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***In vitro* characterization of NPS metabolites produced by human liver microsomes and the HepaRG cell line using liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis: application to furanyl fentanyl**

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Summary

Background: Identification of metabolites is of importance in the challenge of new psychoactive substances (NPS) as it could improve the detection window in biological matrices in clinical and forensic cases of intoxication. Considering the numerous and diverse NPS reported each year, producers increasingly appear today to be targeting non-controlled synthetic opioids, involving fentanyl derivatives such as furanyl fentanyl (Fu-F).

Objective: This work aims to investigate and compare metabolites of Fu-F using two *in vitro* experimental approaches.

Methods: CYP- and UGT-dependent metabolites of Fu-F were investigated by means of analyses of both human liver microsome (HLM) and hepatic (HepaRG) cell line incubates using liquid chromatography with high-resolution mass detection and, subsequently, compared and confronted to recently published data.

Results: Seventeen Fu-F metabolites were produced and several metabolic pathways can be postulated. HLMs and HepaRG cultures appear to be complementary: HepaRG cells produced 9 additional metabolites, but which appear to be minor *in vivo* metabolites. Specific* and/or abundant Fu-F metabolites are dihydrodiol-Fu-F*, norFu-F* and despropionylfentanyl. However, norFu-F seems to be inconstantly observed in *in vivo* cases. Furthermore, a sulfate metabolite present at significant rate in urine obtained from FU-F users was not identified here, as in another *in vitro* study.

Conclusion: HLMs represent an acceptable first choice tool for a single NPS metabolism study in forensic laboratories. Dihydrodiol-Fu-F and despropionylfentanyl could be proposed as reliable metabolites to be recorded in HRMS libraries in order to improve detection of Fu-F users. Nevertheless additional verifications of *in vivo* data remain necessary to confirm relevant blood and urinary metabolites of Fu-F.

Keywords

Furanyl-fentanyl, metabolites, HLM, HepaRG, LC-HRMS

Introduction

New psychoactive substances (NPS) represent significant analytical and interpretive challenges to forensic and clinical toxicologists [1,2]. NPS are new narcotic or psychotropic drugs, in pure form or in preparation, that are not controlled by the 1961 United Nations Single Convention on Narcotic Drugs or the 1971 United Nations Convention on Psychotropic Substances. NPS concern substances that usually (but not exclusively) replicate chemical structures and/or pharmacological effects of classical drugs of abuse (DOA), such as ecstasy, amphetamine, cocaine or cannabis. The use and online availability (on specialized websites) of NPS has rapidly increased over the last decade. For instance, 98 new substances were detected for the first time in the EU in 2015, bringing the number of new substances monitored by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) to more than 560, of which 70 % were detected in the last 5 years [3,4]. Today, producers of NPS increasingly appear to be targeting non-controlled synthetic opioids. Thus, while heroin remains the most commonly abused classical opioid, new synthetic opioids are increasingly misused. In 2014, 18 EU countries reported that more than 10 % of all opioid patients presented problems primarily related to opioids other than heroin [3]. Between 2013 and 2014, over 700 deaths have been related to fentanyl and its analogs (i.e. acetylfentanyl) in the USA. In this country, clandestinely-produced fentanyl is primarily manufactured in Mexico, with its analogs and precursors obtained from distributors in China [4,5]. Among synthetic opioids, fentanyl and fentanyl derivatives have been particularly reported in the literature in relation to their use and associated acute toxicity over the last 4 years worldwide [3,4,6]. Many fentanyl derivatives are highly potent and can be sold as heroin to unaware users, thus standing a high risk of overdose and death, as illustrated with acetylfentanyl which was subject to an EMCDDA–Europol joint report in 2015 [7]. At the same time, the recreational abuse of fentanyl-like substances continues today to be of significant concern, as these substances often exhibit unpredictable outcomes including unconsciousness and respiratory depression, which are typical life-threatening opioid symptoms. [8].

Furanylfentanyl (Fu-F) is a fentanyl derivative recently reported in USA, as well as in EU. Fu-F (N-(1-(2-phenylethyl)-4-piperidinyl)-N-phenylfuran-2-carboxamide) was synthesized and patented in 1986 and has a furanyl ring in place of the methyl group adjacent to the carbonyl bridge (figure 1 and table 1). Fu-F exhibits a pharmacological profile similar to that of fentanyl and other μ -opioid receptor agonists [9]. Fu-F has an ED-50 in mice (0.02 mg/kg) [10] comparable to that of fentanyl (0.016 mg/kg) [11], which is coherent with the hypothesis that Fu-F has similar effects than those of fentanyl, although they have never been studied in humans. According to the US National Forensic Laboratory Information System (NFLIS; a national drug forensic laboratory reporting system that systematically collects results from drug chemistry analyses conducted by participating federal, state and local forensic laboratories across the USA), the first report of Fu-F was recorded in December 2015 in Oregon. From December 2015 to September 2016, 494 submissions to US forensic laboratories identifying Fu-F were reported in NFLIS. The US Drug Enforcement Administration (DEA) has received reports connecting Fu-F to at least 128 confirmed overdose deaths that occurred in 2015 and 2016. A total of 607 drug reports in which Fu-F was identified was submitted to forensic laboratories from December 2015 to September 2016. As a consequence and in order to avoid an imminent hazard to the public safety, Fu-F was placed into schedule I of the United States Controlled Substances Act by the Administrator of the DEA at the end of November 2016 [9, 12-14].

