DEVELOPMENT AND APPLICATION OF ANALYTICAL METHODS FOR FENTANYL ANALOGS IN TRADITIONAL AND ALTERNATIVE MATRICES

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by

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DEVELOPMENT AND APPLICATION OF ANALYTICAL METHODS FOR FENTANYL ANALOGS IN TRADITIONAL AND ALTERNATIVE MATRICES

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DEDICATION

"The answer isn't where you are, it's where you've been."

Breaking Ground – State Champs

I dedicate this work to my grandparents, John and Sharon Palmquist. Your passion for education is infectious. Since the beginning, you have always been intrigued by my studies and endeavors. Your persistent curiosity and enthusiasm mean more than words can describe. Thank you for years of encouragement, support, and motivation.

ABSTRACT

Palmquist, Kaitlyn Bethany, *Development and application of analytical methods for fentanyl analogs in traditional and alternative matrices*. Doctor of Philosophy (Forensic Science), August, 2021, Sam Houston State University, Huntsville, Texas.

Over the last decade, the United States has experienced increasing numbers of overdose deaths attributed to opioid abuse. While the opioid crisis originated with prescription opioids, recent impacts are credited to synthetic novel psychoactive substances (NPS). Synthetic opioids, a category of NPS, can be divided into two categories: fentanyl- and non-fentanyl derivatives. Fentanyl analogs, the focus of this research, not only pose a significant threat to public safety, but also challenges to forensic laboratories due to their high potency, low concentrations, similar molecular structures, and progressive prevalence. To address detection issues faced by forensic toxicologists, it is necessary to develop highly sensitive analytical methods for detecting fentanyl analogs in traditional and alternative biological matrices.

The goals of this study were to 1) develop and validate a data-independent screening method for fentanyl analogs in whole blood and oral fluid for application to postmortem specimens and antemortem oral fluid collected from detainees, respectively; 2) develop and validate a quantitative method for furanyl fentanyl and its metabolites in human and rat plasma for future pharmacological assessment; 3) develop and validate a quantitative method for prevalent fentanyl analogs in whole blood and perform a long-term stability study; and 4) develop and validate a quantitative method for fentanyl analogs in oral fluid for application to antemortem oral fluid samples collected from probationers/parolees.

A data-independent screening method was developed and validated for fentanyl analogs (n=14) in whole blood and oral fluid using liquid chromatography-quadrupole-time-of-flight-mass spectrometry (LC-QTOF-MS). Data were acquired in time of flight (TOF) and All Ions fragmentation (AIF) modes and low limits of detection were achieved. A personal compound database and library (PCDL) was developed for targeted and exogenous compound identification. Postmortem blood samples (n=30) received from National Medical Services (NMS) Labs and oral fluid samples (n=20) collected from detainees in Texas detention centers were screened for fentanyl analogs. In the blood samples, analogs of furanylfentanyl (n=16), 4-ANPP (n=16), cis-3-methylfentanyl (n=4), fentanyl (n=4), norfentanyl (n=2), and valerylfentanyl (n=1) were detected. No fentanyl analogs were detected in the oral fluid samples.

A quantitative method was developed and validated for furanylfentanyl and its metabolites (4-ANPP and furanyl norfentanyl) in human plasma by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using American National Standards Institute/American Standards Board (ANSI/ASB) Standard 036: Standard Practices for Method Validation in Forensic Toxicology. Low limits of detection and small sample volumes (100 μL) were achieved. The method was cross validated in rat plasma for potential application to a pre-clinical pharmacodynamic/pharmacokinetic (PD/PK) study.

A method was developed and validated for the quantification of prevalent fentanyl analogs (n=13) in blood using targeted data acquisition on an LC-QTOF-MS. The method was validated according to ANSI/ASB Standard 036. The method was applied to a long-term stability study assessing fentanyl analog degradation over 9

months at four temperature conditions (-20ºC, 4ºC, 25ºC, and 35ºC). Results described minimal instability under room temperature and refrigerated storage, degradation after 4 freeze/thaw cycles, and instability after 1 week of elevated exposure. Acrylfentanyl had a high degree of instability under most temperature conditions and breakdown mechanism remains undetermined. Authentic forensic blood specimens stored under refrigeration were analyzed 6 months apart to assess stability in postmortem samples. Furanylfentanyl (n=4) and 4-ANPP (n=7) were quantifiable and exhibited percent loss of 0.2-26.8% and 16.3-37.4%, respectively. Loss was attributed to sample source, age, and composition.

The aforementioned data acquisition was utilized to develop and validate a quantification method for fentanyl analogs (n=13) in oral fluid using LC-QTOF-MS by ANSI/ASB Standard 036. The method was applied to authentic oral fluid samples (n=16) received from Redwood Toxicology obtained from probationers/parolees. Oral fluid samples were positive for fentanyl (n=16) and 4-ANPP (n=3) at concentrations of 1.0-104.5 ng/mL and 1.2-5.7 ng/mL, respectively. No fentanyl analogs were detected.

The present work describes sensitive analytical methods for the detection and quantification of fentanyl analogs with proven applicability to forensically relevant samples. In addition, challenges associated with analyte detection, compound differentiation, and drug instability have been addressed. With the constant emergence of novel fentanyl analogs, forensic toxicologists must be proactive with advancement of analytical analyses and sample treatment.

KEY WORDS: Novel synthetic opioids; Fentanyl analogs; LC-QTOF-MS; Blood; Oral fluid

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CHAPTER I

Introduction

Evolution of the Opioid Crisis

In 2017, the United States Department of Health and Human Services declared a public health emergency in response to increasing numbers of opioid deaths coined "the opioid crisis." (1). While the severity of the crisis was seemingly drastic, opioid abuse trends evolved systematically over time. Foundations for opioid abuse began in the late 1990s with the intense promotion of OxyContin® by Perdue Pharma attached to claims disregarding true addiction potential (2). As a result, doctors increased prescription opioid distribution for pain management and parties involved in the opioid market began to prosper (2). Although effective at treating pain, opioids began to cause patients to experience higher rates of addiction, abuse, and overdose deaths (2-4). From 1999-2018, a gradual increase in prescription opioid-related deaths was observed with methadone, oxycodone, and hydrocodone being the most prevalent (5). In addition, the Centers for Disease Control and Prevention (CDC) report more than 232,000 overdose deaths attributed to prescription opioids during that time (6).

Prescription opioid abuse gradually developed into a national issue through increased dispersal of prescriptions at pain management facilities. Once recognized, federal agencies, law enforcement, and medical organizations began implementing stricter regulations and guidelines concerning opioid prescription and circulation (7). With decreased access to prescription opioids, many users began to transition to illicit compounds. Heroin (diacetylmorphine), a semi-synthetic opioid derived from *Papaver somniferum*, is one such compound (8, 9). In a retrospective study conducted by Cicero *et*

al., it was reported that participating heroin users (beginning abuse after 1990) initiated abuse with prescription medications (10). However, participants preferred heroin due to the associated level of "high," ease of use and acquirement, and affordability (10). Dangers associated with heroin abuse include poly-drug use, detrimental physiological effects of adulterants, and increased risk for contracting bacterial infections or bloodborne pathogens (HIV, Hepatitis B, Hepatitis C) (8, 9). In 2010, an increase in the number of overdose deaths involving heroin was observed and persisted until 2015 (5). By 2018, there were approximately 5 times more heroin overdoses than 2010 (8); however, death rates decreased 4.1% between 2017 and 2018 (11).

In 2013, the introduction of synthetic opioids to the illicit drug market began to significantly impact overdose death rates. Synthetic opioids can be fentanyl or non-fentanyl derivatives. Gladden *et al.*, reported a direct correlation between increasing evidential submissions containing illicitly manufactured fentanyl (426%) and synthetic opioid deaths (71%) in the US from 2013-2014 (12). In 2015, the National Forensic Laboratory Information System (NFLIS) described 14,440 reports of seized fentanyl in the US with over 75% from northeastern or midwestern regions (13). In that same year, 2,631 reports of fentanyl analogs were observed (14). Over the next year, reports of seized fentanyl and fentanyl analogs increased to 34,199 and 6,037, respectively, suggesting rapid dissemination in the illicit drug market (14). In a study by O'Donnell *et al.*, fentanyl and fentanyl analogs were detected in 56.3 and 14.0% of cases reported by 10 states, respectively, in the second half of 2016 (15). Of the cases positive for fentanyl or fentanyl analogs, cause of death was attributed to the drug in 97.1 and 93.4%, respectively (15). U-47700, a non-fentanyl derivative, was also observed in 0.8% of cases

(15). Colon-Berezin *et al.*, describes the development of overdose deaths in New York City from 2000-2014, 2016, and 2017 with fentanyl involvement in 2, 44, and 57%, respectively (16). Overdose death rates rose 81% in NYC from 2014-2017, which can be attributed to the rise in synthetic opioid abuse (16).

Progressing to 2018, the CDC reported more than 31,000 overdose deaths attributed to synthetic opioids in the US, accounting for 67% of all opioid-related deaths during that year (17). NFLIS 2018 and 2019 Annual Reports ranked fentanyl as the top narcotic analgesic seized by submitting laboratories with 83,765 and 98,954 reports, respectively (18, 19). While fentanyl analog seizures were not uncommon, specific analog prevalence was varied (18, 19). In 2019, the CDC reported approximately 71,000 overdose deaths in the US, with over 70% occurring due to prescription or synthetic opioids (20). While synthetic opioids have proliferated the opioid crisis, it is apparent that traditional, prescription or semi-synthetic opioids continue to contribute to the leading cause of death of Americans.

In response to the opioid crisis, the US Department of Health and Human Services developed a 5-point strategy to address the pitfalls of the opioid epidemic. As part of this program, resources are provided to improve aspects of addiction (prevention, treatment, recovery), data, pain management, overdose reversing drugs, and research (21). In addition, the CDC presents five strategies for preventing opioid harm. These strategies consist of collecting surveillance and research data, building state, local, and tribal communities, supporting the health system and associated parties, collaborating with law enforcement, and providing outreach and awareness to public consumers (20). While

substantial financial and preventative support have been provided, opioids continue to persist and combatting the ever-evolving crisis remains difficult.

Fentanyl

Dr. Paul Janssen of Janssen Pharmaceutica (Beerse, Belgium) sought to synthesize effective and efficient analgesic medications for pain management, which led to the creation of fentanyl (N-phenyl-N-[1-(2-phenethyl)-4-piperidinyl] propanamide) in 1960 (22, 23). At the time, fentanyl was the strongest available opioid with a potency 50-100 times that of morphine and the fastest onset of action with intravenous administration (22, 24). In addition, fentanyl had a therapeutic index surpassing that of morphine and meperidine by approximately 4 and 50 times, respectively (22, 23). Flourishing in Europe, fentanyl was not as welcomed in the United States due to strenuous approval processes by the Food and Drug Administration (FDA). In 1968, Innovar, a 50:1 formulation of droperidol to fentanyl, was approved for clinical use (22). Fentanyl was then individualized and commercialized in the 1970s for intravenous anesthetic use under the name Sublimaze® (24, 25). With the development of different drug delivery methods, a transdermal patch (Duragesic®), transmucosal lollipops (Oralet and Actiq®), and additional buccal, nasal, and sublingual products were approved as administration methods for medicinal fentanyl (22, 25).

Fentanyl is predominantly used as a surgical anesthetic or chronic pain treatment for opioid tolerant patients. With limited medical use and a high potential for abuse, fentanyl was classified as schedule II under the United States Controlled Substances Act (CSA) (26). Effects associated with use include analgesia, sedation, nausea, bradycardia, hypotension, euphoria, constipation, and respiratory depression (22, 23, 27). Although the

effects of fentanyl are similar to those of traditional (natural and semi-synthetic) opioids, its molecular structure is significantly different. Figure 1.1 displays the structural characterization of fentanyl consisting of four moieties: propionyl, phenyl ring, phenethyl, and piperidinyl positions. Fentanyl analogs are derived from different substituent groups located at or on these moieties.

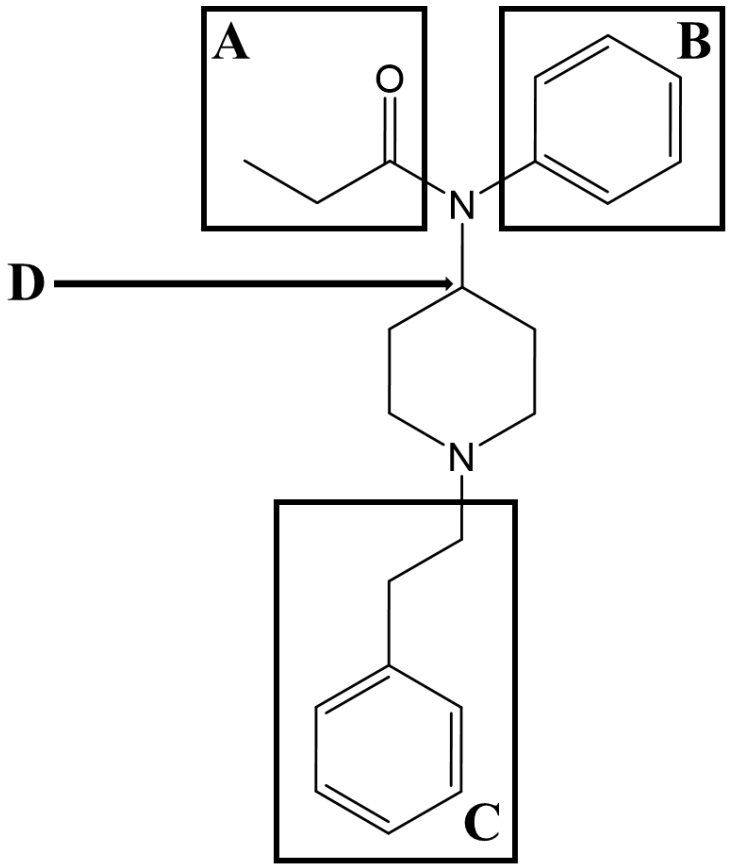

Figure 1.1. Molecular structure of fentanyl with highlighted moieties (A) propionyl, (B) phenyl ring, (C) phenethyl group, and (D) piperidinyl position

Pharmacology

Opioids produce therapeutic and side effects through binding to G protein-coupled receptors known as mu, kappa, and delta (μ, κ, δ) opioid receptors. The μ-receptor is primarily responsible for therapeutic effects of analgesia and sedation, and side effects of respiratory depression, euphoria, constipation, pruritus, bradycardia, and physical dependence (22, 28). Fentanyl binds favorably to the μ-receptor as a full agonist, which not only inhibits the transmission of painful stimuli, but also causes significant respiratory depression. In a study by Volpe *et al.*, the binding affinity of fentanyl was suggested to vary in previously published literature due to variations in experimental design; therefore, cell membrane expressing human recombinant mu-opioid receptor (MOR) was utilized to determine a binding affinity value of 1.346 nM (29). While this is comparable to morphine, fentanyl produces greater opioid effects due to its high lipophilicity for rapid transmission across the blood brain barrier and potency (50-100 times morphine) (29).

The lipophilic nature of fentanyl also allows for transdermal drug delivery. Duragesic® reports an n-octanol:water partition coefficient of 860:1 (27). With high lipid solubility, fentanyl diffuses through the lipids present in the epidermis of the skin. Once in the keratinaceous layer of the epidermis, a fentanyl depot is formed. From this depot, fentanyl releases and slowly passes through the hydrophilic dermis (27, 30). Peak serum concentrations occur 24-72h after initial application and reach steady state after repeated patch applications (application period: 72h) (27). Duragesic® demonstrated mean time to maximum concentration (Tmax) and mean maximum concentrations (Cmax) ranges of 27.5-38.1h and 0.3-2.5 ng/mL, respectively, following 12-100 mcg/h doses (27). Half-life

(t1/2) after patch removal was 17h (27). Variables affecting transdermal absorption of fentanyl include skin thickness, skin condition, and temperature (30).

Intravenous administration of fentanyl is commonly used by medical professionals and illicit drug users. Medicinal dose recommendations for low, moderate, and high dose anesthetic procedures are 2, 2-20, and 20-50 mcg/kg, respectively (31). Pre- and post-operative pain management doses of 50-100 mcg/kg are recommended to be administered intramuscularly (31). Unlike the transdermal route, intravenous administration is characterized by a shorter duration of action and half-life ranging from 2-4h (30, 31) or 3-12h (32), respectively. In addition, peak effect is observed 5-15 mins after injection (33). Fentanyl is recognized to bind to plasma proteins (~80%) (34, 35) and rapidly redistributes into surrounding fats and tissues (31, 36). The recorded volume of distribution for fentanyl is 3-8 L/kg (31, 32). Mean effective dose 50 (ED50) for fentanyl citrate injection is reported as 0.08 mg/kg (33).

Metabolism

Fentanyl metabolism occurs in the liver and is primarily driven by cytochrome P450 CYP3A4 (37). Major metabolic pathways and resulting metabolites are described in Figure 1.2. Production of norfentanyl, an inactive metabolite, through *N*-dealkylation at the piperidine moiety is the predominant metabolic pathway in humans (37). Additional metabolites of despropionylfentanyl, hydroxyfentanyl, and hydroxynorfentanyl are produced in lower abundance (32). Recently, studies by Kanamori *et al.* described additional hydroxylated metabolites using hepatocytes from a liver-humanized mouse and human-induced pluripotent stem cells (38, 39). The predicted metabolites were also elucidated in a study by Wallgren *et al.*, examining cryopreserved hepatocytes and urine

(40). Fentanyl is predominantly eliminated in the urine (85%) with 0.4-6% as unchanged drug and 26-55% as norfentanyl (32). Norfentanyl has been detected and quantified in various biological matrices (blood, liver, vitreous, plasma, urine, kidney, bile, stomach contents, oral fluid) in clinical and forensic applications (41-49).

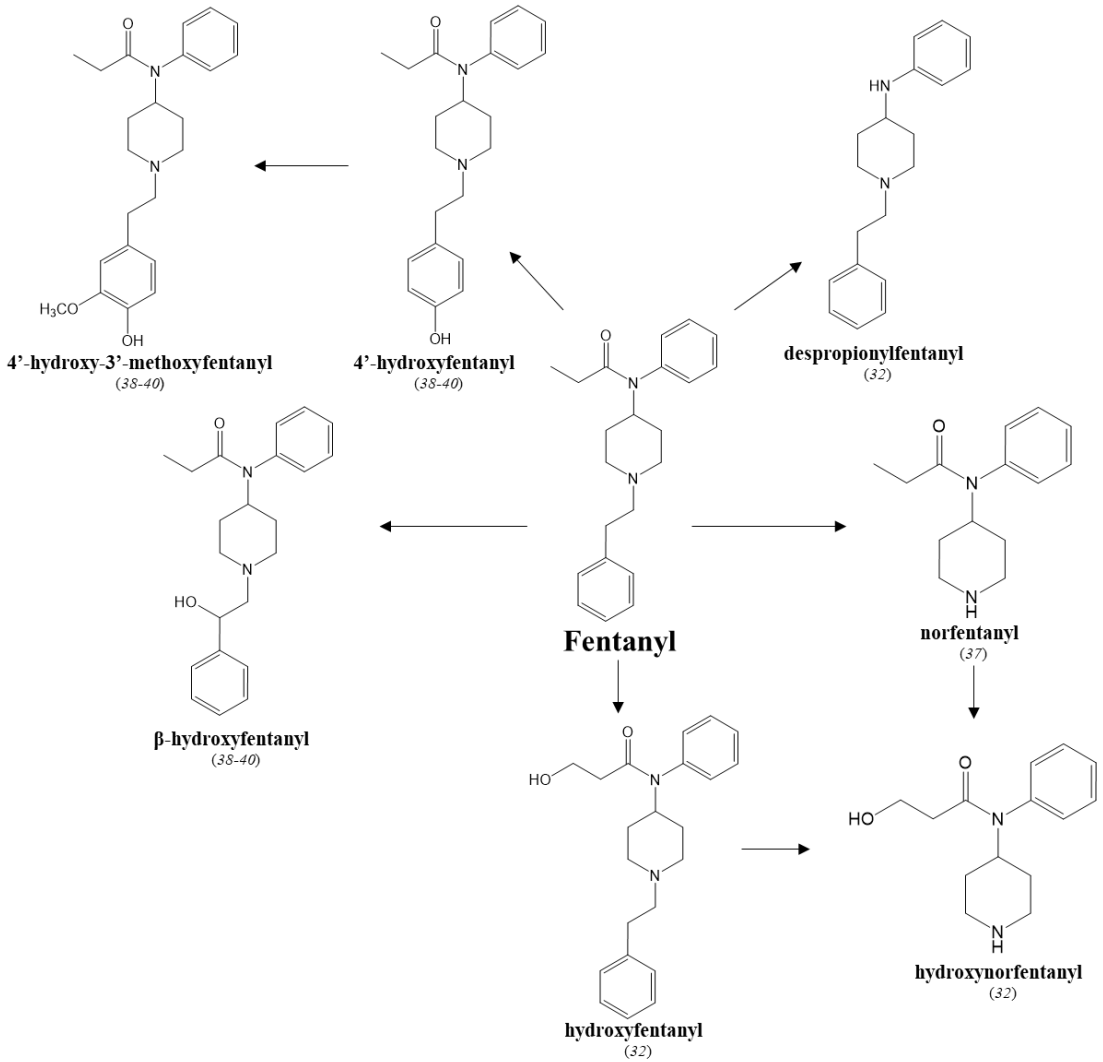

Figure 1.2. Major metabolic pathways of fentanyl as described in the literature (associated references are indicated in parentheses).

Illicit prevalence and distribution

Fentanyl seizures remained steady from 2001-2005 and 2007-2013 (13). In 2006, an isolated fentanyl outbreak occurred through clandestine productions by a single laboratory in Toluca, Mexico, which was abolished with seizure of the laboratory (25). Beginning in 2014, dramatic increases of fentanyl reports were observed (13). Reports of illicitly manufactured fentanyl increased 5-fold from 2013 (978 reports) to 2014 (4,697 reports) and 10-fold from 2014 to 2015 (14,400 reports) (13). Cities reporting the highest number of fentanyl seizures were Cincinnati, OH, Augusta, ME, Pittsburgh, PA, Baltimore, MD, and South Charleston, WV accounting for almost 16% of total seizures in 2015 (13). Fentanyl reports continued to increase in 2016, 2017, 2018, and 2019 with totals of 34,199, 56,530, 83,765, and 98,954, respectively (14, 18, 19, 50). Figure 1.3 displays the increase in illicit fentanyl seizures reported to NFLIS from 2013-2018. Through examination of the available data, fentanyl seizures increased 100-fold over a 7-year period (2013-2019).

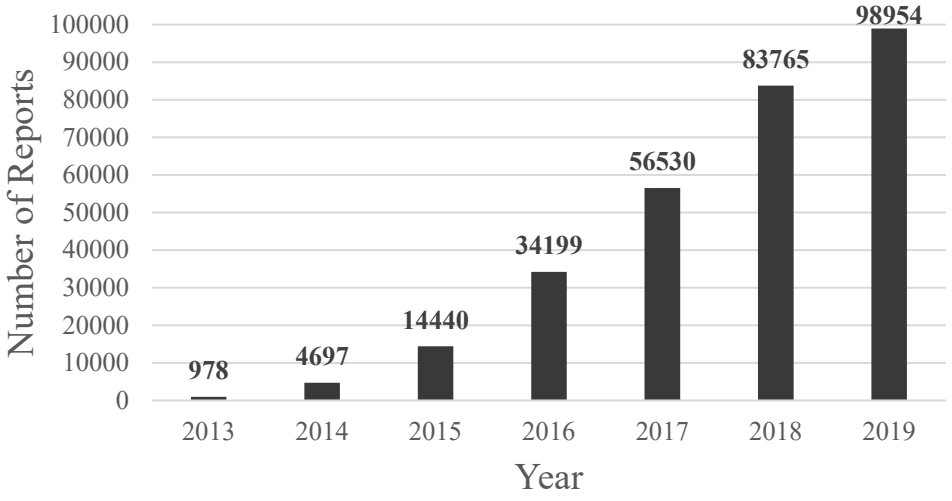

Figure 1.3. Illicit fentanyl seizures reported to NFLIS from 2013-2018 (13, 14, 18, 19, 50)

According to an Intelligence Brief prepared by the Drug Enforcement Administration (DEA), fentanyl sources originated in China and were transported to the United States directly or through Mexico and Canada (51). For the clandestine chemist, fentanyl and precursor compounds were readily and affordably available on the internet as "research chemicals," as well as manufacturing products like pill presses and molds (25). Fentanyl was traditionally trafficked through mixing and distributing with heroin supply; however, with the rising epidemic, clandestine laboratories began producing counterfeit pills laced with fentanyl (51). Often, counterfeit pills can appear as one drug, but contain a different compound entirely. The ambiguity creates danger for naïve drug users unaware of what drugs they are acquiring and abusing. Ultimately, distribution of fentanyl-laced drugs of abuse has directly impacted the increasing number of overdose deaths occurring around the world and in the US.

Fentanyl Analogs

After synthesizing fentanyl in 1960, the Janssen Company continued to develop the molecular structure of fentanyl to produce analogs intended for medical or veterinary use (22, 25). Pharmaceutical analogs developed and utilized prior to 1980 were alfentanil (Alfenta®, Rapifen®), carfentanil (Wildnil), remifentanil (Ultiva®), and sufentanil (Sufenta®, Zalviso™) (32). While initial abuse of pharmaceutical analogs remained low, illicitly manufactured fentanyl analogs began to appear in 1979. In California, a drug known as "China White" infiltrated the illicit community as synthetic heroin. However, no heroin or other known drugs were detectable. After extensive analysis using IR, GC-MS, and HNMR, the compound was determined to be α-methylfentanyl (52). In 1984, 3-methylfentanyl, another fentanyl analog, was responsible for overdose deaths occurring

in Pennsylvania (25). During that same period, approximately 10 additional analogs were associated with overdose deaths, predominantly in California (25, 53).

Since the 1980s, the number of different fentanyl analogs detected in seized drug and forensic casework has increased drastically. As analogs began to appear and cause public health concern, the United States regulated through scheduling (25). However, this did not stop the influx of fentanyl analog occurrences. In Sweden, through monitoring fentanyl analog abuse from 2015-2017 (as part of the STRIDA project) (54, 55), Helander *et al.* discusses observations of replacement drug trends occurring as a result of banishment of existing compounds (54). This drug trend can be compared to the evolution of fentanyl analog abuse in the United States. In an attempt to combat fentanyl analog abuse in the United States, the Drug Enforcement Administration directed a temporary emergency scheduling of all fentanyl-related substances into Schedule I (56). While some early fentanyl analogs were previously scheduled, the emergency order included all isomers, esters, ethers, and salts of the core fentanyl structure. Individual analog scheduling was ineffective at controlling the emergence of novel fentanyl analogs; therefore, a blanket schedule was implemented with the goal of regulating novel substances and protecting the public from associated hazard (56). The temporary schedule was issued from February 2018-2020 (56) and was later extended through May 2021 (57). In a report by Comer *et al.*, challenges associated with blanket scheduling by structure are discussed. Issues include limited knowledge of pharmacological activity and impeding development of alterative analgesics, vaccines, or antibodies against fentanyl analog activity (58).

As previously mentioned, fentanyl analogs are synthetic opioids containing substituent variations on the fentanyl backbone. Nineteen fentanyl analogs were studied in the presented research. Figures 1.4 and 1.5 display the chemical structure of the selected fentanyl analogs (n=19) grouped by moiety alteration location. Chemical properties such as pKa and potency (relative to morphine) are included in the figures.

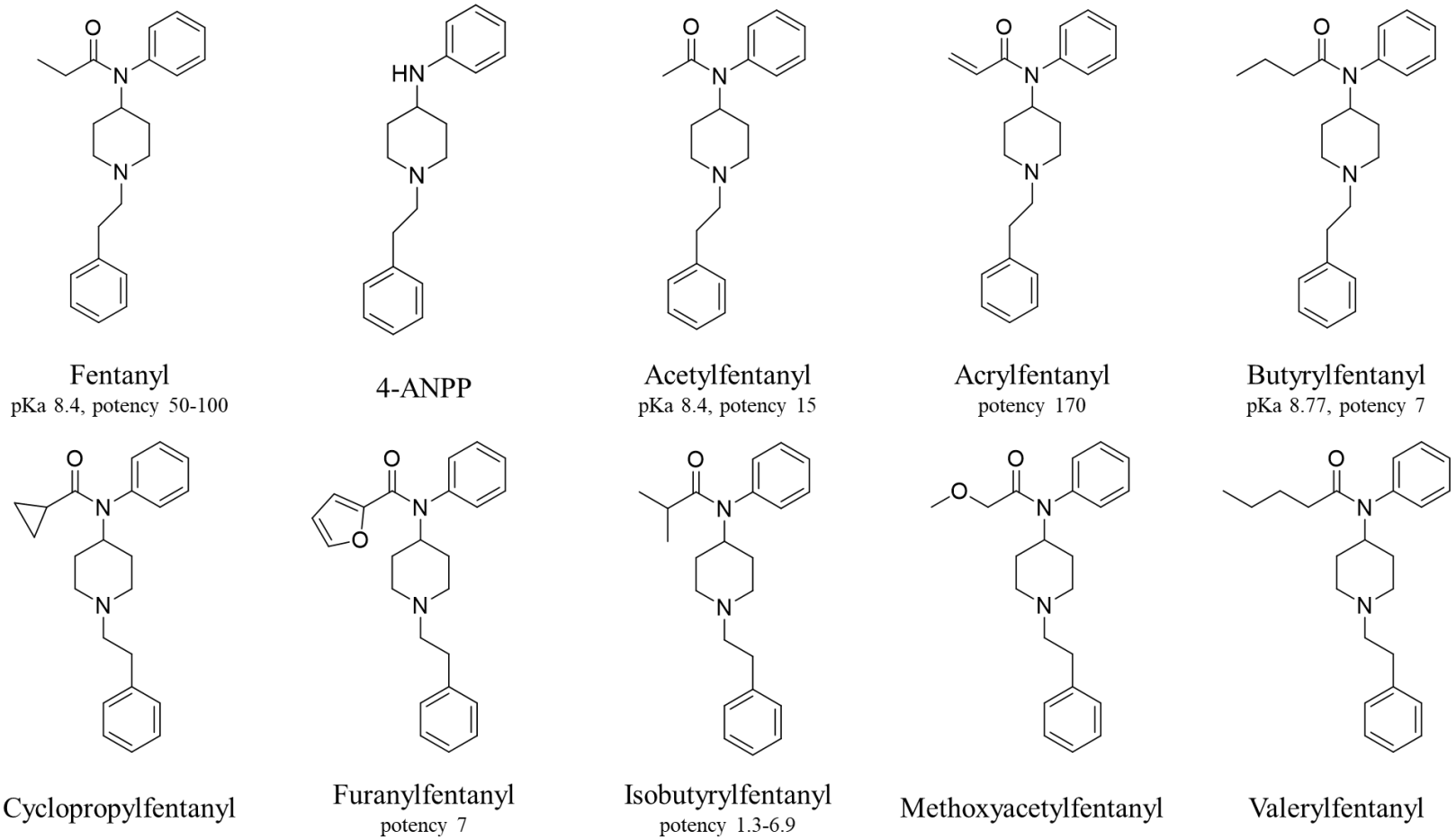

Figure 1.4. Propionyl substituted fentanyl analogs (n=10) investigated in the present study

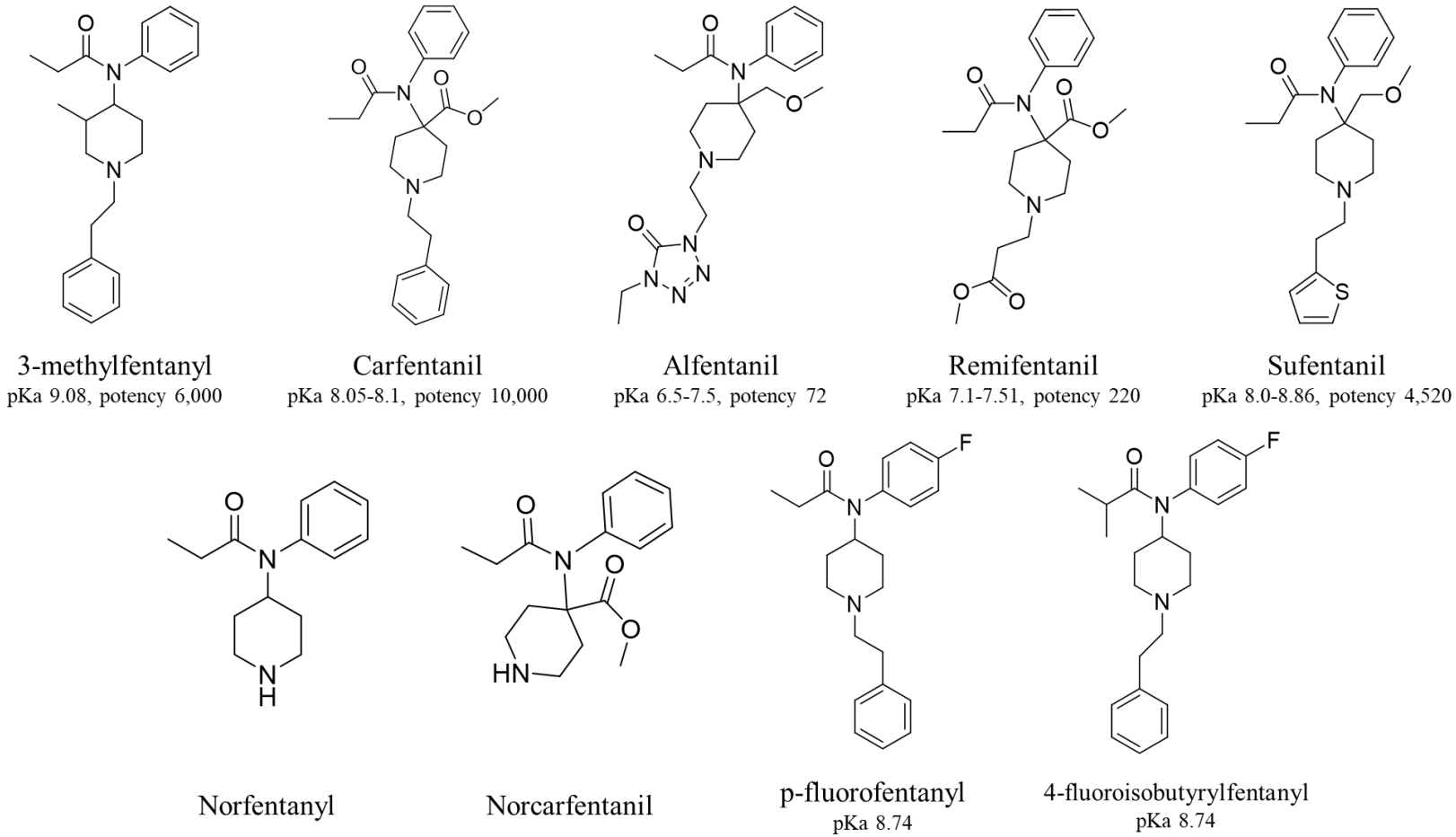

Figure 1.5. Piperidinyl (n=5), phenethyl (n=2), and phenyl (n=2) substituted fentanyl analogs investigated in the present study

Pharmacology and metabolism

Limited pharmacological information is available for fentanyl analogs due to high potencies and safety risks to humans. To date, pharmacokinetic and pharmacodynamic information for novel synthetic opioids (NSOs) has been derived from animal models using rodents or guinea pigs (59-64). In reviews by Conchiero *et al.* and Suzuki *et al.*, fentanyl analog potencies (relative to morphine) were compiled and ranged from 1.3-10,000 and 1.5-10,000, respectively (24, 65). A review by Wilde *et al.* lists fentanyl analog potencies (relative to fentanyl) from 0.3-100 (66). While potencies have been shown to vary, all fentanyl analogs are alkaline compounds and highly lipophilic. Reported pKa and log P values for some fentanyl analogs range from 7.5- 9.08 and 2.8-4.3, respectively (65). Like fentanyl, fentanyl analogs readily cross the blood brain barrier to predominantly act on μ-opioid receptors; therefore, resulting in similar side effects.

