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Qualitative Identification of Fentanyl and Fentanyl Analogs in Horse Serum using
Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS)

By

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THESIS

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Abstract

Highly potent opiates such as Fentanyl and its analogs have a large potential for abuse in horse racing due to their combined pain-relieving properties and their ability to induce Central Nervous System (CNS) stimulatory effects. Recently, a large number of highly potent fentanyl analogs have been synthesized leading to concerns about the ability to detect these compounds in routine analysis. A standard post-race drug testing panel does not currently include many of these novel fentanyl analogs, therefore these may be undetectable in routine testing. Additionally, these compounds are highly potent and their presence in urine and blood samples may evade detection with methods that have high detection levels. Liquid Chromatography - High Resolution Mass Spectrometry (LC-HRMS) is an analytical technique that can efficiently detect drugs in biological matrices without compromising sensitivity and selectivity. Accordingly, an LC-HRMS method for the identification of 43 fentanyl analogs in equine serum was developed and validated. Application of this methodology can allow for better enforcement of anti-doping regulations for the protection of racehorses. The ability to identify fentanyl and fentanyl analogs in serum will support anti-doping measures in horse racing, as well as promote an efficient means of testing in equine drug testing labs.

1.0 Introduction

1.1 Fentanyl and its Influence on Equine Performance

Fentanyl is a substance that can enhance equine athlete performance due to its stimulant and analgesic effects. It predominantly influences autonomic and behavioral responses, and produces moderate analgesic effects [1]. Stimulant effects include an increased heart rate (tachycardia) and locomotor stimulation [2]. Young horses (foals) have been shown to exhibit stimulant behavior when administered high doses of fentanyl, while mature horses exhibit this at lower doses. Significant increases in heart rate have been observed within 15 minutes of fentanyl administration to foals [3]. High doses of fentanyl have been shown to produce significant increases in locomotor activity [4].

Fentanyl poses a challenge in chemical testing as it has a high potency (80-150x more potent than morphine) and produces low concentrations of the primary active component and metabolites in urine [5]. Fentanyl analogs are typically clandestinely synthesized and are similar in structure to fentanyl and have been found to also produce locomotor stimulation. The ability to screen for the presence of fentanyl and fentanyl analogs in biological matrices is necessary to mitigate the use of these illicit substances in equine athletes.

1.2 Research Goals

The goal of this research was to develop an LC-HRMS method to qualitatively identify fentanyl and fentanyl analogs in horse serum. Presently, analytical approaches for equine drug testing can be time-consuming or have limited sensitivity for fentanyl analysis. Additionally, LC-HRMS is suitable for efficiently analyzing substances that are time consuming to include in

a targeted method, such as designer drugs. The study focused on developing and validating an LC-HRMS for qualitative identification of fentanyl and fentanyl analogs in equine serum.

2.0 Background

2.1 Illicit Use of Fentanyl and Fentanyl Analogs

There is an increasing need for analytical methods which can detect fentanyl from both a law enforcement and public health interest. The development and increasing prevalence of fentanyl analogs hinders the attempts of laboratories to detect these substances due to the need for specialized testing. Fentanyl analogs are structurally similar to fentanyl and are also μ-opioid receptor agonists. These receptors are found in brain tissue and the gastrointestinal tract, and are responsible for opiate related effects such as analgesia, euphoria, and respiratory depression [6]. Synthetic analogs are more likely to be illicitly manufactured and are implicated in the increase in opioid deaths in the United States [7].

The Drug Enforcement Administration (DEA) is a federal agency responsible for the enforcement of drug laws in the United States. The National Forensic Laboratory Information System (NFLIS) is a program within the DEA that collects results of drug analyses from forensic laboratories across the country [8]. In the 2019 NFLIS annual report, fentanyl was reported as the 5th most frequently identified drug at the national level. In 2020, it was identified as the 4th most reported drug in the December snapshot report [9]. There is evidence that demand for synthetic opioids is widening since it is emerging in new areas of the world. In 2020, liquid fentanyl was seized in Myanmar during a large drug bust operation. The United Nations Office on Drugs and Crime recognized the bust as a significant seizure that included the discovery of 3,700 liters of the fentanyl analog methylfentanyl by law enforcement agents [10].

The United States Centers for Disease Control and Prevention (CDC) provides reports on various public health issues. This agency reports statistical trends on deaths in the U.S. attributed to synthetic opioids, including rates that vary by region. In 2021, the CDC reported a 1,040% increase in synthetic opioid deaths during the years 2013-2019. The agency also found that the largest increase in synthetic opioid-related deaths occurred in the West (67.9%) during 2018-2019 [11]. Geographic shifts in overdose deaths may implicate the expanding distribution of these types of opioids.

Analytical testing of biological substances for illicit drugs is pivotal in the efforts to protect equine athletes from doping. In horses, fentanyl behaves as a CNS stimulant and an analgesic, therefore it has a high potential for abuse in the horse racing industry [1]. The use of analogs in illegal horse doping is advantageous since laboratories cannot develop targeted methods fast enough to identify novel synthetic drugs. There is evidence these substances are becoming more prevalent in the horse racing industry. In as early as 2015, The Association of Racing Commissioners International (ARCI) issued a press release announcing a trainer's suspension due to horses testing positive for AH-7921. This novel synthetic opioid was detected as a result of the ability of the New York Equine Drug Testing Program to detect these substances. The trainer was penalized to prevent further use of the illegal substance in horse racing [12]. This case highlights the need for sensitive and efficient testing to monitor for novel drugs.

2.2 Clinical use of Fentanyl

Fentanyl is classified as a narcotic analgesic due to its ability to produce analgesic and pain-relieving effects. Its pharmacokinetic effects have been studied in humans and horses and it has been determined that fentanyl is a potent agonist of μ-opioid receptors [3]. Fentanyl was first synthesized in 1960 by Paul Janssen for the treatment of pain in humans. It was approved in 1972 under the commercial name Sublimaze® as an anesthetic, with reports of illicit use occurring as early as the 1980s. Various forms of the drug have emerged, including transdermal patches which have been indicated in overdose deaths since the 1990s [7].

Fentanyl is classified as a short acting analgesic in humans, yet different doses of fentanyl in horses have been found to produce effects that are primarily locomotor and autonomic, with only a slight analgesic effect [1]. Scientific research of fentanyl has sought to determine its potential for clinical use in horses for pain. Research of fentanyl's effects in young foals has shown that changes in behavior are dose dependent, with sedation observed for low doses [3]. Transdermal fentanyl patches have been investigated in adult horses to determine effectiveness in pain management at various doses. Research into transdermal administration of fentanyl to horses indicates that stimulatory effects of the drug may not be a concern. One study found that transdermal delivery of 60 to 67 μg/kg to adult horses was safe and had no adverse effects. However the plasma concentration required for effective analgesia was uncertain [13].

2.3 Fentanyl Analogs

Since 1791, the term "analog" has been used in the sciences to refer to structural and functional similarities. In chemistry, a drug analog can be defined as a compound that has either a structural similarity, a pharmacological similarity, or a structural and pharmacological

similarity to the original drug. It has been proposed that compounds with chemical similarity be identified as "structural analogs" and compounds with common biological properties be identified as "functional analogs" [14]. Fentanyl analogs can be classified as both, since they are highly selective for the μ-opioid receptor and share structural similarities to fentanyl.

In the mid 1970s, Janssen Pharmaceuticals developed synthetic fentanyl analogs sufentanil and alfentanil to provide physicians with multiple options for their patients. In the 1980s, many overdose deaths were associated with the fentanyl analog 3-methylfentanyl, also known as "China White." After the release of the transdermal patch in the 1990s, the abuse of fentanyl led to thousands of overdose deaths due to the misuse of the patches.

In recent years, the demand for fentanyl has only increased as drug traffickers started using it to increase the potency of their heroin product. Post-mortem toxicological analyses on overdose deaths have indicated the presence of N-phenyl-1-(-2-phenylethyl)piperidin-4-amine (4-ANPP). This compound is suspected to be involved with the production of other analogs including acetylfentanyl, butyrylfentanyl, and furanylfentanyl [15]. The most recent synthetic opioid overdose report by the CDC (2019-2020) indicated synthetic opioid deaths accounted for over 82% of opioid related deaths in 2020. The report also noted fentanyl was responsible for the significant increase. The fentanyl analogs acetylfentanyl, furanylfentanyl, and carfentanil were mentioned as challenging to detect in routine testing. As of the date of the report, carfentanil stands as the most potent fentanyl analog detected in the U.S.[16]. However, we cannot conclude this is *the* most potent fentanyl analog that exists due to the limitations in current testing methodologies.

There are four structural features in fentanyl that can be modified to produce an analog, (1) the piperidine ring, (2) the anilinophenyl ring, (3) the 2-phenylethyl substituent, and (4) a

carboxamide moiety linked to the anilino-nitrogen [6]. For example, the figure below highlights the addition of a substituent to the piperidine ring to form carfentanil (**Figure 1**).
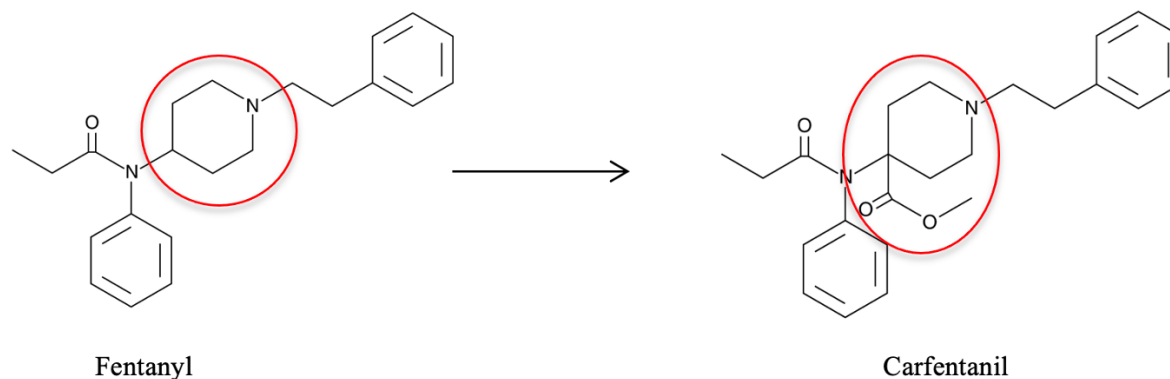

Figure 1. A modification to the piperidine ring in fentanyl results in the analog carfentanil.

Fentanyl's metabolic profile has been investigated extensively, and the data has indicated the initial metabolic transformations involve oxidative *N*-dealkylation, amide hyrdolysis, *N*-oxide formation, and hydroxylation (**Figure 2**).

