

Benzoylfentanyl and parafluorobutyrfentanyl: some analytical and metabolism data

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Summary

Today, New Psychoactive Substances (NPS) are increasingly being related to *post-mortem* cases, and this problem drastically intensifies as a result of the New Synthetic Opioids (NSOs) surge. Among NSOs, fentanyl derivatives (FDs) consist in a myriad of drugs with a high turnover. FDs detection in human biological samples is complicated by low concentrations and usual extensive metabolism. In this challenging context, this manuscript aims to report analytical data about two poorly documented FDs: benzoylfentanyl (BZF) and parafluorobutyrfentanyl (pFBB). In 2018, these FDs were identified in three samples (powders and blotting papers) in France. Nuclear magnetic resonance and liquid chromatography with high-resolution mass spectrometry detection (LC-HRMS) data including observed mass spectrometric fragmentation patterns were obtained. In addition, metabolites were investigated using human liver microsomes incubations and LC-HRMS analysis: norBZF, despropionylfentanyl and a hydroxylated-BZF were identified (and observed in hair) as BZF metabolites; norpFBB, parafluorofentanyl and a hydroxylated-pFBB were identified as pFBB metabolites. In the worrisome context of NSOs detection, these preliminary data can be useful for toxicologists.

Keywords: New Synthetic Opioids, Benzoylfentanyl, Parafluorobutyrfentanyl, NMR, LC-HRMS, metabolites, Human liver microsomes, Hair

Introduction

Today, New Psychoactive Substances (NPS) are increasingly being involved in *post-mortem* cases. This issue is mainly related to the worldwide New Synthetic Opioids (NSOs) surge. [1,2]. Unlike most NPS, NSOs are not only sold on online markets, but also on the usual illicit market where NSOs are sold as heroin, mixed with illicit drugs or counterfeit medications. This higher availability NSOs is causing considerable concern in North America where NSOs contribute to the USA opioid epidemic. NSOs intoxications result in a classical opioid toxidrome. But naloxone dosage used to reverse NSOs toxic effects **has** to be increased owing to high opioid potency of NSOs. **A growing proportion of deaths related to NSOs is observed in the USA since 2013** [1-3]: in 2017, 29 400 out of the 72 000 overdose deaths in the USA were related to fentanyl and NSOs [4]. This increase of fatalities involving NSOs was also observed in Europe since 2015/2016 [5-7].

In addition to a significant challenge for both judicial and public health policy-makers, NSOs represent also an analytical dare for toxicologists [8-12]. NSOs encompass fentanyl derivatives (FDs), benzoamide compounds, and other **opioids derivatives**. Among NSOs, manufacturers (from China and Mexico) are deeply targeting FDs owing to their high profitability and the ease of production of a myriad of drugs derived from fentanyl structure (Figure 1). Several points are available for chemical additions on each region: at α' (e.g. valerylfentanyl) or β' position on the amide group (A), at ortho, meta and/or para positions (e.g. p-fluorofentanyl) on the aniline ring (B), at 2,3 and/or 4 positions (e.g. carfentanil) on the piperidine (C) and at α or β position **on the N-alkyl chain** (Beta-hydroxyfentanyl), and/or at 2', 3' and/or 4' position **on** the phenyl ring *of* the N-alkyl chain (D). In addition and/or in combination, several substituents can occur. For instance, the ethyl group of the amide function can be swap with another chemical function (e.g. furanylfentanyl, benzoylfentanyl). Beyond the difficulty linked to wide range and high turnover, the emergence of FDs is challenging regarding their identification in human biological samples as usual screening tests (i.e. immunochemical tests for opiates detection in urine) are not adapted for FDs detection. Some commercial immunoassays for fentanyl screening in urine can be used for preliminary screening of FDs utilizing the cross-reactivity of such tests [13]. Nevertheless, liquid chromatography with high-resolution mass spectrometry detection methods (LC-HRMS) show better performance in comparative studies for NPS (including FDs) screening assays as higher mass precision greatly facilitates identification of unknown compounds [14]. However, the main pitfall of detection of FDs remains in the needed low detection limits. **This is the**

consequence of low concentrations of these highly potent drugs (i.e. taken in very small doses), short half-time and extensive metabolism. In this context, trying to detect only the parent drug **in blood or urine** could lead to false-negative results even if the LC-HRMS method used is sensitive enough [15-19]. In this way, the identification of FDs metabolites, which would improve the efficiency of toxicological screening libraries, can enlarge the FDs detection window in biological matrices in cases of FDs intake.

In this challenging context, this manuscript aims to report some useful analytical data about two rarely documented FDs: benzoylfentanyl (BZF) and parafluorobutyrfentanyl (pFBBF).

Samples histories

Sample #1: on June 2018, an unknown powder was collected from the SINTES French scheme (Système d'Identification National des Toxiques Et Substances, a scheme intended to document the toxicological composition of illegal substances in circulation in France) and subsequently delivered for analysis to our laboratory. This white powder sample was obtained from a 43-year-old user. This man declared that this 3 g sample was bought on the darknet (China origin, cost 150 euros) as furanylfentanyl, and that **his friend** experienced limited opioid effects using this powder.

