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REVIEW ARTICLE

Old opioids, new concerns: the case of acetylfentanyl

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Abstract Acetylfentanyl is a potent synthetic opioid analgesic that has been increasingly available in America, Europe, Japan, China, and Australia during the last years. It has no approved medical or veterinary use, but it is used illicitly around the world as a substitute of its controlled precursor, fentanyl, as well as of heroin or other related substances in opioid dependent individuals. It is available in retail or "head shops" or over the Internet by companies based mainly in China. Acetylfentanyl is available in the form of powder, tablets, and blotters, while liquid and injectable formulations have been also reported. Acetylfentanyl seizures have dramatically increased during the last 4 years, and its abuse has already caused a number of deaths in the United States, the United Kingdom, Sweden, and Japan, thus leading to its scheduling under the 1961 Single Convention on Narcotic Drugs in the United States, and some European Countries, China, and Japan since 2015. The aim of this review is to summarize the current knowledge about this drug concerning its chemistry, synthesis, prevalence, metabolism, pharmacology, and toxicology, as well as its legal status. Analytical methodologies developed for the determination of acetylfentanyl in biological specimens, as well as published or reported acetylfentanyl related cases, fatal or not, and self reports from drug users are presented.

Keywords Acetylfentanyl · μ-Opioid receptor agonist · Analytical methods · Prevalence · Legislation

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Introduction

During the last decade, an increase in abuse of opioid pain medications, such as fentanyl, has been reported and seems to be fuelling the illicit manufacture of derivatives that are usually more dangerous than those legally produced by pharmaceutical companies. These opioid derivatives are usually produced illicitly and normally replace heroin among street drug users. Because they vary in composition and potency, their use often leads to intoxications and overdose fatalities [1]. However, they are sold and used as substitutes of controlled opioids, synthetic or not. Legal status, availability, and cost, the intention to avoid detection and the user preferences for particular pharmacological properties are some of the motivations for their use [2].

Acetylfentanyl, although first synthesized in 1968 [3], is a newly introduced synthetic compound and a novel psychoactive substance that has invaded the American, European, Japanese, and Australian drug scene in recent years [4, 5]. This fentanyl analogue has not been approved for medical or veterinary use and it has also no industrial or other use in any country worldwide [6]. It is illicitly sold via the Internet from companies mainly based in China and is sometimes purchased as a "research chemical" [5]. Its use is discussed on many drug-user websites and forums [7, 8]. There are no published studies on its safety in human use. Acetylfentanyl has been involved in a number of deaths in the United States, Europe, and Japan [6, 9]. Public and authority concern about the use of this new substance has grown considerably in a short time. Thus, it is controlled as a substance of abuse in the United States, in some European Union member states, and in Norway, China, and Japan [5]. However, the incidence and prevalence of abuse cannot be exactly estimated since it is not routinely tested in forensic toxicology laboratories [6]. The

analysis of acetylfentanyl in biological samples is simple and can be conducted by the same methodology used for fentanyl determination. Thus, analytical methods must be targeted to acetylfentanyl so that this drug can be detected.

In this article we review all the available information on chemistry, synthesis, prevalence, use, metabolism, pharmacology, toxicology, and seizures caused by acetylfentanyl, as well as the methods for its determination in biological samples. Published or reported acetylfentanyl related cases, reported seizures, and the legal status of acetylfentanyl are also presented. All reviewed information was gathered through a detailed search of PubMed and the World Wide Web using the terms "acetylfentanyl", "pharmacology", "toxicology", "seizures", and "legislation".

Chemistry

Acetylfentanyl is a potent synthetic opioid analgesic that belongs to the phenylpiperidine class. It is one more analogue of fentanyl, like its structurally similar α-methylacetylfentanyl, where the phenylpropanamide group has been replaced by the phenylacetamide group (Fig. 1) [5, 9]. The final result is the removal of a methyl group from the structure of fentanyl; acetylfentanyl is also characterized as desmethyl fentanyl. It can be identified as an impurity during the production of fentanyl [5, 10, 11].

Its IUPAC name is *N*-[1-(2-phenylethyl)-4-piperidyl]-*N*-phenylacetamide, while *N*-(1-phenethyl-4-piperidyl)-acetanilide, *N*-phenyl-*N*-[1-(2-phenethyl)-4-piperidyl]-acetamide, desmethyl-fentanyl, fentanyl acetyl analogue, NIH 10485, and MCV 4848 are also used [5, 9, 12–14]. Its CAS number is 3258-84-2. Acetylfentanyl has the molecular formula $C_{21}H_{26}N_2O$ as base (light yellow oil), a molecular weight of 322.44 g/mol, and a boiling point of 453.8 °C, while its melting point has not been determined. Its hydrochloric salt is a pale purple powder with a molecular weight of 358.9 g/mol and a melting point of 256.6 °C [5, 12–14]. Acetylfentanyl, as a base, is slightly soluble in dimethylsulfoxide (DMSO) at 5 mg/mL when warmed [5].

The chemical structure and the specific characteristic groups of acetylfentanyl have been confirmed using nuclear magnetic resonance (NMR), gas chromatography coupled with mass spectrometry (GC–MS), and Fourier transform infrared spectroscopy (FT–IR) techniques [14]. More information on the chemical structure and the analytical parameters of acetylfentanyl can be found in the literature [14–17].