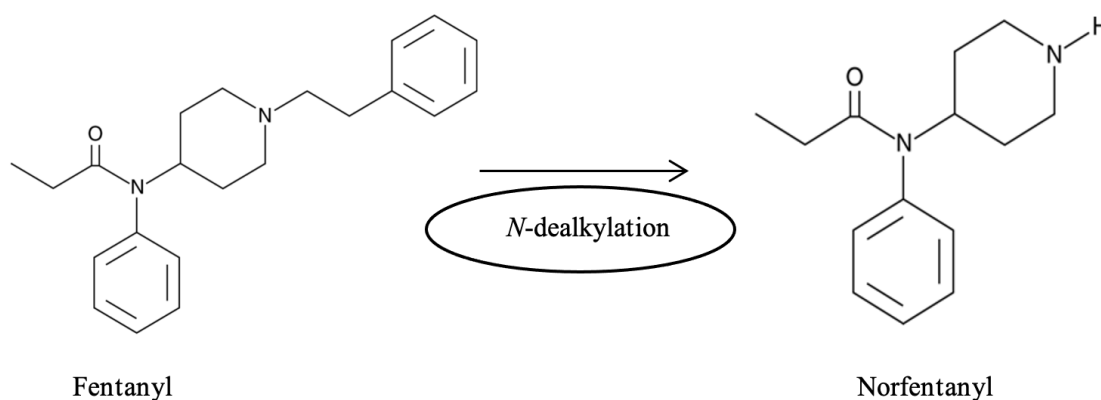Figure 2. Fentanyl is metabolized in the liver through oxidative N-dealkylation at the piperidine ring.

A literature review on the metabolism of fentanyl analogs identified over twenty analogs and their metabolites, potencies, and metabolic pathways. 4-ANPP was a common metabolite among several analogs, including acetylfentanyl, butyrfentanyl, furanylfentanyl, and

methoxyacetylfentanyl [6]. However, our current understanding of the metabolism of analogs is still minimal due to the limitations of testing methodologies. The ability to confirm the presence of analogs and their metabolites will expand our knowledge of their potency, metabolic pathways, and physiological effects.

2.4 Fentanyl Analysis Techniques

There are several analytical approaches used in both equine and human drug testing. Current analytical techniques used for detecting the presence of fentanyl analogs in biological samples predominantly includes immunoassays, Raman spectroscopy, and mass spectrometry. The specificity and sensitivity of each of these techniques varies, including the efficiency of sample preparation and instrument analysis. Successful analysis of fentanyl analogs requires an efficient and sensitive method. The multiple analytical techniques used in drug testing are not all appropriate for novel drug analysis. ELISA is susceptible to false-positives due to cross-reactivity. Targeted LC-MS and GC-MS techniques can be time consuming as each target analyte must be optimized during method development. This can become overwhelming when developing a method for a large class of drug compounds. Additionally, the high specificity of these techniques can cause other drugs and metabolites to go undetected in biological matrices [17].

Enzyme-linked immunosorbent assay (ELISA) is a commonly used analytical technique used to presumptively screen for the presence of drugs. Although immunoassays are susceptible to cross-reactivity, they remain a useful technique for screening. A recent study compared the performance of two types of immunoassays, the ARK Fentanyl II® assay and the Immunalysis SEFRIA® fentanyl assay. It was determined the level of cross-reactivity of the assays was

dependent on the location of the analog modification when compared to fentanyl. The assays were successful at detecting analogs with modifications at the amide group and/or aniline ring. However, when there was a modification to the alkyl portion of the *N*-alkyl chain there was a variability in the ability of each assay to detect the analog. The results indicated that SEFRIA® was more sensitive when compared to ARK Fentanyl II®; the SEFRIA® assay detected 57 of 58 fentanyl analogs while ARK Fentanyl II® detected 51 of 58 fentanyl analogs [18]. The author accurately noted that a high-resolution mass spectrometry assay may be able to confirm fentanyl analogs detected by the ARK Fentanyl II® or SEFRIA® assay. Otherwise, these compounds may have been classified as "false positive" when using a confirmation method with a list containing more common analytes.

Liquid chromatography-mass spectrometry (LC-MS) and gas-chromatography-mass spectrometry (GC-MS) are more sensitive and are frequently used to confirm the presence of drugs in positive immunoassay screens. These techniques involve coupling a chromatographic method to separate analytes (gas chromatography or liquid chromatography) with a mass spectrometer. Mass spectrometry uses either tandem mass spectrometry (MS/MS) or high resolution mass spectrometry (HRMS) to detect analytes. Each offers different advantages and choosing which to use in drug analysis largely depends on the laboratory's testing needs and available equipment.

MS/MS uses a targeted and limited mass range to identify and quantify drugs and is commonly accomplished on a triple quadrupole mass spectrometer using selected reaction monitoring (SRM) or multiple reaction monitoring (MRM). This technique can measure the monoisotopic mass of a molecule to the nearest decimal, providing unit resolution. The general principle of a tandem mass spectrometer involves precursor ion selection (Quadrupole 1),

fragmentation in a collision cell (Quadrupole 2), and fragment filtration (Quadrupole 3). The unique fragments are detected, ultimately producing a peak area which can be used to quantify the analyte of interest.

HRMS has the capability to sensitively screen for all ions generated when operating in full scan or MS/MS mode following fragmentation. This technique has a higher mass resolution, mass accuracy, and a wider mass range relative to unit resolution mass spectrometers. HRMS can measure the monoisotopic mass of a molecule accurately to the nearest three decimal places. The two most common types of HRMS instruments are based on Time of Flight (TOF) or Orbitrap approaches. Due to the ability to resolve spectra, analyte signals can be extracted precisely with a narrow mass window (e.g. millidaltons) from acquired spectra. The capability to extract spectra with a small millidalton window helps resolve interferences and greatly enhances selectivity. Another advantage of HRMS is it allows for retrospective data analysis when using full scan acquisition. This allows the analyst to review previously acquired data to search for compounds not initially included in the method panel while MRM/SRM based methods only look for their targeted compounds.

LC-HRMS can detect multiple drugs in one method, without compromising efficiency. The primary advantage of LC-HRMS is the ability to use full scan data acquisition to detect multiple analytes without the need to optimize precursor and product ions for each target analyte. This makes this technique useful in searching for substances that could not be included in a targeted method, such as designer drugs and other novel compounds.

Testing for fentanyl analogs in human and animal biological substances at trace levels requires specialized methodologies which makes the identification of these drugs challenging. Due to an increasing need to detect these drugs in humans and animals, the CDC developed

opioid material kits containing fentanyl analog standards [19]. Multiple kits were produced

containing a large list of novel opioid standards to support laboratories in their development of

new methods for novel substances.

2.5 Knowledge Gap

Use of fentanyl and its analogs is a risk to the integrity of horseracing and to the equine

and human athletes that compete in it. Fentanyl is a substance with a high potential for abuse in

the sport and the use of novel analogs that share similar pharmacological effects but are

structurally different create regulatory challenges to prevent their misuse. There is an ever

expanding list of fentanyl analogs that are being developed and sensitive and selective analytical

techniques are necessary to detect their use. Accordingly, this study describes the development

and validation of an LC-HRMS method to detect over 40 Fentanyl analogs at low pg/mL levels

in equine serum.

3.0 Materials and Methods

3.1 Chemicals and Materials

3.1.1 Fentanyl Analog Standards and Internal Standards

Fentanyl analog compounds furanyl norfentanil, n-methyl norcarfentanil, β-

hydroxythioacetylfentanyl, remifentanil, norsufentanil, β-hydroxythiofentanyl, thienyl fentanyl,

benzyl fentanyl, para-fluoro methoxyacetyl fentanyl, benzyl acrylfentanyl, β-hydroxy fentanyl,

α'-methoxy fentanyl, tetrahydrofuran fentanyl 3-tetrahydrofurancarboxamide, para-fluoroacetyl

fentanyl, para-methoxy acetyl fentanyl, acetyl fentanyl, ethoxyacetyl fentanyl, thiofentanyl, N-

benzyl furanyl norfentanyl, para-methoxy tetrahydrofuran fentanyl, para-fluoro tetrahyrdofuran

fentanyl, fentanyl methyl carbamate, acrylfentanyl, para-methyl acetyl fentanyl, para-methoxy acrylfentanyl, (+/-)-trans-3-methyl thiofentanyl, furanyl fentanyl 3-furancarboxamide isomer, N,N-dimethylamido-despropionyl fentanyl, meta-fluorofentanyl, 4'-fluorofentanyl, AH-7921, (+/-)-cis-3-methyl thiofentanyl, U-47700, 2'-fluoro ortho-fluorofentanyl, para-methoxy furanyl fentanyl, N-(3-ethylindole) Norfentanyl, furanyl fentanyl, β-methyl fentanyl, para-fluoro furanyl fentanyl, U-48800, para-methylfentanyl, tetrahydrothiophene fentanyl, FIBF, 4'-fluoro, para-fluoro (+/-)-trans-3-methyl fentanyl, para-methyl furanyl fentanyl, senecioylfentanyl, ortho-methoxy butyryl fentanyl, para-chloro furanyl fentanyl, cyclopentenyl fentanyl, 2,3-seco-fentanyl, isopropyl U-47700, (+/-)-cis-3-methyl butyryl fentanyl, isovaleryl fentanyl, MT-45, valeryl fentanyl, para-methyl butyryl fentanyl, para-methoxy valeryl fentanyl, para-chlorobutyryl fentanyl, para-fluoro valeryl fentanyl, para-fluoro cyclopentyl fentanyl, ortho-isopropyl furanyl fentanyl, 2-fluoro MT-45, cyclohexyl fentanyl, para-methyl cyclopentyl fentanyl, para-chloro valeryl fentanyl, heptanoyl fentanyl, and 2,2,3,3-tetramethyl-cyclopropyl fentanyl were provided free of charge by the Cayman Chemical Company as part of the CDC Fentanyl Analog Screening Kit and its expansion packs (Ann Arbor, Michigan, USA).

Internal standards norfentanyl-13C6 oxalate, (+/-)-β-hydroxythiofentanyl-13C6, 4-ANPP-13C6, acryl fentanyl-13C6, furanyl fentanyl-13C6, para-fluorofentanyl-13C6, cyclopropyl fentanyl-13C6, U-48800-13C3,15N2, butyryl fentanyl-13C6, and para-fluorobutyryl fentanyl-13C6 were provided free of charge by the Cerilliant Corporation (Round Rock, Texas, USA) as part of their CDC Opioid Certified Reference Material (CRM) Kit.

