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Quantification and distribution of 4-fluoroisobutyryl fentanyl (4-FiBF) in postmortem biological samples using UHPLC–QqQ-MS/MS

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Abstract

Purpose Development of ultra-high-performance liquid chromatography-triple quadrupole tandem mass spectrometry method for quantification of 4-fluoroisobutyryl fentanyl (4-FiBF) and its distribution in postmortem biological samples in four fatal intoxication cases, which occurred in September 2018, in Poland.

Methods Biological fluids (blood, urine, vitreous humor, bile, gastric content) and tissues (brain, kidney, liver, stomach wall) were extracted with ethyl acetate from alkaline medium (pH 9). Fentanyl-*d*₅ was used as internal standard.

Results The validation parameters were as follows: lower limit of quantification: 0.1 ng/mL (biological fluids) and 0.1 ng/g (solid tissues), intra- and inter-day accuracies and precisions: not greater than 20%; recovery values: 86.9–110%; matrix effect: –13.1–10.4%. Among all tested biological fluid, the highest concentration of 4-FiBF was found in bile (average concentration of 3390 ng/mL) while among the tissues, in liver (average concentration of 1650 ng/g). Furthermore, in collected specimens, there were also found other drugs and new psychoactive substances (NPS) e.g. *N*-ethylpentylon, 4-chloromethcathinone (4-CMC) and α -pyrrolidinoisohexanophenone (α -PiHP). Concentrations and distributions of these substances in postmortem samples have been also detailed. Examinations of seized drug (in case 4) revealed that it included the mix of 4-FiBF and α -PiHP.

Conclusions The developed and fully validated method enabled for determination of 4-FiBF in postmortem biological fluids and tissues. To our knowledge, this is the first report of distribution study of 4-FiBF with other NPS (*N*-ethylpentylon, 4-CMC and α -PiHP) in authentic fatal intoxication cases.

Keywords New psychoactive substance · 4-Fluoroisobutyryl fentanyl · 4-FiBF · Fatal intoxication · Postmortem distribution · UHPLC–QqQ-MS/MS

Introduction

Synthetic opioids pose a high risk of intoxication to consumers and are an example of progressive drug market adaptability. International legal regulations related to the illicit distribution of drugs have resulted in increasing structural modifications to new psychoactive substances (NPS) released onto the global drug market. Since 2009, there have been 57 new synthetic opioids introduced to Europe's

drug market. According to the latest European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) report [1], in 2019, 8 new opioids were seized for the first time, including two totally novel fentanyl analogs. The opioid crisis can also be observed in North America. The present and most deadly wave of fentanyl analog use began in the United States around 2013. Similarly, in Canada, the levels of opioid-related deaths has increased since 2016. Between January and December 2019 alone, there were 77% intoxications involving fentanyl or fentanyl analogs, while seizures of fentanyl increased from about 96 kg in 2015 to more than 2.9 tons in 2018 [2]. Like in North America and Europe, East and Southeast Asia is also observing a large amount of fentanyl analogs reported annually. Japan reported the largest number of NPS, followed by China and Singapore [3]. According to an United Nations Office on Drugs and Crime (UNODC) report (October 2020), the opioid crisis is

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a global complex problem, whose nature seems to be a combination of factors, including lowering costs by organized crime groups through substitution of pharmaceutical opioids with fentanyl analogs and other synthetic opioids [2].

4-Fluoroisobutyl fentanyl (4-FiBF) is one of the fentanyl analogs, which differs from the original drug by the presence of a fluorine atom and isobutyramide in place of the propenamide group. The first official EMCDDA-Europol notification of 4-FiBF was on August 26, 2016 from the Slovenian National Forensic Laboratory. White powder (5 g) was collected as test purchased and shipped from China as a part of the EU co-funded project RESPONSE [4]. As a seizure, 4-FiBF was first identified in November 2016 by German Police in Berlin. From 2016 to 2017 in total, there were 22 other seized drugs reported in Sweden (20), Belgium (1) and the United Kingdom (1) [5]. Due to the varied manufactured formulas in which 4-FiBF can be found (powders, tablets, liquids, blotters and herbal smoking mixtures), there are different ways to take this drug. It can be administered orally, rectally, intranasally via a spray, inhaled by smoking or injected intravenously and intramuscularly. For this reason, 4-FiBF distribution and its concentrations in different tissues and biological fluids can vary significantly [6]. In Europe, one of the first fatal intoxications which was associated with 4-FiBF occurred in September 2016 and was reported by the Swedish STRIDA project. Post-mortem serum concentration of 4-FiBF after intravenous injection was 38 ng/mL [7], however, it is worth noting that the patient was reanimated with naloxone before his death. Twenty-six deaths in the US in which 4-FiBF was detected as one of the fentanyl analogs were also reported during a similar time period [8].