In July 2016, over 40 overdose events caused by crack cocaine contaminated with Fu-F were observed in British Columbia, Canada. [15]. In EU, Fu-F was identified in Sweden in 2015 *via* the STRIDA project. It is noteworthy that the emergence of Fu-F in this country was in part linked to the classification of other fentanyl derivatives (i.e. acetylfentanyl, butyrfentanyl, 4F-butyrfentanyl) as narcotic substances or as harmful to health on August 2015 [16]. Over the course of 4 months in 2015 and 2016, a cluster of seven fatal intoxications involving Fu-F also occurred in Sweden [17]. In addition, the prevalence of Fu-F in opioid-related emergency room admissions and deaths is likely underestimated, as standard immunoassays cannot detect Fu-F.

In fact, one of the main challenges posed by the emergence of NPS with forensic implications like Fu-F is their identification in human biological samples. Trying to detect the parent drug could lead to false-negative results when the delay between consumption and sampling has been too long. The identification of their metabolites could then improve their detection window in biological matrices. In this context, data about the

metabolism of Fu-F can be of interest in the challenging field of analytical detection, especially when the delay between consumption and biological sampling is substantial. Indeed, the identification of Fu-F metabolites, which improves the efficiency of toxicological screening libraries, will enlarge the detection window of Fu-F in biological matrices after Fu-F intake. Data about Fu-F metabolism were recently reported [18-20]. The aim of this work is to investigate and compare possible CYP- and UGT-dependent metabolites of Fu-F by means of (i) analyses of both human liver microsomes (HLMs) and hepatic (HepaRG) cell line incubates using liquid chromatography with high-resolution mass detection (LC-HRMS) and (ii) confrontation to these recently reported data.

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). Ethanol was purchased from VWR International (Fontenay-sous-bois, France). Insulin was obtained from Sigma-Aldrich (Saint Louis, MO, USA). William's E medium supplemented with 10% fetal bovine serum (Lonza, Verviers, Belgium), penicillin-streptomycin, L-glutamine were purchased from Life Technologies (Eugene, OR, USA). William's E medium, penicillin-streptomycin, L-glutamine and trypsin were purchased from ThermoFisher Scientific (Saint Aubin, France). Hydrocortisone hemisuccinate was from Serb (Paris, France). 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. The HepaRG cell line was obtained from a liver tumor of a female patient suffering from hepatocarcinoma [21]. A Fu-F powder was obtained from SINTES scheme [the SINTES (National Detection System of Drugs and Toxic Substances) scheme is intended to document the

toxicological composition of illegal substances in circulation in France]. 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.

Fu-F powder analysis

In order to confirm the absence of other compounds, such as synthesis impurities, and to check the purity of the Fu-F powder, a LC-HRMS analysis (using the method described below) was completed by a nuclear magnetic resonance (NMR) analysis. The NMR spectra were recorded on AVANCE 300 (Bruker Biospin, France) operating at 300 MHz equipped with a 5 mm quadrupole nucleus probe (QNP) probe at 295 K. The ^1H spectra were recorded with 64 scans, a 32-K time-domain data points with 4800 Hz spectral width, an acquisition time of 3.42 s, a relaxation delay of 2 s and a flip angle of 30° . The Free Induction Decays (FIDs) were transformed (0.3 Hz broadening) and the baseline was corrected. For quantification and after thin manual grinding using a mortar, 7.5 mg of the powder was dissolved in 600 μL of methanol- D_4 and the ERETIC (Electronic REference To access In vivo Concentrations) method was used for the determination of absolute concentrations. ERETIC consisted of a digitally generated Gaussian peak (on single point calibration calibrated using a reference sample 15 mg of caffeine in 600 μL de CDCl_3 and a control point using 10 mg of the same substance in 600 μL de CDCl_3) 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 [22].

HLM incubates

HLM incubates were prepared as already reported [23-26]. Once thawed, HLMs (2 mg protein/mL for a final volume of 100 μL) were pre-activated by alamethicin on ice in an intermediate volume of 50 μL in 0.1 M Tris-HCl- MgCl_2 (10 mM MgCl_2 and 100 mM Tris-HCl solution) at pH 7.4. This mixture was added to a 50 μL -dried residue of three different concentrations of a Fu-F methanolic solution (10, 50 and 100 μM). 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 $^\circ\text{C}$ for 60 min and stopped by the addition of 100 μL of methanol. Samples were freeze-dried at -20°C until analysis.

Cell culture incubates

HepaRG cells were seeded at a density of 600.000 cells/well in 12-well plates in William's E medium supplemented with 10 % fetal bovine, 100 U/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL insulin, 2 mM glutamine, and 50 µM hydrocortisone hemisuccinate at 37 °C with 5% humidified CO₂. After 2 weeks, these undifferentiated cells were shifted to the same medium supplemented with 1.7% dimethylsulfoxide (DMSO) for further 2 weeks in order to obtain cells expressing liver-specific functions. Treatments were performed on cells maintained in a medium containing 5% fetal bovine serum and 1% DMSO (used as a vehicle). Experiments included testing of 3 Fu-F concentrations, 3 incubation delays, and the presence or not, of ethanol in the medium (in order to explore the possibility of production of specific metabolites due to reaction with ethanol, like cocaine that forms cocaethylene after ethanol consumption, or methylphenidate that may transform to ethylphenidate [27]). In this way, cells were thus treated with Fu-F (10, 50 or 100 µM), with or without ethanol at 50 mM (i.e. 2.3 g/L) for 6, 24 or 48 hours. Cell culture supernatants were removed and freezed at -20°C until analysis.

