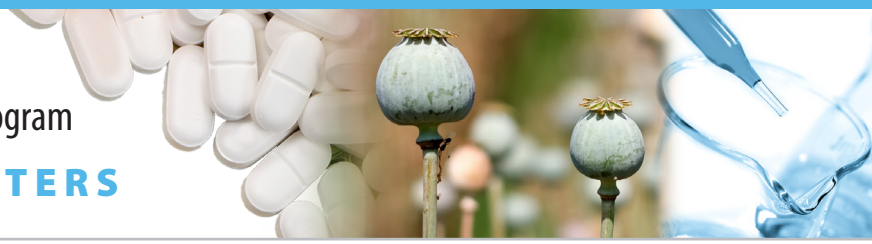
National Laboratory Certification Program
DRUG TESTING MATTERS



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The Importance of Fentanyl Testing in Forensic Toxicology

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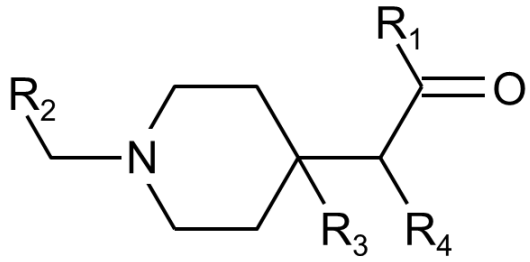
Introduction

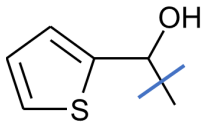
On October 24, 2018, the President of the United States signed Public Law 115-271: SUPPORT for Patients and Communities Act,[1] which required the Secretary of the Department of Health and Human Services (HHS) to determine whether it is justified to add fentanyl to the Mandatory Guidelines for Federal Workplace Drug Testing Programs, based on the reliability and cost-effectiveness of testing.[2] This article provides background information on fentanyl and fentanyl analogs as well as technical aspects on testing.

Fentanyl and Analogs

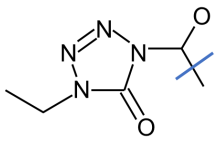
Structurally modifying the basic fentanyl molecule produces analogs of varying potency. ***Table 1*** shows the chemical structure of compounds mentioned herein, including the chemical structures of those approved for human use, the two approved for veterinary use, and 12 illicit fentanyl analogs (although many more are known).

Fentanyl and its legal analogs are Schedule II narcotics under the Controlled Substances Act (CSA).[3] Although several illicit fentanyl analogs, such as cyclopropyl- and isobutyrylfentanyl had been placed in Schedule I earlier, the U.S. Drug Enforcement Administration (DEA) placed all fentanyl analogs that were not already scheduled into CSA Schedule I on February 7, 2018.[4]





-CH(OH)-thiophene



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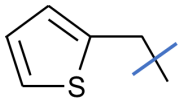

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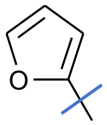