Sample #2: during summer 2018, 5 blotting papers (white color, 3 out of 5 with no inscription or logo, 2 out of 5 with printed black signs that could be part of Arabic script) (Figure 2) were obtained from the Parisian Center of Evaluation and Information on Pharmacodependence and Addictovigilance (CEIP-A). These samples were collected in a specific harm risk reduction program for slammers and chemsexers based on the analysis of NPS they consumed [20].

Sample #3: at the same period, **an** unknown white powder was **sent** to the laboratory by the French Customs Laboratory for identification.

Material and Methods

Chemicals

β -OH-ethyltheophyllin (internal standard), methyl-clonazepam (internal standard), ammonium formate, formic acid, sulfosalicylic acid dihydrate, β -glucuronidase (*Helix pomatia*), alamethicin (*Trichoderma viride*), uridine diphosphate glucuronic acid (UDPGA), glucose-6-phosphate dehydrogenase (G6PD), glucose-6-phosphate (G6P), 5-sulfosalicylic acid, were all purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Tetra-sodium salt of a reduced form of nicotinic acid adenine dinucleotide phosphate (NADPH) was purchased from Roche (Mannheim, Germany). A pool of human liver microsomes (HLMs) at

a concentration of 20 mg of microsomal protein/mL, prepared from livers of 36 donors, including 11 women and 25 men, aged from 31 to 80 years, was purchased from Biopredic (La Bretèche, Saint Grégoire, France) and preserved at -80°C until use. LC-MS grade water, ethanol, methanol and acetonitrile were purchased from Biosolve (Dieuze, France) and all other chemicals were of analytical grade and obtained from common commercial sources.

Powders NMR analysis

A liquid chromatography with high-resolution mass spectrometry detection method (LC-HRMS) (see below) was used to identify FDs in the two powder samples. However, in order to confirm the absence of other compounds, such as synthesis impurities, and to check the purity of the two powders, these assays were completed by a nuclear magnetic resonance (NMR) analysis (already applied in a similar context) [17,19]. The NMR spectra were recorded on AVANCE 300 (Bruker Biospin, France) operating at 300 MHz equipped with a 5 mm quadruple nucleus probe (QNP) probe at 295 K and the ERETIC (Electronic REference To access In vivo Concentrations) method was used for the determination of absolute concentrations of FDs. ERETIC consisted of a digitally generated Gaussian peak inserted into the spectrum of a sample by the software after processing the FID. With ERETIC, the value peak area integrals in the spectrum give directly information about the concentration of protons sample purity [21].

Samples LC-HRMS analysis

In agreement with the literature recommendations [14], a previously reported method [17,19,22,23] was used to investigate NPS presence in the 3 samples. Briefly, the ultra-performance liquid chromatography system consisted of two binary solvent manager LC pumps, a sample manager autosampler and a column manager oven Acquity™. Mass spectrometry data were acquired using a XEVO G2-XS QTOF (Waters, Manchester, UK) instrument controlled with MassLynx™ 4.1 software. Extractions of samples (75 µL of 1 mg/L methanolic solution of powders or 1 out of the 5 blotting papers extract using 500 µL of methanol) were performed using an OASIS HLB on-line column (30 x 2.1 mm, 20 µm) (Waters, Manchester, UK). The mobile phases consisted in 0.2 % ammonia solution (A1) and 100 % methanol (B1) and the elution profile was as follows: 0.5 min, 0% B1, flow rate 2.0 mL/min; 1.0 min, 0% B1, flow rate 0.2 mL/min; 15 min, 100% B1, flow rate 0.2 mL/min; 30 min, 100% B1, flow rate 2.0 mL/min. The chromatographic separation was performed using an ACQUITY HSS C18 column (150 x 2 mm, 1.8 µm) (Waters) in an oven temperature of 50 °C, and mobile phases including ammonium formate buffer 5mM, pH 3 (A2) and acetonitrile in 1 % formic acid (B2); flow rate of 0.4 mL/min was used. Initial concentration of mobile

phase B2 (3 %) held until 0.5 min, increased to 40 % at 25 min, then to 100 % at 26 min, and finally held until 30 min. For detection, mass spectrometric conditions were as follows: positive electrospray ionization interface (ESI+), 20 V as ion spray voltage, source temperature at 140 °C and desolvation temperature at 500 °C with a desolvation gas flow rate of 900 L/h, nitrogen as desolvation gas and argon as collision gas. Conditions for the time of flight mass spectrometer (TOF MSe) scan mode were as follows: scan range 100-1000 m/z for the function 1 and 50-1000 m/z, with a collision energy ramp from 10-40 eV, for the function 2. Data process was performed using ChromaLynx, TargetLynx, MassFragment and MetaboLynx associated softwares (Waters) using a homemade database of more than 1.600 substances including more than 650 (at september 2018) NPS or metabolites (including BZF and pFBF).

