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In vitro and *in vivo* metabolite identification studies for the new synthetic opioids acetylfentanyl, acrylfentanyl, furanylfentanyl and 4-fluoro-isobutyrylfentanyl

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Running head: Metabolism of four new fentanyl analogs

Keywords: Human hepatocytes, Authentic human urine samples, LC-HRMS, Fentanyl analogs, Metabolite identification

Abstract

New fentanyl analogs have recently emerged as new psychoactive substances and have caused numerous fatalities worldwide. To determine if the new analogs follow the same metabolic pathways elucidated for fentanyl and known fentanyl analogs, we performed *in vitro* and *in vivo* metabolite identification studies for acetylfentanyl, acrylfentanyl, 4-fluoroisobutyrylfentanyl and furanylfentanyl. All compounds were incubated at 10 μ M with pooled human hepatocytes for up to 5 h. For each compound, four or five authentic human urine samples from autopsy cases with and without enzymatic hydrolysis were analyzed. Data acquisition was performed in data-dependent acquisition mode during liquid-chromatography high-resolution mass spectrometry analyses. Data was analyzed 1) manually based on predicted biotransformations and 2) with MetaSense software using data-driven search algorithms. Acetylfentanyl, acrylfentanyl and 4-fluoroisobutyrylfentanyl were predominantly metabolized by *N*-dealkylation, cleaving off the phenethyl moiety, monohydroxylation at the ethyl linker and piperidine ring as well as hydroxylation/methoxylation at the phenyl ring. In contrast, furanylfentanyl's major metabolites were generated by amide hydrolysis and dihydrodiol formation while the nor-metabolite was minor or not detected in case samples at all. In general, *in vitro* results matched the *in vivo* findings well, showing identical biotransformations in each system. Phase II conjugation was observed, particularly for acetylfentanyl. Based on our results, we suggest the following specific and abundant metabolites as analytical targets in urine: a hydroxymethoxy and monohydroxylated metabolite for acetylfentanyl; a monohydroxy and dihydroxy metabolite for acrylfentanyl; two monohydroxy metabolites and a hydroxymethoxy metabolite for 4-fluoro-isobutyrylfentanyl and a dihydrodiol metabolite and the amide hydrolysis metabolite for furanylfentanyl.

Keywords: Human hepatocytes, Authentic human urine samples, LC-HRMS, Fentanyl analogs, Metabolite identification

Introduction

Synthetic opioids are a class of new psychoactive substances (NPS) mostly consisting of analogs of the prescription drug fentanyl (Figure 1). Since 2012, they have caused an alarming number of fatalities worldwide [1]. Fentanyl itself is a potent μ -opioid receptor agonist [2] and is estimated to be at least 50 times more potent than morphine [3]. Although therapeutically prescribed as an analgesic since the 1960s, fentanyl has also been abused for its euphoric effects with fatalities occurring due to respiratory depression followed by apnea [4, 5].

A plethora of fentanyl analogs have been developed over the last decades [6], some examples are shown in Figure 1. Approved medications are e.g. alfentanil, sufentanil and remifentanil for use in humans and carfentanil for use in animals. 'Older' fentanyl analogs abused recreationally are 3-methylfentanyl, α -methylfentanyl and β -hydroxyfentanyl [7]. The analogs that recently appeared as NPS are usually generated by modification or replacement of fentanyl's propionyl chain (acetylfentanyl, acrylfentanyl, butyrylfentanyl, isobutyrylfentanyl, furanylfentanyl, ocfentanil) or replacement of the ethylphenyl moiety (isofentanyl, β -hydroxythiofentanyl). Existing variants are further substituted with fluoro, chloro or methoxy substituents at the *N*-phenyl ring [8, 9]. Not only are the fentanyl analogs highly potent, their potencies are also variable, making it difficult for the users to dose the drugs correctly and increasing the risk of accidental overdoses.

To understand and monitor the abuse of fentanyl analogs reliable analytical methods are needed in clinical and forensic laboratories. Urine is still the matrix of choice for drug testing due to the non-invasive nature of sampling, well-established procedure and admissibility. Less than 8% of fentanyl is excreted unchanged. Approximately 85% is excreted within 72 h in feces and urine, the majority as metabolites [10] mainly as norfentanyl generated by *N*-dealkylation at the piperidine nitrogen. Minor metabolites are despropionylfentanyl, which is formed by carboxamide hydrolysis, and hydroxyfentanyl and hydroxynorfentanyl metabolites, both hydroxylated at the propionyl moiety [11, 12].