LC-HRMS analysis

Screening for Fu-F metabolites was performed using a previously reported LC-HRMS method [23-26]. Briefly, after addition of 100 µL of methanol-containing methyl-clonazepam at 1.25 mg/L and β-OH-ethyltheophyllin at 16 mg/L (internal standards), 100 µL of sample (Fu-F powder solution, HLM incubate or cell culture supernatant) were diluted in 400 µL of 3 % 5-sulfosalicylic acid and subsequently centrifuged at 4 °C for 14 min at 32000 g. 75 µL of the supernatant were then injected into the chromatographic system. The liquid chromatography system consisted of two binary solvent manager LC pumps, a sample manager autosampler and a column manager oven Acquity (Waters, Manchester, UK). Extraction was performed using an OASIS HLB on-line column (30 x 2.1 mm, 20 µm) (Waters, Manchester, UK). The chromatographic separation was performed using an ACQUITY HSS C18 column (150 x 2 mm, 1.8 µm, Waters) in an oven at a temperature of 50 °C, and mobile phases including ammonium formate buffer 5mM, pH 3 (A) and acetonitrile in 0.1 % formic acid (B); flow rate of 0.4 mL/min was used. Mass spectrometry data were acquired using a XEVO G2-XS QTOF (Waters, Manchester, UK) instrument controlled with MassLynx 4.1 software. For detection, mass spectrometric conditions were as follows: positive electrospray ionization interface (ESI+), ion spray voltage set at 20 V,

source temperature set 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 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, Manchester, UK). The LC-HRMS acquired data are cross-checked to *in silico* predicted biotransformations using a software algorithm (MetaboLynx™). Briefly, sets of theoretically possible biotransformations of the studied drug (Fu-F) were created based on the structure of the drug, including possible hydroxylation, *O*- or *N*-dealkylation, deamination and glucuronidation transformations. The automated metabolite profiling process was completed through the use of the fragmentation interpretation software tool MassFragment™ 4.1 (Waters), to enable software driven assignment of metabolite structures from fragmentation patterns.

Results and Discussion

For identification and characterization of Fu-F, LC-HRMS together with NMR spectroscopy were applied (figures 1, 2 and 3). No significant organic impurity was detected in the Fu-F powder using LC-HRMS or NMR. In particular, the absence of other fentanyl derivative or furanylfentanyl precursors (that could influence the *in vitro* experiments) was checked. In addition, using the ERETIC method, NMR analysis revealed a Fu-F content of 73 % in the powder (the remaining 27% consist putatively in inorganic compounds) ; this content was subsequently taken into account for HLM and HepaRG cell incubations.

In vitro experiments using HLM and HepaRG incubations and LC-HRMS analysis were performed in order to investigate the potential CYP- and UGT-dependent metabolites of Fu-F.

Regarding the three tested concentrations of Fu-F, a satisfactory metabolite production (highest chromatogram peak area related to metabolite) was observed after incubation of Fu-F (i) at 100 µM with HLM and (ii) at 100 µM for 24 hours with HepaRG (figure 4). It is noteworthy that no difference in the metabolite production rate or the metabolite nature was observed in HepaRG incubates with or without the presence of ethanol (data not shown). This result does not then support the hypothesis of specific metabolite production in case of ethanol co-consumption with Fu-F.

Observed metabolites (n=17) are presented in table 1, and several pathways including hydroxylation, oxidation, hydrolysis of the amide group, *N*-dealkylation and glucuronidation can be postulated (figure 5).

These *in vitro* experiments revealed three main metabolites (based on the intensity of the chromatographic peak areas): M02 (dihydrodiol Fu-F), M03 (norFu-F), and M04 (despropionylfentanyl).

M02 (dihydrodiol Fu-F) is the product of epoxidation and hydration of the furanyl group. This metabolite was the second most dominant metabolite of Fu-F as described by Watanabe et al. using *in vitro* experiments with pooled human hepatocyte incubation [18]. Moreover, M02 was observed at similar concentrations as Fu-F in 44 out of 51 urine samples obtained from Fu-F users [19]. Although M02 was not observed by Labutin et al. in an urine sample of a Fu-F user, two hydroxylated-M02 (m/z 425.2052 and 425.2087) were detected in this case [20].

M03 (norFu-F) results from *N*-dealkylation of Fu-F and its production is coherent with the metabolic pathway of fentanyl and fentanyl derivatives. This nor-metabolite was also significantly detected using pooled human hepatocyte incubation [18]. However, this *in vitro* observation is not in accordance with *in vivo* findings, as M03 was detected in only 1 out of 4 urine samples of Fu-F users [18]. Accordingly, Goggin et al. reported the presence of M03 in only 4 out of 51 urine samples at lower concentrations than those of Fu-F [19].

M04, or despropionylfentanyl [1-(2-phenylethyl)-4-N-anilinopiperidine], is a known fentanyl metabolite resulting from fentanyl amide hydrolysis, that was first reported by Van Rooy et al. [28]. In our study, M04 is the main metabolite produced using HLM or HepaRG. Similarly, Watanabe et al. reported M04 as being the main Fu-F metabolite produced *in vitro* using pooled human hepatocytes, as well as observed *in vivo* in urine samples of Fu-F users [18]. Moreover, M04 was observed at similar concentrations as Fu-F in 42 out of 51 urine samples [19] and its presence was also reported by Labutin et al. in an urine sample of a Fu-F user [20].