-Furanyl

Fentanyl analog	R_1	R_2	R_3	R_4
Fentanyl	-Ethyl	-CH_2-Phenyl	-H	-Phenyl
Modified on R_1				
Acetylfentanyl	**-Methyl**	-CH_2-Phenyl	-H	-Phenyl
Cyclopropylfentanyl	**-Cyclopropyl**	-CH_2-Phenyl	-H	-Phenyl
Furanylfentanyl	**-Furanyl**	-CH_2-Phenyl	-H	-Phenyl
Isobutyrylfentanyl	**-Isopropyl**	-CH_2-Phenyl	-H	-Phenyl
Methoxyacetylfentanyl	**-CH_2-O-CH_3**	-CH_2-Phenyl	-H	-Phenyl
Valerylfentanyl	**-Butyl**	-CH_2-Phenyl	-H	-Phenyl
Modified on R_2 (norfentanyl common metabolite)				
Benzylfentanyl	-Ethyl	**-Phenyl**	-H	-Phenyl
β-hydroxythiofentanyl	-Ethyl	**- CH(OH)-thiophene**	-H	-Phenyl
Thiofentanyl	-Ethyl	***-CH_2-thiophene***	-H	-Phenyl
Modified on R_3				
Carfentanil	-Ethyl	-CH_2-Phenyl	**-C(=O)-O-CH_3**	-Phenyl
Modified on R_4				
3-fluorofentanyl	-Ethyl	-CH_2-Phenyl	-H	**-(meta-F)-phenyl**
Modified on R_1 and R_4				
4-methoxybuturylfentanyl	**-Propyl**	-CH_2-Phenyl	-H	**-(para-OCH_3)-phenyl**
Ocfentanil	**-CH2-O-CH_3**	-CH_2-Phenyl	-H	**-(ortho-F)-phenyl**
Modified on R_2 and R_3				
Alfentanil	-Ethyl	**-(Ethyl-oxo)-tetra-zole**	**-CH_2-O-CH_3**	-Phenyl
Remifentanil	-Ethyl	**-CH_2-C(=O)-O-CH_3**	**-C(=O)-O-CH_3**	-Phenyl
Sufentanil	-Ethyl	**-CH_2-thiophene**	**-CH_2-O-CH_3**	-Phenyl
Modified on R_1, R_2 and R_3				
Thiafentanil	**-CH_2-O-CH_3**	**-CH_2-thiophene**	**-C(=O)-O-CH_3**	-Phenyl

Table 1. Structure of Fentanyl and Fentanyl Analogs Mentioned

Pharmacology

Fentanyl is a powerful synthetic opioid analgesic that can be administered by a variety of routes, including, oral, transdermal, transbuccal, intravenous (IV), and intramuscular (IM). Usually, single IV and IM doses of 25–100 microgram (µg) of fentanyl are administered as needed.[5,6] It is notable that fentanyl can be given by a wide variety of other legitimate methods, including transdermal patch (Duragesic®),[7] oral transmucosal lozenge (Actiq®),[8] sublingual spray (Subsys®),[9] sublingual tablet (Abstral®),[10] nasal spray (Lazanda®),[11] and buccal tablet (Fentora®),[12] to name several.

Figure 1 shows the fundamental metabolic pathways for fentanyl with the percentages of each excretory product found in urine.[13] The major metabolic enzyme involved in the conversion of fentanyl to norfentanyl is CYP3A4.[14] Norfentanyl is also a metabolite of several fentanyl analogs, including benzylfentanyl, thiofentanyl, and β-hydroxythiofentanyl.[14] Thus, the finding of only norfentanyl in urine suggests the use of fentanyl or a fentanyl-related derivative but does not unequivocally prove that fentanyl was the substance used.

