*Journal of Analytical Toxicology*, 2019;00:1–9
doi: 10.1093/jat/bkz081
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# Metabolite Profiling of Ortho-, Meta- and Para-Fluorofentanyl by Hepatocytes and High-Resolution Mass Spectrometry

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## Abstract

New psychoactive substances are emerging on the illegal drug market. Synthetic opioids including fentanyl analogues are of special concern due to their high potency. This indicates the possibility of low drug concentrations in vivo and calls for sensitive analytical methods and identification of the most appropriate analytical targets. In this study the in vitro metabolism of ortho-, meta- and para-fluorofentanyl, three fluorinated derivatives of fentanyl, has been investigated using human hepatocytes and compared to the results from an authentic human urine sample. Based on knowledge on the metabolism of similar fentanyl analogues *N*-dealkylation and hydroxylation was hypothesized to be the most central pathways. The three fluorofentanyl isomers were incubated with pooled human hepatocytes at 1, 3 and 5 h. Liquid chromatography quadrupole time of flight mass spectrometry operating in data-dependent mode was used to analyse the hepatocyte samples, as well as the hydrolysed and non-hydrolysed authentic urine sample. Data were analysed by a targeted approach with a database of potential metabolites. The major metabolite formed in vitro was the *N*-dealkylation product norfluorofentanyl. In addition various hydroxylated metabolites, a *N*-oxide, dihydrodiol metabolites and a hydroxymethoxy metabolite were found. In total, 14 different metabolites were identified for each fluorofentanyl isomer. In the authentic urine sample, three metabolites were detected in addition to the ortho-fluorofentanyl parent compound, with hydroxymethoxy metabolite having the highest abundance followed by norfluorofentanyl and a metabolite hydroxylated on the ethylphenyl ring. This in vitro study showed that the metabolic pattern for ortho-, meta-, and para-fluorofentanyl was close to those previously reported for other fentanyl analogues. We suggest that the hydroxymethoxy metabolite and the metabolite hydroxylated on the ethylphenyl ring should be the metabolites primarily investigated in further studies to determine the most appropriate marker for intake of fluorofentanyl derivatives in urine drug screening for human subjects.

**Key words:** Fluorofentanyl, metabolism, human hepatocytes, high-resolution mass spectrometry

## Introduction

Numerous structural analogs of the opioid fentanyl have entered the illegal drug market in recent decades. These synthetic opioids were originally designed with the purpose of becoming medications used in humans or animals. Some, e.g., remifentanil and alfentanil, are medications approved for human use, while others solely appear as illicit drugs after being produced at clandestine laboratories. New analogs created from existing compounds by substitution with halogens or other functional groups are also adding to the increasing number of potential drugs of abuse. The positional isomers ortho-, meta- and para-fluorofentanyl (o-, m- and p-fluorofentanyl), also named 2-, 3- and 4-fluorofentanyl, respectively, are derivatives of fentanyl with a fluorine atom located at the *N*-phenyl moiety. Para-fluorofentanyl (*N*-(4-fluorophenyl)-*N*-[1-(2-phenylethyl)-4-piperidinyl] propanamide) is classified as a narcotic under the United Nation's Single Convention on Narcotic Drugs (1). The ortho- and meta-derivatives (*N*-(2-fluorophenyl)-*N*-[1-(2-phenylethyl)-4-piperidinyl] propanamide and *N*-(3-fluorophenyl)-*N*-[1-(2-phenylethyl)-4-piperidinyl] propanamide, respectively) were first reported to the EU early warning system of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) in 2016 and are now controlled substances in many European countries as well as in the USA (2, 3).

There have been several reported seizures of these compounds in Europe since 2016, and a case report of a death after intake of ortho-fluorofentanyl has been reported (4). Little research has been performed on the pharmacokinetics and toxicity of these substances. However, in a study on the interaction with cloned human opioid receptors, para-fluorofentanyl was found to be a more potent agonist than fentanyl (5). The high potency indicates the possibility of low drug concentrations *in vivo* and calls for sensitive analytical methods using carefully selected analytical targets to detect a possible intake of these drugs.

In general, metabolites of illicit drugs are formed by complex enzymatic processes. The best way to study these is to use authentic human urine samples. However, due to the limited access to such samples from subjects with a known or suspected drug intake as well as uncertainties related to which drug(s) has been ingested, *in vitro* model systems of drug metabolism have emerged as an important tool. The metabolic pattern of fentanyl and some other fentanyl derivatives has previously been investigated both *in vitro* and in biological samples (6–14) but no studies of metabolism have been performed on ortho-, meta- or para-fluorofentanyl. Based on the knowledge of the metabolic pattern of previously studied fentanyl derivatives, the *N*-dealkylated metabolite and hydroxylated metabolites were expected to be major metabolites.

High resolution mass spectrometry (HR-MS) by liquid chromatography quadrupole time of flight mass spectrometry (LC-QTOF-MS) is a powerful tool for screening for and identification of previous known or unknown compounds like metabolites in metabolite profiling (15). LC-QTOF-MS can be operated in a data-dependent MS/MS mode (Auto-MS/MS), switching between acquiring full spectra MS and MS/MS spectra of ions fulfilling the requirements set in the method. The high-resolution MS data files can be processed in the search for compounds likely to be metabolites of ortho-, meta- and para-fluorofentanyl and the corresponding MS/MS spectra can be used to elucidate their structures.

The primary aim of this study was to investigate the metabolism of ortho-, meta- and para-fluorofentanyl using human hepatocytes and elucidate the structure of the metabolites using HR-MS. Identifying the major metabolites is important as they may serve as analytical targets for urinary drug screening. A secondary aim was to investigate whether the exact position of the fluorine atom had any influence on the degree of formation of the various metabolites.