Other metabolites (table 1) consist in products of hydroxylation or dihydroxylation of Fu-F (M01, M05a to M05d, M09) or of despropionylfentanyl (M08a to M08e), and of *N*-dealkylation (M06, M07). A M08-type metabolite was observed in urine of a Fu-F user [20]. Only one phase 2-metabolite (M010) was detected in this study and corresponds to a glucuronidated form of a hydroxylated Fu-F. No sulfate metabolite was observed, although HepaRG cells have been reported to be capable of sulfatation [29]. In contrast, Watanabe et al. produced a

sulfate metabolite of a hydroxylated despropionylfentanyl (M08-SO₄) using pooled human hepatocytes, that was also detected in urine of Fu-F users [18]. Another sulfate metabolite (m/z 377.1534, C₂₄H₂₈N₂O₄), identified as a despropionylfentanyl-SO₄ (M04-SO₄), was found in 42 out of 51 urine samples by Goggin et al. at relatively high concentrations (mean urine concentration of 90 µg/L versus 34 µg/L for Fu-F) [19]. Some of the identified metabolites appeared poorly produced regarding their related chromatographic peak area / Fu-F chromatographic peak area ratio which were less than 0.02: M01, M05a, M05c, M06, M07, M08b, M08d and M08e.

As a total, our *in vitro* data are coherent with previously published data on other fentanyl derivative metabolism [30-33]. For instance, the metabolism of carfentanil, another potent fentanyl analogue currently used in veterinary medicine, was described with similar *N*-dealkylation and piperidine hydroxylation reactions as the major metabolic steps [30].

From an experimental point of view, the 100 µM Fu-F concentration was determined to be the most efficient for metabolite production by both HLMs and HepaRG and is of the same order than Fu-F concentration used by Watanabe et al. (10 µM) with pooled human hepatocytes [18]. This efficient concentration of Fu-F is also consistent with previously reported concentrations (ranging from 5 to 250 µM) used in similar metabolism studies of fentanyl and other fentanyl derivatives: i.e. 10 µM for fentanyl [31], 100 µM for butyrfentanyl [32], 250 µM for 3-methylfentanyl and isofentanyl [33], and 5 µM for carfentanil [30].

The aim of this work was to characterize as many as possible metabolites of Fu-F using two different *in vitro* approaches, i.e. HLMs and HepaRG cell line, combined with a highly specific and sensitive LC-HRMS method. Both approaches appear to be complementary (figure 4) and in coherence with previously reported data on Fu-F metabolism [18]. Even if the total contribution of CYP and UGT enzymes to xenobiotic metabolism is reported to be in general more than 80% [34], other important enzymatic reactions, such as sulfation, methylation, acetylation or glutathione conjugation, can occur and are not investigated using HLM incubation alone. In this way, the association to HepaRG use is of interest. Indeed, in differentiated HepaRG cells, levels of P450 activities (CYP2C9, CYP3A4, CYP2D6, CYP1A2, CYP2B6 and CYP2E1) and expression of phase 2-enzymes are in accordance with those usually found in primary human hepatocyte cultures [35] and constitute a

supplementary suitable model for studies of liver metabolism of xenobiotics [29,36-38]. In this study, HepaRG cells produced more metabolites than HLMs (9 out of 17), as could have been expected. Nevertheless, all these additional HepaRG-derived Fu-F metabolites appear to be minor *in vivo* metabolites [18-20]. Similar observation concerning the limited contribution of HepaRG toward HLMs into NPS metabolism study has already been reported [39]. Considering the potential drawbacks of cell culture implementation, HLM represent a cost-efficient tool for an easy and rapid NPS metabolism study.

Conclusion

Metabolism studies play an important role in clinical and forensic toxicology. The identification of metabolites is of importance in the challenge of NPS analytical detection as only detecting the parent drug could lead to false-negative results (i) when the delay between consumption and sampling has been too long and (ii) owing to very low blood or urine concentrations generally observed *in vivo*. From an analytical point of view, it is recognized that HRMS, which greatly assists identification of unknown compounds, shows the best performance in comparative studies for drug screening assays [40,41]. Both *in vitro* approaches (HLMs and HepaRG), used in the present study, were complementary as HepaRG cells produce more metabolites of Fu-F than HLMs. However, these additional Fu-F metabolites appear to be of minor interest for *in vivo* applications, as they have been rarely observed in urines of Fu-F users. HLM appears to be an acceptable first choice as an *in vitro* strategy to produce metabolites, particularly for forensic laboratories missing special equipment for cell culture handling.

In vitro data obtained in this work suggest that specific* and/or abundant Fu-F metabolites could be dihydrodiol Fu-F*, norFu-F* and despropionylfentanyl. These results are coherent with those recently obtained *in vitro* by Watanabe et al. [18]. However, norFu-F seems to be rarely observed in real urine specimens [18,19]. Furthermore, sulfate metabolites that were not *in vitro* produced here seem to be present in urine of Fu-F users [19]. As a total, dihydrodiol-Fu-F and despropionylfentanyl could be proposed as reliable metabolites to be recorded in HRMS libraries in order to improve detection of Fu-F users, although additional confrontations to *in vivo* data (from intoxication cases) remain necessary to definitely confirm relevant blood and urinary metabolites of Fu-F.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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Table and figures legends

Figure 1: Fentanyl (**A**), furanyl fentanyl (**B**) [*N*-(1-(2-phenylethyl)-4-piperidinyl)-*N*-phenylfuran-2-carboxamide] and postulated fragmentation pattern. a, h, furan-2-carboxamide cleavage; b, piperidine ring cleavage; c, cleavage between the piperidine ring and the phenethyl rest; d, e, i, degradation of the piperidine ring; f, g, cleavage between the piperidine ring and the *N*-phenylfuran-2-carboxamide; j, elimination of H₂O.

