LITERATURE REVIEW

A Literature Review of the Analytical Toxicology of Fentanyl Derivatives

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Abstract

Introduction: Fentanyl is a synthetic opioid with the potential to cause life-threatening adverse effects in overdose scenarios, including sedation and respiratory depression. The rising prevalence of fentanyl-related substances since the mid-2000s constitutes a serious concern.

Methods: This literature review offers a comprehensive overview of the current information available concerning fentanyl derivative toxicology, including testing methodologies in biological fluids, metabolism, pharmacokinetics and levels found in overdose or deaths associated with their use.

Results: There are significant knowledge gaps in the current literature on fentanyl derivatives, partly due to their extremely low serum concentration. Lower limit of detection figures are typically in the range of 0-1 ng/mL, necessitating the use of highly sensitive testing methodologies. Immunoassays are widely available but limited in their ability to distinguish between derivatives. Gas chromatography-mass spectrometry offers untargeted data acquisition and vast mass spectral libraries; however, this technique has lengthy preparation times and limited sensitivity. Liquid chromatography-mass spectrometry has recently been used with quadrupole time-of-flight or orbitrap technology to offer tentative identification of compounds without library searching. However, the real weapon needed to tackle the ongoing fentanyl crisis is a technique which can assist in the prediction of unknown compounds.

Discussion: The recent advent of a machine learning model applicable to mass spectra offers promising potential to predict the structure and spectra of previously unknown fentanyl analogues. Moreover, increased funding is required to enhance the sensitivity of current fentanyl detection techniques in combating the overdose epidemic.

Keywords: fentanyl, fentanyl derivative, analytical toxicology

Introduction

History and Therapeutic Indications of Fentanyl

Fentanyl was first synthesised by Dr. Paul Janssen in 1960 and was approved for medical use in the USA in 1968[1]. Fentanyl and its analogues have therapeutic uses in analgesia and anaesthesia, especially for cancer and chronic pain patients who experience "breakthrough pain" or develop a tolerance to other opioids[2]. Therapeutic analogues used for human analgesia and anaesthesia include alfentanil, remifentanil and sufentanil. Misuse of fentanyl within the US and Europe has been documented since the 1970s, originally mixed with heroin[3]. The longest documented fentanyl epidemic occurred in Estonia, escalating after the outlaw of opium poppy growth in Afghanistan, originally the world's largest opium poppy supplier[4]. Fentanyl abuse has been particularly on the rise for the past two decades, with an increasing number of deaths related to the abuse of fentanyl-derived opioids. A 2012 report by the European Union Drugs Agency (EMCDDA) suggested the high-risk nature of fentanyls would dissuade opioid users and that the fentanyl crisis may have "built-in breaks" in some respects[5]. However, the number of deaths involving fentanyl and other synthetic opioids is increasing, with 73,838 deaths reported in the US in 2022[6]. Fentanyl analogues are being created at a faster rate than they can be scheduled (i.e. categorised based on abuse potential). In the US, this has led to temporary scheduling orders being placed on fentanyl-related substances in 2018, which has been extended multiple times[7].

Chemistry

Fentanyl is the prototype of the 4-anilidopiperidine class of synthetic opioid analgesics. Its molecular formula is $C_{22}H_{28}N_2O$, and its molecular weight is 336.471 g/mol. Its synthetic name is N-(1-(2-phenethyl)-4 piperidinyl-N-phenyl-propanamide)[8]. Various non-pharmaceutical fentanyl (NPF) derivatives have since been developed by adding various substituents to the basic molecule, enhancing its analgesic potency to 10,000 times that of morphine. Examples of such changes include the replacement of the piperidine ring for pyrrolidine and the replacement of the phenyl group in the phenethyl-part of the molecule for some aromatic heterocycles,

mainly for thiophene and tetrazole[9].

Mechanism of Action

Fentanyl is a selective agonist of mu-opioid receptors. Its rapid onset, duration of action, potency and risk of overdose are attributable to its significant lipid solubility[10]. Mu-opioid receptors are G-protein coupled receptors (GPCRs), comprising a single polypeptide chain with 7 transmembrane domain receptors that interact with heterotrimeric g-proteins[11]. Mu (μ) receptors are involved in the neuromodulation of nociception, respiration, gastrointestinal activity as well as stress, temperature, memory, motivation and endocrine function. Agonism of Mu receptors by fentanyl is responsible for its clinical use in analgesia and anaesthesia but is also responsible for adverse effects experienced by patients, such as opioid-induced constipation, drowsiness and respiratory depression[12].