Synthesis

Several synthetic methods for acetylfentanyl were described initially by Janssen who had synthesized fentanyl from *N*-benzyl-4-piperidone [5, 18, 19]. More recently, an optimized three-step synthetic procedure for acetylfentanyl production with high yields was described (Fig. 2). It begins with the alkylation of the commercially available hydrochloric salt of 4-piperidone monohydrate with 2-(bromoethyl)-benzene in the presence of cesium carbonate (Ce_2CO_3) to furnish the alkylated piperidone, *N*-phenylethylpiperidin-4-one with a yield of 88 %. Then, reductive amination of *N*-phenylethylpiperidin-4-one with aniline mediated by sodium triacetoxyborohydride in the presence of acetic acid yields the 4-piperidineamine precursor, *N*-[1-(2-phenylethyl)-4-piperidinyl]aniline in excellent yield (91 %). Finally, *N*-[1-(2-phenethyl)-4-piperidinyl]aniline is acylated with acetic anhydride in the presence of Hunig's base (diisopropylethylamine) providing acetylfentanyl as light yellow oil with a 98 % yield [17].

Acetylfentanyl is also usually identified as an impurity during the production of fentanyl; it is supposed to be formed during the acylation of aniline nitrogen with propionic anhydride when it is contaminated with acetic

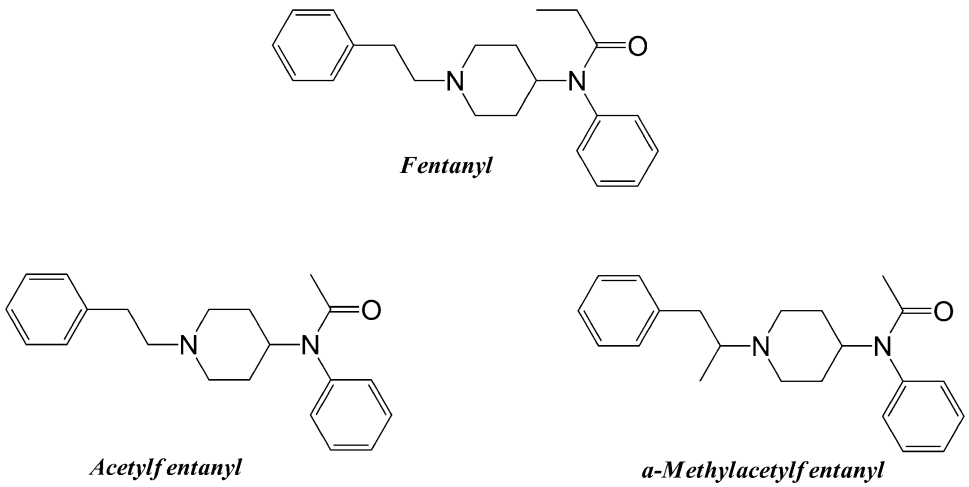

Fig. 1 Structures of fentanyl, acetylfentanyl, and α-methylacetylfentanyl

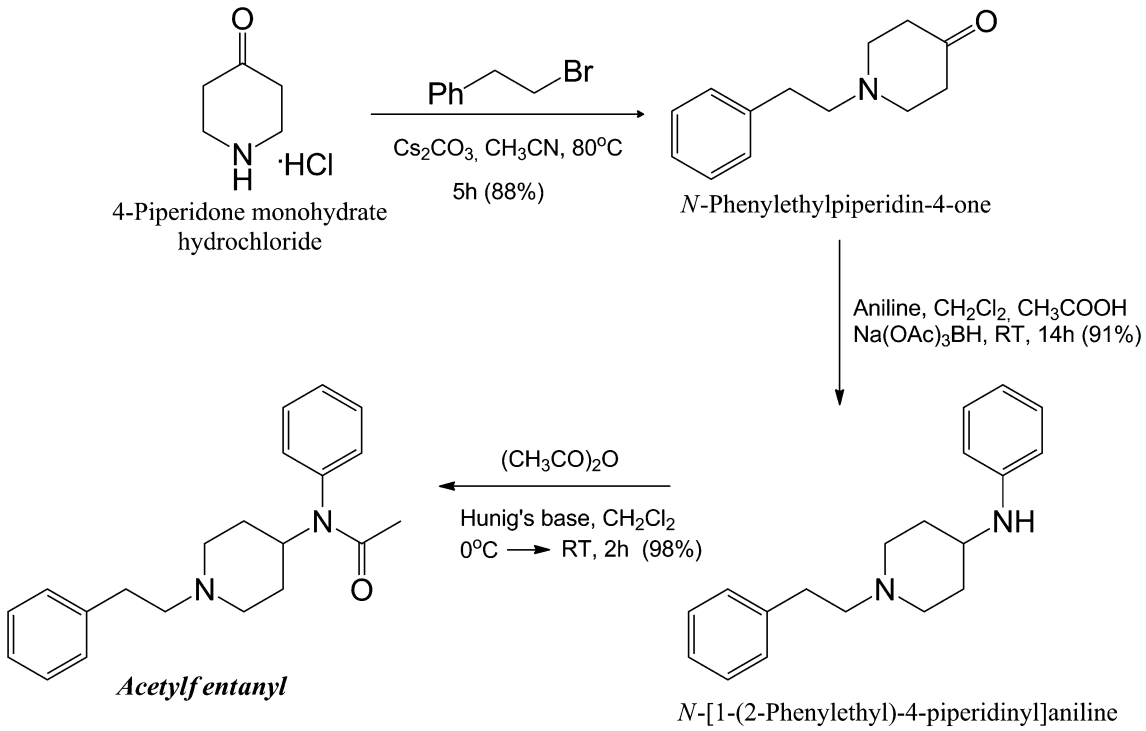

Fig. 2 Synthesis of acetylfentanyl according to Valdez et al. [17]

anhydride [5, 10, 11]. Information on clandestine synthesis of acetylfentanyl is not available, but it is thought to be based on the above reactions.