## Material and Methods

### Chemicals and reagents

Ortho-, meta- and para-fluorofentanyl were purchased from Cayman Chemicals (Ann Arbor, MI, USA). LC-MS grade acetonitrile, formic acid and methanol were purchased from Fisher Scientific (Gothenburg, Sweden). Ammonium formate was obtained from Sigma–Aldrich (Stockholm, Sweden) and 99.5% ethanol from Kemetyl (Haninge, Sweden). Divide; Cryo-preserved human hepatocytes (LiverPool™, 10-donor-pool, Lot nr. RBR) and InVitro Gro HT medium were from BioreclamationIVT (Baltimore, MD, USA). Williams medium E (without L-glutamine and phenol red), L-glutamine 200 mM and Hepes 1 M buffer solution from Gibco® by life technologies™ were purchased from Thermo (Stockholm, Sweden). MilliQ Gradient 10 production unit from Millipore (Billerica, MA, USA) was used to produce high-purity water. β-Glucuronidase/arylsulfatase stock solution (Helix promatia), with activities of 4.5 and 14 U/ml respectively, was purchased from Roche Diagnostics (Mannheim, Germany).

### Preparation of drug solutions and positive control

Stock solutions were diluted in William medium E to form individual drug solutions of 10 µM ortho-, meta- or para-fluorofentanyl with a maximum organic content of 0.2%. A positive control containing a mix of the cytochrome P-450 (CYP) substrates caffeine (CYP1A2), bupropion (CYP2B6), diclofenac (CYP2C9), omeprazole (CYP2C19), dextromethorphane (CYP2D6), chlorzoxazone (CYP2E1) and midazolam (CYP3A4) was diluted in William medium E to a concentration of 10 µM. Internal standards (IS) were diluted in acetonitrile to a final concentration of 300 ng/mL amphetamine-d8 and phenobarbital-d5, 100 ng/mL diazepam-d5 and 50 ng/mL mianserin-d3.

### Incubation with human hepatocytes

The incubation of human hepatocytes with the fluorofentanyl isomers was performed in accordance to the protocol used by Åstrand *et al.* (13). Cryopreserved pooled human hepatocytes were thawed at 37°C and poured into HT medium. After centrifugation (60 g for 5 min at room temperature), the supernatant was removed and the cells were re-suspended in Williams E medium. The cells were centrifuged (60 g for 5 min at room temperature) again, the supernatant was removed and the cells were re-suspended in Williams E medium making a final volume of 2 mL. The concentration of viable cells was evaluated with Trypan blue (0.4% v/v) exclusion dye method. Cells were diluted to 2.0 x $10^6$ cells/mL.

Each fluorofentanyl isomer at a concentration of 5 µM was incubated with $10^5$ cells (100 µL total volume) in 96-well plates in duplicate. The incubations were stopped after 1, 3 and 5 h by adding 100 µL ice cold acetonitrile (including IS) to each well. A negative control (only cells) and a degradation control (only drug) were incubated for 5 h and positive controls were incubated for 0 and 5 h. The controls incubated for 0 h were prepared by adding acetonitrile to the drug immediately before adding the cells. The plates were vortexed and left at −20°C for a minimum of 10 min before centrifugation (1100 g for 15 min at 4°C). Finally 100 µL of the extracts were transferred to an injection plate.

### Preparation of an authentic human urine sample

A urine sample from a patient intoxicated with ortho-fluorofentanyl was used for metabolite profiling and comparison with the *in vitro* results. Clinical information about the case has been presented elsewhere (4). The sample was analyzed with and without enzymatic hydrolysis. For the hydrolyzed sample preparation, 100 µL urine was incubated with 10 µL $\beta$-glucuronidase stock solution at 40°C for 1 h in a water bath and diluted with 300 µL of mobile phase mixture (A/B, 50:50; see later). No pH adjustment was done. The nonhydrolyzed sample was diluted with 310 µL mobile phase mixture. Finally, both samples were filtered using a 13 mm syringe filter with 0.45 µm polytetrafluoroethylene membrane (VWR, Radnor, PA, USA) before injection.

### LC-QTOF analysis

The chromatographic separation of the hepatocyte samples (1 µL injection volume) and the diluted authentic urine sample (5 µL injection volume) was performed by an Agilent 1290 Infinity system equipped with an Acquity HSS T3 column (150 mm x 2.1 mm, 1.8 µm) from Waters (Milford, MA, USA). Separation was achieved using a mobile phase consisting of 0.05% formic acid in 10 mM ammonium formate (A) and 0.05% formic acid in acetonitrile (B). A linear gradient with a flow of 0.50 mL/min starting at 1% B increasing to 40% in 13 min and continuing to 95% in the next 2 min was employed. After a 3.1-min hold at 95% B the column was re-equilibrated for 2.9 min at 1% B, giving a total cycle time of 21 min. Autosampler and column temperatures were set to 7 and 60°C, respectively.

The MS analysis was performed using a 6550 QTOF-MS (Agilent, Santa Clara, CA, USA) with electrospray ionization and iFunnel interface. Positive ionization was used with a fragmentor voltage of 380 V, VCap at 3500 V, gas temperature and flow at 150°C and 18 L/min, respectively, nebulizer pressure at 50 psig and sheath gas temperature at 380°C. Data were acquired in data-dependent Auto MS/MS mode. MS spectra and MS/MS spectra were acquired in the mass range of 100–950 m/z at a rate of 5 Hz and 50–950 m/z at 10 Hz, respectively. The detector was operated in 2 GHz extended dynamic range giving a resolution (m/$\Delta$m at FWHM) of approx. 20,000 at m/z 322.0481. Precursor selection was based on abundance, and an intensity threshold of 5000 counts was applied. After one spectrum from a precursor was acquired, this specific precursor was excluded in 0.03 min. Lock masses 121.0509 and 922.0098 were applied for automated mass correction in all spectra.

The data files were processed by the Agilent MassHunter Qualitative Analysis (B.07.00), using the algorithm Find by Formula. This is a targeted approach with a database with formulas of possible metabolites including mono-, di- and trihydroxylations, carbonylation, dihydrodiol formation, methylation, carboxylation, defluorination, *N*-dealkylations (including loss of the fluorophenyl group), amide hydrolysis, glucuronidation and combinations of these. Only compounds with a mass error within 15 ppm and a peak area above 10,000 were regarded as potential metabolites. In addition, the fragment ions in the MS/MS spectrum associated with the precursor ion of the compound were evaluated. Potential metabolites detected in the negative control, 0 h samples or in the degradation controls were excluded.