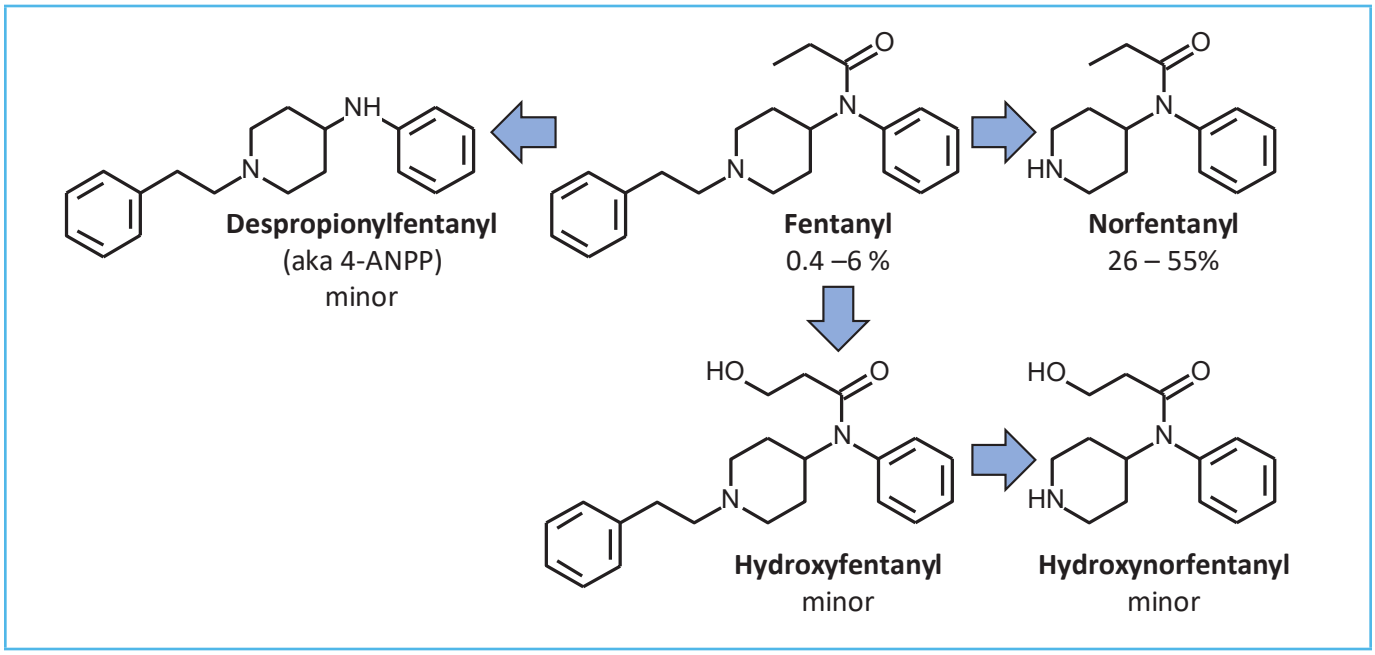

Figure 1. Metabolism of Fentanyl

Similar to fentanyl, licit fentanyl analogs are used for general anesthesia and pain with sufentanil being the most commonly used in healthcare settings.[15] ***Table 2*** presents the potency, pharmacokinetic parameters, and a brief description of legitimate use for fentanyl; its commercially available analogs; and (for comparison purposes) morphine. Thiafentanil, a legal veterinary tranquilizer, is not included in the table because of a paucity of human data.[13,16]

Table 2. Basic Information on Licit Analogs

Parent Drug	Potency Relative to Morphine	Elimination t1/2 (h)	Vd (L/kg)	pKa	Legitimate Use
Fentanyl	100[17]	3–30[5] 3.65[18] 4–12+ (hepatic impairment)[7] 17 (13–22; transdermal)[7]	3–8[5] 0.8–8+ (hepatic impairment)[7]	8.4 (base)[5]	General anesthesia, chronic pain, acute pain
Alfentanil	70[19]	1–2[5] 1.6[18]	0.3–1.0[5] 0.76[5]	6.5 (base)[5]	General anesthesia
Remifentanil	210[20]	0.1–0.27[5]	0.2–0.4[5]	7.1 (base)[5] 7.26[21]	General anesthesia
Sufentanil	500–800[5] 4,500[19]	1.6–5.7[5] 2.73[18]	1.5–3.9[5] 2.9[18]	8.0 (base)[5]	General anesthesia, acute pain
Carfentanil	10,000[17]	5.7[22] (norcarfentanil - 11.8)	?	8.1 (base)[5]	Veterinary tranquilizer for use in large animals, not approved for use in humans
Morphine	1[17]	1.3–6.7[5]	2–5[5]	7.9 (base)[5] 9.6 (acid)[5]	Pain control

pK_a, negative logarithm of the acidity constant; $t_{1/2}$, half-life; V_d, volume of distribution

Adult IV doses for alfentanil are usually 10–100 µg/kg (0.1–7.0 mg/70 kg) and may be followed by 0.5–3.0 µg/kg/min continuous IV infusion. Alfentanil has no metabolites in common with fentanyl.[5]

Anesthetic doses of remifentanil in adults are usually a 1 µg/kg bolus followed by a 0.25–1.0 µg/kg/min infusion. It is notable that the metabolism of remifentanil[5] to GI-90291 involves hydrolysis of a methyl ester function, which can be accomplished by plasma esterases and could substantially reduce parent drug concentrations in unpreserved blood, serum, or plasma specimens.