Prevalence and use

The 37th Expert Committee on Drug Dependence (ECDD) in 2015 reviewed the results extracted from a World Health Organization (WHO) questionnaire on psychoactive substances answered by 65 countries. Fourteen countries had information on acetylfentanyl, whereas no approved medical products containing acetylfentanyl for human or veterinary use are available on the medicinal market in any country. Moreover, there were no reports for any cultural, ceremonial, or religious purposes. Acetylfentanyl is currently being used as a research chemical in two countries (no names available), one of which uses it as a reference standard [20].

Acetylfentanyl is used as a direct substitute of heroin or other related substances by opioid-dependent individuals [5, 20]. Its street names are "fake heroin", "China white" or "white China" (wrongly or erroneously), and the literal "acetylfentanyl" [3, 5]. The drug is usually encountered in powder form, in tablets mimicking pharmaceutical opiate products or pre-loaded paper doses (blotters), while liquid and injectable formulations have been also reported [20, 21]. Acetylfentanyl packages are often labeled and marketed as "not for human consumption", "potpourri", "cleaners", "plant food", "bath salts", "AF-TINCTURE", while a new term "ladybug attractant" has been recently entered in the United States [22]. The amounts offered are consistent with consumer and wholesale demands (0.5–1 kg) [23]. Commonly reported routes of administration of acetylfentanyl include oral ingestion, intravenous injection, or insufflation by using nasal sprays and smoking [5, 20, 23, 24]. In the last case, it has been reported that in Russia acetylfentanyl has been used as a component of homemade "smoking mixtures" [25]. This fentanyl analogue is often mixed with heroin to deceive users, while it has been also reported that it may be sold on the streets as pills containing oxycodone. The use of acetylfentanyl as an opioid substitute or its mixing with illicit and scheduled drugs was facilitated by the fact that it was not scheduled since recently and there were decreased legal repercussions [22].

The actual dose of acetylfentanyl needed to produce a behavioral effect is considered to be in the microgram range, although in Erowid, dosages were supposed to be between 5 and 20 mg [3, 26–29]. A user suggested "…if using iv, then do 100–200 micros as starting iv, roughly 6–8 mg of morphine iv…" [8]. On a different site, a user wrote that he had experimented with several routes of administration. He insufflated 10 mg of acetylfentanyl and reported: "Effects were not immediate, and it took 5–10 min to fully kick in. The effects were typical of this

class of drugs, mildly sedating, nothing crazy. No real euphoria present, as is typical with fentanyl itself, so this wasn't surprising. All in all, 10 mg produced a pleasant buzz that lasted around 90 min before a decline in effects was noticed". He was subsequently insufflated 15 mg acetylfentanyl experiencing similar effects to those of heroin. Vaporization of 10 mg of acetylfentanyl led to lack of its effect, while intravenous administration of 10 mg of the drug "produced very enjoyable and relaxing effects." "And with the aid of two delicious beers… the effects could be felt the rest of the night without any inclination to redose." [7].

The reported illicit activities involving acetylfentanyl include trafficking or Internet imports from abroad and from unknown locations or domestic Internet sales to people who use this substance [20]. On the Internet, acetylfentanyl is sold by companies mainly located in China [20–22, 24], but it can also be purchased in retail or "head shops" or in tobacco outlets in different states in the US, such as Colorado, Florida, Georgia, Rhode Island, and Washington [21, 22]. According to the Drug Enforcement Administration (DEA), fentanyl derivatives, including acetylfentanyl, that have been found in the United States were also traced to clandestine laboratories domestically or in Mexico [1]. Acetylfentanyl use was firstly reported (eight reports) in 2013 in Louisiana, Maine, and North Dakota according to the National Forensic Laboratory Information System (NFLIS). Thirty more reports were registered by NFLIS in 2014 in Florida, Illinois, Louisiana, Maine, New Jersey, Ohio, Oregon, Pennsylvania, and Virginia [21]. Internet monitoring conducted in August 2015 by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) identified at least 23 vendors of acetylfentanyl, 12 of which claimed to ship it from warehouses in China, three within the European Union, four from the United States, and one from India [23].

Acetylfentanyl initially appeared in the drug market of the Russian Federation in smoking mixtures known as "spices" in 2012 [5]. Two years later, the first notification of acetylfentanyl use in Europe was made in Poland in September 2014 after a seizure in March 2014. Its use has been also recorded in four other European Union Member States: Finland, France, Sweden, and the United Kingdom [24].

Metabolism of acetylfentanyl

Patton et al. [30] performed preliminary acetylfentanyl metabolic studies using human liver microsomes and in vivo rodent studies that demonstrated the biotransformation of acetylfentanyl to acetyl norfentanyl by hepatic cytochrome P450. Urine samples from rats that were treated with a toxic dose of acetylfentanyl contained high concentrations of acetylfentanyl and acetyl norfentanyl. Acetyl norfentanyl was considered the major metabolite. However, further toxicokinetic studies are required to elucidate fully the metabolic pathways that are responsible for its detoxification and excretion [30].

Melent'ev et al. [25] identified metabolites of acetylfentanyl in urine of consumers of the drug and suggested possible metabolic pathways for its biotransformation (Fig. 3). *N*-Dealkylated (acetyl norfentanyl) and deacetylated acetylfentanyl were supposed to be the major metabolites of acetylfentanyl after phase I metabolism. However, products hydroxylated at the phenylethyl and piperidine moieties were mainly formed. In the phase II metabolism of acetylfentanyl, conjugates of metabolites hydroxylated by the phenylethyl moiety and methylated hydroxyl-metabolites were identified [25].