FD metabolites investigations (in vitro and in silico)

Metabolites of identified FDs (BZF and pFBF) were *in vitro* investigated by analyzing HLM incubates using LC-HRMS [16,17,19,22,24]. Briefly, HLMs were pre-activated by alamethicin on ice in an intermediate volume of 50 µL in 0.1 M Tris-HCl-MgCl₂ at pH 7.4. This mixture was added to a 50 µL-dried residue of 500 µM of BZF or pFBF methanolic solution. **It should be noted** that (i) these solutions were constituted using powder samples #1 and #3 since no significant organic impurity was detected in these samples and taking in account their BZF or pFBF content determined using NMR analysis and ERETIC method (94% and 91%, respectively, see results section), and that (ii) 500 µM is consistent with previously reported concentrations used in similar metabolism studies of fentanyl and other **FDs** [16,17,25-28]. Fifty µL of a cofactor mixture (5 mM UDPGA, 1.3 mM NADPH, 3.3 mM G6P and 0.5 U/mL G6PD) in 0.1 M Tris-HCl was then added. The enzymatic reaction was performed at 37 °C for 60 min and stopped by the addition of 200 µL of methanol. Samples were frozen at -20°C until analysis. HLM incubate samples were consequently analyzed using the LC-HRMS method described above. Data process was performed using ChromaLynx, TargetLynx, MassFragment and MetaboLynx associated softwares (Waters, Manchester, UK). A software algorithm (MetaboLynx™) was used in order to *in silico* predict BZF and pFBF biotransformations. Main known routes of FDs metabolism includes **N-dealkylation to from norfentanyl** derivatives, hydrolysis to form despropionylfentanyl (ANPP) derivatives and numerous sites of hydroxylation. Many of these phase I metabolites undergo phase II metabolism to form corresponding glucuronides or sulfates. For instance, data about parafluoroisobutyrfentanyl (pFiBF, structurally close to pFBF) metabolism were available since 2017. A total of 17 metabolites were identified: two monohydroxy metabolites and a

hydroxymethoxy metabolite were suggested as specific and abundant pFiBF metabolites [15]. As a consequence, taking into account these data, sets of theoretically possible biotransformations of BZF or pFbF were created, and the automated metabolite profiling process was completed through the use of the fragmentation interpretation software tool MassFragmentTM 4.1 (Waters), to enable software driven assignment of metabolite structures from fragmentation patterns. The LC-HRMS acquired data of HLM incubates are subsequently cross-checked to *in silico* predicted biotransformations: criteria used for identification of the targeted compounds included 3 mDa as exact mass error of the precursor ion, as well as product ions, and +/- 0.25 min as tolerance in retention time for the precursor ion.

Results and Discussion

Samples analysis

NMR spectroscopy and LC-HRMS were applied for identification and characterization of the unknown powders (samples #1 and #2). BZF and pFbF were respectively identified in samples #1 and #3 (Figures 3 and 4; Tables 1 and 2). No significant organic impurity was detected in these powders using LC-HRMS or NMR. In addition, using the ERETIC method, NMR analysis revealed a BZF content of 94 % in sample #1, and a pFbF content of 91 % in sample #3 (the remaining 6 % and 9 %, respectively, consist putatively in inorganic compounds). In addition, pFbF was identified in sample #2 (estimated content of 33 µg/blotting paper).

Benzoylfentanyl (BZF, also known as phenylfentanyl), *N*-phenyl-*N*-[1-(2-phenylethyl)-4-piperidyl]benzamide, results from a swap of the ethyl group of the amide function by a phenyl. Some data about BZF synthesis are available [29] together with urinary detection using RIA [30]. But BZF remains a FD whose physiological and toxicological properties are not documented. In USA (Ohio), BZF was identified in three *post-mortem* cases since June 2018, and was temporary (June 2018), then definitively (December 2018) placed in US Schedule 1 [31,32]. In Europe, seizures of BZF were reported in UK (2017), Hungary (2018) and France (July 2018; Auvergne-Rhône-Alpes region). BZF is controlled in Cyprus since March 2016, Finland since September 2017, Lithuania since December 2017, Sweden since October 2017 and Denmark since May 2018 [33-35].

Parafluorobutyrfentanyl (pFbF, or 4-fluorobutyrfentanyl, 4-FbF), 1-((4-fluorophenyl)(1-phenethylpiperidin-4-yl)amino)butan-1-one, is a fluorinated derivative of butyrfentanyl. Some of physicochemical data for 4-FbF are available [36], but as the same way as BZF, there is a

lack of pharmacological, pharmacokinetic, and toxicological data about pFBB. In particular, pFBB potency ratio to morphine or to fentanyl are unknown [37,38]. According to cross-reactivity, pFBB can be detected in urine using urinary fentanyl immunoassays [13] and solid phase extraction with liquid chromatography-tandem mass spectrometry (LC-MS/MS) applied to pFBB urinary identification has been reported. In USA, several postmortem cases related to pFBB were reported since October 2016 using this LC-MS/MS method [39]. In Europe, seizures of pFBB were reported in Poland (2014), Sweden (2015), Slovenia (2015 and 2016) and Latvia (2018). pFBB is controlled in Cyprus, Finland, Czech Republic, Sweden, Poland, Denmark and France (since 5 September 2017) [40]. The first case of intoxication with pFBB was reported in EU by the Swedish Poison Information Centre in January 2015. **In this non fatal case, disorientation, unsteady, slurred speech together with hypotension were reported** [41]. Two fatalities related to pFBB were reported in Poland in 2016: *post-mortem* concentrations of pFBB were 91 and 112 µg/L in blood and 200 and 414 µg/L in urine [42]. From January 2017, a total of 11 fatalities related to pFBB were reported in UK [43,44].