Fentanyl also causes muscle rigidity in the chest wall via dopaminergic pathways, decreasing respiratory rate and the efficacy of cardiopulmonary resuscitation (CPR)[13]. High or multiple doses of Naloxone, an opioid antagonist, may be required for reversal.

Forensic Toxicology

Forensic toxicology is primarily carried out to determine the role fentanyl plays in drug-related deaths and criminal cases. Low concentrations of fentanyl in postmortem samples often lead to difficulties in detection and interpretation[14]. The minimum effective concentration (MEC) for fentanyl analgesia is approximately between 0.6 - 1.5 ng/mL, while the MEC for anaesthesia is between 10 - 20 ng/mL. The lethal dose for fentanyl is 2mg, however, for a synthetic opioid such as carfentanil, a lethal dose can be just 0.0002mg. This great variation in potency precludes the straightforward detection, identification and cross-comparison of derivatives. These illicitly synthesised fentanyl can also be mixed with other substances such as cocaine, heroin, and ecstasy, amplifying the risk of drug overdose and death, often without the user's knowledge[15].

Aim

The aim of this literature review is to perform a thorough toxicological analysis of fentanyl derivatives, primarily in the legal context, exploring biological testing methods, metabolism, pharmacokinetics and levels found in overdose and deaths associated with their use.

Methodology

Meta-analyses, literature reviews, systematic reviews and case reports on fentanyl and their derivatives were sought from various search engines and websites, including CAS SciFinder, PubMed and Google Scholar. Keywords searched included: fentanyl, fentanyl derivatives, fentanyl analogues, metabolism, fentanyl metabolites, forensic, toxicology, analysis, pharmacokinetics, urine, blood, plasma, gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS). The 'search by structure' feature on Chemical Abstracts Service (CAS) SciFinder enabled access to further papers relevant to specific fentanyl analogues. Studies were excluded if they were deemed irrelevant: eligibility assessment was performed by the independent reviewers and disagreements were resolved by consensus.

Results

Adverse effects: Acute and Chronic

Depending on the route of administration, fentanyl has an onset of action of minutes to hours. Fentanyl overdose is a medical emergency. Acute overdose symptoms include constricted pupils, clammy and cold skin, discoloured or pale skin, nausea and vomiting, choking sounds, slurring or loss of speech, sedation and respiratory depression. Severe overdose can lead to respiratory arrest, cardiac arrest, or a severe anaphylactic reaction, resulting in sudden death. Long-term fentanyl use can result in chronic adverse effects, including opioid tolerance, dependence and addiction[12]. Fentanyl tolerance leads to increased dosage requirements to elicit the desired effects, which increases the risk of overdose.

Prevalence: Statistics

Due to a powerful potency and reduced half-life, fentanyl and its analogues account for many overdoses worldwide. The full extent of the synthetic opioid crisis is presumed to be underreported due to a lack of routine diagnostic monitoring. Drug overdose deaths involving opioids continue to rise, with 80,411 deaths in the U.S. in 2021[6]. Fentanyl has comprised the majority of all drug overdose deaths in the U.S. since 2018, overtaking heroin[6].

Metabolism

As seen in Table 1 , the metabolism routes of fentanyl analogues vary depending on their chemical structure. Fentanyl has various sites for metabolic transformation. It consists of a heterocyclic tertiary aliphatic amine containing two different phenyl rings and an aromatic amide function. Tertiary aliphatic amines are bio transformed through a reversible reaction into tertiary amine oxides. In addition, the tertiary amines undergo N-dealkylation through carbinolamine. When this process occurs on the phenylethyl side chain, a phenylacetaldehyde is also produced, which immediately oxidises into phenylacetic acid. Oxidation at the 2-position of the piperidine ring leads to the production of a carbinolamine. This subsequently transforms into a more stable aminoaldehyde, resulting in ring cleavage. Aromatic rings undergo oxidation, producing the equivalent phenolic derivatives. Moreover, benzylic positions are more prone to oxidation. Amide functions usually undergo hydrolysis, and oxidation of the carbon chain is also frequent.