Figure 2: HRMS chromatogram of the powder and mass spectra (at high and low collision energy) of Fu-F.

Figure 3: ¹H-NMR (MeOD) analysis of the Fu-F powder including signals related to Fu-F [7.55 (3H, m), 7.51 (1H, dd, J 1.75, 0.7Hz), 7.30 (7H, m), 6.29 (1H, dd, J 3.6, 1.75Hz), 5.62 (1H, d, J 3.6Hz), 4.94 (1H, tt, J 12.3, 3.8Hz), 3.68 (2H, d, J 12.3Hz), 3.25 (2H, m), 3.14 (2H, td, J 12.6, 2.0Hz), 3.03 (2H, m), 2.21 (2H, d, 13.6Hz), 1.90 (2H, qd, J 13.0, 3.1Hz)] together with other signals presumably related to powder excipients.

Figure 4: Ratio of metabolites' related chromatographic peak area / Fu-F (substrate) chromatographic peak area at 100 μM for 24 hours with HepaRG and at 100 μM with HLM.

Figure 5: Proposed metabolic pathways of Fu-F from *in vitro* analyses.

Table 1: Metabolites of Fu-F sorted by retention times (RT), the underlying biotransformation, formula, accurate mass of the precursor ions (M+H⁺) and observed MS/MS product ions. Fragments are given sorted by characteristic fragmentation patterns observed. a, h, furan-2-carboxamide cleavage; b, piperidine ring cleavage; c, cleavage between the piperidine ring and the phenethyl rest; d, e, i, degradation of the piperidine ring; f, g, cleavage between the piperidine ring and the *N*-phenylfuran-2-carboxamide; j, elimination of H₂O. The 3 last columns indicate previously reported Fu-F metabolites in literature [18-20] (¹fragmentation patterns not available owing to a too low *in vitro* production; ²at least one of these isomers).