In addition, BZF and pFBB were together identified in 3 hair segments of a French drug abuser victim of fatal carfentanil overdose in December 2017 at hair concentrations of 621, 4460 and 5870 pg/mg for BZF, and 4, 152 and 719 pg/mg for pFBB [45].

Metabolites investigations

Metabolites of BZF and pFBB were *in vitro* investigated by analyzing HLM incubates using LC-HRMS. Three metabolites of each were identified (Figures 5 and 6; Tables 1 and 2).

NorBZF (M01) is the result of *N*-dealkylation (loss of the phenylethyl group) that is one of the classical known routes of FDs metabolism. Amide hydrolysis of BZF leads to despropionylfentanyl (N-phenyl-1-(2-phenylethyl)piperidin-4-amine, or ANPP) (M02) production that is one of the main urinary fentanyl metabolites [26]. M03 is a hydroxylated-BZF: hydroxylation occurs on the phenyl ring of the *N*-alkyl chain, but we were not able to determine the exact position. In addition, we should notify that these three BZF were detected, together with BZF, in hair of the victim of fatal carfentanil overdose, previously cited in the text [45] (Table 1). For pFBB, M01 also consists in norpFBB as a result of *N*-dealkylation. M02 is the demethylation product of the amide group. In fact, this desmethylpFBB is parafluorofentanyl (pFB) that is itself a FD [46]. As well as BZF, M03 is a hydroxylated-pFBB with hydroxylation occurring on the phenyl ring. **Phase II metabolites (glucuronides and sulfates) of BZF and pFBB been researched but not identified.**

In the literature, there is no available data about BZF or pFBF metabolism. The observed metabolites reported here seem to be at low abundance in HLMs extracts (less than 10% of BZF or pFBF chromatographic signals). The also very low abundance of BZF metabolites in hair [45] should be considered in regard of usual very low incorporation of metabolites in hair [47]. These first results about BZF and pFBF metabolites need to be challenged to *in vivo* data (observed in blood and mainly in urine of users) in order to assess if these metabolites can be useful as biomarkers of BZF or pFBF exposition.

Conclusion

When the context suggests a potential opioid related intoxication, toxicologists should be aware of NSOs possibility use, especially when initial toxicology findings are negative. Nevertheless, NSOs detection in biological samples is still challenging. In this way, these preliminary analytical and metabolism data about BZF and pFBF can help.

Conflict of interest

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

Ethical approval

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

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Figures and tables legends

Figure 1: Fentanyl skeleton and regions where substituents occur: **A** amide group, **B** aniline ring, **C** piperidine ring, and **D** *N*-alkyl chain.

Figure 2: Sample #2 consisting in 5 blotting papers: white color, 3 out of 5 with no inscription or logo, 2 out of 5 with printed black signs that could be perhaps part of Arabic script.

Figure 3: BZF chemical structure and ¹H-NMR (MeOD) analysis of the BZF powder (sample #1) including signals related to BZF [7.26 (15H, m, ArH), 3.73 (2H, dm, CH₂, J 12.5Hz), 3.27 (4H, m, CH₂), 3.03 (2H, m, CH₂), 2.31 (2H, dm, CH₂, J 13.6Hz), 1.97 (2H, qd, CH₂, J 11.7, 3.2Hz)], together with other signals presumably related to powder excipients.

Figure 4: pFBB chemical structure and ¹H-NMR (MeOD) analysis of the pFBB powder (sample #3) including signals related to pFBB [7.31 (9H, m, ArH), 4.81 (1H, tt, CH-N), 3.71 (2H, dm, CH₂, J 12.8Hz), 3.32 (2H, m, CH₂), 3.22 (2H, td, CH₂, J 13.0, 2.0Hz), 3.05 (2H, m, CH₂), 2.32 (1H, h, CH, J 6.7Hz), 2.15 (2H, dm, CH₂, J 13.4Hz), 1.73 (2H, qd, CH₂, J 13.4, 3.4Hz), 1.03 (6H, d, (CH₃)₂, J 6.7Hz)], together with other signals presumably related to powder excipients.

Figure 5: Identified metabolites of BZF.

Figure 6: Identified metabolites of pFBB.

Table 1: BZF and metabolites: the underlying biotransformation, formula, observed accurate mass of the precursor ions (M+H⁺), retention time (RT, min), observed MS/MS product ions (fragments are given sorted by characteristic fragmentation patterns observed: a to i in reference to figure 5) and chromatographic peak area of molecular ion ([M+H]⁺) of substances of interest normalized to chromatographic peak area of BZF in HLMs and in 3 hair segments of a drug abuser [45] (nd: not detected).