## Results and Discussion

### Fragmentation pattern of the parent compounds

For each fluorofentanyl analog, 14 potential metabolites were detected in the *in vitro* experiment (Table I). The elucidation of MS/MS spectra from metabolites was based on the fragmentation of the parent compound. The MS/MS spectra of the parent compounds ortho-, meta- and para-fluorofentanyl ($C_{22}H_{27}FN_2O$) with a precursor molecule ion of m/z 355.2180 were identical, containing the fragment ions of m/z 84.0808, 105.0702, 134.0965, 150.0710, 188.1438, 234.1291 and 299.1912 (Figure 1 shows the MS/MS spectrum of the isomer ortho-fluorofentanyl). The fragment ion 84.0808 corresponds to an unchanged piperidine ring and the base peak 105.0702 results from the cleavage between the piperidine ring and the phenetyl moiety. The fragment ion 134.0965 corresponds to the phenethyl moiety and parts of the piperidine ring. The fragment ion 150.0710 corresponds to degradation of the piperidine ring. The fragment ion 188.1438 corresponds to the complete phenethylpiperidine structure. The fragment ion 234.1291 corresponds to cleavage of the piperidine ring. Lastly, a fragment ion 299.1912, corresponding to the elimination of the amide alkyl chain, was detected (Figure 1).

### Identification of the metabolites

Ortho- meta- and para-fluorofentanyl produced metabolites by the same principal metabolic pathways, but with certain differences in retention times (RTs) and absolute chromatographic peak areas (Table I). For simplicity, the metabolites are presented in the text as they originate from one of the parents. The compounds eluted from 4.75 to 12.81 min, and all had a mass error of less than 4.52 ppm. Table I lists all metabolites and parent compounds with their RTs, specific biotransformation, formulas, masses of protonated molecular ions, mass errors, peak areas and diagnostic ions. When the molecular position for the specific biotransformation step could be proposed, the metabolite was given a name in correspondence with a letter- and number-based system given in Figure 1. The metabolites were named using O (ortho), M (meta) or P (para) and numbered 1–14 corresponding to RT order. The MS/MS spectra and proposed fragmentation of the metabolites are shown in the supplementary information (Figure S1). The chromatographic separation of the metabolites of ortho-fluorofentanyl formed *in vitro* is given in Figure 2A. Major metabolites were formed by *N*-dealkylation, hydroxylations at the alkyl chain, ethylphenyl ring and/or piperidine ring and methylation. Detector saturation for the most abundant metabolite (O2/M2/P2) made estimation of relative abundance of the major metabolites impossible.

*N*-dealkylation at the piperidine ring resulting in the loss of the phenethyl moiety and forming norfluorofentanyl (O2/M2/P2) was observed to be the main metabolite *in vitro*. The MS/MS spectra showed one abundant fragment ion at m/z 84.0815, indicating that the piperidine ring was intact in these metabolites. The enzyme CYP3A4 has previously been shown to be responsible for the *N*-dealkylation of fentanyl (16). The metabolite was detected *in vitro* already in the 0 h samples (Table I), but not in the degradation control. This indicates that norfluorofentanyl is rapidly formed. A compound (O1/M1/P1) corresponding to hydroxylation (addition of 15.9949 u, i.e., +O) of the *N*-dealkylated metabolite was also detected. The fragment ion m/z 84.0805 was also dominating these spectra suggesting hydroxylation at the amide alkyl chain or *N*-phenyl ring.

Four different hydroxylated metabolites (addition of 15.9949 u, i.e., +O, when compared to the mass of the parent) with the protonated molecular ion [$C_{22}H_{27}FN_2O$ +H] and m/z 371.2131 were detected eluting from 9.31 to 12.81 min. The most abundant of these metabolites was O12/M12/P12. Unfortunately, an exact structure cannot be elucidated for this metabolite with MS/MS data only.

**Table I.** Ortho-, Meta- and Para-Fluorofentanyl with Proposed Biotransformation, Retention Time (RT), Formula, Accurate Mass of Protonated Compound (m/z), Mass Error of Protonated Compound, Peak Areas in Hepatocyte Samples and in Authentic Urine Sample and Diagnostic Ions