In humans, fentanyl is principally metabolised in the liver by CYP3A4 into norfentanyl. This occurs through oxidative N-dealkylation at the piperidine ring by hepatic CYP3A4 and 3A5 isoenzymes: the main pathway of metabolism. The inactive metabolites and under 10% of the intact molecule are primarily excreted in urine and faeces. Less than 1% is metabolised by alkyl hydroxylation, combined N-dealkylation and hydroxylation, or amide

Table 1. **Analytical Toxicology Data for Fentanyl Derivatives**

Compound	Method	Parent drug/ Metabolites	LLOD	Conc. found	Patient condition	Other	Reference
Acetylfentalnyl	ELISA GC-MS/MS LC-MS/MS	Acetylfentanyl mainly undergoes N-dealkylation to acetyl norfentanyl	0.1-1.0 ng/g	Case 1: In heart whole blood: 155 ng/g In urine: 126 ng/g Case 2: In urine: 570 ng/g	Case 1: death Case 2: survived	Both individuals self-administered mepirapram and acetylfentanyl; case 1 administered intravenously, case 2 administered by inhalation	34, 35
Acrylfentanyl	LC-MS (urine)	Acrylfentanyl metabolites: Nor-acrylfentanyl Hydroxyacrylfentanyl Dihydroxycacrylfentanyl Hydroxymethoxyacrylfentanyl	0.05ng/ mL	Case 1 – 0.3ng/mL Case 2 – 0.95ng/mL Case 3 = 0.32ng/mL	All cases death	Case 3 – 0.95ng/mL furanylfentanyl detected	36
Benzoylfentanyl	NMR LC-HRMS ELISA	BZF metabolites: norBZF, despropionoylfentanyl and a hydroxylated-BZF pFBF metabolites: norpFBF, parafluorofentanyl and a hydroxylated-pFBF					37, 38
Benzoylbenzylfentanyl	LC-QTOF-HR MS	10 Metabolites detected (B1-10) B1-$C_{18}H_{24}N_2O_2$: Amide hydrolysis + benzyl dihydrodiol formation B2-$C_{25}H_{28}N_2O_3$: Benzoyl dihydrodiol formation B3-$C_{18}H_{22}N_2O$: Amide hydrolysis+ benzyl hydroxylation B_4-$C_{18}H_{20}N_2O$: N-dealkylation B5-$C_{25}H_{28}N_2O_3$: Benzyl dihydrodiol formation B6-$C_{18}H_{22}N_2$: Amide hydrolysis B7-$C_{25}H_{26}N_2O_2$: Benzyl hydroxylation B8-$C_{18}H_{22}N_2O$: Amide hydrolysis + N-oxide formation B9-$C_{26}H_{28}N_2O_3$: Benzyl dihydroxylation + methylation B10-$C_{25}H_{26}N_2O_2$: N-oxide formation Most abundant = Normetabolite (B4)					39
Benzodioxole-fentanyl	MS						40
Acetylbenzylfentanyl	LC-QTOF-HR MS	7 metabolites detected (A1-7) A1-$C_{20}H_{26}N_2O_3$: N-Phenyl Dihydrodiol formation A2-$C_{13}H_{18}N_2O$: N-dealkylation A3-$C_{20}H_{26}N_2O_3$: Benzyl Dihydrodiol formation A4-$C_{26}H_{32}N_2O_8$: Hydroxylation + Glucuronidation A5-$C_{20}H_{24}N_2O_2$: Hydroxylation A6-$C_{20}H_{24}N_2O_2$: Benzyl Hydroxylation A7-$C_{20}H_{24}N_2O_2$: N-oxide formation Most abundant = Normetabolite (A2)					39