Internal standards D_3-Morphine-3β-D-Glucuronide (cat. no. M-017) and D_8-Amphetamine (cat. no. A-018) were provided by Cerilliant Corporation. Internal standard D_5-

Furosemide (cat. no. F865002) was provided by Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada).

3.1.2 Reagents

Methanol (Optima® grade, cat. no. A454), Sodium Acetate Trihydrate (ACS reagent grade), Glacial Acetic Acid (HPLC Grade), Sodium Phosphate Monobasic - $NaH_2PO_4 \cdot H2O$ (ACS reagent grade), Sodium Phosphate Dibasic - Na_2HPO_4 (ACS reagent grade), 2-Propanol (Optima® grade), Ammonium Hydroxide (ACS reagent grade), and Methylene Chloride (Optima® grade) were purchased from Fisher Scientific (Waltham, Massachusetts, USA). β-Glucuronidase Enzyme from *Patella vulgata* was purchased from Sigma Aldrich (St. Louis, Missouri, USA). Acetonitrile (>99.0% HPLC grade) and Formic Acid (~98%) was purchased from Budrick and Jackson (Musekgon, Michigan, USA).

3.1.3 Glassware and Materials

Calibrated volumetric flasks of various sizes (Class A Pyrex®), pH Paper (pH 4.0-7.0, cat. No. M95823.PK), Glass culture tubes (12 x 75 mm), and 2 mL Crimp Autosampler Vials (Sun Sri, cat. no. 14-823-418) were purchased from Fisher Scientific (Waltham, Massachusetts, USA). DI Water was provided from a Nanopure® Water system from ThermoFisher Scientific (Waltham, Massachusetts, USA). ThermoFisher Scientific (Waltham, Massachusetts, USA). Pooled control serum was purchased from BioIVT. XtrackT® DAU extraction columns (Lot# 910190-KL) were provided by United Chemical Technologies (Bristol, Pennsylvania, USA).

3.2 Preparation of Stock Solutions

3.2.1 Calibration Standards and Internal Standards

  Fentanyl Analog certified reference materials were provided from an opioid material kit line developed by the United States Centers for Disease Control and Prevention (CDC). The CDC contracted with the Cayman Chemical Company and the Cerilliant Corporation to manufacture and distribute kits to enable labs to screen for synthetic fentanyl compounds. Standards were prepared from the Cayman Fentanyl Analog Screening Kit, Cayman Fentanyl Analog Screening Kit - Emergent Panel Version 1. The kits contained individual vials with 200 µg of powdered reference standard. The powders were reconstituted with 500 µL of methanol for a final concentration of 400 µg/mL and stored at -20°C. The Internal Standard (IS) was prepared from the Cerilliant Corporation's CDC opioid certified reference material kit. This kit included carbon-13 and nitrogen-15 isotopically labeled internal standards.

  Seventy-two individual Cayman reference standards at 400 µg/mL were aliquoted at appropriate volumes to prepare one stock mix that contained each drug at 2 ng/µL. This stock mix was serially diluted to prepare a solution at 0.02 ng/µL (Dilution 1) and 0.002 ng/µL (Dilution 2). These solutions were spiked at the appropriate volume in 1 mL of serum to achieve the desired concentration of each calibrator and QC standards. All of the stock and serial dilutions were prepared in calibrated volumetric flasks and diluted to volume with Methanol. The Internal Standard (IS) was prepared by using 10 Cerilliant standards. These standards were aliquoted at appropriate volumes and diluted with methanol to prepare a solution that contained each drug at 200 ng/mL (**Table 1**).

Table 1. Cerilliant Internal Standards used to prepare a mix which contained each analyte at 200 ng/mL.

Lot Number	Internal Standard	Chemical Formula	Retention Time (Min)
N-126-1ML	Norfentanyl-13C6 oxalate	C8 13C6H2ON2O · C2H2O4	6.18
H-138-1ML	(+/-)-β-Hydroxythiofentanyl-13C6 HCl	C14 13C6H26N2O2S · HCL	7.13
A-175-1ML	4-ANPP-13C6	C13 13C6H24N2	7.65
A-173-1ML	Acryl fentanyl-13C6 HCl	C16 13C6H26N2O · HCl	7.75
F-064-1ML	Furanyl fentanyl-13C6 HCl	C18 13C6H26N2O2 · HCl	8.05
F-068-1ML	para-Fluorofentanyl-13C6	C16 13C6H27N2OF	8.09
C-199-1ML	Cyclopropyl fentanyl-13C6 HCl	C17 13C6H28N2O · HCl	8.15
U-014-1ML	U-48800-13C3,15N2 HCl	C14 13C3H24 15N2OCl2 · HCl	8.30
B-084-1ML	Butyryl fentanyl-13C6	C17 13C6H30N2O	8.47
F-066-1ML	para-Fluorobutyryl fentanyl-13C6	C17 13C6H29FN2O	9.32

A calibration curve was developed using seven concentrations in serum (0.05 ng/mL, 0.1 ng/mL, 0.5 ng/mL, 1 ng/mL, 2.5 ng/mL, 5 ng/mL, and 10 ng/mL). The quality control (QC) standards were prepared at three different levels in serum, representing a Low, Mid, and High concentration across the curve (0.25 ng/mL, 2 ng/mL, and 7 ng/mL, respectively).

3.2.2 Stock Solutions for Solid Phase Extraction Procedure

A solution used for enzymatic hydrolysis was prepared by adding one bottle of β-glucuronidase enzyme (2,000,000 units per vial) to 200 mL of 1.6M acetate buffer, pH 5.0 (Lab Lot#SLCB8351). An IS Spiked Enzyme Solution was prepared by adding 0.4mL of the β-Glucuronidase Enzyme solution per sample (β-Glucuronidase in pH 5.0, 5000 units/mL) and 10μL of IS per mL of Enzyme. This was prepared fresh daily.

A 0.1M Phosphate Buffer, pH 6.0 and 0.6M Phosphate Buffer, pH 6.5 were prepared using the appropriate ratios of Sodium Phosphate Monobasic and Sodium Phosphate Dibasic to achieve the desired concentration. A 1.6M Acetate Buffer, pH 5.0 solution was prepared by adding 136 g of sodium acetate trihydrate to 200 mL of DI water. After mixing, 33 mL of acetic acid was added and the solution was diluted to 1L with DI water. A 1.0M Acetic Acid solution was prepared by adding 57.2 mL of glacial acetic acid to 1L of DI water.

The base solvent 78:20:2 (v:v:v) methylene chloride:2-propanol:ammonium hydroxide was prepared fresh daily by combining 20 mL of 2-Propanol, 2 mL of ammonium hydroxide, and 78 mL of methylene chloride. A reconstitution solvent was prepared to achieve a final concentration of 5% Acetonitrile in Water with Formic Acid.

3.3 Extraction of Fentanyl and Fentanyl Analogs from Serum

Serum samples from a pooled control serum solution were aliquoted at 1 mL into glass culture tubes and then spiked with a β-glucuronidase enzyme solution with IS. The samples were vortexed (Fisher Vortex Genie 2, Fisher Scientific, Waltham, Massachusetts, USA), and the pH was verified to be within 5+/- 0.5 with pH paper. After verifying the pH, the samples were incubated in a water bath set at 37 +/- 5°C for 2 hours (5510 Branson Ultrasonic Water Bath, Fisher Scientific, Waltham, Massachusetts, USA).

Following hydrolysis, the samples were carried through the extraction procedure using XtrackT® DAU columns. The samples were diluted with 1.6 mL of 0.6M Phosphate Buffer, pH 6.5 and briefly vortexed. The pH of 10% of the sample set was verified to be 6 +/- 0.5 and then centrifuged at 4000 rpm at 4°C for 5 minutes (Sorvall Super T21, Kendro Laboratory Products). The columns were placed in a Cerex 48-place solid phase extraction apparatus (Cera Inc.) and conditioned with 3 mL of methanol, followed by 3 mL of 0.1M Phosphate Buffer, pH 6.0. The samples were then loaded onto the conditioned columns and passed through using low-pressure nitrogen at a rate of 1 - 2 mL/min. The columns were rinsed with 3 mL of DI water at a rate of 3 - 5 mL/min, and then acidified with 2 mL of 1M acetic acid at a rate of 3 - 5 mL/min. The columns were dried using high-pressure nitrogen, rinsed with 3 mL of methanol at a rate of 3 - 5 mL/min, and dried again using high pressure nitrogen for 2 minutes.

The base fraction containing fentanyl and fentanyl analogs was collected by passing 1.7 mL of basic solvent 78:20:2 (v:v:v) methylene chloride:2-propanol:ammonium hydroxide through the column at a rate of 1 - 2 mL/min. The base fraction was concentrated by evaporating in a TurboVap Concentrator (TurboVap, Zymark, and Caliper Life Sciences), and then was

reconstituted with 160 uL of 5% acetonitrile in water with formic acid in preparation for LC-HRMS analysis.

3.4 LC-HRMS Methodology

The method was developed using a Thermo Scientific Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) coupled with an Agilent 1260 HPLC (Agilent Technologies, Inc., Santa Clara, California, USA) with an electrospray ionization source.

An ACE® 3 C18 2.1 x 100 HPLC column (Hichrom Ltd., Theale, England, UK) was used to chromatographically separate the analytes of interest. The aqueous mobile phase was Water (LCMS grade, Burdick and Jackson, Muskegon, Michigan, USA) with 0.1% Formic Acid (LCMS grade, Burdick and Jackson, Muskegon, Michigan, USA). The organic mobile phase was Acetonitrile (LCMS grade, Burdick and Jackson, Muskegon, Michigan, USA) with 0.1% Formic Acid (LCMS grade, Burdick and Jackson, Muskegon, Michigan, USA). A reverse phase gradient with a total run time of 12.5 minutes at a flow rate of 0.5 mL/min was used to achieve separation (**Table 2**) with the column maintained at 40°C. The HPLC flow was diverted to waste from start to 0.5 min and back to waste at 14 min.

Table 2. HPLC Gradient

Time (Min)	Organic Mobile Phase (Acetonitrile with 0.1% Formic Acid)	Aqueous Mobile Phase (Water with 0.1% Formic Acid)	Flow Rate (mL/min)
0.0	3%	97%	0.5
2.00	3%	97%	0.5
3.00	20%	80%	0.5
9.00	55%	45%	0.5
10.00	95%	5%	0.5
12.50	95%	5%	0.5
12.51	3%	97%	0.65
14.00	3%	97%	0.65
14.01	3%	97%	0.5
15.00	3%	97%	0.5

The HPLC column autosampler was maintained at a temperature of 40°C. Source parameters were: spray voltage 4000V, sheath gas 50 arbitrary units, auxiliary gas 10 arbitrary units, S-lens RF level 50 arbitrary units, capillary temperature at 300°C, and auxiliary gas heater temperature at 200°C. Full scan spectra (100-850 m/z) were acquired with a mass resolution of 70,000, AGC target of $1e^6$, max IT of 100 milliseconds. All ion fragmentation spectra (56.7-820 m/z) were acquired with a mass resolution of 17,500, AGC target of $5e^5$, and a max IT of 100

milliseconds with a stepped collision energy of 20 and 50 units. Xcalibur software (version

4.0.27.19) from Thermo Scientific (Waltham, Massachusetts, USA) was used to control the LC-

MS system and for data review.