Progressively, this analog became increasingly popular on the drug market, especially due to its easy availability for purchase on the Internet. The eDarkTrends project developed by Lamy et al. [9] revealed that between 2018 and 2019, 4-FiBF was third the most commonly advertised fentanyl analog among darknet customers. Forensic testing of illicit drug seizures in Hong Kong also confirmed this trend. In the years 2016–2018, 4-FiBF along with cyclopropylfentanyl were the mostly detected fentanyl-related substances [10].

A large toxicological study performed by Pardi et al. [11] based on evaluation of 4-FiBF in blood samples from 247 authentic cases in New York City occurring between 2017 and 2018, revealed that postmortem concentration levels of these drug can reach the value even of 331 ng/mL. Compared to the fentanyl, 4-FiBF concentrations are an order of magnitude higher. In part, it can be explained by binding and functional activity data of opioid receptors in the context of fentanyl and its analogs. The half-maximal effective concentration, EC₅₀ (nM), value represents the concentration that causes a half-maximal response of the receptor agonist.

In vitro studies of pharmacodynamics shows that for fentanyl these values are around 28.8, 996 and 347 nM for μ -, δ - and κ - opioid receptors, respectively. In contrast, the values of EC₅₀ for 4-FiBF in context of μ -, δ - and κ - opioid receptors are 115, 2490 and 1330 nM, respectively. Accordingly, the study indicates that more 4-FiBF is needed to achieve half of the maximum receptor response compared to fentanyl. Similar impressions are shared by participants in online forums, who noted that 4-FiBF is long lasting and less euphorogenic when compared to other opioids [6]. This fact might lead users to increase the doses and may be the reason for such high concentrations of this drug in postmortem samples.

Drug detection in health care centers in acute poisoning cases is often performed using commercial immunochemical assays. However, a major limitation is their cross-reactivity. Wharton et al. [12] performed an extended study of the reactivity of fentanyl analogs using immunoassay kits. The investigations showed that analogs with *N*-aryl substitutions (such as 4-FiBF) are relatively poorly detectable by immunochemical methods. Despite its high structural similarity to the fentanyl, *p*-fluoro substituent on the phenyl ring with a methyl group in the acyl fragment of the anilide is not well tolerated in commercially available kits. Furthermore, these NPS exhibited average cross-reactivities of 10–30% across all fentanyl-targeting kits and almost 50% across individual kit types. For this reason, to date, the most sensitive, selective and reliable methods for the determination of fentanyl analogs in biological samples are those based on chromatographic separation with mass spectrometry detection.

The determination of 4-FiBF in biological samples was performed using chromatographic methods: high-performance liquid chromatography coupled with triple quadrupole tandem mass spectrometry (HPLC–QqQ-MS/MS) in blood samples [11, 13, 14]; ultra-high-performance liquid chromatography coupled with ion trap and tandem mass spectrometry (UHPLC–Ion Trap-MSⁿ) in blood, urine and tissues samples [8]; UHPLC coupled with tandem mass spectrometry and triple quadrupole linear ion trap (UHPLC–QTRAP-MS/MS) in blood [15] and hair samples [16]. In turn, the determination of 4-FiBF in seized drugs was performed through the use of gas chromatographic methods: coupled with tandem cold electron ionization mass spectrometry (GC–cold EI-MS/MS) and with vacuum ultraviolet detection (GC–VUV) [17] as well as gas chromatography with infrared detector (GC–IRD) [18].

Case history and pathological findings

The bodies of a 33-year-old male (case 1) and a 38-year-old male (case 2) were discovered in an apartment. No detailed information regarding the circumstances of the incident was available. Internal and external examinations of the corpses

showed edema of the brain tissue, pulmonary emphysema as well as acute gastritis in both the deceased.

An ambulance was called for an unconscious 38-year-old male (case 3). The physician diagnosed sudden cardiac arrest. After transport to the hospital, the man was still unconscious and unresponsive to impulses. Resuscitation was carried out unsuccessfully, and finally, the man's death was confirmed. The autopsy did not reveal any pathological lesions.

A 22-year-old female (case 4) was found in an apartment. According to witnesses' statements, 2 days before her death the woman had been using recreational drugs. The next day, she complained about feeling unwell. Furthermore, the woman had been taking analgesics after an accident and lower limb injury. A plastic bag containing a white powder was found in the woman's apartment. The total weight of seized powder was 57.8 mg. External and internal examination of the corpse revealed cerebral tissue edema, pulmonary emphysema, and acute mucositis.