Table 1. **Analytical Toxicology Data for Fentanyl Derivatives (Cont.)**

Compound	Method	Parent drug/ Metabolites	LLOD	Conc. found	Patient condition	Other	Reference
Butyrfentanyl	LC-MS/MS LC-QTOF	Butyrfentanyl was metabolised to carboxyfentanyl, hydroxyfentanyl, norfentanyl and desbutyrfentanyl Most abundant = Carboxyfentnayl and hydroxyfentanyl	LLOQ:1 ng/mL	In heart blood (9 hours after death): 39 ng/mL In urine (at autopsy): 1100 ng/mL	Death		41
Carfentanil	GC-FID GC-MS LC-MS/MS (blood and urine)	Norcarfentanil		4.2 µg/L .0042 /mL 4.2ng/mL (Blood)	Death		42
Cyclopropylfentanyl	GC-MS HPLC-DAD (Blood/ urine)	Cyclopropyl-norfentanyl, N-methyl cyclopropylnorfentanyl	0.5ng/mL	20.4ng/mL (Blood)	Death		43
Cyclopentylfentanyl	LC-MS-MS	N-dealkylation, mainly alkyl hydroxy metabolites, 4-ANPP (amide hydrolysis), N-oxide and ketone formation	<0.5 ng/mL	0.5-1000 ng/mL (Blood)			24, 44, 45
Methoxyfentanyl	LC-MS (blood)	O-demethylation → O-demethyl- Hydroxylation on ethyl linker → HO-ethyl- (can be precursor → Deethylphenyl-) or phenyl → HO-phenylAbove metabolites combined → HO-HO-ethylphenyl-and HO-ethylphenyl-O-demethyl Cleavage of amide bond → deamide- Hydroxylation on aniline phenyl → Deamide-HO-phenyl Hydroxylation on ethylphenyl linker → Deamide-HO-ethylphenyl O-glucuronidation O-demethyl → O-demthyl-glucoronide		0.022- 0.056 mg/kg (blood)	Death		46
Methoxyacetylfentanyl	LC-MS-MS	Demethylmethoxyacetylfenta nyl (O-demethylation), 4-ANPP (amide hydrolysis), normethoxyacetylfentanyl (N-dealkylation), alkyl/ aryl hydroxy metabolites and phase II conjugates		Mean concentrations for 11 cases quantitatively confirmed was 17.7 ng/ mL		Estimated relative potency to fentanyl: 0.3	24, 44
2,2,3,3-Tetramethyl-cyclopropylfentanyl (hydrochloride)	MS	Monohydroxylations and dihydroxylations and subsequent further oxidation steps					47

Table 1. **Analytical Toxicology Data for Fentanyl Derivatives (Cont.)**

Compound	Method	Parent drug/ Metabolites	LLOD	Conc. found	Patient condition	Other	Reference
Tetrahydrofuranyl fentanyl (THF-F)	GC-MS HPLC-TOF FTIR-ATR GC-(MS)-IR condensed phase	Nortetrahydrofuranylfentanyl (N-dealkylation), alkyl/aryl hydroxy metabolites, ring opening of the tetrahydrofuranyl ring and 4-ANPP (amide hydrolysis); (N-dealkylation is the proposed predominant metabolic step; (hydroxylation of the piperidine ring and the phenylethyl side chain, N-oxidation and amide hydrolysis to 4-ANPP were also observed)			THFF has caused death in at least 15 drug-using individuals to date	Estimated relative potency to fentanyl: approximately 0.2 Highly selective for the mu-opioid receptor Closely related to furanylfentanyl (Differs by bearing a fully saturated furanyl ring, instead of an unsaturated ring)	16, 48, 49
3-phenylpropanoylfentanyl	NMR GC-MS FTIR	Mainly metabolised through N-dealkylation to form nor-metabolites					50
2-fluorofentanyl (ortho-fluorofentanyl)	LC-QTOF-MS		≤0.5ng/ mL	Case 1: Hospitalised. 2.5ng/mL (Serum) Case 2: Autopsy post-mortem: 2.4ng/mL (blood) 3.9ng/mL (urine)	Case 1: Male in early 20s, lost consciousn ess and respiratory function, CPR and Naloxone administer ed. Discharged . Found dead at home a few days later. Case 2: Case 1: Male in early 20s, lost consciousn ess and respiratory function, CPR and Naloxone administer ed. Discharged from hospital.	Case 1: While hospitalised. Additional toxicological findings: (Serum) Ethanol: 1.0 g/L Alprazolam 96 ng/mL Benzoylecgonine: (not quantified) Amphetamine: (not quantified) Paracetamol: (not quantified) Case 2: Autopsy. Additional toxicological findings: Blood Alprazolam: 16 ng/mL Desmethyldiazepam: 3.8 ng/mL (most likely metabolite of previously ingested diazepam) 7-Aminoclonazepam: 34 ng/mL(inactive metabolite of Clonazepam, may be formed post mortem) THC: 21 ng/mL GHB: 22 µg/mL (Urine) Ethanol THCA GHB Concluded that death was caused by ortho-fluorofentanyl.	51`