Due to the rapid procurement of different fentanyl analogs, recent research has focused on the identification and understanding of metabolic pathways. For forensic laboratories, identification of active or inactive metabolites can be critically important when assessing human performance or cause of death, and detection of relevant biomarkers. In general, fentanyl analogs are primarily metabolized by CYP450 enzymes and the production of a nor- metabolite through *N*-dealkylation is frequent (66). That said, various fentanyl analogs have been shown to have unique metabolic pathways increasing potential detection abilities for laboratories.

In a study conducted by Watanabe *et al.*, metabolic pathways of acetylfentanyl, acrylfentanyl, 4-fluoro-isobutyrylfentanyl (4-FIBF), and furanylfentanyl were determined through human hepatocyte incubations and authentic urine profiling. As a

result, 32, 14, 17, and 14 metabolites were identified for acetylfentanyl, acrylfentanyl, 4-FIBF and furanylfentanyl, respectively (67). For acetylfentanyl, abundant metabolites were acetyl norfentanyl (*N*-dealkylation), a monohydroxylated metabolite, and a dihydrol metabolite in hepatocytes. In urine samples, parent compound was detected with the greatest abundance (67). Melent'ev *et al.* also performed structural elucidation of acetylfentanyl metabolites in urine samples. Hydroxylation on the phenylethyl moiety was described as the main metabolic pathway (68). Similar metabolic pathways were described in studies by Kanamori *et al.* examining acetylfentanyl metabolism in pluripotent stem cell-derived hepatocytes and liver-humanized mouse hepatocytes (38, 39). Hydroxyacetyl-fentanyl was described as a novel, minor metabolite in both studies (38, 39) and the involvement of CYP2D6 was suggested (39).

Similar to acetylfentanyl, incubations of acrylfentanyl produced abundant acryl norfentanyl, mono- and di- hydroxylated metabolites in the Watanabe *et al.* study. Parent compound was present in greater abundance than metabolites in urine specimens (67). In urine and hepatocyte samples, 4-FIBF was the first or second most abundant compounds to its nor-metabolite formed by *N*-dealkylation (67). Furanylfentanyl produced 4-ANPP (amide hydrolysis) as its major metabolite followed by its nor- and dihydrol- metabolites (67). These findings are consistent with a study by Goggin *et al.*, which analyzed authentic urine specimens for furanylfentanyl and furanyl norfentanyl and investigated phase II metabolic pathways by performing glucuronide and sulfatase incubations. Metabolites identified were a dihydrol- metabolite, 4-ANPP, and a sulfate metabolite. No glucuronide conjugates were identified (69). Metabolite profiling of methoxyacetylfentanyl was performed by Mardal *et al.*, through incubation with pooled

human hepatocytes. In total, 10 metabolites were identified with major metabolites being *O*-demethyl- (*O*-demethylation) and deamide- (deamination) products (70).

Prior to burytylfentanyl metabolism studies, predicted phase I metabolites of carboxybutyrylfentanyl, hydroxybutyrylfentanyl, desbutrylfentanyl, and norbutyrylfentanyl were detected in several postmortem specimens (71). In a study by Steuer *et al.*, 36 metabolites were identified for butyrylfentanyl when analyzed with human liver microsomes and recombinant CYP450 enzymes (72). For 3-methylfentanyl, 9 phase I and 5 phase II metabolites were identified in rat urine in a study by Meyer *et al.* (73). Similar to previously discussed analogs, *N*-dealkylation and hydroxylation were determined to be major pathways for butyrylfentanyl (72) and 3-methylfentanyl metabolism (73).

Pharmaceutical analogs

Alfentanil, sufentanil, remifentanil, and carfentanil are fentanyl analogs with approved medicinal or veterinary uses. Due to the pharmaceutical nature of these compounds, pharmacological information is available. While illicit use of alfentanil, sufentanil and remifentanil is minimal, carfentanil has been highly abused as part of the opioid crisis; therefore, pharmacological information is crucial.

In a study by Janssens *et al.*, a tail withdrawal reflex (TWR) test was used to determine pharmacokinetic properties of alfentanil in rats. Alfentanil was determined to have a faster action (1 min after administration) and shorter duration of action (3 times) compared to fentanyl (74). Potency was determined to be 72 times that of morphine (74). In humans, alfentanil is administered intravenously in combination with nitrous oxide at dosages ranging from 3-245 μg/kg and 0.5-3 μg/kg/min for induction and maintenance

procedures, respectively (32, 75). Alfentanil has also been administered epidurally and intranasally in children (32). Elimination half-lives are reported as 1-2 h. (32, 75). Alfentanil is metabolized to inactive metabolites by *N*-dealkylation, *O*-dealkylation, ring hydroxylation, and amide hydrolysis and conjugation (32). Noralfentanil is the predominant urinary metabolite and accounts for 31% of dose (32).

Sufentanil has historically been administered as a primary or secondary anesthetic or postoperative analgesic. Intravenous doses range from 1-30 μg/kg to achieve varying levels of general anesthesia and pain management (32). Sufentanil has a potency exceeding 4,000 times the potency of morphine and a high margin of safety of 25,000 (33). Like other pharmaceutical fentanyl analogs, sufentanil has a rapid onset and duration of action. The lethal dose 50 (LD50) has been reported at 17.9 mg/kg (33). In 2018, a 30 μg sufentanil sublingual tablet (SST) was approved for pain management by the FDA, which demonstrates a new method of drug delivery. Reardon *et al.* reviews and describes SST as a promising alternative to traditional methods of treating acute pain due to effective absorption for patients who cannot take medication orally or lack access to parenteral administration methods, applicability to outpatient procedures, and fast onset of action and sustained analgesic effect (76). Primary metabolites of sufentanil are *N*-desalkylsufentanil, *O*-desmethylsufentanil, and an *O*-desmethyl conjugate (32).

Remifentanil is ultra-short acting anesthetic agent available as a hydrochloride salt that requires reconstitution before administration (32, 77). When reconstituted, the formulation includes 1 mg/mL of remifentanil base (78). Typical intravenous doses of remifentanil for anesthesia are 1μg/kg with maintenance of 0.25-1.0 μg/kg/min (32). Potency is predicted to be 20-30 times more than alfentanil (79). Vardanyan and Hruby

discuss remifentanil analgesic action as being reported strongest at 1 min, weakened after 6 min, and gone 12-15 min (33). At a pH of 7.4 (typical pH of blood), remifentanil has an octanol/water partition coefficient of 17.9, suggesting high lipophilicity (77-79). Additional pharmacological properties of remifentanil are half-life of 6-16 min (32), volume of distribution of 0.2-0.4 L/kg (32), pKa of 7.07-7.1 (32, 78, 79), and protein binding at 92% (77). Unlike other fentanyl analogs, remifentanil is rapidly metabolized via hydrolysis by esterases in the blood to form metabolite GI-90291 (remifentanil acid), a carboxylic acid metabolite (78, 79). A secondary metabolite, GI-94219 (norcarfentanil), is formed through *N*-dealkylation (79).

Carfentanil, a highly potent fentanyl analog, was introduced for veterinary use in the 1980s, specifically for immobilization of large, wild animals. Like other fentanyl analogs, carfentanil acts as a μ-receptor agonist and is basic in nature (pKa= 8.1) (32, 80) With a potency 10,000 greater than morphine, carfentanil was not intended for human use (81). However, isotopically labeled 11C-carfentanil has been used in positron emission tomography (PET) studies in humans (82). Recently, in 2018, it was announced that carfentanil was no longer FDA-approved due to the application withdrawal by Wildnil producer Wildlife Laboratories (83). Limited pharmacological information is available for carfentanil in humans; however, animal models have been developed to understand this highly potent compound. Mutlow *et al.*, published the first pharmacological investigation of carfentanil in goats with resulting Tmax and half-life values of 11 min and 5.5 h, respectively (84). Similarly, Cole *et al.*, investigated carfentanil pharmacokinetics in eland and determined peak plasma concentrations (Cmax) at 13.8 min and mean half-life of 7.7 h (85). In a more recent study conducted by Bergh *et al.*,

rats injected (subcutaneous) with carfentanil at various doses were observed to experience decreased body temperature, catalepsy (immobility, flattened posture, splayed hind limbs), and respiratory depression (59). In rats, Cmax values were observed at 15 min and half-life was determined to increase with increasing dose (59). Carfentanil clearance by the kidneys was hindered at higher doses (59). In a study by Feasel *et al.*, carfentanil metabolism was investigated in vivo using human hepatocyte incubations. As a result, twelve total metabolites were identified with two predominant metabolites generated by *N*-dealkylation (norcarfentanil) and monohydroxylation of the piperidine ring (86).

Illicit prevalence

While fentanyl analogs have existed since the late 1900s, abuse of these compounds did not proliferate until 2013. The introduction of novel synthetic opioids required fast action by crime laboratories to develop not only detection and identification methods, but also stringent safety protocols for handling seized drugs. According to a brief by NFLIS, 17,071 cases involving fentanyl or fentanyl analogs were reported in 2015 (14). Over the next year, in 2016, fentanyl-related cases increased to 40,236 reports (14). While the percentage of fentanyl reports in the total remained ~84% over the aforementioned years, the variety and number of fentanyl analog cases increased in 2016. Totals of 8 and 15 different fentanyl analogs were reported in 2015 and 2016, respectively, captured by the NFLIS brief (14). From 2015 to 2016, the number of furanylfentanyl and carfentanil reports increased from zero to 2,273 and 1,251 reports, respectively (14). Additional analogs identified in 2016 were: 3-methylfentanyl, fluoroisobutyrylfentanyl, valerylfentanyl, and acrylfentanyl (14).

In 2017, fentanyl, carfentanil, and furanylfentanyl ranked among the top 25 drugs reported to NFLIS (50). From 2016-2017, reports increased 3,550 and 500% for carfentanil and furanylfentanyl, respectively, in the north eastern region of the US (87). Further, reports of 4-fluoroisobutyrylfentanyl, acrylfentanyl, and cyclopropylfentanyl increased 10,900 (Ohio), 7,035 (Ohio), and 7,700% (Kentucky), respectively (87). Of the top narcotic analgesic reports in 2017, fentanyl analogs (fentanyl, carfentanil, furanylfentanyl, acrylfentanyl, and 4-FIBF) accounted for 71,341 reports and 40.4% percent of total narcotic drug seizures (50).

In 2018, analogs of acetylfentanyl, 4-ANPP, fluoroisobutyrylfentanyl, methoxyacetylfentanyl, and cyclopropylfentanyl were among the top seized narcotic cases (18). Reports for acetylfentanyl, 4-ANPP, FIBF, methoxyacetylfentanyl and cyclopropylfentanyl were 7,148, 2,139, 1,643, 1,057, and 1,011, respectively (18). Carfentanil and furanylfentanyl are not mentioned in the 2018 Annual NFLIS report; therefore, suggesting the difference in prevalent analogs between years. In 2018, there were 52 unique fentanyl-related compounds detected by submitting laboratories, representing the highest number between 2009-2019 (88). In 2019, prevalent analogs continued to shift slightly with acetylfentanyl, 4-ANPP, carfentanil, valerylfentanyl, and fluoroisobutyrylfentanyl being within the top 15 reported narcotic analgesics seized (19). Acetylfentanyl reports were the highest of any fentanyl analog with 12,190 accounting for 6.4% of total narcotic reports (19). Specifically, states of Massachusetts, New Jersey, Ohio, and Pennsylvania had reports greater than 1,000 (88). Additional fentanyl analog reports ranged from 400-5,700 (19). 4-ANPP was most prevalent in New Jersey with 2,713 reports and carfentanil in Ohio with over 1,000 reports (88).

At the present time, limited data are available for fentanyl analog prevalence in 2020. However, in the September 2020 issue of NFLIS Snapshot, fentanyl analogs of fentanyl, 4-ANPP, acetylfentanyl, carfentanil, and valerylfentanyl were present in 24,391 seized drug reports from July to September of that year (89). Chlorofentanyl and ocfentanil were newly reported in the Midwestern and Southern regions of the US, respectively (89). In addition, fentanyl was found in seizures containing xylazine, a veterinary prescription. Of 685 cases, 96% contained a combination of xylazine and fentanyl (89). In the December 2020 issue, fentanyl analogs of 4-ANPP, acetylfentanyl. phenethyl 4-ANPP, carfentanil, and valerylfentanyl were reported with greatest prevalence between October and December (90). Total fentanyl-related compound reports were less than the previous period with 3,336. Of those, 64.5% of those attributed to 4-ANPP detection (90).

Analytical methods

Immunoassay

Routine forensic toxicological analyses often begin with screening. Immunoassay is one screening technique employed by laboratories to presumptively identify illicit substances; however, traditional immunoassays are not amenable to synthetic opioids due to structural differences compared to morphine (25). To routinely screen for fentanyl analogs in biological samples, development of specialized immunoassay antibodies was required. In 2006, Mao *et al.* developed an enzyme-linked immunosorbent assay (ELISA) for fentanyl in horse urine and serum using carboxylfentanyl-thymoglobulin conjugate antibodies for binding, goat- anti rabbit antibody conjugated with alkaline phosphatase for detection, and iron oxide nanoparticles for isolation (91). Analogs of *p*-fluorofentanyl,

thienylfentanyl, and 3-methylthienylfentanyl exhibited cross-reactivity with the anti-fentanyl antibody (>100%), carfentanil (85%), *N*-methylfentanyl (65%), and norfentanyl (50%) (91). Cross-reactivity with α-methylfentanyl, alfentanil, lofentanil, and sufentanil was not observed (91). When used to analyze authentic samples, concentrations were higher than liquid chromatography-tandem-mass spectrometry (LC-MS/MS) results, but were determined to correlate (91). While the developed method was intended for illicit equine doping, it was concluded to have potential applications to human samples.

To investigate fentanyl in human urine, Wang *et al.* developed and validated an automated homogeneous immunoassay (HEIA) targeting fentanyl, despropionylfentanyl, and hydroxyfentanil (Immunalysis Corporation) (92). The limit of detection and cut-off concentration were 1 and 2 ng/mL, respectively. Cross-reactivity of norfentanyl with the reagent antibody was determined to be negligible. The HEIA method was applied to authentic samples, which was determined to be sufficient for fentanyl detection without yielding false negatives (92). Snyder *et al.* evaluated the HEIA method and found cross-reactivities of fentanyl and norfentanyl to be ~70% and >0.1% respectively. Additionally, the HEIA method had 99% agreement with a subsequent LC-MS/MS method (93).

As synthetic opioid abuse began to have a more profound impact on humans, companies began commercializing fentanyl-targeted ELISA kits for forensic use. Research was conducted on the efficacy of those kits and applicability to novel fentanyl analogs in human samples (Table 1.1). Upwards of 30 fentanyl analogs have been analyzed using commercialized kits produced from Neogen®, Immunalysis™, Randox Laboratories Ltd, Thermo Fisher Scientific, and Venture Labs, Inc. Some of these kits have been validated for laboratory use in blood and urine (94-96). Cross-reactivities of

fentanyl analogs with minimal structural alterations (e.g. propionyl substituted analogs) have been described with greatest detection (95-98). Conversely, Wharton *et al.* discusses that analogs with diverse modifications (e.g. piperidine substitutions, rings, or long alkyl chains) have limited IA detection (98). Helander *et al.*, also explains steric hinderance as a possible explanation for low cross-reactivity of some analogs (96). In several studies, cross-reactivity of 4-ANPP and norfentanyl were low or negligible with some kits, suggesting difficult presumptive detection (94, 97, 98). Schackmuth and Kerrigan found norfentanyl cross-reactivity only in a kit targeting norfentanyl (97). While most kits target fentanyl, some are designed to target piperidine substituted analogs such as carfentanil, remifentanil, or alfentanil. Like norfentanyl, detection and cross-reactivity for carfentanil was limited in fentanyl-targeted kits and greatest in carfentanil or piperidine-targeted kits (95, 97, 98).

Table 1.1. Published research investigating fentanyl analog detection by enzyme-linked immunoassay (ELISA)

Screening Kit	Number of Analogs	Matrix	Significant Findings	Reference
Fentanyl ready-to-use (TRU) ELISA kits- Neogen®	6	BL UR	• Two decision points beneficial for flexibility of casework • Limited cross-reactivity for norfentanyl, 4-ANPP, and β-hydroxythiolfentanyl	(94)
Fentanyl Direct ELISA kit- Immunalysis®	9	BL	• Carfentanil showed no detectable cross-reactivity • Furanylfentanyl and 2-fluorofentanyl showed lowest cross-reactivity • Acetylfentanyl and acrylfentanyl showed highest cross-reactivity • ELISA results matched LC-MS/MS confirmation	(95)
Fentanyl ELISA Plate- Randox Carfentanil/Remifentanil ELISA Plate- Randox Fentanyl Group kit- Neogen® Fentanil Group Forensic kit- Neogen® Fentanyl ELISA kit- Immunalysis®	13	UR	• Immunalysis®, Randox, and Neogen® Fentanyl exhibited moderate cross-reactivity to propionyl substituted analogs (except 4-ANPP) and negligible cross-reactivity to piperidine substituted analogs • Randox was the only kit to exhibit cross-reactivity with norfentanyl • Neogen® Fentanil Group kit exhibited strong and poor cross-reactivity to piperidine and propionyl substituted analogs, respectively • No kit exhibited strong cross-reactivity to all substitutions	(97)
ELISA- Fentanyl Assay- Venture Labs, Inc. Carfentanil, Fentanyl, and Fentanil Group ELISA kits- Neogen® Fentanyl Urine Enzyme Immunoassay- Immunalysis® Carfentanil and Fentanyl ELISA kits- Randox	30	UR	• Metabolites, precursors, and carfentanil had the lowest average cross-reactivities across ELISA kits (<10%) • Carfentanil and norcarfentanil were only detected in kits designed to target them • Analogs most structurally similar to fentanyl had greatest cross-reactivity • Analogs with structural diversity (piperidine modifications, rings, or long alkyl chains) were poorly detected	(98)
Thermo DRI® Fentanyl Enzyme Immunonassay ARK™ Fentanyl Assay homogenous enzyme immunoassay Immunalysis® Fentanyl Urine SEFRIA™ Drug Screening Kit	13	UR	• 4-methoxybutyrylfentanyl showed no cross reactivity in any kits; possibly due to steric hinderance • 2-fluorofentanyl showed limited cross-reactivity with DRI kit • Overall, all 3 kits demonstrated promising detectability for target fentanyl analogs	(96)

BL = blood; UR = urine

Overall, none of the commercially available ELISA kits exhibit cross-reactivity for all variations of fentanyl analogs. As addressed by Schackmuth and Kerrigan, this presents a major limitation to immunoassay based screening for all novel psychoactive substances (NPS) due to implementation of multiple kits and associated costs (97). An additional limitation addressed by Abbott *et al.* is the occurrence of false-positives (99). In a study by Abbott *et al.*, case samples analyzed by Neogen® ELISA kits were screening positive for methamphetamine and fentanyl (n=61). When analyzed with a confirmatory method, 33% of those cases did not contain fentanyl. Cross-reactivity experiments determined false positives for fentanyl in urine samples containing high concentrations of methamphetamine (99). To mitigate limitations associated with immunoassay screening, forensic toxicology laboratories have begun transitioning to high resolution mass spectrometry (HRMS) screening methods.

Mass spectral screening

High resolution mass spectrometry provides practical screening opportunities due to accurate mass capabilities. NPS analogs of varying drug-type are often structurally similar or isobaric in nature making detection by traditional detectors more difficult. By coupling chromatographic separation with HRMS, forensic toxicology laboratories have the ability to distinguish, differentiate and identify novel analogs. Data acquisition for HRMS screening can be divided into two categories: data-dependent and data-independent. Data-dependent screening involves predefined thresholds for precursor manipulation and resulting data collection. Conversely, data-independent screening is comprehensive in that all information is collected. Data-dependent (100-103) and data-independent (104, 105) HRMS screening methods have been employed for detecting

fentanyl analogs in various biological matrices. Table 1.2 summarizes published HRMS screening methods for fentanyl analogs.

To the authors knowledge, Krajewski *et al.*, presents the largest fentanyl analog screen, to date. In this study, 174 fentanyl-related compounds (obtained from Fentanyl Analog Screening (FAS) kits from Cayman Chemical and outside purchases) were included in a library system. The validated quadrupole-time-of-flight (QTOF) screening method proved applicable through the analysis of fortified samples (101). Salomone *et al.* also used a QTOF mass spectrometer for the screening of fentanyl analogs in human hair previously testing positive for heroin and its metabolites (105). Fentanyl analogs of fentanyl, norfentanyl, acetylfentanyl, furanylfentanyl, acrylfentanyl, 4-FIBF, cyclopropylfentanyl, butyrylfentanyl and 4-ANPP were identified at pg/mg concentrations (105). In a study by Rab *et al.*, fentanyl and analogs were screened in postmortem blood and urine using an Orbitrap mass spectrometer (100). Fentanyl analogs were identified in 40/84 postmortem cases and analogs of fentanyl, carfentanil, 4-FIBF, and butyrylfentanyl were identified. Of the positive cases, 14 involved combinations of fentanyl analogs (100). Analytical methods utilizing QTOF and Orbitrap mass spectrometers have proved successful in the detection and identification of fentanyl analogs in traditional and alternative biological matrices.

In studies by Noble *et al.* and Gundersen *et al.*, QTOF mass spectrometers were utilized to retrospectively analyze 2,339 blood samples and 1,314 data files, respectively (103, 104). Noble *et al.* detected fentanyl, alfentanil and remifentanil in few samples, but did not detect any novel analogs (104). Alternatively, Gundersen *et al.* identified two fentanyl analogs (fluorofentanyl and cyclopropylfentanyl) that were not previously

identified at initial analysis (103). As NPS continue to emerge and evolve, forensic laboratories must consistently inspect for unknowns and update existing libraries. Due to the collection of vast amounts of data (dependent or independent), the ability to re-analyze data files with NPS libraries through retrospective analysis presents a major advantage to HRMS screening.

Table 1.2. High resolution mass spectrometry screening methods for fentanyl analogs in various biological matrices

Mass Spectrometer	Number of Analogs	Matrix	Screening Type	LOD	Reference
QTOF	n=13 (validation), n=50 (identification)	BL	Data-Independent	0.0005- 0.001 mg/kg	(104)
Orbitrap	Not specified	BL & UR	Data-Dependent	Not specified	(100)
QTOF	n=174 (in library)- FAS kit	PL & UR	Data-Dependent	0.25-2.5 ng/mL (PL), 0.5-5 ng/mL (UR)	(101)
Orbitrap	n=32	n/a	Data-Dependent	0.5 ng/mL	(102)
QTOF	n=88 (synthetic opioids)	BL	Data-Dependent	1 ng/mL	(103)
QTOF	n=12	Hair	Data-Independent	0.2- 1.2 pg/mg	(105)

BL = blood; UR = urine; PL = plasma; LOD = limit of detection

Other qualitative methods

Additional qualitative methods for the detection and identification of fentanyl analogs have been described (Table 1.3). In studies by Adamowicz *et al.* and Seither *et al.*, LC-MS/MS was used to identify 38 and 14 fentanyl analogs, respectively, in blood and urine (106, 107). While tandem mass spectrometry is often used for quantification, it

can serve beneficial for qualitative analysis due to low achievable limits of detection (LOD). Limits of detection reported by the aforementioned studies were as low as 0.01 ng/mL (106, 107). As previously described, LC-QTOF-MS instrumentation is increasing in popularity as a screening tool in forensic laboratories. In a study by Griswold *et al.*, an LC-QTOF-MS qualitative method was utilized to assess the applicability of oral fluid as a matrix for detecting synthetic opioids. A 93% agreement between paired oral fluid and urine specimens was determined, suggesting applicability of the matrix and methodology (108). Shoff *et al.* and Peer *et al.* describe qualitative methods analyzing fentanyl and analogs using ion trap mass spectrometry (45, 109). Ion traps are advantageous because of MSn capabilities for unknown compound identification and structural elucidation that could be relevant for the evolving fentanyl analogs market. Shoff *et al.* was able to achieve limits of detection of 0.1-5.0 ng/mL for 13 fentanyl analogs and other opioids in blood, urine and postmortem tissues (109). The method was used to analyze approximately 500 postmortem samples for fentanyl analogs. A total of 375 cases were positive for fentanyl and/or one (or more) fentanyl analog, and 60% of these cases were also positive for heroin or cocaine (109). The following six fentanyl analogs were detected: β-hydroxythiofentanyl, acetylfentanyl, carfentanil, furanylfentanyl, para-fluoroisobutyrylfentanyl, and butyrylfentanyl. 4-ANPP was also detected in specimens positive for furanylfentanyl (109).

Table 1.3. Additional qualitative methods for fentanyl analogs

Analog Number	Matrix	Extraction	Instrumentation	LOD (ng/mL)	Reference
n=38	BL	LLE	LC-MS/MS	0.01- 0.2	(106)
n=10	OF, UR	LLE	LC-QTOF-MS	1.0-2.0	(108)
fentanyl, norfentanyl	UR	SPE	Direct-MS (ion trap)	10.0	(45)
n=14	BL, UR	SPE	LC-MS/MS	0.01-0.5	(107)
n=13	BL, UR, PM tissues	SPE	LC-Ion trap-MS	0.1-5	(109)

BL = blood; OF = oral fluid; UR = urine; PM = postmortem; LLE = liquid-liquid extraction; SPE = solid-phase extraction

Quantitative analyses

With the proliferation of synthetic opioid abuse, research has developed quantitative analytical methods for various fentanyl analogs in different biological matrices (Table 1.4). While gas chromatography-mass spectrometry (GC-MS) is considered a standard practice for detection and quantification of drugs of abuse in forensic toxicology laboratories, fentanyl analog detection is often performed using liquid chromatography-tandem-mass spectrometry (LC-MS/MS). LC-MS/MS techniques are more applicable due to increased sensitivities capable of detecting highly potent compounds. Lower limits of quantification (LOQ) have been reported as low as 4 pg/mL (110). Table 1.4 displays GC-MS (n=3), LC-MS/MS (n=28), paper spray-MS/MS (n=1) and direct MS/MS (n=1) methods for the quantification of over 30 different fentanyl analogs. While LC-HRMS is frequently used for screening, limited quantitative methods

exist using that type of instrumentation. Viaene *et al.*, sought to determine if the LC-QTOF-MS was a suitable replacement for quantitative analysis by liquid chromatography- triple quadrupole- mass spectrometry (LC-QQQ-MS) through dual validation of 16 opioids (including fentanyl and norfentanyl) in human plasma (111). As a result, suitability of instrumentation was determined to be compound-, parameter-, and concentration-dependent. Sensitivity of the LC-QTOF-MS was observed 10-fold higher; however, the authors address pitfalls associated with this finding due to higher sensitivity being generally reported by multiple reaction monitoring acquisition of LC-QQQ-MS. Poor repeatability was observed for norfentanyl on both instruments, but additional validation parameters were acceptable. Fentanyl was acceptably analyzed on both detectors. Ultimately, methods were fully validated on both instruments with minor issues (e.g. selectivity, matrix effects, etc.), suggesting applicability for quantitative analysis (111).