Table 2: pFBB and metabolites: the underlying biotransformation, formula, observed accurate mass of the precursor ions (M+H⁺), retention time (RT, min), observed MS/MS product ions (fragments are given sorted by characteristic fragmentation patterns observed: a to j in reference to figure 6) and chromatographic peak area of molecular ion ([M+H]⁺) of substances of interest normalized to chromatographic peak area of pFBB in HLMs.

Figure 1

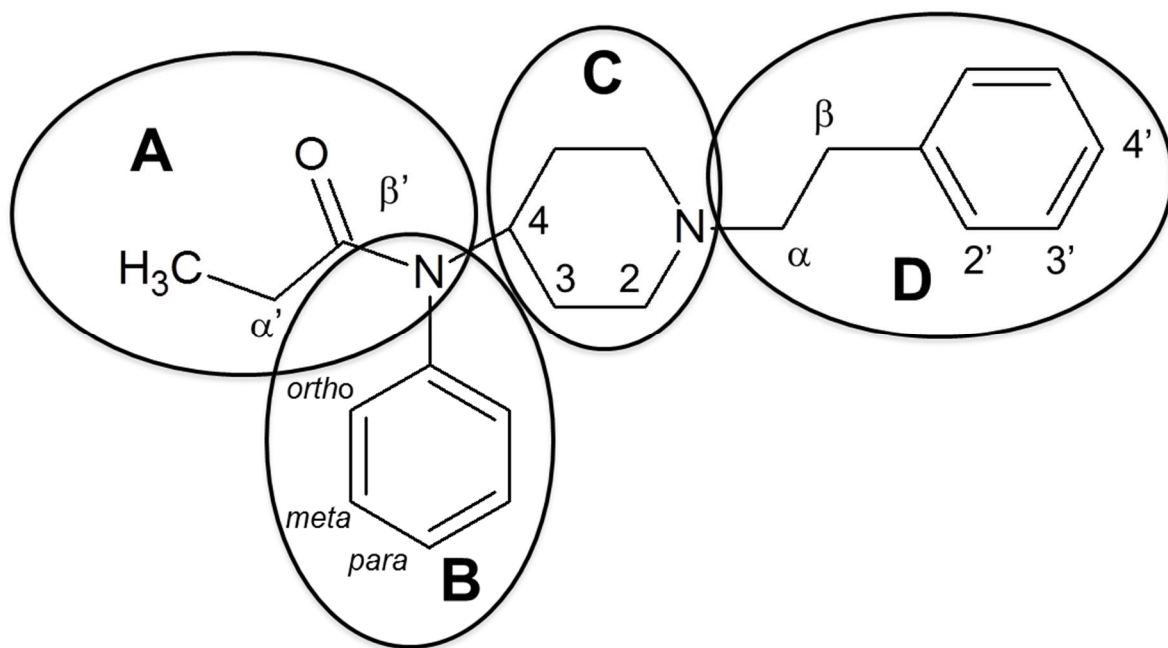


Figure 2

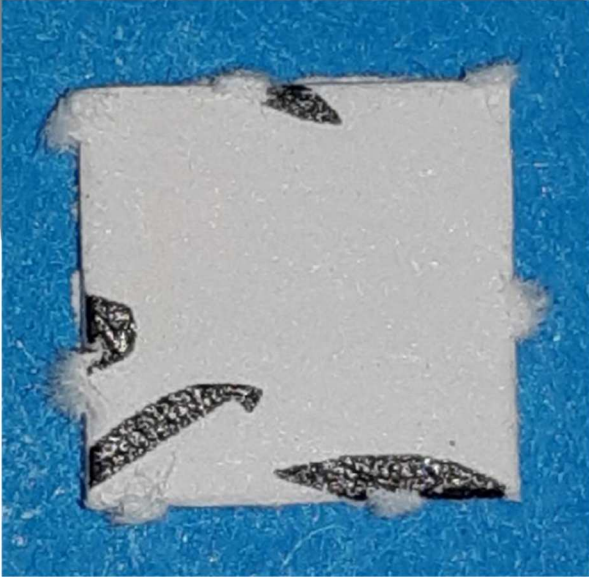


Figure 3

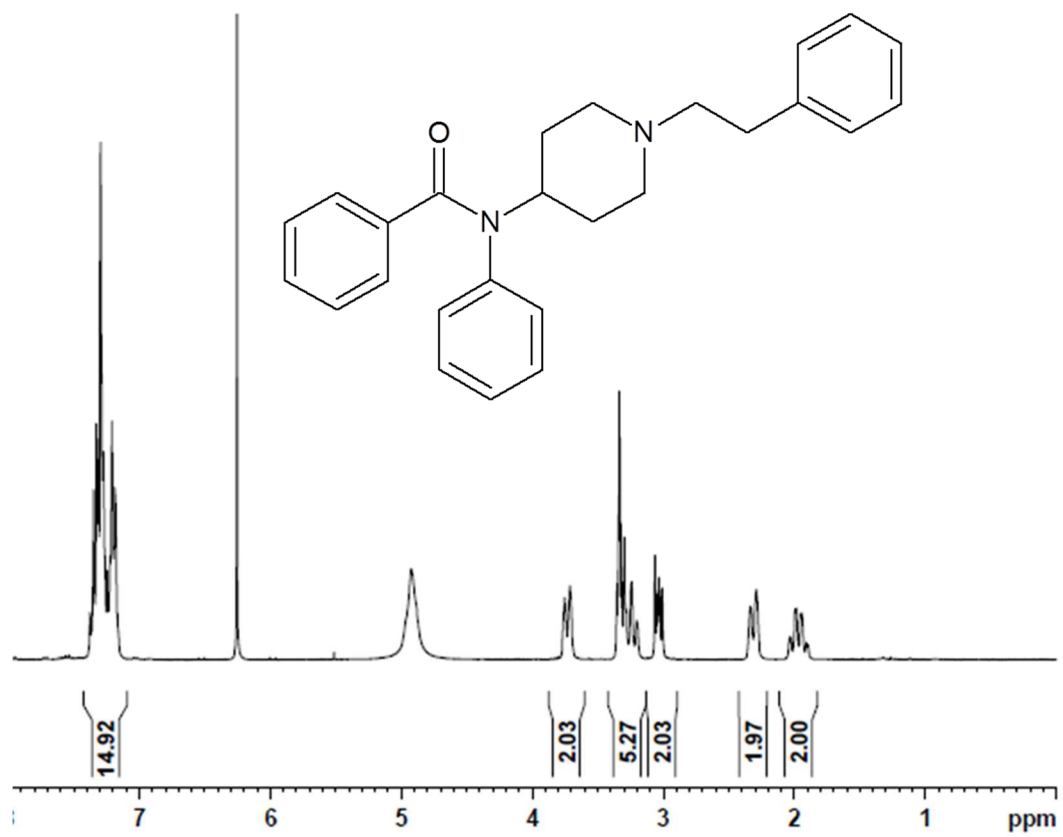


Figure 4

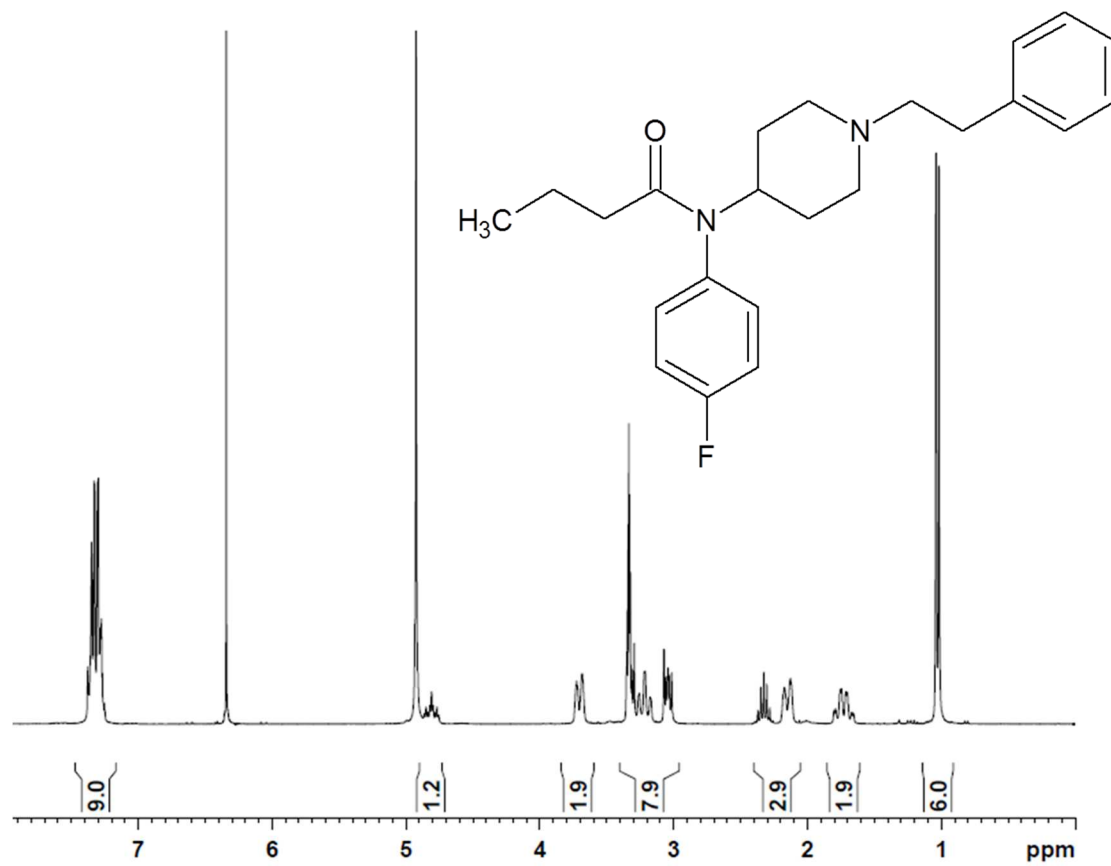


Figure 5

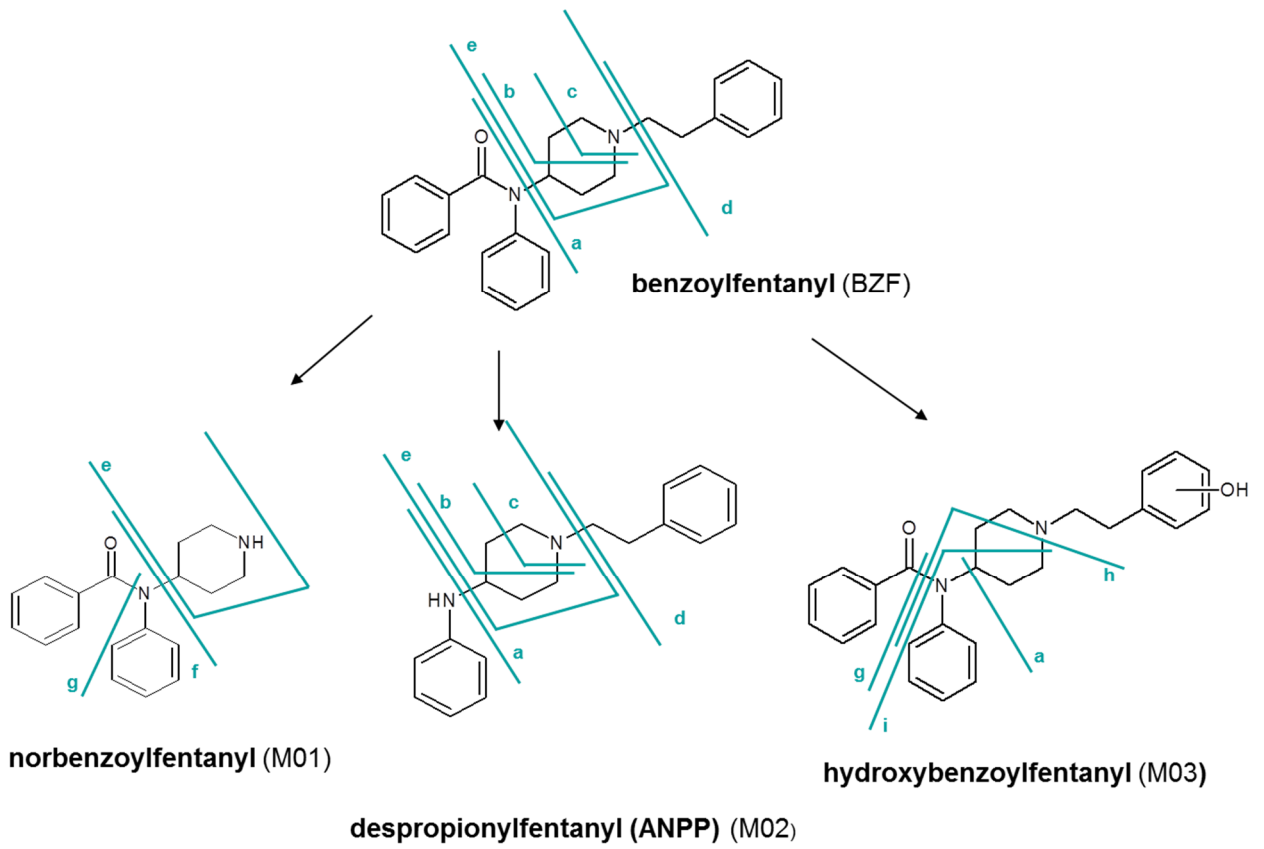


Figure 6