In each case, the participation of third parties in the deaths were excluded. For further toxicological analysis, biological fluids and tissues were collected. In cases 1 and 2: blood, urine, vitreous humor, bile, brain, kidney, liver and gastric content; in case 3: blood, vitreous humor, bile, brain, kidney, liver and stomach wall; in case 4: blood, urine, vitreous humor, brain, liver and gastric content. All the blood samples were collected in tubes containing sodium fluoride as the preservative agent. Toxicological investigations found 4-FiBF in all collected postmortem materials along with other drugs and NPS. Analysis for low molecular volatile compounds did not reveal the presence of ethanol and its congeners (methanol, acetone, and isopropanol) in any of the reported cases.

This paper aims to apply an UHPLC–QqQ-MS/MS method for quantification of 4-FiBF and its distribution in authentic postmortem biological fluids (blood, urine, bile, vitreous humor, gastric content) and tissues (brain, liver, kidney, stomach wall) in four fatal intoxications which occurred in September 2018 in Poland.

Materials and methods

Chemicals

Water (Chromasolv® LC–MS), methanol (Chromasolv® LC–MS), ethyl acetate, formic acid and fentanyl- d_5 were purchased from Sigma-Aldrich (Steinheim, Germany); ammonium formate from Sigma-Aldrich (Bangalore, India); ammonium carbonate from Fluka (*Buchs*, Switzerland); 4-fluoroisobutryl fentanyl (4-FiBF) from Chiron (Trondheim, Norway). Standard solutions of 4-FiBF and internal

standard (IS) fentanyl- d_5 , at concentration of 100 µg/mL were prepared in methanol.

Biological material

Drug-free blank blood samples used for the development and validation of the method were obtained from Regional Blood Donation Center, while blank urine and tissues (brain, kidney, liver and stomach wall) were collected during autopsies performed in Department of Forensic Medicine. Blank samples were screened prior to spiking to ensure that they were free from drugs. Forensic biological fluids (blood, urine, bile, vitreous humor, gastric content) and tissues (brain, liver, kidney, stomach wall) were sent to our laboratory, for toxicological analyses.

Working solutions, calibration curve, and quality control samples

The stock and standard solutions were stored at $-20\text{ }^{\circ}\text{C}$. Standard solutions were diluted with methanol to obtain working standard solutions at the following concentrations for 4-FiBF: 1, 2, 5, 10, 20, 50, 100, 200 and 500 ng/mL. Calibration points and quality control (QC) samples were prepared by mixing the appropriate 4-FiBF working solution with blank whole blood, urine, and homogenates of brain, kidney, liver and stomach wall samples. The final concentrations of the calibrators were 0.1 (lower limit of quantification; LLOQ), 0.2, 0.5, 1, 2, 5, 10, 20 and 50 (upper limit of quantification; ULOQ) ng/mL (biological fluids) or ng/g (solid tissues) for 4-FiBF. Quality control samples were prepared by spiking blank human whole blood, urine, and homogenates of brain, kidney, liver and stomach wall to yield final concentration of 0.2 (low QC), 2.0 (medium QC) and 20 (high QC) ng/mL or ng/g.

Sample preparation

A 0.5-mL volume of biological fluid (blood, urine, bile, vitreous humor, gastric content) or 500 mg of homogenized tissue sample (brain, liver, kidney, stomach wall) was transferred into 10-mL plastic vials. Next, 20 µL of methanolic IS solution (fentanyl- d_5 , 100 ng/mL) and 0.5 mL of 0.5 M ammonium carbonate buffer (pH 9) were added. Liquid–liquid extraction (LLE) with 2 mL of ethyl acetate was carried out for 10 min. The samples were centrifuged for 10 min ($2540\times g$ at $4\text{ }^{\circ}\text{C}$). The organic phase was transferred into 2-mL Eppendorf tubes and evaporated to dryness under a stream of inert nitrogen gas at $40\text{ }^{\circ}\text{C}$. The dry residues were dissolved in 50 µL of methanol. The solution was then transferred into glass inserts of autosampler vials and analyzed by UHPLC–QqQ-MS/MS. Because the concentrations of 4-FiBF in most biological

fluids and tissues were markedly above ULOQ (50 ng/mL), the assay was repeated. Blood, urine, vitreous humor and brain samples were diluted with water (LC–MS grade) tenfold, while bile, kidney, liver, stomach wall and gastric content samples were diluted with water tenfold twice, finally obtaining a 100-fold dilution. To homogenize the tissue samples, 1 g of solid specimen (brain, kidney, liver, stomach wall) was transferred to plastic tube (12 mL) and mixed with 1 mL of water (LC–MS grade). The tube was placed in a glass beaker containing ice cubes. The contents of the tube was homogenized using a Q55 sonicator (QSonica, Newtown, USA).