Table 1. **Analytical Toxicology Data for Fentanyl Derivatives (Cont.)**

Compound	Method	Parent drug/ Metabolites	LLOD	Conc. found	Patient condition	Other	Reference
4-fluoro-isobutyrylfentanyl (4F-iBF)	Paper 1: LC-HRMS HPLC-TOF GC-MS-IR Paper 2: MS, LC-MS/MS	Paper 2: solvent = chloroform-d Metabolites: Isobutyrylfentanyl hydrochloride, nor-4-fluoro-isobutyrylfentan yl (nor-4F-iBF) hydrochloride, nor-4-chloro-isobutyrylfentan yl (nor-4Cl-iBF) hydrochloride, and nor-isobutyrylfentanyl (nor-iBF) hydrochloride				GC-MS analysis of 4F-iBF and 4F-BF (4-fluoro-butyrfentanyl) display very similar mass spectrometry fragmentation patterns. Analytical reference standards or access to reference spectra required for both substances to distinguish between them. ELISA may not distinguish between 4F-iBF and fentanyl due to structural similarity	52, 53
4-chloro-isobutyrfentanyl (4Cl-iBF)				Serum: 5-45 ng/mL Urine: 11-136 µg/mmol creatinine		Acrylfentanyl, 4F-iBF, THF-F	54
2-Furanylfentanyl (2-Fu-F)	LC–MS-MS		LLOQ: 0.100 ng/mL	Blood concentration ranges of 0.15-0.30 ng/mL. Case 3: levels of 2-Fu-F: 8.7 ng/mL (heart blood), 5.5 ng/mL (femoral blood) Approx 30 ng/mL (vitreous humor)	Case 3: Death.	Research and testing completed in the context of cases of DUID (Driving Under the Influence of Drugs) and in post-mortem cases. Case 3: Cause of death was combined effects of heroin, fentanyl, diphenhydramine and 2-Fu-F. Accidental death.	55
Valerylfentanyl	MS-MS GC-MS LC-MS/MS	Paper 2: 4-Anilino-N-phenethylpiperidi ne (4-ANPP) Hydroxylated valerylfentanyl Valeryl norfentanyl	0.05 ng/mL LLOQ: 0.10 ng/mL	0.10 - 21 ng/mL (post-mortem blood)	4 selected cases, all deceased	Not detected as the sole intoxicant in these cases. Other opiates, cocaine, benzodiazepines and ethanol were most commonly detected.	56, 57
3-Methylcrotonylfentanyl (3-MCF) C24H30N2O	UHPLC-QTO F-MS	6 Metabolites detected (A1-A6) A1-C24 H30N2O2: Hydroxylation A2-C16H22N2O: N-dealkylation A3-C24H28N2O3: Carboxylation A4- C24H28N2O3: Carboxylation A5-C24H28N2O2: Carbonylation A6- C24H30N2O3: Dihydroxylation Abundance: A1>A3>A5>A2>A6>A4 and A3> A6> A4> A1> A2> A5 after 30 and 240 min					58

Blank data indicates no available data as of 18/04/2023

hydrolysis to the inactive compounds hydroxyfentanyl, hydroxynorfentanyl, and despropionylfentanyl[16].

Drug-drug Interactions

Due to its metabolism, fentanyl should not be combined with CYP3A4 inhibitors, including macrolide antibiotics, azole-antifungal agents, or protease inhibitors. This inhibition will decrease fentanyl's degradation to inactive norfentanyl. CYP3A4 inducers such as carbamazepine and phenytoin will increase fentanyl clearance, reducing its effect. Fentanyl increases serotonin levels, so combination with any MAO inhibitors, SSRIs or any drug that increases serotonin levels can cause serotonin toxicity[17]. Fentanyl can also reduce the clearance of sedative drugs such as midazolam[18].

Analytical Confirmation and Methodologies Used: Concentrations in Biofluids

Biofluids typically used in fentanyl analysis include blood, urine and saliva. Prior to analysis, biofluid samples are prepared/purified by either solid-phase extraction (SPE) or liquid-liquid extraction (LLE) methods. Methodologies used for analysis depend on the type of biofluid for analysis, the sensitivity and selectivity of the analytical method, and the required detection limit.

As seen in Table 1, the lower limit of detection (LLOD) ranges for fentanyl derivatives are typically extremely low (0-1 ng/mL), meaning analytical methods to determine fentanyls in biofluids must have a high sensitivity. Immunoassays are antibody-based methods commonly used to screen samples for fentanyl. These methods include lateral flow assays (LFAs), heterogeneous immunoassays, such as enzyme-linked immunosorbent assays (ELISAs), and homogeneous immunoassays, such as enzyme multiplied immunoassay technique (EMIT)[19]. Despite their ubiquity in fentanyl toxicology labs, immunoassays are limited in their utility. Standard immunoassays are unable to detect new opioids, are limited to a set number of drugs, have limited cross-reactivity, and cannot distinguish between derivatives of fentanyl. Specific assays can be utilised, but often are not used routinely, and require confirmation with specific chromatographic techniques[20]. LFAs are the fastest and least expensive option, however, they are not as sensitive as other immunoassays. Wang et. al developed a high throughput homogeneous enzyme immunoassay (HEIA) that can detect fentanyl in urine at a cut-off concentration of 2 ng/ml, offering much greater sensitivity than LFAs[21].

