Evaluation of Acetylfentanyl Following Suspected Heroin Overdose When Complicated by the Presence of Toxic Fentanyl and Alprazolam Concentrations

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ABSTRACT

A 34-year-old male was reported to be snorting a white powder that was believed to contain heroin. Toxicological analysis revealed free morphine (356 µg/L), fentanyl (34.7 µg/L), alprazolam (64.9 µg/L), and acetylfentanyl (32.9 µg/L) in femoral blood and 6-monoacetylmorphine (6-MAM, <10.0 µg/L) in vitreous fluid. Norfentanyl was only detected in stomach contents (<1.00 µg/total). Heroin, fentanyl, and acetylfentanyl were also detected in solid dose evidence submitted by law enforcement. The fentanyl and alprazolam concentrations might normally be associated with a fatal outcome and are supported with the distribution of fentanyl and alprazolam being consistent with an acute intoxication. In addition, the presence of 6-MAM and a free versus total morphine ratio of 67.9% provide supporting evidence of a rapid death following intranasal (IN) administration. However, the presence of illicit acetylfentanyl complicates toxicologic interpretation due to overlapping recreational and fatal concentrations of this compound reported in the literature as well as a potential for postmortem redistribution (PMR). Reported acetylfentanyl concentrations have also varied when presented with significant fentanyl concentrations and underscore the need to consider a wide range of illicit opioid compounds when investigating drug-related deaths. Based on our comprehensive toxicologic analysis, the results suggest an acute intoxication primarily by IN administration of acetylfentanyl and fentanyl. In addition, we suggest the presence of alprazolam, 6-MAM, and a percentage free morphine is also consistent with rapid death. The cause of death was officially attributed to an acute combined intoxication of acetylfentanyl, fentanyl, alprazolam, and heroin, with the manner of death as accidental. *Acad Forensic Pathol.* 2019 9(3-4): 191-199

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INFORMATION

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INTRODUCTION

Polypharmaceutical intoxication is a widespread problem in the United States (1-3). Recreational abuse of fentanyl and its analogs (also known as “fentalogs”) has dominated the illicit drug scene by virtue of its near-ubiquitous presence in seized drug material, in illicitly synthesized street drugs intended for consumption, and in biological samples from human performance and postmortem toxicology casework (4, 5). Due to their relatively high potency, lethality, and addiction liability as opioid receptor agonists, the “fentalogs” have unfortunately become more routine in the postmortem setting. In particular, the Nassau County Medical Examiner has experienced a sharp rise not only in opioid-related fatalities within the last several years but also in fatalities explicitly attributed to fentanyl and its analogs. In particular, acetylfentanyl was first reported by our laboratory in May of 2015 but had been reported elsewhere in the United States as early as 2013 (6). The analgesic potency of acetylfentanyl has been shown to be less than that of fentanyl and only slightly (approximately 5 to 15 times) more potent than heroin (7). This pharmacological difference may be due, in part, to the loss of a single methyl group from fentanyl (**Figure 1**).

Given the nature of polydrug abuse, toxicologic interpretation is often complicated by other substances that might also be present in toxic or lethal concentrations. In addition, the added coingestion of fentanyl analogs

like acetylfentanyl adds an extra layer of complexity when determining which substance was ultimately responsible for the fatal outcome due to limited information concerning its pharmacology. Like fentanyl, acetylfentanyl may also be subject to postmortem redistribution (PMR) based on its lipophilic nature and volume of distribution (7), and postmortem toxicological results should be interpreted accordingly.

In order to aid our toxicological investigation, we also utilized the ratio of free morphine to total morphine as a means of evaluating the extent of survival time following administration. Historically, a free:total morphine ratio between 51% and 75% has been correlated with a rapid death following heroin use (8). Because of its rapid conversion to 6-monoacetylmorphine (6-MAM) followed by deacetylation to morphine, detection of 6-MAM also serves as a useful adjunct for determining heroin exposure (8). More recently, evidence of heroin use has become useful in cases where a combination of heroin and fentanyl/fentanyl analog ingestion was indicated in the cause of death and can potentially provide insight with respect to the decedent’s intent of illicit drug abuse. In many such cases, the decedent was likely expecting to take their “typical” dose of heroin but was unknowingly exposed to fentanyl or its analogs solely by itself or in combination with heroin. Opioid-naïve individuals with limited tolerance are thus at the highest risk for fatal intoxication from even slight variations in dosing inherent with heroin use.