| ID | Biotransformation | RT[1] (min) | Formula | m/z[1] | Mass error[1] (ppm) | Peak area in hepatocyte samples at various time points (two parallels at each time point, marked #1 and #2, respectively) | | | | | | | | Peak area in urine sample | | Diagnostic ions (m/z) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 0 h #1 | 0 h #2 | 1 h #1 | 1 h #2 | 3 h #1 | 3 h #2 | 5 h #1 | 5 h #2 | Hyd[2] | Nonhyd[3] | |
| O1 | *N*-Dealkylation + | 4.76 | $C_{14}H_{19}FN_2O_2$ | 267.1489 | −4.52 | ND | ND | 9.2E+04 | 1.0E+05 | 1.5E+05 | 1.5E+05 | 1.4E+05 | 1.6E+05 | ND | ND | 84.0815 |
| P1 | monohydroxylation at the | 4.86 | | 267.1500 | −1.84 | ND | ND | 2.8E+04 | 2.8E+04 | 4.3E+04 | 4.2E+04 | 4.7E+04 | 5.0E+04 | | | |
| M1 | amide group | 4.75 | | 267.1502 | −1.43 | ND | ND | 8.6E+04 | 7.8E+04 | 1.1E+05 | 1.1E+05 | 1.5E+05 | 1.4E+05 | | | |
| O2 | *N*-Dealkylation | 7.55 | $C_{14}H_{19}FN_2O$ | 251.1559 | 0.96 | 3.0E+05 | 3.0E+05 | Sat | Sat | Sat | Sat | Sat | Sat | 4.4E+04 | 5.5E+04 | 84.0805 |
| P2 | | 7.62 | | 251.1556 | −0.27 | 2.3E+05 | 2.4E+05 | Sat | Sat | Sat | Sat | Sat | Sat | | | |
| M2 | | 7.57 | | 251.1555 | −0.50 | 2.1E+05 | 2.4E+05 | Sat | Sat | Sat | Sat | Sat | Sat | | | |
| O3 | Dihydrodiol: At the *N*-alkyl | 7.98 | $C_{22}H_{29}FN_2O_3$ | 389.2230 | −1.41 | ND | ND | 3.1E+04 | 3.1E+04 | 1.0E+05 | 9.3E+04 | 9.9E+04 | 1.1E+05 | ND | ND | 164.0864, |
| P3 | chain | 8.02 | | 389.2237 | 0.14 | ND | ND | 6.9E+04 | 7.3E+04 | 2.4E+05 | 2.2E+05 | 3.0E+05 | 2.9E+05 | | | 207.1297 |
| M3 | | 7.98 | | 389.2230 | −1.40 | ND | ND | 3.6E+04 | 3.1E+04 | 9.0E+04 | 8.3E+04 | 1.0E+05 | 9.1E+04 | | | |
| O4 | Dihydroxylation: at the | 8.22 | $C_{22}H_{27}FN_2O_3$ | 387.2071 | −1.99 | ND | ND | ND | ND | ND | ND | 4.5E+04 | 4.4E+04 | ND | ND | NDI |
| P4 | *N*-alkyl chain, piperidine ring | 8.35 | | 387.2069 | −2.68 | ND | ND | ND | ND | ND | ND | 2.4E+04 | 2.5E+04 | | | |
| M4 | and/or the amide chain | 8.29 | | 387.2072 | −1.51 | ND | ND | ND | ND | ND | ND | 5.1E+04 | 4.7E+04 | | | |
| O5 | Dihydrodiol: at the *N*-alkyl | 8.39 | $C_{22}H_{29}FN_2O_3$ | 389.2250 | −2.74 | ND | ND | ND | ND | ND | ND | 1.6E+04 | 1.8E+04 | ND | ND | 164.0875 |
| P5 | chain | 8.44 | | 389.2226 | −2.17 | ND | ND | ND | ND | ND | ND | 4.6E+04 | 3.7E+04 | | | |
| M5 | | 8.41 | | 389.2224 | −3.13 | ND | ND | ND | ND | ND | ND | 1.9E+04 | 1.5E+04 | | | |
| O6 | Dihydroxylation: at the | 9.10 | $C_{22}H_{27}FN_2O_3$ | 387.2071 | −2.25 | ND | ND | ND | ND | ND | ND | 1.8E+04 | 1.9E+04 | ND | ND | NDI |
| P6 | *N*-alkyl chain, piperidine ring | 9.10 | | 387.2069 | −1.81 | ND | ND | ND | ND | ND | ND | 7.6E+03 | 8.3E+03 | | | |
| M6 | and/or the amide chain | 9.09 | | 387.2068 | −1.94 | ND | ND | ND | ND | ND | ND | 7.5E+03 | 7.2E+03 | | | |
| O7 | Monohydroxylation: at the | 9.31 | $C_{22}H_{27}FN_2O_2$ | 371.2127 | −0.60 | ND | ND | 1.9E+06 | 1.9E+06 | 2.3E+06 | 2.1E+06 | 1.9E+06 | 2.1E+06 | ND | ND | 84.0803, 105.0690, |
| P7 | amide alkyl chain | 9.40 | | 371.2129 | −0.41 | ND | ND | 5.5E+05 | 4.5E+05 | 6.6E+05 | 6.4E+05 | 6.6E+05 | 6.6E+05 | | | 134.0960, 150.0697, |
| M7 | | 9.35 | | 371.2128 | −0.59 | 9.3E+04 | 9.3E+04 | 1.7E+06 | 1.3E+06 | 1.4E+06 | 1.4E+06 | 1.7E+06 | 1.5E+06 | | | 188.1434, 299.1908 |
| O8 | Monohydroxylation: at | 9.81 | $C_{22}H_{27}FN_2O_2$ | 371.2131 | 0.0 | ND | ND | 6.2E+05 | 6.7E+05 | 7.3E+05 | 6.5E+05 | 4.9E+05 | 6.4E+05 | 1.2E+04 | 4.1E+03 | 84.0808, 121.0646, |
| P8 | phenethyl moiety | 9.78 | | 371.2128 | −0.22 | ND | ND | 9.8E+05 | 8.3E+05 | 1.2E+06 | 1.2E+06 | 1.3E+06 | 1.2E+06 | | | 150.0688, 204.1376 |
| M8 | | 9.77 | | 371.2131 | −0.07 | ND | ND | 7.1E+05 | 5.0E+05 | 5.4E+05 | 5.4E+04 | 5.4E+05 | 4.8E+05 | | | |
| O9 | Methylation + | 10.11 | $C_{23}H_{29}FN_2O_3$ | 401.2257 | 4.47 | ND | ND | 5.7E+04 | 5.9E+04 | 6.6E+04 | 6.0E+04 | ND | 5.6E+04 | 6.0E+04 | 6.0E+04 | 119.0487, 151.0749, |
| P9 | dihydroxylation: at the | 10.08 | | 401.2242 | 1.28 | ND | ND | 1.4E+05 | 1.2E+05 | 1.6E+05 | 1.6E+05 | 1.2E+05 | 1.3E+05 | | | 164.0867, 234.1503 |
| M9 | phenethyl moiety | 10.08 | | 401.2227 | −0.61 | ND | ND | 4.8E+04 | 3.3E+04 | ND | 3.4E+04 | 3.5E+04 | ND | | | |
| O10 | Dihydroxylation: At the phenethyl moiety and | 10.17 | $C_{22}H_{27}FN_2O_3$ | 387.2080 | 0.07 | ND | ND | ND | ND | ND | ND | 5.6E+04 | 5.5E+04 | ND | ND | 121.0643, 164,0863 |
| P10 | 2-position of piperidine ring | 10.21 | | 387.2071 | −2.51 | ND | ND | ND | ND | ND | ND | 1.1E+04 | 1.2E+04 | | | |
| M10 | and/or amide group | 10.20 | | 387.2066 | −2.94 | ND | ND | ND | ND | ND | ND | 1.1E+04 | 1.1E+04 | | | |

(Continued)