As mentioned previously, specific ELISAs can also be used to detect fentanyls, however this process is limited by its manual nature. ELISA testing additionally requires confirmation by mass spectrometry (MS), a technique which is often not available in hospital laboratories[19]. GC-MS offers the ability to obtain untargeted data, which can be searched in vast mass spectral data libraries to identify compounds in biological samples. However, sensitivity remains an issue. Typically, detection values range between 1-10 ng/ml is not sufficient for detection of the more potent fentanyl analogues, which are often found in incredibly low concentrations[22]. Additionally, GC-MS methods are not able to directly analyse non-volatile, polar or thermally labile drugs, necessitating the use of lengthy sample preparation techniques and thus limiting the application of GC-MS to routine rapid testing.

Liquid chromatography with tandem mass spectrometry (LC-MS/MS), on the other hand, has high sensitivity and offers relatively rapid detection. LC-MS/MS can detect fentanyl in plasma samples with a lower limit of qualification (LLOQ) of 0.05ng/ml and norfentanyl at 0.25ng/ml[23]. Fogarty et al. developed a technique using LC-MS/MS that has allowed the pharmacological effects of methoxyacetylfentanyl and cyclopropylfentanyl to be associated with quantitative values in samples, a breakthrough for two of the more elusive fentanyl derivatives[24]. This achievement illustrates the potential for LC-MS/MS to offer insights into other derivatives. However, currently, a universal library for LC-MS/MS does not exist, as does for GC-MS, and many forensic laboratories do not have MS/MS capabilities. Additionally, LC-MS/MS is often targeted, it will only detect substances for which the method is specifically designed. It is also time-consuming; results are rarely produced in sufficient time to contribute to the real-time care of patients or detection of outbreaks. Overall, this technique is useful in the profiling of illicit fentanyl compounds, however, it is not capable of solely conquering the ongoing threat of synthetic opioid creation.

Liquid chromatography electron ionisation mass spectrometry (LC-EI-MS) is a promising testing alternative. LC-MS offers the advantage of injection at room temperature (circumventing the thermal degradation obstacle) of compounds dissolved in aqueous solution, however there is no universal library available for LC-MS/MS. GC-MS offers an extensive library searching capability, which can be employed to full advantage with the myriad fragmentations produced by EI. Put simply, LC-EI-MS combines the advantages of these two techniques, overcoming the limitations of using either in isolation. This technique is rapid and highly sensitive, with the ability to determine fentanyl in plasma between 0.02–10ng/ml and to determine fentanyl and norfentanyl in urine in the range 0.1–50 and 0.102–153 ng/ml, respectively[25].

Other modern testing methodologies have been developed for the detection of fentanyl analogues in various settings, both for forensic toxicology laboratories and for use on the field. Thermal desorption direct analysis in real-time mass spectrometry (TD-DART-MS) is a fentanyl detection technique with potential applications in mobile laboratories, emergency vehicles and hospitals. This approach may be more effective than current ELISA screening and GC/MS analysis techniques, as it offers greater sensitivity. Ion mobility spectrometry (IMS) is another technique offering greater sensitivity than current colourimetric techniques.This approach can also be used to detect fentanyl even in the presence of heroin, making it particularly advantageous for use on

the field[26].

Legislation and Education

Regulation. Fentanyl and its analogues are subject to both international treaties and the laws of individual countries. Fentanyl has been internationally controlled under the 1961 Single Convention since 1964. In the United States, the Drug Enforcement Administration (DEA) schedules drugs, including fentanyl, under the Controlled Substances Act of 1970 and the Controlled Substances Analogue Enforcement Act of 1986[27]. On February 6, 2018, a proactive class-wide scheduling of fentanyl-related substances was initiated, leading to a dramatic fall in fentanyl analogues in the marketplace. The United States Congress has temporarily extended this scheduling, which is to expire in December of 2024. The imminent expiration date has led to renewed pressure for a class-based scheduling strategy and increased research into fentanyl-related substances[27].

Impact on Society. Given the reduced cost, increased potency, more straightforward synthesis and lack of agricultural requirements of fentanyl in comparison to heroin, illicit drug distributors have recently demonstrated increased preference of synthetic opioids over non-synthetic opioids. The price of fentanyl has been reported to be 1% that of heroin in some cases, substantially decreasing expenses. Opium poppy growers will most likely be made obsolete by the rise of synthetic opioids. Farm prices in Mexico have decreased between 60-80%, and Afghanistan's massive opium poppy economy is at risk of the same demise[28].

