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Recommended methods for the **Identification and Analysis of Fentanyl and its Analogues in Biological Specimens**

MANUAL FOR USE BY NATIONAL DRUG ANALYSIS LABORATORIES

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Laboratory and Scientific Section
UNITED NATIONS OFFICE ON DRUGS AND CRIME
Vienna

Recommended Methods for the Identification and Analysis of Fentanyl and its Analogues in Biological Specimens

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Note

Operating and experimental conditions are reproduced from the original reference materials, including unpublished methods, validated and used in selected national laboratories as per the list of references. A number of alternative conditions and substitution of named commercial products may provide comparable results in many cases. However, any modification has to be validated before it is integrated into laboratory routines.

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Contents

1. Introduction

1.1 Background

The Belgian pharmacologist and pharmaceutical chemist Paul Janssen developed fentanyl in 1960. It was at the time a revolutionary compound with a potency much greater than that of the natural opioid morphine, and a significantly greater safety margin than other pharmaceutical synthetic opioids such as dextromoramide, meperidine and phenoperidine. Under Janssen's leadership, a deeper understanding of the structure/activity relationships led to the development of the very potent analgesics sufentanil, alfentanil, lofentanil and carfentanil, which could also be used for anaesthesia [1]. These drugs have been successfully used clinically for over fifty years, and have made possible complex surgeries and the successful management of chronic pain for millions of patients worldwide. Fentanyl, together with codeine, methadone and morphine are four opioid analgesics on the World Health Organization's (WHO) List of Essential Medicines [2].

In California, United States in 1979, a series of deaths occurred in people who injected drugs that were subsequently attributed to an analogue of fentanyl, *alpha*-methylfentanyl. Initially discovered and patented by Janssen [3], *alpha*-methylfentanyl had neither been evaluated in humans for safety or efficacy, nor approved as a pharmaceutical drug [4,5]. This was the first recorded instance of a completely novel clandestinely synthesized opioid, and earned the sobriquet "designer drug". Through the early 1980s *alpha*-methylfentanyl was quickly joined by a series of other fentanyl analogues including: *para*-fluorofentanyl, 3-methylfentanyl, *beta*-hydroxyfentanyl, and a number of others that were identified in deaths in the west coast of the United States.

Subsequently, the use of fentanyl itself became widespread due to its availability worldwide in injectable, sublingual and transdermal forms, and it also became subject to diversion and abuse. There were isolated cases of fentanyl and its analogues being mixed with heroin in the street drug supply, such as in the "Tango and Cash" product sold in New York City in 1991, resulting in deaths and hospitalizations. Fentanyl was also recognized as a problem in Europe in 2012 [6,7]. At the beginning of 2014, the availability of fentanyl in the United States had started to increase exponentially [8], with crime laboratories reporting more than 4,500 fentanyl cases in 2014 and over 14,400 in 2015. Around the same time, a group of fentanyl new

psychoactive substances (NPS) began to appear in the street drug market, beginning with acetylfentanyl, and butyrylfentanyl. By 2017, 15 fentanyl analogues and two precursor chemicals had been placed under international control and more than 20 individual substances had been detected in deaths. They have been routinely detected in drug markets in Europe and North America, and several more have been encountered in smaller, more isolated incidents, along with other non-morphine or fentanyl-related opioid receptor agonists. Because of their potency and toxicity in overdose, the ease with which they can be manufactured and distributed, and their proliferation in international markets, this manual has been prepared to assist public health, forensic, and clinical laboratories with the identification, analysis and quantification of novel fentanyl analogues found in overdoses, poisonings and death investigation casework.

1.2 Purpose and use of the manual

The present manual is one in a series of similar UNODC publications dealing with the identification and analysis of various types of drugs under international control. These manuals are the outcome of a programme pursued by UNODC since the early 1980s, aimed at the harmonization and establishment of recommended methods of analysis for national drug analysis laboratories. The present manual is the first to deal specifically with the analysis of the growing class of fentanyl-related compounds and focuses on testing in biological tissues and fluids, to assist with investigation of fentanyl-related poisonings and deaths. Many of these analytical considerations also apply to analysis of seized drugs. The first part of the manual discusses the pharmacology and toxicology of fentanyl and its analogues, which is an especially important consideration for this drug class, because the potency of the substances can vary widely. This is followed by a discussion of validated analytical methodologies, using different analytical techniques and instrumental modes of operation.

In line with the overall objective of this series of UNODC publications, this manual suggests approaches that may assist drug analysts in the selection of methods appropriate to the sample under examination, and the range of technologies and resources that might be available in their laboratories. Therefore, the methods described here should be understood as practical guidance, that is, minor modifications to suit local circumstances should normally not change the validity of the results. The choice of the methodology and approach to analysis, as well as the decision as to whether or not additional methods are required, remain with the analyst and may also be dependent on the availability of appropriate instrumentation and the level of legally acceptable proof in the jurisdiction within which the analyst works. The reader should be aware that there are a number of other methods, including those published in forensic science literature, which may also produce acceptable results. However, any new method that is about to be used in a laboratory must be validated and/or verified prior to routine use. Attention is also drawn to the importance of the availability of

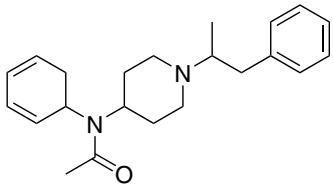
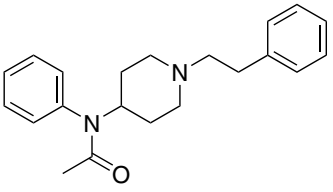
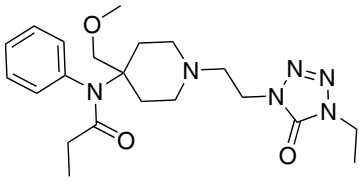
reference materials and literature on drugs of abuse and analytical techniques. Moreover, the analyst must of necessity keep abreast of current trends in drug analysis, consistently following current analytical and forensic science literature. UNODC Laboratory and Scientific Section welcomes observations on the contents and usefulness of the present manual. Comments and suggestions may be addressed to: Laboratory and Scientific Section, United Nations Office on Drugs and Crime, Vienna International Centre, P.O. Box 500, 1400 Vienna, Austria. Fax: (+43-1) 26060-5967. E-mail: Lab@unodc.org. All manuals, as well as guidelines and other scientific-technical publications may be requested by contacting the address above or can be accessed online at https://www.unodc.org/unodc/en/scientists/index.html.

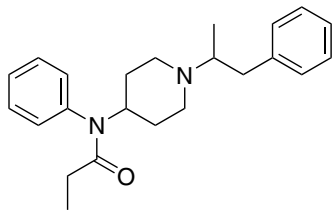
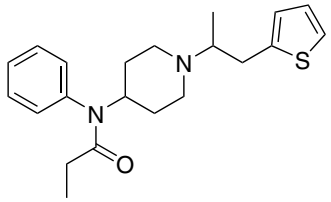
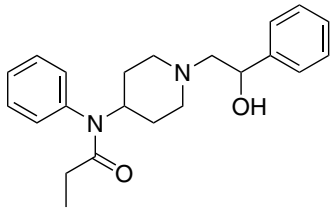
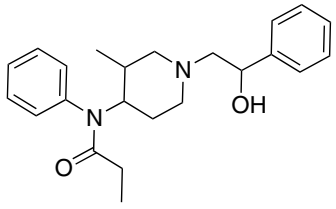
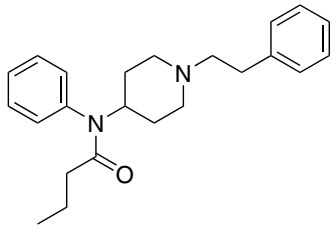
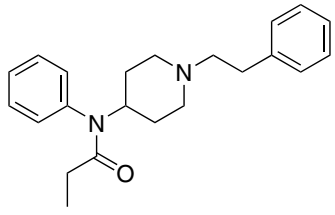
2. General aspects

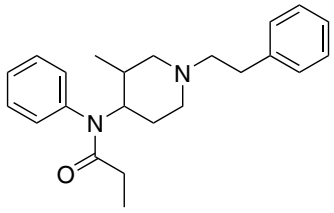
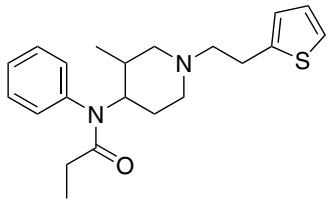
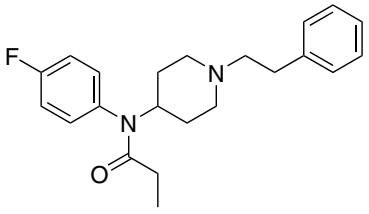
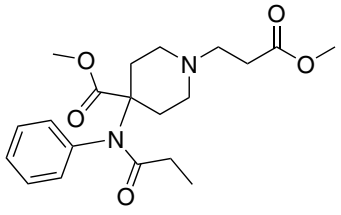
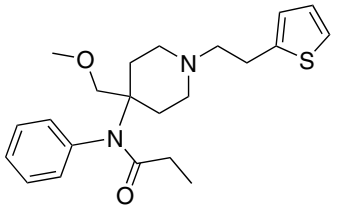
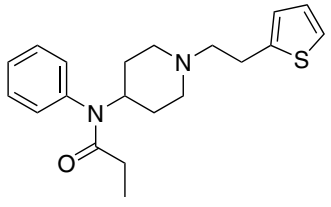
2.1 Definition of fentanyl and analogues

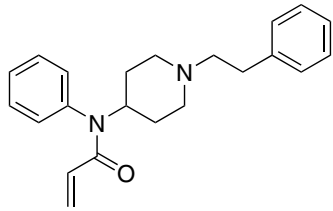
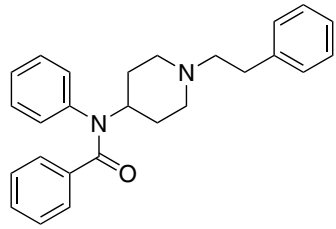
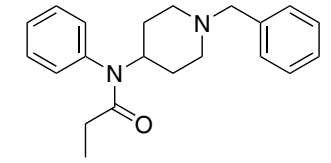
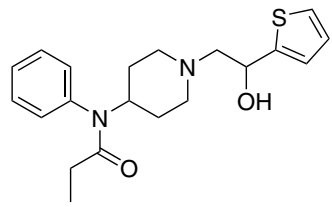
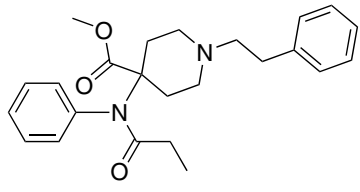
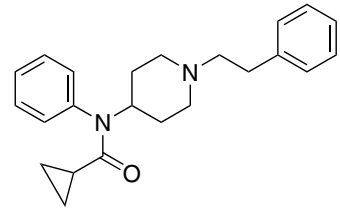
Table 1 includes a list of fentanyl and its analogues that are under international control and a secondary list of fentanyl-related new psychoactive substances that have been reported to the UNODC Early Warning Advisory, as well as newly emerging fentanyls recently seen in forensic casework in several jurisdictions.

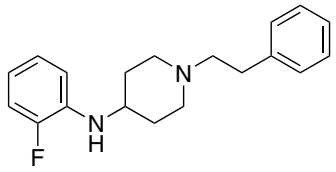
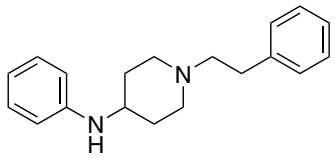
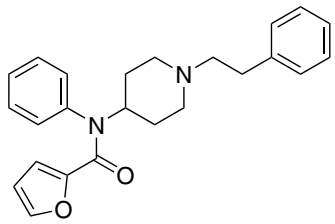
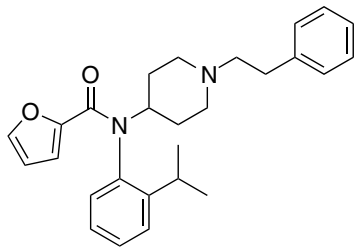
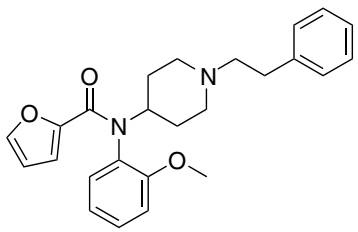
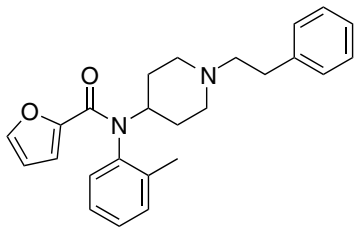
Table 1. List of fentanyl substances under international control and controlled fentanyl analogues reported to the UNODC Early Warning Advisory on New Psychoactive Substances as of September 2017.

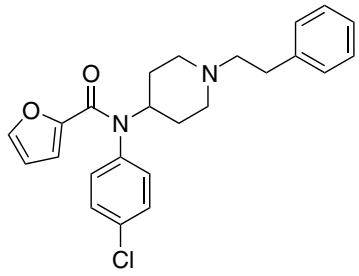
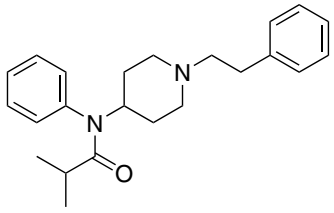
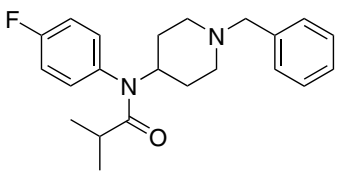
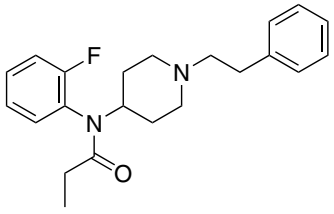
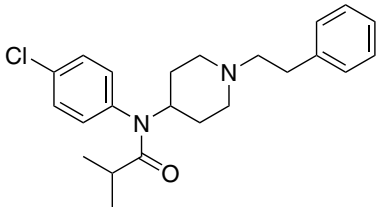
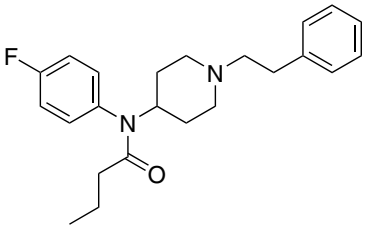
Name: Acetyl-*alpha*-methylfentanyl **Alternate name:** *N*-phenyl *N*-(1-(1-phenylpropan-2-yl)piperidin-4-yl)acetamide **CAS no.** 101860-00-8 **M.W.** 336.48 **Control:** 1961, Schedule I/IV	
Name: Acetylfentanyl **Alternate name:** *N*-[1-(2-Phenylethyl) -4-piperidyl]-*N*-phenylacetamide **CAS no.** 3258-84-2 **M.W.** 322.45 **Control:** 1961, Schedule I/IV	
Name: Alfentanil **Alternate name:** *N*-(1-(2-(4-ethyl-5-oxo-4,5-dihydro-1*H*-tetrazol-1-yl)ethyl)-4-(methoxymethyl) piperidin-4-yl)-*N*-phenylpropionamide **CAS no.** 71195-58-9 **M.W.** 416.53 **Control:** 1961, Schedule I	