**Table I.** Continued

| ID | Biotransformation | RT[1] (min) | Formula | m/z[1] | Mass error[1] (ppm) | Peak area in hepatocyte samples at various time points (two parallels at each time point, marked #1 and #2, respectively) | | | | | | | | Peak area in urine sample | | Diagnostic ions (m/z) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 0 h #1 | 0 h #2 | 1 h #1 | 1 h #2 | 3 h #1 | 3 h #2 | 5 h #1 | 5 h #2 | Hyd[2] | Nonhyd[3] | |
| O11 | Dihydroxylation at piperidine ring and amide group or | 10.28 | $C_{22}H_{27}FN_2O_3$ | 387.2077 | −2.25 | ND | ND | ND | ND | ND | ND | 4.7E+04 | 6.5E+04 | ND | ND | 105.0690, 164.0871, 186.1276 |
| P11 | *N*-oxide + hydroxylation at | 10.64 | | 387.2077 | −0.91 | ND | ND | ND | ND | ND | ND | 6.8E+04 | 6.8E+04 | | | |
| M11 | the amide group | 10.52 | | 387.2070 | −2.08 | ND | ND | ND | ND | ND | ND | 6.6E+04 | 6.2E+04 | | | |
| O12 | Monohydroxylation: at | 10.54 | $C_{22}H_{27}FN_2O_2$ | 371.2128 | −0.79 | ND | ND | 3.8E+06 | 3.8E+06 | 4.2E+06 | 3.7E+06 | 3.2E+06 | 3.6E+06 | ND | ND | 105.0695, 150.0710, |
| P12 | phenethyl linker or piperidine | 10.53 | | 371.2129 | −0.12 | ND | ND | 4.3E+06 | 3.4E+06 | 4.6E+06 | 4.5E+06 | 4.4E+06 | 4.5E+06 | | | 160.0864, 186.1277, |
| M12 | ring | 10.55 | | 371.2130 | −0.28 | ND | ND | 5.6E+06 | 3.8E+06 | 4.2E+06 | 4.4E+06 | 5.1E+06 | 4.4E+06 | | | 204.1380, 353.2020 |
| O | Parent compound (fluorofentanyl) | 11.56 | $C_{22}H_{27}FN_2O$ | 355.2180 | 0.03 | Sat | Sat | Sat | Sat | Sat | Sat | Sat | Sat | 4.3E+04 | 1.2E+04 | 84.0808, 105.0702, 134.0965, 150.0710, |
| P | | 11.51 | | 355.2178 | −0.57 | Sat | Sat | Sat | Sat | Sat | Sat | Sat | Sat | | | 188.1438, 234.1291, |
| M | | 11.55 | | 355.2179 | −0.25 | Sat | Sat | Sat | Sat | Sat | Sat | Sat | Sat | | | 299.1912 |
| O13 | Dihydroxylation at phenethyl moiety and amide group or | 11.84 | $C_{22}H_{27}FN_2O_3$ | 387.2080 | −0.23 | ND | ND | 1.6E+05 | 1.7E+05 | 2.9E+05 | 2.7E+05 | 2.5E+05 | 2.9E+05 | ND | ND | 164.0867, 207.1279 |
| P13 | *N*-oxide + hydroxylation at | 12.02 | | 387.2068 | −3.18 | ND | ND | 1.7E+04 | 1.8E+04 | 2.5E+04 | 2.3E+04 | 2.1E+04 | 2.5E+04 | | | |
| M13 | the amide group | 11.99 | | 387.2073 | −1.84 | ND | ND | 3.0E+04 | 2.5E+04 | 3.8E+04 | 3.5E+04 | 4.0E+04 | 3.6E+04 | | | |
| O14 | *N*-oxide: at the piperidine | 12.66 | $C_{22}H_{27}FN_2O_2$ | 371.2129 | 0.00 | ND | ND | 9.9E+05 | 1.0E+06 | 1.6E+06 | 1.5E+06 | 1.4E+06 | 1.6E+06 | ND | ND | 105.0714, 150.0700, |
| P14 | ring | 12.81 | | 371.2130 | 0.01 | ND | ND | 3.5E+05 | 3.3E+05 | 5.3E+05 | 5.2E+05 | 5.8E+05 | 5.8E+05 | | | 164.0868 |
| M14 | | 12.79 | | 371.2129 | −0.79 | ND | ND | ND | 3.4E+05 | 5.2E+05 | 5.2E+05 | 6.3E+05 | 5.8E+05 | | | |

[1]Measured at 5 h and sample #2 (except for metabolite 2 measured at 1 h).

[2]Hydrolyzed.

[3]Not hydrolyzed.

Abbreviations: ID = identification (O = ortho, M = meta, P = para; the number that follows represents a consecutive numbering of the metabolites according to their RTs); ND = not detected; NDI = no diagnostic ions found; Sat = detector saturated.

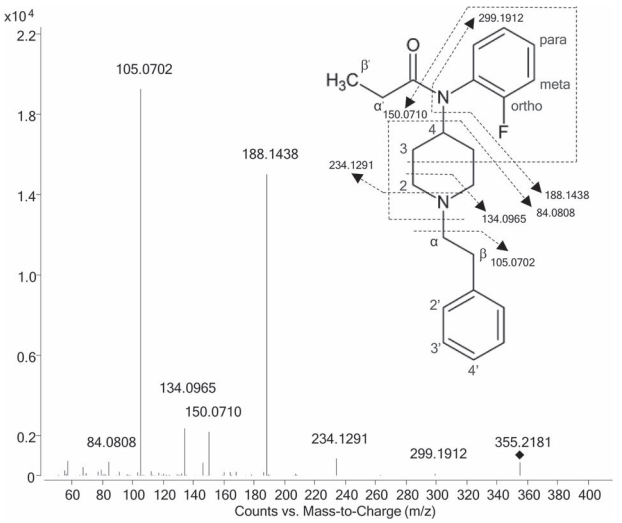


**Figure 1.** MS/MS spectrum of ortho-fluorofentanyl, suggested explanation of fragmentation and a positional system for indicating the position of substituents.