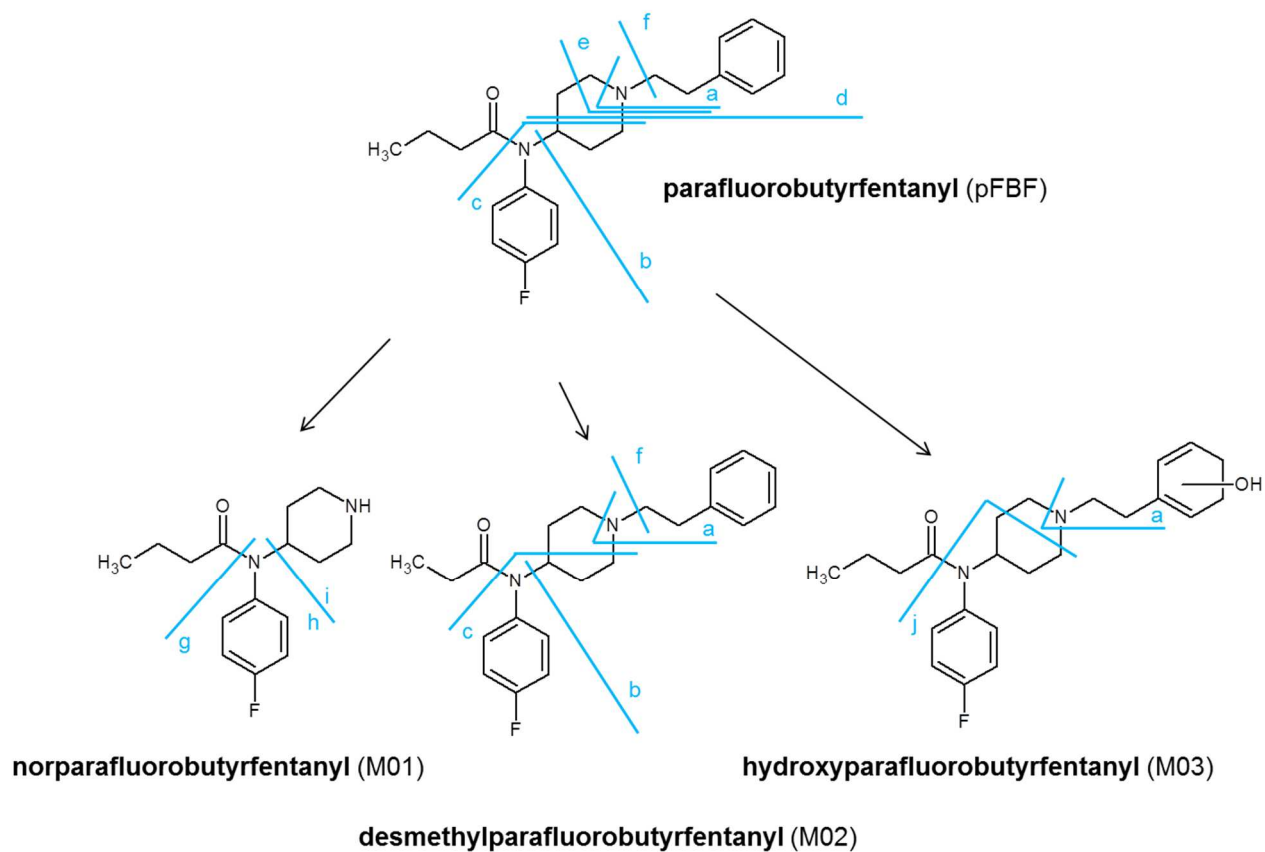


Table 1: BZF and metabolites: the underlying biotransformation, formula, observed accurate mass of the precursor ions (M+H+), retention time (RT, min), observed MS/MS product ions (fragments are given sorted by characteristic fragmentation patterns observed: a to i in reference to figure 5) and chromatographic peak area of molecular ion ([M+H]⁺) of substances of interest normalized to chromatographic peak area of BZF in HLMs and in 3 hair segments of a drug abuser [45] (nd: not detected).

	Name	Biotransformation	Formula	[M+H] ⁺	RT	a (-H ₂ O)	b	c	d	e	f	g	h	i	HLMs	Hair
Parent	BZF	none	C ₂₆ H ₂₈ N ₂ O	385.2274	7.23	188.1439	146.0970	134.0970	105.0704	84.0813					100	100/ 100/ 100
