆	94 $\pm$ 4
NorAcetyl Fentanyl	110 $\pm$ 1
NorAcetyl Fentanyl- ¹³ C ₆	104 $\pm$ 10
Fentanyl	104 $\pm$ 18
Fentanyl-d ₅	95 $\pm$ 8
Norfentanyl	107 $\pm$ 4
Norfentanyl-d ₅	118 $\pm$ 9

Table 4

Ion suppression or enhancement percentages calculated at the high and low QC concentrations using ten different lots of whole blood extracts and run in duplicate compared to six un-extracted samples.

Drug Concentration	Fentanyl 0.0005 mg/L	Norfentanyl 0.0005 mg/L	Acetyl Fentanyl 0.001 mg/L	Acetyl Norfentanyl 0.001 mg/L
% suppression/enhancement	1	-25	-18	-4
% CV	15	14	14	14
Concentration	0.040 mg/L	0.040 mg/L	0.080 mg/L	0.080 mg/L
% suppression/enhancement	-4	-3	-10	-8
% CV	14	7	12	9

Table 5

Decedent's Demographics, case histories, toxicology findings and the causes and manners of death for fentanyl and acetyl fentanyl cases.

Key to abbreviations: M (male), F (female), W (white), B (black), Dec'd (decedent), IV (intravenous), ACLS (advanced cardiac life support), ER (emergency room), ICU (intensive care unit), PB (peripheral blood), 6-AM (6-acetylmorphine), VH (vitreous humor), UR (urine), HB (heart blood), AB (admission blood), BE (benzoylecgonine).

	Demographics	History	Toxicology Results	Cause and Manner of Death
1	46 W/M	Dec'd found dead on bedroom floor. History of prescription drug abuse.	Morphine (free) 0.03 mg/L PB Morphine (total) 0.06 mg/L PB 6-AM present PB Fentanyl 0.020 mg/L PB Acetyl Fentanyl 0.009 mg/L PB Cocaine 0.07 mg/L PB BE 0.97 mg/L PB	Intoxication by heroin, fentanyl, acetyl fentanyl and cocaine Accident
2	39 W/M	Dec'd found dead in bed. Used needles, spoon and white powder nearby. History of heroin and cocaine abuse.	Morphine (free) negative PB Morphine (total) 0.02 mg/L PB 6-AM present VH, UR Fentanyl 0.015 mg/L PB Acetyl Fentanyl 0.012 PB mg/L	Intoxication by heroin, fentanyl and acetyl fentanyl Accident
3	41 W/M	Dec'd found dead in bathroom. Spoon, needle and syringe nearby. History of drug abuse.	Morphine (free) <0.02 mg/L PB Morphine (total) <0.02 mg/L PB 6-AM present PB Alprazolam 0.03 mg/L PB Fentanyl 0.019 mg/L PB Acetyl Fentanyl 0.006 mg/L HB	Intoxication by heroin, fentanyl, acetyl fentanyl and alprazolam Accident
4	40 W/M	Dec'd found dead in hotel room. Syringe and powder nearby. History of heroin abuse.	Morphine (free) 0.02 mg/L PB Morphine (total) 0.03 mg/L PB 6-AM present PB Fentanyl 0.004 mg/L PB	Intoxication by heroin and fentanyl Accident
5	44 W/M	Dec'd found dead in bathroom (in same house as case below). White powdery substance on bedroom table.	Morphine (free) 0.02 mg/L PB Morphine (total) 0.04 mg/L PB 6-AM present VH, UR Methadone 0.32 mg/L PB Alprazolam 0.03 mg/L PB Fentanyl 0.007 mg/L PB	Intoxication by heroin, fentanyl, methadone and alprazolam Accident
6	40 W/F	Dec'd found dead in bedroom (in same house as case above). White powdery substance on bedroom table.	Morphine (free) 0.05 mg/L PB Morphine (total) 0.08 mg/L PB 6-AM present PB Methadone 0.40 mg/L PB Alprazolam 0.03 mg/L PB Fentanyl 0.038 mg/L HB	Intoxication by heroin, fentanyl, methadone and alprazolam Accident
7	53 W/M	Dec'd found dead in chair. Syringe, spoon and three baggies of white powder nearby. History of IV drug abuse.	Morphine (free) 0.10 mg/L PB Morphine (total) 0.09 mg/L PB 6-AM present PB, VH, UR Fentanyl 0.004 mg/L PB Methamphetamine 0.27 mg/L PB	Intoxication by heroin, fentanyl and methamphetamine Accident
8	40 W/M	Dec'd found dead on bathroom floor. Spoon and syringe nearby.	Morphine (free) 0.02 mg/L PB Morphine (total) 0.06 mg/L PB 6-AM present PB Hydromorphone (free) <0.02 PB Hydromorphone (total) 0.04 PB Fentanyl 0.015 mg/L PB	Intoxication by heroin, fentanyl and hydromorphone Accident
9	38 B/M	Dec'd moved out of wife's house two weeks prior due to domestic violence injunction. He returned to wife's house while she was at work to walk the dog. Wife returned from work and found dec'd dead on bathroom floor. Syringe and tourniquet nearby. History of IV drug	Morphine (free) <0.02 mg/L PB Morphine (total) 0.03 mg/L PB 6-AM present VH, UR Fentanyl 0.007 mg/L PB	Intoxication by heroin and fentanyl Accident