Table 1.4. Quantitative analytical methods for fentanyl analogs

Analogs	ISTD (matched)	Matrix	Extraction	Instrumentation	LOQ (LOD*)	Application	Reference
Fentanyl Norfentanyl	Fentanyl-D5 Norfentanyl-D5	PL OF	PP	LC-MS/MS	30 ng/mL (PL) 45 ng/mL (OF)	11 paired PL and OF samples	(49)
Acrylfentanyl Carfentanil Fentanyl FIBF Furanylfentanyl	Carfentanil-D5 Fentanyl-D5	OTC powder slurries	n/a	Paper spray-MS/MS	2.3- 7.4 ng/g	Qual/Quant analysis of tablets	(112)
4-ANPP Acetylfentanyl Acetyl norfentanyl Alfentanil β-hydroxyfentanyl β-hydroxythiofentanyl Butyrylfentanyl Butyrylfentanyl carboxy-metab Butyryl norfentanyl Carfentanil Cyclopropylfentanyl Cyclopropyl norfentanyl Despropionyl *p*-fluorofentanyl Fentanyl Furanylfentanyl Furanyl norfentanyl Furanylethyl fentanyl Methoxyacetylfentanyl Methoxyacetyl norfentanyl Norfentanyl Phenylacetyl fentanyl Sufentanil Valerylfentanyl carboxy-metab	Acetyl norfentanyl-D5 Fentanyl-D5	BL UR Hair	LLE SPE	LC-MS/MS	2-6 ng/L (BL/UR) 11- 21 pg/g (Hair)	42 PM BL, UR, or Hair from 27 fatalities	(113)
4-ANPP Fentanyl Norfentanyl	Fentanyl-D5 Norfentanyl-D5	LV	QuEChERS SPE LLE	LC-MS/MS	0.4- 0.5 µg/kg	12 PM LV samples	(114)

(continued)

Analogs	ISTD (matched)	Matrix	Extraction	Instrumentation	LOQ (LOD*)	Application	Reference
3-methylfentanyl 4-ANPP Acetylfentanyl Acetyl norfentanyl β-hydroxyfentanyl Butyrylfentanyl Carfentanil Cyclopropylfentanyl Fentanyl Furanylfentanyl Methoxyacetylfentanyl Norcarfentanil Norfentanyl *p*-fluorofentanyl Remifentanil acid Remifentanil Sufentanil	Acetylfentanyl-$^{13}C_6$ Acetyl norfentanyl-$^{13}C_6$ Fentanyl-D5 Norfentanyl-D5 Sufentanil-D5	BL PL	SPE	LC-MS/MS	50-100 pg/mL (BL) 2- 5 pg/mL (PL)	44 PM BL samples. 18- PL samples from patients receiving IV fentanyl	(48)
Alfentanil Fentanyl Norfentanyl Remifentanil Sufentanil (and other opioids)	Fentanyl-D5 Norfentanyl-D5 Sufentanil-D5	Serum PL PM tissues	SPE	LC-MS/MS	0.1- 1.0 ng/mL	13 PM cases containing fentanyl (n=4) and norfentanyl (n=3)	(115)

(continued)

Analogs	ISTD (matched)	Matrix	Extraction	Instrumentation	LOQ (LOD*)	Application	Reference
4-FIBF Acetylfentanyl Acrylfentanyl Alfentanil Butyrylfentanyl Crotonylfentanyl Fentanyl Furanylfentanyl Methoxyacetylfentanyl Norfentanyl Ocfentanil THFF Valerylfentanyl (and other synthetic opioids)	Fentanyl-D5	Hair	SPE	LC-MS/MS	0.15-1.0 pg/mg	17 hair samples from fentanyl analog users	(116)
4-ANPP α-methylfentanyl Acrylfentanyl β-hydroxyfentanyl β-methylfentanyl Butyrylfentanyl Carfentanil Cyclopentylfentanyl Cyclopropylfentanyl Fentanyl FIBF Furanylfentanyl Isobutyrylfentanyl Methoxyacetylfentanyl Norfentanyl *o*-fluorofentanyl *p*-fluorofentanyl *p*-methylfentanyl THFF	Acrylfentanyl-D5 Butyrylfentanyl-D5 Cyclopentylfentanyl-D5 Cyclopropylfentanyl-D5 Fentanyl-D5 Furanylfentanyl-D5 Isobutyrylfentanyl-D5 Methoxyacetylfentanyl-D5 Norfentanyl-D5 THFF-D5	BL	SPE	LC-MS/MS	Not Specified	42 case samples involving cyclopropyl- or methoxyacetylfentanyl	(117)

(continued)

Analogs	ISTD (matched)	Matrix	Extraction	Instrumentation	LOQ (LOD*)	Application	Reference
Acetylfentanyl	Acetylfentanyl-13C6	BL LV UR VH PM tissue	LLE	GC-MS	Not Specified	2 case reports	(118)
4-ANPP Furanylfentanyl	Fentanyl-D5	BL UR Bile CSF GC	SPE	LC-MS/MS	0.03 ng/mL (FuF)* 0.1 ng/mL (4-ANPP)*	1 case sample to derive postmortem redistribution	(119)
4-ANPP Acetylfentanyl Acetyl norfentanyl Acrylfentanyl Alfentanil β-hydroxyfentanil β-hydroxythiofentanyl Butyrylfentanyl & carboxy metab Butyryl norfentanyl Carfentanil Cyclopropylfentanyl Despropionyl *p*-fluorofentanyl Fentanyl Furanylfentanyl Furanyl norfentanyl Methoxyacetylfentanyl Methoxyacetyl norfentanyl Norfentanyl Ocfentanil *p*-fluorobutryrylfentanyl Phenacetylfentanyl Remifentanil Sufentanil trans-3-methyl norfentanyl THFF Valerylfentanyl carboxy metab	Fentanyl-D5	Hair	SPE	LC-MS/MS	0.5 pg.mg	Application of method to hair collected from drug addicts under withdrawal treatment (n=97) and postmortem hair samples (n=20).	(120)

(continued)

Analogs	ISTD (matched)	Matrix	Extraction	Instrumentation	LOQ (LOD*)	Application	Reference
Alfentanil α-methylfentanyl cis-3-methylfentanyl Fentanyl Norfentanyl *p*-fluorofentanyl Remifentanil Sufentanil trans-3-methylfentanyl (and other opioids)	Fentanyl-D5	BL UR	LLE	LC-MS/MS	Fentanyl: 0.0001 mg/L; norfentanyl: 0.007 mg/L; sufentanil: 0.0002 mg/L	Simultaneous screening and quantification	(121)
2-furanylfentanyl 4-ANPP 4-methoxybutyrylfentanyl Acrylfentanyl Alfentanil Carfentanil Despropionyl-2-fluorofentanyl Fentanyl Methoxyacetylfentanyl Norfentanyl Ocfentanil Remifentanil Sufentanil Valerylfentanyl	Alfentanil-D3 Carfentanil-D5 Fentanyl-D5 Norfentanyl-D5 Remifentanil-C6	BL UR	LLE	LC-MS/MS	0.10-0.40 ng/mL (BL)	211 urine samples from ER or addiction patients. Retrospective Analysis	(122)

(continued)

Analogs	ISTD (matched)	Matrix	Extraction	Instrumentation	LOQ (LOD*)	Application	Reference
Acetyl fentanyl β-hydroxythiofentanyl Butyryl fentanyl Carfentanil Fentanyl Furanylfentanyl *p*-fluoroisobutyrylfentanyl	Acetylfentanyl-13C6 β-hydroxythiofentanyl-D5 Butyrylfentanyl-D5 Fentanyl-D5 Furanylfentanyl-D5	BL Serum LV BR	SPE	LC-MS/MS	0.5 ng/mL (0.1 ng/mL-carfentanil) *	312 authentic samples previously testing positive for fentanyl or analogs	(123)
3 fluorofentanyl 3-methylfentanyl Acetylfentanyl Acetyl norfentanyl Alfentanil Butyrylfentanyl Butyryl norfentanyl Carfentanil Fentanyl Furanylfentanyl Furanyl norfentanyl Methoxyacetylfentanyl Norcarfentanil Norfentanyl Ocfentanil Sufentanil	Acetylfentanyl-D5 Fentanyl-D5 Norfentanyl-D5	Hair	LLE	LC-MS/MS	1-2.5 pg/mg	Application to a case study-intoxication due to snorting "China White"	(124)

(continued)

Analogs	ISTD (matched)	Matrix	Extraction	Instrumentation	LOQ (LOD*)	Application	Reference
Cyclopropylfentanyl Crotonylfentanyl Methacrylfentanyl *o*-methylacrylfentanyl *p*-methylacrylfentanyl	Cyclopropylfentanyl-D5	BL	SPE	LC-MS/MS	1 ng/mL	31 BL samples. Chromatographic separation of isobaric fentanyl analogs	(125)
4-ANPP Hydroxynorfentanyl Fentanyl Norfentanyl	Hydroxynorfentanyl-D5 Fentanyl-D5 Norfentanyl-D5	PL UR	LLE	LC-MS/MS	0.02- 0.67 ng/mL (PL) 0.08-1.28 ng/mL (UR)	Clinical plasma (n=1,280) and urine samples (n=128)	(126)
Acetylfentanyl Butyrylfentanyl Furanylfentanyl Ocfentanil	Methadone-D3	BL	PP	GC-MS	0.5-1 ng/mL	50 BL samples	(127)
Furanylfentanyl (other NSOs)	AH-7921-D3	BL	SPE	LC-MS/MS	0.5 ng/mL*	20 PM BL samples	(128)
2-furanylfentanyl 4-ANPP 4-methylphenethyl acetylfentanyl α-methylfentanyl Acrylfentanyl β-hydroxythiolfentanyl butyrylfentanyl/Isobutyrylfentanyl Carfentanil fluoroisobutyrylfentanyl (FIBF) *o*-fluorofentanyl *p*-fluorofentanyl *p*-fluorobutyrylfentanyl *p*-methoxybutyrylfentanyl Valerylfentanyl (other NSOs)	4-methyl-phenethylacetylfentanyl-D5 α-methylfentanyl-D3 acetylfentanyl-$^{13}C_6$ β-hydroxythiofentanyl-D5 *p*-fluorofentanyl-D5	BL	SPE	LC-MS/MS	0.1-0.5 ng/mL	2,758 PM BL cases	(129)

(continued)

Analogs	ISTD (matched)	Matrix	Extraction	Instrumentation	LOQ (LOD*)	Application	Reference
(±)-*cis/trans*-3-Methylfentanyl 4-ANPP 4-FIBF Alfentanil Acetylfentanyl Acetyl norfentanyl Acrylfentanyl β-Hydroxythiofentanyl Butyrylfentanyl Carfentanil Cyclopropylfentanyl Fentanyl Furanylfentanyl Isobutyryl fentanyl Methoxyacetylfentanyl Norcarfentanil Norfentanyl *o*-fluorofentanyl Ocfentanil *p*-fluorobutyrylfentanyl *p*-fluorofentanyl Remifentanil Sufentanil Valeryl fentanyl (other NSOs)	Fentanyl-D5 Norfentanyl-D5	BL	LLE	LC-MS/MS	0.05- 0.2 ng/mL	3 BL samples	(130)

(continued)

Analogs	ISTD (matched)	Matrix	Extraction	Instrumentation	LOQ (LOD*)	Application	Reference
3-methyllthiofentanyl 4-ANPP 4-fuoroisobutyryl fentanyl α-methylfentanyl Acetylfentanyl Acetyl norfentanyl Acrylfentanyl Alfentanil β-hydroxyfentanyl β-hydroxy-3-methylfentanyl β-hydroxythiofentanyl Butyrylfentanyl Carfentanil *cis*-3-methylfentanyl Cyclopropylfentanyl Fentanyl Furanylfentanyl Isobutyrylfentanyl Methoxyacetylfentanyl Norfentanyl Norcarfentanil Ocfentanil *p*/*o*-fluorofentanyl *p*-fluorobutyryl fentanyl (PFBF) Remifentanil Remifentanil acid Sufentanil *trans*-3-methylfentanyl Thiofentanyl THFF Valeryl fentanyl (other NSOs)	Fentanyl-d5 Norfentanyl-D5	Hair	cryogenic grinding	LC-MS/MS	2-5 pg/mg	Application to authentic hair samples	(131)
Acetylfentanyl Acetyl norfentanyl	Acetyfentanyl-D5 Acetyl norfentanyl-D5	UR	SPE	LC-MS/MS	1.06-1.62 ng/mL	Metabolism study in rats	(132)

(continued)

Analogs	ISTD (matched)	Matrix	Extraction	Instrumentation	LOQ (LOD*)	Application	Reference
4-ANPP Acetylfentanyl Acrylfentanyl Alfentanil Butyrylfentanyl Carfentanil *cis*-3-methylfentanyl Fentanyl Furanylfentanyl Norfentanyl *p*-fluorobutyrylfentanyl sufentanil (traditional & synthetic opioids)	Fentanyl-D5 Norfentanyl-D5 Sufentanil-D5	Hair	SPE	LC-MS/MS	1 or 10 pg/mg	64 hair samples (3-5 cm)	(133)
Acetylfentanyl Acetyl norfentanyl Fentanyl Norfentanyl	Acetyl fentanyl-$^{13}C_6$ Acetyl norfentanyl-$^{13}C_6$ Fentanyl-D5 Norfentanyl-D5	BL	SPE	LC-MS/MS	0.0005-0.001 mg/L	Investigation of PM tissue distribution	(134)
2-furanylfentanyl 3-methylfentanyl α-methylfentanyl Acrylfentanyl Alfentanil Carfentanil Cyclopropylfentanyl Fentanyl Isobutyrylfentanyl Lofentanil Methoxyacetylfentanyl Norcarfentanil Norfentanyl Norlofentanil Norsufentanil Ocfentanil Sufentanil	$^{2}H_5$-Acrylfentanyl $^{13}C_6$-Alfentanil $^{2}H_5$-Carfentanil $^{2}H_5$-Cyclopropylfentanyl $^{2}H_5$-Fentanyl $^{2}H_5$-Furanylfentanyl $^{2}H_5$-Isobutyrylfentanyl $^{2}H_5$-Methoxyacetylfentanyl $^{2}H_5$-Norcarfentanil $^{2}H_5$-Norfentanyl $^{2}H_3$-Norlofentanil $^{2}H_5$-Norsufentanil $^{2}H_5$-Ocfentanil $^{2}H_5$-Sufentanil	DBS	Solvent extraction	LC-MS/MS	0.127- 0.704 ng/mL*	Fortified PM BL for evaluation of application	(135)
Carfentanil	Fentanyl-D5	BL	PP	LC-MS/MS	10 ng/mL	13 case samples	(136)

(continued)

Analogs	ISTD (matched)	Matrix	Extraction	Instrumentation	LOQ (LOD*)	Application	Reference
3-methylfentanyl α-methylfentanyl Alfentanil Carfentanil Fentanyl Lofentanil Sufentanil	$^{13}C_6$-Alfentanil $^{2}H_5$-Carfentanil $^{2}H_5$-Fentanyl $^{2}H_5$-Sufentanil	DBS	SPE	SPE-MS/MS	0.15-0.66 g/mL*	n/a	(137)
2-furanylfentanyl 3-methylfentanyl Acetylfentanyl Carfentanil Fentanyl Norfentanyl	Acetylfentanyl-$^{13}C_6$ 2-furanylfentanyl-D5 Fentanyl-D5 Norfentanyl-D5	BL VH	SPE	LC-MS/MS	0.1-1 ng/mL	PM (n=98) and DUID (n=26) cases	(44)
Alfentanil Fentanyl Norfentanyl Sufentanil	Fentanyl-D5	UR	LLE	GC-MS	0.5 ng/mL	Application to clinical samples	(138)
Fentanyl Norfentanyl	Fentanyl-D5 Norfentanyl-D5	BL UR	SPE	LC-MS/MS	0.0025 ng/mL (fentanyl) (UR) 0.005 ng/mL (norfentanyl) (UR) 0.01 ng/mL (BL)	n/a	(139)

(continued)

Analogs	ISTD (matched)	Matrix	Extraction	Instrumentation	LOQ (LOD*)	Application	Reference
2-furanylfentanyl 4-ANPP Acetylfentanyl Acrylfentanyl Alfentanil β-hydroxyfentanyl Benzylfentanyl Butyrylfentanyl Carfentanil *cis*-3-methylfentanyl Cyclopentenylfentanyl Cyclopropylfentanyl Fentanyl Fentanyl carbamate Methoxyacetylfentanyl *N*-methyl norfentanyl Norfentanyl Norcarfentanil *p*-fluoroacrylfentanyl *p*-fluorofentanyl *p*-fluoroisobutyrylfenanyl *p*-methoxyacetylfentanyl Phenylfentanyl Sufentanil *trans*-3-methylfentanyl	2-furanylfentanyl-D5 Acetylfentanyl-$^{13}C_6$ Carfentanil-D5 Cyclopropylfentanyl-D5 Fentanyl-D5 Methoxyacetylfentanyl-D5 Norfentanyl-D5 *p*-fluorofentanyl-D5 *p*-FIBF-D7	BL	SPE	LC-MS/MS	0.025, 0.125 ng/mL	Achieved baseline separation of nine structural or stereo isomers and one isobar. Application to PM and DUID case samples	(140)

(continued)

Analogs	ISTD (matched)	Matrix	Extraction	Instrumentation	LOQ (LOD*)	Application	Reference
2-fluorofentanyl 3-fluorofentanyl 4-chloroisobutyrylfentanyl 4-fluorobutyrylfentanyl 4-fluorofentanyl 4-FIBF 4-methoxybutrylfentanyl Acrylfentanyl Acetylfentanyl Acetylnorfentanyl Alfentanil Benzodioxolefentanyl Butyrylfentanyl Carfentanil Cis-3-methylfentanyl Cyclopropylfentanyl Despropionyl-2-fluorofentanyl Fentanyl Furanylfentanyl Methoxyacetylfentanyl Norfentanyl Norcarfentanil Ocfentanil Remifentanil Valerylfentanyl	4-fluorobutyrylfentanyl-D7 4-methoxybutyrylfentanyl-D7 Acetylfentanyl-$^{13}C_6$ Fentanyl-D5 Furanylfentanyl-D5 Norfentanyl-D5	BL	LLE	LC-MS/MS	4.0-20.0 pg/mL	Determination of fentanyl in 2 PM BL samples	(110)

(continued)

Analogs	ISTD (matched)	Matrix	Extraction	Instrumentation	LOQ (LOD*)	Application	Reference
4-ANPP Acetylfentanyl Acetylfentanyl 4-methylphenethyl Acrylfentanyl Alfentanil Butyrylfentanyl Butyryl norfentanyl Carfentanil (±)-*cis*-3-methyl fentanyl Despropionyl *p*-fluorofentanyl Fentanyl Furanylfentanyl Furanyl norfentanyl Isobutyrylfentanyl Norfentanyl *p*-fluorobutyrylfentanyl *p*-fluoroisobutyryl fentanyl *p*-methoxyfentanyl Remifentanil acid Remifentanil Sufentanil Valerylfentanyl	Acetylfentanyl-$^{13}C_6$ Fentanyl-D5 Norfentanyl-D5	BL	SPE	LC-MS/MS	0.1-0.5 ng/mL	725 BL from unintentional overdose deaths	(141)

ISTD = internal standard; LOQ = limit of quantification; LOD = limit of detection; PL = plasma; OF = oral fluid; OTC = over the counter; BL = blood; UR = urine; LV = liver; PM = postmortem; VH = vitreous humor; CSF = cerebrospinal fluid; GC = gastric contents; BR = brain; DBS = dried blood spots; PP = protein precipitation; LLE = liquid-liquid extraction; SPE = solid-phase extraction

Of the published methods presented in Table 1.4, whole blood (n=20) and solid-phase extraction (n=20) are the most utilized quantitative analyses for fentanyl analogs. However, traditional liquid-liquid extractions are often employed (n=10). Although more expensive, solid-phase extraction (SPE) methods can be advantageous due to amenability to a wide range of compounds, intensified sample clean up to mitigate matrix effects, and ability to automate the extraction process. Shaner *et al.* presents two automated SPE procedures for fentanyl analogs in urine (142). The automated processes (off-line 96-well plate, or on-line SPE) were determined successful in isolating fentanyl analogs from urine, with the off-line method having greater recoveries (142). Automation can be beneficial for increasing the efficiency of toxicological analyses and decreasing error associated with the analyst.

Blood and urine are traditional biological fluids analyzed by forensic toxicologists. While these matrices are highly addressed in the literature, alternative matrices such as oral fluid, dried blood spots, hair, and postmortem tissues have also been investigated. Table 1.4 depicts several methods quantifying fentanyl analogs in hair (n=6) reaching limits of quantification as low as 0.15 pg/mg (116). The developed methods have also proven applicable to authentic ante- and postmortem hair samples with concentrations in the sub pg/mg range (113, 116, 120, 124, 131, 133). On the contrary, limited analytical methods are available for quantifying fentanyl analogs in oral fluid and dried blood spots. Hair provides a wide window of detection or past exposure history due to drug deposition in the hair over time. Oral fluid, on the other hand, can demonstrate recent drug use. Dried blood spots can provide similar information to whole blood, but requires a safer, simplified collection process. While alternative matrices originate from

the same individual, they each provide different information about drug abuse and exposure.

Case reports

Quantitative analyses are often applied to acute or fatal intoxications. Table 1.5 displays fentanyl analog related case studies (n=27) reported in the literature. The described cases involve men and women ranging from age 18-66. Often, case information on overdose deaths involves an individual with substance abuse history and powdery substances and/or drug paraphernalia found on scene. Common autopsy findings associated with accidental fentanyl-related drug overdoses are pulmonary and cerebral edema and congestion of the respiratory tract, which are typical observations of any opioid overdose. Opioid-induced intoxications frequently involve administration of naloxone, an agent that blocks or reverses opioid action, which was associated with several of the presented case studies (54, 55, 124, 140, 143).

Some of the most common fentanyl analogs detected in the presented case studies are fentanyl (n=14), acetylfentanyl (n=13), furanylfentanyl (n=12), butyrylfentanyl (n=7), and carfentanil (n=7) at concentration ranges of 0.93-993 ng/mL, 0.11-285 ng/mL, 0.34-148 ng/mL, 0.16-220 ng/mL, and 0.09-12.1 ng/mL, respectively, in traditional biological matrices. 4-ANPP was also quantified in several cases. Non-traditional postmortem tissues have also been analyzed to understand postmortem tissue distribution and redistribution of select fentanyl analogs (71, 119, 134). Other classes of drugs of abuse common between the presented case studies are ethanol, cannabinoids (Δ9-tetrahydrocannabinol (THC) and metabolites), traditional opioids (codeine, 6-monoacetylmorphine (6-MAM), morphine, buprenorphine, hydrocodone,

hydromorphone), synthetic opioids (U-47700, MT-45), synthetic cathinones (α-pyrrolidinovalerophenone (PVP), α-pyrrolidinobutiophenone (PBP)), benzodiazepines (alprazolam, diazepam, and synthetics), stimulants (cocaine, benzoylecgonine, methamphetamine, amphetamine, methylphenidate), and other (pregabalin, gabapentin, diphenhydramine, quinine, butalbital).

Table 1.5. Fentanyl analog intoxication and fatality case reports

n	Age	Sex	Fentanyl Analog Concentration (ng/mL)	Matrix	Other Drugs Present	Notes	Reference
5	19-25	M	Butyrylfentanyl: 0.6- 65.6 4-fluorobutyrylfentanyl: 9.5-15 Fentanyl: 1.5- 993	Serum UR	Three cases contained pregabalin. Two cases also contained combinations of *N*-ethylbuphedrone, 3-MeO-PCP, and α-PBP. Cannabis, MT-45, and flubromazepam were also detected in individual cases.	Intoxications- often involving decreased or loss of consciousness. Additional symptoms included disorientation, agitation, odd behavior, slurred speech, etc.	(143)
14	21-40	13M 1F	Acetylfentanyl: 0.6- 51.6 4-methoxybutyrylfentanyl: 1.3- 11.0 Furanylfentanyl: 4.4- 148 Additional fentanyl analogs: Fentanyl, Butyrylfentanyl, 4-fluorobutyrylfentanyl	Serum	Additional opioids of oxycodone and morphine-3-glucuronide were detected. Some reported drugs of abuse were flubromazolam, 7-amino-clonazepam, pregabalin, amphetamine, ethanol, cannabis, and cocaine	Intoxications- hospital care ranging from 1-28 days. Eight cases received naloxone treatment.	(55)
1	28	M	Acetylfentanyl: 235 (BL), 2400 ng/g (LV), 131 (VF), 234 (UR)	BL LV VH UR	Ibuprofen and tadalafil were quantified in blood. Positive results for 4-ANPP and oxandrolone were detected in urine. Testosterone and epitestostrone were quantified in urine	Found unresponsive with torniquet around arm and syringe nearby. Decedent had a history of substance abuse. Autopsy revealed pulmonary edema and mild diffuse cerebral edema.	(144)
42	19-63	35M 7F	Methoxyacetylfentanyl: 0.21- 39.9 Fentanyl: 0.93-22 Norfentanyl: 0.31- 5.5 FIBF: 7.5- 18 Acetylfentanyl: 0.31 Acrylfentanyl: 0.64- 2.1 Furanylfentanyl: 0.52	BL UR VH	Ethanol, mitragynine, cocaine (and BZE), morphine, delta 9-THC (and metabolites), methamphetamine, amphetamine, 6-MAM, codeine, U-47700, various benzodiazepines, etc.	Fatalities- drug paraphernalia often found on scene	(117)

(continued)

n	Age	Sex	Fentanyl Analog Concentration (ng/mL)	Matrix	Other Drugs Present	Notes	Reference
2	20, 50	1M 1F	Acetylfentanyl: 192- 285 (BL), 1,100 ng/g (LV-1), 620 ng/g (BR-1), 2,720-3,420 (UR), 140 (VH-2)	BL LV UR BR VH	n/a	Both decedents were pronounced dead at the scene and had a history of drug abuse	(118)
1	53	M	Furanylfentanyl: 2.7- 11.8 (BL), 71.3 (UR), 7.7 (Bile), 2.6 (CSF), 40.1 ng/g (GC) 4-ANPP: 50.4- 93.5 (BL), 171.7 (UR), 41.9 (Bile), 10.2 (CSF), 24.2 ng/g (GC)	BL UR Bile CSF GC	n/a	Decedent found dead on the scene with needle inserted into arm. White powder discovered at the scene. Autopsy revealed multivisceral congestion, and pulmonary and cerebral edema.	(119)
7	24-36	M	Furanylfentanyl: 0.38- 2.74 ng/g Fentanyl: 0.00038-0.0012 ng/g	BL UR	Δ^9-THC, pregabalin, buprenorphine, clonazepam, promethazine, methylphenidate, amphetamine, gabapentin, alprazolam, diazepam, etc.	All but one descendant was found dead in his home. One decedent was found lying in a ditch. Paraphernalia was often found at the scene. Pulmonary edema was a common diagnosis at autopsy.	(145)
11	19-51	10M 1F	Acrylfentanyl: 0.5- 2.1 4-chloroisobutyrylfentanyl: 5.1 4-FIBF: 38 THFF: 45	Serum UR	6 cases did not indicate detection of other substances in urine. Drugs detected in remaining cases were NPP, flunitrazolam, oxazepam, temazepam, 4Cl-α-PVP, ephylone, amphetamine, etc.	CNS depression and tachycardia were observed at the time of hospital admission in 90% of the presented intoxication cases. Naloxone was administered in 6 cases.	(54)
25	21-54	22M 3F	Carfentanil: 0.09-4.004 (BL), 0.03-12.163 (UR), 0.021-0.098 (Serum) Fentanyl: 1-3.1 (BL) Norfentanyl: <0.1 (BL) Additional fentanyl analogs: Butyrylfentanyl, 4-FBF, furanylfentanyl, Alfentanil, and 4-ANPP	BL UR Serum	Some additional drug classes quantified were ethanol, benzodiazepines, opioids, stimulants, and cannabis	Combinations of carfentanil with additional fentanyl analogs was seen more frequently than cases with just carfentanil.	(146)

(continued)

n	Age	Sex	Fentanyl Analog Concentration (ng/mL)	Matrix	Other Drugs Present	Notes	Reference
1	43	M	1 Month after OD: 3-fluorofentanyl (150 pg/mg), Furanylfentanyl (40 pg/mg), Fentanyl (37 pg/mg) 1 year after OD: 3-fluorofentanyl (25-80 pg/mg), Furanylfentanyl (310-500 pg/mg), Fentanyl (620-760 pg/mg), Acetylfentanyl (1 pg/mg), Methoxyacetylfentanyl (500-600 pg/mg) and Carfentanil (2.5-3 pg/mg)	Hair	6-MAM and methadone was detected before and after hospitalization	Hospitalized for opioid overdose after snorting "China White." The patient was found unconscious with respiratory depression. He received naloxone. No toxicology testing was performed. Due to a history of substance abuse, methadone treatment was initiated.	(124)
1	23	M	Furanylfentanyl: 1.9-2.8 (BL), <0.20 (VH), 55,000 ng (GC) 4-ANPP: 4.3-5.8 (BL), <0.20 (VH), >40 ng/g (LV)	BL UR VH GC LV	n/a	Decedent found unresponsive on bedroom floor with pill-suspected to be oxycodone. Screening of decedent's blood indicated no oxycodone. Counterfeit pill contained furanylfentanyl.	(147)
1	24	M	Acetylfentanyl: 250-260 (BL), 1000 ng/kg (LV), 240 (VH), 2,600 (UR)	BL LV VH UR	n/a	Decedent found unresponsive in bedroom after a night out. Decedent was found with drug paraphernalia and had a history of opioid abuse- previously overdosing twice. Autopsy revealed edema and congestion of the respiratory tract.	(148)
1	44	M	Butyrylfentanyl: 58-97 (BL), 320 ng/g (LV), 40 (VH), 670 (VH), 170 mg (GC) Acetylfentanyl: 32-38 ng/mL (BL), 110 mg/g (LV), 38 mg/mL (VH), 540 ng/mL (UR)	BL LV VH UR GC	n/a	Decedent was found unresponsive in bathroom with a syringe and plunger (additional paraphernalia was found elsewhere). The decedent was supposed to begin suboxone treatment days after his overdose. At autopsy, the lungs presented evidence of edema and congestion.	(149)

(continued)

n	Age	Sex	Fentanyl Analog Concentration (ng/mL)	Matrix	Other Drugs Present	Notes	Reference
19	18-40	M	Furanylfentanyl: 2.5- 76.0 Butyrylfentanyl: 0.33- 26.0 Acetylfentanyl: 0.62	BL	U-47700 was also detected and quantified in 16 samples. 4-ANPP was detected in 5 samples. Additional drugs detected in some samples were amphetamine, ethanol, mephedrone, citalopram, Δ^9-THC, diphenhydramine, tramadol, 3-MeO-PCP, quinine, oxycodone, hydrocodone, etc.	Most decedents were known to have a history of drug abuse. Powders were often found at the scene, sometimes including a label of contents.	(128)
3	47-58	M 2F	Fentanyl: 2.0- 8.0 Remifentanil: 0.4 Sufentanil: 0.3	BL	n/a	Two cases were determined to have therapeutic concentrations of pharmaceutical fentanyls. In only one case did the patient die- fentanyl may have played a role in her death.	(130)
2	35, 51	1M 1F	Fentanyl: 8.02 pg/mg Sufentanil: 31.48- 183.91 pg/mg	Hair	n/a	Case 1 involved a female undergoing anesthesia for surgery. One month after operation, hair was analyzed and contained fentanyl. Case 2 involved a chronic drug user. Hair was sampled and cut into 3 time segments. Sufentanil was detected in all segments.	(131)
1	44	M	Furanylfentanyl: 1.6	BL	MMMP, THC, THC-COOH, mirtazapine, paliperidone, quetiapine were all quantifiable. Hydroxybenzoylpaliperidone and 4-ANPP were present but not quantified.	Resuscitation was attempted at the scene but failed. White powders and tube containing colored liquid were present on the scene. White froth in the respiratory tract, pulmonary edema and congestion were observed at autopsy.	(150)

(continued)

n	Age	Sex	Fentanyl Analog Concentration (ng/mL)	Matrix	Other Drugs Present	Notes	Reference
6	23-48	4M 2F	Fentanyl: 30.7- 185 Norfentanyl: 10.3- 21.4	UR	diazepam, nordiazepam, alprazolam, mirtaepine, ethanol, hydrocodone, promethazine, imipramine, desipramine, amitriptyline, nortriptyline, methadone, butalbital, and diphenhydramine	Cases involved individuals with history of substance abuse or chronic pain. Two cases involved fentanyl patches- one on the skin and the other in the mouth. The manner of death determined in all cases was accidental.	(45)
2	45, 53	2F	Butyrylfentanyl: 3.7- 220 (BL), 9.8-32 (VH), 590-4,000 (GC), 63-93 ng/g (BR), 39-41 ng/g (LV), 49-260 (Bile), 2-64 (UR) Acetylfentanyl: 21- 95 (BL), 68 (VH), 28,000 (GC), 200 (BR), 160 ng/g (LV), 330 (Bile), and 8.0 (UR) Acetyl norfentanyl: 1.2 (BL), 8.9 (GC)	BL VH GC BR LV Bile UR	Alprazolam and ethanol were detected in case #2	In both cases, drug paraphernalia was not discovered at the scene. At autopsy, both decedents had dark colored lungs displaying signs of edema and frothy liquid	(151)
14	26- 55	12M 2F	Acetylfentanyl: 0.006- 0.6 mg/L Fentanyl: 0.004- 0.038 mg/L	BL VH	Morphine, 6-MAM, cocaine (and BZE), alprazolam, methamphetamine, hydromorphone, oxycodone, and oxymorphone	Decedents (often with substance abuse history) were found unresponsive with paraphernalia in proximity	(134)
1	28	M	Concentrations not reported Fentanyl analogs detected: carfentanil, fentanyl, furanylfentanyl, and para-fluoroisobutyrylfentanyl	BL	U-47700 and its metabolite were identified in the sample. Alprazolam was also quantified.	Individual found unresponsive in a vehicle with a needle on his lap. Police found two additional bags in the car containing suspected heroin. Upon recovery, the individual told police that he took a "bump of heroin" and passed out in the car.	(107)

(continued)

n	Age	Sex	Fentanyl Analog Concentration (ng/mL)	Matrix	Other Drugs Present	Notes	Reference
13	25-62	8M 5F	Carfentanil: 0.0104- 0.617 Furanylfentanyl: 0.61 Fentanyl: 1.1- 2.9	BL	Additional drug categories of opioids, cannabis (and metabolites), and benzodiazepines were present	Decedents were often found unresponsive in their home with paraphernalia on site. All causes of death were ruled an accident.	(136)
12	19-41	9M 3F	Concentrations not reported Fentanyl analogs detected: β-hydroxythiofentanyl, Acetylfentanyl, *p*-fluoroisobutyrylfentanyl, Butyrylfentanyl, Furanylfentanyl, and Carfentanil	BL UR PM tissues	Drug categories detected: ethanol, cannabinoids, stimulants, benzodiazepines, opioids, etc.	Decedents were found unresponsive in various situations and all manners of death were determined to be accidental.	(109)
5	31-54	2M 3F	Fentanyl: 7.3-35 (BL), 5.1 (VH) Norfentanyl: 0.26-0.53 (BL) Acetylfentanyl: 2.2-7.2 (BL), 1.3 (VH) 3-methylfentanyl: 1.7-2.6 (BL), 0.65 (VH) Furanylfentanyl: 5.5-8.7 (BL), 30 (VH) Carfentanil: 0.33-1.9 (BL)	BL VH	Opioids, cannabinoids, and benzodiazepines were detected in the case samples	Four cases were fatalities in which the descendants were found unresponsive, and autopsy often revealed increased lung weights. In one DUID case, the individual admitted to snorting brown heroin before being involved in a car accident.	(44)
7	44-66	7M	Methoxyacetylfentanyl: 0.025- 0.19 Norfentanyl: 0.5- 3.7 Acetylfentanyl: 0.11-7.7 Fentanyl: 3.8- >20 Cyclopropylfentanyl: 0.063- 2.5 *cis*-3-methylfentanyl: 0.17- 1.3 *trans*-2-methylfentanyl: 0.042- 1.0 β-hydroxyfentanyl: 0.21 *N*-methyl-norfentanyl: >20 Benzylfentanyl: 0.95 Butyrylfentanyl: 0.16	BL	Additional analogs: β-hydroxyfentanyl, 4-ANPP. Other drugs of abuse detected: THC (and metabolites), ethanol, morphine, codeine, 6-MAM, BZE	Five cases were overdose fatalities. In two of the fatalities, no illicit drugs or ethanol was found on scene. Two other cases involved hospitalizations prior to death. The article also presents two DUID cases in which the individuals were involved in motor vehicle collisions (rear-end and unoccupied vehicles). One individual was suspected to be under the influence of alcohol, while the other received 6 doses of naloxone.	(140)

(continued)

n	Age	Sex	Fentanyl Analog Concentration (ng/mL)	Matrix	Other Drugs Present	Notes	Reference
1	23	M	Butyrylfentanyl: 39-66 (BL), 110 ng/g (muscle), 57 ng/g (LV), 160 ng/g (K), 3100 ng/g (lung) 590 ng/g (spleen), 550 ng/g (fat)	BL PM tissues	Butyrylfentanyl metabolites were identified in PM samples.	Traces of white powder and a tube were found with the deceased male. At autopsy, traces if white powder were found in the nose and CT scans revealed brain edema.	(71)
2	25, 34	M	Furanylfentanyl: 0.34 Carfentanil: 0.12- 1.3	BL VH UR	Morphine, hydromorphone, 6-MAM, and hydrocodone were detected in case 1. Benzoylecgonine was detected in case 2.	Case 1 was discovered in a running care with a brown substance nearby. Case 2 was discovered in a tent also with a brown colored substance on scene. Both decedents had a history of abuse and displayed ventricular hypertrophy at autopsy.	(152)

M = male; F = female; UR = urine; BL = blood; LV = liver; VH = vitreous humor; BR = brain; CSF = cerebrospinal fluid; GC = gastric contents; PM = postmortem; 3-MeO-PCP = 3-methoxyphencyclidine; BZE = benzoylecgonine; THFF = tetrahydrofuranfentanyl; 4Cl-α-PVP = 4-chloro-alpha-pyrrolidinovalerophenone; MMMP = 2-methyl-4-methylthio-2-morpholinopropiophenone; THC-COOH = carboxy-tetrahydrocannabinol

In a case report by Martucci *et al.*, two intact and one dissolved blue colored tablets suspected to be oxycodone 30 mg were discovered near the decedent and in his vomit, respectively. The pill was analyzed by GC-MS and screened positive for furanylfentanyl. Although the pill had visual characteristics of an oxycodone tablet, it did not contain any oxycodone. In addition, the decedent had previously admitted to using oxycodone, not fentanyl analogs (147). As previously discussed, illicit drug manufacturers are synthesizing counterfeit pills that look like traditional medications but contain fentanyl; therefore, presenting major danger to naïve drug users. The present case study represents a situation in which a fentanyl analog was disguised as oxycodone and resulted in fatality.