Chromatographic and spectrometric conditions

Analyses were performed using an UHPLC, Shimadzu Nexera X2 (Kyoto, Japan). The separation was done using a Kinetex XB-C18 column (150 × 2.1 mm i.d., particle size 2.6 µm; Phenomenex, Torrance, CA, USA) with the thermostat set at 40 °C. The mobile phase consisted 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient elution was carried out at a constant flow of 0.4 mL/min. The gradient applied was as follows: 0 min, 5% B; 12 min, 98% B; 14 min, 98% B; and 15 min, 5% B. Return to the initial gradient compositions (95% A/5% B) was performed for 5 min. The injection volume was 2.0 µL.

Detection of the investigated compounds was achieved using a QqQ-MS/MS, Shimadzu 8050 (Kyoto, Japan). The spectrometer was equipped with an electrospray ionization (ESI) source; determination of the investigated substances was carried out in the multiple reaction monitoring (MRM) mode. The following MS parameters were fixed: nebulizing gas flow, 3 L/min; heating gas flow, 10 L/min; interface temperature, 250 °C; desolvation line temperature, 200 °C; heat block temperature, 350 °C; and drying gas flow, 10 L/min. A summary of precursor and product ions, collision energies, dwell time, Q1–Q3 pre-bias

voltages, and retention time for each compound is presented in Table 1.

Validation

Validation of the method included examination of selectivity, linearity, precision and accuracy, carryover, limit of detection and quantification, recovery and matrix effect.

Selectivity

Ten different lots of blank whole blood, and five different lots of urine, brain, kidney, liver, stomach wall samples from different origin were tested for possible endogenous interference peaks at the retention times of 4-FiBF and IS.

Linearity

Linearity was evaluated by analysis of 4-FiBF working solutions with blank whole blood, urine, brain, kidney, liver and stomach wall in final concentrations of 0.1, 0.2, 0.5, 1, 2, 5, 10, 20 and 50 ng/mL or ng/g. Linear calibration model was applied. The coefficient of determination (R^2) were determined. According to the acceptance criteria used, the coefficient of determination should meet the condition: $R^2 \geq 0.995$.

Precision and accuracy

The intra-day and inter-day precision and accuracy were estimated by replicating analysis ($n = 5$) of QC samples at three concentration levels: 0.2, 2.0 and 20 ng/mL or ng/g. Precision was defined as relative standard deviation (RSD%), while accuracy was expressed as mean relative error (RE%).

Carryover

To investigate the carryover, three samples of each matrix type without analytes were analysed after a calibration sample at the 4-FiBF concentration in highest calibration level

Table 1 Multiple reaction monitoring (MRM) conditions used in the high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (HPLC–ESI–MS/MS) method for quantification of 4-fluoroisobutyl fentanyl (4-FiBF) in biological samples

Compound	Precursor ion (m/z)	Product ion (m/z)	Dwell time (msec)	Q1 pre-bias (V)	Collision energy (V)	Q3 pre-bias (V)	Retention time (min)
Fentanyl- d_5	342.1	188.3	7.0	10.0	25.0	19.0	5.68
		105.2*		10.0	39.0	16.0	
4-FiBF	369.4	188.2*	2.0	17.0	25.0	18.0	6.31
		105.2		16.0	41.0	16.0	
		150.1		10.0	34.0	14.0	

*Ions selected for quantitative analysis

of 50 ng/mL or 50 ng/g. Unacceptable carry over was when peak area ratio in a zero sample exceeded 20% of the area ratio observed for the LLOQ samples.

The LLOQ and the LOD

The LLOQ was defined as the concentration at which the RSD% and RE% do not exceed 20% and 15%, respectively [19]. The limit of detection (LOD) was considered to be the lowest concentration of the sample for which the signal-to-noise ratio met the condition at least: $S/N \geq 3$.

Recovery and matrix effect

The recovery and matrix effect were evaluated at each of the three different concentrations: 0.2, 2.0 and 20 ng/mL or ng/g. The recovery (% , $n = 5$) was determined by comparing the response of extracted analyte in spiked blank matrix with the response of analyte spiked after the extraction of blank matrix. The matrix effect (% bias, $n = 5$) was determined by comparing the response of analyte spiked after the extraction of blank matrix with the response of analyte in neat solution [20].