Doses of 2–8 µg/kg of sufentanil produce profound analgesia whereas doses of 8–30 µg/kg cause deep general anesthesia. It is notable that the N-dealkylated product of sufentanil is the same as N-dealkylated alfentanil, making distinction between the two forensically impossible when only the N-dealkylated metabolite is detected in any matrix.[5]

Prevalence

The 2020 National Forensics Laboratory Information System annual report noted that methamphetamine, cannabis/tetrahydrocannabinol (THC), cocaine, and heroin accounted for 64% of the total number of drug analyte findings based on more than 1.2 million drug reports. These drugs are currently tested under the Mandatory Guidelines. Among non–heroin-related narcotic analgesics, fentanyl (9.1%) was the most frequently reported, but other fentanyl analogs were also reported, including despropionylfentanyl (i.e., 4-ANPP; 0.86%), acetylfentanyl (0.35%), carfentanil (0.11%), and valerylfentanyl (0.04%).[23] Acetylfentanyl is a fentanyl analog in its own right, but it has been suggested that recent findings are more likely to be an artifact from illicit fentanyl production.[24] Similarly, despropionylfentanyl is a by-product and metabolite of fentanyl and several fentanyl analogs.[13]

Data from 24 Ohio counties indicate that fentanyl itself plays a larger role in the epidemic than the analogs. Fentanyl was involved in 253 of 281 (90%) unintentional deaths caused by fentanyl and its analogs that were identified in 24 Ohio counties in January to February 2017.[25] DEA drug seizure data show a similar picture. In the first half of 2021, fentanyl accounted for 89% of the 2,199 findings of fentanyl, fentanyl analogs, and other new opioids. The fentanyl-related compounds 4-ANPP and acetylfentanyl were identified 75 (3.4%) and 57 (2.6%) times whereas the most prevalent fentanyl analog p-fluorofentanyl was identified 118 times (5.4%).[26]

According to the National Institute on Drug Abuse (NIDA), U.S. overdose deaths have increased from 44,000 in 2013 to 92,000 in 2020. During the same period, overdoses involving other synthetic narcotics, a category dominated by fentanyl, have increased from 3,100 to 57,000,[27] and it has been suggested that illicitly manufactured fentanyl is primarily responsible for the increase.[28] Data from the State Unintentional Drug Overdose Reporting System from the first half of 2019 indicate that fentanyl was involved in 62% of all overdose deaths.[29]

As noted above, all fentanyl-related substances not already listed were placed into Schedule I of the CSA on February 7, 2018.[4] Placement was based on the substantial similarity between the chemical makeup and effects of these substances and detailed the types of base molecule substitutions that would result in immediate placement into Schedule I. Since that time, there has been a substantial decrease in the number and variety of fentanyl analogs in postmortem and driving under the influence of drugs cases. According to postmortem data from North Carolina, the number of cases with at least one fentanyl analog peaked in August 2017 with 125 and declined to less than five cases identified in September 2018.[30]

The presence of fentanyl and analogs in the workplace drug testing population has not been well-studied but HHS-certified laboratories that perform fentanyl testing for non-regulated workplace testing and other reasons estimate the positivity rate at <0.1%–5%.[31] To further investigate the prevalence of fentanyl use in the Federal Workplace Drug Testing Program, HHS arranged for a certified laboratory to perform random pulse testing of deidentified workplace urine specimens in 2017 and 2019; see ***Table 3***. In total, seven samples (or 0.16%) were confirmed positive for fentanyl. In 2019, 11 fentanyl analogs were included in the confirmation method, but no positive specimens were identified.