Statement of the Problem

Over a historically short period of time, traditional and synthetic opioids have made a drastic impact on drug abuse culture and overdose deaths in the United States. Fentanyl and fentanyl analogs have recently played a major role in propagating those devastating numbers and continue to pose significant challenges to forensic laboratories due to their high potency, low concentrations, similar molecular structures, and ever evolving prevalence. To combat the opioid crisis, forensic laboratories must stay current with popular drug trends and associated analytical methodologies for the detection of emerging novel psychoactive substances. To address these issues, the present research began by developing data independent HRMS screening techniques for fentanyl analogs in whole blood and oral fluid. Next, quantitative methodologies were optimized and validated to further toxicological knowledge associated with pharmacokinetics, pharmacodynamics, and compound stability of fentanyl analogs. Finally, a quantitative

method for fentanyl analogs in oral fluid was developed for applicability and progression of routine analyses of alternative matrices in forensic toxicology laboratories.

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CHAPTER II

Data-Independent Screening Method for 14 Fentanyl Analogs in Whole Blood and Oral Fluid Using LC-QTOF-MS[1]

This dissertation follows the style and format of *Journal of Analytical Toxicology*.

[1] Palmquist, K.B., and Swortwood, M.J. (2019) Data-independent screening method for 14 fentanyl analogs in whole blood and oral fluid using LC-QTOF-MS. *Forensic Science International*, **297**, 189-197. Reprinted with permission of publisher.

Abstract

Recently, fentanyl analogs account for significant number of opioid deaths in the United States. Routine forensic analyses are often unable to detect and differentiate these analogs due to low concentrations and presence of structural isomers. A data-independent screening method for 14 fentanyl analogs in whole blood and oral fluid was developed and validated using liquid chromatography-quadrupole-time-of-flight mass spectrometry (LC-QTOF-MS). Data were acquired using Time of flight (TOF) and All Ions Fragmentation (AIF) modes. The limits of detection (LOD) in blood were 0.1-1.0 ng/mL and 0.1-1.0 ng/mL in TOF and AIF modes, respectively. In oral fluid, the LODs were 0.25 ng/mL and 0.25-2.5 ng/mL in TOF and AIF modes, respectively. Matrix effects in blood were acceptable for most analytes (1-14.4%), while the nor- metabolites exhibited ion suppression >25%. Matrix effects in oral fluid were -11.7-13.3%. Stability was assessed after 24 hours in the autosampler (4^{o}C) and refrigerator (4^{o}C). Processed blood and oral fluid samples were considered stable with -14.6-4.6% and -10.1-2.3% bias, respectively. For refrigerated stability, bias was -23.3- 8.2% (blood) and -20.1- 20.0% (oral fluid). Remifentanil exhibited >20% loss in both matrices. For proof of applicability, postmortem blood (n=30) and oral fluid samples (n=20) were analyzed. As a result, six fentanyl analogs were detected in the blood samples with furanyl fentanyl and 4-ANPP being the most prevalent. No fentanyl analogs were detected in the oral fluid samples. This study presents a validated screening technique for fentanyl analogs in whole blood and oral fluid using LC-QTOF-MS with low limits of detection.

KEY WORDS: Fentanyl analogs; Postmortem blood; Oral fluid; Novel synthetic opioids; High resolution mass spectrometry

Introduction

Fentanyl (N-phenyl-N-[1-(2-phenethyl)-4-piperidinyl] propanamide) is a synthetic opioid analgesic with 50-100 times the potency of morphine (1-7). Due to its chemical properties, fentanyl distributes more favorably into the tissues and is effective at low doses; therefore, leading to lower concentrations in the blood (7, 2). In addition, fentanyl acts as a full μ-agonist, which activates μ-receptors in the brain resulting in a decrease in the transmission of painful stimuli. For these reasons, fentanyl is often prescribed as a slow-release pain management treatment given orally or transdermally in the form of Actiq ® (lollipop) or Duragesic ® (patch), respectively (1, 2, 8, 7, 4, 5). However, fentanyl not only has a high potential for abuse, but it also exhibits dangerous side effects such as respiratory depression and bradycardia that can lead to fatal overdose (1, 4, 7). Under the Controlled Substances Act of the United States Drug Enforcement Administration (DEA), fentanyl is listed as a Schedule II substance (9).

Synthetic analogs of fentanyl (with potencies up to 10,000 times that of morphine) were rapidly abandoned for clinical use, and are now being produced in clandestine laboratories (1, 3, 4, 8, 5, 10). Recently, fentanyl analogs have flooded the American illicit drug market as cutting agents for heroin under the names “China White” or “Synthetic Heroin” (1, 5, 6, 4). In an effort to diminish the opioid epidemic, the DEA temporarily placed all fentanyl analogs under Schedule I until February 2020 with the possibility of an additional year extension (11). Given that drug users are often unaware of the purity, potency, and chemical composition of their heroin supply, the introduction of these combinations is resulting in synthetic opioid- and heroin-related deaths around the world, particularly in the US (4, 12, 13, 1, 5).

With the emergence of new synthetic fentanyl analogs, the analytical differentiation of structural isomers is essential to identify the toxicant in an acute or fatal overdose. In addition, high sensitivity techniques are required to detect low dose fentanyl analog concentrations in biological matrices such as blood and oral fluid. Oral fluid is an alternative matrix that requires non-invasive collection and represents recent drug use. For these reasons, oral fluid can be a beneficial matrix for antemortem drug testing for workplace cases, treatment facilities, and drivers expected of being under the influence (14). Previous research suggests that detection of clandestine opioids in oral fluid is comparable to detection in urine (14), suggesting its applicability as a matrix of interest.

Previous research has developed and validated detection methods using both gas chromatography (GC) and liquid chromatography (LC) coupled to a variety of mass analyzers. Initial fentanyl analog studies determined retention indices for alkane analogs of fentanyl (8) and valid methods for the simultaneous detection of few fentanyl analogs (15) using GC-MS. However, GC-MS methods may require derivatization of some analytes (15) and lack sensitivity (5). Numerous liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods were developed and validated for the simultaneous detection and quantification of opioids in postmortem specimens (2), the detection of fentanyl and its metabolites in blood and urine (7), the ability to detect and quantify opioids using two mass analyzers (16), and the screening of opioids in postmortem fluids and tissues and its application to authentic samples (5). However, the present study seeks to develop a data-independent screening technique for fentanyl analogs in various matrices that have been isolated by solid phase extraction (SPE). Data-independent screening and SPE were effective for fentanyl analogs in various studies. Noble *et al.*

developed and validated a data-independent screening method for 50 fentanyl analogs in whole blood by LC-QTOF-MS using reference materials and predictive fragmentation patterns (17). Strayer *et al.* and Shaner *et al.* utilized manual and automatic SPE methods when analyzing fentanyl analogs in blood, respectively (18, 6).

To continue to address the emergence of new, low concentration analogs, this study presents a fully optimized SPE method utilizing small sample volumes and a comprehensive, validated screening method for fentanyl analogs (fentanyl, alfentanil, acetylfentanyl, butyrylfentanyl, remifentanil, carfentanil, cis-3-methylfentanyl, 4-ANPP, furanylfentanyl, isobutyrylfentanyl, norcarfentanil, valerylfentanyl, norfentanyl, and sufentanil) in whole blood and oral fluid using liquid chromatography-quadrupole-time-of-flight mass spectrometry (LC-QTOF) analysis. The application of a screening method in conjunction with the production of an in-house library is beneficial for the detection and identification of unknown compounds using data-independent acquisition. Through this non-targeted processing, samples can be data-mined to elucidate emerging novel psychoactive substances (NPS).

Methods

Chemicals and reagents

Certified reference standards of cis-methylfentanyl, 4-ANPP, acetylfentanyl, alfentanil, butyrylfentanyl, carfentanil, fentanyl, furanylfentanyl, isobutyrylfentanyl, norcarfentanil, norfentanyl, remifentanil, sufentanil, and valerylfentanyl were purchased as methanolic solutions from Cerilliant (Round Rock, Texas). Deuterated internal standards of carfentanil-D5, fentanyl-D5, and norfentanyl-D5 were also purchased from Cerilliant. LC-MS grade acetonitrile and formic acid (>95%) were purchased from Fisher

Scientific (Hampton, New Hampshire) and Sigma-Aldrich (St. Louis, Missouri), respectively, for mobile phase preparation. Ultra pure water was produced by a Millipore Direct-Q ® 3UV (Burlington, Massachusetts) purification system. Acetic Acid and dichloromethane used in extraction were from Mallinckrodt Pharmaceuticals (St. Louis, Missouri). Dibasic sodium phosphate and monobasic sodium phosphate used in phosphate buffer (100 mM, pH 6.0) preparation were purchased from Sigma-Aldrich (St. Louis, Missouri). Ammonium hydroxide, hexane, ethyl acetate, and methanol, used in sample preparation and extraction were purchased from J.T. Baker (Center Valley, Massachusetts). CEREX® Clin II SPE cartridges were obtained from SPEware (Baldwin Park, California). Whole bovine blood was purchased from Quad Five (Ryegate, Montana). Quantisal extraction buffer was purchased from Immunalysis Corporation (Pomona, California). Oral fluid was collected via expectoration from drug-free users and pooled for method development and validation after verification by LC-MS/MS.

Standard preparation

Reference standards were diluted with LC-MS grade methanol to working standards at 1000 ng/mL. Serial dilutions and mixtures were prepared as needed for optimization and validation experiments. A mixed methanolic solution of all D0 analytes was prepared at 25 ng/mL and serially diluted to 5 and 2.5 ng/mL. An internal standard (ISTD) solution was prepared at 50 ng/mL. Quality controls were prepared at 5 ng/mL containing all analytes. Diluted standards were labeled and stored at -20°C in amber vials.

Sample preparation and extraction

Whole bovine blood (250 µL) was fortified with the mixed working standards (50 µL of D0 and 12.5µL of ISTD). Samples were buffered with 2.5 mL phosphate buffer, centrifuged for 5 minutes at 2500 g, loaded onto SPE columns, and allowed to flow through under low pressure on a SPEWare System 48™ CEREX® Pressure Processor (Baldwin Park, California). The columns were washed with deionized water (1 mL) and 1M acetic acid (1 mL), and then dried under maximum flow nitrogen for 5 mins. Columns were washed consecutively with hexane (1 mL), ethyl acetate (1 mL), methanol (1 mL), and dichloromethane (1 mL). Analytes were then eluted with 1 mL of 5% ammonium hydroxide in ethyl acetate (v/v) and dried at 50°C under nitrogen in the BiotageTurboVap LV® (Charlotte, NC). Extracts were reconstituted in 50 µL of 50:50 mobile phase.

Oral fluid samples were analyzed according to a validated synthetic opioid protocol (19) with a few minor changes. Briefly, pooled oral fluid (250 µL) was mixed with 3 parts Quantisal buffer (total 1 mL) and fortified with the mixed working standards (50 µL of D0 and 12.5µL of ISTD). Samples were diluted with 2 mL phosphate buffer, loaded onto SPE columns, and allowed to flow through under low pressure. Columns were washed with deionized water and acetic acid, and then dried under nitrogen before washing with hexane, ethyl acetate, and methanol. Analytes were eluted with 1 mL of 5% ammonium hydroxide in ethyl acetate (v/v), similar to the blood extraction. The acidic/neutral fraction was not collected for this assay. Eluents were evaporated to dryness at 50°C under nitrogen and reconstituted in 100 µL of 90:10 mobile phase.

A blank (matrix only) and a negative control (ISTD only) were analyzed first in every batch of samples. A positive control (neat standard at 5 ng/mL) was analyzed daily to verify retention time and instrument performance.

Instrumentation

Analyses were performed using an Agilent Technologies 1290 Infinity liquid chromatograph (Santa Clara, Californiа) coupled to an Agilent Technologies 6530 Accurate Mass Time-of-Flight mass spectrometer. Chromatography was achieved using an Agilent Poroshell 120 EC-C18 column (2.1 x 100mm x 2.7 μm) with matching guard column (2.1x 5 mm x 2.7 μm) in a thermostatically controlled column compartment (35°C). The aqueous and organic mobile phases used were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), respectively. A gradient elution system was used at a 0.4 mL/min flow rate starting at 10% B and increased to 25%B over 2 min. Over the next 5 min, the gradient increased to 50% B before switching to 90% B with a 2 min hold. The column was then allowed to re-equilibrate at starting mobile phase conditions for 2 min. The first 0.25 and last 4 min were diverted to waste. The mass spectrometer was equipped with a Jet Stream electrospray ionization source operating in positive mode under the following conditions: drying gas (N2) temperature- 300^{o}C; drying gas flow rate- 13 L/min; nebulizer pressure- 45 psi; sheath gas temperature- 350^{o}C; sheath gas flow rate- 12 L/min; capillary voltage- 3000V; nozzle voltage- 0V; fragmentor voltage- 150V; skimmer voltage- 65V. Analysis was performed with two separate injections as the QTOF-MS was operated in two modes: Time of Flight (TOF) mode and All Ions Fragmentation (AIF) mode. For TOF mode, all ions from 100-1000 m/z were allowed to pass to the detector and no collision energies were applied. For AIF mode, three

experiments with increasing collision energies (0, 10, 20 eV) were cycled to acquire spectra of fragmentation variation for each analyte. Data were acquired with an acquisition rate of 3 spectra/s and an MS scan rate of 333.3 ms/spectrum. Reference ion solutions were monitored for mass (ppm) correction within each injection.

Software and data processing

Chromatographic and mass spectral information for each compound were using MassHunter Qualitative Analysis Software (B.07.00) and stored to a Personal Compound Database and Library (PCDL). The PCDL contains the following information for 14 fentanyl analogs and 3 internal standards: molecular formula, accurate mass, retention time, and mass spectra (produced from collision energies of 0, 10, 20, 30 eV). Qualification in TOF mode was based the following formula matching parameters: (1) ±0.35 min from the retention time; (2) ±15 ppm from the exact mass; and (3) absolute peak area >10,000 counts. Qualification in AIF mode was based on the following formula matching parameters: (1) ±0.35 min from the retention time; (2) ±15 ppm from the exact mass; and (3) absolute peak area >5,000 (blood) or >2,500 (oral fluid); as well as, the following ion confirmation parameters: (1) S/N >3; (2) presence of two diagnostic ions; (3) ion coelution score > 60; and (4) ±0.15 min of expected retention time. Two additional PCDLs were created containing 138 fentanyl analogs and other drugs of abuse (synthetic opioids, synthetic benzodiazepines, synthetic hallucinogens, and commonly encountered drugs of abuse), respectively. The additional drugs of abuse PCDL contained mass, retention time, and spectral information. Compound detection was performed using the same parameters used to qualify the target fentanyl analogs.

Method validation

Two methods (blood and oral fluid) were developed and validated based on SWGTOX qualitative analysis guidelines (20). Parameters assessed were limit of detection (LOD), interferences, ion suppression/enhancement, carryover, and stability.

Limit of detection

Samples were prepared at 1, 0.5, 0.25, and 0.1 ng/mL (blood) and 2.5, 1, 0.5, and 0.25 ng/mL (oral fluid) in three sources of matrix and assessed in duplicate on three days. The LOD was determined as the lowest concentration in which the analyte was "qualified" in the MassHunter Qualitative Analysis software.

Interferences and carryover

To assess matrix interferences, blank sources of blood (n=10) and pooled oral fluid (n=3) were extracted as above and analyzed using the TOF and AIF methods. The resulting data were assessed to ensure no peaks met qualification criteria for analytes of interest.

To assess stable isotope interferences, negative samples (ISTD only), extracted as above, were analyzed to ensure no peaks met qualification criteria for the non-deuterated analytes of interest. Subsequently, high concentration neat samples with no ISTD were analyzed to ensure no response for the deuterated compounds.

To assess interferences from other commonly encountered analytes, four interference mixes containing 32 drugs (Δ9-tetrahydrocannabinol, alprazolam, amobarbital, amphetamine, amitriptyline, butalbital, caffeine, carbamazepine, carisoprodol, cocaine, codeine, cotinine, cyclobenzaprine, dextromethorphan, diazepam, diphenhydramine, hydrocodone, hydromorphone, ketamine, methadone, nicotine,

nordiazepam, oxazepam, oxycodone, pentobarbital, phencyclidine, phenobarbital, propoxyphene, secobarbital, tetrahydrocannabinolic acid, tramadol, and zolpidem) were extracted and analyzed using the TOF and AIF modes. The resulting data were analyzed for to ensure no peaks met qualification criteria for analytes of interest.

Carryover was assessed by injecting a blank after a high concentration neat standard (100 ng/mL). The resulting data from the blank were analyzed for analyte contamination.

Matrix effects (ion suppression/enhancement) and recovery

Matrix effects and recovery were analyzed using post extraction addition (21). Ten sources of blood and three pooled sources of oral fluid were fortified with D0 and ISTD solutions before and after extraction. Concentrations of the solutions were 5 ng/mL and 10 ng/mL (in matrix), respectively. Neat solutions (n=5 blood; n=3 oral fluid) were also prepared at the same concentrations. For matrix effects, the mean peak areas of each analyte in the post-extraction samples were compared to the mean peak areas of neat standards. Ion suppression/enhancement were considered acceptable within ±25%. For recovery, the mean peak areas of each analyte in the pre-extraction samples were compared to the mean peak areas of post-extraction samples.

Stability

Autosampler stability was assessed by comparing fresh processed samples from three sources of each matrix at 5 ng/mL to the same samples that had been stored in the autosampler for 24 h at 4°C in duplicate. Mean relative peak areas (analyte/ISTD) of both sets of samples were compared. Internal standards were paired based on structural

similarities and retention time. Autosampler stability was determined to be acceptable with bias ±20%.

Refrigerated stability in blood and oral fluid was assessed by comparing fresh extracted samples from three sources for each matrix at 5 ng/mL (in matrix) to 24 hour refrigerated (4°C) samples from the same sources in duplicate. Mean relative peak areas of both sets of samples were compared. Analytes were considered stable if peak area within ±20%.

Authentic specimen collection and handling

Anonymized postmortem blood samples (n=30) were obtained from National Medical Services (NMS) Labs (Willow Grove, PA). All specimens were extracted and screened as described above. Results were compared and verified to those previously reported by NMS. Authentic oral fluid samples (n=20) were obtained from arrestees under an Institutional Review Board (IRB) approved protocol from a Texas adult detention center. All subjects gave written informed consent prior to collection. Oral fluid samples were collected using a Quantisal® collection device (Immunalysis Corporation), refrigerated (4°C), extracted (1 mL of 1:3 oral fluid:Quantisal extraction buffer), and analyzed within 72 h as described above.

Results and Discussion

Method development

Fentanyl analogs were identified based on (1) accurate mass, (2) retention time and (3) qualified product ions (Table 2.1).

Table 2.1. Analyte information used for identification and library matching

Analyte	Chemical Formula	Exact Mass	Retention Time (min)	Precursor Ion $[M+H]^+$	Isotope & product ions
Norfentanyl-D5	$C_{14}H_{15}D_5N_2O$	237.18895	2.385	238.1948	239.1994, 182.1673, 155.1222, 84.0804, 56.0496
Norfentanyl	$C_{14}H_{20}N_2O$	232.15756	2.402	233.1637	234.1674, 177.1373, 150.0921, 84.0806, 56.0496
Norcarfentanil	$C_{16}H_{22}N_2O_3$	290.16304	2.728	291.1692	292.1736, 259.1431, 231.1504, 175.1229, 146.0954, 142.0849, 113.0597
Remifentanil	$C_{20}H_{28}N_2O_5$	376.19982	3.138	377.2098	378.2109, 317.1849, 261.1590, 228.1222, 202.1216, 146.0955, 113.0595
Acetylfentanyl	$C_{21}H_{26}N_2O$	322.20451	3.515	323.2113	324.2156, 188.1439, 134.0961, 105.0705, 84.0811
Alfentanil	$C_{21}H_{32}N_6O_3$	416.25359	4.063	417.2606	418.2639, 385.2347, 314.1860, 268.1768, 197.1282, 99.0581
4-ANPP	$C_{19}H_{24}N_2$	280.19395	4.080	281.2002	282.2049, 188.1436, 134.0965, 105.0701, 55.0548
Fentanyl-D5	$C_{22}H_{23}D_5N_2O$	341.25155	4.165	342.2577	343. 2628, 188.1444, 105.0709, 84.0821
Fentanyl	$C_{22}H_{28}N_2O$	336.22016	4.182	337.2271	338.2297, 216, 1372, 188.1426,105.0691
Furanylfentanyl	$C_{24}H_{26}N_2O_2$	374.19943	4.405	375.2059	376.2098, 188.1437, 105.0703, 84.0814
Carfentanil-D5	$C_{24}H_{25}D_5N_2O_3$	399.25703	4.730	400.2633	401.2682, 368.2377, 340.2440, 284.2171, 246.1493, 134.0962, 113.0598
Carfentanil	$C_{24}H_{30}N_2O_3$	394.22564	4.747	395.2322	396.2369, 335.2112, 279.1844, 246.1484, 134.0959, 113.0592
3-methylfentanyl	$C_{23}H_{30}N_2O$	350.23581	4.747	351.2424	352.2469, 230.1546, 202.1598, 134.0969, 105.0703
Butyrylfentanyl	$C_{23}H_{30}N_2O$	350.23581	4.850	351.2425	352.2468, 188.1436, 134.0961, 105.0702
Isobutyrylfentanyl	$C_{23}H_{30}N_2O$	350.23581	4.850	351.2425	352.2468, 188.1436, 134.0961, 105.0702
Sufentanil	$C_{22}H_{30}N_2O_2S$	386.20280	5.227	387.2095	388.2138, 355.1845, 238.1263, 206.0998, 140.1065, 111.0259
Valerylfentanyl	$C_{24}H_{32}N_2O$	364.25146	5.620	365.2579	366.2621, 188.1433, 105.07002, 57.0706

The optimized chromatographic method was able to detect all compounds within 7 minutes with baseline resolution for most analytes. Analytes that did coelute were ensured to have different exact masses. However, cis-methylfentanyl, butyrylfentanyl, and isobutyrylfentanyl have the same exact mass; therefore, separation of these analytes is required for detection. When analyzed individually and superimposed, butyryl- and isobutyrylfentanyl were distinguished based on retention time, which was also reported by Kahl *et al.* (22). However, when analyzed together, the optimized chromatography was unable to distinguish the isomers. The analytes were joined for detection purposes. No peer reviewed literature has reported the ability to separate these analytes from the same mixture using LC-MS/MS. Cis-methylfentanyl and butyryl/isobutyryl fentanyl were separated; however, full baseline resolution was not achieved (Figure 2.1).

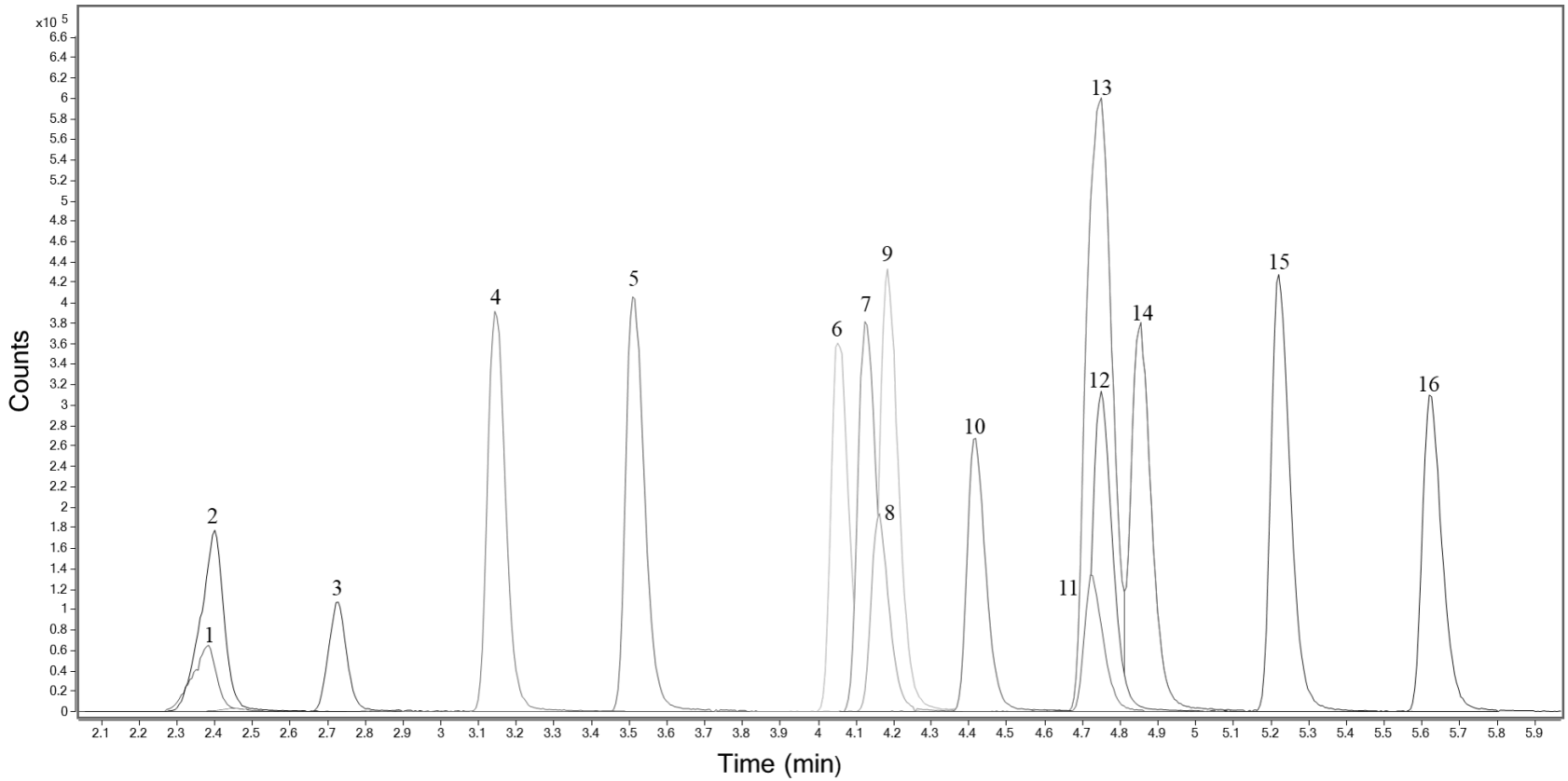

Figure 2.1. Optimized chromatogram of an extracted quality control sample (5 ng/mL) acquired in Time-of-Flight mode. Fentanyl analog and deuterated internal standards are labeled as follows: (1) norfentanyl-D5, (2) norfentanyl, (3) norcarfentanil, (4) remifentanil, (5) acetylfentanyl, (6) alfentanil, (7) 4-ANPP, (8) fentanyl-D5, (9) fentanyl, (10) furanylfentanyl, (11) carfentanil-D5, (12) carfentanil, (13) cis-methylfentanyl, (14) butyryl/isobutyryl fentanyl, (15) sufentanil, (16) valerylfentanyl

A solid phase extraction (SPE) procedure was optimized for the isolation of fentanyl analogs from whole blood and oral fluid. Blood volume, internal standard volume, elution solvent, and reconstitution volume were all addressed as part of the optimization. Final extraction conditions were 250 µL of blood or oral fluid mixture (1:3 oral fluid:Quantisal extraction buffer), 12.5 µL of internal standard, an elution solvent of 5% ammonium hydroxide in ethyl acetate, and reconstitution volumes of 50 µL (blood) or 100 µL (oral fluid).

Data-independent acquisition was achieved using two injections modes: TOF and AIF. The TOF mode proved to have lower LODs for all analytes based on the detection of a precursor ion. While these detection limits were lower, the benefit of having a second injection utilizing AIF mode is fragmentation of the precursor ion. AIF mode can assist with the analysis of a true unknown by obtaining a collaborative spectra with product ions produced at various collision energies. In a study conducted by Caspar *et al.*, it was determined that the combination of a high resolution full scan (HRFS) and AIF acquisition was preferred to a targeted method due to simple integration of new analytes and ability to retrospectively analyze data (23). A data-dependent acquisition requires abundance of a known precursor ion above a threshold; therefore, an unknown or low concentration analyte could pass through undetected. With data-independent screening, all ions passing through to the detector are stored within the data file, which can be data-mined or retrospectively analyzed. Noble *et al.* utilized data-independent screening for the retrospective analysis of fentanyl analogs in 2339 whole blood samples (17). The present study also utilized retrospective analysis by searching for U-47700 metabolites identified by Krotulski *et al.* in authentic samples confirmed for U-47700 (24). While

reference materials for N-desmethyl-U-47700 and N,N-didesmethyl-U-47700 were available in the laboratory, reference materials for the hydroxylated metabolites were not. Retrospective analysis was performed and these metabolites were found within several samples based on precursor ion and manual analysis of fragmentation patterns.

Method validation

LOD data are presented in Table 2.2. Overall, the LOD values ranged from 0.1-1 ng/mL in blood and 0.25-2.5 ng/mL in oral fluid. The blood and oral fluid values were comparable to the LODs reported in methods developed by Shoff *et al.* and Kahl *et al.*, and Griswold *et al.*, respectively (5, 14, 22). In addition, the range of values presented in this method are similar to those discussed in various opioid quantification methods (16, 25). Due to the potency and lipophilic nature of fentanyl related compounds, the resulting concentrations in blood and oral fluid are often low. The lowest concentration in an overdose case was reported by Shanks *et al.* as 0.01 ng/mL (26). The Swedish STRIDA project has reported intoxication case studies of fentanyl, acetylfentanyl, carfentanil, furanylfentanyl, 4-methoxybutyrfentanyl, acrylfentanyl, 4-chloroisobutyrfentanyl, 4-fluoroisobutyrlfentanyl, butyrylfentanyl and tetrahydrofuranfentanyl concentrations in various matrices (serum, urine, and blood) as low as 1.1 ng/mL, 0.14 ng/mL, 0.11 ng/mL, 0.15 ng/mL, 1.3 ng/mL, 0.5 ng/mL, 5 ng/mL, 5 ng/mL, 0.6 ng/mL, and 5 ng/mL, respectively (27, 28, 1). Sofalvi *et al.* reported postmortem and DUID concentrations of fentanyl analogs in blood and vitreous humor ranging from 0.11-15 ng/mL and 0.11-9.8 ng/mL, respectively (29). Although oral fluid has been analyzed qualitatively for fentanyl analogs in overdose emergencies (14), limited reports have been published discussing quantitative data for this matrix. Fatal concentrations of fentanyl analogs in

blood are often higher than those in acute intoxications (10, 30-35, 5). While some of these reported concentrations are lower than the LOD in this study, those analytes were detected in combination with ethanol and other drugs of abuse that may have attributed to cause of death (26, 5, 36-39). Death investigations often provide drug use history and physical scene information indicating opioid use (30, 35, 40, 31, 34, 33, 38). If fentanyl analogs are suspected but missed in preliminary immunoassays or screens, samples may be refluxed to more targeted or sensitive confirmatory techniques. Most concentrations reported in the literature are above the LODs presented in this study, suggesting that the method is sensitive, reliable, and practical for detecting fentanyl analogs in acute or fatal intoxications.

Table 2.2. Limit of detection (ng/mL) of fentanyl analogs using two data acquisition modes by LC-QTOF

	Whole Blood		**Oral Fluid**	
Analyte	*TOF Mode*	*AIF mode*	*TOF Mode*	*AIF Mode*
Norfentanyl	0.5	>1.0	0.25	2.5
Norcarfentanil	1.0	1.0	0.25	0.25
Remifentanil	0.25	1.0	0.25	0.5
Acetylfentanyl	0.25	1.0	0.25	0.5
Alfentanil	0.1	0.5	0.25	0.25
4-ANPP	0.1	>1.0	0.25	2.5
Fentanyl	0.1	1.0	0.25	0.5
Furanylfentanyl	0.25	0.5	0.25	0.5
Carfentanil	0.1	0.25	0.25	0.5
Cis-methylfentanyl	0.25	0.1	0.25	0.25
Butyryl/Isobutyryl fentanyl	0.25	0.1	0.25	0.25
Sufentanil	0.1	0.25	0.25	0.25
Valerylfentanyl	0.1	0.1	0.25	0.25

TOF = time-of-flight; AIF = All Ions fragmentation

No interferences from the matrices, stable isotope internal standards, or commonly encountered analytes were detected. No carryover was observed.