Pharmacology and toxicology of acetylfentanyl

Acetylfentanyl possesses opioid-like in vitro and in vivo binding to μ-opioid receptors, as well as μ-opioid receptor agonist effects [5, 9, 31]. In vivo animal studies revealed that acetylfentanyl produced 17.5-fold more potent analgesic response than morphine and threefold less potent response than fentanyl in mice. The ED_{50} dose for acetylfentanyl, fentanyl, and morphine was found to be 0.021, 0.0061, and 0.33 mg/kg, respectively [32]. Aceto et al. [33] reported antinociceptive effects of acetylfentanyl in mice using the tail flick and phenylquinone writhing tests. Particularly, after subcutaneous administration, its ED_{50} values in the tail flick and phenylquinone writhing tests were found to be 0.3 and 0.05 mg/kg, respectively, being more potent than morphine (ED_{50} at 5.8 and 0.23 mg/kg, respectively). Acetylfentanyl was inactive as an antagonist against morphine's antinociceptive effects in the mouse tail flick test at doses up to 30 mg/kg subcutaneously administered [33].

Controlled preclinical pharmacological and toxicological studies on acetylfentanyl are limited, while such clinical studies have not been conducted [5]. The available information related to the use and the effects of this drug is scarce and originates from Internet drug forums and a limited number of recently published poisoning cases. The pharmacological effects reported by acetylfentanyl users include alterations in mood, drowsiness, euphoria, respiratory depression, suppression of cough reflex, miosis, and impaired gastrointestinal motility [5, 9, 24].

Acute toxicity studies in mice showed that the LD_{50} of acetylfentanyl was 9.3 mg/kg, which is about seven and 50 times lower than the respective LD_{50} of fentanyl and morphine. Significant bleeding in the small intestines of

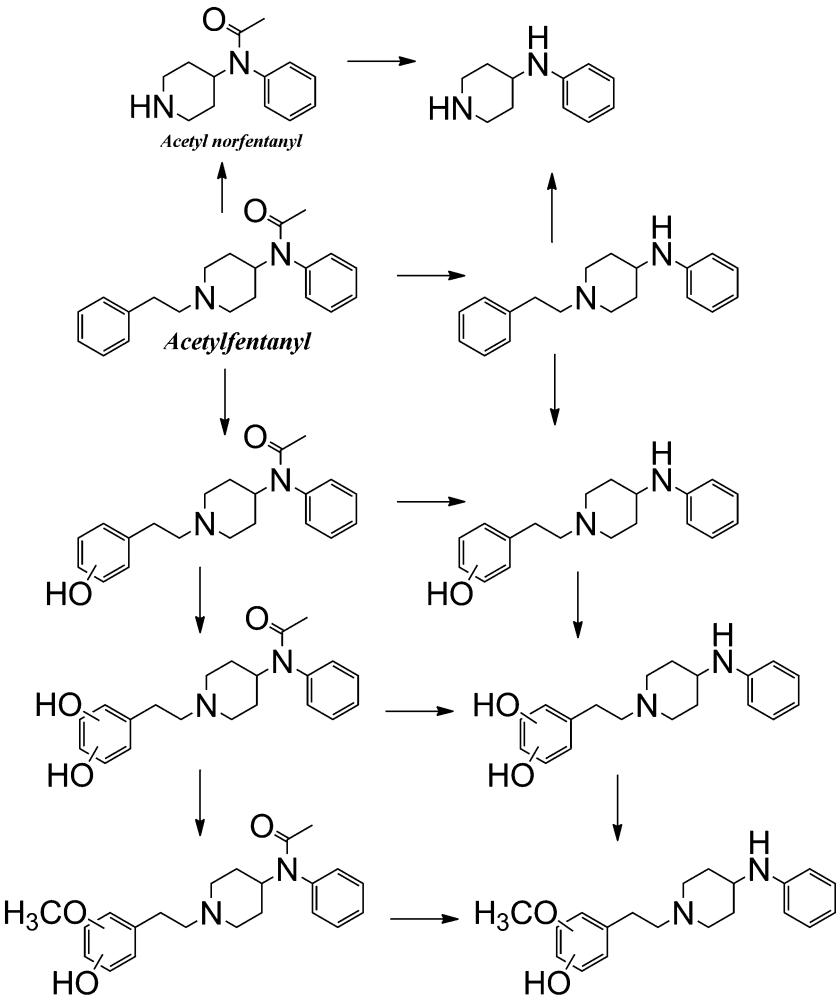

Fig. 3 Biotransformation pathways of acetylfentanyl in humans, as suggested by Melent'ev et al. [25]

mice was observed in acetylfentanyl-administered mice [5, 9]. The LD_{50}/ED_{50} ratio for acetylfentanyl is 442.9, far less than morphine's (1424.2) or fentanyl's (10,163.9), suggesting a far narrower safety index for acetylfentanyl than for these two other opioids [5, 32].

Limited preclinical studies showed that acetylfentanyl completely suppressed the withdrawal signs in morphine-dependent monkeys, thereby demonstrating cross dependence to morphine. This suggests that opiate-dependent individuals, such as heroin addicts, could find relief during withdrawal by acetylfentanyl use [5, 9, 32]. Preclinical tests of abuse liability in animals or humans appear to be lacking [31].

Because acetylfentanyl is a μ-opioid receptor agonist, naloxone, the classic opioid antagonist, can reverse potentially fatal acetylfentanyl-induced respiratory depression in cases of overdose. The same reversal effect is observed with fentanyl and other synthetic opioids [34–36]. Because of the increased potency of acetylfentanyl, larger doses of naloxone might be needed to achieve the above reversal effect [37]. Furthermore, expansion of community-based programs providing opioid-overdose prevention services, including distribution of and training in the use of naloxone, might be an effective strategy to help reduce acetylfentanyl-related overdose deaths [35, 36, 38]. It is also recommended that emergency departments and emergency medical services treat suspected acetylfentanyl overdoses, like the opioid ones, according to standard protocols [34, 36]. It has to be mentioned here that since drug abusers obtain this synthetic fentanyl analogue through illicit sources, its identity, purity, and quantity are uncertain leading to severe health risks [22, 32]. Adverse reactions in humans can appear after acetylfentanyl use or abuse, and in many cases they can lead to deaths due to overdose [5]. Users reported that: "It also tends to induce a higher ratio of respiratory depression to subjective effects than heroin, making them intrinsically more dangerous, particularly with repeated dosing." [7].