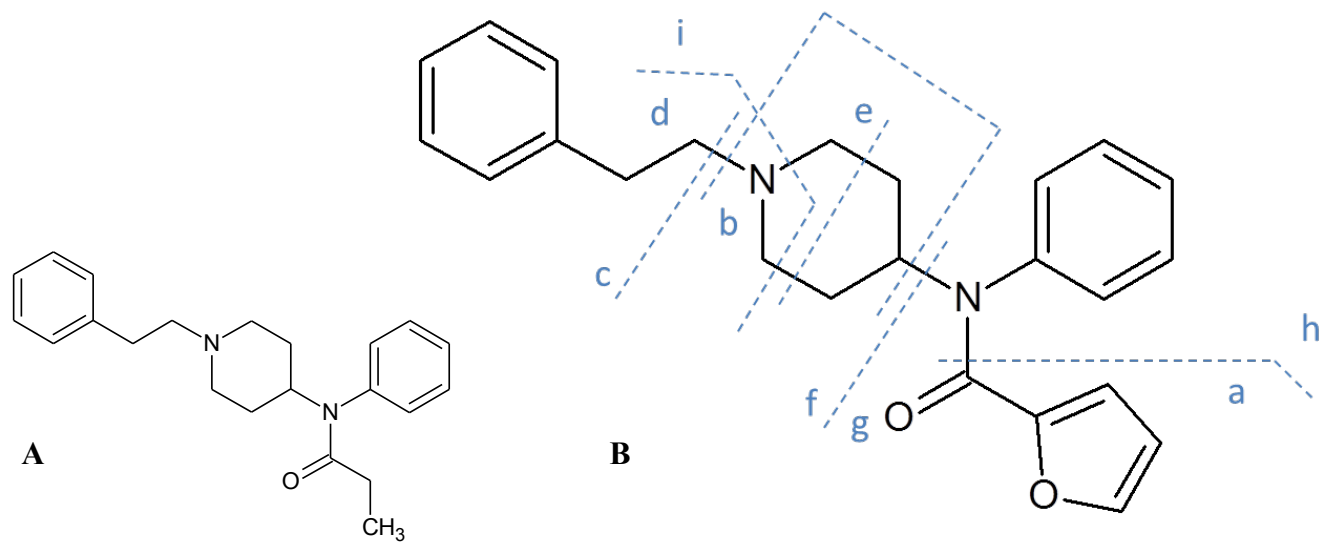


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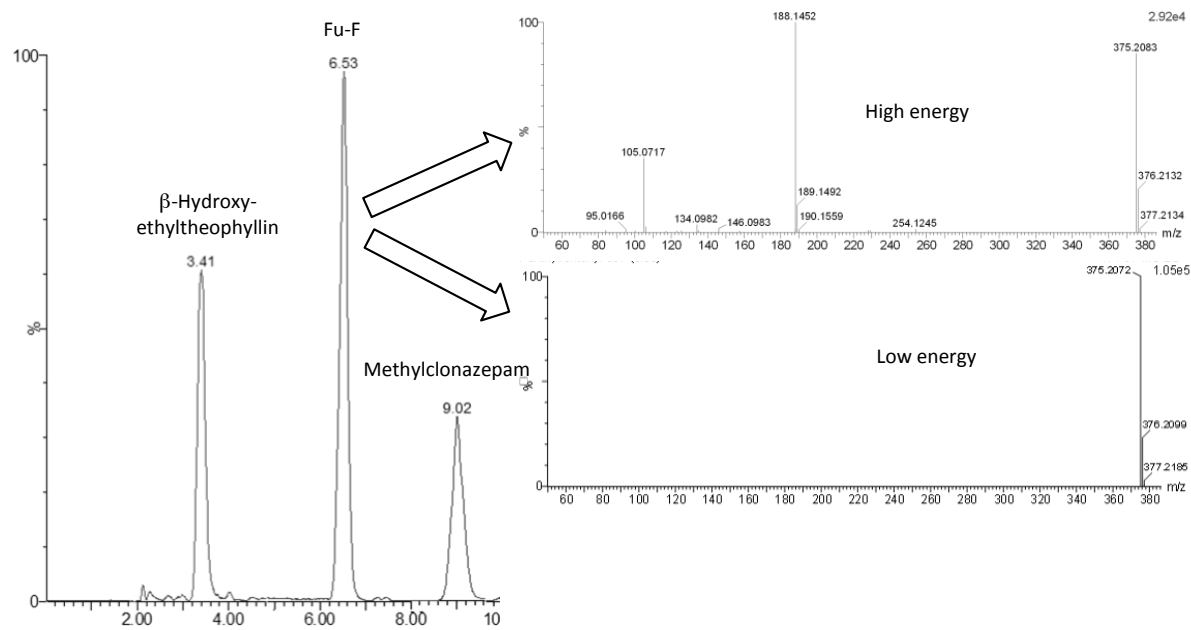


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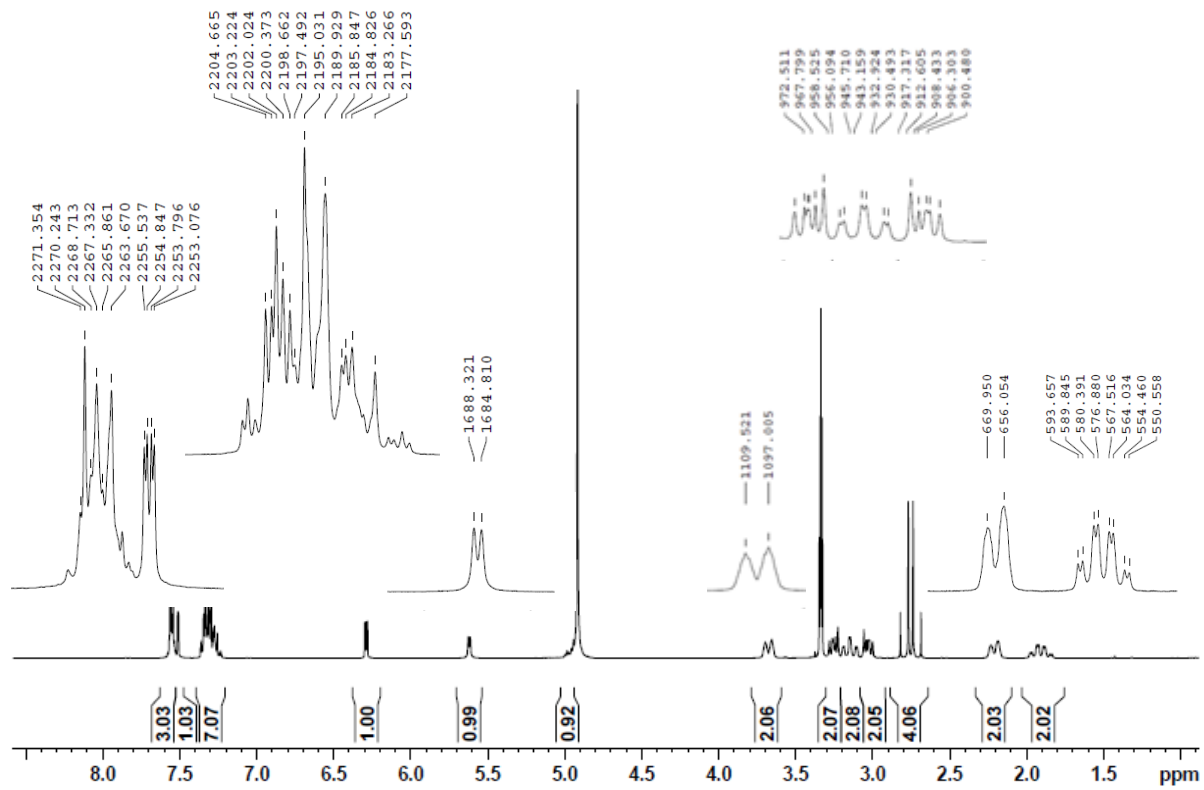