M01	NorBZF	<i>N</i> -dealkylation (loss of the phenylethyl)	C ₁₈ H ₂₀ N ₂ O	281.1648	5.27					84.0813	198.0919	105.0340			7	3/ 2/ 1
M02	Despropionylfentanyl (ANPP)	Amide hydrolysis	C ₁₉ H ₂₄ N ₂	281.2012	6.4	188.1439	146.0970	134.0970	105.0704	84.0813					4	4/ 2/ 1
M03	HydroxyBZF	Hydroxylation of phenyl ring of the <i>N</i> -alkyl chain	C ₂₆ H ₂₈ N ₂ O ₂	401.2229	6.7	204.1388 (186.1283)						105.0340	144.0813	132.0813	5	1/ 1/ nd

Table 2: pFBB and metabolites: the underlying biotransformation, formula, observed accurate mass of the precursor ions (M+H+), retention time (RT, min), observed MS/MS product ions (fragments are given sorted by characteristic fragmentation patterns observed: a to j in reference to figure 6) and chromatographic peak area of molecular ion ([M+H]+) of substances of interest normalized to chromatographic peak area of pFBB in HLMs.

	Name	Biotransformation	Formula	[M+H] ⁺	RT	a (-H ₂ O)	b	c	d	e	f	g	h	i	j	HLMs
Parent	pFBB	none	C ₂₃ H ₂₉ N ₂ OF	369.2342	7.17	248.1451	188.1439	150.0719	146.097	134.097	105.0699					100
M01	NorpFBB	<i>N</i> -dealkylation (loss of the phenylethyl)	C ₁₅ H ₂₁ N ₂ OF	265.1716	5.35							195.1298	182.0981	84.0813		4
M02	DesmethylpFBB	Demethylation (amide group)	C ₂₂ H ₂₇ N ₂ OF	355.218	6.62	234.1294	188.1439	150.0719			105.0699					1
M03	HydroxypFBB	Hydroxylation of phenyl ring of the <i>N</i> -alkyl chain	C ₂₃ H ₂₉ N ₂ O ₂ F	385.2291	6.7	204.1388, (186.1283)									164.0876	2