Seizures

An increasing number of acetylfentanyl seizures have been reported worldwide during recent years. According to reports issued by national, federal, state, and local forensic laboratories in the United States, acetylfentanyl was identified in 10 and 40 exhibits in 2013 and 2014, respectively [9]. The total number of seizures has been increased internationally from nine to 280 from 2013 to the mid-2015 [20].

In April 2013, approximately 3 kg of acetylfentanyl in powder form and 12,400 tablets containing acetylfentanyl were seized in Montreal, Canada [1, 5, 21, 39]. The seized quantity was supposed to be huge and could be used for the production of millions of dosage units, since a typical dose of acetylfentanyl is in the microgram range. In September 2013, another seizure was reported in Louisiana, the United States. A month later, acetylfentanyl was found to be the primary detected substance in suspected oxycodone tablets purchased in Rhode Island. Eight additional seizures of acetylfentanyl have been reported in Georgia, Florida, Colorado, and Washington [21].

Seizures of acetylfentanyl have also been reported in European countries, such as Belgium, Finland, France, Germany, Poland, Sweden, Norway, and the United Kingdom. Most had been made at street level and at national borders, while two were at the scene of death. In September 2013, seizures of acetylfentanyl in powder (beige, green, and blue color) and tablet (labeled as CDN/80, A/512, PHANTOM/100) forms were recorded in Germany [40]. In March 2014, 20.2 g of a white powder containing acetylfentanyl, 4-anilino-*N*-phenethylpiperidine, and a precursor of fentanyl were seized by Polish customs, while the rest of the seizures were conducted in other European countries during 2015. These include 13 seizures of acetylfentanyl in the form of white, pink, or pale-beige powder (0.42–28 g in Sweden, France, and the United Kingdom), four seizures of 3–290 mL in liquid form, including nasal sprays (Norway and Finland), and four seizures of tablets (Germany and Sweden). In the case of 28 g of white powder seizure, acetylfentanyl powder had been shipped to France by express freight from China. In most seizures, acetylfentanyl was the only substance identified. However, one seizure of tablets contained both acetylfentanyl and fentanyl, and one seizure of a nasal spray (Nasonex®) contained acetylfentanyl, U-47700 and butyrfentanyl, while in another seizure of a liquid, U-47700 along with acetylfentanyl was detected [23, 24, 41–45]. In October 2015, the Norwegian federal police reported a seizure of a 10 mL liquid in a green bottle labeled as "AF-TINCTURE". The bottle was seized in July 2015 by the customs at Ålesund Airport and identified by mass spectrometry (MS) to contain acetylfentanyl [41].

Acetylfentanyl was also identified in herbal products that were sold in Japan through the Internet and were seized from November 2013 to May 2014. The products appeared as alternatives to controlled substances such as traditional narcotics and designer drugs [5, 46]. It has been also reported that Dharmachems and Shouguang Huatian Co. Ltd., companies located in China, were offering acetylfentanyl, with the former as pure substance at a "negotiable" price and the latter within a product labeled as "W-15 W-18 Etizolam Diclazepam Flubromazepam Acetylfentanyl" [5].

In November 2015, acetylfentanyl was seized for the first time in West Australia in the tablet form. Toxicological analysis showed the presence of this substance in 180 tablets disguised as "PEZ" lollies, a popular candy in the United States. Thus, anyone who possessed such tablets was advised to destroy them [47, 48].

Intoxications and fatal cases related to acetylfentanyl

The abuse of acetylfentanyl has led to intoxications, fatal or not. It has been used either alone or in mixtures with heroin or other drugs leading to severe adverse effects, sometimes lethal [5, 22]. Abusers who reach emergency rooms show symptoms resembling those caused by heroin overdose (lethargy and disorientation, shallow breathing, bradycardia, and hypotension). In many cases, patients are unaware of using acetylfentanyl, and they usually claim that they have used heroin or oxycodone. In these cases, the drug screening is negative for opiates and the patients do not respond to standard naloxone doses. The suspicions for acetylfentanyl use arise from positive enzyme-linked immunosorbent assays (ELISA) for fentanyl where acetylfentanyl shows cross reactivity, and the confirmation is normally made with gas chromatography–mass spectrometry (GC–MS) methods [22].

The risk of overdose fatalities increases as acetylfentanyl has a narrow margin of safety. Several deaths have been reported in North America and Europe after the use of this substance alone or in combination with other drugs of abuse [31]. Since 2012, 12 acetylfentanyl related deaths have been recorded in Russia, while the DEA has reported at least 52 confirmed fatalities associated with acetylfentanyl occurring in the United States during the years 2013–2015. These fatalities were observed in California, North Carolina, Florida, Louisiana, Maine, Maryland, New Jersey, North Dakota, Ohio, Oregon, Pennsylvania, Rhode Island, Virginia, and Wisconsin [9, 21, 24]. Five more deaths were reported in the cities of Jefferson Parish and New Orleans [12]. Between March and June 2013, 14 overdose deaths were reported in Rhode Island [12, 21, 34, 49]. The 14 dead acetylfentanyl users aged from 19 to 57 years; ten of them

were male. Law enforcement agents seized drug paraphernalia, such as needles and syringes, suggesting intravenous drug use. Crack pipes and cut straws were found at the scenes of deaths of nine acetylfentanyl related cases, while track marks were observed in three decedents. All of these victims had a known history of drug abuse, while half of them had a history of opioid use. The results of toxicological analyses using GC–MS showed, additionally to acetylfentanyl, the presence of other drugs including other opioids, cocaine, benzodiazepines, and ethanol. None of them was found positive for fentanyl. In no case was any evidence found of naloxone administration [34, 50, 51]. One more acetylfentanyl related case was reported in Rhode Island by the end of the year 2013 [21].