Opioid users may convert from heroin to fentanyl due to its high potency, increased availability and lower cost[29]. Given that fentanyl has a duration of action approximately 1/2-1/3 that of heroin, synthetic opioids must be administered more frequently to avoid withdrawals, bearing an increased risk of blood-borne illnesses and overdoses[30]. A cross-sectional risk behaviour survey in Estonia found that 62% of participants who injected fentanyl as their primary drug had contracted Human Immunodeficiency Virus (HIV). The survey also demonstrated increased likelihood to share needles, reuse needles and use discarded needles for intravenous injection31. In general, the literature illustrates that fentanyl, and its analogues may become more prevalent than heroin due to increased accessibility for intravenous drug users.

Education and Awareness. Prior to 2017, the highest overdose death mortality rates in Europe were held by Estonia for over a decade. Fentanyl has been a large contributor to this epidemic. Estonia's declining drug overdose mortality rates have been due to many factors, including the distribution of take-home naloxone kits, needle exchange programmes, free and confidential HIV screening and antiretroviral treatment (ART) for HIV positive users, and the closedown of a large producer & distributor of illicit fentanyl. The "Break the Cycle" initiative was introduced in Tallinn, and due to its success was introduced to New York. This programme was developed as a motivational-interview-based initiative. The programme aims to discourage experienced people who inject drugs (PWIDs) from showing other drug users how to inject drugs for the first time, as almost all IV drug users require assistance from experienced PWIDs for their first injection. These programmes and tools have been invaluable in tackling Estonia's opioid epidemic, and their implementation should be considered in other countries[4].

Existing Gaps in our Knowledge. An exhaustive review of all relevant information concerning fentanyl derivatives was precluded by lack of research, and occasionally lack of access to existing literature, on some of the more elusive analogues. Choice of testing methodology and medium for fentanyl detection varied greatly in the articles reviewed, making a comparison of fentanyl levels difficult. More sensitive technology is needed to investigate LLOD values for all fentanyl analogues and to associate their pharmacological effects with levels found in samples.

Discussion

As illustrated by Table 1, fentanyl is often extensively metabolised through demethylation, hydroxylation, N-dealkylation, and amide hydrolysis to form a variety of metabolites. Fentanyl metabolism is primarily mediated by CYP3A4, although other cytochrome P450 (CYP) isoenzymes may make minor contributions32. The main site of metabolism is the liver. The primary metabolite of fentanyl is the norfentanyl form, a nontoxic and inactive piperidine N-dealkylated compound. Other minor metabolites (less than 1%) identified include despropionylfentanyl, hydroxyfentanyl, and hydroxynorfetanyl. All fentanyl metabolites have negligible pharmacological activity.

Our findings have illustrated that typical detection concentrations for fentanyl derivatives are in the low nanogram range, necessitating highly sensitive detection techniques. Fentanyls and their derivatives can be detected using immunoassays, although this technology is limited by inability to detect novel opioids or distinguish between fentanyl derivatives. LFAs are the fastest and least expensive option, however, they are not as sensitive as other immunoassays. The high throughput homogeneous enzyme immunoassay (HEIA) developed by Wang et al. in comparison, offers much greater sensitivity[21].

Certain fentanyl derivatives, such as methoxyacetylfentanyl and cyclopropylfentanyl (Table 1), are present in such low concentrations that they lie outside the scope of routine drug testing. These analogues can only be detected by extremely sensitive techniques, such as that developed by Fogarty et al. using LC-tandem mass spectrometry. This technique has allowed the pharmacological effects found in case reports to be associated with quantitative values found in postmortem specimens, enabling much more effective investigation into methoxyacetylfentanyl and cyclopropylfentanyl overdose[24]. This new technique, similar to the HEIA devised by Wang et al., illustrates how current detection technologies can be enhanced to

yield more data on potent fentanyl derivatives. Further research is needed to develop similar techniques for the analysis of other elusive fentanyl analogues, such as benzodioxolefentanyl and 3-fluorofentanyl, which remain largely uninvestigated, as seen in Table 1.