Matrix effects and recoveries for each analyte are summarized in Table 2.3. Recovery data ranged from 57.2-103.0% and 71.0-116.5% for blood and oral fluid, respectively. Matrix effects in blood were considered acceptable for most analytes (1-14.4%), while the nor- metabolites exhibited ion suppression >25%. This was not determined to be problematic as the sensitivity of the present method is sufficient to identify parent drug without the need for incorporation of metabolites. Sofalvi *et al.* quantified norfentanyl in ante- and postmortem blood samples with concentrations ranging from 0.11-3.5 ng/mL and 0.10-3.7 ng/mL, respectively (29). Poklis *et al.*, quantified nor- metabolites in postmortem redistribution analyses with concentrations exceeding 1 ng/mL (41). Similarly, Chatterton *et al.*, quantified norfentanyl in postmortem blood samples with mean femoral, iliac, and subclavian blood concentrations of 4.6, 4.6, and 7.4 ng/mL, respectively (42). The literature mostly reports concentrations of norfentanyl in blood at or above the described LOD. Although the matrix effects of these analytes exceeds the threshold of ±20%, the presented method should be sufficiently sensitive to detect the nor- metabolites at forensically relevant concentrations. In oral fluid, matrix effects ranged from -11.7- 13.3%, suggesting acceptability. Overall, when comparing each analyte to its respective internal standard, the matrix effects values were similar, thus suggesting suitable compensation for matrix effects and extraction recovery.

Table 2.3. Matrix effects and recovery for fentanyl analogs in whole blood (n=10) and oral fluid (n=3) resulting from post extraction addition at 5 ng/mL

	Whole Blood		**Oral Fluid**	
Analyte	*Matrix Effects (%)*	*Recovery (%)*	*Matrix Effects (%)*	*Recovery (%)*
Norfentanyl	-35.2	103.0	-11.7	104.7
Norfentanyl D5	-37.8	90.3	-5.3	107.2
Norcarfentanil	-28.2	98.7	1.2	116.5
Remifentanil	14.4	86.5	2.5	88.8
Acetyl fentanyl	3.1	80.2	-6.6	79.3
Alfentanil	1.0	89.3	1.0	84.8
4-ANPP	9.1	80.3	-8.7	71.0
Fentanyl D5	7.3	71.1	-3.9	85.9
Fentanyl	4.6	74.6	-3.9	83.3
Furanyl fentanyl	4.7	62.8	-6.3	79.8
Carfentanil D5	7.2	69.4	-5.1	84.9
Carfentanil	4.4	76.3	-6.4	83.9
Cis-methyl fentanyl	4.0	75.5	-2.5	82.0
Butyryl/Isobutyryl fentanyl	2.7	65.6	-4.1	82.2
Sufentanil	4.8	63.6	-2.2	87.5
Valeryl fentanyl	2.2	57.2	13.3	88.7

Analyte stability is detailed in Table 2.4. For autosampler stability, bias results were -14.6- 4.2% for blood and -10.1- 2.3% for oral fluid. All analytes remained stable in the processed samples. For refrigerated stability, bias results were -23.3- 8.2% for blood and -20.1- 20.0% for oral fluid. All analytes remained stable in matrix in the refrigerator, except for remifentanil with a >20% loss in both matrices. The loss of remifentanil may be attributed to its known rapid metabolism as reported in blood (43). Analyte stability was assessed based on relative peak area to one of the three internal standards targeted in this study. While deuterated internal standards for each analyte were not investigated, those targeted were paired to analytes based on similar molecular structure and retention time. Further stability studies should be performed using matched internal standards or quantitative analyses.

On occasion, processed samples may have to be stored for more than 24 h in forensic laboratories that do not operate seven days a week. For each stability study, longer time intervals should be examined to ensure stability of these analytes in various temperatures and conditions. Shorter (24 h) time intervals were chosen in this study due to safety protocols and practices in the laboratory.

Table 2.4. Processed and refrigerated sample stability bias values resulting from the analysis of three matrix sources in duplicate

		Whole Blood		**Oral Fluid**	
Analyte	Paired ISTD	*Processed Stability*	*Refrigerated Stability*	*Processed Stability*	*Refrigerated Stability*
		Bias (%, n=3 in duplicate at 5ng/mL)			
Norfentanyl	Norfentanyl-D5	-1.0	-7.2	-0.1	-1.0
Norcarfentanil	Norfentanyl-D5	2.7	-5.5	2.3	-1.7
Remifentanil	Norfentanyl-D5	-6.9	**-23.3**	0.4	**-20.1**
Acetylfentanyl	Fentanyl-D5	-10.0	-7.3	-0.7	-6.9
Alfentanil	Fentanyl-D5	-14.6	-16.1	-10.1	-16.1
4-ANPP	Fentanyl-D5	2.5	-2.4	2.0	-12.9
Fentanyl	Fentanyl-D5	-1.3	-11.4	-1.8	-1.3
Furanylfentanyl	Carfentanil-D5	-4.0	5.5	-3.4	7.4
Carfentanil	Carfentanil-D5	0.8	8.2	-3.5	4.3
Cis-methyl fentanyl	Carfentanil-D5	4.2	6.5	-0.7	-0.2
Butyryl/Isobutyryl fentanyl	Carfentanil-D5	2.9	5.7	2.2	20.0
Sufentanil	Carfentanil-D5	4.6	-6.9	1.5	16.3
Valerylfentanyl	Carfentanil-D5	-0.6	-6.6	-1.1	-1.0

Authentic case samples

In the postmortem blood samples (n=30), the following were detected: furanyl fentanyl (n=16), 4-ANPP (n=15), cis-methylfentanyl (n=4), fentanyl (n=2), norfentanyl (n=2) and valerylfentanyl (n=1). Another synthetic opioid, U-47700 and its metabolites (N-desmethyl-U47700 and N,N-didesmethyl-U47700) were also detected (n=15). Additional illicit and over the counter substances detected are listed in Table 2.5.

Table 2.5. Authentic postmortem blood sample findings

	Analytes Detected	
Sample Number	*Fentanyl Analogs*	*Other Findings (Detected by Present Method)*
1	4-ANPP* Furanylfentanyl	Alprazolam, Caffeine, Diphenhydramine, N-desmethyl-U-47700*, N,N-didesmethyl-U-47700*, U-47700
2	Cis-methyl fentanyl	Caffeine, Diphenhydramine
3	None	Nicotine/ Cotinine
4	None	Cotinine, Etizolam, N-desmethyl-U-47700*, N,N-didesmethyl-U-47700*, U-47700
5	4-ANPP* Furanylfentanyl	Cotinine
6	4-ANPP* Furanylfentanyl	Cotinine
7	None	N-desmethyl-U-47700*, N,N-didesmethyl-U-47700*, U-47700, 7-hydroxymitragynine*, Mitragynine
8	Cis-methyl fentanyl	6-MAM, Buprenorphine, Cocaine*, Codeine, Cotinine, Diphenhydramine, Morphine
9	None	Alprazolam*, Diphenhydramine, N-desmethyl-U-47700*, N,N-didesmethyl-U-47700*, U-47700
10	4-ANPP Furanylfentanyl	Nicotine/Cotinine, Diphenhydramine, Morphine
11	None	Caffeine, Diazepam, Diphenhydramine, Hydrocodone, Morphine, N-desmethyl-U-47700*, N,N-didesmethyl-U-47700*, U-47700
12	Cis-methyl fentanyl	Cotinine/Nicotine, Pseudoephedrine
13	4-ANPP Furanylfentanyl	Alprazolam*, Cotinine, Oxycodone, N-desmethyl-U-47700*, N,N-didesmethyl-U-47700*, U-47700
14	4-ANPP* Furanylfentanyl	Cotinine/Nicotine, Cyclobenzaprine, Lorazepam, Morphine, Oxycodone
15	Cis-methyl fentanyl	Caffeine

(continued)

	Analytes Detected	
Sample Number	*Fentanyl Analogs*	*Other Findings (Detected by Present Method)*
16	4-ANPP* Furanylfentanyl	Caffeine, Cyclobenzaprine, Diphenhydramine*, Ketamine*, Morphine, 6-MAM
17	4-ANPP Furanylfentanyl	Caffeine, Cocaine, Cotinine/Nicotine, Diphenhydramine*, Hydrocodone, Morphine, Oxycodone, N-desmethyl-U-47700*, N,N-didesmethyl-U-47700*, U-47700
18	4-ANPP Furanylfentanyl	Caffeine, Diphenhydramine, 7-hydroxymitragynine*, Mitragynine, Oxycodone, ZolpidemN-desmethyl-U-47700*, N,N-didesmethyl-U-47700*, U-47700
19	4-ANPP* Furanylfentanyl	Cotinine, Amitriptyline*, Morphine
20	None	Cocaine, Etizolam*, 7-hydroxymitragynine*, Mitragynine*, N-desmethyl-U-47700*, N,N-didesmethyl-U-47700*, U-47700
21	4-ANPP* Furanylfentanyl Valerylfentanyl*	Alprazolam, Caffeine, Cocaine, Dextromethorphan, Oxycodone
22	4-ANPP Furanylfentanyl	Diphenhydramine, N-desmethyl-U-47700*, N,N-didesmethyl-U-47700*, U-47700
23	Fentanyl Norfentanyl (low abundance)*	Cyclobenzaprine, N-desmethyl-U-47700*, N,N-didesmethyl-U-47700*, U-47700
24	Fentanyl Furanylfentanyl Norfentanyl (low abundance)	Alprazolam, Caffeine, Cotinine, Morphine, Oxycodone*
25	None	Cotinine/Nicotine, N-desmethyl-U-47700*, N,N-didesmethyl-U-47700*, U-47700
26	Furanylfentanyl	Cotinine/Nicotine, Caffeine, Cocaine
27	4-ANPP Furanylfentanyl	Alprazolam*, Diphenhydramine, N-desmethyl-U-47700*, N,N-didesmethyl-U-47700*, U-47700
28	None	Cyclobenzaprine, Etizolam*, Diphenhydramine*, Pseudoephedrine*, N-desmethyl-U-47700*, N,N-didesmethyl-U-47700*, U-47700
29	4-ANPP* Furanylfentanyl	Ketamine*
30	None	Cotinine/Nicotine, Caffeine, 7-hydroxymitragynine*, Mitragynine, N-desmethyl-U-47700*, N,N-didesmethyl-U-47700*, U-47700

*Indicates a compound detected by the present method, but not reported by the previous laboratory

Most fentanyl analogs detected were consistent with the findings previously determined by the submitting laboratory. However, the present method did not include para-fluoro-fentanyl, which was indicated in one of the samples. Conversely, the present method detected valeryl fentanyl in a sample, which was not previously reported. In some instances, 4-ANPP was detected, but not indicated by the previously laboratory, suggesting the sensitivity of the present method. One limitation of the present method is that some compounds detected by the previous laboratory are not available in the PCDL, therefore, were undetected by the present method. In addition, the present method is more suitable for the analysis of basic drugs, which may limit the ability to detect drugs more acidic in nature.

In the oral fluid samples (n=20), there were no fentanyl analogs detected. Morphine or heroin was indicated in 6 cases. Additional drugs of abuse detected are summarized in Table 2.6.

Table 2.6. Frequency of common drugs of abuse detected in the authentic oral fluid samples (n=20)

Drug of Abuse	Prevalence
Methamphetamine	16
Amphetamine	14
Cocaine	6
Morphine/Heroin	6
Codeine	2
Alprazolam	1

Conclusion

A comprehensive screening method for the detection of fentanyl analogs in whole blood and oral fluid using LC-QTOF instrumentation was developed and validated according to SWGTOX guidelines. The goal of this study was to not only detect similar structure compounds using high resolution mass spectrometry, but also to detect forensically relevant concentrations of fentanyl analogs in biological matrices. As a result, all fourteen fentanyl analogs were successfully detected and limits of detection were as low as 0.1 ng/mL, suggesting the high sensitivity associated with this method. Although limited case reports are published describing fentanyl analog concentrations in oral fluid, the present method is believed suitable for the effective screening of this matrix for the aforementioned reasons. The method has also proved applicable to additional drugs of abuse through the analysis of an authentic case samples and the use of an expanded PCDL. Data-independent screening methods via LC-QTOF provide useful data for forensic laboratories because they allow for non-targeted analysis and offer the ability to retrospectively analyze pre-existing data files as NPS are added to libraries; therefore, serving as an essential tool in the investigation of known and unknown compounds.

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CHAPTER III

Quantification of Furanylfentanyl and its Metabolites in Human and Rat Plasma Using LC-MS/MS[2]

This dissertation follows the style and format of *Journal of Analytical Toxicology*.

[2] Palmquist, K.B., and Swortwood, M.J. (2020) Quantification of Furanylfentanyl and its Metabolites in Human and Rate Plasma Using LC-MS/MS. *Journal of Analytical Toxicology*, **44**, 589-595. Reprinted with permission of publisher.

Abstract

Fentanyl analogs (novel and traditional) continue to impact the ever-growing opioid epidemic. Furanylfentanyl (FuF) is one analog equipotent to fentanyl that has documented involvement in thousands of intoxication and fatality cases around the world. Due to its prevalence, toxicologists need to improve detection and understanding of this analog. A method for the quantification of FuF and its metabolites (4-ANPP, furanyl norfentanyl (FuNorF)) in a small volume (100 µL) of human plasma by LC-MS/MS was developed and validated according to ANSI/ASB Standard. The method was cross validated in rat plasma for a future pharmacokinetic (PK)/pharmacodynamic (PD) study. In human plasma, calibration ranges were 0.025- 25 ng/mL (FuF and 4-ANPP) and 0.5-25 ng/mL (FuNorF). Limits of detection were 0.0125 ng/mL (FuF and 4-ANPP) and 0.25 ng/mL (FuNorF). Lower limits of quantification coincided with lowest calibrator concentrations of 0.025 ng/mL (FuF and 4-ANPP) and 0.5 ng/mL (FuNorF). Precision and bias values were determined to be acceptable for all analytes. Matrix effects were acceptable for all analytes (-8.6-25.0%), except FuNorF with suppression >25%. Extraction recoveries ranged from 84.5- 98.1%. No carryover or endogenous interferences were observed. Qualitative interferences with 4-ANPP were observed from some n-acyl substituted fentanyl analogs predicted to be low-concentration standard impurities. Analytes were stable under all conditions and dilution integrity was sustained. The method was successfully cross-validated in rat plasma with acceptable bias (-7.4-8.4%), precision (within-run <19%CV and between-run <12.6%CV), matrix effects (-9.3- 17.2%, except FuNorF with >25% suppression), recoveries (79.2- 94.5%), and dilution integrity (1/2 and 1/10).

KEY WORDS: Novel synthetic opioids; Fentanyl analogs; Furanylfentanyl; LC-MS/MS

Introduction

Fentanyl analogs represent a group of novel synthetic opioids (NSOs) contributing to the exponential increase in overdose deaths in the United States (1-4). Although first synthesized to develop alternatives for fentanyl, few analogs have legitimate medical use (1, 5, 3). For this reason, the Drug Enforcement Administration (DEA) began to schedule emerging analogs as they appeared in the illicit drug market (1, 6). Fentanyl analog prevalence remained gradual until 2013 when law enforcement and forensic laboratories began to see an influx of cases (7, 8) Over the years since then, novel fentanyl analogs have continued to emerge and instigate death (2, 9, 1, 3, 10-12). In 2018, the DEA placed a temporary blanket schedule on all analogs containing the fentanyl backbone structure until February 2020 (13). The rapid pace at which these analogs enter forensic toxicology laboratories makes detection, quantification and understanding of these compounds difficult.

One such analog is furanylfentanyl (N-(1-(2-phenylethyl)-4-piperidinyl)-N-phenyl-furan-2-carboxamine), which was synthesized in 1986, but not detected by forensic laboratories until 2016 (14, 11, 4, 12, 15). In 2017, FuF ranked among the top 25 drugs identified by laboratories submitting to the National Forensic Laboratory Information System (NFLIS) with an estimate of almost 5,000 cases (16). FuF is classified as a schedule I substance (17) with a potency suspected as being equipotent to fentanyl (18). Numerous case studies around the world have been reported involving FuF (14, 19-21, 2, 3, 10). However, this analog is often involved in polydrug intoxications (14, 2, 22, 20, 3, 10, 23, 19, 11). For example, Kahl *et al.* analyzed 321 postmortem cases and FuF was detected with other drugs in 62 of those cases, while it was the only finding

in a single case (23). Additionally, Guerrieri *et al.* presented 7 fatalities in Sweden involving FuF (5 polydrug intoxications) at concentrations ranging from 0.38-2.74 ng/g. The authors also suggest that FuF contributed to cause of death; however, defining a lethal concentration is difficult to do with limited information on pharmacodynamic/pharmacokinetic (PD/PK) interactions and individual user's opioid tolerance (2).

In order to address gaps in the knowledge surrounding PD/PK properties of FuF, metabolite detection is imperative. Few metabolism studies have been conducted on FuF. Goggin *et al.* examined FuF metabolites through hydrolysis studies and the analysis of high-resolution mass spectrometry data resulting from FuF positive urine specimens. The major metabolites identified in more than 80% of the specimens were a dihydrol metabolite, 4-anilino-N-phenethyl-piperidine (4-ANPP), and an ANPP-sulfate conjugate. Furanyl norfentanyl (FuNorF) was identified as a minor metabolite in some samples (24). In addition, Watanabe *et al.* performed in vivo and in vitro metabolism studies using human hepatocyte incubations and the analysis of postmortem urine samples (analyzed with and without hydrolysis). As a result, the authors suggest major metabolite formation by amide hydrolysis and dihydrol formation (25). Ultimately, both metabolism studies agree on the formation of a dihydrol metabolite, 4-ANPP, and FuNorF (minor). Due to difficult synthesis and instability, the dihydrol- metabolite is not currently commercially available.

Previous research has successfully quantified FuF and 4-ANPP in various biological matrices using LC-MS/MS (3, 10, 21, 24, 2, 23, 11, 9) and GC-MS (5, 14). As previously mentioned, 4-ANPP is known in the toxicological community as a metabolite

(24, 25), as well as a precursor for the illicit production of fentanyl (26). Presently, detection of 4-ANPP is indicative of metabolism and lends to determination of relevant biomarkers of FuF use. To the authors' knowledge, there are limited studies quantifying furanyl norfentanyl, a minor metabolite (9, 24). Additionally, while FuF has not been studied in humans, few PD/PK parameters have been determined in animal models (18). The objective of this study is to develop and validate a quantitative method for FuF and its metabolites in human and rat plasma using LC-MS/MS for a future controlled drug administration study in an animal model. In order for FuF (and other fentanyl analogs) to be better understood by the forensic toxicology community, analytical methods should detect and quantify metabolites for determining parent: metabolite ratios and relevant biomarkers associated with fentanyl analog use (intoxication or fatal). These methods should then pair analytical information with behavioral observation to derive PD/PK data in animals (rats) to be extrapolated to the human system.

Methods

Chemicals and reagents

Certified reference standards of FuF, 4-ANPP and corresponding deuterated internal standards (FuF -D5 and 4-ANPP-D5) were purchased from Cerilliant (Round Rock, Texas). A certified reference standard of FuNorF was purchased from Cayman Chemical (Ann Arbor, Michigan). Pooled human plasma containing K2 EDTA anticoagulant was purchased from Innovative Research (Novi, Michigan) and pooled Sprague-Dawley rat plasma preserved with sodium heparin was from Bio IVT (Medford, Massachusetts). Mobile phase preparation required deionized water produced by a Millipore Direct-Q® 3UV (Burlington, Massachusetts), and LC-MS grade acetonitrile

and formic acid (>95%) from Fisher Scientific (Hampton, New Hampshire). Extraction was performed using CEREX® Clin II SPE cartridges from Tecan (Baldwin Park, California). Di- and monobasic sodium phosphate used for the preparation of phosphate buffer (100 mM, pH 6.0) were purchased from Sigma-Aldrich (St. Louis, Missouri). Acetic acid used in the extraction process was obtained from Mallinckrodt Pharmaceuticals (St. Louis, Missouri). Additional extraction solvents of hexane, ethyl acetate, methanol, and ammonium hydroxide were purchased from J.T. Baker (Center Valley, Massachusetts).

Standard preparation

Individual working solutions were combined to prepare the highest mixed analyte calibrator at 250 ng/mL for all analytes. Serial dilutions were then performed to prepare mixed methanolic calibrators at concentrations of 100, 50, 25, 10, 5, 1, and 0.25 ng/mL. When fortified in plasma (100 µL), analytes were at a final concentration of 25, 10, 5, 2.5, 1, 0.5, 0.1, and 0.025 ng/mL, although the 0.1 and 0.025 ng/mL calibrators were dropped for the FuNorF curve. Individual working solutions were combined to prepare mixed methanolic quality controls (QCs) at concentrations of 200, 20, and 15/0.75 ng/mL, which corresponded to concentrations of 20 (High QC, HQC), 2 (Medium QC, MQC), and 1.5/0.075 (Low QC, LQC) ng/mL when fortified in plasma. FuF and 4-ANPP were prepared at 0.75 ng/mL in the LQC solution. The concentration of FuNorF was increased to 15 ng/mL during LQC preparation by fortifying with a calculated volume of the methanolic working solution. QC solutions were prepared from the same stock solutions as calibrators but on different days by different individuals. An internal

standard (ISTD) solution was prepared at a concentration of 20 ng/mL in methanol (2 ng/mL in plasma). All solutions were stored in the freezer (-20ºC) in amber vials.

Sample preparation and extraction

Samples were extracted based on the procedure developed by Smith et al (27). Briefly, plasma (100 µL samples, calibrators, or QCs) were fortified with 900 µL of phosphate buffer (100 mM, pH 6.0) and ISTD. Samples were loaded onto SPE cartridges on a SPEWare System 48™ CEREX® Pressure Processor (Baldwin Park, California) and allowed to flow through under pressure. The cartridges were washed with ultra-pure deionized water and 1M acetic acid and then dried under maximum pressure for 5 mins. Following additional washes of hexane, ethyl acetate, and methanol, analytes were eluted with 1 mL of 5% ammonium hydroxide in 80:20 dichloromethane:isopropanol (v/v). Eluents were evaporated to dryness at 50°C under nitrogen in the BiotageTurboVap LV® (Charlotte, North Carolina). Extracts were reconstituted in 50 µL of 75:25 mobile phase (A:B), with 5 µL injected into the LC-MS/MS system.

Instrumentation

Analysis was performed using an Agilent Technologies 1290 Infinity II liquid chromatograph (Santa Clara, California) coupled to an Agilent Technologies Ultivo Triple Quadrupole System equipped with JetStream electrospray ionization operated in positive mode. Separation was achieved using an Agilent Infinity Poroshell 120 EC-C18 column (2.1 x 100 mm x 2.7 µm) with matching guard column (2.1 x 5 mm x 2.7 µm) at a constant temperature of 35°C. Mobile phase composition consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). At a flow rate of 0.4 mL/min, gradient elution went from 25% B to 50% B over 3 mins. After 3 mins, the composition

switched to 90% B for 2 mins. The column was then re-equilibrated for 2 mins at starting conditions for a total runtime of 7.2 min. The Ultivo mass spectral conditions were as follows: drying gas temperature 350°C, drying gas flow 12 L/min, nebulizer pressure 25 psi, sheath gas temperature 350°C, sheath gas flow 12 L/min, nozzle voltage 0V, and capillary voltage 2500V. Analytes were detected using dynamic multiple reaction monitoring (dMRM) of one quantifier and one qualifier transition (Table 3.1). Data were analyzed using Agilent MassHunter Quantitative Analysis (Version B.09.00).

Table 3.1. Mass spectrometry parameters: Q1- quadrupole 1, Q3- quadrupole 3

Analyte	Q1 mass (m/z)	Q3 mass (m/z)	Fragmentor Voltage (V)	Collision Energy (eV)	Retention Time (min)	ISTD
Furanyl norfentanyl	271.2 271.2	**188.1** 55.0	102	9 41	0.952	Furanyl fentanyl-D5
4-ANPP	281.2 281.2	**188.1** 104.9	102	9 29	2.073	4-ANPP-D5
Furanyl fentanyl	375.2 375.2	**188.2** 104.9	122	13 37	2.195	Furanyl fentanyl-D5
4-ANPP-D5	286.2 286.2	**188.1** 104.9	107	9 33	2.046	-
Furanyl fentanyl-D5	380.2 380.2	**188.1** 104.9	127	17 41	2.185	-

Quantifying transitions are bolded

Method validation for human plasma

The presented method was validated according to Academy Standards Board: Standard Practices for Method Validation in Forensic Toxicology (28). Parameters assessed were calibration model, bias, precision, limits of detection (LOD), lower limits of quantification (LLOQ), ionization suppression/enhancement, interference studies, carryover, stability, and dilution integrity.

Calibration models were determined using 6 (FuNorF) or 8 (FuF, 4-ANPP) non-zero calibrators over 5 days in human plasma matrix-matched samples for the linear ranges found in Table 3.2. Models were selected based on residual plots and R^2 values >0.99.

Table 3.2. Limit of detection, lower limit of quantification, and calibration model information for FuF and its metabolites in human plasma

Analyte	LOD (ng/mL)	LLOQ (ng/mL)	Calibration Range (ng/mL)	Slope or quadratic constants (mean ± std dev, n=5)	y-intercept (mean ± std dev, n=5)	R^2 (range, n=5)
Furanyl norfentanyl	0.25	0.5	0.5- 25	0.028 ± 0.003	-0.0017 ± 0.0002	0.991- 0.998
4-ANPP	0.0125	0.025	0.025- 25	-0.03 ± 0.05 2.90 ± 0.04	0.009 ± 0.002	0.998- 0.999
Furanylfentanyl	0.0125	0.025	0.025- 25	1.03 ± 0.02	0.0019 ± 0.0003	0.995- 0.997

Precision (within- and between-run) and bias were determined using three QC concentrations (n=3) over 5 runs in human plasma. Values (%CV and %Bias, respectively) were acceptable within ± 20%.

LOD was determined based on sufficient identification criteria of peak shape, signal to noise ≥ 3, retention time matching (± 0.1 min), and ion ratio confirmation (± 20%). LLOQ was defined as the lowest non-zero calibrator. In addition to defined identification criteria, the LLOQ was also required to have acceptable precision and bias (within ± 20%) and signal to noise ≥ 10. LOD and LLOQ were assessed in triplicate over 3 days.

Ion suppression and enhancement were determined using post-extraction addition (29). Five samples of pooled human plasma were fortified with low and high QCs and ISTD before and after extraction. Five neat samples were prepared at the same concentrations. Matrix effects were determined by comparing peak areas of post-extraction samples to neat samples. Extraction recovery was also assessed by comparing peak areas of pre- and post-extraction samples. Matrix effects were determined acceptable within ± 25%.

Matrix and stable isotope interferences were evaluated daily through the analysis of blank (no ISTD) and negative samples (ISTD only). A high concentration sample (at 50 ng/mL) without the addition of ISTD was also analyzed. To assess exogenous interferences, mixes containing 32 commonly encountered drugs of abuse (D9-tetrahydrocannabinol, alprazolam, amobarbital, amphetamine, amitriptyline, butalbital, caffeine, carbamazepine, carisoprodol, cocaine, codeine, cotinine, cyclobenzaprine, dextromethorphan, diazepam, diphenhydramine, hydrocodone, hydromorphone,

ketamine, methadone, nicotine, nordiazepam, oxazepam, oxycodone, pentobarbital, phencyclidine, phenobarbital, propoxyphene, secobarbital, tetrahydrocannabinolic acid, tramadol, and zolpidem) were analyzed on the present method for qualitative interferences at 10,000 ng/mL. In addition, a mix containing 10 alternative fentanyl analogs (fentanyl, norfentanyl, norcarfentanil, butyrylfentanyl, alfentanil, acetylfentanyl, cis-methylfentanyl, valerylfentanyl, sufentanil, and carfentanil) was analyzed neat and extracted (fortified in blank and LQC samples) to assess qualitative and quantitative interferences at 20 ng/mL. Qualitative interferences were evaluated based on retention time and ion ratios (±20%). Quantitative interferences were considered negligible with acceptable quantification bias (±20%) of the target analyte.

Carryover was measured by reinjection of a blank after the highest calibrator over 3 days. Carryover was negligible if analyte response (in the blank reinject) was less than 10% of analyte response in the lowest calibrator.

Dilution integrity and stability were assessed in triplicate. For stability, QCs at two concentration levels were assessed fresh (t_0) and after 48h in the autosampler (4ºC) to determine processed sample stability. Analytes were determined to be stable with acceptable bias (± 20%) compared to t0. For dilution integrity, 1/2 and 1/10 dilutions with blank matrix were performed on samples fortified with the high QC. Dilution integrity was sustained with acceptable precision and bias values (± 20%).

Cross-validation for rat plasma

A cross-validation was performed to ensure proper quantification of analytes in rat plasma against calibration curves prepared with human plasma. Over 3 runs, low (n=3), medium (n=3), and high (n=3) QCs prepared in drug-free rat plasma were

extracted and analyzed to ensure that acceptable precision and bias criteria were met. Matrix effects and recovery were assessed as described above through the comparison of pre (n=3), post (n=3), and neat (n=3) samples at low and high QC concentrations. Ionization suppression/enhancement values were compared between human and rat matrix sources. Stability of target analytes in rat plasma at two concentrations were assessed fresh and after 3 freeze/thaw cycles (-20ºC). Dilution integrity was also assessed in rat plasma at factors of 1/2 and 1/10 of the highest calibrator. Stability and dilution integrity were acceptable with precision and bias values within ± 20%.

Results and Discussion

Method validation (human plasma)

Calibration curves were prepared based on working ranges of 0.025- 25 ng/mL (FuF and 4-ANPP) and 0.5- 25 ng/mL (furanyl norfentanyl). Ranges were selected based on predicted low concentrations of target analytes applicable to an animal model. However, concentrations of FuF and 4-ANPP have been described in the literature to be within that range in human blood (11, 2, 30, 21, 10, 23, 19, 22, 14). While, blood-to-plasma (B/P) ratios have not been reported for the target analytes, B/P ratios for fentanyl and other analogs have been described to range from 0.6-1.3 (31). To the authors' knowledge, Strayer *et al.* is the only published study of reporting furanyl norfentanyl in blood; however, concentration values were not reported (9). While some target analyte concentrations in blood fall outside of the working range of the present study (11, 21, 3, 23), proper sample dilution can account for this limitation. Calibration models were selected based on homoscedasticity of residual plots. Models for FuF and FuNorF were determined to be linear with $1/x^2$ weighting, while 4-ANPP required a quadratic model

and 1/x weighting. The R^2 values for all analytes exceeded 0.991 during validation. Total calibration model data is presented in Table 3.2.

The results for bias and precision at three concentrations are displayed in Table 3.3. Bias values ranged from -5.4- 9.7% for all analytes. Within-run precision ranged from 0.52- 17.2% CV, 0.05- 6.9% CV, and 1.7- 13.9% CV at low, medium, and high QC concentrations, respectively. Between run precision was <±10.8% for all analytes at all concentrations. Overall, all bias and precision values were within the acceptable range described by the ASB Guidelines (28).

Table 3.3. Precision and bias results in human plasma at three QC concentrations

	Bias (%, n=15)			**Maximum Within-Run Precision (%CV, n=3)**			**Between-Run Precision (%CV, n=15)**		
	LQC[a]	*MQC*[b]	*HQC*[c]	*LQC*	*MQC*	*HQC*	*LQC*	*MQC*	*HQC*
Furanyl norfentanyl	5.9	9.7	9.5	17.2	14.2	13.9	10.3	10.8	7.4
4-ANPP	-4.0	-2.0	-4.6	6.8	6.4	10.2	6.1	4.3	6.9
Furanyl fentanyl	2.4	3.7	-5.4	3.1	3.2	3.3	2.7	2.1	2.5

[a]Low QC concentration: 0.075 ng/nL (1.5 ng/mL for furanyl norfentanyl)

[b]Medium QC concentration: 2 ng/mL

[c]High QC concentration: 20 ng/mL

The LOD were determined to be 0.0125 ng/mL (FuF and 4-ANPP) and 0.25 ng/mL (FuNorF). The LLOQ were determined to be 0.025 ng/mL (FuF and 4-ANPP) and 0.5 ng/mL (FuNorF) (Table 3.2). Quantifying and qualifying transitions at the LLOQs are

depicted in Figure 3.1. Bias and precision values resulting from LLOQ experiments ranged from -9.4- 1.5% and 0.06- 0.09%CV, respectively. All detection, identification, bias and precision criteria were met. The presented LOD and LLOQ for FuF and 4-ANPP are lower than previously published studies (30, 21, 23, 9, 32). To the authors' knowledge, these are the lowest LOD/LLOQ reported for these analytes in blood/plasma. Conversely, Strayer *et al.* presented furanyl norfentanyl LOD/LLOQ values of 0.058 and 0.25 ng/mL, respectively, which exceed the sensitivity of the present method for that analyte (9). However, the presented LOD/LLOQ values in this report are predicted to be sufficient for application in a PD/PK study.

Matrix effects and recovery data are detailed in Table 3.4. Analytes exhibited acceptable ion suppression and enhancement in both QC concentration levels ranging from -8.6- 25.0%. Analytes with matched deuterated internal standards had comparable matrix effects values. Furanyl norfentanyl was suppressed >25% in both QC levels. However, ion suppression/enhancement outside the acceptable range was reproducible in pooled plasma and did not interfere with detection or other validation parameters such as, LOD and LLOQ. Extraction recoveries ranged from 84.5- 98.1% encompassing all analytes.