3.5 Validation Parameters

Validation experiments were carried out over several days to evaluate the following

method parameters: limit of detection (LOD), limit of quantitation (LOQ), linearity, accuracy,

precision, recovery, and matrix effects. The validation plan can be referenced in **Table 3**.

Table 3. Validation Plan

Day 1	Day 2	Day 3
Experiments: Linearity, Accuracy, and Precision	**Experiments: Linearity, Accuracy, Precision, Recovery, and Matrix Effects**	**Experiments: Linearity, Accuracy, Precision, Stability at RT**
Calibrators - 7 Negative Control - 1 QC Low - 6 QC Mid - 6 QC High - 6 *Total Number of Samples = 26*	Calibrators - 7 Negative Control - 1 Positive Control - 1 (2 ng/mL) QC Low - 6 QC Mid - 6 QC High - 6 Post Extraction Spike Samples - 18 Neat Standards - 18 *Total Number of Samples = 18*	Calibrators - 7 Negative Control - 1 QC Low - 6 QC Mid - 6 QC High - 6 *Total Number of Samples = 29*

A calibration curve consisting of seven data points (0.05 ng/mL, 0.1 ng/mL, 0.5 ng/mL,

1 ng/mL, 2.5 ng/mL, 5 ng/mL, and 10 ng/mL) was prepared and extracted to determine linear

range and regression coefficients (R^2). The QC standards as a set of six replicates at the low

(0.25 ng/mL), mid (2 ng/mL), and high level (7 ng/mL) were prepared (18 samples total) and

extracted to evaluate bias (%) and precision (% Coefficient of Variation). Each of these

experiments were repeated on three separate days.

A post-extraction spike method was used to assess the recovery and matrix effects. Six replicates were prepared at the QC low, mid, and high level and were spiked in after the base fraction was dried down (18 samples total). A set of neat standards were prepared at the equivalent final concentration for comparison. Matrix effects were determined by determining the peak area ratio of post-extraction spiked samples to neat standards and percent recovery was determined by calculating the peak area ratio of post-extraction spiked samples to pre-extraction spiked QCs.

The LOD and LOQ were determined after evaluating all of the observed ions for each analog. The LOD was established by confirming (1) chromatography represented a gaussian peak shape, (2) presence of the parent ion, and (3) at least one unique fragment detected. The LOQ was established by confirming (1) chromatography represented a gaussian peak shape, (2) presence of the parent ion, and (3) at least two (preferably unique) fragment ions detected.

3.6 Data Analysis

A linear regression analysis for each analog was performed by plotting the concentration of seven calibrators against the instrument response. A best fit weighting factor of 1/x was used for all compounds. The relative standard deviation (%RSD) of the internal standard was calculated to assess the variability of the extraction. The error (%) of each calibrator was determined by comparing the theoretical concentration compared to the actual concentration.

$$\% \, RSD \, of \, Internal \, Standard = 100 \, \times \frac{Standard \, Deviation \, IS \, Area \, Count}{Average \, IS \, Area \, Count}$$

$$\% \, Error \, = \, 100 \, \times \frac{Calculated \, Concentration \, - \, Theoretical \, Concentration}{Theoretical \, Concentration}$$

The accuracy (% Bias) of the methodology was determined by calculating the average concentration of six QC replicates and subtracting the true value and dividing that by the true value. Precision (% CV) was determined by calculating the standard deviation of six QC replicates and dividing that by the average. These calculations were repeated for each QC level (Low, Med, High).

$$Bias \ = 100 \ \times \frac{Average\ Concentration \ - \ Theoretical\ Concentration}{Theoretical\ Concentration}$$

$$Precision \ = \ 100 \ \times \frac{Standard\ Deviation\ Concentration}{Average\ Concentration}$$

Matrix effects were determined by calculating the ratio of the peak area from post-extracted spiked samples to the peak area from neat standards at equivalent concentrations. Recovery was determined by calculating the ratio of the average peak area of extracted samples to the peak area of post-extracted spiked samples.

4.0 Results

A total of 68 fentanyl analogs (**Table 4**) were evaluated against qualitative and quantitative criteria: presence of the accurate mass parent ion (M+H), retention time comparable to known standard, presence of at least one product ion, elution order, $R^2 \geq 0.95$, and gaussian peak shape.

Table 4. Observed product ions and retention time (min) of 68 Fentanyl analogs. Product ions that are shared among different analogs are bolded.

Fentanyl Analog	Chemical Formula	Retention Time (min)	Precursor Ion (M+H)	Observed Product Ions (m/z)
Furanyl norfentanil	C16H18N2O2	6.31	271.1441	**188.0706**
N-methyl Norcarfentanil	C17H24N2O3	6.53	305.18597	**245.16484** 189.13862 158.09643 **113.05971**
β-Hydroxythioacetylfentanyl	C19H24N2O2S	6.53	345.16313	327.1524 **192.08422** 111.02661
Remifentanil	C20H28N2O5	6.95	377.2071	285.15975 228.12308 116.0706 **113.05971**
Norsufentanil	C16H24N2O2	7.02	277.1911	**245.16484** 184.13321 128.10699 96.08078
β-Hydroxythiofentanyl	C20H26N2O2S	7.15	359.1788	341.1682 285.1420 **192.08415** 146.09643
Thienyl fentanyl	C19H24N2OS	7.22	329.1682	180.08413 97.01140
Benzyl fentanyl	C21H26N2O	7.25	323.21179	233.07847 **174.12773** **91.05423**
para-fluoro Methoxyacetyl fentanyl	C22H27N2O2F	7.36	371.2129	**331.2129** **188.14316** **105.07019**
Benzyl Acrylfentanyl	C21H24N2O	7.38	321.1961	**174.12759** 138.09134 **91.05423**
β-hydroxy fentanyl	C22H28N2O2	7.39	353.2224	**335.2118** 279.18558 204.13829 186.12773
α'-methoxy Fentanyl	C23H30N2O2	7.40	367.2380	344.49262 **331.20926** **188.14282** **105.06999**

Fentanyl Analog	Chemical Formula	Retention Time (min)	Precursor Ion (M+H)	Observed Product Ions (m/z)
Tetrahydrofuran fentanyl 3-tetrahydrofurancarboxamide	C24H30N2O2	7.44	379.2380	**335.14847** **188.14303** **105.07012**
para-fluoroacetyl fentanyl	C21H25FN2O	7.47	341.2024	220.11324 **188.14311** **105.07018**
para-methoxy acetyl fentanyl	C22H28N2O2	7.49	353.22235	346.89558 **188.1432** 162.09107 **105.07017**
Acetyl fentanyl	C21H26N2O	7.50	323.21179	**188.14321** **105.07015**
Ethoxyacetyl fentanyl	C23H30N2O2	7.60	367.23800	309.13188 205.08576 **188.14310** 146.09648 **105.07020**
Thiofentanyl	C20H26N2OS	7.61	343.18390	245.16484 194.0998 **111.02660**
N-benzyl furanyl norfentanyl	C23H24N2O2	7.68	361.1911	174.12772 **91.05466**
para-methoxy tetrahydrofuran fentanyl	C25H32N2O3	7.72	409.2486	363.11681 **188.14307** **123.07976** **105.07019**
Para-fluoro tetrahydrofuran fentanyl	C24H29N2O2F	7.74	397.2286	**188.14316** **105.07015**
para-methyl methoxyacetyl fentanyl	C23H30N2O2	7.74	367.2380	252.96886 **188.14301** **105.07017**
fentanyl methyl carbamate	C21H26N2O2	7.74	339.2067	**188.14326** 146.09649 **105.07021**
Acrylfentanyl	C22H26N2O	7.77	335.2118	**188.14338** **105.06988**
para-methyl acetyl fentanyl	C22H28N2O	7.90	337.2274	**188.14317**
para-methoxy acrylfentanyl	C23H28N2O2	7.99	365.2224	323.1454 **188.14323** **105.07016**
(±)-trans-3-methyl thiofentanyl	C21H28N2OS	8.00	357.1995	**259.17968** 208.11525 **111.02651**

Fentanyl Analog	Chemical Formula	Retention Time (min)	Precursor Ion (M+H)	Observed Product Ions (m/z)
Furanyl fentanyl 3-furancarboxamide isomer	C25H28N2O3	8.06	405.2173	**188.14319** **105.07019**
N,N-Dimethylamido-despropionyl fentanyl	C22H29N3O	8.07	352.23834	**188.14325** 72.04500
meta-fluorofentanyl	C22H27N2OF	8.08	355.2180	**299.19180** 234.12890 **188.14338** 105.06990
4'-fluorofentanyl	C22H27N2OF	8.10	355.218	**299.19151** 216.13816 **206.13816** **123.06066** 103.05458
AH 7921	C16H22Cl2N2O	7.87	329.1182	**284.05968** **172.9549** 95.08577
U-47700	C16H22Cl2N2O	7.87	329.1182	**284.05972** **172.95492** 81.07036
(±)-cis-3-methyl thiofentanyl	C21H28N2OS	8.11	357.1995	**259.17968** 208.11541 **111.02652**
2'-fluoro ortho-fluorofentanyl	C22H26F2N2O	8.22	373.2086	356.23671 **206.13386**
para-methoxy furanyl fentanyl	C25H28N2O3	8.24	405.2173	**188.14319** **105.07019**
N-(3-ethylindole) Norfentanyl	C24H29N3O	8.24	376.23834	245.16514 144.08066 189.13847
Furanyl fentanyl	C24H26N2O2	8.28	375.2067	**188.14338**
β-methyl fentanyl	C23H30N2O	8.29	351.2431	202.15903 119.08544 **91.05462**
para-fluoro furanyl fentanyl	C24H25FN2O2	8.29	393.1973	317.19579 **188.14303** **105.07003**
U-48800	C17H24Cl2N2O	8.33	343.13385	298.07547 158.97593 112.11224
para-methylfentanyl	C23H30N2O	8.54	351.2431	253.07279 230.15262 **188.14302** **105.07015**