Isenschmid et al. [52] reported 18 confirmed deaths due to acetylfentanyl in the United States between July 2013 and April 2014. The demographics for these cases included seven cases from Louisiana, three cases from California, and one case each from Arizona, Florida, Massachusetts, New Hampshire, New Jersey, Pennsylvania, Vermont, and New Brunswick (Canada). Sixteen of the decedents were male, one was female, and in one case the sex was not provided, and the average age was 33 years (19–54 years, $n = 15$). Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis determined acetylfentanyl in blood samples of all the victims. The blood concentration for these 18 cases ranged from 0.58 to 730 ng/mL (average range: 160 ng/mL). In only two cases, acetylfentanyl concentration was below 1 ng/mL, while in 13 cases it exceeded 100 ng/mL. In one case, where acetylfentanyl was quantified in blood and the liver, its concentrations were 150 ng/mL and 1900 ng/g, respectively. Other scheduled drugs were also detected, such as methylone (one case), 4-methylethcathinone (4-MEC) (one case), phenazepam (two cases), cannabinoids (six cases), cocaine (two cases), opiates (two cases), methamphetamine (one case), benzodiazepines (five cases), alprazolam (two cases), antidepressants/antipsychotics (five cases), and diphenhydramine (one case). Fentanyl was detected in two cases at concentrations of 0.67 and 22 ng/mL, respectively. Naloxone that was also detected in five cases, which was probably due to resuscitative efforts. The fact that acetylfentanyl concentrations were higher than those quantified in fentanyl-related deaths can incriminate this designer drug for the cause of deaths, while in two cases where acetylfentanyl concentrations were below 1 ng/mL, the presence of heroin or 4-MEC can explain the cause of deaths [52].

Cunningham et al. [53] reported the death of a 28-year-old recreational drug user that occurred in 2014. The decedent was found dead on the bathroom floor with a syringe nearby. External examination revealed foamy secretions at the mouth and needle track marks along the interior side of the elbow. The autopsy revealed mild diffuse cerebral and pulmonary edema, while neither contributory disease nor physical injury was identified. Toxicological analysis of subclavian blood using LC–MS/MS confirmed the presence of acetylfentanyl (235 ng/mL), tadalafil (79 ng/mL), and ibuprofen (3.3 ng/mL). Acetylfentanyl was also determined in the liver (2400 ng/g), vitreous humor (131 ng/mL), and urine (234 ng/mL) along with acetyl norfentanyl, 4-anilino-*N*-phenethyl-4-piperidine, testosterone, epitestosterone, and oxandrolone. The cause of death was determined to be acetylfentanyl intoxication, and the manner was accidental. Acetylfentanyl concentration in blood was judged to be sufficient to cause fatal respiratory depression. The presence of the detected medicines and hormones was not contributory to the death [53].

Poklis et al. [54] presented seven deaths recorded in the United States. The decedents were six white men aged between 28 and 55 years and a 26-year-old woman. Each of them had a history of drug abuse, mainly heroin. The decedents were found unresponsive or dead at home or in hotel rooms, and paraphernalia such as syringes, needles, spoons, razor blades, and straws, as well as powders and oxycodone pills were also found at the different scenes of deaths. In one case, resuscitation attempts were made, and the victim was transferred to an emergency room, but he died the next day. Toxicological analyses of post-mortem blood, urine, bile, and liver and brain tissues were performed by an ultra-performance liquid chromatography–tandem mass spectrometric (UPLC–MS/MS) method. The results confirmed the presence of acetylfentanyl at concentrations ranging from 6 to 600 ng/mL, while morphine, 6-monoacetylmorphine, oxycodone, oxymorphone, fentanyl, cocaine, benzoylecgonine, and alprazolam were also identified. The cause of death was suggested to be intoxication by acetylfentanyl alone or along with fentanyl and/or alprazolam, and the manner of death accidental [54].

Zhang et al. [55] reported more three acetylfentanyl related deaths in Baltimore, Maryland in 2015. All three decedents, who had previous histories of drug abuse, were male (Hispanic, Caucasian, and African American), aged 17, 23, and 36 years, respectively, and they were found unresponsive at home. In one case, pills, powders, and drug paraphernalia were found at the scene of his death, while in two cases resuscitation attempts and/or treatment with naloxone were unsuccessful. Acetylfentanyl was determined in their blood at much higher concentrations as compared to those typically detected in fentanyl intoxications. The concentrations of acetylfentanyl determined in heart blood were found to be 500, 700, and 180 ng/mL. In the case of the highest acetylfentanyl concentration (700 ng/mL), the heart blood concentration was within 35 % of the femoral blood concentration. The liver and the

vitreous humor concentrations were close to those in blood, while higher concentrations of the drug were detected in urine showing that this specimen is suitable for screening acetylfentanyl use. In two out of three cases, α-pyrrolidinovalerophenone (α-PVP) and benzoylecgonine (1.3 μg/mL) were also detected in the urine of the Caucasian male and in the blood of the African American, respectively [55].

McIntyre et al. [56] presented the death of a 24-year-old man in San Diego, California. The descendent had a history of heroin abuse and was found dead at his parents' house. A complete autopsy was conducted 25 h after the decedent was found and revealed three apparent recent needle marks in the left elbow pit and forearm. Lung edema and congestion were also observed, while in his left arm vein and his lungs small amounts of foreign material were detected microscopically, being consistent with his chronic or prior intravenous drug abuse. Other significant findings were not observed. GC–MS analysis of postmortem samples showed the presence of acetylfentanyl in peripheral blood, central blood, vitreous humor, urine, and the liver at concentrations of 260, 250, 240, 2600 ng/mL, and 1000 ng/g, respectively. The syringe found at the scene of death was collected and analyzed; it was confirmed to contain only acetylfentanyl. Acute acetylfentanyl intoxication was determined as the cause of his death, while the manner of death was reported as accidental [56].