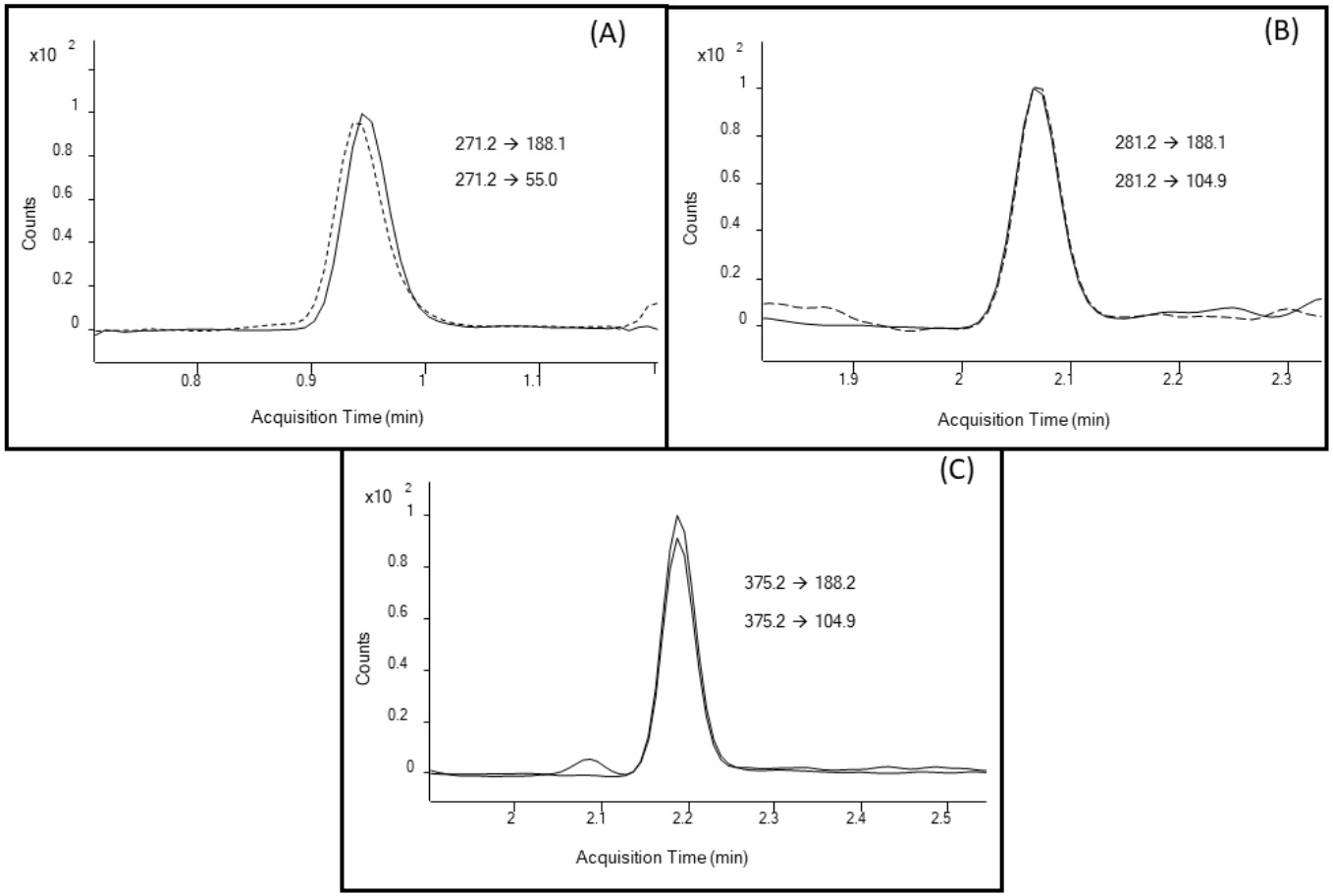

Figure 3.1. Extracted ion chromatogram of quantifier (solid) and qualifier (dashed) ions of FuNorF (A), 4-ANPP (B), and FuF (C) at limit of quantitation concentrations (0.5, 0.025, and 0.025 ng/mL, respectively

Table 3.4. Matrix effects (ionization suppression/enhancement) and recovery data at two concentrations

	Matrix Effects (%, n=5)		**Recovery (%, n=5)**	
	LQC[a]	*HQC*[b]	*LQC*	*HQC*
Furanyl norfentanyl	-49.2	-35.6	93.7	89.3
4-ANPP	8.3	-3.9	98.1	90.2
Furanyl fentanyl	-8.6	-5.0	92.7	86.6
4-ANPP-D5	25.0	15.8	97.4	84.5
Furanyl fentanyl-D5	-8.2	-7.2	95.3	86.0

[a]Low QC concentration: 0.075 ng/mL (1.5 ng/mL for furanyl norfentanyl); [b]High QC Concentration: 20 ng/mL

Matrix and stable-isotope interferences were negligible in blank and negative samples. No interferences were detected qualitatively from other common drugs of abuse. While conducting the interference experiments, samples containing fentanyl analog mix at 20 ng/mL (without targets) and fentanyl analog mix plus LQC were extracted and analyzed (n=2) by the present method. Qualitative interferences were indicated at the retention time of 4-ANPP by presence of a peak with acceptable ion ratios. Analogs included in the mixture were analyzed individually to determine which caused the qualitative interference. N-acyl substituted fentanyl analogs of fentanyl, acetylfentanyl, valerylfentanyl, and butyrylfentanyl were responsible. However, not all n-acyl substituted fentanyl analogs produced an interference, such as FuF. Standards were checked for contamination, which was determined to be negligible. The mechanism of the interference is predicted to be impurity presence at low concentrations in the standard. In

the 20 ng/mL fentanyl analog mix samples, response of 4-ANPP impurity ranged from 464- 630, which equated to 57-78% of the LLOQ response. It should be noted that as the concentration of n-acyl substituted analog increased, the impurity contribution also increased. It should be noted that exogenous interferences are not an issue for the practicality of the present method due to controlled intravenous drug-administration to drug-free rats.

Carryover response was less than 10% of the lowest calibrator response for all analytes analyzed in triplicate; therefore, no carryover is observed.

Processed stability resulted in bias ranges of -3.7- 1.6% and -7.1- 1.2% (compared to t0) in low and high QCs after 48 h in the autosampler (4ºC). All analytes remained stable under these conditions. For dilution integrity, 1/2 and 1/10 dilutions were performed on the HQC resulting in an expected concentration of 10 ng/mL in human plasma. Bias values ranged from -17.1- 2.9% and -11.8-1.6% for 1/2 and 1/10 for all analytes, respectively, suggesting acceptability.

Cross-validation (rat plasma)

The present method was cross validated in rat plasma in order to prove applicable for quantification of FuF and its metabolites as related to an animal model study involving controlled drug administration (Supplemental Table 3.1). Acceptable matrix effects were determined with ranges of -3.5- 17.2% and -9.3- 4.9% in the low and high QCs, respectively. FuNorF still exhibited ion suppression >25% in rat plasma. Extraction recovery values were sufficient, ranging from 77.0-94.5%. Precision and bias of rat plasma generated QCs quantified on human plasma calibration curves presented acceptability. Bias values ranged from -7.4- 4.4% for all analytes at three QC

concentrations. Within-run and between-run precision values were <19.0%CV and <12.6%CV, respectively. Stability after 3 freeze/thaw cycles (-20ºC) was acceptable with %bias ranges of -2.1-4.7% and -5.2- -1.3% in low and high QCs, respectively, for all analytes. Dilution integrity bias values (for both factors) were also acceptable ranging from -19.1- -5.7%.

Conclusion

The presented quantification method for FuF and its metabolites in human plasma by LC-MS/MS was successfully developed and validated according to ASB Guidelines. The method was cross validated in rat plasma for a future controlled administration animal study for the determination of pharmacological data. In addition, detection and quantification of metabolites was achieved to assist with determination of parent:metabolite ratios and forensically relevant biomarkers.

To ensure effectiveness of the present method for an animal model application, low sample volumes and limits of quantification were achieved. Following controlled administration of FuF and behavioral observation, unknown pharmacokinetic and pharmacodynamic information will be derived. This PD/PK modeling will assist the forensic toxicology community with understanding pharmacological mechanisms of a representative fentanyl analog, FuF.

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CHAPTER IV

Long-term Stability of 13 Fentanyl Analogs in Blood[3]

This dissertation follows the style and format of *Journal of Analytical Toxicology*.

[3] Palmquist, K.B., and Swortwood, M.J. (2021) Long-term Stability of 13 Fentanyl Analogs in Blood. *Journal of Analytical Toxicology*, bkab051. Reprinted with permission of publisher.

Abstract

Fentanyl analogs continue to play a major role in proliferating the opioid epidemic in the United States. With high rates of overdose deaths, forensic laboratories experience backlogs, which may lead to false negative results due to drug instability. To address this issue, a quantitative method was validated for fentanyl analogs (3-methylfentanyl, 4-anilino-N-phenethylpiperidine (4-ANPP), 4-fluoro-isobutyrylfentanyl (4-FIBF), acetylfentanyl, acrylfentanyl, butyrylfentanyl, carfentanil, cyclopropylfentanyl, fentanyl, furanylfentanyl, methoxyacetylfentanyl, *p*-fluorofentanyl, and valerylfentanyl) in blood using liquid chromatography- quadrupole-time-of-flight mass spectrometry (LC-QTOF-MS) and used to assess long-term stability under various temperature conditions (-20°C, 4°C, ~25°C, and 35°C) for 9 months. Authentic specimens were also analyzed 6 months apart for applicability to postmortem blood. Method validation resulted in calibration ranges of 1-100 ng/mL and limits of detection (LOD) of 0.5 ng/mL. Precision and bias were acceptable (within ±7.2% coefficient of variation (CV) and ±15.2%, respectively). Matrix effects exhibited ion enhancement for all analytes, except carfentanil and 4-ANPP in low quality control (>25%). For long-term stability, fentanyl analogs (except acrylfentanyl) remained stable under room temperature and refrigerated conditions at low and high concentrations (81.3- 112.5% target) for 9 months. While most fentanyl analogs remained stable frozen, degradation was observed after 2 weeks (4 freeze/thaw cycles). At elevated temperatures, most analytes were stable for 1 week (74.2-112.6% target). Acrylfentanyl was unstable after 24h under elevated (70% loss) and room temperatures (53-60% loss), 48-72h refrigerated (28-40% loss), and 4 weeks frozen (22% loss). In authentic bloods (n=7), initial furanylfentanyl (FuF) and 4-ANPP

concentrations were 1.1-3.6 and 1.4-6.4 ng/mL, respectively. Percent loss of FuF and 4-ANPP over 6 months were 16.3- 37.4% and 0.2- 26.8%, respectively. Samples suspected to contain fentanyl analogs are recommended to be stored refrigerated or frozen with limited freeze/thaw cycles. Due to instability, in the event of an acrylfentanyl overdose, samples should be analyzed immediately or stored frozen with analysis within 1 month.

KEY WORDS: Fentanyl analogs; LC-QTOF-MS; Stability

Introduction

Fentanyl analogs continue to drive the high rates of overdose deaths plaguing the United States (US). According to the Centers for Disease Control and Prevention (CDC), the number of overdose deaths occurring in the 12-month period before May 2020 was over 81,000 (1). During that time, deaths due to illicitly produced fentanyl increased 38.4% from the preceding period (1). From a seized drug standpoint, the National Forensic Laboratory Information System (NFLIS) described over 24,000 reports for fentanyl and fentanyl analogs (4-anilino-N-phenethylpiperidine (4-ANPP), acetylfentanyl, carfentanil, and valerylfentanyl) from July 1-Sept 30, 2020, suggesting continued prevalence (2). While fentanyl analog seizures and deaths have been predominately documented in northeastern/midwestern states (3-8), the CDC reports 98% increase in opioid-related deaths in 10 western states in early 2020 (1). While efforts for the opioid epidemic have been hindered by the COVID-19 pandemic, measures for surveillance and prevention by the CDC remain. Additionally, the temporary scheduling of fentanyl-related substances in the US was extended to May 2021 (9).

Increased backlog is a major concern for forensic toxicology laboratories in regions with high rates of opioid-related fatalities. Following an overdose death, forensic samples may be shipped and stored for extended periods of time before analysis; thus, jeopardizing the detectability of critical drugs of abuse. To account for these potential discrepancies, drug stability should be considered. Short-term stability of fentanyl analogs has been adequately assessed. Fogarty *et al.* evaluated stability of 18 fentanyl analogs in blood and found all analogs stable (except norfentanyl) under frozen conditions (-20°C and -80°C) for 30 days (10). Moody *et al.* investigated the 30-day

stability of fentanyl analogs and other novel synthetic opioids in blood, serum, and urine under room temperature (with- and without light exposure), refrigerated and frozen conditions. For blood and serum, analytes (except acrylfentanyl and MT-45) were stable for at least 2 weeks. For urine, all analytes were stable under refrigeration or frozen conditions for 30 days, and at room temperature for 1 week (11). Jung *et al.* found fentanyl analogs (except remifentanil) were stable in urine for 30 days at 4°C and -20°C. In addition, some instability was observed in blood; therefore, frozen storage was recommended (12). Multiple studies have shown fentanyl analog stability in processed extracts of various matrices ranging from 6-72 hours (13-18) and after 3 freeze/thaw cycles (11, 14).

While short-term stability assessments of fentanyl analogs have been conducted, limited data on long-term stability exist. Extended stability information is critically important when laboratories are faced with backlog. To the authors' knowledge, Kahl *et al.* presents the only long-term stability data for fentanyl analogs to date. In this study, fentanyl and 6 analogs were stored in blood under refrigeration (4°C) and analyzed periodically over 9 months. All analytes were stable (13). The purpose of the present study is to expand upon available literature to determine the long-term stability of 13 prevalent fentanyl analogs (Supplementary Figure 4.1) in blood under four temperature conditions: frozen (-20°C), refrigerated (4°C), room temperature (~25°C), and elevated temperature (35°C). Assessment of temperature can provide information on shipping, handling, and storage conditions for fentanyl-containing samples. Recommendations on case prioritization to ensure analytical accuracies can also be provided.

Experimental

Chemicals and reagents

Certified reference standards of 3-methylfentanyl, 4-ANPP, 4-ANPP-D5, 4-fluoro-isobutyrylfentanyl (4-FIBF), acetylfentanyl, acrylfentanyl, butyrylfentanyl, carfentanil, carfentanil-D5, cyclopropylfentanyl, fentanyl, fentanyl-D5, furanylfentanyl, methoxyacetylfentanyl, *p*-fluorofentanyl, *p*-fluorofentanyl-D3, and valerylfentanyl were purchased from Cerilliant Corporation (Round Rock, Texas). Bovine blood containing sodium fluoride and potassium oxalate preservatives was obtained from Quad Five (Ryegate, Montana). For sample preparation, phosphate buffer (100 mM, pH 6.0) components di- and monobasic sodium phosphate were acquired from Sigma-Aldrich (St. Louis, Missouri). For extraction, acetic acid and dichloromethane were purchased from Mallinckrodt Pharmaceuticals (St. Louis, Missouri) and hexanes, ethyl acetate, methanol, and ammonium hydroxide from J.T. Baker (Center Valley, Massachusetts). For mobile phase preparation, deionized water was produced by Millipore Direct-Q® 3UV (Burlington, Massachusetts). LC-MS grade acetonitrile and additive formic acid (>95%) were acquired from Fisher Scientific (Hampton, New Hampshire). Commonly encountered drugs used to assess interferences (Δ9-tetrahydrocannabinol, alprazolam, amobarbital, amphetamine, amitriptyline, butalbital, caffeine, carbamazepine, carisoprodol, cocaine, codeine, cotinine, cyclobenzaprine, dextromethorphan, diazepam, diphenhydramine, hydrocodone, hydromorphone, ketamine, methadone, nicotine, nordiazepam, oxazepam, oxycodone, pentobarbital, phencyclidine, phenobarbital, propoxyphene, secobarbital, tetrahydrocannabinolic acid, tramadol, and zolpidem) were purchased from Cerilliant Corporation (Round Rock, TX).

Preparation of standards and blood

Certified reference materials of fentanyl analogs at 0.1 mg/mL (except acetylfentanyl at 0.05 mg/mL) were used to separately prepare the highest calibrator and (high) quality control (QC, HQC) at 2500 and 2000 ng/mL, respectively, in methanol. Serial dilutions were performed to create calibrator solutions at 1250, 625, 250, 125, and 25 ng/mL and medium- and low quality controls (MQC, LQC) at 1000 and 75 ng/mL, respectively. An internal standard (ISTD) mixture was prepared at 250 ng/mL in methanol using diluted stock solutions at 1000 ng/mL. All solutions were stored in amber vials in the freezer (-20°C). For stability, bovine blood (100 mL) was fortified with aqueous standard mixes to achieve low and high concentrations of 10 and 80 ng/mL, respectively, as blood QCs. Blood aliquots were transferred into three glass Vacutainer™ tubes per condition and stored in the following ways: room temperature (~25°C), refrigerated (4°C) and elevated (35°C). QCs subjected to frozen conditions (-20°C) were placed in plastic Falcon® tubes for storage. Frozen samples were allowed to thaw before each analysis, then refrozen. Condition temperatures were monitored and determined to be consistent. To establish t_0, blood aliquots (LQC n=3, HQC n=3) were analyzed immediately. At preceding sampling intervals, one aliquot was removed from each tube (n=3) of the temperature conditions at both concentration levels. Samples and calibrators were analyzed after 24 h, 48 h, weekly (for 5 weeks), and then monthly (for 9 months) for 16 timepoints. An additional timepoint of 72 h was monitored for acrylfentanyl. Analyte concentrations were compared to t0 and analytes were considered stable if compounds quantified within ±20%. To investigate degradation products, acrylfentanyl was fortified

into bovine blood (5 mL) at a concentration of 50 ng/mL and stored at room temperature. Aliquots (n=3) were analyzed immediately (t0) and after 4, 8, 12, and 24 h.

Extraction

Samples (blank, negative, fortified) were extracted using a previously published method (16) and analyzed against fresh calibration curves and QCs at 16 timepoints. Briefly, blood (250 µL) was fortified with standard mixes (10 µL calibrator or 10 µL QC, and 10 µL ISTD) and buffered (100 mM phosphate buffer, pH 6.0). Samples were loaded onto CEREX® Clin II SPE cartridges from Tecan (Baldwin Park, California) and extracted using an SPEWare System 48™ CEREX® Pressure Processor (Baldwin Park, California). Columns were washed with aqueous solutions, dried, then washed with organic solvents. Analytes were eluted with 5% ammonium hydroxide in ethyl acetate. Eluents were evaporated at 50°C under nitrogen in the Biotage TurboVap LV Evaporator (Charlotte, North Carolina) and reconstituted in 50 µL of mobile phase (0.1% formic acid in DiH2O: 0.1% formic acid in acetonitrile 75:25, v/v).

Instrumentation

Analysis was performed using an Agilent Technologies 1290 Infinity liquid chromatograph (Santa Clara, California) coupled to a 6530 Accurate Mass Time-of-Flight Mass Spectrometer (Santa Clara, California). Compounds were ionized using Jetstream electrospray ionization in positive mode. Mobile phase consisted of 0.1% formic acid in deionized water (A) and 0.1% formic acid in acetonitrile (B). Separation was achieved using gradient elution of 75:25 (A:B) to 64:36 (A:B) over 6 mins at a 0.4 mL/min flow rate. After elution, composition switched to 90% B for 2 mins and back to starting composition for re-equilibration for 2 mins. Total run time was 10 mins.

Separation occurred on an Agilent Infinity Poroshell 120 EC-C18 column (2.1 x 100 mm, 2.7 µm) with matching guard column (2.1 x 5 mm, 2.7 µm) held at 35ºC. Chromatography is described in Supplementary Figure 4.2. Operating source conditions were as follows: drying gas temperature 300°C, drying gas flow 13 L/min, nebulizer pressure 45 psi, sheath gas temperature 350°C, sheath gas flow 12 L/min, nozzle voltage 0 V, capillary voltage 3000 V and fragmentor voltage 150 V. Data acquisition was performed in targeted mode for quantification. Additional data were acquired in Auto-MS/MS and TOF modes to identify possible degradation products. Precursor ions were targeted in the first quadrupole and fragmented with collision energies ranging from 5-25 eV (Table 4.1). All resulting fragment ions (100-1000 m/z) were collected. Quantification was performed by monitoring two ion transitions (one quantifier and one qualifier) manually selected (based on abundance and reproducibility) from the mass spectrum. Data analysis was performed using Agilent MassHunter Quantitative Analysis (Version B.09.00).

Table 4.1. Mass spectral parameters for analyte quantification

Analyte	Precursor Ion (*m/z*)	Retention Time (min)	Collision Energy (eV)	Quantifier Ion (*m/z*)	Qualifier Ion (*m/z*)	Paired ISTD
Methoxyacetylfentanyl	353.2224	1.60	20	188.1434	105.0702	Fentanyl-D5
Acetylfentanyl	323.2118	1.80	25	188.1429	105.0700	Fentanyl-D5
Acrylfentanyl	335.2118	2.50	20	188.1434	105.0708	Fentanyl-D5
4-ANPP	281.2012	2.60	20	188.1439	105.0705	4-ANPP-D5
Fentanyl	337.2274	2.66	25	188.1437	105.0704	Fentanyl-D5
Furanylfentanyl	375.2067	3.01	20	188.1438	105.0701	Fentanyl-D5
p-fluorofentanyl	355.2180	3.06	25	188.1427	105.0698	*p*-fluorofentanyl-D3
Cyclopropylfentanyl	349.2274	3.17	25	188.1430	105.0694	Fentanyl-D5
3-methylfentanyl*	351.2431	3.55	25	202.1596	105.0705	Carfentanil-D5
Carfentanil	395.2329	3.60	15	335.2114	246.1483	Carfentanil-D5
Butyrylfentanyl*	351.2431	3.79	25	188.1436	105.0709	Fentanyl-D5
4-fluoroisobutyrylfentanyl (4-FIBF)	369.2337	4.10	25	188.1437	105.0700	*p*-fluorofentanyl-D3
Valerylfentanyl	365.2587	5.40	25	188.1433	105.0697	Fentanyl-D5
4-ANPP-D5	286.2326	2.50	20	188.1435	105.0698	-
Fentanyl-D5	342.2586	2.60	25	188.1430	105.0698	-
p-fluorofentanyl-D3	358.2368	3.00	25	188.1435	105.0701	-
Carfentanil-D5	400.2643	3.56	5	340.2433	246.1490	-

ISTD- internal standard; *isobaric compounds

Method validation

The method was validated using Academy Standards Board: Standard Practices for Method Validation in Forensic Toxicology (19) as a guideline. Linearity was determined by analyzing 6 non-zero calibrators on 5 days with R2 values >0.9955. Precision (within- and between-run, % coefficient of variation (CV)) and bias (%bias) were assessed in LQC, MQC, and HQC (n=3) over 5 runs with acceptability within ±20%. Limits of detection (LOD) were analyzed in three sources, in triplicate, over three runs. Chromatographic and identification criteria of signal/noise ≥3, retention time (±0.1 min), and ion ratios (±20%) were assessed. Lower limits of quantification (LLOQ) were evaluated in the same manner and defined by acceptable identification (signal/noise ≥10) and quantification criteria (precision and bias within ±20% of target) as the lowest calibrator. Matrix effects were determined using post-extraction addition (20) of ten blood sources. Ion suppression/enhancement was calculated by dividing mean analyte peak areas from extracted to neat samples, subtracting one, and converting to a percentage. Acceptable matrix effects were ±25%. Endogenous interferences were analyzed in 5 sources of blank blood without ISTD. Stable isotope interferences were determined in blank samples with ISTD (n=5) and a fortified sample without ISTD. Exogenous interferences were assessed by analyzing mixes containing 32 commonly encountered drugs at 400 ng/mL in blood with LQC. Interferences were negligible if LQC quantified within ±20% target concentration. To assess carryover, a blank was reinjected after the highest calibrator. Carryover was insignificant if analyte response in the reinjection was <10% of the lowest calibrator. Processed stability was assessed in LQC and HQC by comparing fresh concentrations to results after 48h storage in the

autosampler (4°C). For short-term stability, LQC and HQC were fortified in blood and stored at room temperature (~25°C) for 24h (n=3) and refrigerated (4°C) for 72h (n=3). Analytes were stable within ±20% bias.

Results and Discussion

Method validation

Linear models with 1/x2 weighting were prepared at a working range of 1-100 ng/mL for all analytes. Calibration curves resulted in average R2 values ≥0.9955. LLOQ were equivalent to the lowest calibrator at 1 ng/mL and LOD were 0.5 ng/mL for all analogs. While fentanyl analog concentrations in blood have reported lower than the detection limits presented (13, 21-25), reports within or exceeding the range are also abundant (10, 13, 23, 24, 26-29); therefore, the linear range was sufficient for authentic sample quantification and long-term stability applications.

Validation data are presented in Table 4.2. Within- and between- run precision was acceptable with ranges of 0.2-7.2 and 2.8-6.3%CV, respectively, encompassing three concentration levels. Bias values were ±15.2%. Acceptable ion enhancement was exhibited by all analogs in LQC and HQC (0.1-18.0%), except 4-ANPP and carfentanil in LQC (25.4 and 30.0%, respectively). Matrix effects were compensated by matched deuterated ISTDs. No endogenous or exogenous interferences were observed through the analysis of daily blanks and negatives or drug-fortified samples. Additionally, no carryover was observed.

Table 4.2. Validation data for fentanyl analogs in blood

Analyte	Linear range (ng/mL), R^2 (n=5)	LOD/LLOQ (ng/mL)	Max within-run precision (n=15, %CV)			Between-run precision (n=15, %CV)			Bias (n=15, %)			Matrix Effects (%)	
			LQC[a]	MQC[b]	HQC[c]	LQC	MQC	HQC	LQC	MQC	HQC	LQC	HQC
Methoxyacetylfentanyl	1-100, 0.997	0.5/1	4.6	4.2	3.9	4.4	3.8	3.4	-10.8	-6.3	-6.5	14.7	6.7
Acetylfentanyl	1-100, 0.996	0.5/1	4.5	4.8	5.5	4.1	3.9	3.7	-12.5	-9.2	-9.9	14.6	8.4
Acrylfentanyl	1-100, 0.997	0.5/1	4.0	2.9	3.7	2.9	3.0	2.9	-10.9	-5.6	-6.7	13.4	6.1
4-ANPP	1-100, 0.995	0.5/1	5.7	4.7	3.4	6.3	5.4	3.2	-6.2	-5.2	-6.4	25.4*	4.3
Fentanyl	1-100, 0.997	0.5/1	7.2	3.4	4.5	4.7	3.7	3.2	-10.5	-6.7	-7.6	13.8	6.2
Furanylfentanyl	1-100, 0.996	0.5/1	5.0	5.5	4.0	3.8	5.2	4.0	-9.5	-5.3	-8.4	11.1	5.8
p-fluorofentanyl	1-100, 0.998	0.5/1	3.2	2.9	3.1	4.0	3.3	3.2	-10.1	-5.5	-3.2	16.2	8.2
Cyclopropylfentanyl	1-100, 0.998	0.5/1	4.3	4.8	3.5	3.6	4.5	3.9	-13.4	-6.6	-5.5	12.8	6.9
3-methylfentanyl	1-100, 0.996	0.5/1	5.2	4.1	1.6	4.3	3.8	3.0	-7.0	4.7	15.2	3.9	0.1
Carfentanil	1-100, 0.997	0.5/1	5.0	3.6	4.3	3.7	3.3	3.6	-12.7	-5.6	0.3	30.0*	17.1
Butyrylfentanyl	1-100, 0.998	0.5/1	4.8	4.5	5.2	3.8	4.4	4.8	-10.8	-4.8	-4.4	13.1	9.2
4-FIBF	1-100, 0.997	0.5/1	4.2	4.7	4.5	3.6	3.2	3.7	-12.7	-7.1	1.5	18.0	9.4
Valerylfentanyl	1-100, 0.999	0.5/1	3.6	4.5	2.8	2.8	4.6	3.6	-9.5	-2.8	-1.5	15.4	7.9

[a]Low QC (LQC) concentration = 3ng/mL; [b]Medium QC (MQC) concentration = 40 ng/mL; [c]High QC (HQC) concentration = 80 ng/mL; *Ion enhancement outside of acceptable criteria, matched ISTD

In processed extracts stored in the autosampler (4°C), all analytes were stable for 48h. Fortified blood samples (with LQC and HQC) were stored at room temperature (~25°C) and under refrigeration (4°C) for 24 and 72hrs, respectively. All analytes were stable at two concentrations under refrigeration with bias values ±8.21%. At room temperature, all analytes were stable (±7.6% bias), except acrylfentanyl with bias of -37.7 and -38.1% in LQC and HQC, respectively.

Long-term stability

For long-term stability, target concentrations were defined as the mean concentration resulting from initial (t0) analysis. Stable compounds quantified within ±20% of the target concentration. Percent loss was determined by subtracting percent target from 100% (baseline concentration). Instability was observed outside of the defined threshold.

Stability at room temperature (~25°C)

At room temperature, fentanyl analogs (except acrylfentanyl) were stable for 9-months at low and high concentrations (Figures 4.1A and 4.2A). Over 9-months, percent target values were 81.3- 112.5% with maximum percent loss of 18.7%. Acrylfentanyl was unstable (percent loss of 60.3 and 53.1% in LQC and HQC, respectively) after 24h. To the authors' knowledge, acrylfentanyl stability is addressed in one other which concluded stability in blood at room temperature for 1 day (11). Acrylfentanyl was undetectable after 3 days in LQC and 21 days in HQC (Figure 4.3).