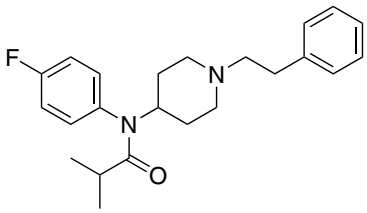
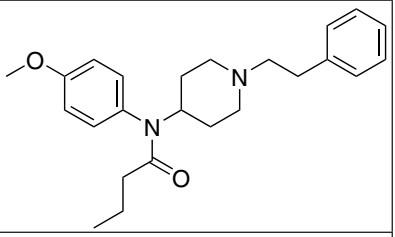
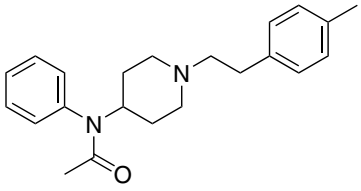
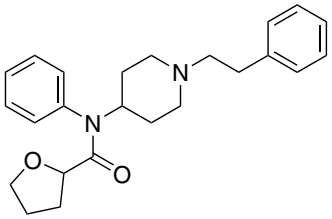
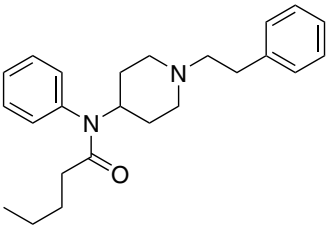
Name: *alpha*-Methylfentanyl **Alternate name:** *N*-phenyl-*N*-(1-(1-phenylpropan-2-yl)piperidin-4-yl)propionamide **CAS no.** 79704-88-4 **M.W.** 350.51 **Control:** 1961 Schedule I/IV	
Name: *alpha*-Methylthiofentanyl **Alternate name:** *N*-phenyl-*N*-(1-(1-(thiophen-2-yl) propan-2-yl)piperidin-4-yl)propionamide **CAS no.** 103963-66-2 **M.W.** 356.53 **Control:** 1961 Schedule I/IV	
Name: *beta*-Hydroxyfentanyl **Alternate name:** *N*-(1-(2-hydroxy-2-phenylethyl) piperidin-4-yl)-*N*-phenylpropionamide **CAS no.** 78995-10-5 **M.W.** 352. 48 **Control:** 1961 Schedule I/IV	
Name: *beta*-Hydroxy-3-methylfentanyl **Alternate name:** *N*-(1-(2-hydroxy-2-phenylethyl)-3-methylpiperidin-4-yl)-*N*-phenylpropionamide **CAS no.** 78995-14-9 **M.W.** 366.51 **Control:** 1961 Schedule I/IV	
Name: Butyrylfentanyl **Alternate name:** *N*-(1-phenethylpiperidin-4-yl)-*N*-phenylbutyramide **CAS no.** 1169-70-6 **M.W.** 350.51 **Control:** 1961 Schedule I	
Name: Fentanyl **Alternate name:** *N*-(1-phenethylpiperidin-4-yl)-*N*-phenylpropionamide **CAS no.** 437-38-7 **M.W.** 336.48 **Control:** 1961 Schedule I	

Name: 3-Methylfentanyl **Alternate name:** *N*-(3-methyl-1-phenethylpiperidin-4-yl)-*N*-phenylpropionamide **CAS no.** 42045-86-3 **M.W.** 350.51 **Control:** 1961 Schedule I/IV	
Name: 3-Methylthiofentanyl **Alternate name:** *N*-(3-methyl-1-(2-(thiophen-2-yl) ethyl)piperidin-4-yl)-*N*-phenylpropionamide **CAS no.** 86052-04-2 **M.W.** 356.53 **Control:** 1961, Schedule I/IV	
Name: *para*-Fluorofentanyl **Alternate name:** *N*-(4-fluorophenyl)-*N*-(1-phenethylpiperidin-4-yl)propionamide **CAS no.** 1189887-65-7 **M.W.** 354.47 **Control:** 1961 Schedule I/IV	
Name: Remifentanil **Alternate name:** methyl 1-(3-methoxy-3-oxopropyl)-4-(*N*-phenylpropionamido) piperidine-4-carboxylate **CAS no.** 132875-61-7 **M.W.** 376.45 **Control:** 1961 Schedule I	
Name: Sufentanil **Alternate name:** *N*-(4-(methoxymethyl)-1-(2-(thiophen-2-yl)ethyl) piperidin-4-yl)-*N*-phenylpropionamide **CAS no.** 56030-54-7 **M.W.** 386.55 **Control:** 1961 Schedule I	
Name: Thiofentanyl **Alternate name:** *N*-phenyl-*N*-(1-(2-(thiophen-2-yl) ethyl)piperidin-4-yl)propionamide **CAS no.** 1165-22-6 **M.W.** 342.50 **Control:** 1961 Schedule I/IV	

Substances not under international control as of September 2017	
Name: Acrylfentanyl **Alternate name:** *N*-(1-phenethylpiperidin-4-yl)-*N*-phenylacrylamide **CAS no.** 82003-75-6 **M.W.** 334.46	
Name: Benzoylfentanyl **Alternate name:** *N*-(1-phenethylpiperidin-4-yl)-*N*-phenylbenzamide **CAS no.** N/A **M.W.** 384.52	
Name: Benzylfentanyl **Alternate name:** *N*-(1-benzylpiperidin-4-yl)-*N*-phenylpropionamide **CAS no.** 1474-02-8 **M.W.** 322.45	
Name: *beta*-Hydroxythiofentanyl **Alternate name:** *N*-(1-(2-hydroxy-2-(thiophen-2-yl)ethyl)piperidin-4-yl)-*N*-phenylpropionamide **CAS no.** 1474-34-6 **M.W.** 358.50	
Name: Carfentanil **Alternate name:** methyl 1-phenethyl-4-(*N*-phenylpropionamido)piperidine-4-carboxylate **CAS no.** 59708-52-0 **M.W.** 394.52	
Name: Cyclopropylfentanyl **Alternate name:** *N*-(1-phenethylpiperidin-4-yl)-*N*-phenylcyclopropanecarboxamide **CAS no.** N/A **M.W.** 348.49	

Name: Despropionyl-2-fluorofentanyl **Alternate name:** *N*-(2-fluorophenyl)-1-phenethylpiperidin-4-amine **CAS no.** 864422-91-3 **M.W.** 298.41	
Name: Despropionylfentanyl (4-anilino-*N*-phenethylpiperidine) **Alternate name:** 1-phenethyl-*N*-phenylpiperidin-4-amine **CAS no.** 21409-26-7 **M.W.** 280.42	
Name: 2-Furanylfentanyl **Alternate name:** *N*-(1-phenethylpiperidin-4-yl)-*N*-phenylfuran-2-carboxamide **CAS no.** 101345-66-8 **M.W.** 374.48	
Name: 2-Furanylfentanyl *ortho*-2-isopropylphenyl analogue **Alternate name:** *N*-(2-isopropylphenyl)-*N*-(1-phenethylpiperidin-4-yl)furan-2-carboxamide **CAS no.** N/A **M.W.** 416.57	
Name: 2-Furanylfentanyl *ortho*-2-methoxyphenyl analogue **Alternate name:** *N*-(2-methoxyphenyl)-*N*-(1-phenethylpiperidin-4-yl)furan-2-carboxamide **CAS no.** N/A **M.W.** 404.51	
Name: 2-Furanylfentanyl *ortho*-2-methylphenyl analogue **Alternate name:** *N*-(1-phenethylpiperidin-4-yl)-*N*-(o-tolyl)furan-2-carboxamide **CAS no.** N/A **M.W.** 388.51	

Name: 2-Furanylfentanyl *para*-chlorophenyl analogue **Alternate name:** *N*-(4-chlorophenyl)-*N*-(1-phenethylpiperidin-4-yl)furan-2-carboxamide **CAS no.** N/A **M.W.** 408.93	
Name: Isobutyrylfentanyl **Alternate name:** *N*-(1-phenethylpiperidin-4-yl)-*N*-phenylisobutyramide **CAS no.** 117332-90-8 (HCl) **M.W.** 350.51	
Name: Isobutyryl-F-fentanyl *N*-benzyl analogue **Alternate name:** *N*-(1-benzylpiperidin-4-yl)-*N*-(4-fluorophenyl)isobutyramide **CAS no.** N/A **M.W.** 354.47	
Name: *ortho*-Fluorofentanyl **Alternate name:** *N*-(2-fluorophenyl)-*N*-(1-phenethylpiperidin-4-yl)propionamide **CAS no.** N/A **M.W.** 354.47	
Name: *para*-Chloroisobutyrylfentanyl **Alternate name:** *N*-(4-chlorophenyl)-*N*-(1-phenethylpiperidin-4-yl)isobutyramide **CAS no.** N/A **M.W.** 384.95	
Name: *para*-Fluorobutyrylfentanyl **Alternate name:** *N*-(4-fluorophenyl)-*N*-(1-phenethylpiperidin-4-yl)butyramide **CAS no.** 244195-31-1 **M.W.** 368.50	

Name: *para*-Fluoroisobutyrylfentanyl **Alternate name:** *N*-(4-fluorophenyl)-*N*-(1-phenethylpiperidin-4-yl)isobutyramide **CAS no.** N/A **M.W.** 368.50	
Name: *para*-Methoxybutyrylfentanyl **Alternate name:** *N*-(4-methoxyphenyl)-*N*-(1-phenethylpiperidin-4-yl)butyramide **CAS no.** N/A **M.W.** 380.53	
Name: *para*-Methylphenethylacetylfentanyl **Alternate name:** *N*-(1-(4-methylphenethyl) piperidin-4-yl)-*N*-phenylacetamide **CAS no.** 1071703-95-1 (HCl) **M.W.** 336.48	
Name: Tetrahydrofuranylfentanyl **Alternate name:** *N*-(1-phenethylpiperidin-4-yl)-*N*-phenyltetrahydrofuran-2-carboxamide **CAS no.** N/A **M.W.** 378.52	
Name: Valerylfentanyl **Alternate name:** *N*-(1-phenethylpiperidin-4-yl)-*N*-phenylpentanamide **CAS no.** 122882-90-0 **M.W.** 364.53	

2.2 Nomenclature

The most formal system for naming chemical compounds follows nomenclature rules designated by the International Union of Pure and Applied Chemistry (IUPAC). However, the spelling and identity of substituent placement may vary depending on accepted practices, which may vary from country to country, leading to confusion regarding analyte identity. The fentanyl analogues have generic endings of *yr*-, *yrl*-, and *oyl*-, which are used interchangeably. Occasionally, alternative names arise from

multiple numerical conventions, such as valerylfentanyl, which is also known as pentanoyl or pentanylfentanyl. Arene substitution pinpoints the position of substituents other than hydrogen in relation to each other on an aromatic hydrocarbon; *ortho*-, *meta*-, and *para*- and 2-, 3-, and 4- refer to the same placement, respectively, of those substituents (for example, 2-fluorofentanyl is *ortho*-fluorofentanyl). For consistency, this manual uses *ortho*-, *meta*- and *para*- designations. Esoteric notations include Greek symbols that can either be represented as the symbol or its name (for example, *alpha*-methylfentanyl = α-methylfentanyl). For consistency, this manual spells out the "alpha" and "beta" notations.

2.3 Fentanyl products in pharmaceutical form and in combination with other substances

Pharmaceutical preparations of fentanyl are currently available as oral transmucosal lozenges, effervescent buccal tablets, sublingual tablets and sprays, nasal sprays, transdermal patches and injectable formulations [9]. Brand names for fentanyl include Actiq® (lozenge), Duragesic® (transdermal patch), Fentora™ (buccal tablet), Abstral® (sublingual tablet), Subsys™ (sublingual spray), Sublimaze® (injection), Lazanda® (nasal spray), and Ionsys™ (transdermal device). Sufentanil, and carfentanil brand names are Sufenta® and Wildnil™, respectively and brand names for alfentanil include Alfenta® and Rapifen®.

The popularity of fentanyl progressed beyond the use/abuse of pharmaceutical preparations, to the abuse of illicitly manufactured fentanyl, and eventually resulted in the proliferation of fentanyl analogues. Fentanyl and its analogues are typically seen in powder form, which can be used as is or mixed with another substance and then smoked or taken by the intranasal or intravenous route. They can also be pressed into tablets, often as counterfeit forms of other opioid pharmaceuticals (for example, hydrocodone or morphine pills), or other drug classes (alprazolam tablets), or mixed into an intranasal spray [9–12]. The abuse of fentanyl and its analogues is popular due to its potent narcotic analgesic effects. These substances may act as a substitute for, or even enhance the effect of heroin and other natural and/or semi-synthetic opioids. It has been estimated that 41 per cent of the approximately 7,100 heroin-related deaths in the United States between 2012 and 2014 involved fentanyl [13]. A number of countries, including Canada, Sweden, Estonia, Germany, the United Kingdom, Finland and Greece have reported almost 1,500 fentanyl-related fatalities between 2005 and 2014 [6,14,15]. In March 2015, the United States Drug Enforcement Administration (DEA) issued a warning of fentanyl-laced heroin [16], and a number of other regions in the United States reported increases in illicit fentanyl-related deaths [17–22].

One of the challenges of developing validated methods for the analysis and identification of fentanyl and its analogues in such a rapidly changing and dynamic market is that analytical reference materials may often not be commercially available. To assist

the work of analysts, there are a number of online resources and databases (refer to table 2), which may contain reference mass spectra and other analytical data to support the identification of novel and emerging fentanyl analogues.

Table 2. Selection of online mass spectral and drug resources

UNODC	https://www.unodc.org/LSS/Home/NPS
SWGDRUG	http://swgdrug.org/
Southern Association of Forensic Scientists	http://forendex.safs1966.org/index.php/home/index
European Project Response to Challenges in Forensic Drug Analyses	http://www.policija.si/apps/nfl_response_web/seznam.php
Cayman Chemicals	https://www.caymanchem.com/Home
Spectral Database for Organic Compounds SDBS	http://sdbs.db.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi
NPS Data Hub	https://www.nps-datahub.com/
Designer Drugs Online MS Database	https://db12.designer-drugs.de/login.pl
New Synthetic Drug Database	http://nsddb.eu/
Data Search System for New Psychoactive Substances	http://npsdb.nihs.go.jp/Search/Default_e.aspx
Mass Bank	http://www.massbank.jp/?lang=en
Pubchem	https://pubchem.ncbi.nlm.nih.gov/
Russian drug database	http://www.aipsin.com/article/aipsin/ips/drugs/mass_spectra/msdb_description.pdf

3. Safety aspects of handling fentanyl exhibits

3.1 Good laboratory practice

Laboratory policies should be put into place to ensure safe work environments. In addition, laboratories should have the facility and protocols in place to perform the drug testing service(s) offered. The handling of biological materials exposes personnel to infection hazards from, among others, hepatitis and HIV; all personnel should therefore take the necessary precautions and adhere to safety procedures such as wearing gloves and other protective clothing. See *Section VI*. Laboratory accommodation, environment and safety in the Recommended Guidelines for Quality Assurance and Good Laboratory Practices, for additional recommendations [23].

The risk of clinically significant exposure of fentanyl and its analogues to emergency responders is extremely low, as incidental dermal absorption resulting in opioid toxicity is unlikely. The American College of Medical Toxicology (ACMT) and American Academy of Clinical Toxicology (AACT) advise that the use of basic personal protective equipment (PPE), such as nitrile gloves and a N95 respirator, are sufficient protection to avoid exposure to fentanyls either by dermal exposure or inhalation [24].

Availability and use of naloxone in analytical facilities

Emergency personnel and other professionals who may encounter fentanyl or fentanyl analogues should be trained to recognize the symptoms of opioid intoxication, such as depressed respiratory function, confusion, and loss of alertness [24]. In addition, these professionals should have ready access to naloxone (for example, Narcan®), a medication used to block the effects of opioids, as well as training for its administration.

4. Pharmacology and toxicology of fentanyl and its analogues

4.1 Overview

Fentanyl, a potent narcotic analgesic, was developed in 1960. It is used in the treatment of breakthrough cancer pain and other chronic pain, and in surgical anaesthesia. Fentanyl activates the same receptors as morphine, and is therefore classified as an opioid. Fentanyl belongs to a family of drugs based on a phenylethylpiperidine backbone, and possesses multiple sites for the addition or substitution of various chemical functional groups to produce subfamilies of compounds with similar or even higher analgesic or toxic effect.

Fentanyl and its analogues are characterized by potencies typically higher than that of morphine—up to several thousand times in the case of carfentanil or remifentanil, but typically of the order of ten to five hundred times morphine's potency. They typically have a short duration of action, rapid onset of effect, high lipid solubility, few cardiovascular risks, and in contrast to morphine, low histamine release [25]. Pharmaceutical fentanyls are known to have a significant first pass effect, being rapidly metabolized in the liver. Subsequently, these are not administered therapeutically by the enteric route but instead by transmucosal methods, such as sublingual or buccal routes (lozenge, "lollipop", or spray), in the management of breakthrough pain. For the treatment of chronic pain, fentanyl is administered by a transdermal patch. For surgical use and anaesthesia, fentanyls are administered by intramuscular or intrathecal methods as a nerve block, or by intravenous methods [26]. Pharmaceutical preparations of fentanyl have been diverted for abuse, with users applying multiple patches, or extracting, chewing, or smoking gel reservoirs from fentanyl patches [27–31].

Other pharmaceutical fentanyls (alfentanil, remifentanil, sufentanil, and carfentanil—the latter approved only for veterinary use) only have applications in surgical anaesthesia as a result of their potency, and have historically been less subject to diversion and abuse [32]. Starting in the 1980s there have been incidents of clandestine and illicit synthesis of analogues of fentanyl. Some of these are taken directly from the Janssen patents of the 1960s and thereafter, but others are completely novel and have not previously been described. For those novel drugs, little is known in terms of strength of receptor binding or analgesic response in animals or man, and they represent a significant threat to public health and safety.