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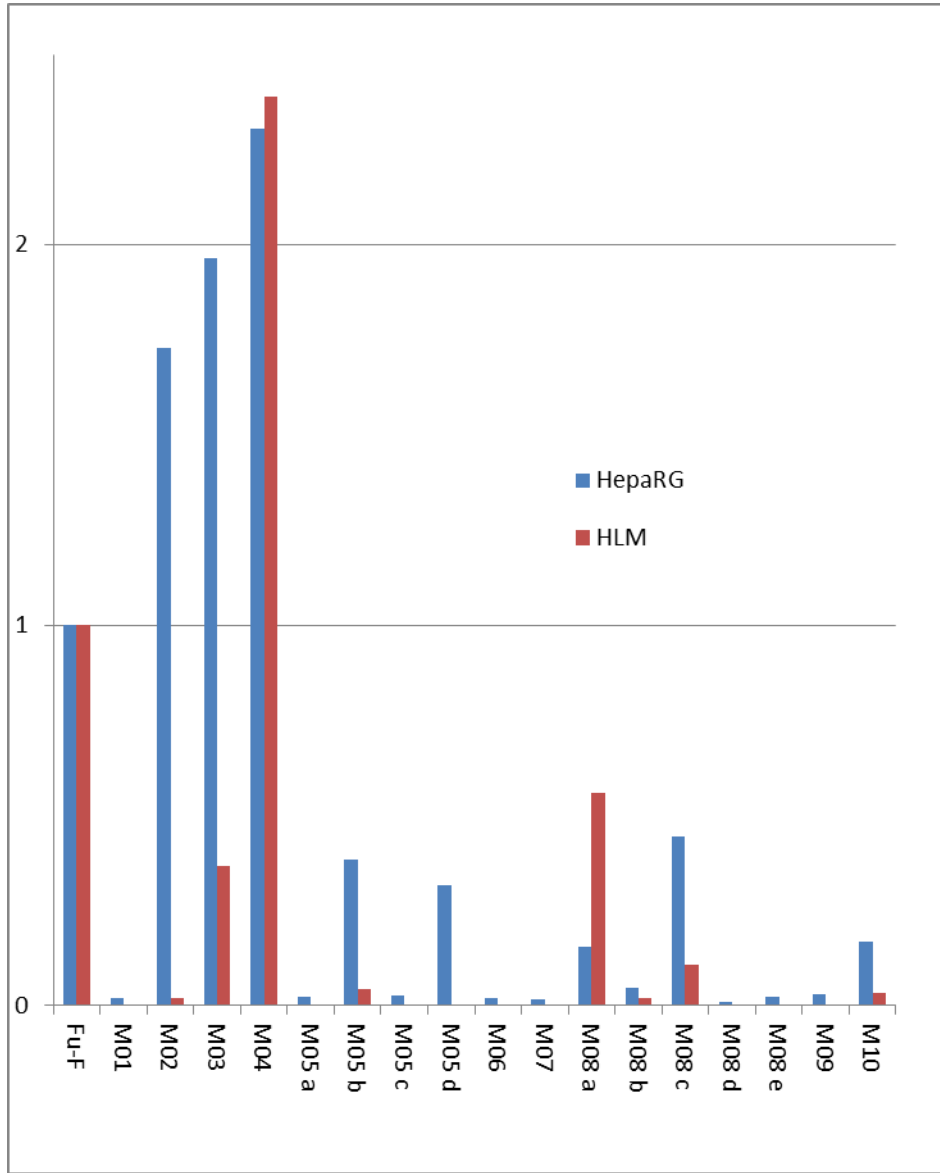


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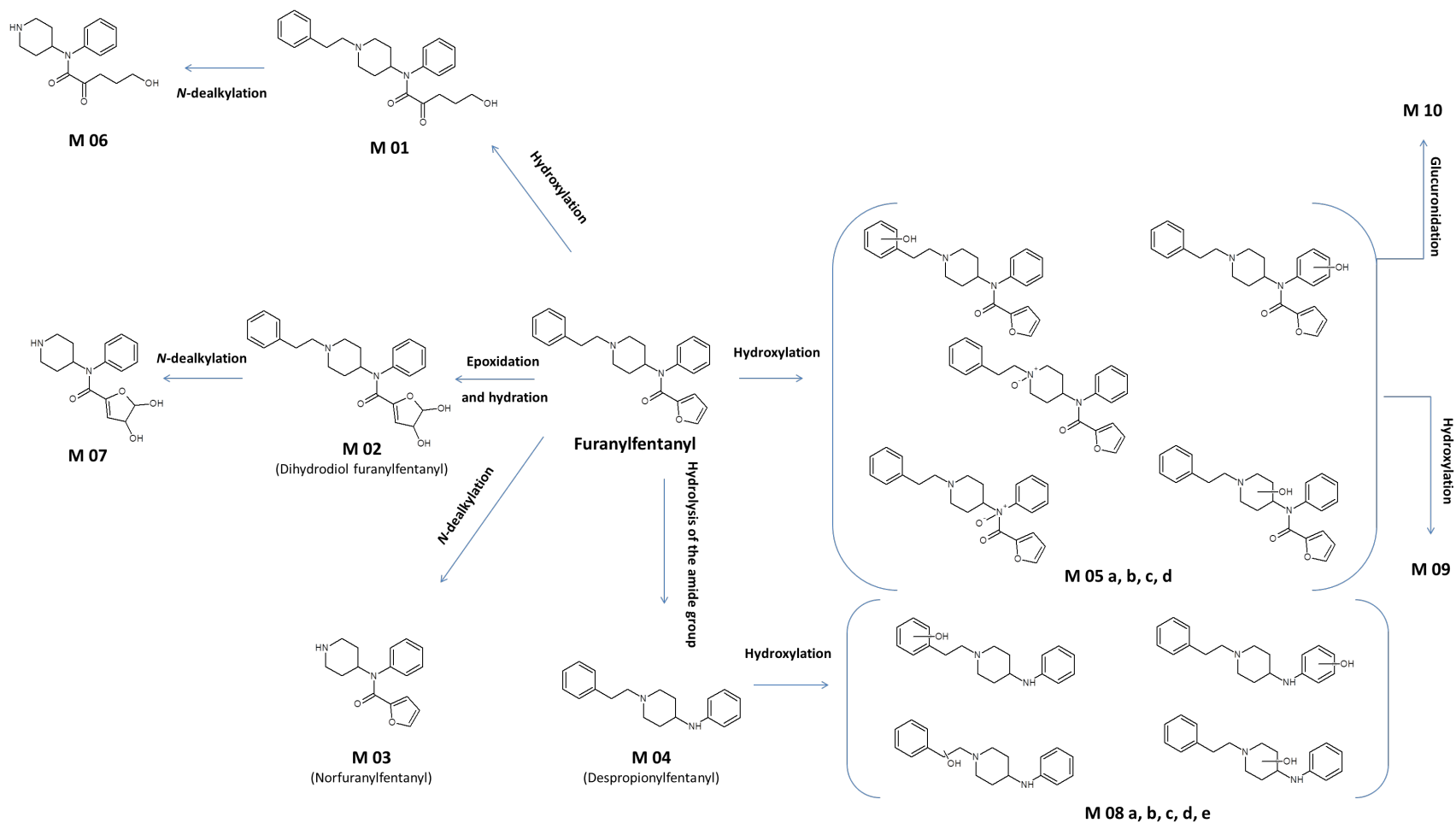