Figure 1: Chemical structure comparison of fentanyl and acetylfentanyl.

Alprazolam is a short-acting benzodiazepine and commonly used in the treatment of anxiety and panic disorders (9). Its relatively short duration of action facilitates the need for more frequent dosing in the chronic user and lends itself to a higher addiction and dependence liability than with longer acting benzodiazepines such as diazepam or nordiazepam. Peak plasma concentrations following a 1 mg oral dose ranged from 12 to 22 $\mu\text{g/L}$, with concentrations changing by 10 to 12 $\mu\text{g/L}$ for each daily dosage change of 1 mg/day (10). Therapeutic doses depend on the purpose of use; 0.75 to 4 mg is typically prescribed for anxiety and 6 to 9 mg for panic attacks and panic disorders (11). Administered alone, the potential for an acute overdose is low but may substantially increase when taken in combination with other potent respiratory depressants. Alprazolam's respiratory depressant actions are modulated via the GABA_A benzodiazepine receptor site, while fentanyl and acetylfentanyl's depressant actions are modulated via mu-opioid receptors in a dose-dependent fashion (12, 13). Taken together, lower-than-expected alprazolam concentrations may still lend it toward producing toxicologically significant effects. In the past, the significance of combined opioid and benzodiazepine toxicity has been debated. However, evidence exists that demonstrates an increased risk of combined intoxication in a dose-dependent fashion (14). In addition, the Centers for Disease Control and Prevention recently conducted an epidemiological evaluation and recommended that clinicians avoid prescribing both opioids and benzodiazepines together owing to the potential for increased toxicity (15). Here, we report a case of typically life-threatening fentanyl and alprazolam concentrations (detected in multiple biological specimens) when taken together due to their potential additive effects on different receptor systems that ultimately contribute to profound respiratory and central nervous system depression but where the cause of death was also attributed to intranasal (IN) use of acetylfentanyl.

CASE HISTORY

The decedent was a 34-year-old white male who had completed drug rehabilitation and was last seen alive by his mother toward the evening of the terminal event. The next morning, the decedent's mother found him

unresponsive on the floor in his bedroom. Emergency medical services were called, and the decedent was given naloxone with no response. Advanced cardiac life support protocol was initiated en route to the emergency room (ER), but the decedent had never responded and cardiac stand still was confirmed ten minutes later by bedside ultrasound. There were no signs of trauma or intravenous drug abuse stigmata found, and no admission blood samples were drawn. The ER charge nurse had informed the medical examiner's office that detectives had earlier presented a list of medications that were found at the residence: penicillin, gabapentin, and suboxone. Evidence submitted to the toxicology laboratory included two clear vials, one amber vial, and one red straw, with the straw containing white powdered residue. A full autopsy was conducted and revealed no significant external injuries. Internal examination revealed significant pulmonary congestion and edema.

METHODS

Postmortem Testing Protocol

Specimens collected from autopsy included cardiac blood in polypropylene containers containing potassium oxalate and femoral blood in gray-top vacutainer tubes containing sodium fluoride and potassium oxalate. Urine, liver, brain, vitreous, and stomach contents were collected in polypropylene containers that did not contain any additives. The specimens were submitted to the Nassau County Medical Examiner Toxicology Laboratory for a general unknown toxicologic analysis. All specimens were collected ~12 hours following the terminal event and were stored in refrigeration at all times except for sample preparation. The general unknown screen performed included a drug of abuse panel by enzyme-multiplied immunoassay technique and screens for acidic, weak acidic, weak alkaline, and alkaline drugs by gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS). Quantitation of ethanol and other volatiles is performed using headspace gas chromatography with a flame ionization detector. In addition, screening and confirmation of fentanyl and its analogs are performed using liquid chromatography/tandem mass spectrometry (LC/MS-MS).

Acetylfentanyl Quantitation

Using a method previously described by the author, acetylfentanyl was isolated from case specimens by means of an alkaline liquid/liquid extraction scheme (16). In short, an aliquot of the specimen is mixed with a strong base to convert acetylfentanyl to a non-ionized state. A volume of nonpolar solvent is added into which the nonionized drug will transfer. The resultant solvent layer is evaporated to dryness, reconstituted in mobile phase, and analyzed by LC/MS-MS. Acetylfentanyl was quantified by LC/MS-MS using fentanyl-D₅ as the internal standard (Figure 2). Instrument performance was verified using matrix controls.