4.2 Mechanism of action and toxicity

The pharmacological activity of fentanyl and its analogues is due to their activation of the opioid receptors, with primary activity resulting from activation of the mu receptor, which is the main receptor sub-type involved in pain signaling [33]. Besides their analgesic properties, mu-opioid receptor agonists cause pupillary constriction, constipation, decreased consciousness, euphoria and dose-dependent respiratory depression, which can lead to death. The euphoric effects of opioid agonists can lead to habituation and dependence. The delta-opioid receptors also contribute to analgesia, as well as sedation, impairment of cognition, depressed mood, and motor incoordination and impairment, while the kappa-opioid receptor mediates pain sensation, sedation, cognitive impairment, effects on mood, diuresis, and temperature control. Opioids with significant kappa receptor agonist binding also cause hallucinatory or dissociative effects. Overall, the typical side effect profile of opioid agonist use includes respiratory depression, sedation, sleepiness, dizziness, nausea, vomiting, fatigue, headache and constipation. Tolerance to the analgesic and euphoric effects of opioids develops quickly, with doses frequently requiring adjustment for effective pain management. Cessation of opioid agonist use leads to a withdrawal syndrome, characterized by drug craving, dysphoria, anxiety, insomnia, irregular heart rate, loss of appetite, diarrhoea, sweating, nausea and vomiting [34].

The main mechanism of fatal opioid overdose is respiratory depression, leading to pathological indicators such as froth in the airways, and cerebral and pulmonary oedema. A distended urinary bladder is often observed at autopsy in opioid-induced death. Fentanyl and its analogues have high potency compared to morphine. Poor control of dose, poly-drug use, and patterns of repeated use are most likely contributors to the high rates of overdose, respiratory depression and death associated with these drugs [35].

4.3 Relative potency of fentanyl and its analogues

Fentanyl and its analogues were originally developed as safer alternatives to morphine, with fewer side effects. Fentanyl itself has been demonstrated to have an analgesic potency of 50-100 times that of morphine, but with a therapeutic index approximately five times higher. Besides those fentanyl analogues that have been approved for human use such as fentanyl, remifentanil, alfentanil and sufentanil, few other fentanyl analogues have been evaluated for pharmacokinetic parameters in humans, including potency, binding affinity to mu-, delta-, and kappa-opioid receptors, or receptor efficacy. Due to the multiple positions at which substitutions can be made to the fentanyl core structure, such as replacement of or substitution on the phenyl or piperidinyl rings, alteration of chain length or substitution on the alkyl chain, and halogenation on multiple sites, a very large number of permutations, and consequently fentanyl analogues, are possible. These structural changes can

dramatically affect the analgesic activity and potency of such compounds, in addition to altering their legal status. Table 3 summarizes the analgesic activity and potency relative to morphine for fentanyl and selected analogues. The median effective dose (ED_{50}) is the amount of drug that produces a therapeutic response or desired effect in half of the test subjects.

Table 3. Analgesic activity (ED_{50}) and analgesic potency relative to morphine for specified fentanyl analogues

Compound	*Analgesic activity ED_{50} (mg/kg)*	*Analgesic potency relative to morphine*	*Reference*
Morphine	13.9	1	[36]
Butyrylfentanyl	0.047-0.220	7	[37]
Furanylfentanyl	0.02	7	[38]
Acetylfentanyl	0.021	16	[37]
para-Fluorofentanyl	0.021	16	[37]
alpha-Methylfentanyl	0.0058	56.9	[37]
Alfentanil	0.044	75	[32]
Acrylfentanyl	0.082	170	[36]
Remifentanil	0.73	220	[39]
Fentanyl	0.062	224	[36]
Sufentanil	0.00071	4520	[40]
(+) *cis*-3-Methylfentanyl	0.00058	5530	[40]
Carfentanil	0.00032	10030	[40]
Isobutyrylfentanyl	0.048-0.261	1.3-6.9	[37]

Note that many of these data are derived from a selection of non-human animal models with different endpoints for analgesia, thus accounting for differences in findings. In aggregate however, these data give some sense of the relative potency of a range of analogues. All of the fentanyl analogues for which information is available have stronger analgesic activity relative to morphine [41]. However, these pre-clinical studies do not provide insight into toxicity of the fentanyl analogues, such as degree of respiratory depression or unconsciousness, and should only be used for cumulative understanding of relative potency to morphine.

As demonstrated in table 3, all fentanyl analogues have greater analgesic potency than morphine, and some can be more potent than fentanyl itself. For example, (+) *cis*-and *trans*-3-methylfentanyl are approximately 7,000 and 1,000 times more potent than morphine respectively [42], and carfentanil has an analgesic potency of more than 10,000 times that of morphine in a mouse model [37]. These differences, which result in a greater demand for analytical sensitivity, have implications for the

detectability of these very potent drugs in toxicological specimens. Some traditional analytical techniques such as liquid chromatography with UV visible detection (LC/UV) and gas chromatography with flame ionization detection (GC/FID) are unsuitable for fentanyl analysis.

The variability in potency and half-life also have an impact on medical intervention. For example, acrylfentanyl is more potent than fentanyl, with a longer duration of action [43]. Consequently, acrylfentanyl may require a longer period of symptomatic support in the emergency room, or repeated doses of naloxone.

4.4 Metabolism

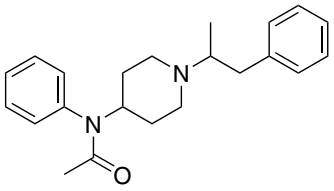
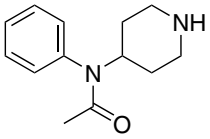
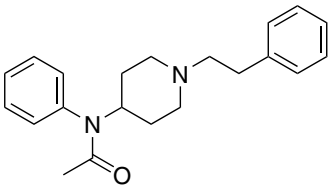
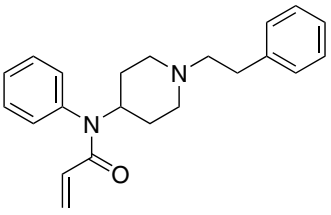
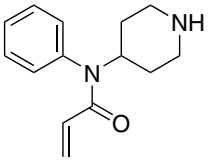
Identification of known metabolites of drugs can help prolong the detection window. For many fentanyl analogues, their metabolites may only have been postulated in the literature and not yet confirmed in authentic casework. However, the prediction of metabolites using known metabolic pathways yields likely scenarios that may be helpful in adding to toxicological assays, if reference material permits. As noted earlier, fentanyl has a phenylethylpiperidine core in its structure, and since many of its analogues typically incorporate variations on this core, they follow the established metabolic pathways of fentanyl.

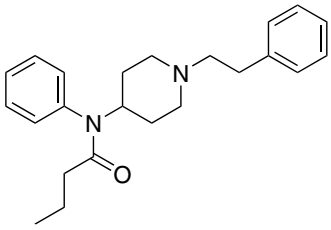
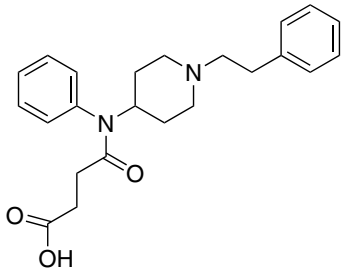
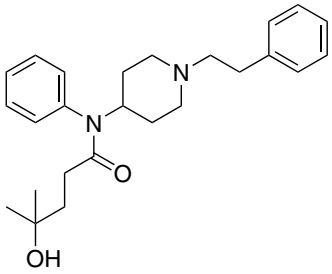
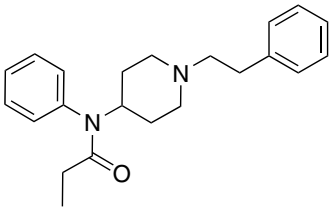
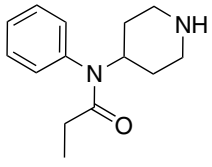
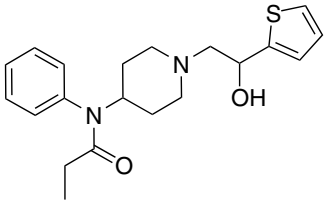
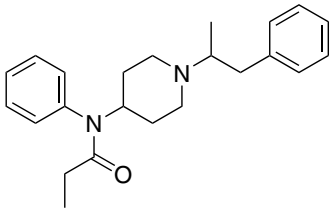
The major route of metabolism for fentanyl is *N*-dealkylation on the piperidine nitrogen to norfentanyl, its primary inactive metabolite, with minor pathways involving amide hydrolysis to despropionylfentanyl and alkyl hydroxylation to hydroxyfentanyl [44]. Other fentanyl analogues share these general pathways. The metabolism of 3-methylfentanyl, studied in rats and with recombinant human isoenzymes, identified *N*-dealkylation to 3-methylnorfentanyl followed by hydroxylation of the alkyl and aryl moiety, and hydroxylation of the propanamide side chain, as the predominant pathways [45]. Acetylfentanyl is metabolized in humans predominantly by hydroxylation on the phenyl moiety to hydroxyacetylfentanyl, with formation of noracetylfentanyl being a minor pathway [46]. Acrylfentanyl and *para*-fluoro-isobutyrylfentanyl are predominantly metabolized by *N*-dealkylation, cleaving off the phenethyl moiety, mono-hydroxylation at the ethyl linkage and piperidine ring, as well as hydroxylation/methoxylation at the phenyl ring [47]. The major metabolites of furanylfentanyl were generated by amide hydrolysis and dihydrodiol formation [48]. For butyrylfentanyl, hydroxylation and carboxylation predominated while the nor-metabolite and desbutyryl metabolite were minor [49,50]. For carfentanil, *N*-dealkylation and mono-hydroxylation of the piperidine ring were the predominant routes of metabolism [51]. Table 4 shows known metabolites of the major fentanyls and highlights those metabolites that are common to more than one drug.

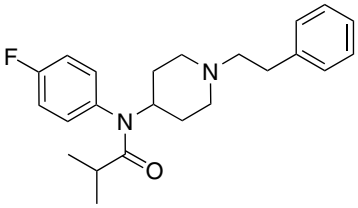
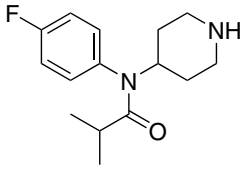
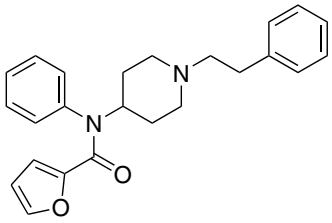
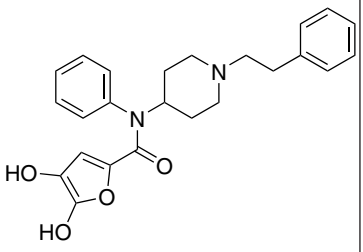
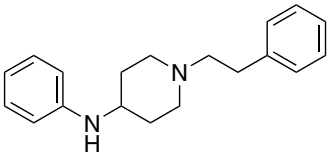
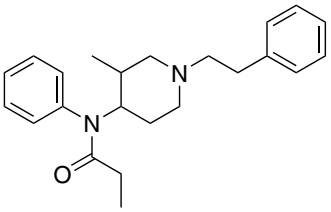
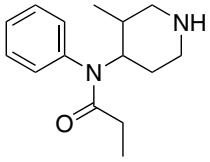
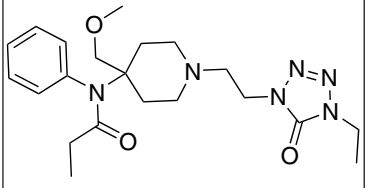
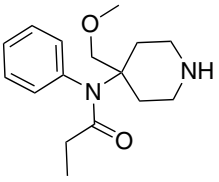
Based on the above considerations, it is likely that other fentanyl analogues whose fate has not been studied, are similarly metabolized through the same phase I reactions to their respective nor-metabolites via *N*-dealkylation on the piperidine ring,

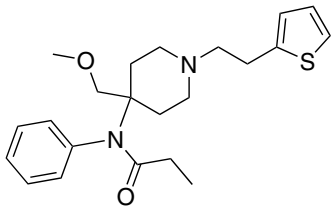
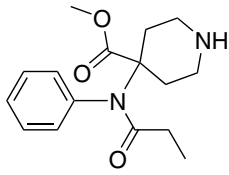
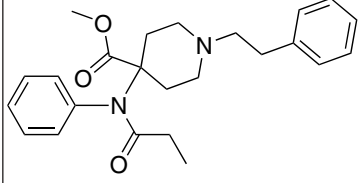
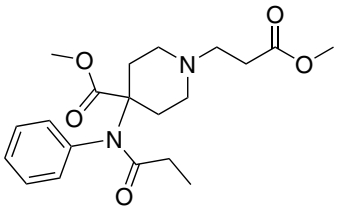
and via other common metabolic pathways including hydroxylation, dihydroxylation, and carboxylation, often in combination with *N*-dealkylation. Hydroxylated metabolites are frequently conjugated through phase II reactions, making hydrolysis of urine samples advisable prior to analysis [47]. Due to similar structures and shared metabolic pathways, the metabolism of many fentanyl variants can result in shared metabolites, which may complicate interpretation of toxicological data. For example, norfentanyl will be detected as a metabolic product of fentanyl, *alpha*-methylfentanyl, and *beta*-hydroxythiofentanyl [52,53]. The metabolism of both alfentanil and sufentanil share the common *N*-desalkyl metabolite. 4-ANPP (4-anilino-*N*-phenethylpiperidine; despropionylfentanyl) is frequently detected in fentanyl-related cases and is both a precursor to the manufacturing of illicit fentanyl and its analogues [44], as well as a shared metabolite of several; therefore, it offers limited specificity in terms of identifying what substance was ingested.

Table 4. Key fentanyl analogues and their known and common metabolites

Common name/ abbreviation	*Structure*	*Metabolite*
Acetyl-*alpha*-methylfentanyl		 Acetylnorfentanyl [47]
Acetylfentanyl		
Acrylfentanyl		 Acrylnorfentanyl [47]

Butyrylfentanyl		 Carboxybutyrylfentanyl [50]  Hydroxybutyrylfentanyl [50]
Fentanyl		 Norfentanyl [52,54]
beta-Hydroxythiofentanyl		
alpha-Methylfentanyl		

para-Fluoroisobutyryl-fentanyl		 *para*-Fluoroisobutyrylnorfentanyl [47]
2-Furanylfentanyl		 Furanylfentanyldihydrodiol [47]  Amide hydrolysis of furanylfentanyl [47]
3-Methylfentanyl		 3-Methylnorfentanyl [45]
Alfentanil		 Noralfentanil [55]

Sufentanil		 Norcarfentanil [51]
Carfentanil		
Remifentanil		

5. Analysis of fentanyl and its analogues and their metabolites in biological specimens

5.1 Priority fentanyl-related analytes in analytical casework

The focus of this manual is on both currently available fentanyl analogues approved for human or veterinary use (fentanyl, sufentanil, alfentanil, remifentanil, and carfentanil), and illicit clandestinely synthesized fentanyl analogues. As of September 2017, the list includes those fentanyl analogues that are under international control and other fentanyl analogues that have been reported to the UNODC Early Warning Advisory on New Psychoactive Substances, as well as newly emerging fentanyls recently seen in forensic casework (see table 1).

5.2 General structural considerations for selecting analytical methods

The fentanyl molecule is composed of three main parts: a phenylalkyl moiety, a piperidinyl ring, and a propylalkylamide moiety. Numerous fentanyl analogues are produced by modifications made on the propylalkylamide moiety of fentanyl, by altering the chain length on the arylalkylpiperidinyl group, or substituting the aromatic substituent on the phenylalkyl moiety. Fentanyl is basic drug with a pKa of 8.4. Other fentanyl analogues had reported pKas as follows: alfentanil 6.5, remifentanil 7.1 and sufentanil 8.0. Extraction efficiency is greatest when the pH of the solution is at approximately ±2 units of the pKa, at which point the drug will be either completely ionized or unionized. In liquid-liquid extraction protocols, the unionized form of the drug will be readily extracted into an organic solvent, and in the case of fentanyl all of the drug would be expected to be in the unionized form at two pH units above the pKa (pH ~ 10.4). In solid phase extraction methods using cation exchange, the optimal pH range would be two units below the pKa (pH ~ 6.4), resulting in the drug being ionized and allowing it to bind to the sorbent and be retained.