	Demographics	History	Toxicology Results	Cause and Manner of Death
		abuse.		
10	50 W/M	Dec'd found dead on bathroom floor. Small red plastic baggie in toilet and syringe found in pocket. Recently released from prison. History of heroin abuse.	Morphine (free) 0.10 mg/L HB Morphine (total) 0.24 mg/L HB 6-AM present VH, UR Fentanyl 0.016 mg/L HB	Intoxication by heroin and fentanyl Accident
11	55 W/M	Dec'd found unresponsive in recliner. 911 called, ACLS initiated and transported to Admitted to ICU and died next day. Oxycodone filled the day before with 60 pills, 30 remained.	Oxycodone 0.08 mg/L AB Oxymorphone (total) 0.02 mg/L AB Alprazolam <0.02 mg/L AB Acetyl Fentanyl 0.31 mg/L PB	Intoxication by acetyl fentanyl and oxycodone Accident
12	26 B/F	Dec'd found dead in bathroom, sitting on toilet with needle in arm. History of IV heroin abuse.	Morphine (free) 0.03 mg/L PB Morphine (total) 0.07 mg/L PB Acetyl Fentanyl 0.40 mg/L PB	Intoxication by acetyl fentanyl and morphine Accident
13	30 W/M	Dec'd found dead in bed in motel room. Last seen alive the night before by brother and seemed intoxicated. Needle marks in left arm, spoon and straws nearby. History of IV heroin abuse.	Alprazolam 0.02 mg/L PB Acetyl Fentanyl 0.56 mg/L PB	Intoxication by acetyl fentanyl and alprazolam Accident
14	28 W/M	Dec'd found dead in bed in motel room. Razor blades, white powdery substances, straws, syringes and lighter nearby. History of heroin abuse.	Alprazolam 0.23 mg/L PB Acetyl Fentanyl 0.60 mg/L PB	Intoxication by acetyl fentanyl and alprazolam Accident

Average peripheral blood drug opioid concentrations and ranges (i) in parenthesis) in mg/L for heroin, fentanyl and acetyl fentanyl related fatalities

Table 6

Type of case	Morphine (Free)	Morphine Total)	Fentanyl	Norfentanyl	Acetyl Fentanyl	Acetyl Norfentanyl
Heroin, Fentanyl and Acetyl Fentanyl Cases 1–3	0.01 (none –0.030)	0.03 (none – 0.060)	0.018 (0.015–0.021)	0.002 (0.001–0.003)	0.008 (0.006–0.012)	0.001 (0.001–0.002)
Heroin and Fentanyl Cases 4–10	0.04 (none –0.10)	0.08 (0.030–0.90)	0.012 (0.004–0.027)	0.001 (0.0002–0.003)		
Acetyl Fentanyl Cases 11–14					0.467 (0.31–0.60)	0.053 (0.002–0.086)

Table 7

Tissue Distributions of Fentanyl, Norfentanyl, Acetyl Fentanyl and Acetyl Norfentanyl (concentrations shown in units of mg/L or mg/Kg).

Case	Drug	PB	HB	Vitreous	Brain	Liver	Bile	Urine
1	Fentanyl	0.021	0.032	0.029	0.108	0.210	0.320	1.200
	Norfentanyl	0.003	0.004	0.005	0.003	0.028	0.250	0.840
	Acetyl Fentanyl	0.009	0.026	0.031	0.080	0.180	0.140	0.980
	Acetyl Norfentanyl	0.002	0.002	0.002	0.005	0.011	0.340	0.470
2	Fentanyl	0.015	0.014	0.018	0.047	0.052	0.061	0.011
	Norfentanyl	0.003	0.002	0.002	0.001	0.007	0.032	0.056
	Acetyl Fentanyl	0.012	0.006	0.001	0.037	0.029	0.023	0.003
	Acetyl Norfentanyl	0.001	0.001	0.001	0.006	0.005	0.023	0.017
3	Fentanyl	0.019	0.016	0.012	0.077	0.084	0.025	0.015
	Norfentanyl	0.008	0.003	0.004	0.004	0.002	0.010	0.026
	Acetyl Fentanyl	0.006	0.002	0.004	0.021	0.036	0.022	0.015
	Acetyl Norfentanyl	0.001	0.001	0.001	0.004	0.004	0.013	0.032
4	Fentanyl	0.005	0.019	0.008	–	0.040	0.081	0.034
	Norfentanyl	0.001	0.001	0.001	–	0.004	0.003	0.012
5	Fentanyl	0.005	0.020	0.029	0.140	0.062	0.062	0.042
	Norfentanyl	0.0002	0.001	0.001	0.0003	0.003	0.003	0.006
6	Fentanyl	0.027	0.038	0.032	0.110	0.340	0.150	0.064
	Norfentanyl	0.003	0.005	0.002	0.001	0.014	0.019	0.038
8	Fentanyl	0.015	0.041	0.008	0.120	0.098	0.046	0.051
	Norfentanyl	0.002	0.005	0.001	0.002	0.012	0.029	0.120
11	Acetyl Fentanyl	0.310	0.700	0.290	1.100	0.690	0.540	6.200
	Acetyl Norfentanyl	0.063	0.086	0.025	0.064	0.190	0.300	3.400
12	Acetyl Fentanyl	0.400	0.400	0.100	1.700	1.800	0.100	–
	Acetyl Norfentanyl	0.003	0.004	0.001	0.005	0.020	0.015	–
13	Acetyl Fentanyl	0.560	0.980	0.300	1.100	1.200	0.800	0.130
	Acetyl Norfentanyl	0.002	0.004	0.002	0.007	0.016	0.095	0.100
14	Acetyl Fentanyl	0.600	0.670	0.900	1.400	1.900	4.800	16.000

Case	Drug	PB	HB	Vitreous	Brain	Liver	Bile	Urine
	Acetyl Norfentanyl	0.036	0.034	0.019	0.044	0.160	1.400	18,000

PB, peripheral blood; HB, heart blood; – indicates specimen was not available for analysis; tissue distribution studies were not conducted on Cases 7, 9 and 10.