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Fentanyl Analog	Chemical Formula	Retention Time (min)	Precursor Ion (M+H)	Observed Product Ions (m/z)
Tetrahydrothiophene fentanyl	C24H30N2OS	8.56	395.21516	**188.14323** **105.07021**
FIBF	C23H29N2OF	8.60	369.2337	312. 21706 **188.14338** **105.06990**
4'-fluoro, para-fluoro (±)-trans-3-methyl fentanyl	C23H28F2N2O	8.64	387.2243	377.26843 220.14967 123.06100 **103.05453**
para-methyl furanyl fentanyl	C25H28N2O2	8.65	389.2224	241.07351 **188.14361**
Senecioylfentanyl	C24H30N2O	8.73	363.24309	281.20139 **188.14342** **105.07008** 83.04962
ortho-methoxy Butyryl fentanyl	C24H32N2O2	8.79	381.2537	375.73251 343.18095 **188.14338** **105.06988**
para-chloro furanyl fentanyl	C24H25ClN2O2	8.85	409.1677	**188.14314** **105.07033**
Cyclopentenyl fentanyl	C25H30N2O	8.86	375.24309	**188.14329** **105.07014**
2,3-seco-Fentanyl	C22H30N2O	8.89	339.24309	313.17909 204.13824 120.08094
Isopropyl U-47700	C18H26Cl2N2O	8.91	357.1495	312.091 172.95513 81.07036
(±)-cis-3-methyl butyryl fentanyl	C24H32N2O	8.99	365.2587	202.15822 **105.07033**
Isovaleryl fentanyl	C24H32N2O	8.99	365.2587	281.20052 **188.14309** **105.07016**
MT-45	C24H32N2	9.03	349.26383	**181.10104** 166.07759 **103.05455** 87.0921
Valeryl fentanyl	C24H32N2O	9.06	365.2587	**281.20157** **244.16741** **188.14306** **105.07017**
para-methyl Butyryl fentanyl	C24H32N2O	9.15	365.25874	295.21709

Fentanyl Analog	Chemical Formula	Retention Time (min)	Precursor Ion (M+H)	Observed Product Ions (m/z)
				244.17037 **188.14319** **146.09631** **105.07017**
para-methoxy valeryl fentanyl	C25H34N2O2	9.31	395.2693	328.52486 **188.14312** **105.07021**
para-chlorobutyryl fentanyl	C23H29ClN2O	9.33	385.2041	**281.10499** **188.14300** **105.07018**
para-fluoro valeryl fentanyl	C24H31FN2O	9.37	383.2493	**299.19162** **188.14312** **105.07015**
para-fluoro cyclopentyl fentanyl	C25H31FN2O	9.48	395.2493	**299.19097** 229.07321 **188.14336** **105.07019**
ortho-isopropyl furanyl fentanyl	C27H32N2O2	9.60	417.2537	342.20755 **188.14303** **105.07014**
2-fluoro MT-45	C24H31FN2	9.63	367.2544	199.09161 179.08542 169.16992
Cyclohexyl fentanyl	C26H34N2O	9.69	391.2744	**281.20123** **188.14338** **105.07021**
para-methyl cyclopentyl fentanyl	C26H34N2O	9.90	391.27439	295.21743 **188.14323** **105.07014** 69.07054
para-chloro valeryl fentanyl	C24H31ClN2O	9.97	399.2198	287.14639 **188.14332** **105.07021**
Heptanoyl fentanyl	C26H36N2O	10.54	393.29004	240.23209 **188.1432** **105.07019**
2,2,3,3-tetramethyl-cyclopropyl fentanyl	C27H36N2O	10.58	405.2900	125.0961 **281.20123** **188.14338**

Forty-three fentanyl analogs were confirmed; the linearity, retention time stability (% CV),

LOD, and LOQ of these analogs are shown in **Table 5**.

Table 5. Linearity and retention time stability of analytes that met criteria: $R^2 \geq 0.95$; linear range 0.05-10 ng/mL

Fentanyl Analog	RT Stability (% CV)	Linear Regression Equation	R^2	LOD (ng/mL)	LOQ (ng/mL)
Furanyl norfentanyl	0.13	Y = 2.258x – 0.0813	0.9929	0.10	0.10
ß-Hydroxythioacetylfentanyl	0.13	Y= 3.404x + 0.0046	0.9839	2.5	2.5
N-methyl Norcarfentanil	0.13	Y = 2.655x – 0.0212	0.9967	0.05	0.10
Norsufentanil	0.11	Y = 7.652x + 0.1192	0.9768	0.05	0.10
beta-Hydroxythiofentanyl	0.12	Y = 3.064x – 0.0637	0.9893	0.50	0.50
Thienyl fentanyl	0.12	Y = 0.792x – 0.0474	0.9893	0.50	0.50
Benzyl fentanyl	0.13	Y = 8.492x – 0.1007	0.9938	1.0	1.0
para-fluoro Methoxyacetyl fentanyl	0.11	Y = 5.064x – 0.0276	0.9915	0.05	0.10
Benzyl Acrylfentanyl	0.10	Y = 4.936x – 0.1947	0.9910	0.10	0.10
beta-hydroxy Fentanyl	0.10	Y = 4.964x + 0.0038	0.9872	0.50	0.50
alpha'-methoxy Fentanyl	0.12	Y = 5.494x – 0.0944	0.9930	1.0	0.10
para-Fluoroacetyl fentanyl	0.10	Y = 3.595x + 0.0769	0.9821	0.05	0.10
Thiofentanyl	0.12	Y = 2.836x – 0.1141	0.9896	0.10	0.10
Fentanyl Methyl Carbamate	0.10	Y = 4.010x + 0.1132	0.9785	0.05	0.10
Acrylfentanyl	0.11	Y = 2.798x – 0.0697	0.9888	0.05	0.10
AH 7921	0.11	Y = 2.137x – 0.0811	0.9913	0.10	0.10
U-47700	0.11	Y = 3.913x – 0.2041	0.9785	0.10	0.10
para-methyl Acetyl fentanyl	0.10	Y = 3.006x – 0.114	0.9917	0.05	0.10
para-methoxy Acrylfentanyl	0.10	Y = 2.733x – 0.0837	0.9950	0.05	0.10
(+)-trans-3-methyl Thiofentanyl	0.10	Y = 1.956x – 0.0867	0.9755	0.50	0.50
Furanyl fentanyl 3-furancarboxamide isomer	0.10	Y = 3.718x – 0.1244	0.9890	0.05	0.10
N,N-Dimethylamido-despropionyl fentanyl	0.09	Y =7.612x – 0.3289	0.9867	0.05	0.10

Fentanyl Analog	RT Stability (% CV)	Linear Regression Equation	R^2	LOD (ng/mL)	LOQ (ng/mL)
(+)-cis-3-methyl Thiofentanyl	0.10	Y = 1.636x – 0.0609	0.9795	0.50	0.50
2'-fluoro ortho-Fluorofentanyl	0.10	Y = 1.337x – 0.0912	0.9829	0.50	0.50
para-methoxy Furanyl fentanyl	0.09	Y = 3.217x – 0.1187	0.9940	0.05	0.10
Furanyl fentanyl	0.09	Y = 3.146x – 0.1526	0.9906	0.10	0.10
para-fluoro Furanyl fentanyl	0.09	Y = 1.824x – 0.0689	0.9943	0.10	0.10
para-Methylfentanyl	0.09	Y = 5.204x – 0.3055	0.9916	0.05	0.10
Tetrahydrothiophene fentanyl	0.10	Y = 2.578x – 0.1374	0.9718	0.10	0.50
4'-fluoro, para-fluoro (+)-trans-3-methyl Fentanyl	0.10	Y = 1.684x – 0.0399	0.9822	0.50	0.50
para-methyl Furanyl fentanyl	0.10	Y = 2.278x – 0.1468	0.9925	0.50	0.50
ortho-methoxy Butyryl fentanyl	0.10	Y = 1.841x – 0.1347	0.9965	0.50	0.50
para-chloro Furanyl fentanyl	0.09	Y = 1.261x – 0.0764	0.9959	0.10	0.50
Cyclopentenyl fentanyl	0.09	Y = 1.313x – 0.0672	0.9953	0.10	0.50
2,3-seco-Fentanyl	0.09	Y = 8.617x + 0.9351	0.9635	0.05	0.10
Isopropyl U-47700	0.08	Y = 1.940x – 0.2363	0.9846	0.10	0.10
para-methoxy Valeryl fentanyl	0.09	Y = 2.103x – 0.1866	0.9945	0.10	0.50
para-chlorobutyryl fentanyl	0.09	Y = 1.272x – 0.1281	0.9981	0.10	0.10
para-fluoro Valeryl fentanyl	0.09	Y = 1.250x – 0.3320	0.9975	0.50	0.50
para-fluoro Cyclopentyl fentanyl	0.09	Y = 0.751x – 0.0788	0.9969	0.10	0.10
Cyclohexyl fentanyl	0.09	Y = 0.470x – 0.0339	0.9837	0.05	0.10
para-methyl Cyclopentyl fentanyl	0.09	Y = 1.061x + 0.0024	0.9727	0.50	1.0
para-chloro Valeryl fentanyl	0.10	Y = 0.379x – 0.1323	0.9993	0.50	0.50

The linearity of the compounds was reviewed to determine which were able to meet the

minimum criteria of $R^2 \geq 0.95$, as well as assessing other factors such as reviewing the

variability of the internal standard response (% RSD) and % error in concentration of the

calibrators. Most anlaytes had regression coefficients above 0.98 as shown in representative data

from n-methyl norcarfentanil, benzyl fentanyl, and benzyl acrylfentanyl **Figures 3-5.**