Table 3. Pulse Testing Summary Results

Year	Assay	Total specimens	Specimens ≥ 1 ng/mL (%)	Confirmed by LC-MS/MS (%)
2017	Microgenics DRI®	1,083	3 (0.27%)	2 (0.19%)
	Neogen ELISA[a]	1,056	3 (0.27%)	3 (0.27%)
2018	ARK Diagnostics	2,158	8 (0.37%)	2 (0.09%)

[a] Cutoff was 0.75 ng/mL.

Current Technology

Information provided by HHS-certified laboratories in 2017 and 2018 indicated that a majority (83%)[31] have the ability to analyze urine specimens for fentanyl/norfentanyl. An estimate of the cost to implement fentanyl into the Federal Workplace Drug Testing Program ranged from $0.18–$1.94 per specimen, mainly because of reagent costs for the initial test.

Currently, the immunoassays used in laboratories are targeted toward the parent compound fentanyl and show little to no cross-reactivity to the major metabolite, norfentanyl. To identify fentanyl properly, a new immunoassay that cross-reacts with norfentanyl might be needed because up to 30% of chronic pain patients treated with fentanyl were only positive for norfentanyl in urine. The remainder were positive for fentanyl (39%) or fentanyl and norfentanyl (31%).[32] In addition, cross-reactivity with norfentanyl would allow detection of fentanyl analogs primarily metabolized to norfentanyl.

Fentanyl immunoassays appear to show limited cross-reactivity toward most fentanyl analogs. Helander et al.[33] tested the cross-reactivity for 12 illicit fentanyl analogs using three different fentanyl immunoassay kits. Some cross-reactivity was observed for most analytes using any kit (median 72%, Q1–Q3 49%–82%). For each assay, at least 80% cross-reactivity was observed for three to five analogs, but >80% cross-reactivity across all assays was only observed for butyrylfentanyl. Furthermore, no cross-reactivity was observed for 4-Methoxybutyrylfentanyl with any kit. When challenged with 20 authentic urine samples confirmed positive for fentanyl analogs by LC-MS/MS, all kits tested positive for at least 19 (95%) of the samples.

It is also concerning that only 33%–66% of the positive initial test results from the pulse study were confirmed by mass spectrometry (see ***Table 3***), and this finding raises questions about the effectiveness of immunoassay screening for fentanyl. These results are at odds with a study in which 149 of 152 positive screening results in clinical specimens were confirmed by LC-MS/MS for fentanyl (i.e., a 98% confirmation rate).[34] For comparison, the confirmation rate for the most common initial test assay, tetrahydrocannabinolic acid, is estimated at >95%.

Urine

Even though fentanyl is a common analyte in urinary drug testing, only a few papers report quantitative data, as seen in ***Table 4***. The main resource in the literature appears to be the study by Cummings et al. where concentrations from 77,000 specimens from patients treated with a transdermal patch were recorded.[35] The data show that average concentrations of norfentanyl are 5 times higher than those of fentanyl and that the samples span a wide range of concentrations.

Table 4. Concentrations in Urine

Study	n	Population	Fentanyl pg/mg	Norfentanyl pg/mg
Cummings et al., 2016[35]	77,018	Transdermal patch patients	Mean 86 [range <2–2,100]	Mean 442 [range <10–2,500]
DePriest et al., 2010[36]	221[a]	Chronic pain	Median 22 [range 0.5–600]	Median 26 [range 0.5–1,800]

[a] n=192 for norfentanyl.