Other Quantitations

Fentanyl and norfentanyl were quantified by LC/MS-MS (16). Alprazolam was quantified by LC/MS (17). In both cases, instrument performance was verified using matrix controls. Both 6-MAM and free morphine were quantified using solid-phase extraction (SPE) with oxime and trimethylsilyl (BSTFA-TMS) derivatization followed by GC/MS analysis (18). For total morphine, enzyme hydrolysis with β -glucuronidase in a water bath at 65°C preceded SPE. Complete hydrolysis was confirmed using a morphine-3-glucuronide control (19).

Solid Dose Evidence Testing

Evidentiary materials recovered from postmortem investigative scenes may be submitted to the toxicology laboratory. The laboratory utilizes GC/MS to qualitatively identify drugs from evidence such as unknown powders and pills. Two prepared standard drug mixes containing common drugs and chemicals are used to positively identify and confirm compounds routinely encountered within postmortem evidence items. A small sample of evidence obtained from the scene is also diluted in the appropriate solvent prior to GC/MS analysis and analyzed in the same batch with the two prepared standard drug mixes. Compounds detected in case evidence that are not contained within the standard mixes are confirmed by a secondary confirmatory analysis with the use of certified reference material. The confirmation of salts and/or diluents is not routinely conducted.

RESULTS

The decedent's femoral blood was negative for ethanol and other volatiles. Urine immunoassay was presumptive positive for opiates, benzodiazepines, and tetrahydrocannabinol. Although the decedent was alleged to have had a prescription for suboxone, buprenorphine was not detected via immunoassay. Preliminary analysis of liver specimens revealed the presence of

Figure 2: Total ion chromatogram of acetylfentanyl with m/z 323.2 $\rightarrow$ 188.0 and 323.2 $\rightarrow$ 105.0, fentanyl-D₅ with m/z 342.3 $\rightarrow$ 188.0

fentanyl, despropionylfentanyl (4-ANPP), acetylfentanyl, and alprazolam. Of note, 4-ANPP is a precursor and known impurity of illicit fentanyl preparations, as well as an inactive metabolite of fentanyl as well as acetylfentanyl (20, 21). Gabapentin was not detected (despite being alleged to having a prescription), nor were any other acidic, weak acidic, or neutral drugs. Femoral blood fentanyl and alprazolam were quantified using LC/MS-MS and LC/MS, respectively. Norfentanyl is routinely quantified with fentanyl and was only detected in stomach contents for this case. Acetylfentanyl was quantified in femoral blood by LC/MS-MS. Based on these results (34.7 µg/L fentanyl, 64.9 µg/L alprazolam, 32.9 µg/L acetylfentanyl), a complete distribution was performed (**Table 1**).

Free morphine (femoral blood 356 µg/L) and 6-MAM (vitreous fluid <10.0 µg/L) were quantified by GC/MS. Total morphine was determined in femoral blood (524 µg/L) after incorporating the enzyme hydrolysis step prior to extraction.

Analysis of postmortem evidentiary material by GC/MS was initially found to contain heroin, fentanyl, and acetylfentanyl. All were confirmed by chromatographic comparison with the contents of the standard mixes or with purchased certified reference material.

DISCUSSION

In this case, our analysis revealed potentially toxic levels of acetylfentanyl, fentanyl, and alprazolam. In instances where multiple substances might precipitate a fatal outcome, questions arose as to which toxic agent was primarily responsible. Results from the analysis of morphine, in addition to the presence of 6-MAM, also support the possibility of a “hot shot” heroin overdose. To this end, a complete distribution of acetylfentanyl, fentanyl, alprazolam, and determination of free to total morphine was performed.