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carboxamide cleavage; b, piperidine ring cleavage; c, cleavage between the piperidine ring and the phenethyl rest; d, e, i, degradation of the piperidine ring; f, g, cleavage between the piperidine ring and the N-phenylfuran-2-carboxamide; j, elimination of H₂O. The 3 last columns indicate previously reported Fu-F metabolites in literature [18-20] (¹fragmentation patterns not available owing to a too low *in vitro* production; ²at least one of these isomers).

	Name	Biotransformation	Formula	M+H ⁺	RT	a	b	c	d	e	f	g	h	i	j	[18]	[19]
Fu-F	Furanylfentanyl	none	C ₂₄ H ₂₆ N ₂ O ₂	375.2067	6.45		84	105	134	146	188		281			✓	✓
M01	HO-Fu-F-M	hydroxylation (and cleavage) of the furanyl ring	C ₂₄ H ₃₀ N ₂ O ₃	395.2329	5.35			105	134	146	188						
M02	Dihydrodiol-Fu-F	dihydrodiol furanyl fentanyl	C ₂₄ H ₂₈ N ₂ O ₄	409.2122	5.41			105	134	146	188	188				✓	✓
M03	NorFu-F	<i>N</i> -dealkylation (loss of the phenylethyl)	C ₁₆ H ₁₈ N ₂ O	271.1441	4.55	95	84				188					✓	
M04	Despropionylfentanyl	amide hydrolysis (loss of the 2-furanyl cetone)	C ₁₉ H ₂₄ N ₂	281.2012	6.36		84	105	134	146	188					✓	✓
M05a	HO-Fu-F (a)	hydroxylation	C ₂₄ H ₂₆ N ₂ O ₃	391.2016	5.51			121			204					✓ ²	
M05b	HO-Fu-F (b)	hydroxylation	C ₂₄ H ₂₆ N ₂ O ₃	391.2016	5.9						204			240	373	✓ ²	
M05c	HO-Fu-F (c)	hydroxylation	C ₂₄ H ₂₆ N ₂ O ₃	391.2016	6.8						204			240		✓ ²	
M05d	HO-Fu-F (d)	hydroxylation	C ₂₄ H ₂₆ N ₂ O ₃	391.2016	7.18			105			204			240		✓ ²	
M06	Nor-HO-Fu-F-M	<i>N</i> -dealkylation (loss of the phenylethyl), hydroxylation (and cleavage) of the furanyl ring	C ₁₆ H ₂₂ N ₂ O ₃	291.1703	3.43	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>		
M07	NorDihydrodiol-Fu-F	<i>N</i> -dealkylation (loss of the phenylethyl), of dihydrodiol furanyl fentanyl	C ₁₆ H ₂₀ N ₂ O ₄	305.1496	3.43	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>	✓	
M08a	HO-Despropionylfentanyl (a)	amide hydrolysis (loss of the 2-furanyl cetone), hydroxylation	C ₁₉ H ₂₄ N ₂ O	297.1970	4.16			105	134	146	188					✓ ²	
M08b	HO-Despropionylfentanyl (b)	amide hydrolysis (loss of the 2-furanyl cetone), hydroxylation	C ₁₉ H ₂₄ N ₂ O	297.1970	5.3			121			204				281	✓ ²	
M08c	HO-Despropionylfentanyl (c)	amide hydrolysis (loss of the 2-furanyl cetone), hydroxylation	C ₁₉ H ₂₄ N ₂ O	297.1970	5.68				134	146	204					✓ ²	
M08d	HO-Despropionylfentanyl (d)	amide hydrolysis (loss of the 2-furanyl cetone), hydroxylation	C ₁₉ H ₂₄ N ₂ O	297.1970	6.51	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>	✓ ²	

M08e	HO-Despropionylfentanyl (e)	amide hydrolysis (loss of the 2-furanyl cetone), hydroxylation	C ₁₉ H ₂₄ N ₂ O	297.1970	7.01	/	/	/	/	/	/	/	/	/	/	✓ ²	
M09	Di-HO-Fu-F	di-hydroylation	C ₂₄ H ₂₆ N ₂ O ₄	407.1965	6.57	/	/	/	/	/	/	/	/	/	/		
M10	Glu-HO-Fu-F	hydroxylation, glucuronidation	C ₃₀ H ₃₄ N ₂ O ₉	567.2343	5.11									240	391		