The DEA reported recently that acetylfentanyl was detected in fatal cases (no number stated) that occurred during January 2014 in Sampson, Person, and Transylvania counties of North Carolina. This synthetic drug caused more seven deaths in North Carolina between February and August 2014. The DEA also reported 14 deaths in Louisiana and three deaths in California, Oregon, and Pennsylvania, respectively [21].

An alert was issued on the 10th of September 2015 by the EMCDDA after 22 confirmed acetylfentanyl related deaths in the European Union that were reported by the European Union Early Warning System Network. Twenty of these cases were reported in Sweden [23], while two deaths were reported in the United Kingdom [23, 24]. Seventeen of the decedents were male, aged within the range of 21 and 56 years, and five were female between 22 and 55 years of age. In half of the cases, where the causes of deaths were available, acetylfentanyl use was reported to be the main cause of death or a major contributory factor to death [23, 24]. In the first case reported in the United Kingdom in March 2014, the decedent was a 56-year-old male who bought the acetylfentanyl powder via the Internet. Toxicological analysis of postmortem femoral blood samples using GC–MS and LC–MS showed the presence of acetylfentanyl (1.2 μg/mL), mirtazapine (0.15 μg/mL), olanzapine (<0.05 μg/mL), pregabalin (3.7 μg/mL), sertraline (0.39 μg/mL), and alcohol (11 mg/dL propably due to postmortem production). Ischemic heart disease and acute heart failure after acetylfentanyl overdose were reported as the cause of his death [24]. The second case was reported in Cardiff, Wales, in February 2015. The 37-year-old male, a father of three children former opium and heroin addict, and known user of methamphetamine, was found dead at home by his wife after self-administration of acetylfentanyl, probably via intravenous injection, to "get off heroin". It was reported that acetylfentanyl in the powder form was purchased via an Internet site based in China. The toxicology report revealed high concentrations of acetylfentanyl in femoral blood and traces of amphetamine, buprenorphine, and phenazepam, as well as loperamide and methamphetamine in urine. The death was attributed to the toxic effects of the drug cocktail (acetylfentanyl, phenazepam, buprenorphine, and amphetamines) [24, 57].

Hisatsune et al. [58] reported a fatal case due to acute acetylfentanyl intoxication in Japan in 2015. Screening of the postmortem blood sample, after the appropriate pretreatment, using liquid chromatography–quadrupole time-of-flight/mass spectrometry (LC–QTOF/MS) system, detected acetylfentanyl and α-pyrrolidinohexiophenone (α-PHP), a new synthetic cathinone, while quantification of these two recreational drugs was performed by a GC–MS method. Blood concentration levels of acetylfentanyl and α-PHP were determined at 69 and 413 ng/mL, respectively. Some hydroxylated and deacetylated metabolites of acetylfentanyl were also detected. Finally, the synergy of acetylfentanyl with α-PHP was supposed to be the cause of death [58].

Determination of acetylfentanyl in biological specimens

The presence of acetylfentanyl in biological specimens or seized materials cannot be differentiated from its Schedule II precursor fentanyl by immonoassays, such as ELISA. Thus, positive screening tests are followed by confirmatory tests with different chromatographic techniques combined with MS detection to identify fentanyl, acetylfentanyl or other fentanyl analogues clearly [5, 59]. Ruangyuttikarn et al. [60] showed that acetylfentanyl had good cross reactivity with fentanyl, as percent binding at 2 ng/mL of standard fentanyl was comparable to that of acetylfentanyl at 1.8 ng/mL [60]. Wang et al. [59] used a fentanyl immunoassay to detect fentanyl, its metabolites, acetylfentanyl, and other fentanyl analogues in urine of a patient population in San Francisco. Acetylfentanyl showed cross reactivity with fentanyl at concentrations higher than 2.5 ng/mL [59].

Both liquid–liquid extraction (LLE) and solid-phase extraction (SPE) techniques have been used for the isolation of acetylfentanyl. The United States Centers for

Disease Control and Prevention Health Advisory Alert suggested a routine pretreatment procedure that included *n*-butyl chloride LLE after alkalinization of the samples followed by acidic back extraction [61].

Patton et al. [30] developed an LC–MS/MS method that provided low limit of quantification (LOQ) (1 ng/mL) and high levels of accuracy and reproducibility for acetylfentanyl and acetyl norfentanyl. Prior to analysis urine samples were subjected to β-glucuronidase treatment and then to SPE extraction, while analytes were eluted with methanol/acetonitrile/ammonium hydroxide (50:50:2, v/v/v) [30].

Zhang et al. [55] developed a GC–MS method for the determination of acetylfentanyl in biological specimens. The pretreatment procedure included an initial alkaline LLE of acetylfentanyl from specimens with *n*-butyl chloride/ether, then a back extraction into sulfuric acid, and finally alkalinization and extraction with methylene chloride. The developed method was sensitive, linear from 25 to 800 ng/mL, and it was successfully applied to postmortem specimens (heart and femoral blood, urine, liver, kidney, and vitreous humor) of three fatal cases reported in Baltimore, Maryland [55].

Another GC–MS method for the determination of acetylfentanyl in biological specimens has been described by McIntyre et al. [56]. Specimens were pretreated through alkaline LLE with 1-chlorobutane. The limit of detection (LOD) and LOQ values were determined to be 50 and 100 ng/mL, respectively. Both LOD and LOQ could be substantially lowered by the use of a more sensitive calibration; quantitative and qualifier ions were distinctly measurable at concentrations as low as 1.0 ng/mL. The method was applied to postmortem specimens (blood, vitreous humor, urine, and liver) of an acetylfentanyl related fatal case [56].