Results

Validation process

Under the chromatographic conditions, the m/z transitions of $369.4 \rightarrow 188.2$, $369.4 \rightarrow 105.2$, $369.4 \rightarrow 150.1$ and $342.1 \rightarrow 188.3$, $342.1 \rightarrow 105.2$ were selected for optimal monitoring of 4-FiBF and fentanyl- d_5 , respectively. No interfering ion current signals were observed at the retention times of 4-FiBF or IS. Chromatograms of the blank blood sample, 4-FiBF at concentration of LOQ and real analysis of the blood sample in case 1 (diluted tenfold) are presented in Fig. 1. Product ion spectra of 4-FiBF and fentanyl- d_5 (IS) in the tested blood sample are presented in Fig. 2. Chromatograms of blank samples and 4-FiBF at concentration of LOQ in five types of matrix: urine, brain, kidney, stomach wall and liver are presented in Fig. 3. The linear concentration range was from 0.1 to 50 ng/mL (for biological fluids) and 0.1 to 50 ng/g (for solid tissues). Analysis of higher concentrations of 4-FiBF in the samples cause the saturation of the detector, thus, ULOQ was determined as a concentration of 50 ng/mL or 50 ng/g. A summary of calibration curve parameters for each biological matrix is presented in Table 2. The LLOQ was 0.1 ng/mL (for biological fluids) and 0.1 ng/g (for solid tissues). The analysis of the sample spiked with a lower concentration (0.05 ng/mL and 0.05 ng/g) showed that the signal from analyte was also visible but the RSD exceeded 20%. Because lower concentration

samples were not analyzed, 0.05 ng/mL and 0.05 ng/g was determined as the LOD of the method. Intra-day and inter-day precisions (RSD%) and accuracies (RE%) examined at 4-FiBF concentrations of low, medium and high QC, did not exceed 20%. Recovery and matrix effect values were in the range 86.9–110% and –13.1–10.4%, respectively. Furthermore, there were no substances carried over between samples. A summary of the validation parameters is presented in Table 2.

Toxicological analysis of biological samples

Distribution of 4-FiBF in postmortem biological fluids (blood, urine, bile, vitreous humor, gastric content) and tissues (brain, liver, kidney, stomach wall) in four fatal intoxication cases is presented in Table 3. Determination of 4-FiBF in vitreous humor, bile, and gastric content was performed on urine, blood, and gastric homogenate calibration curves, respectively. The average concentration of 4-FiBF in the tested specimens was as follows: 140 ng/mL in the blood (range 76.1–257 ng/mL), 642 ng/mL in the urine (range 289–1000 ng/mL), 117 ng/mL in the vitreous humor (range 89.9–150 ng/mL), 3390 ng/mL in the bile (range: 1100–5410 ng/mL), 3310 ng/mL in the gastric content (range 2280–3990 ng/mL), 134 ng/g in the brain (range 94.5–176 ng/g), 1650 ng/g in the liver (range 1400–2040 ng/g) and 588 ng/g in the kidney (range 388–811 ng/g). In case 3, the concentration of 4-FiBF in the stomach wall was 1900 ng/g. Among all tested biological fluids, the highest concentrations of 4-FiBF were found in the bile and gastric content, while among tested tissues, in the liver. The lowest percentage of total body distribution of 4-FiBF was in the blood, vitreous humor and brain. Further toxicological analysis of collected blood and urine samples in the four intoxication cases showed the presence of other drugs and NPS such as amphetamine and *N*-ethylpentylone in cases 1, 2 and 3 and *cis*-tramadol with metabolites, α -pyrrolidinoisohexanophenone (α -PiHP) and 4-chloromethcathinone (4-CMC) in case 4. The quantification of these NPS was performed by the validated methods previously published [21, 22]. Detailed toxicological findings with concentrations of these substances and distribution in biological samples are presented in Table 4. Examination of the seized white powder in a plastic bag (from case 4) revealed that it included a mix of 4-FiBF (40.25 mg) with α -PiHP (1.84 mg).

Discussion

The most widely used method for the determination of 4-FiBF in biological samples is LC–MS, which are characterized by high levels of selectivity, sensitivity and accuracy. These parameters are essential for reliable verification of

Fig. 1 Multiple reaction monitoring (MRM) chromatograms of **a** blank blood sample; **b** 4-fluoroisobutyl fentanyl (4-FiBF) at the concentration of limit of quantification (LOQ): 0.1 ng/mL; **c** real

analysis of blood sample in case 1 (diluted tenfold): 4-FiBF at concentration of 10.9 ng/mL (final concentration of 109 ng/mL in whole blood)

fentanyl analog ingestions. A comparison of the methods applied for analysis of biological fluids in cases of 4-FiBF intoxication is presented in Table 5. Most of the articles described the methods with the use of triple quadrupole [11, 13, 14], however, ion trap [8] and triple quadrupole linear ion trap [15] systems have also been applied. Furthermore, the most widespread ion source was ESI, while detection of 4-FiBF was performed based on its fragmentation in the MRM mode [11, 13–15] or auto MSⁿ [8]. All methods concerned on determination of 4-FiBF in whole blood samples