The addition of an oxygen to fragment ion m/z 188.1438 forming m/z 204.1380 (with water loss to m/z 186.1277) and the fragment ions m/z 150.0710 and 164.0864 indicate hydroxylation in the 2-position. The fragment ion m/z 353.2020 can correspond to an elimination of $H_2O$ from the metabolite which favors an interpretation towards aliphatic hydroxylation, and the fragment ion m/z 105.0695 may have been formed from a $\beta$-hydroxy-metabolite. Therefore we propose that O12/M12/P12 is a monohydroxy metabolite with the hydroxy group at either the piperidine ring or at the ethyl linker.

The second most abundant monohydroxylated metabolite was O7/M7/P7. Fragment ion m/z 188.1434 corresponds to an intact phenethylpiperidine structure and together with m/z 299.1908 (Figure 1) it indicates hydroxylation on the amide alkyl chain. Traces of this metabolite were detected in the 0 h sample of meta-fluorofentanyl. The Monohydroxylated metabolite O8/M8/P8 was hydroxylated at the phenethyl moiety as indicated by the fragment ions m/z 121.0646 (mass of phenethyl moiety +O). Hydroxylation at the phenyl ring (position 2′, 3′ or 4′) or a $\beta$-hydroxy at the *N*-alkyl chain is most probable as α-hydroxy-metabolites are not known to exist (intermediate to the *N*-dealkylation pathway). The last monohydroxylated metabolite O14/M14/P14 elutes after the parent drug (Table I) which is unexpected for a more polar compound. These late eluting metabolites have been described previously for *N*-oxide metabolites of fentanyl analogs (7, 10) and in an *in vitro* study of the metabolism of nicotine (17). Based on its RT and mass we propose O14/M14/P14 to be an *N*-oxide (Figure 3). Also, fragment ions m/z 105.0700 and m/z 164.0868 indicate an unchanged phenethyl moiety and *N*-phenyl ring, which is consistent with an *N*-oxide, as shown with similar compounds in previous studies (7, 13).

Five di-hydroxylated metabolites (addition of 2x15.9949 u, i.e., $+O_2$, when compared to the mass of the parent) with the protonated molecular ion [$C_{22}H_{27}FN_2O_3$ +H] and m/z 387.2078 were detected per parent *in vitro*. O13/M13/P13 was already detected in the 1 h samples while the other four were only present in the 5 h samples. No di-hydroxylated metabolites were detected in the authentic urine sample. The most abundant O13/M13/P13 with fragment ion at m/z 164.0867 and lack of m/z 188.1434 indicates an unchanged *N*-phenyl ring and that hydroxylation has occurred at the amide alkyl chain, phenethyl moiety or at the 2-position of the piperidine ring. Fragment ion m/z 207.1279 can be formed by loss of the amide alkyl chain and the phenethyl moiety (with one carbon left; $C_{12}H_{16}FN_2$), see supplementary information (Figure S1). This opens up for a second interpretation that includes a monohydroxylated *N*-oxide or a water loss from an *N*-oxide with dihydrodiol. O11/M11/P11 shows a fragment of m/z 186.1276, which indicates that the first hydroxylation is at position 2 at the piperidine ring (after water loss similar to O12/M12/P12) and the fragments with m/z 105.0700 and 164.0868 suggesting an intact *N*-phenyl ring and phenethyl moiety and therefore the second hydroxylation at the amide alkyl chain. The presence of a fragment ion at m/z 207.1247 means that the first oxidation can be an *N*-oxide (as shown for O13/M13/P13). The third di-hydroxylated metabolite O10/M10/P10 was detected and the fragment ion m/z 121.0643 indicates hydroxylation on the phenethyl moiety. The location of the second hydroxyl group could not be determined by the MS/MS spectrum but 164.0863 indicates position 2 at the piperidine ring. The MS/MS spectrum of O4/M4/P4 and O6/M6/P6 did not contain any diagnostic ions which could help interpret the position of the hydroxyl groups.

Two compounds corresponding to dihydrodiol metabolites were detected, O3/M3/P3 and O5/M5/P5. Theoretically a dihydrodiol can be located on the ethylphenyl ring or the *N*-phenyl ring. The common fragment ion of m/z 164.0864/164.0875 suggests that the *N*-phenyl ring is unchanged in both compounds and that the ethylphenyl ring is the target of the biotransformation. Watanabe *et al.* suggested in a study of the metabolism of 4-fluoro-isobutyrylfentanyl that a water loss from one of the dihydrodiol hydroxyl groups leads to re-aromatization and that this is a favorable reaction that explains the absence of the expected fragment ion m/z 139.0754 but the presence of fragment ions m/z 121.0640/121.0633 (7). The exact position of the dihydrodiol hydroxyl groups on the ethylphenyl ring could not be determined from the fragmentation pattern. Finally a compound corresponding to a hydroxylated and methoxylated metabolite with the protonated molecular ion [$C_{23}H_{29}FN_2O_3$ +H] was detected, O9/M9/P9. The fragment ion at m/z 151.0749 suggests the presence of hydroxyl and a methoxy group at the phenethyl moiety (4′-hydroxy-3′-methoxy-fluorofentanyl). The presence of m/z 119.0487 is caused by the loss of methanol.

## Metabolites detected in an authentic urine sample

Three of the metabolites found *in vitro* were also detected in the authentic ortho-fluorofentanyl urine sample. These were norfluorofentanyl (O2), 2′, 3′ or 4′-hydroxy-fluorofentanyl (O8) and either 4′-hydroxy-3′-methoxy-fluorofentanyl or 3′-hydroxy-4′-methoxy-fluorofentanyl (O9). The chromatographic separation of the metabolites of ortho-fluorofentanyl in the authentic urine sample is shown in Figure 2B. To enable comparisons between MS/MS spectra of metabolites detected in both urine and *in vitro* samples these spectra are shown as supplementary information (Figure S2). In urine, O9 had the highest abundance, in contrast to the *in vitro* experiment where this metabolite was of low abundance compared to the other metabolites. Norfluorofentanyl (O2) and 2′, 3′ or 4′-hydroxy-fluorofentanyl (O8) was the second and third most abundant metabolite in the authentic urine sample.