Modification and substitution of the fentanyl core structure to yield other fentanyls has resulted in multiple isomers with the same molecular formula and exact mass, but which differ in the arrangement of atoms within the molecules. The challenge of isomer differentiation is that identification is not always possible based solely on mass spectral means, especially when standard reference materials are not available. Isomers produce the same molecular ion and often the same base peak, and frequently have similar fragmentation patterns. In order to distinguish isomers that do not have unique fragmentation, they must be resolved chromatographically or differentiated using unique transitions, where they exist.

5.3 Testing and analysis

Sampling and sample types—specimens from living individuals/deceased individuals

Blood/serum/plasma

In living subjects, whole blood is generally collected from the antecubital vein. Plasma is the liquid portion of the blood derived when all the blood cells are separated from whole blood. Serum is the liquid portion of the blood collected after clotting and removal of the clot. Drug concentrations in blood reflect the amount of drug present at the time of collection. Many factors influence the degree to which the blood or serum plasma concentration reflects the effects on the individual, including tolerance, postmortem redistribution, acute versus chronic use, and receptor activation. Whole blood specimens should be collected into a tube that contains both a preservative and anticoagulant (for example, sodium fluoride and potassium oxalate). The blood-to-plasma ratio for fentanyl is reported to be 0.8-1.0, and for norfentanyl 1.0-1.3. Additional blood-to-plasma ratios have been reported for alfentanil (0.6) and sufentanil (0.6-0.7) [56]. Postmortem specimens will vary in quality and homogeneity depending on a number of factors. Ideally, femoral or peripheral blood is best sampled and analysed for forensic toxicology purposes.

Urine

Urine, as a biological sample, can be used to demonstrate previous exposure or historical drug exposure. Typically, after absorption, drugs or drug metabolites are present in urine at higher concentrations than in the blood. In urine, the target analytes are often the metabolites of the parent drugs, which are often inactive. The higher concentrations of drugs in urine have the advantage of extending the period over which prior drug use can be detected.

Additional specimens from deceased individuals

Tissues

Tissue specimens, such as brain, liver, kidney, or muscle tissue from near the site of injection, can be tested in postmortem investigations. In the absence of blood or

urine, such as occurs after putrefaction, tissues can often be used to detect and in some cases quantify a suspected drug. Liver is the optimal sample in the absence of blood or urine. Drug concentrations determined from tissue analysis are difficult to interpret. Prior to analysis, it is recommended that tissue samples are homogenized using established protocols.

Vitreous humour

Vitreous humour, which is a gel-like substance behind the lens and in front of the retina of the eye, is another alternative specimen recommended for analysis in postmortem toxicology casework. Vitreous humour is an anatomically isolated site, making it less susceptible to putrefactive changes such as postmortem redistribution. Often only 2-3mL can be collected for analysis. Concentrations of drugs in vitreous humour are often similar to those in blood; however interpretation should be done with caution.

Stability and storage

Fentanyl has been demonstrated to be stable in plasma at room temperature for 6 hours and for 6 months at -20°C [57]. In fluoridated blood, fentanyl was stable for 1 week at room temperature [58]. In urine, fentanyl and norfentanyl were stable for 24 hours at room temperature or 4°C [59]. Acetylfentanyl, alfentanil, *beta*-hydroxythiofentanyl, butyrylfentanyl, carfentanil, fluorofentanyl, fluoroisobutyrylfentanyl furanylfentanyl, *alpha*-methylfentanyl, *para*-methylphenethylacetylfentanyl, *para*-methoxybutyrylfentanyl, norfentanyl and valerylfentanyl, were all stable in sodium fluoride/potassium oxalate preserved human blood for at least 30 days at room temperature, refrigerated or frozen. Acrylfentanyl was stable for only one day at room temperature, seven days refrigerated, or one month frozen. 4-Anilino-*N*-phenethylpiperidine (4-ANPP) was stable for fourteen days at room temperature, and for thirty days refrigerated or frozen. Stability indicates losses of less than 20 per cent over the period specified [60]. Best practices for detection of fentanyl in toxicological specimens call for them to be stored refrigerated or frozen until the time of analysis.

Screening methods for fentanyls

Immunoassays

Immunoassays are commonly used for preliminary screening of biological specimens to presumptively identify the presence of drugs. Immunoassays provide a quick, cost-efficient and sensitive screening method that allows for automation and high throughput. The principle involves the binding of the target (antigen) to an antibody coupled with a colour change reaction for detection. Depending on the specificity of the antibody and the degree of homology of the related drugs, an immunoassay may or may not give a positive reaction with other drugs that are chemically related to the principal target. As reflected below, various immunoassays have limited cross-reactivity with many of the fentanyl analogues, and for others their cross-reactivity is unknown. Consequently, immunoassays cannot be used to

reliably rule out the presence of fentanyl analogues in any given case, and other more sensitive and specific chromatographic/mass spectrometric methods as described below should be considered.

Known cross-reactivity of fentanyl analogues on common immunoassay tests

Fentanyl and fentanyl analogue cross-reactivity in urine was described using an enzyme-linked immunosorbent assay (ELISA) developed by Janssen Life Sciences. The nine analogues included in the study and their cross-reactivities at 2 ng/mL, were as follows: acetylfentanyl (111%), *para*-methylfentanyl (93%), *para*-fluorofentanyl (87%), butyrylfentanyl (77%), (±) *trans*-3-methylfentanyl (50%), *alpha*-methylfentanyl (19%) and (±) *cis*-3-methylfentanyl (3%). Benzylfentanyl and alfentanil did not cross-react [61].

The cross-reactivity of fentanyl analogues has been evaluated using the Neogen® fentanyl ELISA with the following results: Less than 1% cross-reactivity was noted for 4-ANPP, *beta*-hydroxythiofentanyl and norfentanyl in blood and urine, with the exception of 1.4% cross-reactivity for *beta*-hydroxythiofentanyl in urine. Other fentanyl analogues showed some cross-reactivity in blood/urine respectively: acetylfentanyl (31%/55%), butyrylfentanyl (66%/78%) and furanylfentanyl (63%/106%) [62]. Fentanyl, *para*-fluorofentanyl, furanylfentanyl, butyrylfentanyl and 4-ANPP were evaluated for cross-reactivity on fentanyl ELISA and enzyme multiple immunoassay technique (EMIT) kits. Cross-reactivities in phosphate buffered saline on the ELISA kit, and in urine on the EMIT kit, respectively were butyrylfentanyl (90%/88%), furanylfentanyl (77%/74%) and *para*-fluorofentanyl (47%/61%).

5.4 Sample preparation

The following examples illustrate typical approaches to sample preparation from published validated methods.

Liquid-liquid extraction

1. One mL of sample (blood, urine, liver and gastric contents) was combined with 2 mL of deionized (DI) water and vortex mixed. The sample was made alkaline with the addition of 1 mL of concentrated ammonium hydroxide (28-30%) followed by vortex mixing and the addition of 6 mL of 1-chlorobutane. The sample was subsequently mixed for 10 minutes, followed by centrifugation and the addition of 200 mg of sodium sulfate to suppress emulsion formation, followed by another centrifugation step. The organic layer was transferred to a new tube followed by the addition of 2 mL of 1.0 M hydrochloric acid. After mixing for 10 minutes, the organic layer was aspirated to waste and the aqueous layer was made alkaline with 1 mL of concentrated ammonium hydroxide. The sample was vortex mixed and 3 mL of 1-chlorobutane was added followed by 10 minutes of

mixing and subsequently transferring the organic layer to a new tube. The method was applied to cases containing butyrylfentanyl and acetylfentanyl [63].

2. One mL of sample (blood) was made basic by the addition of 300 µL of tris buffer (pH 11) and extracted into 500 µL of ethyl acetate in a vortex mixer. The sample was centrifuged and the organic layer was transferred to a new tube followed by the addition of 75 µL of ammonium acetate (10 mM, 0.1% formic acid, pH 3.2) prior to evaporation. 75 µL of acetonitrile was added to the residue followed by 5 minutes of sonication and centrifugation. The method was applied to cases containing 3-methylfentanyl [64].

3. Five hundred µL of sample (plasma or urine) was combined with 500 µL of an aqueous potassium carbonate solution (pH 12.3) and the sample was vortex mixed. 7 mL of n-hexane and ethyl acetate (7:3) was subsequently added, followed by mixing for 15 minutes, centrifugation, and transfer of the organic layer for analysis with GC-MS [65].

4. One mL of sample (blood, urine or vitreous humour) or 1 gram of a 1:4 tissue homogenate was extracted using n-butyl chloride and ammonium hydroxide with an acidic back extraction. The method was specifically targeted to acetylfentanyl [66].

Solid phase extraction

1. Five hundred µL of sample (blood) was pretreated with 2 mL of 0.1 M phosphate buffer (pH 6) and centrifuged. A reverse phase and ion exchange column (130 mg) was conditioned with 3 mL methanol, 3 mL deionized water (DI) and 1 mL phosphate buffer. After the sample was applied, the column was washed with 1.5 mL DI water, 0.5 mL 0.1M acetic acid and 1.5 mL methanol. The sample was eluted with 2 mL of ethyl acetate, acetonitrile and ammonium hydroxide (78:20:2) and dried to completion at 40°C. The method was used to detect U-47700 (3,4-dichloro-*N*-((1S,2S)-2-(dimethylamino)cyclohexyl)-*N*-methylbenzamide) and furanylfentanyl [67].

2. Five hundred µL of sample (blood or urine) was pretreated with 2 mL of potassium phosphate buffer (pH 6) followed by 15 minutes of sonication, and centrifugation for 10 minutes. A Cerex Trace B (Tecan®, Männedorf, Switzerland) column (35 mg) was treated with 1 mL of methanol followed by 1 mL phosphate buffer. After the sample was applied, the column was washed with 2 mL of DI water and 2 mL 100mM acetic acid, followed by drying and subsequent washes with 1 mL methanol and 1 mL ethyl acetate. The sample was eluted with 1.2 mL of ethyl acetate, methanol and ammonium hydroxide (93:5:2) in 600 µL aliquots. The method was suitable for 16 opioid compounds, including *para*-methoxybutyrylfentanyl, acetylfentanyl, butyrylfentanyl, carfentanil, despropionylfentanyl, fentanyl, furanylfentanyl, isobutyrylfentanyl *N*-desmethyl-U-47700, norfentanyl, *para*-chlorofentanyl, *para*-fluorobutyrylfentanyl, *para*-fluoroisobutyrylfentanyl, U-47700, valerylfentanyl, and *beta*-hydroxythiofentanyl [68].

3. One mL of sample (blood) was pretreated with 2 mL of 0.1M phosphate buffer (pH 6) followed by vortex mixing and centrifugation. A solid phase column was conditioned with 3 mL of methylene chloride, isopropanol, ammonium hydroxide (78:20:2), 3 mL of methanol, 3 mL of DI water and 1 mL of phosphate buffer. After application of the sample, the column was washed with 3 mL of DI water, 1 mL of 1.0 M acetic acid, 2 mL of hexane, and 3 mL of methanol. The sample was eluted with 3 mL of methylene chloride, isopropanol, ammonium hydroxide (78:20:2) and dried to completion at 40°C. The method was applied to fentanyl-positive cases [19].

5.5 Gas chromatography/mass spectrometry screening (GC/MS)

The following is an example of a screening method for common drugs of abuse including, fentanyl, sufentanil, alfentanil and norfentanyl in urine using an Agilent 7890 Gas Chromatograph coupled to an Agilent 5975A mass spectrometer (Agilent Technologies, Santa Clara, California, United States). The limits of detection were 2 ng/mL for alfentanil and 5 ng/mL for fentanyl and sufentanil, and mass spectra information is provided in table 5 [69].

GC-MS operating conditions		
GC oven conditions:	140°C for 0.4 minutes, increased to 280°C at a rate of 35°C/min, then 118°C/min to 320°C and held for 1.5 minutes, (total time, 6.2 min)	
Column:	5% phenylmethylpolysiloxane (J&W DB-5), 10 m X 0.18 mm. i.d., 0.18 µm film thickness	
Injection parameters:	Splitless mode	
	Injector temp:	270 °C
Carrier gas:	Helium, flow rate:	0.7 mL/min
Detector:	Ionization mode:	Electron impact (EI) mode, 70 eV
	Scan parameters:	SIM/SCAN mode (m/z 51-550)
Limit of detection:	2 ng/mL for alfentanil 5 ng/mL for fentanyl and sufentanil	

Note that GC/MS methods typically have detection limits in the 1-10 ng/mL range for fentanyl and its metabolites and analogues, which are typically too high to detect toxicologically significant concentrations of the more potent compounds, and are not recommended when alternatives are available.

Table 5. Characteristic mass spectral ions and retention times for selected fentanyls

Substance	*Retention time (min)*	*Characteristic ions (m/z)*
Fentanyl-D5	4.798	250, 194
Norfentanyl-TMS	3.230	*155*, 154, 247, 289, 304
Fentanyl	4.800	*245*, 146, 189, 202
Sufentanil	4.927	140, 187, 238, 289
Alfentanil	5.185	*289*, 222, 268, 359

5.6 Liquid chromatography/high-resolution mass spectrometry (LC-HRMS) screening

The following is an example of a screening method using a Thermo Scientific Dionex Ultimate 3000 RSLC ultra-high-performance liquid chromatography (Idstein, Germany) coupled to a Bruker Daltonics AmaZon Speed™ ion trap mass spectrometer (Bremen, Germany). The method was developed for the qualitative identification of 44 opioid-related compounds with a detection limit of 0.1 to 5 ng/mL for all analytes (mass spectral data shown in table 6) [70].

LC-MS operating conditions	
LC:	
Column:	Thermo Scientific Acclaim® RSLC 120 C18 column (2.2 µm, 2.1 X 100 mm)
Mobile phase:	(A) 2 mM ammonium formate, 0.1% formic acid, 1% acetonitrile in water
	(B) 2 mM ammonium formate, 0.1% formic acid, 1% water in acetonitrile
Gradient:	Started at 99% A, was adjusted to 1% A over 8 minutes and then held for 3 minutes to allow for re-equilibration
Flow rate:	500 µL/min
Injection volume:	5 µL
MS:	
Detection and ionization mode:	Positive electrospray ionization scanning mode for a mass range of 70-800 m/z

Table 6. Targeted precursor ions for selected fentanyls and opioid-related compounds.

Compound	*Precursor ion* (MS^2)[a]	*Precursor ion* (MS^3)[b]
3-Methylfentanyl	351.19	–
6-*O*-Acetylmorphine	328.08	–
Acetaminophen	151.91	110
Acetylfentanyl	323	188
Acetylsalicyclic acid	178.92	137
Alfentanil	417.15	–
beta-Hydroxythiofentanyl	359.22	341
Buprenorphine	468.27	–
Butyrylfentanyl	351	188
Carfentanil	395.15	335.2
Codeine	300.04	–
Desproprionyl fentanyl	281	188
Dihydrocodone	302.08	–
EDDP	278.03	–
Fentanyl	337.16	–
Furanylfentanyl	375	188
Heroin	370.14	–
Hydrocodone	300.08	–
Hydromorphone	285.99	–
Ibuprofen	206.95	207
Meperidine	248.02	–
Methadone	310.1	265
Mitragynine	399.19	238
Morphine	286	–
Naloxone	328.1	310
Naltrexone	342.04	324
Naproxen	230.89	184.8
N-Desmethyltramadol	250	232
Norbuprenorphine	414.4	–
Norcodeine	286.03	–
Norfentanyl	233.07	–
Normorphine	271.96	–
Noroxycodone	302.12	284
Noscapine	414	220
O-Desmethyltramadol	250	232

Oxycodone	316.1	298
Oxymorphone	301.97	284
para-Fluorobutyrylfentanyl	369	188
para-Fluoroisobutyrylfentanyl	369	188
Salicylic acid	136.96	93.2
Sufentanil	387.1	–
Tramadol	264	246
U-47700	329	284
W-18	422	273

[a] parent ion targeted for MS^2, [b] parent ion targeted for MS^3

5.7 Targeted analysis/confirmatory and quantitative methods

Liquid chromatography with UV detection (HPLC/UV)

No published methods using this platform are considered to be suitable for the detection of even toxic concentrations of fentanyl and its analogues, and HPLC/UV should not be used for toxicological applications.

Gas chromatography mass spectrometry (GC/MS)

The following is an example of a method using an Agilent 6890 GC coupled to a 5973 mass spectrometer (Agilent Technologies, Santa Clara, California, United States). The linear range for the method was 0.5-50 ng/mL with limits of detection at 0.08 ng/mL for all compounds with the exception of alfentanil at 0.04 ng/mL. The retention time and mass spectral information is shown in table 7 [55].