[11, 13–15], however, Shoff et al. [8] applied their method for analysis of both, whole blood and urine. All methods described to date have used fentanyl-*d*₅ as an IS. Due to its structural similarity to the determined 4-FiBF, it was possible to achieve very good recovery values ranging from 73.6% (in the work of Strayer et al.) [13] up to 99.7% (in the method described by Qin et al.) [15]. The lowest LOQ of the method (0.05 ng/mL) with the smallest sample volume (100 µL) was achieved by Qin et al. [15]. However, the authors were the only ones who applied UHPLC–QTRAP–MS/MS,

Fig. 2 Product ion spectra of 4-FiBF and fentanyl-*d*₅ in authentic human blood; collision energy: **a** 10, **b** 20, and **c** 35 V

which allows for much higher sensitivity through the availability of trapping ions before its further fragmentation. Shoff et al. [8] and Adamowicz et al. [14] determined only LOD of their methods, which were 0.5 ng/mL and 0.13 ng/mL, respectively. Nevertheless, it is difficult to compare both LOD and LOQ values reported by the authors due to the different injection volumes used. Most of the UHPLC–MS/MS procedures described to date, as well as our method, achieved a LOQ at the concentration of 0.1 ng/mL. However, the injection volumes ranged from 2.0 μ L (presented method) to 5.0 μ L [8, 15] to as much as 10 μ L [11, 14]. Of the techniques described to date, more than half have been based on the use of solid-phase extraction (SPE) as a sample preparation procedure [8, 11, 13]. The most important highlights of this procedure is very good purification of the sample as well as obtaining a clear extract with concentrated analyte content. However, this preanalytical sample procedure is complicated and time consuming. Furthermore, the SPE techniques described by Shoff et al. [8] and Strayer et al. [13] required 1000 μ L of biological material. In this paper, as well as the two previous described methods [14, 15], an application of LLE technique in alkaline medium (pH 9–11)

with ethyl acetate as the organic phase was achieved. This technique of sample pre-treatment is fast and simple, while reducing of multi-stage preparation procedure allows to obtain better precision, accuracy and recovery values.

The results of toxicological analysis presented in this paper are the most detailed characterization of 4-FiBF concentrations in various biological samples. The reports on postmortem concentrations are limited to serum, blood and urine concentrations [7, 11]. Helander et al. [7] reported fatal ingestion of a 250-mg dose of 4-FiBF by a 27-year-old male. Toxicological analysis of biological material collected 3.5 h after intoxication revealed 4-FiBF concentrations of 38 ng/mL and 31 ng/mL in the serum and urine, respectively. In turn, Pardi et al. [11] presented 247 cases in which the presence of 4-FiBF in blood samples was found. Concentrations of this synthetic fentanyl analog in postmortem blood samples ranged from 0.1 up to 331 ng/mL. The 4-FiBF concentrations described in this paper (76.1–257 ng/mL) are within those previously reported. The presented four fatal intoxications were associated with rapid (within minutes) loss of consciousness and death after the drug administration. Our results suggest that the

Fig. 3 MRM chromatograms of blank samples and 4-FiBF at concentration of LOQ in five types of matrix: urine, brain, kidney, stomach wall and liver

Table 2 Calibration curves and validation parameters of the method for quantification of 4-FiBF

Biological matrix	Calibration curve		Validation parameters									
	The linear concentration range (ng/mL) or (ng/g)	Calibration regression equation	The coefficient of determination (R^2)	LLOQ (ng/mL)	ULOQ (ng/mL)	Concentration level (ng/mL)	Intraday		Interday		Recovery (%) [*]	Matrix effect (%) [*]
							Precision (%) [*]	Accuracy (%) [*]	Precision (%) [*]	Accuracy (%) [*]		
Whole blood	0.1–50	$y=0.747746x$	0.999	0.1	50	0.2	6.4	1.6	6.9	8.2	102	2.1
						2.0	1.4	1.0	1.2	9.4	101	0.6
						20.0	2.4	5.0	5.5	8.7	105	4.6
Urine	0.1–50	$y=0.406508x$	0.998	0.1	50	0.2	5.2	− 9.8	5.6	− 1.8	98.6	− 1.4
						2.0	6.7	− 6.6	6.9	2.5	110	10.4
						20.0	12.2	− 6.5	5.6	− 5.7	108	7.9
Brain	0.1–50	$y=0.364710x$	0.999	0.1	50	0.2	6.0	3.3	1.7	0.8	95.9	− 4.1
						2.0	7.7	3.3	4.9	5.3	106	5.5
						20.0	6.1	6.1	0.8	4.9	109	8.8
Kidney	0.1–50	$y=0.367046x$	0.998	0.1	50	0.2	2.7	2.7	6.1	3.0	97.2	− 2.8
						2.0	5.5	8.5	6.6	− 1.6	105	5.2
						20.0	7.1	− 8.6	11.5	− 3.8	97.4	− 2.6
Liver	0.1–50	$y=0.300368x$	0.998	0.1	50	0.2	13.8	5.7	10.8	10.2	86.9	− 13.1
						2.0	3.3	14.6	6.0	13.2	95.1	− 4.9
						20.0	10.3	16.8	5.0	15.2	98.5	− 1.5
Stomach wall	0.1–50	$y=0.418985x$	0.999	0.1	50	0.2	1.2	− 19.5	9.6	− 13.7	90.2	− 9.8
						2.0	5.8	− 17.2	8.9	− 14.4	97.8	− 2.2
						20.0	5.6	− 16.7	11.9	− 15.1	99.7	− 0.3