A UPLC–MS/MS method was developed by Poklis et al. [54] for the determination of acetylfentanyl, fentanyl, and their nor-metabolites in biological fluids and tissues. The analytes were isolated via SPE with dichloromethane/isopropanol/ammonia (78:20:2, v/v/v) and analyzed by the UPLC–MS/MS system using gradient mobile phase consisting of 10 mM ammonium formate/0.1 % formic acid in water and methanol with a flow rate of 0.6 mL/min. The method was linear for the concentration range 0.001–0.100 μg/mL, while the LOQ was set at 0.001 μg/mL for acetylfentanyl. The developed method was applied to postmortem specimens of decedents in the United States and provided information on the distribution of acetylfentanyl in tissues. In three cases, the relatively low concentrations of acetylfentanyl and its metabolite in blood, the brain and liver as compared to those in urine and bile indicate a delayed death, while in four cases the higher concentrations of acetylfentanyl in the brain and liver than in urine and bile reveal rapid deaths [54].

High-performance liquid chromatography (HPLC)–MS and GC–MS methods have been also used for the identification of acetylfentanyl metabolites in urine samples. They were isolated from urine using fractionation of extracts by LC. They were identified by their molecular ions recorded by HPLC–MS with chemical ionization (CI) and by the complete electron ionization spectra after GC–MS analysis. Pretreatment of urine samples using SPE and acetylation or acylation (pentafluoro-derivatives) of urine extracts for the detection of hydroxylated metabolites and their *N*-deacetylated analogues were necessary steps prior to GC–MS analysis [25].

Hisatsune et al. [58] detected acetylfentanyl and α-PHP in a postmortem blood sample at a screening test using LC–QTOF/MS after deprotonization of the sample with methanol. The separation was performed with an L-2 ODS column using two mobile phases at a flow rate of 0.10 mL/min as follows: A) 5 % methanol/10 mM ammonium acetate buffer and B) 95 % methanol/10 mM ammonium acetate buffer [58]. The use of the LC–TOF/MS technique renders specific and sensitive forensic tests, challenging low concentrations of acetylfentanyl in biological fluids and tissues that would be typically missed by the use of other routine screening methodologies and technologies. It can also prevent from false positive findings from fentanyl analogues and their compounds [62]. Quantification of acetylfentanyl was conducted with GC–MS after the appropriate pretreatment of the samples that included alkaline LLE with ethyl acetate. Blood concentration levels of acetylfentanyl and α-PHP were determined at 69 and 413 ng/mL, respectively. Accurate mass measurements for searching main metabolites could detect metabolites of acetylfentanyl and confirmed its expected metabolic pathways, i.e. hydroxylation and deacetylation [58].

Legal status

Acetylfentanyl has been recently recommended to be controlled in the United States as a Schedule I drug under the 1961 Single Convention on Narcotic Drugs as consistent with Article 3, paragraph 3 (iii) of that Convention in that the substance is liable to be abused and produces effects similar to those of α-methylacetylfentanyl (Schedule I) [5, 31, 62, 63].

Acetylfentanyl, as well as its optical, positional, and geometric isomers and their salts are Schedule I controlled drugs under the Federal Controlled Substances Act in Canada as fentanyl analogues, since July 2015 [9, 12, 21, 41]. In Japan and China, the drug has been a controlled substance since July 2014 and October 2015, respectively. In the United States, acetylfentanyl was temporarily placed into Schedule I in 21 May 2015 [12, 41]. As a psychoactive

substance, it is under drug control legislation in Austria (annex 1 of the Narcotic Substances Regulation), Estonia (under Control Regulation, listed 8.06.2015), Cyprus, Ireland (Misuse of Drugs Regulations 1988; S.I. 328 or 1988), Latvia, Lithuania, Norway, Poland, and Sweden. In Sweden and Finland, acetylfentanyl is a controlled substance since August and September 2015, respectively [12, 41]. Belgium, Bulgaria, Croatia, Czech Republic, Denmark, France, Germany, Greece, Hungary, Italy, Luxembourg, Malta, Netherlands, Portugal, Romania, Slovakia, Slovenia, Spain, and Turkey have not included acetylfentanyl in controlled substances [12, 41]. Acetylfentanyl scheduling and its placement into Schedule I of the United Nations Controlled Substances Act are necessary to prevent imminent hazards to the public society. Thus, persons who manufacture, distribute, import, export, engage in research, possess, or propose to handle acetylfentanyl should be subjected to administrative, civil, and criminal sanctions/penalties [21].

Conclusions

Acetylfentanyl is used as a substitute of fentanyl, heroin, and other scheduled opioids, and it has invaded the international drug arena during recent years. It has μ-opioid analgesic properties mimicking the effects of fentanyl due to its structural similarity to the latter. Although it was first synthesized in 1968, it has recently gained the attention of authorities worldwide due to human intoxications and fatalities; it has a significant potential for overdose. Acetylfentanyl is available online or in retail shops in various forms, while it is often used with other recreational drugs. Its use has been reported in the Unites States, Europe, Australia, and Japan leading to intoxications and fatalities. Several published and reported fatal cases have eventually led governmental authorities to specifically schedule acetylfentanyl and cover the legal loophole as they did for other drugs. Similar actions are expected to be taken worldwide, along with increased public vigilance by hospitals, law enforcement, and medical examiners. This could prevent not only the increase of acetylfentanyl abuse, but also related intoxications and deaths.

Compliance with ethical standards

Conflict of interest There are no financial or other relations that could lead to a conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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