GC-MS operating conditions		
GC oven conditions:	100°C for 1 minute, increased to 200°C at a rate of 42°C/min, then 15°C/min to 280°C and held for 12 minutes,	
Column:	5% phenylmethylsilicone (J&W DB-5), 30m X 0.25 mm. i.d., 0.5 mm film thickness	
Injection parameters:	Splitless mode	
	Injector temp:	270 °C
Carrier gas:	Helium, flow rate:	0.7 mL/min
Detector:	Ionization mode:	EI mode: 70 eV
	Scan parameters:	SIM mode

Table 7. Retention times and mass spectral ions used for targeted identification and quantification of selected fentanyl compounds

Compound	*Retention time (mins)*	*Ions (m/z) (ions in bold used for quantification)*
Norfentanyl (pentafluoropropionic)	12.8	**150**, 322, 229
Fentanyl	17.7	**245**, 146, 189
Sufentanil	18.5	**289**, 140, 187
Alfentanil	21.1	**289**, 222, 268
Fentanyl-D5	17.7	**250**, 151, 194

A. Example of a method using an Agilent 7890 gas chromatograph coupled to a 5975 mass spectrometer (Agilent Technologies, California, United States). Chromatographic separation was achieved using a Zebron ZB5-MS (15 m X 0.25 mm X 0.25 µm; Phenomenex, Torrance, California, United States) capillary column. The injection port was 250°C in splitless mode. The temperature programme was as follows: 100°C ramped at 20°C/minute until 290°C and held for 2 minutes. The mass spectrometer was operated in SIM mode for butyrylfentanyl. The linear range was 10-250 ng/mL with a limit of detection at approximately 2 ng/mL [63].

B. Example of a method using an Agilent 6890 gas chromatograph coupled to a 5973 mass spectrometer (Agilent Technologies, California, United States). Chromatographic separation was achieved using a RTX ms-1 column (15 m X 250 µm X 0.25 mm; Restek, Bellefonte, Pennsylvania, United States). The injection port was set to 250°C in splitless mode. The oven temperature programme was as follows: 100°C to 280°C at 20°C/minute, which was held for 1 minute, resulting in a 10 minute run time. The mass spectrometer was operated in SIM mode for acetylfentanyl. The linear range of the method was 125-2000 ng/mL with a limit of detection at 62.5 ng/mL [66].

As noted below, toxicologically significant effects of fentanyl and its analogues occur at concentrations below 1 ng/mL, so if a laboratory only has access to GC-MS technology, it would be essential for it to establish its own limits of detection and ensure that they are appropriate for the intended purpose of analysis.

Liquid chromatography tandem mass spectrometry (LC/MS/MS)

The following is an example of method using an Alliance® HT 2705 LC system coupled to a Quattro Premier mass spectrometer (Waters, Massachusetts, United States). The mass spectrometer was operated with positive electrospray ionization in multiple reaction monitoring (MRM) mode for fentanyl, norfentanyl, alfentanil, remifentanil, sufentanil and 3-methylfentanyl (see table 8) and the method had a linear range from 0.1-50 ng/mL [65].

LC-MS operating conditions		
LC:		
Column:	Xterra® MS C18 column (3.5 µm, 2.1 X 150 mm) and Xterra® MS C18 column guard (3.5 µm, 2.1 X 10 mm) (both Waters Corporation) set to 60°C	
Mobile phase:	(A) 0.15% formic acid in water	
	(B) 0.15% formic acid in acetonitrile	
Gradient:	Gradient started at 10% B at 0.00 minutes, and was increased to 27% B over 0.50 minutes, held for 8 minutes, and returned to starting conditions within 0.10 minute.	
	Column allowed to equilibrate for 5 minutes (total run sample, 13.5 min)	
Flow rate:	0.3 mL/min	
MS:		
Detection:	Ionization mode:	Positive electrospray ionization
	Scan parameters:	Multiple reaction monitoring (MRM) mode
Linear range	0.1-50 ng/mL	

Table 8. MRM transitions, MS conditions and retention times for the targeted identification and quantification of selected fentanyl compounds. (q) indicates ion used for quantification.

Compound	*Parent ion (m/z)*	*Product ion (m/z)*	*Cone volts (V)*	*Collision energy (eV)*	*Retention time (min)*
Alfentanil	417.3	268.6 (q)	30	20	4.93
		197.7	30	20	
Fentanyl	337.2	188.5 (q)	35	25	4.87
		105.6	35	35	
Sufentanil	387.6	238.2 (q)	30	20	6.72
		355.7	30	20	
3-Methylfentanyl	351.5	202.4 (q)	40	25	5.70
		105.6	40	35	
Remifentanil	377.1	317.0 (q)	25	15	4.05
		345	25	15	
Norfentanyl	233	84.0 (q)	25	20	3.61
		176	25	15	
Fentanyl-D5	342.2	188.5 (q)	35	35	4.84
		105.6	35	35	
Norfentanyl-D5	238	84.0 (q)	25	20	3.59
		181.9	25	15	

A. Example of a method using an Agilent 1260 liquid chromatograph system coupled to a 6460 tandem mass spectrometer (Agilent Technologies). Chromatographic separation was achieved using a Poroshell 120 EC-C18 analytical column (2.7 μm, 2.1 X 100 mm) set to 40°C, using 5mM ammonium formate and 0.1% formic acid in water (A) and acetonitrile with 0.1% formic acid (B). The gradient started at 20% B at 0.00 minutes, was held for 0.5 minutes and then increased to 55% B at 8 minutes, followed by an increase to 90% B at 8.50 minutes. The column was allowed to equilibrate for 3 minutes, resulting in 11.50-minute run time. The mass spectrometer was operated in positive electrospray ionization and in (MRM) mode. The method was developed for 16 novel opioid compounds (4-methoxybutyrylfentanyl, acetylfentanyl, butyrylfentanyl, carfentanil, despropionylfentanyl, fentanyl, furanylfentanyl, isobutyrylfentanyl, *N*-desmethyl U-47700, norfentanyl, *para*-chlorofentanyl, *para*-fluorobutyrylfentanyl, *para*-fluoroisobutyrylfentanyl, U-47700, valerylfentanyl, *beta*-hydroxythiofentanyl) and had limits of detection ranging from 0.01 to 0.5 ng/mL in blood and urine [68].

B. Example of a method using an Agilent 1100 series high performance liquid chromatograph coupled to a 6430 tandem mass spectrometer (Agilent, California, United States). Chromatographic separation was achieved using a ZORBAX Eclipse Plus C18 column (3.5 μm, 4.6 x 100 mm; Agilent, California, United States) operated at 40°C, using 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The gradient started at 40% B adjusting to 10% B over five minutes and returning to 40% B at 5.5 minutes with a total run time of 10 minutes. The mass spectrometer was operated in positive electrospray ionization and MRM mode for U-47700, U-50488 (*rel*-3,4-dichloro-*N*-methyl-*N*-[(1R,2R)-2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide) and furanylfentanyl with a linear range of 5-500 ng/mL for U-47700 and U-50488 and 1-100 ng/mL for furanylfentanyl. The limit of detection was 0.5 ng/mL for all compounds [67].

Liquid chromatography/high-resolution mass spectrometry

Analyses were performed on a Waters Acquity I class UPLC coupled to Waters G2-S Q-TOF (Waters, Milford, United States). Chromatographic separation was achieved using an Acquity BEH C18 UPLC column (1.7 μm, 2.1 x 100 mm; Waters, Milford, United States) operated at 50°C 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient started at 5% B, was held there for 1 minute, and increased to 15% B at 6 minutes, Subsequently, the gradient was increased to 100% B at 7 minutes, held isocratically from 7 to 7.8 minutes and then returned to initial conditions over 0.1 minute with a total run time of 10 minutes. The mass spectrometer operated in MS^E mode with the quadrupole in scanning mode and switching between low collision energy (6 eV) for full scan data and a collision energy ramp from 15 to 30 eV for fragmentation data. The limit of quantitation and limit of detection were 0.104 ng/mL and 0.031 ng/mL for fentanyl and 0.037 ng/mL and 0.011 ng/mL for norfentanyl, respectively [71].

6. Reference data for the interpretation of toxicological findings of fentanyl and selected analogues

6.1 Fentanyl

Numerous incidents of fentanyl-related fatalities have been reported, with blood fentanyl concentrations ranging from 3.0-28 ng/mL; in comparison, recommended serum concentrations range from 1-2 ng/mL for analgesia and 10-20 ng/mL for anaesthesia [26,56]. Case series of reported fentanyl concentrations in adults who died from transdermal, intravenous, and oral abuse of fentanyl patches were 18 ng/mL (range 4-54), 59 (3-383) and 28 (7-97), respectively [29,72]. In fentanyl-only related deaths, average blood concentrations have been increasing, with a 2003 case series reporting an average result of 9 ng/mL [73], and a 2014 case series reporting an average of 30 ng/mL [74]. A cluster of 9 fentanyl-laced heroin deaths were reported in Australia with reported average and median femoral blood concentrations of 18 and 29 ng/mL (range <1-45) accompanied by morphine, 140 and 80 ng/mL (range 20-400 ng/mL) [75]. The combination of heroin and fentanyl has become a routine toxicological finding in suspected opioid overdose death investigations, but even fentanyl by itself is posing an increased risk of overdose and death to recreational users.

6.2 3-Methylfentanyl and *alpha*-methylfentanyl

Deaths and intoxications attributed to 3-methylfentanyl and *alpha*-methylfentanyl were reported in the late 1970s to 1980s. Emergency physicians in the United States became suspicious of "China White" after an unusual increase in narcotic overdoses appearing in the emergency department (ED) coupled with "routine drugs of abuse" screens presenting negative for opiates despite reversal of effects by naloxone [76,77]. The causal agent for those cases was analytically confirmed to be 3-methylfentanyl, but another 15 deaths were caused by *alpha*-methylfentanyl [78]. In 122 fentanyl-related deaths during the 1980s, the average concentration of fentanyls found in blood (including fentanyl, *alpha*-methylfentanyl, and 3-methylfentanyl) was 3.0 +/- 3.1 ng/mL [79]. Although this range appears to be consistent with concentrations recommended in serum for

management of analgesia using fentanyl, this average includes findings of *alpha*-methylfentanyl and (+) 3-methylfentanyl, which are both more potent by a factor of 1.1 and 16 respectively than fentanyl [79,80]. Concentrations of *alpha*-methylfentanyl in reported fatalities averaged 7 ng/mL (2-11) [56]. More than 110 3-methylfentanyl fatalities were reported in 2005-2006 in Estonia where the mean combined concentration of *cis* and *trans* isomers among cases was 1.9 ng/mL, which is 10 times lower than the mean fentanyl concentration in fentanyl-related fatalities [81]. For cases only involving 3-methylfentanyl, *cis*-3-methylfentanyl ranged from 0.06-2.59 (n=50) and *trans*- ranged from 0.19-1.91 (n=49). In deaths involving 3-methylfentanyl in Cuyahoga County, Ohio, United States in 2016, concentrations were in the range of 0.15-1.7 ng/mL [82].

6.3 Acetylfentanyl

Acetylfentanyl gained popularity in the United States in 2013 after 14 reported deaths in Rhode Island; subsequently reports of acetylfentanyl related overdose fatalities were reported in Pennsylvania, California, Louisiana, North Carolina, and Oregon [83,84]. Forty-one acetylfentanyl-related overdose deaths in Pennsylvania reported blood concentrations with median and mean of 11 and 169 ng/mL, (range 0.13-2100 ng/mL); all but one case was ruled as multiple drug toxicity (acetylfentanyl in blood = 170 ng/mL) with fentanyl itself being detected in 26 cases and heroin in 22 [85]. Peripheral blood acetylfentanyl concentrations reported in four cases were 310-600 ng/mL when acetylfentanyl was the only drug present, and 6-12 ng/mL when fentanyl concentrations were 15-21 ng/mL (n=3) [86]. Acetylfentanyl was confirmed in a series of non-fatal intoxications presenting to the ED in Sweden between April and November 2015; serum concentrations (n=7) ranged from 0.6-51.6 ng/mL [10]. In Europe, acetylfentanyl was confirmed in 1 death in 2013, 2 in 2014, and 29 in 2015; in 19 of the deaths, acetylfentanyl was the cause of or contributor to death [87].

6.4 Butyrylfentanyl

Butyrylfentanyl was first reported by the Early Warning System operated by the EMCDDA and Europol from seizures in Poland in summer 2013, before its appearance in the United States in 2014 [12]. Sweden reported its first seizures in April 2014; two intoxications presenting to the emergency department had serum concentrations of 0.9 and 0.6 ng/mL, with concomitant fentanyl concentrations of 4.3 and 10.2 ng/mL [12]. An intoxication with butyrylfentanyl in the United States involved an 18 y/o M who survived an overdose but who suffered from clinically significant hemoptysis, acute lung injury, hypoxic respiratory failure, and diffuse alveolar hemorrhage after snorting a white powder; which was subsequently identified as butyrylfentanyl by gas chromatography and mass spectrometry [88]. Butyrylfentanyl may undergo postmortem redistribution, based on analysis of butyrylfentanyl in multiple specimens, including paired cardiac and peripheral blood samples; peripheral blood results ranging from 3.7-99 ng/mL in 4 deaths [50,63,89].

6.5 Furanylfentanyl

In 2016, Helander et. al. reported furanylfentanyl serum concentrations of 4.4-148 ng/mL in non-lethal intoxications presenting to the ED, however, survival of furanylfentanyl intoxication in these cases may be attributed to prompt treatment and respiratory support, as these antemortem concentrations overlap with those seen in deaths [10]. Furanylfentanyl was confirmed in eight postmortem cases with blood concentrations ranging from 2.5-76 ng/mL (mean and median; 26 + 28 and 12.9 ng/mL, respectively) [67]. Over the course of four months in 2015 and 2016, a cluster of seven fatal intoxications involving furanylfentanyl occurred in Sweden, where furanylfentanyl intoxication, either alone or in combination with other substances, was determined to be the cause of death with concentrations ranging from 0.43-2.89 ng/mL. [90].

6.6 Carfentanil

Carfentanil was identified in drug seizures in Latvia in 2013 [91]. In July 2016, carfentanil emerged on the recreational drug scene in combination with heroin in the United States. It has subsequently been implicated in a large number of overdoses and deaths. The first reports were in Michigan, Ohio and Florida. In Northeast Ohio, there were over 25 deaths within a 30-day period attributed to carfentanil; between two state counties there were 165 reported deaths from carfentanil alone or in combination with heroin and fentanyl [82]. Carfentanil blood concentrations were in the range between 0.11-0.88 ng/mL. When approximately 500 postmortem cases were submitted for analysis at Miami-Dade County Medical Examiner's Office in a six-month period, 134 cases were positive for carfentanil, and of those, 104 were initially missed by gas chromatography mass spectrometry [70]. In a four-month span in the last half of 2016, carfentanil was identified in 262 postmortem blood specimens, with mean and median concentrations of 0.193 and 0.098 ng/mL (range 0.010 to 2.0) [92]. Carfentanil has also been reported in impaired driving cases; six cases were reported out of West Palm Beach, Florida, with concentrations ranging from 0.0115-0.125 ng/mL in blood [93]. Other non-fatal intoxications (n=1) and impaired driving cases (n=2) had serum and blood results of 0.6 and 0.013-0.043 ng/mL [94,95]. These reports of carfentanil detection in the United States are some of the first reports in literature of carfentanil being intentionally sold for recreational abuse; the only previous report was an accidental exposure during an attempt to tranquilize large animals [96].

6.7 Acrylfentanyl

Acrylfentanyl followed the emergence of carfentanil and furanylfentanyl; its first reported identification in literature was in Denmark in May 2016 [97]. Acrylfentanyl has been detected in at least 20 non-fatal intoxications; in eight of those cases, acrylfentanyl was confirmed in serum (0.5-2.1 ng/mL) [15]. Between April and

October 2016, a series of 40 lethal intoxications occurred in Sweden, in which the presence of acrylfentanyl was determined to be the main, or a contributing cause of death; concentrations ranged from 0.01-5.27 ng/mL [98]. Of those 40 cases, only two involved acrylfentanyl as the sole intoxicant and acrylfentanyl was confirmed at 0.01 and 0.02 ng/mL. In 2016, a total of 47 analytically confirmed deaths associated with acrylfentanyl occurred in Europe; Sweden accounted for 43 of those cases, but Denmark (n=1) and Estonia (n=3) also reported deaths. In addition to the three listed countries, Finland, Latvia and Slovenia have also reported detections of acrylfentanyl [36].