Figure 3. Linearity of N-methyl Norcarfentanil.
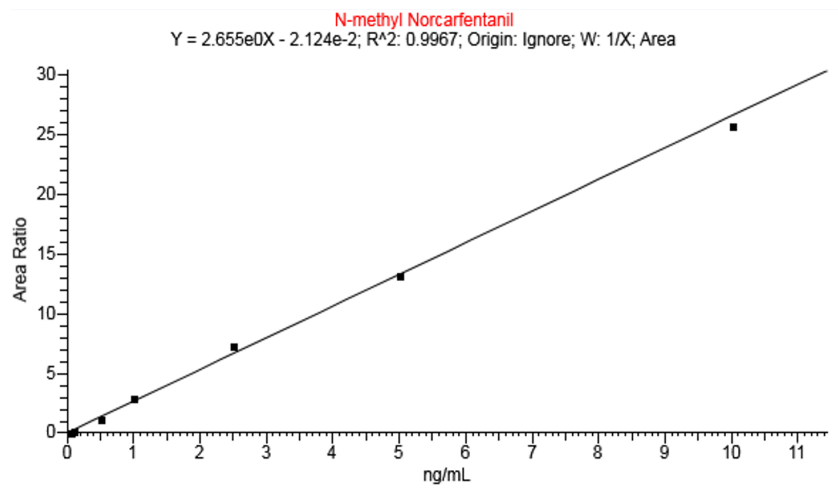
Figure 4. Linearity of Benzyl fentanyl.
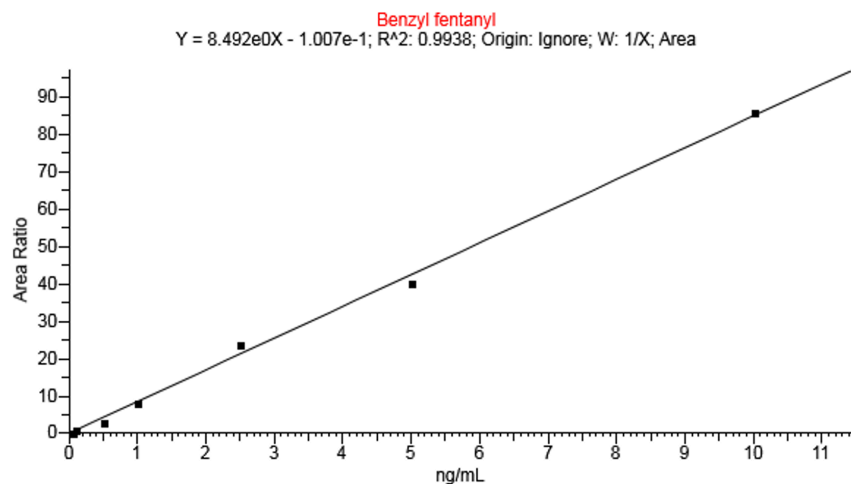
Figure 5. Linearity of Benzyl acrylfentanyl.

After evaluating linearity, the precision and bias was determined at the QC Low, Mid, and High level. The criteria for each variable were evaluated with most compounds having < 20% (**Table 6)**.

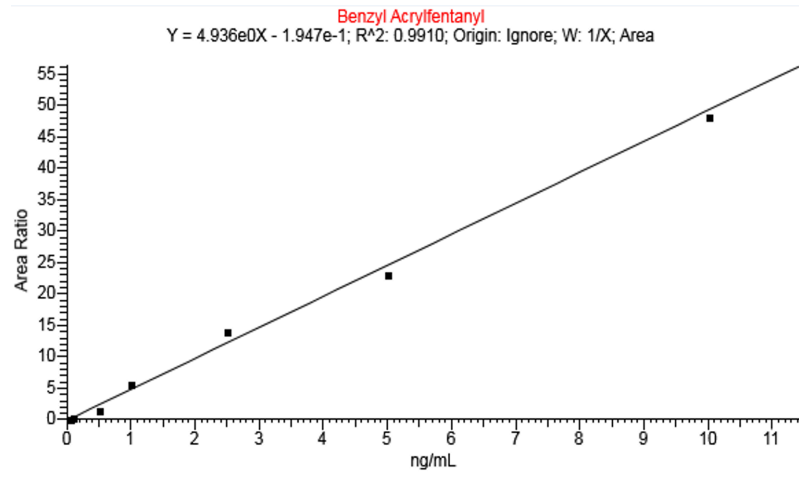**Table 6**. Intra-day Precision (%CV) and Accuracy (% Bias). The values that did not meet the 20% criteria are **bolded**.

Fentanyl Analog	QC Low - 0.25 (ng/mL)		QC Mid - 2.0 (ng/mL)		QC High - 7.0 (ng/mL)	
	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)
Furanyl norfentanyl	6.08	-17.73	2.61	-8.55	5.55	-11.28
ß-Hydroxythioacetylfentanyl	3.64	4.73	4.40	15.30	4.04	0.27
N-methyl Norcarfentanil	10.56	3.47	4.60	11.85	5.82	-6.39
Norsufentanil	**19.57**	19.33	13.54	-10.93	**21.39**	3.12
beta-Hydroxythiofentanyl	4.35	-3.00	3.96	11.53	2.95	6.17
Thienyl fentanyl	16.86	3.07	9.61	16.28	**38.26**	15.00
Benzyl fentanyl	18.03	-12.87	12.75	18.22	16.10	**25.85**
para-fluoro Methoxyacetyl fentanyl	13.96	-9.93	12.84	11.78	15.44	**21.77**
Benzyl Acrylfentanyl	19.17	-4.00	7.23	16.84	6.01	6.47

Fentanyl Analog	QC Low - 0.25 (ng/mL)		QC Mid - 2.0 (ng/mL)		QC High - 7.0 (ng/mL)	
	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)
beta-hydroxy Fentanyl	11.96	-8.00	11.19	12.73	14.72	19.07
alpha'-methoxy Fentanyl	15.51	-6.53	11.13	13.73	12.52	**21.11**
para-Fluoroacetyl fentanyl	14.48	**20.07**	4.51	10.29	2.84	1.55
Thiofentanyl	15.60	-13.73	7.29	15.93	4.48	9.23
Fentanyl Methyl Carbamate	9.96	14.20	7.24	14.19	4.32	-1.31
Acrylfentanyl	5.66	-7.67	3.74	9.04	3.64	5.08
AH 7921	8.18	-12.27	3.90	1.82	5.57	6.88
U-47700	3.87	-16.33	4.42	-8.17	5.50	-6.40
para-methyl Acetyl fentanyl	8.66	-14.33	7.35	8.23	4.27	13.80
para-methoxy Acrylfentanyl	10.53	-11.53	8.25	13.52	5.06	13.90
(+)-trans-3-methyl Thiofentanyl	12.92	3.33	11.83	6.87	**27.78**	-9.18
Furanyl fentanyl 3-furancarboxamide isomer	7.63	-7.73	4.23	4.24	4.38	6.00
N,N-Dimethylamido-despropionyl fentanyl	6.68	16.60	7.67	10.42	7.68	15.11
(+)-cis-3-methyl Thiofentanyl	13.09	5.13	7.32	12.57	**34.57**	-3.48
2'-fluoro ortho-Fluorofentanyl	11.61	-11.27	8.08	0.72	**27.00**	4.67
para-methoxy Furanyl fentanyl	8.69	-12.27	7.77	7.42	6.34	17.97
Furanyl fentanyl	7.33	-9.73	5.73	5.42	5.48	8.84
para-fluoro Furanyl fentanyl	10.02	-6.27	4.26	8.47	10.12	9.60
para-Methylfentanyl	8.70	**-20.53**	9.27	3.08	7.57	**23.00**
Tetrahydrothiophene fentanyl	13.80	-5.33	9.63	**40.63**	7.78	6.64
4'-fluoro, para-fluoro (+)-trans-3-methyl fentanyl	16.68	**24.33**	7.47	**30.67**	13.33	-0.12
para-methyl Furanyl fentanyl	10.48	-19.40	10.70	4.86	8.58	**30.68**
ortho-methoxy Butyryl fentanyl	14.43	-3.60	8.32	18.90	7.20	**20.35**
para-chloro Furanyl fentanyl	10.36	**-20.53**	9.13	7.22	11.64	**21.26**
Cyclopentenyl fentanyl	11.41	-10.20	9.42	14.99	11.85	19.24

Fentanyl Analog	QC Low - 0.25 (ng/mL)		QC Mid - 2.0 (ng/mL)		QC High - 7.0 (ng/mL)	
	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)
2,3-seco-Fentanyl	**27.96**	**-72.13**	10.28	4.63	11.91	**26.81**
Isopropyl U-47700	9.62	-9.13	10.13	-2.02	17.09	7.95
para-methoxy Valeryl fentanyl	9.81	-3.07	10.50	5.56	10.59	**35.46**
para-chlorobutyryl fentanyl	16.11	-7.13	8.51	3.93	9.21	**23.71**
para-fluoro Valeryl fentanyl	8.38	**58.53**	6.33	3.78	9.92	**21.64**
para-fluoro Cyclopentyl fentanyl	9.66	-4.07	13.74	-0.75	14.11	**27.24**
Cyclohexyl fentanyl	18.63	**-32.80**	16.91	-7.08	15.27	**33.95**
para-methyl Cyclopentyl fentanyl	19.19	**-53.93**	15.62	-9.27	13.30	**43.25**
para-chloro Valeryl fentanyl	14.40	**72.32**	10.78	-1.46	11.49	**28.24**

Percent recovery and matrix effects (matrix factor ratio) were evaluated at the QC Low, Mid, and High level. The data was reviewed to determine if it was consistent for each drug (**Table 7**).

Table 7. Recovery and Matrix Effects.

Fentanyl Analog	Recovery (%)			Matrix Effects		
	0.25 ng/mL	**2.0 ng/mL**	**7.0 ng/mL**	**0.25 ng/mL**	**2.0 ng/mL**	**7.0 ng/mL**
Furanyl norfentanyl	55.3	62.6	74.3	1.10	1.04	1.53
ß-Hydroxythioacetylfentanyl	45.8	49.8	46.6	1.31	1.24	0.95
N-methyl Norcarfentanil	64.1	68.9	79.8	1.15	1.04	1.20
Norsufentanil	47.0	48.3	57.4	0.91	1.10	1.99
beta-Hydroxythiofentanyl	39.6	42.1	43.1	1.23	1.30	0.96
Thienyl fentanyl	5.84	6.98	8.33	1.02	0.96	0.85
Benzyl fentanyl	65.4	75.3	87.7	0.99	1.31	0.95