Blood results involving fentanyl may be subject to PMR, though the extent of this phenomenon is not well defined with acetylfentanyl (21). Nevertheless, reported blood concentrations alone may be subject to misinterpretation and should be avoided, particularly in cases involving an extended postmortem interval. Thus, cardiac and femoral blood sites, as well as liver, brain, and stomach contents, were analyzed for fentanyl and acetylfentanyl. The cardiac-to-femoral (C/F) and tissue-to-femoral (T/F) ratios may help to serve not only as a reference for future case comparisons or interpretation but also as a useful tool for examining the potential for PMR (22). However, it should be noted that the C/F and T/F ratios alone for any drug may only be indicative of site location dif-

Table 1: Case Distribution Results

Drug	Femoral blood (µg/L)	Cardiac blood (µg/L)	Brain (µg/kg)	Liver (µg/kg)	Stomach contents (µg/total)	Vitreous (µg/L)	Urine (µg/L)
Fentanyl	34.7	36.1	54.5	52.4	2.20	NA	NA
Norfentanyl	ND	ND	ND	ND	<1.00	NA	NA
Alprazolam	64.9	61.1	105	208	1.18	NA	NA
Free morphine	356	285	153	519	13.4	QNS	NA
Total morphine	524	467	NA	NA	NA	NA	ND
6-MAM	NA	NA	NA	NA	NA	<10.0	ND
Acetylfentanyl	32.9	24.4	46.9	55.3	1.07	NA	NA

NA - Not analyzed

ND - Not detected

QNS - Quantity not sufficient

ferences between the two tissues and may or may not be an accurate representation of PMR. In fact, these site location differences may also be representative of incomplete distribution following an acute overdose, though the extent of the contribution of incomplete distribution versus the postmortem interval (and subsequently PMR) resulting in changes toward C/F and T/F ratios is often difficult, if not impossible, to determine in certain scenarios. The C/F ratio for fentanyl (1.04) was greater than one, but the C/F ratio for acetylfentanyl (0.742) was less than one. The T/F ratios for fentanyl were 1.57 L/kg for brain and 1.51 L/kg for liver, while T/F ratios for acetylfentanyl were 1.42 L/kg for brain and 1.68 L/kg for liver. The C/F ratios indicate that there may or may not be a high potential for PMR of fentanyl, but the C/F and T/F ratios for acetylfentanyl possibly suggest minimal potential and were consistent with postmortem cardiac blood, femoral blood, and tissue concentrations reported by Poklis et al. and McIntyre et al. (23, 24). In particular, McIntyre et al. had calculated similar ratios for acetylfentanyl and suggested that T/F ratios <5 L/kg are indicative of little to no propensity for redistribution (21, 23-25). Changes in sample integrity and blood/tissue drug concentrations owing to decomposition processes and other postmortem phenomena, the drugs' specific physicochemical properties (pK_A , V_D , protein binding affinity, etc.) and interindividual pharmacokinetic differences might contribute to the wide range and potential overlap of reported T/F values (1.6-97 L/kg) (22) used for interpretation of a drug's potential for PMR.

As previously mentioned, 4-ANPP is considered an inactive metabolite of fentanyl and acetylfentanyl, as well as a known impurity of illicit fentanyl or fentanyl analog preparations. However, it is still unclear whether acetylfentanyl is additionally an active, minor metabolite of fentanyl or included as a standalone analog or from an impurity in the synthesis of illicit fentanyl. Outside of evidence submitted with a postmortem case, one cannot assume that the presence of acetylfentanyl is solely from ingestion. A brief retrospective analysis of all fentanyl-positive cases from 2018 that were received in Nassau County found that 39.5% contained acetylfentanyl. Of those cases, 10%

had postmortem evidentiary material found to contain both acetylfentanyl and fentanyl, while no cases had postmortem evidentiary material with only fentanyl or only acetylfentanyl. In only one case, acetylfentanyl was present without fentanyl in the toxicological analysis—interestingly enough, this case did not have any accompanying postmortem evidence. These findings warrant the need for more research with respect to fentanyl and acetylfentanyl metabolism as well as their presence in seized drug material. Acetylfentanyl concentrations in a postmortem case that are higher than, or similar to, fentanyl may provide some indication that ingestion of acetylfentanyl occurred. However, given that all of our casework from 2018 where acetylfentanyl was detected had indicated the compound in the cause of death, it is apparent that it still plays an important role as a potent, pharmacologically active respiratory depressant, regardless of its potential *in vivo* formation, through cross-contamination of illicit fentanyl products or through impurities in illicit fentanyl synthesis.