LLOQ lower limit of quantification, ULOQ upper limit of quantification

^{*} $n=5$

Table 3 Distribution of 4-FiBF in postmortem samples (biological fluids and tissues)

Case number	Blood (ng/mL)	Urine (ng/mL)	Vitreous humor (ng/mL)	Bile (ng/mL)	Brain (ng/g)	Kidney (ng/g)	Liver (ng/g)	Stomach wall (ng/g)	Gastric content (ng/mL)
Case 1	109	635	150	5410	176	811	1400	— ^a	3670
Case 2	76.1	1000	89.9	1100	94.5	388	1620	—	3990
Case 3	257	—	128	3670	154	564	2040	1900	—
Case 4	119	289	101	—	112	—	1540	—	2280

^aNot determined

distribution of 4-FiBF is very rapid, and concentrations of the xenobiotic in the liver, bile, and kidney are many orders of magnitude higher than in blood concentrations. However, in the case of co-administered cathinone analogs (4-CMC and *N*-ethylpentylone), the differences were not as significant. Nevertheless, taking into account the coincidence of 4-FiBF with cathinones (which are unstable in blood samples [21, 23–25]), and the high concentrations of 4-FiBF in the liver, it is appropriate to collect the fragment of this tissue together with blood samples for further toxicological studies. The elimination of 4-FiBF occurs rapidly, and the urinary concentrations are extremely high. It appears to be inversely proportional to the blood concentration—in our cases, as the blood concentration of 4-FiBF decreased, an increase in the urinary concentration was observed, which may suggest that this is the main pathway of elimination of this compound from the body. An interesting finding was observed in the case of quantification of 4-FiBF in the gastric contents and stomach wall, as particularly high concentrations of this substance were found there. The results are even more surprising, as all four decedents ingested 4-FiBF by inhalation before their deaths (this fact was described in detail by the witnesses). Therefore, it seems that the presence of 4-FiBF in the gastrointestinal tract was the result of saliva or smoke swallowing while smoking.

The examination of the seized white powder (from case 4) revealed the presence of a mix of two NPS belonging to the group of synthetic cathinones (α -PiHP) and fentanyls (4-FiBF). To our knowledge, such an unusual combination of these two drugs has not been reported to date. The local

nature of this trend seems to be confirmed by the fact that in 2020 Adamowicz et al. [14] reported a fatal intoxication case which occurred in Poland. The determined concentrations of 4-FiBF along with α -PiHP in the postmortem blood sample were 74 ng/mL and 56 ng/mL, respectively. In our cases, the intoxication with a mixture of 4-FiBF and α -PiHP, and in addition with 4-CMC and tramadol was found. However, the blood concentrations of 4-FiBF and α -PiHP described in this paper's UHPLC–QqQ-MS/MS analysis (119 ng/mL and 6.1 ng/mL, respectively) differed from those obtained by Adamowicz et al. [14]. These differences were most likely due to the different proportions of psychoactive substances in the drugs used.

Conclusions

An UHPLC–QqQ-MS/MS method for 4-FiBF determination has been evaluated and fully validated. The method described in this paper was applied in 4-FiBF determination in four fatal intoxications. The distribution study of 4-FiBF as well as other NPS (*N*-ethylpentylone, α -PiHP and 4-CMC) was performed on a wide range of postmortem biological fluids (blood, urine, vitreous humor, bile, gastric content) and tissues (brain, kidney, liver, stomach wall). Examinations of the seized drug (in case 4) revealed that it included a mix of 4-FiBF and α -PiHP, which is the first report to our knowledge of such an unusual combination of these two drugs on the global drug market.