Fentanyl analogs have been detected in urine.[37,38] In a postmortem study,[37] concentrations of cyclopropyl fentanyl (n=11, median 38 ng/mL), methoxyacetylfentanyl (n=3, median 843 ng/mL), furanylfentanyl (n=1, 84 ng/mL), and acetylfentanyl (n=5, median 2,800 ng/mL) were reported. Similarly, urinary concentrations from intoxication cases were reported for furanylfentanyl (n=2, 179 & 1,430 ng/mL), acetylfentanyl (n=8, median 700 ng/mL, range 2.4-3,200), and 4-methoxybutyrylfentanyl (n=3, mean 348 ng/mL, range 16–1000).[38]

Oral Fluid

The plasma binding of fentanyl and its analogs appears to be favorable for transfer of these compounds to oral fluid. Except for alfentanil, the pK_a values are favorable for so-called ion trapping.[39] For drugs exhibiting ion trapping, concentrations are generally as easily detected in oral fluid as in blood and blood products such as serum and plasma because of the slightly lower pH of oral fluid and ionized condition of the drug causing it to become "trapped" or to accumulate.

A mean oral fluid/plasma ratio of 3.0 has been reported for fentanyl,[40] and similar detection rates in urine and oral fluid have been shown in at least two studies.[41,42]

Data on concentrations in oral fluid are very limited, as seen in ***Table 5***. That said, fentanyl concentrations appear to be higher than those of norfentanyl with mean and median concentrations in the low ng/mL range.[43,44] Several methods designed to detect fentanyl analogs have been published,[45,46] but concentrations from authentic cases are lacking.

Table 5. Concentrations in Oral Fluid

Study	n	Population	Device	Fentanyl ng/mL	Norfentanyl ng/mL
Bista et al., 2015[43]	163[a]	Cancer pain patients	Salivette	Mean 4.8 [range 0.012–38]	Mean 0.34 [range 0.004–4.2]
Heltsey et al., 2011[44]	424[b]	Chronic pain patients	Quantisal	Median 6.6 [range 0.2–5,300]	Median 1.6 [range 0.5–130]

[a] Collected from 56 participants. [b] n=148 for norfentanyl

Hair

In a study of heroin users and individuals positive for opiates, 98 were positive for fentanyl and 154 for norfentanyl.[47] Interestingly, 146 were also positive for 4-ANPP and 108 for acetyl fentanyl. The latter is interesting, because acetyl fentanyl potentially could serve as a marker for illicitly manufactured fentanyl.

Table 6. Concentrations in Hair

Study	n	Population	Fentanyl pg/mg	Norfentanyl pg/mg
Salomone et al., 2020[47]	198[a]	Heroin users or opiate positive[b]	Median 95 [range 0.3–8,600]	Median 38 [range 0.3–320]
Palamar et al., 2019[48]	39[c]	Heroin users[b]	Median 440 (Q1–Q3 170–1,100)	Median 26 (Q1–Q3 15–67)
Ramírez Fernández et al., 2020[49]	16[d]	Fentanyl analog users	Median 62 (Q1–Q3 35–187)[e]	Median 6.2 (Q1–Q3 1.8–16)[e]

[a] n=154 for norfentanyl. [b] Palamar 2019 & Salomone 2020 might have specimens in common. [c] n=36 for norfentanyl. [d] n=14 for norfentanyl. [e] Segmented analysis. Max concentration from each donor used for calculations.

Fentanyl analogs have also been successfully measured in hair. Furanyl fentanyl was identified with a median concentration of 6 pg/mg (n=87, range LOQ-590).[47] In addition, 3-fluorofentanyl, butyrylfentanyl, carfentanil, methoxyacetylfentanyl, ocfentanil, tetrahydrofuranfentanyl, and valerylfentanyl have also been identified in hair samples.[49-51]

Conclusions

Fentanyl is a potent synthetic opioid that has been a driving factor behind the growing number of overdose deaths in the last few years. Fentanyl can be detected by immunoassays and quantified by chromatographic techniques in urine, oral fluid, and hair. The main metabolite is norfentanyl, which unfortunately does not cross-react with fentanyl in most immunoassays. In urine, norfentanyl concentrations are generally higher than fentanyl concentrations whereas the opposite is true in oral fluid and hair specimens.

Fentanyl analogs are far less common than fentanyl as drugs of abuse but appear to be detectable and quantifiable in urine, oral fluid, and hair by chromatographic techniques. However, only limited cross-reactivity with fentanyl immunoassays is observed.

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