Fentanyl Analog	Recovery (%)			Matrix Effects		
	0.25 ng/mL	2.0 ng/mL	7.0 ng/mL	0.25 ng/mL	2.0 ng/mL	7.0 ng/mL
para-fluoro Methoxyacetyl fentanyl	73.0	79.5	87.8	1.11	1.27	0.99
Benzyl Acrylfentanyl	40.1	45.8	46.7	1.04	1.00	0.89
beta-hydroxy Fentanyl	63.3	72.9	78.5	1.16	1.28	0.96
alpha'-methoxy Fentanyl	66.0	72.6	77.6	1.04	1.27	0.96
para-Fluoroacetyl fentanyl	65.8	77.5	84.9	0.99	1.14	0.93
Thiofentanyl	30.2	38.6	40.7	0.99	1.12	0.91
Fentanyl Methyl Carbamate	66.8	76.2	82.7	1.07	1.28	0.93
Acrylfentanyl	53.8	67.7	72.9	0.95	1.13	0.93
AH 7921	45.3	59.6	69.4	0.93	1.58	1.29
U-47700	45.3	59.6	69.4	0.93	1.58	1.29
para-methyl Acetyl fentanyl	61.3	77.3	85.5	0.67	1.02	0.90
para-methoxy Acrylfentanyl	58.5	81.4	90.4	0.75	0.95	0.83
(+)-trans-3-methyl Thiofentanyl	18.6	24.5	24.7	0.92	1.18	0.86
Furanyl fentanyl 3-furancarboxamide isomer	51.9	67.0	69.6	0.71	0.90	0.78
N,N-Dimethylamido-despropionyl fentanyl	58.4	81.8	93.4	0.63	1.10	0.92
(+)-cis-3-methyl Thiofentanyl	17.9	23.5	24.8	0.91	1.22	0.87
2'-fluoro ortho-Fluorofentanyl	16.1	21.9	24.4	0.65	1.06	0.82
para-methoxy Furanyl fentanyl	55.3	71.8	76.0	0.58	0.80	0.74
Furanyl fentanyl	47.2	63.9	66.3	0.65	0.77	0.71
para-fluoro Furanyl fentanyl	47.2	61.4	62.4	0.68	0.87	0.77
para-Methylfentanyl	38.0	51.5	58.3	0.41	0.76	0.75
Tetrahydrothiophene fentanyl	29.4	63.6	69.6	0.80	1.04	0.73
4'-fluoro, para-fluoro (+)-trans-3-methyl Fentanyl	31.9	42.2	43.0	0.48	0.92	0.76
para-methyl Furanyl fentanyl	48.3	62.2	64.7	0.39	0.68	0.64
ortho-methoxy Butyryl fentanyl	21.0	32.0	38.9	0.30	0.65	0.69

Fentanyl Analog	Recovery (%)			Matrix Effects		
	0.25 ng/mL	2.0 ng/mL	7.0 ng/mL	0.25 ng/mL	2.0 ng/mL	7.0 ng/mL
para-chloro Furanyl fentanyl	38.9	57.1	58.7	0.36	0.59	0.53
Cyclopentenyl fentanyl	38.3	58.9	61.7	0.67	0.82	0.59
2,3-seco-Fentanyl	35.9	51.5	65.4	0.49	0.88	1.13
Isopropyl U-47700	24.4	41.2	45.8	0.62	1.69	1.20
para-methoxy Valeryl fentanyl	33.2	49.5	55.4	0.15	0.34	0.42
para-chlorobutyryl fentanyl	22.0	37.4	44.0	0.18	0.39	0.43
para-fluoro Valeryl fentanyl	16.0	24.5	29.1	0.18	0.43	0.47
para-fluoro Cyclopentyl fentanyl	33.0	48.0	54.4	0.15	0.32	0.39
Cyclohexyl fentanyl	33.6	50.0	53.6	0.08	0.21	0.30
para-methyl Cyclopentyl fentanyl	27.5	43.0	48.2	0.06	0.15	0.23
para-chloro Valeryl fentanyl	10.7	24.1	28.7	0.07	0.18	0.23

5.0 Discussion

Detection of low-level concentrations of fentanyl analogs in biological matrices such as serum require highly sensitive, selective and reproducible analytical and extraction methods. To achieve these goals in biological matrices, highly reproducible extraction approaches combined with highly sensitive liquid chromatography - mass spectrometry-based analysis are desired. Accordingly, an LC-HRMS method was developed and validated to detect over 43 fentanyl analogs.

The retention time stability of the compounds was determined by calculating the Coefficient of Variation (% CV). It ranged from 0.08% - 0.13%, indicating that the retention times of each of the compounds has minimal variability. The 43 analogs which passed the minimum coefficient criteria ($R^2 \geq 0.95$) had an R^2 range from 0.96 - 1.00. At least half of these

analogs had an $R^2 = 0.99$, and 9 analogs had an $R^2 > 0.99$ (N-methyl norcarfentanil, para-methoxy acrylfentanyl, ortho-methoxy butyryl fentanyl, para-chloro furanyl fentanyl, cyclopentenyl fentanyl, para-chlorobutyryl fentanyl, para-fluoro valeryl fentanyl, para-fluoro cyclopentyl fentanyl, and para-chloro valeryl fentanyl). This data indicates out of the total 68 analogs evaluated, approximately 63% passed the minimum criteria for the linear regression analysis (weighting factor = $1/x$). Analogs which failed to meet the minimum R^2 criteria are shown in **Table 8**.

Table 8. These 14 Fentanyl analogs failed due to not meeting the minimum R^2 criteria ($R^2 \geq 0.95$).

Analog	R^2
Remifentanil	0.9395
para-methoxy acetyl fentanyl	0.9184
Acetyl fentanyl	0.9075
Ethoxyacetyl fentanyl	0.9126
N-benzyl furanyl norfentanyl	0.9321
para-methoxy tetrahydrofuran fentanyl	0.9472
para-fluoro tetrahydrofuran fentanyl	0.9231
para-methyl methoxyacetyl fentanyl	0.9379
N-(3-ethylindole)Norfentanyl	0.8806
beta-methyl fentanyl	0.8908
U-48800	0.9129
ortho-isopropyl furanyl fentanyl	0.5408
2-fluoro MT-45	0.7910
heptanoyl fentanyl	0.8387

When reviewing the chromatography for the analogs that had an $R^2 < 0.95$, there were a few that either had co-elution present or the primary peak was never found. 2-fluoro MT-45

exhibited poor chromatography with split peaks while ortho-isopropyl furanyl fentanyl and

heptanoyl fentanyl both had the parent ion missing. The remaining 11 analogs passed the R^2

criteria, however upon reviewing the chromatograms, all failed due to the presence of significant

co-eluting peaks. Analogs which failed as a result of poor chromatography are shown in **Table

9**.

Table 9. These 11 Fentanyl analogs failed chromatography (split peaks, co-elution, and/or absent parent ion).

Analog	R^2
Tetrahydrofuran fentanyl 3-tetrahydrofurancarboxamide	0.9924
meta-fluorofentanyl	0.9904
4'-fluorofentanyl	0.9904
(+/-)-cis-3-methyl butyryl fentanyl	0.9952
Isovaleryl fentanyl	0.9815
Valeryl fentanyl	0.9666
para-methyl butyryl fentanyl	0.9952
2,2,3,3-tetramethyl-cyclopropyl fentanyl	0.9989
FIBF	0.9845
Senecioylfentanyl	0.9881
MT-45	0.9978

Several analogs co-eluted due to having the same molecular weight and retention time.

Additionally, poor chromatography (e.g., broad peaks, split peaks, non-gaussian peak shape)

made it impossible to confirm identification of these compounds. In future versions of the

method, it would be best to prepare a separate stock mix of these analogs. A revision of the

chromatographic conditions may also be necessary to achieve separation. As shown in **Figure 6**,

fentanyl analogs (+/-)-cis-3-methyl butyryl fentanyl, isovaleryl fentanyl, and valeryl fentanyl

exhibited significant co-elution due to their shared molecular weight 365.2587 and similar retention times.

Figure 6. Co-eluting compounds (+/-) cis-3-methyl butyryl fentanyl, isovaleryl fentanyl, and valeryl fentanyl
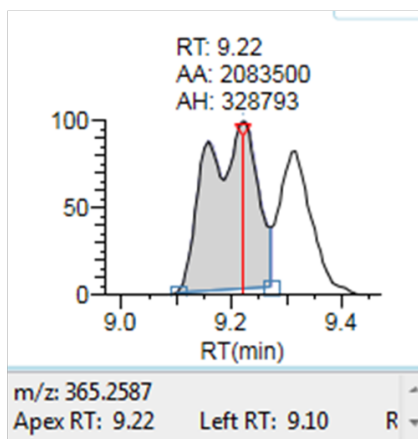

1. (+) cis-3-methyl Butyryl fentanyl
2. Isovaleryl fentanyl
3. Valeryl fentanyl

It was possible to identify analogs that had the same molecular weights if their retention times were unique enough to distinguish elution order. For example, α'-methoxy fentanyl, ethoxyacetyl fentanyl, and para-methyl methoxyacetyl fentanyl had equal molecular weights, however we were able to identify each due to their known elution order, as well as evaluating other criteria, such as locating the presence of product ions that were not shared among the analogs (α'-methoxy fentanyl - 344.49262, ethoxyacetyl fentanyl - 309.13188, and para-methyl methoxyacetyl fentanyl - 252.96886) (**Figure 7**). This methodology was applied to identify sets of fentanyl analogs that shared molecular weights, as presented in **Table 10**.

Figure 7. Chromatograms of α'-methoxy fentanyl, ethoxyacetyl fentanyl, and para-methyl methoxyacetyl fentanyl
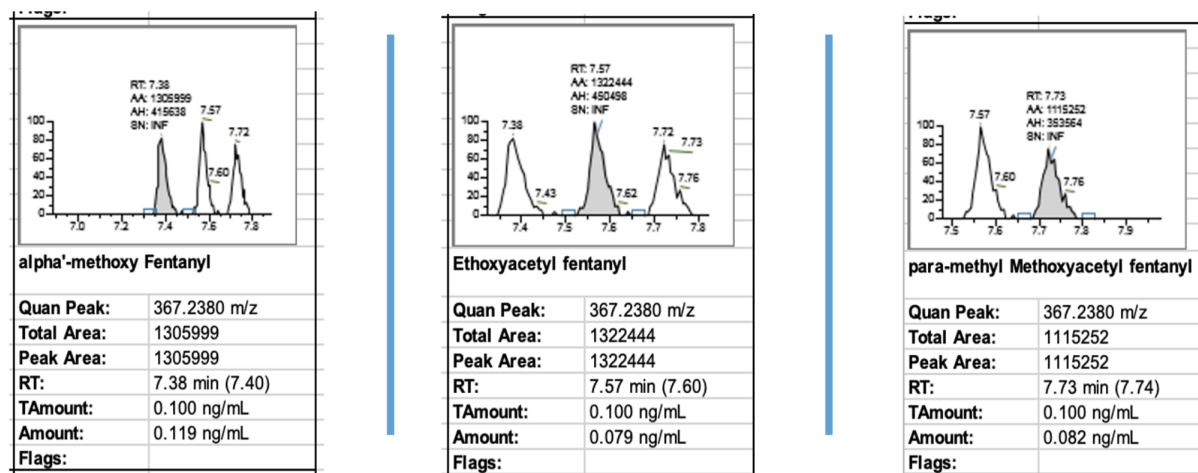
Table 10. Fentanyl analogs confirmed with the same molecular weights.