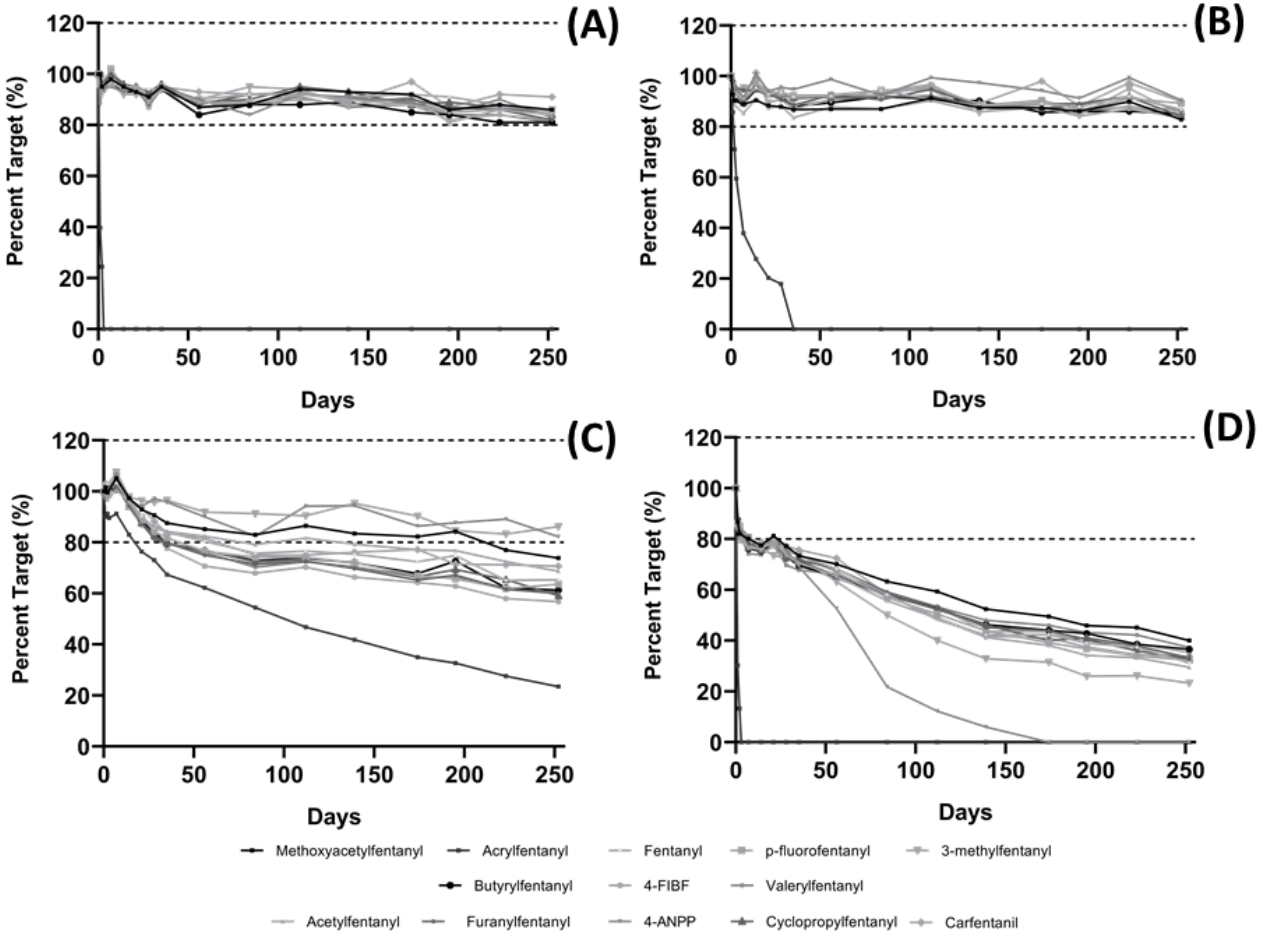

Figure 4.1. Fentanyl analog stability at (A) room temperature, (B) refrigerated temperature, (C) frozen temperature, and (D) elevated temperature in LQC (10 ng/mL) blood samples

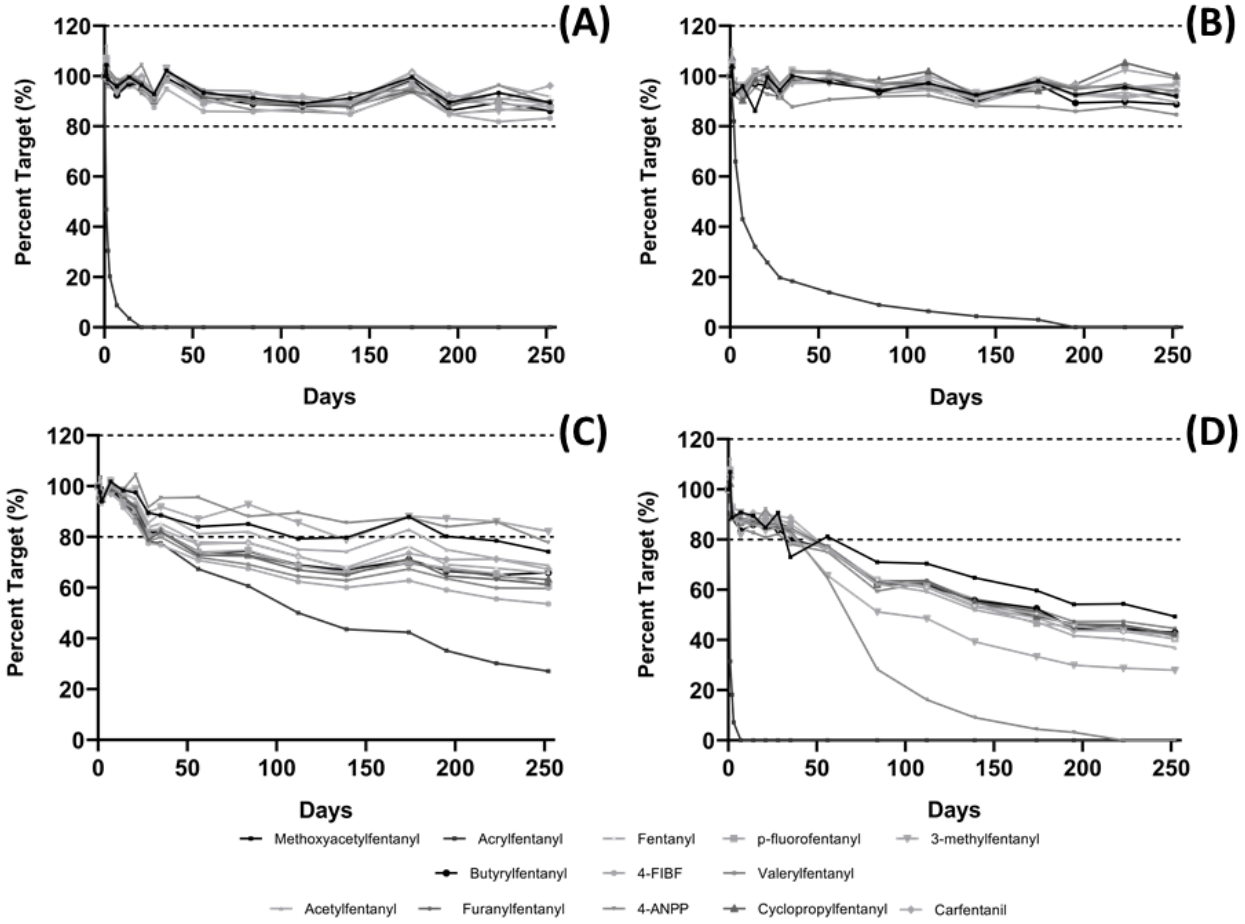

Figure 4.2. Fentanyl analog stability at (A) room temperature, (B) refrigerated temperature, (C) frozen temperature, and (D) elevated temperature in HQC (80 ng/mL) blood samples

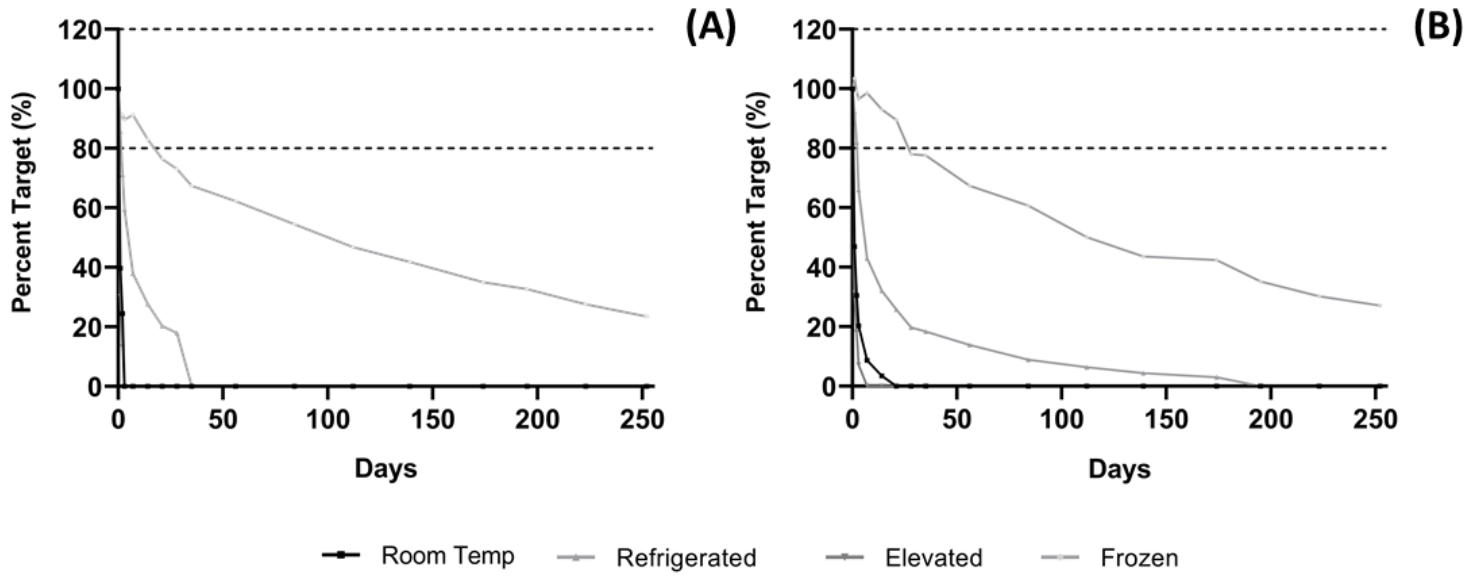

Figure 4.3. Acrylfentanyl stability in (A) LQC (10 ng/mL) and (B) HQC (80 ng/mL) blood samples subjected to room, refrigerated, frozen, and elevated temperatures

Stability at refrigerated temperature (4°C)

Similarly, fentanyl analogs (excluding acrylfentanyl) remained stable under refrigeration for 9-months (Figures 4.1B and 4.2B). Percent target values were 82.9-111.1% with maximum percent loss of 17.1%. These results are consistent with Kahl et al (13). Acrylfentanyl was unstable in LQC after 48h (percent loss 28.9%) and in HQC after 72h (percent loss 34.0%), which differs slightly from a published timeframe of 1 week under refrigeration (11). While accurate concentrations are lost after a few days, acrylfentanyl remained detectable for 35 days in LQC and 6 months in HQC (Figure 4.3). Refrigeration represents optimal storage conditions.

Stability at frozen temperature (-20°C)

Stability data of fentanyl analogs stored frozen are shown in Figures 4.1C and 4.2C. At both concentrations, analytes remained stable for at least 21 days. Acrylfentanyl instability was observed after 21 days in LQC and 28 days in HQC (Figure 4.3). Overall, degradation is apparent after day 7, which equates to 4 freeze/thaw cycles. While the present study agrees with previously published literature signifying fentanyl analog stability after 3 freeze/thaw cycles (11, 14), it is recommended for laboratories not to exceed 4 freeze/thaw cycles to prevent such degradation. After 9-months, fentanyl analog stability varies. For example, analogs such as 4-ANPP and 3-methylfentanyl remained stable for at least 8 months. Conversely, analogs such as furanylfentanyl, *p*-fluorofentanyl, butyrylfentanyl, cyclopropylfentanyl, 4-FIBF, and valerylfentanyl demonstrated variable degradation between 1-2 months in LQC and HQC. Under frozen conditions, although unstable, acrylfentanyl was detectable for the entire study (Figure

4.3). In a forensic laboratory, samples may remain frozen without freeze/thaw cycles before analysis. Fentanyl analog stability in frozen samples may differ.

Stability at elevated temperature (35°C)

To simulate potential temperatures experienced during shipping/handling, fentanyl analogs were stored in a heated bead bath. To the authors' knowledge, this is the first study to examine fentanyl analog stability at elevated temperatures. Most analytes were stable within 1 week (Figures 4.1D and 4.2D), with greater instability observed in LQC. In HQC, analogs (except acrylfentanyl) remained stable for 1 month. After 35 days, degradation is observed for most analogs, suggesting greater instability with prolonged heat. 4-ANPP exhibited rapid degradation after day 56, eventually becoming undetectable. Like room temperature, acrylfentanyl was unstable after 24h with nearly 70% loss at both concentrations. Acrylfentanyl was undetectable within the first week of analysis (3-7 days) (Figure 4.3). In the event of known exposure to elevated temperatures, it is recommended to analyze fentanyl-containing samples within 1 week.

Acrylfentanyl degradation

An intensive stability experiment was performed to investigate the breakdown products of acrylfentanyl. Over 24 h of room temperature storage (26ºC), acrylfentanyl concentration decreased by 90.7%. Degradation half-life in blood was determined to be 7 h (419 min). Analyzing data collected in full scan and Auto MS/MS acquisition modes yielded no detectable breakdown products. While it can be predicted that resulting breakdown products were missed due to instability, more research into this mechanism is required.

Authentic sample analysis

Anonymous postmortem blood samples (n=7) known to contain fentanyl analogs were received from a reference laboratory and stored under refrigeration. Stability was assessed between the time of initial analysis (t0, n=1) and 6 months (n=1). Authentic concentrations and stability data are presented in Table 4.3. Samples contained 4-ANPP (n=7) and furanylfentanyl (n=3) with initial concentrations of 1.1-6.4 and 1.3-3.6 ng/mL, respectively. After 6 months, percent loss ranged from 0.2-26.8% and 16.3-37.4% for 4-ANPP and furanylfentanyl, respectively. A paired t-test was conducted to compare mean 4-ANPP concentrations before and after 6-months. There was no significant difference between the initial mean 4-ANPP concentration (M= 3.2; SD= 4.2) and the mean 4-ANPP concentration after 6-months (M= 2.7; SD= 3.0); $t(6)= 2.38$, $p>0.05$. However, some samples demonstrated >20% loss for both analytes. While this variation does not directly correlate with the presented stability data, differences such as blood source (bovine vs. human), storage container (glass vacutainer vs. plastic microcentrifuge tube), and sample age and composition (postmortem) could account for differences. In one sample, furanyl fentanyl was detected, but <LLOQ in blood after 6 months. This is important to note because it demonstrates the ability to lose valuable information due to low concentrations and analog degradation over time.

Table 4.3. 6 month refrigerated stability of postmortem blood samples (n=7)

Sample Number	4-ANPP			Furanylfentanyl		
	Initial Concentration (ng/mL)	*Final Concentration (ng/mL)*	Percent Loss (%)	*Initial Concentration (ng/mL)*	*Final Concentration (ng/mL)*	Percent Loss (%)
1	3.1	2.5	21.7	2.8	2.1	23.6
2	5.6	5.3	5.1	-	-	-
3	2.7	2.7	0.2	1.1	<LLOQ	-
4	1.4	1.1	22.4	-	-	-
5	6.4	4.7	26.8	3.6	2.3	37.4
6	1.4	1.2	18.9	1.3	1.1	16.3
7	1.6	1.3	20.0	-	-	-

Conclusion

Fentanyl analogs continue to proliferate overdose deaths occurring in the US, which indirectly increases backlog experienced by forensic laboratories. To ensure accurate analyses, analyte stability needs to be understood. A quantification method for 13 fentanyl analogs in blood using LC-QTOF-MS was developed, validated, and applied to a long-term stability study. Prior to this study, limited information on long-term fentanyl analog stability existed. The present study not only expanded upon previous literature, but also determined novel stability information for blood stored under various temperature conditions.

Fentanyl analogs (except acrylfentanyl) were stable for 9 months under room and refrigerated temperatures. Analyte degradation was observed in frozen samples after 4 freeze/thaw cycles. Though analytes were not unstable, it is recommended to limit freeze/thaw cycles to maintain drug integrity. At elevated temperatures (exceeding ~25°C), fentanyl analogs were stable within one week. Acrylfentanyl had the highest degree of instability of all target analytes. Immediate analysis of cases suspected of involving acrylfentanyl would be ideal; however, may not be possible. In the event of an acrylfentanyl overdose, samples should be stored frozen with analysis within 1 month.

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CHAPTER V

Quantification of Fentanyl Analogs in Oral Fluid Using LC-QTOF-MS[4]

This dissertation follows the style and format of *Journal of Analytical Toxicology*.

[4] Palmquist, K.B., and Swortwood, M.J. (2021) Quantification of Fentanyl Analogs in Oral Fluid Using LC-QTOF-MS. Submitted to *Journal of Forensic Sciences.*

Abstract

Oral fluid is a valuable alternative matrix for forensic toxicologists due to ease of observed collection, limited biohazardous exposure, and indications of recent drug use. Limited information is available for fentanyl analog prevalence, interpretation, or analysis in oral fluid. With increasing numbers of fentanyl-related driving under the influence of drug (DUID) cases appearing in the United States, development of detection methods is critical. The purpose of the present study was to develop and validate a quantitative method for fentanyl analogs in oral fluid (collected via Quantisal™) using liquid chromatography-quadrupole-time-of-flight-mass spectrometry (LC-QTOF-MS). Validation resulted in limits of detection and quantification ranging from 0.5-1 ng/mL. Established linear range was 1-100 ng/mL for all analytes, except acetyl fentanyl at 0.5-100 ng/mL ($R2 > 0.994$). Within- and between-run precision and bias were considered acceptable with maximum values of ±15.2%CV and ±14.1%, respectively. Matrix effects exhibited ionization enhancement for all analytes with intensified enhancement at a low concentration (9.3-47.4%). No interferences or carryover was observed. Fentanyl analogs were stable in processed extracts stored in the autosampler (4ºC) for 48h. The validated method was used to quantify fentanyl analogs in authentic oral fluid samples (n=17) from probationers/parolees. Fentanyl and 4-ANPP concentrations were 1.0-104.5 ng/mL and 1.2-5.7 ng/mL, respectively.

KEY WORDS: Fentanyl analog; Liquid-chromatography-quadrupole-time-of-flight-mass spectrometry (LC-QTOF-MS); Oral fluid; Novel synthetic opioids; Alternative matrix; Forensic toxicology

Introduction

Traditionally, blood and urine are matrices of interest for forensic toxicological analyses; however, oral fluid has presented as a viable alternative matrix for drug detection. Due to proximity, drugs of abuse are passively transferred from blood to salivary glands (1, 2). However, successful diffusion is dependent on characteristics of the drug (chemical composition, protein binding, lipophilicity, ionization state, pKa), the oral fluid (composition, pH), and biological structure (mucosal membrane) (1, 2). Advantages associated with oral fluid collection include fast, non-invasive methods performed on-site, limited biohazardous risk (compared to traditional matrices), commercialization of collection devices, and observed collection to avoid adulteration (1, 2). Additionally, oral fluid can be beneficial for detecting parent compounds indicative of recent drug use, compared to metabolites found predominately in urine (2). According to the Recommendations for Toxicological Investigation of Drug-Impaired Driving and Motor Vehicle Fatalities- 2017 Update, oral fluid and blood are preferred specimens for investigating driving under the influence of drugs (DUID) cases (3). In addition to DUID cases, oral fluid can provide useful for drug detection in clinical settings (therapeutic drug monitoring), workplace testing, and probation or parole requirements.

In recent years, fentanyl detection has not only increased in the seized drug and postmortem toxicology communities, but also in antemortem DUIDs. In the DUID-toxicology community, drugs of interest are classified into tiers based on frequency of encounter and ease of routine analysis (3). In 2017, fentanyl classification was upgraded to Tier I, due to increased detection in multiple matrices by forensic laboratories. Of the 70 participating labs, 18 indicated fentanyl in the top ten frequently detected drugs in

DUID casework (3). Fentanyl analogs were also added to Tier II, which encompasses compounds with limited prevalence that are not amenable to routine forensic analyses and require advanced instrumental analysis (3). In that same year, Tiscione *et al.* report carfentanil as the most detected drug in DUID blood samples (55 of 145 cases), second to ethanol, in south Florida (4). More recently, Rohrig *et al.* reports a drastic increase in the percentage of fentanyl related DUIDs reported in various geographical locations (north- and southeast, midwest) in the United States. Twenty case studies of individuals driving under the influence of fentanyl are also reported with concentrations in blood ranging from 2.0-16 ng/mL (5). Increasing reports of fentanyl and fentanyl analog reports in DUID cases presents a major public safety issue that requires the need for routine, advanced forensic toxicological analyses.

Previous research suggests the applicability of analyzing novel synthetic opioids (fentanyl and non-fentanyl derivatives) in oral fluid (6-11). Typical forensic analyses for fentanyl analogs in oral fluid include analytical screening techniques by liquid chromatography coupled to quadrupole-time of flight-mass spectrometry or tandem-mass spectrometry (LC-QTOF-MS or LC-MS/MS) (6-8), but limited quantification studies are available. In the study by Arantes *et al.*, a quantitative method for 50 analytes, including fentanyl, in oral fluid was developed using LC-MS/MS; however, no fentanyl analogs were analyzed (11). Morato *et al.* presents a quantitative method for 30 drugs of abuse (including alfentanil, fentanyl, furanyl fentanyl, norcarfentanil, remifentanil, and sufentanil) in oral fluid using Touch Spray-MS, an ambient ionization technique not commonly utilized in forensic laboratories (10). The goal of the present study was to develop and validate a quantitative method for fentanyl analogs in oral fluid using LC-

QTOF-MS. Previous research was expanded upon through the inclusion of prevalent fentanyl analogs and advanced analytical instrumentation. For proof of applicability, the method was applied to authentic oral fluid samples collected from probationers/parolees.

Methods

Chemicals and reagents

Certified reference standards of fentanyl analogs (3-methylfentanyl, 4-ANPP, 4-fluoroisobutyrylfentanyl, acetyl fentanyl, acrylfentanyl, butyrylfentanyl, carfentanil, cyclopropylfentanyl, fentanyl, furanylfentanyl, methoxyacetylfentanyl, *p*-fluorofentanyl, and valerylfentanyl) and deuterated internal standards (ISTD) (4-ANPP-D5, carfentanil-D5, fentanyl-D5, and *p*-fluorofentanyl-D3) were purchased from Cerilliant Corporation (Round Rock, Texas). Di- and monobasic sodium phosphate solids used in the preparation of phosphate buffer (100 mM, pH 6) were purchased from Sigma-Aldrich (St. Louis, Missouri). Organic solvents of hexane, ethyl acetate, and methanol were purchased from J.T. Baker (Center Valley, Massachusetts). Acetic acid and ammonium hydroxide used during extraction were from Mallinckrodt Pharmaceuticals (St. Louis, Missouri) and J.T. Baker (Center Valley, Massachusetts), respectively. Deionized water was produced in-house using a Millipore Direct-Q® 3UV (Burlington, Massachusetts). Mobile phase components of LC-MS grade acetonitrile and additive formic acid (>95%) were purchased from Fisher Scientific (Hampton, New Hampshire). For oral fluid preparation, blank oral fluid was collected from drug-free, anonymized volunteers and Quantisal™ extraction buffer was acquired from Immunalysis Corporation (Pomona, California).

Standard preparation

Methanolic reference materials were used to prepare the highest calibrator mix at 1000 ng/mL. Serial dilutions were performed to make 6 additional calibrators at 500, 250, 100, 50, 10 and 5 ng/mL, resulting in concentrations of 0.5, 1, 5, 10, 25, 50 and 100 ng/mL in oral fluid. ISTD mix was prepared at 100 ng/mL in methanol, which equates to 10 ng/mL when fortified in oral fluid. Quality control (QC) mixes were prepared separately in the same manner as described above. QC concentrations were 800, 400, and 15 ng/mL (80, 40, and 1.5 ng/mL in oral fluid). Although calibration range varied for analytes of interest, low quality control (LQC) was prepared at 3 times the lowest limit of quantification (LOQ).

Extraction

A previously validated solid phase extraction procedure was utilized with modification (6). Sample volume was decreased to 400 µL (100 µL oral fluid:300 µL extraction buffer). Extraction was performed using a SPEWare System 48™ CEREX® Pressure Processor (Baldwin Park, California). Briefly, samples were fortified (10 µL calibrator, QC, and/or ISTD), buffered (100 mM phosphate buffer, pH 6), loaded on CEREX® Clin II SPE cartridges (Baldwin Park, California), washed with aqueous solutions (DiH2O, 1M acetic acid), dried under nitrogen, and then washed with organic solvents (hexane, ethyl acetate, methanol). The described wash steps were included for optimal cleanup of endogenous matrix and exogenous extraction buffer components. Elution solvent was 5% ammonium hydroxide in ethyl acetate (v/v). Samples were placed in a Biotage TurboVap LV Evaporator (Charlotte, North Carolina) for drying

under nitrogen at 50°C. Analytes were reconstituted in 100 µL of mobile phase (75:25 0.1% formic acid in deionized water: 0.1% formic acid in acetonitrile) for analysis.

Instrumentation

Instrumental parameters, chromatography, and data analysis were applied from a previously published method (12). Briefly, an Agilent Technologies 1290 Infinity liquid chromatograph coupled to an Agilent Technologies 6530 Accurate Mass Time-of-Flight Mass Spectrometer (Santa Clara, California) equipped with Jetstream electrospray ionization was utilized. Source conditions were: positive mode, drying gas temperature 300°C, drying gas flow 13 L/min, nebulizer pressure 45 psi, sheath gas temperature 350°C, sheath gas flow 12 L/min, nozzle voltage 0V, capillary voltage 3000V and fragmentor voltage 150 V. For analysis, 10 µL of extract was injected on a Poroshell 120 EC-C18 column (2.1 x 100 mm, 2.7 µm) with matching guard and separated using a gradient elution comprised of (A) 0.1% formic acid in deionized water and (B) 0.1% formic acid in acetonitrile. Total run time was 10 min. Data were acquired using targeted acquisition and analyzed using MassHunter Quantitative Analysis (Version B.09.00). Targeted analysis is a data-dependent approach to quantitative analysis by high resolution mass spectrometry. Precursor ions were included in a targeted list to be selected for fragmentation. Quantification resulted from detecting quantifying and qualifying ion transitions (Table 5.1).

Table 5.1. Targeted acquisition parameters for fentanyl analog quantification (12)

Analyte	Precursor Ion (*m/z*)	Retention Time (min)	Quantifier Ion (*m/z*)	Collision Energy (eV)	Qualifier Ion (*m/z*)	Paired Internal Standard
Methoxyacetylfentanyl	353.2224	1.60	188.1434	20	105.0702	Fentanyl-D5
Acetylfentanyl	323.2118	1.80	188.1429	25	105.0700	Fentanyl-D5
Acrylfentanyl	335.2118	2.50	188.1434	20	105.0708	Fentanyl-D5
4-ANPP	281.2012	2.60	188.1439	20	105.0705	4-ANPP-D5
Fentanyl	337.2274	2.66	188.1437	25	105.0704	Fentanyl-D5
Furanylfentanyl	375.2067	3.01	188.1438	20	105.0701	Fentanyl-D5
p-fluorofentanyl	355.2180	3.06	188.1427	25	105.0698	*p*-fluorofentanyl-D3
Cyclopropylfentanyl	349.2274	3.17	188.1430	25	105.0694	Fentanyl-D5
3-methylfentanyl	351.2431	3.60*	202.1596	25	105.0705	Carfentanil-D5
Carfentanil	395.2329	3.60	335.2114	15	246.1483	Carfentanil-D5
Butyrylfentanyl	351.2431	3.90*	188.1436	25	105.0709	Fentanyl-D5
4-fluoroisobutyrylfentanyl (4-FIBF)	369.2337	4.10	188.1437	25	105.0700	*p*-fluorofentanyl-D3
Valerylfentanyl	365.2587	5.40	188.1433	25	105.0697	Fentanyl-D5
4-ANPP-D5	286.2326	2.50	188.1435	20	105.0698	-
Fentanyl-D5	342.2586	2.60	188.1430	25	105.0698	-
p-fluorofentanyl-D3	358.2368	3.00	188.1435	25	105.0701	-
Carfentanil-D5	400.2643	3.56	340.2433	5	246.1490	-

*Updated retention times from previously published method (12)

Method validation

The present quantitative method was validated by evaluating parameters such as limit of detection (LOD), lower limit of quantitation (LLOQ), calibration model, precision, bias, interferences, carryover, ionization suppression/enhancement, processed sample stability, and dilution integrity. Validation experiments and acceptance were assessed using ANSI/ABS Standard 036: Standard Practices for Method Validation in Forensic Toxicology as a guideline (13).

Limits of detection were assessed in three sources of oral fluid in triplicate over three different days. For acceptability, analytes were evaluated in terms of adequate peak shape, reproducible retention times (±0.5 min), ion ratios (within ±30%) and signal to noise ratios (S/N >3.3). Lower limits of quantification were evaluated in three matrix sources, in triplicate over three days. Criteria for acceptability were the same as LOD with the following additions: suitable precision and bias (±20 %CV or bias, respectively) and S/N>10.

Calibration models were determined using 6 non-zero calibrators for all analytes (except acetyl fentanyl with 7 calibrators) fortified in five sources of oral fluid over five days. Coefficients of determination (R2) values were acceptable if >0.99. In accordance with calibration runs, precision (within- and between-run) and bias were evaluated at three concentration levels (low- medium- and high- quality controls: LQC, MQC, HQC), in triplicate, over five days (n=15). As described above, acceptable precision and bias were within ±20 %CV and %bias, respectively. For samples exceeding the validated working range, dilution integrity experiments were performed at factor of 1:2. Dilution integrity was sustained with bias values within ±20%.

Three types of interferences were evaluated: matrix, stable isotope, and commonly encountered analytes. Matrix and stable isotope interferences were assessed by analyzing blank and negative (blank fortified with ISTD) oral fluid sources (n=5) for the presence of target analytes. To ensure that non-deuterated analytes did not interfere with ISTD identification, a high concentration sample (200 ng/mL) without ISTD was analyzed for the presence of deuterated compounds. To determine interference from commonly encountered compounds, four mixes (containing basic, neutral, and acidic drugs) at 150 ng/mL in oral fluid were fortified into LQC samples. Quantitative interferences were considered negligible with accurate quantification of target analytes in LQC (±20%). Compounds included in the interference mixes were Δ9-tetrahydrocannabinol, alprazolam, amobarbital, amphetamine, amitriptyline, butalbital, caffeine, carbamazepine, carisoprodol, cocaine, codeine, cotinine, cyclobenzaprine, dextromethorphan, diazepam, diphenhydramine, hydrocodone, hydromorphone, ketamine, methadone, nicotine, nordiazepam, oxazepam, oxycodone, pentobarbital, phencyclidine, phenobarbital, propoxyphene, secobarbital, tetrahydrocannabinolic acid, tramadol, and zolpidem. Carryover was determined by comparing analyte signal in the reinjection of a blank after highest calibrator to signal in the lowest calibrator. If the reinject signal was less than 10% of the lowest calibrator, carryover was negligible.

Ionization suppression/enhancement was evaluated using post-extraction addition (14) of LQC and HQC in 10 different sources of blank oral fluid. Comparison of analyte peak area in post samples to neat samples was used to calculate matrix effects (%). Ion enhancement and suppression were observed in analytes with matrix effects >0% and <0%, respectively. Acceptable matrix effects were within ±25%. For values exceeding

acceptability, ionization suppression/enhancement of matched ISTD were analyzed for comparability.

Processed sample stability was performed by reinjecting LQC (n=3) and HQC (n=3) extracts stored in the autosampler at 4°C for 48 hrs. Fresh (t0) and processed (48hr) concentrations were compared. Analyte quantification within ±20% bias was considered stable.

Results and Discussion

Method validation

Lower limits of quantification (LLOQ) were determined to be 1 ng/mL for all analytes, except acetylfentanyl at 0.5 ng/mL. Limits of detection (LOD) were 0.5 ng/mL for acetylfentanyl, methoxyacetylfentanyl, furanylfentanyl, *p*-fluorofentanyl, 3-methylfentanyl, carfentanil, butyrylfentanyl, 4-FIBF, and valerylfentanyl. LOD were 1 ng/mL for acrylfentanyl, 4-ANPP, fentanyl, and cyclopropylfentanyl. Linear ranges were 1-100 ng/mL for fentanyl analogs (0.5-100 ng/mL for acetylfentanyl). The National Safety Council (NSC) recommends fentanyl cutoff values of 1 and 0.5 ng/mL in oral fluid for screening and confirmation, respectively, which are consistent with this method (3). Calibration models were linear with a 1/x2 weighting, achieving coefficient of determination values >0.994 for all analytes. Detection limits and linearity data are presented in Table 5.2. Dilution integrity was sustained at a 1:2 dilution factor in MQC with a percent bias not exceeding -15.5%. Limited reports of fentanyl analog concentrations in oral fluid in forensic casework exist; however, traditional opioids have been shown to have increased concentrations in oral fluid (compared to blood) due to their weak basic nature (2). If this relationship were extrapolated to fentanyl analogs, the

presented range and detection limits are determined to be suitable based on reported concentrations of fentanyl analogs in blood (15-25). Nonetheless, knowledge of fentanyl interpretation in oral fluid is inadequate (2) and requires further investigation.

Table 5.2. Calibration parameters, limits of detection, and lower limits of quantification for target fentanyl analogs

Analyte	LOD (ng/mL)	LLOQ (ng/mL)	Calibration Range (ng/mL)	Mean Slope (n=5)	Mean y-intercept (n=5)	Mean R^2 (n=5)
Methoxyacetylfentanyl	0.5	1	1- 100	1.47	0.03	0.997
Acetylfentanyl	0.5	0.5	0.5- 100	1.47	0.007	0.997
Acrylfentanyl	1	1	1- 100	0.92	0.006	0.998
4-ANPP	1	1	1- 100	0.81	0.01	0.994
Fentanyl	1	1	1- 100	0.84	0.004	0.995
Furanylfentanyl	0.5	1	1- 100	0.94	0.02	0.997
p-fluorofentanyl	0.5	1	1- 100	0.95	0.02	0.997
Cyclopropylfentanyl	1	1	1- 100	0.79	0.005	0.997
3-methylfentanyl	0.5	1	1- 100	4.43	0.04	0.998
Carfentanil	0.5	1	1- 100	3.06	0.04	0.997
Butyrylfentanyl	0.5	1	1- 100	1.35	0.007	0.997
4-fluoroisobutyrylfentanyl (4-FIBF)	0.5	1	1- 100	1.24	0.02	0.998
Valerylfentanyl	0.5	1	1- 100	4.06	0.03	0.999

LOD- limit of detection; LLOQ- lower limit of quantification

Acceptable precision and bias data are presented in Table 5.3. Bias ranges were -14.1 to 1.1%, -9.6 to 4.2%, and -8.2 to 4.6% in LQC, MQC, and HQC, respectively. Over five runs, within-run and between run precision were within ±15.2 and ±12.3%CV, respectively, incorporating all concentration levels. Maximum within-run precision values were 15.2, 7.4, and 8.1%CV for LQC, MQC, and HQC, respectively. Ultimately, all precision and bias data were acceptable (within ±20%).

Table 5.3. Precision and bias validation results

	Maximum within-run precision (%CV, n= 3)				**Between-run precision (%CV, n=15)**				**Bias (%, n=15)**			
Analyte	**LQC*	*†MQC*	*‡HQC*	*LLOQ§*	*LQC*	*MQC*	*HQC*	*LLOQ§*	*LQC*	*MQC*	*HQC*	*LLOQ§*
Methoxyacetylfentanyl	8.4	5.0	8.1	5.2	6.6	6.5	8.1	4.8	-13.4	-5.7	-7.6	-1.0
Acetylfentanyl	7.8	7.4	7.4	6.1	8.4	5.4	7.1	7.9	-6.3	-6.6	-7.2	-0.8
Acrylfentanyl	14.9	2.9	5.4	8.7	9.8	3.4	5.8	7.5	-9.8	-5.7	-6.3	3.6
4-ANPP	8.2	4.8	3.8	9.0	9.1	4.8	5.3	6.6	-3.7	4.2	-2.9	1.0
Fentanyl	7.1	4.2	5.4	7.8	7.7	4.1	5.3	7.1	-3.0	-4.4	-5.5	10.0
Furanylfentanyl	15.2	3.5	5.2	8.6	12.3	4.0	6.0	7.4	-7.9	-3.0	-3.9	-5.5
p-fluorofentanyl	7.8	3.8	7.2	9.3	8.2	5.0	5.8	7.8	-10.8	-4.4	-5.8	-1.0
Cyclopropylfentanyl	12.3	4.2	6.1	9.1	8.1	3.8	6.3	7.0	-5.8	-2.2	-2.2	1.4
3-methylfentanyl	10.1	4.9	5.8	3.5	8.0	5.3	5.8	3.1	-10.9	-4.1	-2.6	-2.2
Carfentanil	6.7	5.4	4.1	5.2	5.9	5.0	7.5	9.4	-14.1	-9.6	-8.2	-0.1
Butyrylfentanyl	6.0	3.3	4.7	6.5	7.3	3.2	5.7	4.6	1.1	3.7	4.3	1.4
4-fluoroisobutyrylfentanyl (4-FIBF)	14.9	4.0	5.7	5.1	8.6	4.5	5.7	8.7	-6.4	2.1	4.6	-0.3
Valerylfentanyl	5.0	3.8	5.3	4.1	5.5	3.7	5.9	4.1	-6.4	-3.6	-1.8	2.7

*LQC- low quality control: 1.5 ng/mL; †MQC- medium quality control: 40 ng/mL; ‡HQC- high quality control: 80 ng/mL; §LLOQ-lower limit of quantification: 0.5-1 ng/mL. Replicates of LLOQ were n=9 for between-run precision and bias.