6.8 Other fentanyl analogues

Besides the compounds described above, there have been sporadic reports of several other fentanyl analogues, including *para*-fluorobutyrylfentanyl, *para*-fluoroisobutyrylfentanyl, *para*-methoxybutyrylfentanyl, *ortho*-fluorofentanyl and ocfentanil. Specific case reports for these analytes, as well as for previously listed compounds, are listed in annex 1. Several new fentanyl analogues have appeared in 2015 and 2016. The Miami-Dade County Medical Examiner Office reported the detection of new related compounds between 2015 and 2016, including *beta*-hydroxythiofentanyl (n=9), *para*-fluoroisobutyrylfentanyl (n=22), and U-47700 (n=4) [2]. Fatal intoxications with fentanyl analogues in Sweden between 2015 and 2016 primarily involved acetylfentanyl (n=34) and acrylfentanyl (n=43); in addition, *para*-fluoroisobutyrylfentanyl (n=14), furanylfentanyl (n=10), *para*-chloroisobutyrylfentanyl (n=3), tetrahydrofuranfentanyl (n=5), *para*-methoxybutyrylfentanyl (n=2), *para*-fluorobutyrylfentanyl (n=1) and butyrylfentanyl (n=1) have also been reported [4].

Fentanyl and its analogues have been detected in hospitalizations, impaired drivers, and in death investigation casework. The triad of opioid symptoms, CNS depression, miotic pupils, and respiratory depression are common clinical signs seen in cases presenting to EDs. Additionally, signs of intoxication (drowsiness, inattention, slowed reflexes) leading to cognitive and psychomotor impairment have been documented. The impairing effects of opioids can also be exacerbated when combined with other CNS depressants such as alcohol or benzodiazepines. Fentanyl and its analogues should be considered by hospital staff, emergency responders, medical examiners, death investigators and police officers when investigating suspected opioid intoxications, especially when hospital immunoassay-based tests are negative, or presumptive positive results cannot be confirmed.

References

[1] P. A. J. Janssen, in *Opioids in Anesthesia*, Butterworth Publishers, 1984, p. 37–44.

[2] World Health Organization, *WHO model list of essential medicines. (2017)* [online]. Available at: http://www.who.int/medicines/publications/essential-medicines/en/ [Accessed 22 Sep.2017].

[3] P. A. J. Janssen, *1-Aralkyl-4-(n-Aryl-Carbonyl Amino)-Piperidines and Related Compounds*, 1965, US3164600 A.

[4] G. L. Henderson, *J. Forensic Sci.* 1988, *33*, p. 569–575.

[5] R. M. Baum, *Chemical and Engineering News* 1985.

[6] J. Mounteney, I. Giraudon, G. Denissov, P. Griffiths, *International Journal of Drug Policy* 2015, *26*, p. 626–631.

[7] European Monitoring Centre for Drugs and Drug Addiction, *Fentanyl in Europe: EMCDDA Trendspotter Study*, 2012.

[8] United States Department of Justice Drug Enforcement Administration, Diversion Control Division National Forensic Laboratory Information System, *NFLIS Brief: Fentanyl, 2001-2015*, 2017.

[9] United States Department of Justice Drug Enforcement Administration, Diversion Control Division, Drug and Chemical Evaluation Section, *Fentanyl*, 2016.

[10] A. Helander, M. Bäckberg, O. Beck, *Clinical Toxicology* 2016, *54*, p. 324–332.

[11] A. Helander, M. Bäckberg, P. Signell, O. Beck, *Clinical Toxicology* 2017, *55*, p. 589–599.

[12] M. Bäckberg, O. Beck, K.-H. Jönsson, A. Helander, *Clinical Toxicology* 2015, *53*, p. 609–617.

[13] R. G. Frank, H. A. Pollack, *New England Journal of Medicine* 2017, *376*, p. 605–607.

[14] R. Kronstrand, H. Druid, P. Holmgren, J. Rajs, *Forensic Science International* 1997, *88*, p. 185–195.

[15] Canadian Centre on Substance Candian Community Epidemiology Network on Drug Use, *CCENDU Bulletin: Novel Synthetic Opioids in Counterfeit Pharmaceuticals and Other Illicit Street Drugs*, 2016.

[16] United States Department of Justice Drug Enforcement Administration, *2016 National Drug Threat Assessment Summary*, 2016.

[17] Cuyahoga F., Hamilton L. *Increases in Fentanyl-Related Overdose Deaths—Florida and Ohio*, 2013–201 [18] R. M. Gladden, *MMWR Morb Mortal Wkly Rep* 2016, *65*, DOI 10.15585/mmwr.mm6533a2.

[19] D. Lee, C. W. Chronister, W. A. Broussard, S. R. Utley-Bobak, D. L. Schultz, R. S. Vega, B. A. Goldberger, *Journal of Analytical Toxicology* 2016, *40*, p. 588–594.

[20] L. J. Marinetti, B. J. Ehlers, *Journal of Analytical Toxicology* 2014, *38*, p. 592–598.

[21] J. G. Thompson, A. M. Baker, A. H. Bracey, J. Seningen, J. S. Kloss, A. Q. Strobl, F. S. Apple, *Journal of Forensic Sciences* 2007, *52*, p. 978–981.

[22] S. C. Wong, J. A. Curtis, W. E. Wingert, *Journal of Forensic Sciences* 2008, *53*, p. 495–498.

[23] United Nations, *Recommended Guidelines for Quality Assurance and Good Laboratory Practices: Manual for Use by National Laboratories*, 1995.

[24] American College of Medical Toxicology (ACMT) and American Academy of Clinical Toxicology (AACT), *ACMT and AACT Position Statement: Preventing Occupational Fentanyl and Fentanyl Analog Exposure to Emergency Responders*, 2017.

[25] A. Giorgetti, C. Centola, R. Giorgetti, *Human Psychopharmacology: Clinical and Experimental* 2017, *32*, e2605.

[26] T. H. Stanley, *The Journal of Pain* 2014, *15*, p. 1215–1226.

[27] C. K. M. W. Schauer, J. A. D. Shand, T. M. Reynolds, *Basic & Clinical Pharmacology & Toxicology* 2015, *117*, p. 358–359.

[28] A. M. Young, J. R. Havens, C. G. Leukefeld, *Harm reduction journal* 2010, *7*, p. 24.

[29] K. L. Woodall, T. L. Martin, B. A. McLellan, *Journal of Forensic Sciences* 2008, *53*, p. 222–225.

[30] European Monitoring Centre for Drugs and Drug Addiction, *The Internet and Drug Markets*, 2016.

[31] K. A. Marquardt, R. S. Tharratt, *J. Toxicol. Clin. Toxicol.* 1994, *32*, p. 75–78.

[32] R. S. Vardanyan, V. J. Hruby, *Future Medicinal Chemistry* 2014, *6*, p. 385–412.

[33] B. L. Kieffer, *Trends in pharmacological sciences* 1999, *20*, p. 19–26.

[34] I. Buajordet, A.-C. Naess, D. Jacobsen, O. Brørs, *European Journal of Emergency Medicine* 2004, *11*, p. 19–23.

[35] Stanley, T.H., *Anesthesia for the 21st century, Proceedings (Baylor University. Medical Center)*, 2000-1, volume 13, issue 1, p. 7-10.

[36] I. Ujváry, R. Jorge, R. Christie, T. Le Ruez, H. V. Danielsson, R. Kronstrand, S. Elliott, A. Gallegos, R. Sedefov, M. Evans-Brown, *Forensic Toxicology* 2017, *35*, p. 232–243.

[37] Y. Higashikawa, S. Suzuki, *Forensic Toxicology* 2008, *26*, p. 1–5.

[38] B.-S. Huang, R. C. Terrell, K. H. Deutsche, L. V. Kudzma, N. L. Lalinde, *N-Aryl-N-(4-Piperidinyl)Amides and Pharmaceutical Compositions and Method Employing Such Compounds*, 1986, US4584303 A.

[39] P. M. Wax, C. E. Becker, S. C. Curry, *Annals of Emergency Medicine* 2003, *41*, p. 700–705.

[40] W. Van Bever, C. Niemegeers, K. Schellekens, P. Janssen, *Arzneimittel-Forschung* 1976, *26*, p. 1548–51.

[41] Y. Zhu, B. Ge, S. Fang, Y. Zhu, Q. Dai, Z. Tan, Z. Huang, X. Ghen, *Yaoxue Xuebao [Acta Pharmaceutica Sinica]* 2001, *16*, p. 199–210.

[42] W. Van Bever, C. Niemegeers, P. Janssen, *Journal of Medicinal Chemistry* 1974, *17*, p. 1047–1051.

[43] M. Essawi, *Pharmazie* 1999, *54*, p. 307–308.

[44] E. Schneider, K. Brune, *Naunyn-Schmiedeberg's archives of pharmacology* 1986, *334*, p. 267–274.

[45] M. R. Meyer, J. Dinger, A. E. Schwaninger, D. K. Wissenbach, J. Zapp, G. Fritschi, H. H. Maurer, *Anal Bioanal Chem* 2012, *402*, p. 1249–1255.

[46] A. B. Melent'ev, S. S. Kataev, O. N. Dvorskaya, *J Anal Chem* 2015, *70*, p. 240–248.

[47] S. Watanabe, S. Vikingsson, M. Roman, H. Green, R. Kronstrand, A. Wohlfarth, *AAPS J* 2017, DOI 10.1208/s12248-017-0070-z.

[48] M. M. Goggin, A. Nguyen, G. C. Janis, *Journal of Analytical Toxicology* 2017, *41*, p. 367–375.

[49] A. E. Steuer, E. Williner, S. N. Staeheli, T. Kraemer, *Drug Testing and Analysis* 2017, *9*, p. 1085–1092.

[50] S. N. Staeheli, M. R. Baumgartner, S. Gauthier, D. Gascho, J. Jarmer, T. Kraemer, A. E. Steuer, *Forensic Science International* 2016, *266*, p. 170–177.

[51] M. G. Feasel, A. Wohlfarth, J. M. Nilles, S. Pang, R. L. Kristovich, M. A. Huestis, *AAPS J* 2016, *18*, p. 1489–1499.

[52] S. Sato, S. Suzuki, X.-P. Lee, K. Sato, *Forensic Science International* 2010, *195*, p. 68–72.

[53] L. M. Labay, T. J. Gallagher, M. Friscia, D. Buzby, F. X. Diamond, B. D. Holsey, S. A. Keeling, B. K. Logan, *National Association of Medical Examiners Annual Meeting, Minneapolis MNMeeting 2016* 2016.

[54] D. E. Feierman, J. M. Lasker, *Drug Metab. Dispos.* 1996, *24*, p. 932–939.

[55] S. Strano-Rossi, I. Alvarez, M. J. Tabernero, P. Cabarcos, P. Fernández, A. M. Bermejo, *J Appl Toxicol* 2011, *31*, p. 649–654.

[56] R.C. Baselt. *Disposition of Toxic Drugs and Chemicals in Man*, 11th edition, Biomedical Publications, Seal Beach, CA, 2017, ISBN 978-0-692-77499-1

[57] Y.-W. Chang, H.-T. Yao, Y.-S. Chao, T.-K. Yeh, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2007, *857*, p. 195–201.

[58] R. Karinen, E. L. Oiestad, W. Andresen, G. Wethe, A. Smith-Kielland, A. Christophersen, *J Anal Toxicol* 2010, *34*, p. 420–428.

[59] F. Badoud, E. Grata, L. Perrenoud, L. Avois, M. Saugy, S. Rudaz, J.-L. Veuthey, *J Chromatogr A* 2009, *1216*, p. 4423–4433.

[60] B. K. Logan, NMS Labs Stability Data, personal communication

[61] W. Ruangyuttikarn, M. Y. Law, D. E. Rollins, D. E. Moody, *Journal of analytical toxicology* 1990, *14*, p. 160–164.

[62] N. B. Tiscione, K. Wegner, *Journal of Analytical Toxicology* 2017, DOI 10.1093/jat/bkx005.

[63] I. M. McIntyre, A. Trochta, R. D. Gary, J. Wright, O. Mena, *J Anal Toxicol* 2016, *40*, p. 162–166.

[64] I. Ojanperä, M. Gergov, I. Rasanen, P. Lunetta, S. Toivonen, E. Tiainen, E. Vuori, *Am J Forensic Med Pathol* 2006, *27*, p. 328–331.

[65] S. Cooreman, C. Deprez, F. Martens, J. Van Bocxlaer, K. Croes, *J Sep Sci* 2010, *33*, p. 2654–2662.

[66] C. Fort, B. Curtis, C. Nichols, C. Niblo, *J Anal Toxicol* 2016, 40, p. 754–757.

[67] A. L. A. Mohr, M. Friscia, D. Papsun, S. L. Kacinko, D. Buzby, B. K. Logan, *Journal of Analytical Toxicology* 2016, *40*, p. 709–717.

[68] J. Seither, L. Reidy, *Journal of Analytical Toxicology* 2017, p. 1–5.

[69] S. Strano-Rossi, A. M. Bermejo, X. de la Torre, F. Botrè, *Analytical and Bioanalytical Chemistry* 2011, *399*, p. 1623–1630.

[70] E. N. Shoff, M. E. Zaney, J. H. Kahl, G. W. Hime, D. M. Boland, *Journal of Analytical Toxicology* 2017, p. 1–9.

[71] J. Viaene, K. Lanckmans, B. Dejaegher, D. Mangelings, Y. Vander Heyden, *Journal of Pharmaceutical and Biomedical Analysis* 2016, *127*, p. 49–59.

[72] T. L. Martin, K. L. Woodall, B. A. McLellan, *J Anal Toxicol* 2006, *30*, p. 603–610.

[73] J. J. Kuhlman, R. McCaulley, T. J. Valouch, G. S. Behonick, *Journal of analytical toxicology* 2003, *27*, p. 499–504.

[74] D. D. Lung, N. P. Lemos, *Forensic Toxicology* 2014, *32*, p. 118–125.

[75] L. N. Rodda, J. L. Pilgrim, M. Di Rago, K. Crump, D. Gerostamoulos, O. H. Drummer, *Journal of Analytical Toxicology* 2017, *41*, p. 318–324.

[76] M. Martin, J. Hecker, R. Clark, J. Frye, D. Jehle, E. J. Lucid, F. Harcheiroad, *Annals of Emergency Medicine* 1991, *70*, p. 158–164.

[77] J. Hibbs, J. Perper, C. L. Winek, *JAMA* 1991, *265*, p. 1011–1013.

[78] T. C. Kram, D. A. Cooper, A. C. Allen, *Analytical chemistry* 1981, *53*, 1379A–1386A.

[79] G. L. Henderson, *Journal of Forensic Sciences* 1991, *36*, p. 422–433.

[80] S. Vuckovic, M. Prostran, M. Ivanovic, L. Dosen-Micovic, Z. Todorovic, Z. Nesic, R. Stojanovic, N. Divac, Z. Mikovic, *Current medicinal chemistry* 2009, *16*, p. 2468–2474.

[81] I. Ojanperä, M. Gergov, M. Liiv, A. Riikoja, E. Vuori, *Int. J. Legal Med.* 2008, 1*22*, p. 395–400.

[82] S. Sofalvi, H. E. Schueler, E. S. Lavins, C. K. Kaspar, I. T. Brooker, C. D. Mazzola, D. Dolinak, T. P. Gilson, S. Perch, *Journal of Analytical Toxicology* 2017, p. 1–11.

[83] M. J. Lozier, M. Boyd, C. Stanley, L. Ogilvie, E. King, C. Martin, L. Lewis, *Journal of Medical Toxicology* 2015, *11*, p. 208–217.

[84] M. C. Mercado, S. A. Sumner, M. B. Spelke, M. K. Bohm, D. E. Sugerman, C. Stanley, *Pain Med* 2017, DOI 10.1093/pm/pnx015.

[85] J. B. Dwyer, J. Janssen, T. M. Luckasevic, K. E. Williams, *Journal of Forensic Sciences* 2017, DOI 10.1111/1556-4029.13517.

[86] J. Poklis, A. Poklis, C. Wolf, M. Mainland, L. Hair, K. Devers, L. Chrostowski, E. Arbefeville, M. Merves, J. Pearson, *Forensic Science International* 2015, *257*, p. 435–441.