LC-EI-MS is another promising testing alternative, combining the advantages of LC-MS with EI and GC-MS to offer a rapid and highly sensitive detection technique. However, the real challenge of combating the ongoing fentanyl crisis lies in the detection and prediction of unknown compounds. LC high resolution mass spectrometry (LC-HRMS) quadrupole time-of-flight or orbitrap technology offers several benefits in the detection of fentanyl, including tentative identification of compounds without library searching, and the untargeted acquisition of data that can be applied to analysis of new synthetic substances and the elucidation of their metabolic pathways. However, this technology is not readily available in most clinical laboratories.

Machine learning models are among the most promising new techniques in the prediction or classification of unknown samples as fentanyl analogues, alongside their current role of enhancing the accuracy of overdose analysis. Machine learning involves extracting patterns from mass spectra of known fentanyl analogues to assist in the prediction of unknown fentanyl derivatives[38]. Traditional detection of fentanyl via library matching is greatly enhanced through such models, and machine learning random forest models offer even more significant improvement. Such models have recently been applied to fentanyl analogue detection using sensing technologies such as infrared spectra and surface-enhanced Raman spectra. However, Koshute et al. have been the first to apply machine learning to general fentanyl analogue detection using mass spectra, a more prominent technology in forensic toxicology laboratory analyses[33]. This study has illustrated that random forest models can achieve 99% probability of detection (PD) and 1% probability of false alarm (PFA) against unseen spectra, offering a hugely significant improvement over standard detection techniques such as library matching. Further validation of the model developed by Koshute et al. would be greatly beneficial, potentially through the evaluation of earlier models upon analogues that have been assigned a later date of emergence. Further effort is also needed to adapt the approach to time series of mass spectra, a highly prominent technique in forensic toxicology. These enhancements have the potential to dramatically improve the accuracy of fentanyl detection analysis and are well worth investigating.

Conclusion

We reviewed the current literature available as of April 2023 on fentanyl derivative toxicology. Our findings indicate that fentanyl analogues are currently most often detected in urine and blood, as well as hair and saliva. Fentanyl is extremely potent, minute quantities can elicit powerful effects. This property often leads to challenges in fentanyl detection, as many analogues are present in concentrations that fall outside the range of routine testing. Highly sensitive testing methodologies are needed to perform accurate analogue analysis. The high throughput HEIA developed by Wang et al. offers much higher sensitivity than standard LFAs used for fentanyl detection. However, immunoassay techniques in general are severely limited in sensitivity. LC-MS is a far more sensitive option and has recently been used in conjunction with electrospray ionisation to further enhance fentanyl detection in forensic toxicology laboratories. Other techniques such as TD-DART-MS and IMS are more suited to use on the field in comparison to LC-MS.

The "designer" aspect of fentanyl and its analogues presents another challenge. Testing is necessitated for myriad structures, some of which may be novel, unknown, or yet to be reported in the literature. Machine learning has the potential to provide extremely useful insight into such analogues. The machine learning models described by Koshute et al. have potential application in the prediction of structure and spectra of fentanyl analogues that have not yet been observed but could potentially be synthesised. This enhancement of their model would be highly valuable in combating possible future threats. However, further work is needed on the reliable prediction of mass spectra from chemical structures before this can be achieved. ◄

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Declarations

This article was anonymised following submission and subsequently reviewed and accepted by an independent team of editors and peer reviewers as per the TSMJ's peer review and article acceptance protocol. The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Key for Analytical Techniques

LC	Liquid Chromatography
GC	Gas Chromatography
MS	Mass Spectometry
LC-MS-MS	Liquid Chromatography Tandem Mass Spectrometry
LC-HRMS	Liquid Chromatography with High-Resolution Mass Spectrometry
LC-QTOF-HRMS	Liquid Chromatography Quadrupole Time of Flight with High-Resolution Mass Spectrometry
UHPLC-QTOF-MS	Ultra-High Performance Liquid Chromatography Quadrupole Time of Flight Mass Spectrometry
HPLC-TOF	High Performance Liquid Chromatography Time-Of-Flight
HPLC-DAD	High Performance Liquid Chromatography with Diode-Array Detection
GC-MS	Gas Chromatography – Mass Spectrometry
GC-MS-IR	Gas Chromatography - Mass Spectrometry - Infrared Spectroscopy
GC-FID	Gas Chromatography - Flame Ionization Detection
FTIR	Fourier Transform Infrared Spectroscopy
FTIR-ATR	Fourier Transform Infrared Spectroscopy Attenuated Total Reflectance
NMR	Nuclear Magnetic Resonance
ELISA	Enzyme-Linked Immunosorbent Assay
LOD	Limit of Detection
LOQ	Limit of Quantification
B	Blood
U	Urine
S	Saliva