Table 4 Detailed toxicological findings in four fatal intoxication cases with concentrations and distribution in biological fluids and tissues of other drugs and new psychoactive substances

Case No. age/sex	Further toxicological findings										Outcome
	Substance	Concentration Biological fluids (ng/mL) and tissues (ng/g)									
		Blood	Urine	Vitreous humor	Bile	Brain	Kidney	Liver	Stomach wall	Gastric content	
Case 1 33y/M	<i>N</i> -Ethylpentylone	64.7	2590	270	2250	362	289	152	– ^a	2980	Fatal intoxication
	Amphetamine	4.1	235	9.2	106	33.8	25.5	25.3		77.4	
Case 2 38y/M	<i>N</i> -Ethylpentylone	95.7	8580	261	636	217	254	260	–	220	Fatal intoxication
	Amphetamine	3.0	438	9.2	22.9	15.4	21.9	14.5		6.7	
Case 3 38y/M	<i>N</i> -Ethylpentylone	20.1	–	133	176	128	37.4	68.7	5.5	–	Fatal intoxication
	Amphetamine	1.9		4.5	40.1	18.4	8.1	20.2	4.7		
Case 4 22y/F	α-PiHP	6.1	31.7	2.5	–	7.8	–	0.0	–	246	Fatal intoxication
	4-CMC	4.2	437	13.9		2.1		2.0		57.3	
	<i>O</i> -Desmethyltramadol	1.2	26.3	2.1		1.4		2.8		1.9	
	<i>cis</i> -Tramadol	1.2	45.0	2.4		3.3		3.0		6.1	
	<i>N</i> -Desmethyltramadol	0.5	35.3	1.7		1.1		1.4		3.5	

α-PiHP *α*-pyrrolidinoisohexanophenone, 4-CMC 4-chloromethcathinone^aNot determined

Table 5 Comparison of determinations of 4-FiBF in biological samples by liquid chromatography coupled with mass spectrometry methods

Biological sample (volume)	Sample preparation	Detector (mode)	Injection volume (μL)	Recovery (%) / internal standard	Precision/ accuracy (%)	LOQ (ng/mL) or (ng/g)	Concentrations in biological samples (ng/mL) or (ng/g)	Year	References
Whole blood Urine (1000 μL)	SPE (Clean Screen Dau)	ESI/Ion trap (auto MS ⁿ)	5.0	— ^a Fentanyl- <i>d</i> ₅	— ^a	— ^a	— ^a	2017	[8]
Whole blood (1000 μL)	SPE (Clean Screen Dau)	ESI/triple quadrupole (MRM)	—	73.6–87.9 Fentanyl- <i>d</i> ₅	88.0–92.1 ^b 4.7–19.0 ^c	0.1	—	2018	[13]
Whole blood (100 μL)	LLE (pH 9.2) with ethyl acetate	ESI/QTRAP (MRM)	5.0	88.1–99.7 Fentanyl- <i>d</i> ₅	3.6–11.3 ^b 86.6–100.5 ^c	0.05	—	2019	[15]
Whole blood (200 μL)	LLE (pH 11) with ethyl acetate	—/triple quadrupole (MRM)	10.0	82.2 Fentanyl- <i>d</i> ₅	3.2–4.6 ^d 6.8–13.2 ^c	—	74.0	2019	[14]
Whole blood (500 μL)	SPE (Clean Screen Xcel I)	ESI/triple quadrupole (MRM)	10.0	— ^a Fentanyl- <i>d</i> ₅	1.8–12.0 ^b 4.3–7.3 ^c	0.1	0.1–331.0	2020	[11]
Whole blood Urine Vitreous humor Bile Gastric content (500 μL) Brain homogenate Kidney homogenate Liver homogenate Stomach wall homogenate (500 mg)	LLE (pH 9) with ethyl acetate	ESI/triple quadrupole (MRM)	2.0	101–105 (blood) Fentanyl- <i>d</i> ₅	1.2–6.9 ^d 1.0–9.4 ^c (blood)	0.1	76.1–257 289–1000 89.9–150 1100–5410 2280–3990 94.5–176 388–811 1400–2040 1900	2021	Presented study

LOQ limit of quantification, SPE solid-phase extraction, LLE liquid–liquid extraction, QTRAP triple quadrupole linear ion trap

^aInformation was not detailed in article

^bCoefficient of variation

^cRelative error

^dRelative standard deviation

Declarations

Conflict of interest The authors declare no conflicts of interest (including financial and personal) that might appear to influence the work reported in this paper.

Ethical approval All procedures performed in this study were in accordance with the ethical standards of the national committee and with the 1964 Declaration of Helsinki. Biological fluids (blood, urine, bile, vitreous humor, gastric content) and tissues (brain, liver, kidney, stomach wall) collection from decedents was made by judicial authorities, and the samples were sent to our institute for toxicological analysis at their request. The blank biological materials used to develop the method were collected in accordance with Ethical Committee approval

(No. 333/14). This article does not contain any studies with living human participants or animals performed by any of the authors.

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