[87] R. Jorge, R. Christie, A. Almeida, M. Evans-Brown, A. Gallegos, R. Sedefov, D. Dudek, *Acetyl Fentanyl: EMCDDA-Europol Joint Report on a New Psychoactive Substance N-Phenyl-N-[1-(2-Phenethyl)Piperidin-4-Yl]Acetamide (Acetylfentanyl)*, European Monitoring Centre For Drugs And Drug Addiction, Luxembourg, 2016.

[88] J. B. Cole, J. F. Dunbar, S. A. McIntire, W. E. Regelmann, T. M. Slusher, *Pediatrics* 2015, *135*, e740–e743.

[89] J. Poklis, A. Poklis, C. Wolf, C. Hathaway, E. Arbefeville, L. Chrostowski, K. Devers, L. Hair, M. Mainland, M. Merves, et al., *Journal of Analytical Toxicology* 2016, *40*, p. 703–708.

[90] D. Guerrieri, E. Rapp, M. Roman, H. Druid, R. Kronstrand, *Journal of Analytical Toxicology* 2017, DOI 10.1093/jat/bkw129.

[91] European Monitoring Centre for Drugs and Drug Addiction, *New Drugs in Europe, 2012*, 2012.

[92] K. G. Shanks, G. S. Behonick, *Journal of Analytical Toxicology* 2017, p. 1–7.

[93] N. B. Tiscione, R. Miller, X. Shan, D. Tate Yeatman, *Journal of Analytical Toxicology* 2017, p. 1–6.

[94] S. Müller, S. Nussbaumer, G. Plitzko, R. Ludwig, W. Weinmann, S. Krähenbühl, E. Liakoni, *Clinical Toxicology* 2017, p. 1–2.

[95] N. B. Tiscione, K. G. Shanks, *TOXTALK* 2016, *40*, 14–17.

[96] A. V. George, J. J. Lu, M. V. Pisano, J. Metz, T. B. Erickson, *Am J Emerg Med* 2010, *28*, p. 530–532.

[97] T. Breindahl, A. Kimergård, M. F. Andreasen, D. S. Pedersen, *Drug Testing and Analysis* 2017, *9*, p. 415–422.

[98] D. Guerrieri, E. Rapp, M. Roman, G. Thelander, R. Kronstrand, *Forensic Science International* 2017, DOI 10.1016/j.forsciint.2017.05.010.

[99] T. J. Gillespie, A. J. Gandolfi, T. P. Davis, R. A. Morano, *Journal of analytical toxicology* 1982, *6*, p. 139–142.

[100] I. Takase, T. Koizumi, I. Fujimoto, A. Yanai, T. Fujimiya, *Legal Medicine* 2016, *21*, p. 38–44.

[101] S. M. Cunningham, N. A. Haikal, J. C. Kraner, *Journal of Forensic Sciences* 2016, *61*, S276–S280.

[102] I. M. McIntyre, A. Trochta, R. D. Gary, M. Malamatos, J. R. Lucas, *Journal of Analytical Toxicology* 2015, *39*, p. 490–494.

[103] K. Yonemitsu, A. Sasao, S. Mishima, Y. Ohtsu, Y. Nishitani, *Forensic Science International* 2016, *267*, e6–e9.

[104] M. Rojkiewicz, M. Majchrzak, R. Celiński, P. Kuś, M. Sajewicz, *Drug Testing and Analysis* 2017, *9*, p. 405–414.

[105] A. Helland, W. R. Brede, L. S. Michelsen, P. O. M. Gundersen, H. Aarset, J. E. Skjølås, L. Slørdal, *Journal of Analytical Toxicology* 2017, p. 1–2.

[106] F. E. Dussy, S. Hangartner, C. Hamberg, C. Berchtold, U. Scherer, G. Schlotterbeck, D. Wyler, T. A. Briellmann, *Journal of Analytical Toxicology* 2016, *40*, p. 761–766.

[107] V. Coopman, J. Cordonnier, M. De Leeuw, V. Cirimele, *Forensic Science International* 2016, *266*, p. 469–473.

[108] D. M. Swanson, L. S. Hair, S. R. Strauch Rivers, B. C. Smyth, S. C. Brogan, A. D. Ventoso, S. L. Vaccaro, J. M. Pearson, *Journal of Analytical Toxicology* 2017, p. 1–5.

Annex I. Synopsis of toxicologically confirmed cases involving fentanyl and its analogues

Drug	*History*	*Clinical symptoms/autopsy findings*	*Drug results (ng/mL unless specified)*	*Reference*
3-Methylfentanyl	30 y/o M found deceased in his residence after recent release from prison. Drug paraphernalia was found on scene.	Inflammation and fatty degeneration in the liver	**Blood:** 0.4 (cis) + hydroxyzine 700, fluoxetine 400, clozapine 200, amphetamine 32 mg/L	[64]
	41 y/o F found deceased in her residence; drug paraphernalia was found on scene.	N/A	**Blood:** 0.3 (*cis*) + EtOH, 0.077 g/dL, amphetamine 650	[64]
	35 y/o M found deceased in a public restroom; drug paraphernalia was found on scene.	Cirrhosis and fatty degeneration of the liver	**Blood:** 0.9 (*cis*) + diazepam 100, nordiazepam 300, morphine 30	[64]
alpha-methylfentanyl	28 y/o M located by paramedics in respiratory paralysis. Pronounced dead at hospital.	Hyperemia, pulmonary oedema, congestive hepatosplenomegaly, and a prominent enlarged lymph node in the porta hepatis.	**Blood:** 3.1 **Liver:** 78 **Bile:** 6.4	[99]
Acetylfentanyl	28 y/o M prescribed oxycodone and pregabalin. In ED after reportedly using fludiazepam.	Apneic, IV naloxone administered during ambulance transport.	**Urine:** 685 ng/mmol creatinine + urine: flubromazolam, pregabalin and oxycodone	[10]
	27 y/o M taken to ED after reported use of acetylfentanyl.	Miotic pupils. Intubation and ventilator support required due to aspiration. 28-day ICU stay due to recurring severe agitation, delirium and hyperthermia.	**Serum:** 19.1 **Urine:** 85.5 ng/mmol creatinine	[10]
	22 y/o M taken to ED after ingestion of unknown NPS, clonazepam and alcohol.	7-day hospital stay. Pronounced rhabdomyolysis.	**Serum:** 14.8 **Urine:** 154 ng/mmol creatinine + 7-amino clonazepam and EtOH	[10]

Drug	*History*	*Clinical symptoms/autopsy findings*	*Drug results (ng/mL unless specified)*	*Reference*
Acetylfentanyl (*Cont'd*)	37 y/o M taken to ED after insufflating suspected acetylfentanyl. Individual was prescribed lorazepam.	Miotic pupils. IV naloxone given in ambulance.	**Serum:** 51 **Urine:** 235 ng/mmol creatinine + 7-AMC, diclazepam, lorazepam, amphetamine, tramadol, O-desmethyltramadol, methiopropamine, oxycodone and 2-AI	[10]
	20 y/o M taken to ED after oral ingestion of powder. Reported pregabalin, buprenorphine, clonazepam, and amphetamine use.	Miotic pupils. Naloxone administered but insufficient response. 2-day hospital stay.	**Serum:** 0.6 **Urine:** 0.9 ng/mmol creatinine + 7-AMC and pregabalin	[10]
	21 y/o M taken to ED after ingesting acetylfentanyl and maybe benzodiazepines.	Miotic pupils. IV naloxone administered in ambulance. 3-day hospital stay.	**Serum:** 32.7 **Urine:** 30 .9 ng/mmol creatinine + 2C-P, 4-FMC, 7-AMC, and 4-OH-alprazolam	[10]
	35 y/o F taken to ED. Suspected alprazolam and unknown NPS use.	Mydriasis. 2-day hospital stay.	**Serum:** 4.4 + clonazolam, oxazepam, amphetamine, 7-amino-nitrazepam, nitrazepam, and EtOH	[10]
	40 y/o M taken to ED after suspected MDPV and PV use prior to death.	Apneic, tachycardic and cerebral hemorrhage progressing to cerebral oedema.	**Serum:** 4.7 **Urine:** 47 ng/mmol creatinine + 4F-PV8, *alpha*-PVT, dextromethorphan, amphetamine and fentanyl	[10]
	34 y/o M found dead in bed in supine position; vomit beneath head. Snoring at least 12 h before death. Police found "designer drugs" and insufflation straws.	Heavy organs (L and R lung, liver and brain were 590, 780, 1610, 1430 g). Cardiac hypertrophy with interstitial fibrosis and fatty infiltration of R ventricle indirectly contributed to death.	**Cardiac blood:** 270	[100]

Drug	*History*	*Clinical symptoms/autopsy findings*	*Drug results (ng/mL unless specified)*	*Reference*
Acetylfentanyl (*Cont'd*)	28 y/o M found unresponsive on bathroom floor with tourniquet around his arm and syringe nearby. Decedent had history of substance abuse, including anabolic steroids.	Needle track marks and foamy secretions from mouth. Pulmonary oedema and mild diffuse cerebral oedema.	**Subclavian blood:** 235 **Liver:** 2400 ng/g **Vitreous fluid:** 131 **Urine:** 234	105
	24 y/o M found unresponsive by mother with uncapped syringe containing acetylfentanyl and rubber tourniquet. Hx of heroin abuse, with 2 previous overdoses.	Lungs oedematous and congested (R 610 g and L 580 g). 3 recent punctures in L forearm and antecubital fossa noted at autopsy.	**Peripheral blood:** 260 **Central blood:** 250 **Liver:** 1000 ng/g **Vitreous fluid:** 240 **Urine:** 2600	[102]
	Early 30s y/o M found unresponsive at home, pronounced dead at hospital. Hx of methamphetamine abuse. Acetylfentanyl and 4-MeO-PV8 detected in pale brown-white powder and syringe.	Congested lungs and other organs, eyelid, capsula cordis and pleura petechiae and fluidity of heart blood. 2 recent forearm needle marks.	**Femoral blood:** 153 **Central blood:** 239 **Urine:** 240 **Gastric contents:** 880 + 4-MeO-PV8: femoral blood 389; central blood 960; urine 245; gastric contents 500. + 7-aminonitrazepam 7700; + phenobarbital 30. + methylphenidate, chlorpromazine and risperidone.	[103]
	20 y/o M found deceased in bed. EMS pronounced dead at scene. Hx of illicit drug use.	No significant trauma. No anatomic cause of death.	**Femoral blood:** 192 **Heart blood:** 285 **Liver:** 1100 ng/g **Brain:** 620 ng/g **Urine:** 3420 + methoxetamine and fluoxetine in heart blood.	[66]

Drug	*History*	*Clinical symptoms/autopsy findings*	*Drug results (ng/mL unless specified)*	*Reference*
Acetylfentanyl (*Cont'd*)	50 y/o F found unresponsive in bed. EMS pronounced dead. History of seizures, EtOH and prescription (hydrocodone, tramadol, gabapentin, celecoxib, cyclobenzaprine, venlafaxine, clonidine, buspirone, hydroxyzine and chlordiazepoxide abuse).	External examination and toxicology testing only.	**Femoral blood:** 255 **Heart blood:** 210 **Vitreous fluid:** 140 **Urine:** 2720 + heart blood venlafaxine, nordiazepam and chlordiazepoxide; femoral blood 2000 venlafaxine.	[66]
	64 y/o M; death ruled acetylfentanyl toxicity.	N/A	**Blood:** 170	[85]
Butyrylfentanyl	30 y/o M in ED after reported MT-45 and flubromazepam use.	Decreased consciousness and tachycardia.	**Urine:** 8 + N-ethylbuphedrone, flubromazepam, 3-MeO-PCP, methiopropamine, MT-45, *alpha*-PBP, *alpha*-PPP, and 4F-PVP	[12]
	24 y/o M in ED after reported drug intake.	Sudden unconsciousness, apnoea, respiratory depression, miotic pupils, hypothermia, and suspected cardiac arrest. Required 2-day hospitalization.	**Serum:** 0.9 **Urine:** 65.6 + fentanyl serum 4.3; urine 993; + cannabis and pregabalin	[12]
	23 y/o M in ED after nasal spray butyrylfentanyl and AB-FUBINACA.	Agitation, odd behaviour, unpleasant feelings. Sudden unconsciousness, respiratory depression, myoclonus, dilated pupils, hyperthermia, hypertension and tachycardia.	**Serum:** 0.6 **Urine:** 2.0 + fentanyl serum10.2; urine 118; + N-ethylbuphedrone, 3-MeO-PCP, 4-MeO-PCP, *alpha*-PBP and pregabalin	[12]

Drug	*History*	*Clinical symptoms/autopsy findings*	*Drug results (ng/mL unless specified)*	*Reference*
Butyrylfentanyl (*Cont'd*)	44 y/o M found unresponsive on bathroom floor. Syringe and drug paraphernalia at scene. Decedent had history of heroin abuse.	Needle marks in antecubital fossae, forearms, L wrist, and ankles. Recent puncture on L antecubital fossa. Lungs oedematous and congested (R 890 g and L 815 g).	**Peripheral blood:** 58 **Central blood:** 97 **Liver:** 320 ng/g **Vitreous fluid:** 40 **Urine:** 670 + peripheral blood acetylfentanyl 38; central blood 32; liver 110 ng/g; vitreous 38; urine 540 and BZE	[63]
	53 y/o F found unresponsive in bathroom. No drug paraphernalia. History of smoking, prescription drug abuse, and psychiatric disorders hospitalization.	Lungs oedematous and congested (L 450 g and R 510 g); mild atherosclerosis and L concentric ventricular myocardial hypertrophy.	**Peripheral blood:** 99 **Central blood:** 220 **Gastric:** 590 **Brain:** 93 ng/g **Liver:** 41 ng/g **Vitreous fluid:** 32 **Bile:** 260 **Urine:** 64	[89]
	45 y/o F found unresponsive in bed. History of anxiety, bipolar, disorder, previous suicide attempts and prescription drug and alcohol abuse. Only prescribed medications and expected pill counts found.	Lungs oedematous and congested (L 570 g and R 680 g); mild L ventricular myocardial hypertrophy and mild nephrosclerosis.	**Peripheral blood:** 3.7 **Central blood:** 9.2 **Gastric:** 4000 **Brain:** 63 ng/g **Liver:** 39 ng/g **Vitreous fluid:** 9.8 **Bile:** 49 **Urine:** 2 + acetylfentanyl peripheral blood 21; central blood 95; gastric 28,000; brain 200 ng/g; liver 160 ng/g; vitreous 68; bile 330; urine 8 + peripheral blood alprazolam 40 and 0.11% EtOH.	[89]

Drug	*History*	*Clinical symptoms/autopsy findings*	*Drug results (ng/mL unless specified)*	*Reference*
Butyrylfentanyl (*Cont'd*)	23 y/o M found unresponsive in bathroom. Tray with white powder and tube located at scene.	Cerebral oedema and moderate full bladder. Residual white powder in nose. Fresh abrasions, bruising and fresh bleedings.	**Peripheral blood:** 66 **Central blood:** 39 **Muscle:** 110 ng/g **Kidney:** 160ng/g **Liver:** 57 ng/g 120% increase in femoral blood concentrations taken 19 h apart, and 55% increase in heart blood.	[50]
Furanylfentanyl	22 y/o M taken to ED after using furanylfentanyl by nasal spray.	Received SC and IV naloxone.	**Serum:** 148 **Urine:** 85.2 ng/mmol creatinine + 5-EAPB, MDPHP	[10]
	22 y/o M taken to ED after using nasal spray containing unknown drug and prescribed clonazepam.	Apnoeic and cyanotic. IV naloxone. Required 2-day stay in hospital.	**Serum:** 4.4 **Urine:** 9.2 ng/mmol creatinine + *para*-methoxybutyrylfentanyl:serum 11; urine 51.3 ng/mmol creatinine) +MDPHP, pregabalin, and clonazepam	[10]
	26 y/o M with history of drug abuse found deceased in home. Drug paraphernalia found on scene.	Cerebral and pulmonary oedema.	**Blood:** 1.05 + THC, mirtazapine and metabolite, pregabalin 6.0 mcg/mL, buprenorphine and metabolite	[90]
	36 y/o M with history of drug abuse found deceased at home. Package of drug Lyrica (pregabalin) was found with four tablets missing.	Pulmonary oedema and froth in the airways. Postmortem examination excluded severe traumatic injuries.	**Blood:** 2.89 + pregabalin 14 mcg/mL	[90]