No interferences were observed from endogenous sources, internal standards, or target analytes. Exogenous interferences were assessed at a concentration 100 times the LQC (150 ng/mL in oral fluid). At this level, no qualitative interferences were observed and LQC quantified accurately, suggesting no quantitative interferences. No carryover was observed.

Ionization suppression/enhancement was assessed at low and high QC concentrations (Table 5.4). For LQC, ion enhancement was observed with matrix effects ranging from 9.3 to 47.4% for target compounds. Only 3-methylfentanyl had acceptable matrix effects in LQC. In HQC, ion enhancement values were acceptable for all analytes (except carfentanil) with values ranging from 4.0 to 23.8%. Matrix effects for carfentanil were 28.0% with matched ISTD (carfentanil-D5) enhancement at 30.2%. Some enhancement observed at both concentration levels is outside of the acceptable ±25% range described by ANSI/ASB Standard 036. However, target analytes with matched deuterated ISTD exhibit comparable matrix effects values and limits of detection were still considered acceptable. Matrix effects were reproducible in ten oral fluid sources with %CV values ranging 3.3 to 10.6 and 1.4 to 5.1% in LQC and HQC, respectively. One possible explanation for increased matrix effects is the presence of Quantisal™ extraction buffer. Desrosiers & Huestis describe the potential for interference from buffers and surfactants with LC-MS/MS analysis (2). Immunalysis, the manufacturer of the Quantisal™ collection device, suggests performing sample clean-up on oral fluid samples to prevent interference from the pre-existing extraction buffer (26).

Table 5.4. Ion enhancement observed for fentanyl analogs in oral fluid

Analyte	Matrix Effects (%)			
	**LQC (n=10)*	*%CV*	*†HQC (n=10)*	*%CV*
Methoxyacetylfentanyl	31.4‡	4.6	14.1	2.3
Acetylfentanyl	31.6‡	4.5	11.5	2.3
Acrylfentanyl	36.7‡	3.3	12.7	2.0
4-ANPP	36.0‡	10.6	23.8	3.4
Fentanyl	28.0‡	6.5	11.0	3.0
Furanylfentanyl	25.9‡	7.3	12.4	2.0
p-fluorofentanyl	27.6‡	9.8	14.2	2.4
Cyclopropylfentanyl	28.0‡	8.0	13.4	2.4
3-methylfentanyl	9.3	4.2	4.0	2.3
Carfentanil	47.4‡	3.8	28.0‡	1.4
Butyrylfentanyl	31.6‡	4.2	12.9	1.7
4-fluoroisobutyrylfentanyl (4-FIBF)	39.5‡	3.9	14.5	2.0
Valerylfentanyl	30.2‡	3.3	12.4	2.0
4-ANPP-D5	42.6‡	7.2	25.0	4.2
Fentanyl-D5	31.3‡	4.1	10.2	2.1
Carfentanil-D5	48.4‡	4.6	30.2‡	5.1
p-fluorofentanyl-D3	35.8‡	4.1	14.2	2.4

*LQC- low quality control: 1.5 ng/mL; †HQC- high quality control: 80 ng/mL; ‡Ion enhancement exceeding acceptable range (±25%)

To account for delayed analysis time that may occur in a forensic lab, processed sample stability was assessed by storing sample extracts in the autosampler (4°C) for 48h. LQC and HQC extracts were analyzed at t0 and after 48h. Analytes quantified within ±6.3% of target concentrations (LQC and HQC) after 48h, indicating stability in the autosampler.

Authentic sample analysis

Authentic (anonymized) oral fluid samples (n=17) collected from probationers/parolees with Quantisal™ devices were received from Redwood Toxicology (Santa Rosa, CA). Upon arrival, samples were stored immediately under refrigeration (4°C). For proof of applicability, samples were extracted and quantified using the present method. Quantification results are presented in Table 5.5. Fentanyl (n=16) and 4-ANPP (n=3) were detected. Fentanyl concentrations ranged from 1.0 to 104.5 ng/mL and 4-ANPP concentrations ranged from 1.2 to 5.7 ng/mL. The total and extracted ion chromatograms of sample 10 are displayed in Figure 5.1. Without knowledge of user tolerance, route of administration, or oral fluid:blood ratios, it is difficult to predict corresponding blood fentanyl concentrations or intoxication from these oral fluid results. No additional fentanyl analogs were detected. Analysis by the initial laboratory resulted in quantifiable fentanyl concentrations ranging 3 to 90 ng/mL. Some samples exceeded the panel's upper limit of quantification (100 ng/mL). 4-ANPP was not indicated by the submitting laboratory because it is not targeted in the panel. When comparing results between labs, the concentrations determined by the present method were lower than initial analysis with percent loss ranging 7.2 to 90.5% (Table 5.5). These discrepancies could be due to sample age (analyte stability in oral fluid) or degradation during shipping and/or storage. However, fentanyl concentrations in the samples were detectable above cutoff values defined by the NSC and Redwood Toxicology (1 ng/mL) (3, 27).

Table 5.5. Quantification results for authentic oral fluid samples received from Redwood Toxicology

Sample Number	Analyte	Concentration: present method (ng/mL)	Concentration: initial lab (ng/mL)	Fentanyl Percent Loss (%)
1	Fentanyl	1.0	11	90.5
2	Fentanyl	104.5	>100	-
	4-ANPP	1.2	-	-
3	Fentanyl	90.5	>100	>9.5
4	Fentanyl	14.6	17	14.0
5	Fentanyl	17.4	21	17.0
6	Fentanyl	4.2	5	15.5
7	Fentanyl	11.0	48	77.0
8	Fentanyl	35.5	>100	>64.5
9	Fentanyl	1.5	4	61.8
10	Fentanyl	92.8	>100	>7.2
	4-ANPP	3.3	-	-
11	None detected	-	3	>66.7
12	Fentanyl	2.0	3	32.8
13	Fentanyl	25.3	90	71.8
14	Fentanyl	6.3	12	47.4
15	Fentanyl	70.8	>100	>29.2
16	Fentanyl	3.5	15	76.4
17	Fentanyl	45.9	71	35.4
	4-ANPP	5.7	-	-

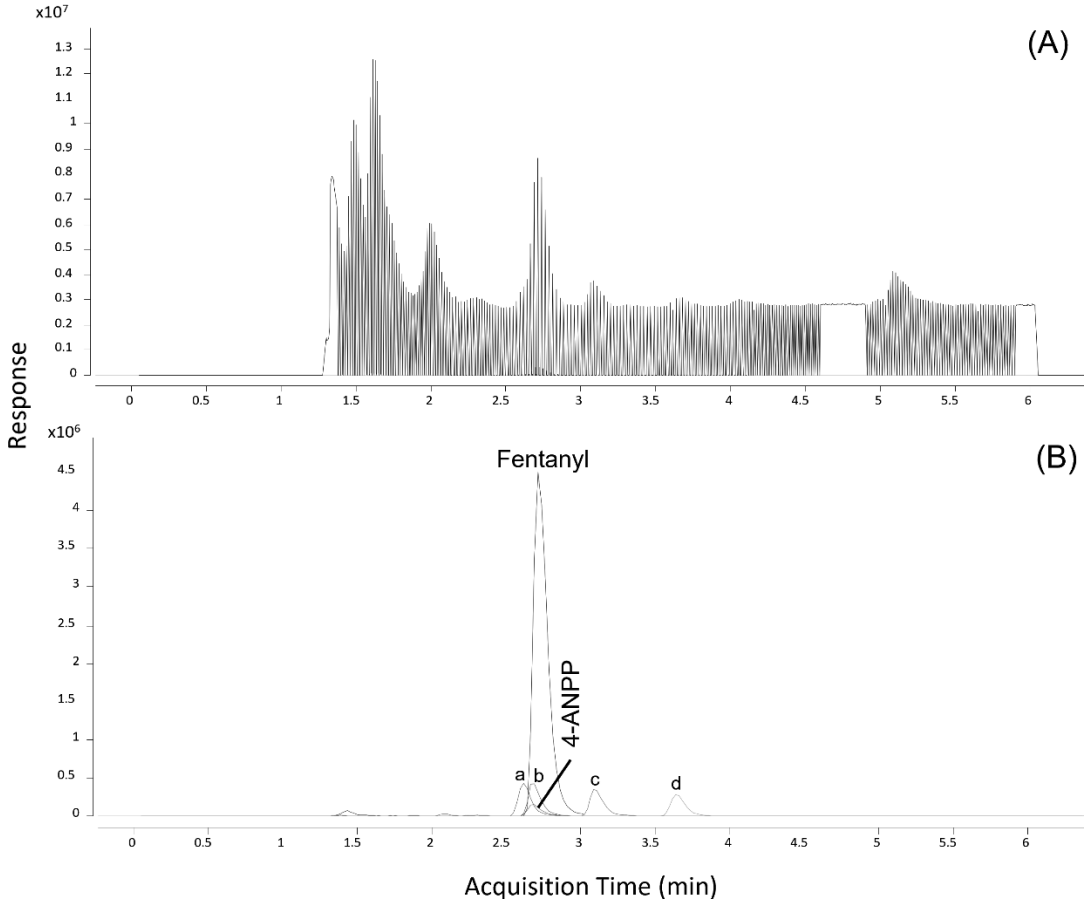

Figure 5.1. Total ion chromatogram (A) and extracted ion chromatogram (B) resulting from the analysis of authentic oral fluid sample 10. Fentanyl and 4-ANPP were detected and quantified. Internal standards were present: 4-ANPP-D5 (a), fentanyl-D5 (b), p-fluorofentanyl-D3 (c), and carfentanil-D5 (d).

Conclusion

As fentanyl analogs continue to thrive in the illicit drug community, detection and quantification of those compounds in alternative matrices is important for addressing progressing public health concerns. Oral fluid is a viable alternative matrix because it allows for rapid, easy, and on-site collection that can be applied to forensic casework such as DUID, workplace drug testing, pain management administration and probation/parole protocols. To the authors knowledge, this study presents the first method for quantification of multiple fentanyl analogs in oral fluid using LC-QTOF-MS. The method was fully validated and applied to authentic samples for fentanyl analog quantification. While limited fentanyl analogs were detected, the present method remains relevant for the evolving use of fentanyl in the United States and analysis of oral fluid in the forensic toxicology community.

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CHAPTER VI

Conclusions

The opioid crisis continues to plague the United States with sizeable numbers of drug intoxications and overdose fatalities, and fentanyl analogs hold responsibility in its proliferation. With no end in sight, forensic toxicologists must stay current with evolving drug trends dealing with these dangerous compounds. Development of advanced analytical methods capable of identifying unknowns, distinguishing similar structure compounds, and detecting low concentrations in traditional and alternative biological matrices is essential. Data-independent screening with high resolution mass spectrometry (HRMS) can be a valuable tool for identifying known or unknown novel psychoactive substances (NPS) by capturing vast amounts of analytical data to be analyzed presently or retrospectively. Pairing HRMS with the creation of in-house libraries also presents promise for efficient screening of samples and identification of novel substances. The present work demonstrated data-independent screening applicability in authentic blood and oral fluid samples.

While blood is habitually analyzed in forensic laboratories, oral fluid is an alternative matrix with indications of recent drug use and advantages of easy, safe collection without the need for trained phlebotomists. The present work describes a novel quantification method for fentanyl analogs in oral fluid. Although no fentanyl analogs were detected in forensic samples, analyte detection and quantification in oral fluid can be advantageous for forensic laboratories beginning to incorporate routine oral fluid testing for driving under the influence of drugs (DUID) cases, workplace drug testing, pain management/monitoring facilities, and probation/parole protocols.

Forensic toxicologists must work to thoroughly understand newly emerging drugs of abuse. Properties such as compound pharmacology and stability are often unknown for NPS, but essential for toxicological analysis and data interpretation. In the present study, method development and validation were performed to quantify furanylfentanyl and its metabolites in human and rat plasma as a preliminary step to determining unknown pharmacological activity. Through animal modeling and pre-clinical studies, pharmacological profiling can be extrapolated to the human system for greater understanding of intoxication impairment.

Understanding drug stability is critical for forensic laboratories experiencing backlog. Often case samples may be stored for weeks to months before analysis. When considering the low concentrations at which fentanyl analogs are typically present, potential degradation experienced during testing delays may result in false negatives. Forensic laboratories need to understand NPS stability in order to properly store and prioritize case samples to mitigate degradation. The present study determined the long-term stability of fentanyl analogs in blood over a 9-month period. Acrylfentanyl demonstrated extreme degradation in blood reaching instability as early as 24h at room and elevated temperatures. In the event of a suspected acrylfentanyl intoxication, samples should be analyzed immediately or stored frozen with analysis within 1 month. Other fentanyl analogs were determined to be stable for 9 months stored at room temperature and under refrigeration. Under frozen conditions, sample degradation was observed after 4 freeze/thaw cycles. When exposed to elevated temperatures, fentanyl analogs remained stable for 1 week; however, greater instability was observed with prolonged heat

exposure. Based on these observations, freeze/thaw cycles should be limited and storage under refrigeration is recommended for preservation of stability.

The presented work describes highly sensitive analytical methods for the detection and quantification of fentanyl analogs in traditional and alternative biological matrices. Methods were fully validated in accordance with published guidelines and standards recognized by the forensic toxicology community. Limits of detection were achieved in the sub-ng/mL range; thus, capable of detecting forensically relevant concentrations. Utilization of the described methods will assist the forensic toxicology community with overcoming the challenges associated with fentanyl analog detection, and ultimately, combating the opioid crisis.

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Wilson, A.S., Stiller, R.L., Davis, P.J., Fedel, G., Chakravorti, S., Israel, B.A., *et al.* (1997) Fentanyl and alfentanil plasma protein binding in preterm and term neonates. *Anesthesia and Analgesia*, **84**, 315- 318.

Zawilska, J.B., Kuczyńska, K., Kosmal, W., Markiewicz, K. and Adamowicz, P. (2021) Carfentanil – from an animal anesthetic to a deadly illicit drug. *Forensic Science International*, **320**, 110715.

Zhang, Y., Sheng, Z., Hua, Z., Liang, C., Cai, Z., Wang, R., *et al.* (2020) Simultaneous separation and determination of 32 fentanyl-related substances, including seven sets of isomeric fentanyl analogues, by ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry. *Journal of Separation Science*, **43**, 3735-3747.

APPENDIX A

Supplemental Information for Chapter III

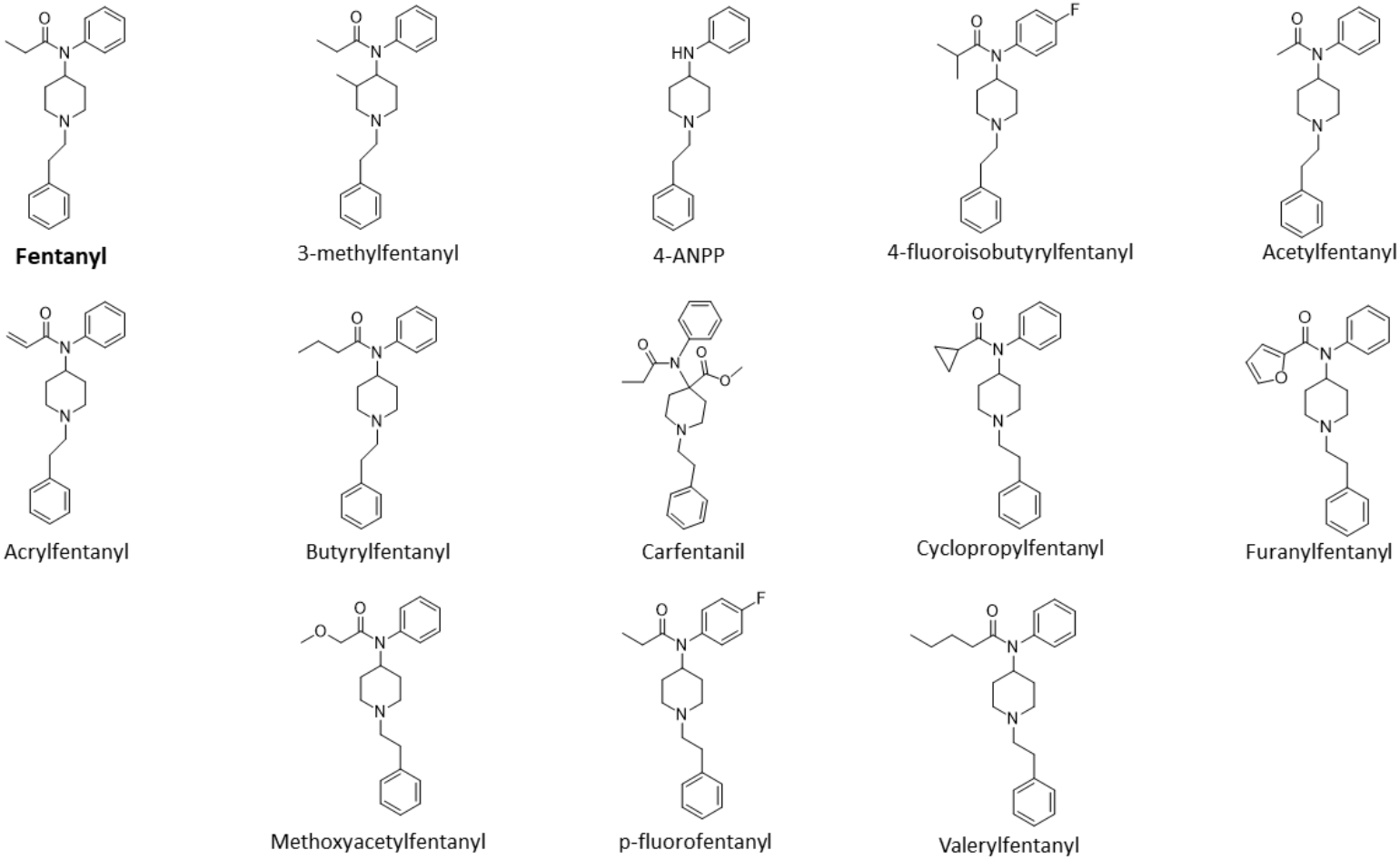
Supplemental Table 3.1. Matrix effects, recovery, bias, precision, stability, and dilution integrity data at various QC concentration levels in rat plasma

	Matrix Effects (%, n=3)		**Recovery (%, n=3)**		**Bias (%, n=9)**			**Maximum Within-Run Precision (%CV, n=3)**			**Between-Run Precision (%CV, n=9)**			**Freeze/thaw stability, 3 cycles (%bias, n=3)**		**Dilution Integrity (n=3)**	
	LQC[a]	*HQC*[c]	*LQC*	*HQC*	*LQC*	*MQC*[b]	*HQC*	*LQC*	*MQC*	*HQC*	*LQC*	*MQC*	*HQC*	*LQC*	*HQC*	*1/2 (%bias, %CV)*	*1/10 (%bias, %CV)*
Furanyl norfentanyl	-40.9	-34.5	82.7	79.2	1.8	8.4	-0.5	10.3	19.0	8.0	12.6	8.3	9.9	-5.8	-5.2	-11.0, 5.0	-11.5, 12.4
4-ANPP	17.2	-9.3	83.7	93.3	-2.5	-0.4	-7.4	4.4	5.1	2.7	4.6	3.2	2.5	4.7	-3.9	-19.1, 3.9	-13.9, 5.7
Furanyl fentanyl	-2.3	0.2	81.7	85.2	4.4	4.4	-5.3	2.8	4.4	2.1	2.2	2.9	1.6	-2.1	-1.3	-13.0, 3.4	-5.7, 3.8
4-ANPP-D5	15.7	4.9	87.5	94.5	-	-	-	-	-	-	-	-	-	-	-	-	-
Furanyl fentanyl-D5	-3.5	-3.4	82.8	86.5	-	-	-	-	-	-	-	-	-	-	-	-	-

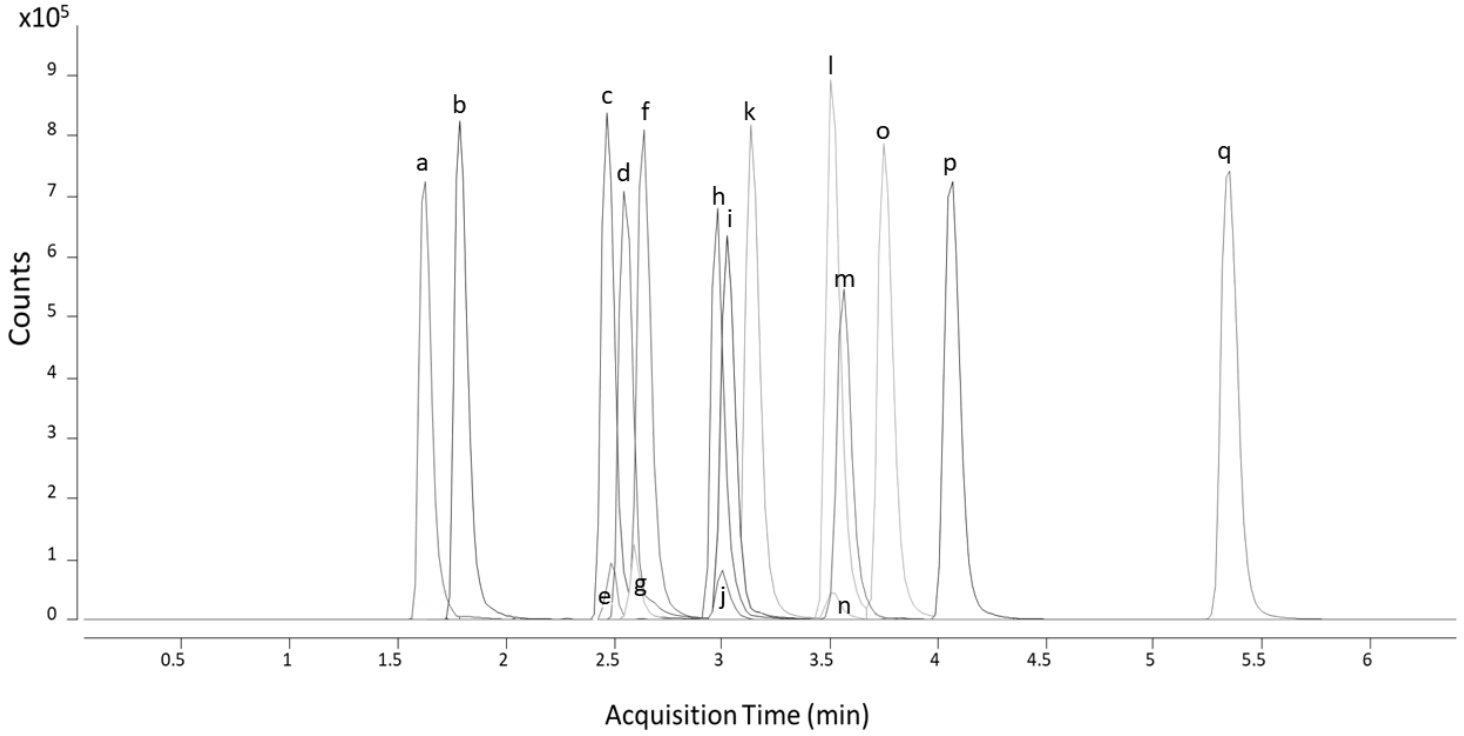
[a]Low QC concentration: 0.075 ng/nL (1.5 ng/mL for furanyl norfentanyl); [b]Medium QC concentration: 2 ng/mL; [c]High QC concentration: 20 ng/mL

APPENDIX B

Supplemental Information for Chapter IV



Supplementary Figure 4.1. Molecular structures of target fentanyl analogs in alphabetical order



Supplementary Figure 4.2. Chromatogram of fentanyl analogs (a) methoxyacetylfentanyl, (b) acetylfentanyl, (c) acrylfentanyl, (d) 4-ANPP, (e) 4-ANPP-D5, (f) fentanyl, (g) fentanyl-D5, (h) furanylfentanyl, (i) *p*-fluorofentanyl, (j) *p*-fluorofentanyl-D3, (k) cyclopropylfentanyl, (l) 3-methylfentanyl, (m) carfentanil, (n) carfentanil-D5, (o) butyrylfentanyl, (p) 4-fluoroisobutyrylfentanyl, and (q) valerylfentanyl in HQC (80 ng/mL).

VITA

KAITLYN B. PALMQUIST

EDUCATION

PhD Sam Houston State University, Huntsville, TX 2016- 2021
PhD in Forensic Science
GPA: 4.0

BS Towson University, Towson, MD 2012-2016
BS in Forensic Chemistry- Trace Evidence/Drug Analysis
Summa Cum Laude, Honors Graduate
GPA: 3.932

RESEARCH EXPERIENCE

Graduate Research 2016- 2021
Sam Houston State University (Advisor: Dr. Madeleine Swortwood)

- Developed a data independent screening method for fentanyl analogs in whole blood and oral fluid using liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF-MS)
- Developed and optimized a solid phase extraction (SPE) procedure for fentanyl analogs from whole blood and oral fluid
- Built an in-house Personal Compound Database and Library (PCDL) using certified reference materials of common drugs of abuse and novel psychoactive substances
- Performed method validations according to the SWGTOX guidelines
- Applied validated methods to authentic case samples (IRB approved): postmortem blood; antemortem oral fluid from arrestees
- Analyzed fentanyl analog isomers using GC-MS for chromatographic separation unachievable by LC-MS
- Developed a dynamic multiple reaction monitoring (dMRM) acquisition method for the detection of furanyl fentanyl and its metabolites in human plasma using an Ultivo LC-QQQ system
- Performed a cross-validation of quantitative method for furanyl fentanyl and its metabolites in rat plasma using Ultivo LC-QQQ system
- Developed a targeted, quantitative method for fentanyl analogs in whole blood and using liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF-MS)
- Conducted a long-term (9 month) stability study for fentanyl analogs in whole blood under various temperature conditions; determined optimal storage conditions
- Analyzed stability of authentic postmortem blood samples containing fentanyl analogs

- Developed a targeted, quantitative method for fentanyl analogs in oral fluid using liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) for authentic sample analysis
- Performed method validations according to ANSI/ASB Standard 036: Standard Practices for Method Validation in Forensic Toxicology
- Performed instrument maintenance on LC-MS/MS (cleaned ionization source, prepared reference ion and calibration solutions, unclogged lines, changed frit and guard column, replaced needle seat)

Capstone Research Title: Screening of Fentanyl Analogs in Whole Blood Using LC-QTOF Analysis

EMPLOYMENT HISTORY

Doctoral Teaching Fellow Fall 2019- Spring 2021

Sam Houston State University, Huntsville, TX

- Instructor for Introduction to Forensic Science, online undergraduate course
- Answer student questions related to course function or materials
- Design and update Blackboard site
- Administer Honors Contracts

Crime Lab Intern

Johnson County Sheriff's Office Criminalistics Laboratory, Olathe, KS Summer 2017

- Organized database information
- Contacted agencies to follow-up on CODIS report information
- Assisted with specialized projects in multiple forensic disciplines (firearms, latent prints, crime scene investigation)
- Disposition of evidence from resolved cases, received experience in LIMS

Graduate Assistant 2016- 2019

Sam Houston State University, Huntsville, TX

- Teaching Assistant for instrumental and toxicology laboratories: prepare reagents and materials, assist with laboratory procedure
- Aided the Department of Forensic Science with administrative tasks
- Lab maintenance and organization: equipment and chemical inventory upkeep, cleaning glassware, restock disposable materials
- Instrument maintenance (Agilent GC-MS): cleaning ion source
- Performed the annual calibration of pipettes and balances
- Conducted demonstrations for high school students via tours and academic community engagement (ACE) projects
- Creative Development: construction and display of department poster

Learning Assistant in General Chemistry Lab Fall 2014- Fall 2015

Towson University, Towson, MD

- Helped students perform proper laboratory techniques
- Answered student's questions pertaining to laboratory work and chemistry
- Assisted in chemistry related calculations and measurements
- Signed-off on properly completed laboratory notebooks
- Provided weekly tutoring sessions to students

Toxicology Lab Intern Summer 2015

Office of the Chief Medical Examiner, Baltimore, MD

- Performed routine maintenance of laboratory equipment (Agilent GC-MS): instrument tuning, changing the septum, replacing the inlet material, filling solvent vials, emptying waste vials
- Aided in the annual calibration of pipettes
- Completed training for designed laboratory standard operating procedures
- Assisted in a validation study for a designed standard operating procedure dealing with sympathomimetic amines in postmortem samples; utilized assisted liquid-liquid extractions; prepared calibration standards and control samples; analyzed samples on Agilent GC-MS; constructed calibration curves; performed data analysis on ChemStation software
- Contributed to a method development for the preparation of postmortem specimens for new instrumentation (GEM Premier 400 Co-Oximeter)

FIELD EXPERIENCE

Missing Persons Search Participant

Forensic Science Student Organization, Towson University, Towson, MD

- Kentucky Body Search (May 2015): assisted Jodi Powers Search and Rescue in the search for human remains. Work consisted of grid line searches through wooded terrain, probing soil for sub-surface disturbances, review of terrain for artificial landforms, and interpretation of aerial maps. Team members included a forensic anthropologist, K-9 cadaver dog handler, SAR teams, and homicide detectives.
- Catonsville Body Search (June 2015): assisted Baltimore County Police Department's Homicide Division with the search for human remains in a wooded location in Catonsville, MD. Human remains were flagged, collected and sent to the Office of the Chief Medical Examiner for identification. Crime lab personnel were on the scene.

PUBLICATIONS

Palmquist KB, Swortwood MJ. Quantification of Fentanyl Analogs in Oral Fluid using LC-QTOF-MS. Submitted to *Journal of Forensic Sciences*.

Palmquist KB, Swortwood MJ. Long-Term Stability of 13 Fentanyl Analogs in Blood. *Journal of Analytical Toxicology* 2021; doi: https://doi.org/10.1093/jat/bkab051

Palmquist KB, Swortwood MJ. Quantification of Furanyl Fentanyl and its Metabolites in Human and Rat Plasma using LC-MS/MS. *Journal of Analytical Toxicology* 2020; **44**(6): 589- 595.

Truver MT, **Palmquist KB**, Swortwood MJ. Oral Fluid and Drug Impairment: Pairing Toxicology with Drug Recognition Expert Observations. *Journal of Analytical Toxicology* 2019; **43**(8): 637-643.

Palmquist KB, Swortwood MJ. Data-independent screening method for 14 fentanyl analogs in whole blood and oral fluid. *Forensic Science International* 2019; **297**: 189-197.

PRESENTATIONS

K.B. Palmquist*, M.J. Swortwood. Long-term Stability of Fentanyl Analogs in Blood. ORAL PRESENTATION. Crossing Forensic Borders Global Lecture Series. May 2021.

K.B. Palmquist*, M.J. Swortwood. Long-Term Stability Assessment of Fentanyl Analogs in Blood using Liquid Chromatography/Quadrupole Time-of-Flight/Mass Spectrometry (LC-QTOF-MS). ORAL PRESENTATION. American Academy of Forensic Sciences Annual Scientific Meeting. Virtual Conference. Feb 2021.

K.B. Palmquist, M.J. Swortwood. Quantification of Furanylfentanyl and its Metabolites in Human and Rat Plasma by LC-MS/MS. Midwest Association for Toxicology and Therapeutic Drug Monitoring (MATT)/Southwestern Association of Toxicologists (SAT) Combined Annual Meeting. Kansas City, MO. April 2020. – CANCELLED DUE TO COVID-19

K.B. Palmquist*, M.T. Truver* Detection and Quantification of Synthetic Opioids in Oral Fluid. ORAL PRESENTATION. American Academy of Forensic Sciences National Institute of Justice Forensic Science Research and Development Symposium. Baltimore, MD. Feb 2019

K.B. Palmquist*, M.J. Swortwood. Liquid Chromatography/Quadrupole Time-of-Flight (LC-QTOF) Screening for Fentanyl Analogs in Whole Blood and Oral Fluid. POSTER PRESENTATION. American Academy of Forensic Sciences Annual Scientific Meeting. Baltimore, MD. Feb 2019.

K.B. Palmquist*, M.J. Swortwood. Screening of Fentanyl Analogs in Whole Blood using LC-QTOF Analysis. ORAL PRESENTATION. Society of Forensic Toxicologists Annual Meeting. Minneapolis, MN. Oct 2018.

HONORS AND AWARDS

Educational Research Award- Doctorate, Society of Forensic Toxicologists	2021
Sparks Veasey, III, MD, JD Academic Prize in Forensic Science, SHSU	2021
Forensic Science Foundation, Student Scholarship Award (2021)	2020
Ravens Scholar, Sam Houston State University	2020
3MT Finalist, Sam Houston State University	2019
Best Toxicology Poster, AAFS Annual Meeting (2019)	2019
Forensic Science Foundation, Student Scholarship Award (2019)	2018
Forensic Science Foundation, Lucas Grant Recipient	2017
Forensic Science Scholarship, Sam Houston State University	2016-2021
PolyED Award for Outstanding Achievement in Organic Chemistry	2015
Nominee: Merck Outstanding Performance in Organic Chemistry	2014
Dean's List at Towson University	2012-2016

PROFESSIONAL AFFILIATIONS

Society of Forensic Toxicologists, Student Affiliate	2017-Present
American Academy of Forensic Sciences, Student Affiliate	2017-Present
Secretary: Society of Forensic Science, SHSU	Fall 2019- Spring 2021
Marketing Director: Society of Forensic Science, SHSU	Spring 2019
Society of Forensic Science, Sam Houston State University	2016-2021
President: FSSO, Towson University	Spring 2016
Vice President: FSSO, Towson University	Spring 2015- Fall 2016
Forensic Science Student Organization (FSSO) member, Towson University	2013-2016
American Chemical Society	2015-2016