Accidental and suicidal (acute) overdose of opiates and other synthetic opioid compounds may ensue secondary to fatal respiratory and central nervous system depression. Analyzing parent:metabolite ratios of fentanyl and norfentanyl may also help determine acute exposure, with Ruan et al. suggesting a ratio of greater than one (26). Norfentanyl was only present in stomach contents at <1.00 µg/total, but fentanyl was reported at 34.7 µg/L in femoral blood. In this case, the presence of fentanyl and the almost complete absence of norfentanyl suggest an acute exposure. We were unable to determine parent:metabolite ratios for acetylfentanyl as the laboratory does not currently analyze for its main metabolites, thus limiting the potential for determining simultaneous acute exposures of fentanyl with acetylfentanyl. Potential metabolites of acetylfentanyl for future consideration include noracetylfentanyl, hydroxyacetylfentanyl, or β-hydroxyacetylfentanyl (23, 27). For stomach-drug concentrations, Fattinger et al. directly compared drug input into systemic circulation following oral and nasal dosing with cocaine and found that ~30% of systemic exposure came from the nasal mucosa, while the other 70% was absorbed through the gastrointestinal

tract following the first-pass effect (28). Thus, either ion trapping or subsequent oral ingestion following IN administration may explain the stomach fentanyl, norfentanyl, and acetylfentanyl concentrations. Given that the decedent was recently released from rehabilitation, a prolonged period of abstinence may have contributed to reduced opioid tolerance and a higher likelihood of acute exposure, even if fatal concentrations typically reported for fentanyl or acetylfentanyl may be higher than what is presented here.

Our morphine findings are also suggestive of a “hot shot” heroin overdose. The percent free morphine determined was 67.9%, with 6-MAM detected in vitreous fluid (<10.0 µg/L). A high percentage of free morphine along with confirmation of 6-MAM has been reported as indicators for a rapid death following heroin exposure (8). The low concentration of 6-MAM determined in the vitreous fluid along with an absence of 6-MAM and morphine in the urine may be indicative of incomplete distribution following ingestion. Holmgren et al. reported that vitreous morphine concentrations relative to femoral blood concentrations may also help determine rapid death from heroin use (29); however, a free morphine evaluation in the vitreous was not feasible in this case due to insufficient sample quantity. It is also likely that high fentanyl and acetylfentanyl concentrations contributed to a rapid death as evidenced by the high percentage of free morphine.

Alprazolam also likely contributed to a rapid demise due to its potent respiratory depressant activity via the GABA_A benzodiazepine receptor site (12). Its therapeutic index is generally high, as is the case for most benzodiazepines, but its use in tandem with other potent respiratory depressants can lower this threshold. Consequently, a concentration of 64.9 µg/L in femoral blood is typically considered to be in the “therapeutic range.” Determination of alprazolam ratios with its primary metabolite, α -hydroxyalprazolam, may provide better insight as to the nature or frequency of use (acute versus chronic). However, this metabolite is not currently included in our analysis. Reported concentrations in acute intoxications attributed to alprazolam have been reported between 60 and 170 µg/L

spread out in both clinical and postmortem settings, with lower concentrations accounting from changes to the dosing regimen or the concomitant ingestion of other central nervous system depressants (30–34). The potential additive effects of alprazolam with fentanyl, acetylfentanyl, and heroin can thus lead to untoward effects at lower-than-expected blood concentrations. With this information combined with the measured distribution, the authors determined (insofar as we could) that alprazolam played a significant role in precipitating this fatal outcome.

CONCLUSION

When taken together, the results suggest an acute intoxication primarily by IN administration of acetylfentanyl and fentanyl. In addition, we suggest that the presence of alprazolam, 6-MAM, and a high percentage of free morphine are consistent with rapid death. The cause of death was officially attributed to an acute combined intoxication of acetylfentanyl, fentanyl, alprazolam, and heroin, with the manner of death as accidental. Prolonged abstinence and limited access to traditional drugs of abuse resulting from rehabilitation may have led to the loss of opioid tolerance and subsequent misuse of alprazolam as well as what the decedent might have believed to be only heroin. The now common adulteration of heroin with fentanyl or fentanyl analogs such as acetylfentanyl in addition to the wide availability of benzodiazepines such as alprazolam appears to have led to a rapid demise.

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