## Differences between metabolites of ortho-, meta- and para-fluorofentanyl

When comparing the RTs and chromatographic peak areas of the corresponding metabolites from the three fluorofentanyl isomers,

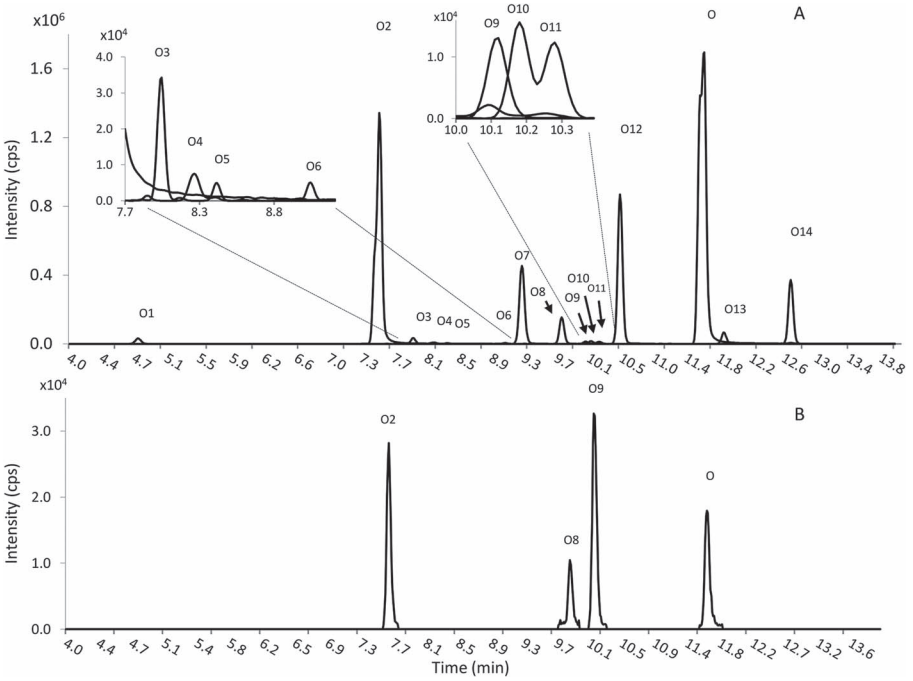


**Figure 2.** Extracted ion chromatogram (EIC) of ortho-fluorofentanyl and metabolites from 5 h incubation with human hepatocytes (A) and the hydrolyzed authentic urine sample (B). Letter and numbers on top of the peaks correspond to those used in Table I; O represents ortho-fluorofentanyl and O1-O14 represents its metabolites. Magnified EICs of the minor metabolites O3, O4, O5, O6, O9, O10 and O11 are also displayed.

there were some notable differences. The three parent compounds eluted within 0.06 min from each other with para-fluorofentanyl eluting first (Table I). Such a small difference could be a result of instrumental instability, but the two IS (amphetamine-d8 and midazolam-d3) showed excellent RT stability (within 0.01 min) between the samples, indicating that the difference between the parent compounds is real. The retention order varied from metabolite to metabolite (Table I), and the RT difference varied from 0.02 min (O12/M12/P12) to 0.37 min (O11/M11/P11). The position of the fluorine atom obviously affects the RT more for some metabolites than for other. There were also differences in peak areas between isomers, but without any clear pattern. Metabolites from all three parent compounds were formed by the same principle pathways even though the difference in peak areas for some of the metabolites was considerable; e.g., for O13/M13/P13 it was 10-fold. As relative peak areas between metabolites probably vary between individuals, they can most likely not be used as an identification parameter.

## Metabolic pathways

The general metabolic pattern for the three fluorofentanyl isomers could be suggested as shown for ortho-fluorofentanyl in Figure 3. Metabolites were formed through *N*-dealkylation and/or oxidations. In addition, a pathway of hydroxylation and methylation via dihydrodiol forming O9/M9/P9 could be proposed. The same pathway was shown by. Watanabe *et al*., which suggested that the dihydrodiol compound can be metabolized by the enzyme cathechol-O-methyltransferase to a metabolite containing a hydroxyl group and methylation of a second hydroxyl group giving O9/M9/P9, possibly through an intermediate catechol compound (7). However, such a dihydroxylated precursor of O9/M9/P9 could not be detected among the metabolites. The main *in vitro* metabolites norfluorofentanyl (O2/P2/M2) and hydroxyl fluorofentanyl (O7/P7/M7, O8/P8/M8, O12/P12/M12 and O14/P14/M14) were consistent with findings in previous studies on fentanyl and some other analogs (6–8, 10). Glucuronidated metabolites were not observed. This is consistent with previous studies performing *in vitro* experiments of fentanyl analogs with hepatocytes. Watanabe *et al*. detected only one glucuronidated metabolite from the fentanyl analogs acetylfentanyl, acrylfentanyl, furanylfentanyl and 4-fluoro-isobutyrylfentanyl. Carboxylated metabolites have been detected in previous studies of fentanyl analogs, e.g., 2,2,3,3-tetramethyl-cyclopropylfentanyl (13) and crotonylfentanyl (14). No metabolites of this type were detected for fluorofentanyl. The amide hydrolysis product, fluoro-4-anilino-*N*-phenylpiperidine, was detected in the degradation control, 0 h sample and with a declining peak area throughout the experiment. This finding indicates that the compound is not formed *in vitro* which is in contrast to other studies of similar fentanyl analogs where amide hydrolysis is a significant metabolic pathway (7–9). The relative low number of metabolites detected in the authentic urine sample compared to the *in vitro* study can at least partly be attributed to the low drug concentration in the urine sample and the simple dilute-and-shoot sample preparation.

Norfluorofentanyl (O2/P2/M2) may be a suitable marker of drug intake as it was the most abundant metabolite found *in vitro* and was also detected in the authentic urine sample. However, norfluorofentanyl might not be specific enough to be used as a single analytical target. Future fentanyl analogs could potentially produce this metabolite as well, and a more specific marker will be necessary to unambiguously identify drug exposure. O8/P8/M8 includes the whole structure of its parent drug and might therefore be a better candidate. Unfortunately, even though abundant *in vitro*, only traces were detected in the authentic urine sample. O9/P9/M9 is another specific marker and according to the results of the authentic urine sample likely to be in relative high abundance.