Molecular Weight	Compound 1	RT	Compound 2	RT	Compound 3	RT
323.2118	Benzyl Fentanyl	7.25	Acetyl Fentanyl	7.50	n/a	n/a
367.2380	α'methoxy fentanyl	7.40	Ethoxyacetyl fentanyl	7.60	Para-methyl methoxyacetyl fentanyl	7.74
375.2067	Furanyl fentanyl 3-furancarboxamide isomer	8.06	Furanyl fentanyl	8.28	n/a	n/a
351.2431	Beta-methyl fentanyl	8.29	Para-methyl fentanyl	8.54	n/a	n/a
391.2744	Cyclohexyl fentanyl	9.69	Para-methyl cyclopentyl fentanyl	9.90	n/a	n/a
353.2224	Beta-hydroxy fentanyl	7.39	Para-methoxy acetyl fentanyl	7.49	n/a	n/a
357.1995	(+/-)-trans-3-methyl thiofentanyl	8.00	(+/-)-cis-3-methyl thiofentanyl	8.11	n/a	n/a

The LOD ranged from 0.05 ng/mL - 2.5 ng/mL. The LOQ ranged from 0.10 ng/mL - 2.5 ng/mL. We were able to detect down to the lowest point on the curve, for at least 15 of the 43 fentanyl analogs that passed minimum criteria, and quantitate at 0.10 ng/mL for 25 analogs. Typically, the reason we had to raise the level for either detection or quantitation had to do with poor chromatography and/or a lack of unique product ions. One validation study that used a

protein precipitation method which validated 13 fentanyl analogues in whole blood using data independent acquisition and retrospective analysis of samples reported an LOD of 0.0005 mg/kg (0.5 ng/mL) for acrylfentanyl and furanyl fentanyl. In our study, we were able to detect down to 0.05 ng/mL (0.00005 mg/kg) for acrylfentanyl and 0.10 ng/mL (0.0001 mg/kg) for furanyl fentanyl [20]. Another study which used HRMS to evaluate 9 fentanyl analogs in plasma and urine reported the lowest reportable limit as 0.25 ng/mL (LRL_{100}). In the set of 9 analogs, they evaluated one fentanyl analog that was also included in our study - furanyl fentanyl [21]. For this compound, they were only able to detect down to 2.50 ng/mL, whereas in our study we were able to detect down to 0.10 ng/mL and also quantitate at this same level.

The precision (%CV) and bias (%) results indicated 23 analogs failed to meet the criteria $x < 20\%$ criteria for one or both parameters. Norsufentanil, thienyl fentanyl, (+)-trans-3-methyl thiofentanyl, (+)-cis-3-methyl thiofentanyl, 2'-fluoro ortho -fluorofentanyl, and 2,3-seco-fentanyl failed precision. A majority of analogs failed to meet the criteria for the Bias parameter (12 total) at the QC high level (7.0 ng/mL). The most significant failures were para-fluoro valeryl fentanyl failing at 56%, 2,3-seco fentanyl failing at 72%, para-methyl cyclopentyl fentanyl failing at 54%, and para-chloro valeryl fentanyl failing at 72%. 2,3-seco-fentanyl was the only analog that failed both precision and bias. A table of each analog that failed either precision or bias is presented in Table 11.

Table 11. Fentanyl analogs which failed precision and/or bias criteria $x < 20\%$.

Fentanyl Analog	QC Low - 0.25 (ng/mL)		QC Mid - 2.000 (ng/mL)		QC High - 7.000 (ng/mL)	
	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)
Norsufentanil	**Fail** **(19.57)**	Pass	Pass	Pass	**Fail** **(21.39)**	Pass
Thienyl fentanyl	Pass	Pass	Pass	Pass	**Fail** **(38.26)**	15.00
Benzyl fentanyl	Pass	Pass	Pass	Pass	Pass	**Fail** **(25.85)**
para-fluoro Methoxyacetyl fentanyl	Pass	Pass	Pass	Pass	Pass	**Fail** **(21.77)**
α'-methoxy Fentanyl	Pass	Pass	Pass	Pass	Pass	**Fail** **(21.11)**
para-Fluoroacetyl fentanyl	Pass	**Fail** **(20.07)**	Pass	Pass	Pass	Pass
(+)-trans-3-methyl Thiofentanyl	Pass	Pass	Pass	Pass	**Fail** **(27.78)**	Pass
(+)-cis-3-methyl Thiofentanyl	Pass	Pass	Pass	Pass	**Fail** **(34.57)**	Pass
2'-fluoro ortho-Fluorofentanyl	Pass	Pass	Pass	Pass	**Fail** **(27.00)**	Pass
para-Methylfentanyl	Pass	**Fail** **(-20.53)**	Pass	Pass	Pass	**Fail** **(23.00)**
Tetrahydrothiophene fentanyl	Pass	Pass	Pass	**Fail** **(40.63)**	Pass	Pass
4'-fluoro, para-fluoro (+)-trans-3-methyl fentanyl	Pass	**Fail** **(24.33)**	Pass	**Fail** **(30.67)**	Pass	Pass
para-methyl Furanyl fentanyl	Pass	Pass	Pass	Pass	Pass	**Fail** **(30.68)**

Fentanyl Analog	QC Low - 0.25 (ng/mL)		QC Mid - 2.000 (ng/mL)		QC High - 7.000 (ng/mL)	
	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)
ortho-methoxy Butyryl fentanyl	Pass	Pass	Pass	Pass	Pass	Fail (20.35)
para-chloro Furanyl fentanyl	Pass	Fail (-20.53)	Pass	Pass	Pass	Fail (21.26)
2,3-seco-Fentanyl	Fail (27.96)	Fail (-72.13)	Pass	Pass	Pass	Fail (26.81)
para-methoxy Valeryl fentanyl	Pass	Pass	Pass	Pass	Pass	Fail (35.46)
para-chlorobutyryl fentanyl	Pass	Pass	Pass	Pass	Pass	Fail (23.71)
para-fluoro Valeryl fentanyl	Pass	Fail (58.53)	Pass	Pass	Pass	Fail (21.64)
para-fluoro Cyclopentyl fentanyl	Pass	Pass	Pass	Pass	Pass	Fail (27.24)
Cyclohexyl fentanyl	Pass	Fail (-32.80)	Pass	Pass	Pass	Fail (33.95)
para-methyl Cyclopentyl fentanyl	Pass	Fail (-53.93)	Pass	Pass	Pass	Fail (43.25)
para-chloro Valeryl fentanyl	Pass	Fail (72.32)	Pass	Pass	Pass	Fail (28.24)

The recovery at the QC Low level (0.25 ng/mL) ranged from 10.7% - 73.0%, Mid-level (2.0 ng/mL) ranged from 6.98% - 81.4%, and High level (7.0 ng/mL) ranged from 8.33% - 90.4%. There were a greater number of analytes at the high level where the recovery was close to 90%, and a greater number at the low level which had recoveries < 20%. The matrix effects were also evaluated at 0.25 ng/mL, 2.0 ng/mL, and 7.0 ng/mL. At 0.25 ng/mL, the results

ranged from 0.06 - 1.31, at 2.0 ng/mL the results ranged from 0.15-1.69, and at 7.0 ng/mL the

results ranged from 0.23 – 1.99. Of the 43 fentanyl analogs evaluated, 16 had a matrix effect

value 1.00 +/- 0.05 at one or two levels assessed. These results are presented in Table 12. A

study which evaluated the matrix effects of 9 fentanyl analogs in plasma and urine using LC-

QTOF reported 10.1% for furanyl fentanyl at 15 ng/mL, indicating ion suppression. Similarly,

we also observed ion suppression, with a matrix effect ratio of 0.71 at 7.0 ng/mL [21].

Table 12. 16 fentanyl analogs which had a matrix effect value 1.00 +/- 0.05.

Fentanyl Analog	Matrix Effects		
	0.25 ng/mL	2.0 ng/mL	7.0 ng/mL
Thienyl fentanyl	1.02	0.96	n/a
Benzyl fentanyl	0.99	n/a	0.95
Benzyl acrylfentanyl	1.04	1.00	n/a
alpha'-methoxy fentanyl	1.04	n/a	0.96
Para-fluoroacetyl fentanyl	0.99	n/a	n/a
Thiofentanyl	0.99	n/a	n/a
Furanyl norfentanyl	n/a	1.04	n/a
N-methyl norcarfentanil	n/a	1.04	n/a
Para-methyl acetyl fentanyl	n/a	1.02	n/a
Para-methoxy acrylfentanyl	n/a	0.95	n/a
2'-fluoro ortho fluorofentanyl	n/a	1.06	n/a
Tetrahydrothiophene fentanyl	n/a	1.04	n/a
Beta-hydroxythioacetyl fentanyl	n/a	n/a	0.95
Beta-hydroxythiofentanyl	n/a	n/a	0.96
Para-fluoro methoxyacetyl fentanyl	n/a	n/a	0.99
Beta-hydroxy fentanyl	n/a	n/a	0.96

The most common observed product ions across the 68 analogs evaluated was 188 and 105; these product ions were observed in 43 of the total analogs analyzed. Other product ions which were shared among different analogs are **bolded** in **Table 4**. In the validation study that used a protein precipitation method to evaluate fentanyl analogues in whole blood, these same product ions were also commonly observed among 18 fentanyl analogs analyzed when using known reference standards [20]. Additionally, the researchers in this study developed a library of expected mass precursors and predicted ions derived from an understanding of common fentanyl fragmentation patterns (such as the example provided in Figure 1). In their library, they identified valeryl fentanyl as having product ions 178, 188, 244, and 281. In our research, we also identified valeryl fentanyl product ions 188, 244, and 281. Once again here, we observe the common product ion 188. Researchers have attempted to use accurate mass measurements with HRMS to better understand the fragmentation patterns of fentanyl and its analogs. One such study was able to confirm 188 as a characteristic ion of fentanyl derivatives [22]. Understanding which product ions are common among analogs will assist in further developing fentanyl screening methods.

6.0 Conclusion

We evaluated 68 fentanyl analogs in serum using LC-HRMS against qualitative and quantitative criteria. We were able to assess validation parameters (linearity, LOD, LOQ, Matrix Effects, and Recovery) for 43 fentanyl analogs which passed initial confirmation criteria. In addition, we were able to identify product ions for all 68 analogs, which will aid in further development and research of screening and quantifying fentanyl analogs using LC-HRMS. Although we were only able to confirm 43 of 68 analogs analyzed, we were able to identify issues that could be remedied in the next version of the method, such as addressing poor chromatography and co-eluting compounds. Further, we were successful in acquiring useful data for a large number of fentanyl analogs, in addition to being successful with meeting validation criteria for a portion of the analogs analyzed.

Our research supports the use of Liquid Chromatography - High Resolution Mass Spectrometry (LC-HRMS) as an analytical technique that can be used to qualitatively identify fentanyl and fentanyl analogs in horse serum. The ability to confirm these substances in biological matrices will promote a more efficient means of testing in equine drug testing labs, which will ultimately serve to support anti-doping measures in horse racing.

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