Drug	*History*	*Clinical symptoms/autopsy findings*	*Drug results (ng/mL unless specified)*	*Reference*
Furanylfentanyl (*Cont'd*)	37 y/o M found unconscious in a ditch; among possessions included an empty strip of zopiclone sleeping pills.	Visceral congestion and chronic inflammation of liver.	**Blood:** 0.95 + carbamazepine, venlafaxine, promethazine, alimemazine, methylphenidate, acetaminophen, pregabalin 34 mcg/mL, amphetamine, 7-AMC	[90]
	26 y/o M with history of drug abuse was found deceased at home. Drug paraphernalia was found at scene.	Pulmonary and cerebral oedema.	**Blood:** 0.43	[90]
	26 y/o M with history of drug abuse was found deceased at home. Three nasal sprays suspected to contain fentanyl were found.	Cerebral and pulmonary oedema and froth in the airways.	**Blood:** 0.78 + carbamazepine, pregabalin, gabapentin, norbuprenorphine, fentanyl, alimemazine, alprazolam, diazepam, methylphenidate,	[90]
	27 y/o M with a history of suicide attempts was found deceased at home.	Enlarged liver with steatosis and incipient cirrhosis.	**Blood:** 1.15	[90]
	24 y/o M, recently discharged (3 days prior) from an addiction treatment centre, was found deceased at home. Drug paraphernalia found on scene.	Chronic bronchitis and signs of aspiration. Lungs were congested and oedematous.	**Blood:** 0.40 + fentanyl 1.27	[90]
	24 y/o M with history of substance abuse was found unresponsive with syringe in arm.	N/A	**Blood:** Positive + U-47700 59, 4-ANPP and quinine	[67]

Drug	*History*	*Clinical symptoms/autopsy findings*	*Drug results (ng/mL unless specified)*	*Reference*
Furanylfentanyl (*Cont'd*)	36 y/o M found unresponsive in bathroom with syringe cap in mouth.	N/A	**Blood:** 26 + U-47700 135, EtOH, 4-ANPP, and quinine	[67]
	33 y/o M found deceased; suspected drug overdose.	N/A	**Blood:** 56 + U-47700 167, morphine, 48, 4-ANPP, 6-MAM, and quinine	[67]
	29 y/o M found unresponsive; suspected heroin OD.	N/A	**Blood:** 76 + U-47700 490, 4-ANPP, quinine	[67]
	40 y/o M with history of heroin/ opioid abuse was found deceased.	N/A	**Blood:** 2.5 + U-47700 105, 4-ANPP, quinine	[67]
	30 y/o M with history of substance abuse found unresponsive at home.	Pulmonary oedema, gastritis, and chronic hepatitis.	**Blood:** 6.1 + *delta* 9-THC 1, tramadol 75, O-desmethyl tramadol 40, butyrylfentanyl 0.33	[67]
	32 y/o M with history of drug abuse found unresponsive at home.	N/A	**Blood:** 12.9 + THCC 13, diphenhydramine 140, acetylfentanyl 0.65, naloxone	[67]
	26 y/o M with history of drug abuse found unresponsive at home.	Pulmonary and cerebral oedema.	**Blood:** 6.2 + alprazolam 25, hydrocodone 120, hydromorphone 1.8, dihydrocodeine 21, venlafaxine 83, O-desmethylvenlafaxine 400, THCC 7.2, diphenhydramine 90	[67]

Drug	*History*	*Clinical symptoms/autopsy findings*	*Drug results (ng/mL unless specified)*	*Reference*
para-Methoxybutyrylfentanyl	29 y/o F taken to ED after suspected use of unknown NPS and prescribed tramadol.	Miotic pupils. Required 2-day stay in hospital.	**Serum:** 1.3 **Urine:** 5.1 ng/mmol creatinine + butyrylfentanyl, *para*-fluorobutyrylfentanyl, cannabis, nordiazepam tramadol, O-desmethyltramadol.	[10]
	28 y/o M taken to ED after suspected unknown opioids and benzodiazepines intake.	Miotic pupils. IV naloxone. Required 2-day hospital stay.	**Serum:** 3.1 + *para*-fluorobutyrylfentanyl, alprazolam, 4-OH-alprazolam, 7-AMC, BZE, clonazepam, cocaine, O-desmethyltramadol, diazepam, oxazepam	[10]
	34 y/o M taken to ED after oral ingestion unknown tablets and prescribed fentanyl.	Temporarily apnoeic. Displayed miotic pupils. IV naloxone. Required 2-day hospital stay.	**Urine:** 11.9 ng/mmol creatinine + *para*-fluorobutyrylfentanyl, clonazolam, GHB and fentanyl	[10]
para-Fluorobutyryl fentanyl	25 y/o M in ED after *para*-fluorobutyrylfentanyl, pregabalin and clonazepam intake.	Disorientation, unsteadiness, slurred speech and hypotension.	**Serum:** 15.0 **Urine:** 9.5 + urine: pregabalin; tramadol, O-desmethyltramadol, 4-OH-alprazolam, oxazepam	[12]
	26 y/o M found deceased at home. 4-Fluorobutyrylfentanyl detected in e-liquid for e-cigarette.	N/A	**Blood:** 91 **Urine:** 200 **Liver:** 902 ng/g **Kidney:** 411ng/g **Brain:** 248 ng/g **Gastric:** 8450	[104]

Drug	*History*	*Clinical symptoms/autopsy findings*	*Drug results (ng/mL unless specified)*	*Reference*
para-Fluorobutyryl fentanyl (*Cont'd*)	25 y/o F found deceased at home.	N/A	**Blood:** 112 **Urine:** 414 **Liver:** 136 **Kidney:** 197	[104]
para-Fluoroiso-butyrylfentanyl	27 y/o M discovered lifeless with syringe reportedly containing *para*-fluoroisobutyrylfentanyl next to him. Transferred to hospital but declared dead 43 hrs after arriving to hospital.	Unconsciousness, apnea, and asystole. Repeated naloxone unsuccessful. CT scans showed cerebral oedema.	**Serum:** 38 **Urine:** 31	[11]
ortho-Fluorofentanyl	The first of 2 20-something y/o Ms hospitalized with miosis and respiratory dysfunction after exposure to white powder obtained from a foreign source by mail.	Loss of consciousness and respiratory distress. Responded to naloxone administration.	**Serum:** 2.5 + EtOH, alprazolam 96, BZE, amphetamine, acetaminophen	[105]
	Second M found deceased at home a few days after being discharged from the hospital. Drug paraphernalia found on scene.	Autopsy performed 3 days after decedent was found.	**Blood:** 2.4 + alprazolam 16, nordiazepam 3.8, 7-AMC 34, Delta-9 THC 21, GHB 22 **Urine:** 3.9 + EtOH, THCC, GHB	[105]
Ocfentanil	24 y/o M found deceased at home. Drug paraphernalia, brown powder identified as ocfentanil, found at scene. History of illicit drug use. Nasal swabs indicated ocfentanil.	Lung and brain congestion. Distended bladder (300 mL).	**Peripheral blood:** 9.1 **Heart blood:** 27.9 **Urine:** 480 + peripheral blood: quetiapine <10, citalopram 130, THC 2.8 and >5 THCC.	[106]

Drug	*History*	*Clinical symptoms/autopsy findings*	*Drug results (ng/mL unless specified)*	*Reference*
Ocfentanil (*Cont'd*)	17 y/o M found deceased at home after snorting a brown powder identified as ocfentanil purchased online. Drug paraphernalia at scene. History of illicit drug use. Nasal swabs indicated ocfentanil.	N/A	**Femoral blood:** 15.3 **Cardiac blood:** 23.3 **Liver:** 31.2 mcg/kg **Kidney:** 51.2 mcg/kg **Brain:** 37.9 mcg/kg **Vitreous humour:** 12.5 **Urine:** 6.0 **Bile:** 13.7 + peripheral blood: acetaminophen 45 mg/L and caffeine 230	[107]
Carfentanil	16 y/o M presented to ED after being discovered unconscious.	Individual hypotensive, tachycardic, hypopneic, and cyanotic. IV administration of naloxone.	**Serum:** 0.6 **Urine:** 13	[94]
	24 y/o M found unconscious behind wheel after crashing vehicle into pole in a parking lot.	Naloxone successfully administered and individual regained consciousness.	**Blood:** 0.013 + fentanyl <1.0, acetylfentanyl 0.39	[95]
	29 y/o M found unconscious behind wheel of vehicle at drive-through food establishment.	Naloxone successfully administered and individual regained consciousness.	**Blood:** 0.043 + 6-MAM <5.0, morphine <5.0	[95]
	28 y/o M found unconscious by police officer in driver seat of vehicle. A needle was observed in subject's lap and two clear plastic baggies containing suspected heroin also discovered. Subject transferred to local hospital and released after treatment.	N/A	**Blood:** Positive + alprazolam 55, fentanyl <0.5, positive furanylfentanyl, *para*-fluoroisobutyrylfentanyl, U-47700, and its metabolite	[68]

Drug	*History*	*Clinical symptoms/autopsy findings*	*Drug results (ng/mL unless specified)*	*Reference*
Carfentanil (*Cont'd*)	34 y/o M found deceased in a running vehicle. Drug paraphernalia found at scene.	Autopsy findings unremarkable except for mild hypertensive heart disease with left ventricular hypertrophy and mild hepatic steatosis.	**Heart blood:** 1.3 + furanylfentanyl 0.34, fentanyl 6, morphine total <20, hydromorphone total <20	[108]
	25 y/o M found deceased in his residence. Drug paraphernalia found at scene.	Autopsy findings unremarkable except for mild left ventricular hypertrophy	**Heart blood:** 0.12, 460 BZE (peripheral blood)	[108]
Acrylfentanyl	29 y/o M taken to ED after use of acryl fentanyl using a nasal spray.	Anxiety, dizziness, paresthesia, tremor, and clammy skin.	**Serum:** 1.3 **Urine:** 0.2 mcg/mmol creatinine	[11]
	35 y/o M taken to ED after use of acrylfentanyl using a nasal spray.	Miotic pupils, clammy skin, nausea, and vomiting. IV naloxone administered.	**Serum:** 0.6 **Urine:** 0.2 mcg/mmol creatinine	[11]
	51 y/o M taken to ED after use of acrylfentanyl using a nasal spray.	Miotic pupils, clammy skin; naloxone administered in ED.	**Urine:** 0.7	[11]
	29 y/o M taken to ED after reported PCP use.	Psychotic behaviour on admission to ED, increasing agitation and delirium at hospital, tachypnea, foam at mouth, clammy skin.	**Serum:** 1.0 **Urine:** 7.5 mcg/mmol creatinine	[11]
	23 y/o M taken to ED after reported use of a fentanyl analogue via nasal spray.	CNS depression and apnoea at ambulance arrival; anxiety and miotic pupils in hospital; naloxone administered by paramedics.	**Serum:** 2.1 **Urine:** 10.5 mcg/mmol creatinine	[11]

Drug	*History*	*Clinical symptoms/autopsy findings*	*Drug results (ng/mL unless specified)*	*Reference*
Acrylfentanyl (*Cont'd*)	27 y/o M taken to ED after reported use of Ephylone.	Miotic pupils; naloxone administered by paramedics, additional unknown dose in ED and then continuous infusion.	**Serum:** 0.7 **Urine:** 0.6 mcg/mmol creatinine + NPP, flunitrazolam, oxazepam, temazepam	[11]
	38 y/o M taken to ED after use of acryl fentanyl using a nasal spray.	Miotic pupils, cyanosis; IV naloxone administered by paramedics.	**Serum:** 0.8 **Urine:** 5.2 mcg/mmol creatinine + 4Cl-*alpha*-PVP, ephylone, and amphetamine	[11]
	19 y/o F taken to ED after use of acrylfentanyl using a nasal spray.	CNS depression, miotic pupils, apnea; 0.4 mg naloxone administered IV by paramedics.	**Serum:** 1.3 **Urine:** 6.4 mcg/mmol creatinine	[11]
	28 y/o M taken to ED after ingesting green pills.	Miotic pupils. Intubation and ventilation.	**Serum:** 0.5 **Urine:** 10.2 + *para*-chloroisobutyrylfentanyl: serum 5.1;urine 11.5, + NEH, fentanyl	[11]
	25 y/o M with past history of drug abuse was found deceased in residence. A nasal spray bottle marked "A" was found next to the body.	Examination detected presence of secretions in trachea, nose, and mouth, cerebral and pulmonary oedema (lungs 1292 g).	**Blood:** 0.02	[98]
	48 y/o M found unconscious in drug-treatment facility. Resuscitiation efforts revived a pulse in the individual, who was subsequently transferred to a hospital. Individual died morning after admission; a second unconscious patient survived and declared they were crushing and snorting tablets.	Severe arteriosclerosis, hypoxia-damaged myocardium, chronic liver inflammation, presence of blood and fluid in the lungs, and traces of an old infarct in the left-brain hemisphere. Lungs weighed 1225 g.	**Blood:** 0.32 + mirtazapine 94, desmethyl mirtazapine 31, citalopram 350, O-desmethylcitalopram 74 blood, pregabalin 8.3 mcg/mL, methylphenidate 4 , ritalin 190, positive n-ethylnorhexedrone	[98]

Drug	*History*	*Clinical symptoms/autopsy findings*	*Drug results (ng/mL unless specified)*	*Reference*
Acrylfentanyl (*Cont'd*)	22 y/o M was found deceased at home by his girlfriend; the girlfriend witnessed the victim inhaling a nasal spray.	Secretion in the airways, severe pulmonary oedema, acute congestion of lungs and liver, moderate congestion of internal organs, presence of inhaled blood and stomach content in the lungs. Lungs weighed 1374 g.	**Blood:** 0.82 + 7-AMC 105, nordiazepam 32	[98]
	45 y/o F found deceased at her residence.	Autopsy report indicated a chronic cholecystitis with gallstones, minor brain swelling, blood and fluid congestion in the lungs, along with pleural and pericardial effusion. Lungs weighed 1378 g.	**Blood:** 3.0 + bupropion 230, hydroxybupropion 5200, quetiapine 130, fluoxetine 1800, norfluoxetine 1700, dihydropropiomazine 100, diazepam 95, nordiazepam 120, and zopiclone 80	[98]
	35 y/o M found deceased at home. Green tablets marked "acrylic".	Autopsy report described a mild, fatty liver, and coronary artery calcification. Lungs weighed 1360 g.	**Blood:** 0.01 + EtOH	[98]
Tetrohydrofuranyl-fentanyl	26 y/o M taken to ED after intranasal application of "fentanyl" nasal spray.	Decreasing consciousness, miotic pupils, and respiratory depression. Naloxone administered.	**Serum:** 45 **Urine:** 136 + flunitrazolam	[11]

Abbreviations in annex

ED = Emergency Department, EMS = Emergency Management Services, ICU = Intensive Care Unit, Hx = History, 4-OH-alprazolam = Hydroxy-alprazolam, NPS = New Psychoactive Substances, IV = intravenous, SC = subcutaneous, EtOH = Ethanol, 7-AMC = 7-amino clonazepam, 6-MAM = 6-monoacetylmorphine, 2-AI = 2-aminoindane, MDPV = methylenedioxypyrovalerone, PV = pyrovalerone, 2C-P = 2,5-dimethoxy-4-(*n*)-propylphenethylamine, 5-EAPB = 1-(benzofuran-5-yl)-*N*-ethylpropan-2-amine, 4-FMC= 4-fluoromethcathinone/flephedrone, 4F-PV8 = 4-fluoro-*alpha*-pyrrolidinoheptiophenone, *alpha*-PVT = *alpha*-pyrrolidinopentiothiophenone, MDPHP = *alpha*-pyrrolidinoheptanophenone, 4-MeO-PV8 = 1-(4-methoxyphenyl)-2-(pyrrolidin-1-yl)-heptan-1-one, 3-MeO-PCP/4-MeO-PCP = 3-methoxyphencyclidine/4-methoxyphencyclidine, *alpha*-PBP = *alpha*-pyrrolidinobutiophenone, *alpha*-PPP = alpha-pyrrolidinopropiophenone, 4F-PVP = 4-fluoro-pyrrolidinovalerophenone, BZE = benzoylecgonine, 3-MMC = 3-methylmethcathinone, 4-FMA = 4-fluoromethamphetamine, 4F-butyrylfentanyl = 4-fluoro butyrylfentanyl, 4Cl-*alpha*-PVP = 4-chloro-*alpha*-pyrrolidionovalerophenone, GHB = *gamma*-hydroxybutyric acid, NPP = 2-(isopropylamino)-1-phenylpentan-1-one; NEH = N-ethylnorhexedrone, 4-ANPP = 4-anilino-*N*-phenethylpiperidine, THC = tetrahydrocannabinol, THCC = 11-nor-carboxy-*delta*-9-tetrahydrocannabinol.

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