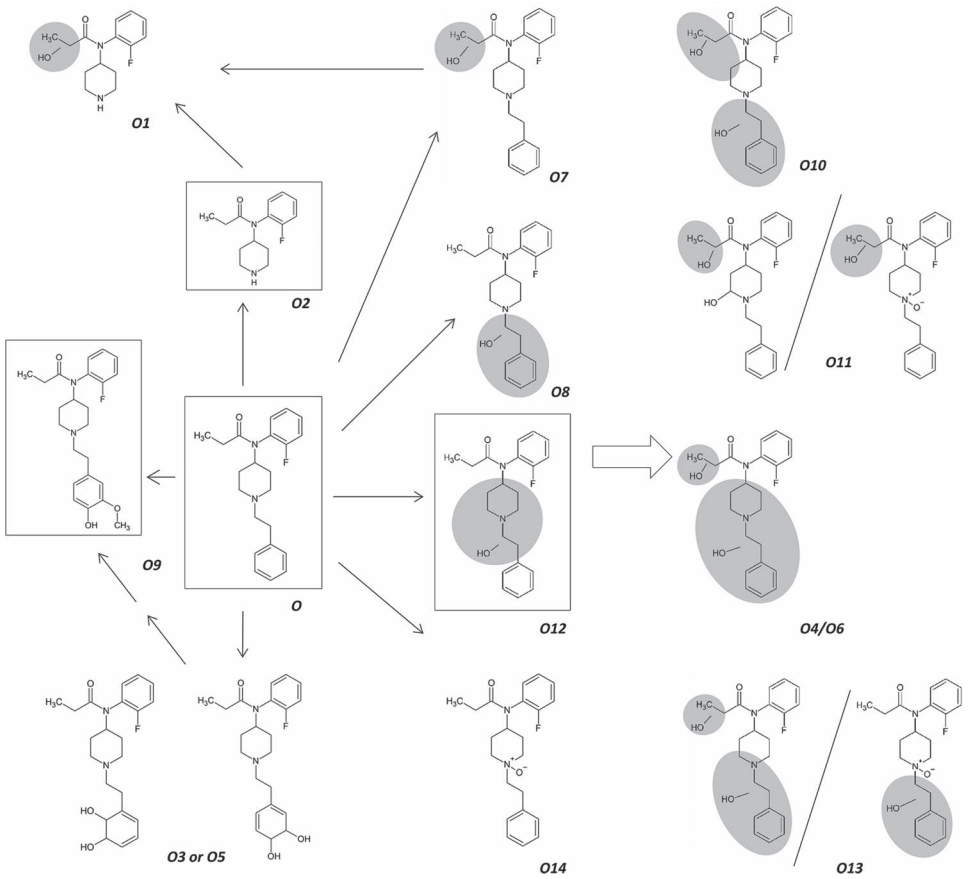


**Figure 3.** Metabolic pathway of ortho-fluorofentanyl (O). Compounds in frames were detected both in the *in vitro* study and in the authentic urine sample. The others were detected in the *in vitro* study, only. Highlighted parts of the molecules indicate possible positions of hydroxylation. The numbering of the metabolites corresponds to those used in Table I.

The elimination half-lives of ortho- meta- and para-fluorofentanyl are not studied, but it is reasonable to believe that they would be similar to fentanyl itself, and thus to be somewhere between 3 and 12 hours (18). Detection times in urine might be extended by using the O9/P9/M9 and/or O8/P8/M8 as markers, but this must be confirmed by analyses of several positive samples.

### Strengths and weaknesses

Human hepatocytes were chosen over human liver microsomes in this *in vitro* model, as they are living cells and contain all endogenous enzymes, cofactors, drug transporters and drug-binding proteins to mimic human drug metabolism. However, due to extrahepatic drug metabolism and transport as well as inter-individual differences, the in vitro results may differ from those obtained *in vivo*. Even though only one authentic urine sample was available, the results indicate that there are differences both in the range of metabolites and the number detected. However, just having one authentic urine sample available is clearly a weakness of this study and a definite recommendation on the most appropriate marker to choose when analyzing human urine cannot be given.

Compounds corresponding to the hydroxylated metabolites of diclofenac, omeprazole and midazolam were detected in the positive control samples incubated for 5 h, showing that the cells were functional. No glucuronidated metabolites were detected in the *in vitro* study or in the authentic sample, which may be due to a very limited formation of these and/or lack of detection capacity with the analytical instrument used. The similar peak areas observed for the same metabolite in the hydrolyzed and nonhydrolyzed authentic urine sample indicate that glucuronidation is not taking place, but again, caution should be exercised in the interpretations as only one sample was available.

The use of LC-QTOF-MS or other comparable HR-MS instrumentation is a well suited approach for acquiring identification data from *in vitro* experiments, as accurate masses and MS/MS spectra can be used to tentatively elucidate the structures. However, complete determination of the structures is not possible without synthesizing and characterizing (by e.g., nuclear magnetic resonance spectroscopy and chromatography) a range of possible candidates and comparing these with those detected *in vitro*.

## Conclusions

Fourteen metabolites for each of the three parent compounds ortho- meta- and parafluorofentanyl were formed after incubation with hepatocytes and detected by LC-QTOF-MS analysis. There were no principal differences in which metabolites were formed by the three positional isomers. The detected metabolites were in accordance with the expectations based on *in vitro* data from other similar fentanyl analogs and included norfluorofentanyl, an *N*-oxide at the piperidine ring, hydroxylated and methylated metabolites. The most abundant metabolite *in vitro* was norfluorofentanyl which was also detected in the authentic urine sample together with a monohydroxylated

metabolite and a both hydroxylated and methylated metabolite. The current approach, using hepatocytes and HR-MS, is a relatively cost-effective and straight-forward tool to generate information on the metabolism and identify potential targets for metabolite of illicit drug detection in urine samples, thereby being able to determine both the specific substances ingested and increasing the time window for detection after intake.

## Funding

This work was supported by the Vinnova (the Psychomics project, Eurostar Project ID 10628) and Strategic Research Area in Forensic Sciences (Strategiområdet forensiska vetenskaper, grant number 2016:7) at Linköping University.

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