To date, information about metabolism of fentanyl analogs, which have been found as drugs of abuse, is limited to a few studies: So far, metabolism of α -methylfentanyl in rat urine [13], 3-methylfentanyl in rat urine [14], isofentanyl in rat urine [14], butyrylfentanyl in human blood, tissues and urine [15] and acetylfentanyl in human liver microsomes and rat urine [16] and human urine [17] have been investigated. Some analogs, such as α -methylfentanyl, 3-methylfentanyl, isofentanyl and acetylfentanyl, were metabolized to the nor-metabolite, similar to fentanyl. Minor metabolites were produced by hydroxylation, dihydroxylation and carboxylation, often in combination with *N*-dealkylation. For acetylfentanyl, however,

hydroxylation and hydroxylation/methoxylation at the phenethyl moiety dominated over *N*-dealkylation. For butyrylfentanyl, hydroxylation and carboxylation dominated, while the nor- and desbutyryl metabolite were minor.

Based on the current state of knowledge, we hypothesize that suitable analytical targets in urine for new fentanyl analogs can often, but not always, be predicted based on fentanyl's metabolic pathway demonstrating the need to perform individual metabolism studies. Therefore, the aims of this study were to perform metabolism studies for four new fentanyl analogs, acetylfentanyl, acrylfentanyl, 4-fluoro-isobutyrylfentanyl and furanylfentanyl. We analyzed authentic human urine samples that were available from casework and also incubated the drugs with human hepatocytes. Sample analysis was performed under identical conditions with liquid chromatography high-resolution mass spectrometry (LC-HRMS) allowing for direct comparison between hepatocyte and urine samples and producing accurate mass measurements for metabolite molecules and fragment ions. Incubation with human hepatocytes provides *in vitro* metabolite profiles that are often similar to what is found in humans [18, 19]. Individual incubation under controlled conditions eliminates analogs as potential source of metabolites. However, *in vitro* models are inherently limited running the risk of missing metabolites or misinterpreting their relevance. Conversely, authentic human urine samples contain the real human metabolites formed from the whole organism. They usually come, however, with limited information about the intake itself, e.g. time before sampling, identity and purity of the drug or co-consumption of other compounds. A combination of both systems, complementing advantages and compensating for disadvantages, seems to be the road forward.

Materials and Methods

Chemicals and reagents

Acetylfentanyl, acrylfentanyl, furanylfentanyl and 4-fluoro-isobutyrylfentanyl were purchased as powders from Cayman Chemical (Ann Arbor, USA), diclofenac from Sigma (Stockholm, Sweden). Cryopreserved hepatocytes LiverPool (10-donor-pool), InVitro Gro HT and InVitro Gro KHB media were obtained from Bioreclamation IVT (Brussels, Belgium). Liquid chromatography mass spectrometry grade acetonitrile, water, methanol and formic acid were from Fisher Scientific (Gothenburg, Sweden), ammonium formate from Fluka (Sigma-Aldrich, Stockholm, Sweden), ammonium acetate and acetic acid from Merck (Darmstadt, Germany). The β -glucuronidase/sulfatase mixture (*Helix pomatia*) was purchased from Roche (Mannheim, Germany). Ultra-pure water was prepared in-house using a MilliQ Gradient 10 production unit (Millipore, Billerica, USA).

Incubation with human hepatocytes & sample preparation

Cryopreserved human hepatocytes were thawed at 37 °C and transferred into InVitro Gro HT medium. The solution was centrifuged at 100g for 4 min, the supernatant aspirated and the hepatocyte pellet suspended in Krebs–Henseleit buffer (KHB). After centrifugation at 60 g for 4 min and aspiration of the supernatant the pellet was re-suspended in KHB. Using Trypan Blue (0.4% v/v) dye exclusion method, cell viability was determined as 100%. Drugs were incubated at 10 μ mol/L with human hepatocytes (125,000 cells/125 μ L total volume/well) in a 96-well plate at 37 °C for 1, 3 and 5 h. The reaction was quenched by 125 μ L ice-cold acetonitrile. Organic solvent content was $\leq$ 0.4%. A positive control with diclofenac, a negative control without drugs and degradation controls without hepatocytes were included. After centrifugation the supernatant was stored at -20°C. Samples were injected undiluted onto the liquid chromatography-quadrupole time-of-flight mass spectrometer (LC-QTOF); injection volume was 1 μ L.

Authentic human urine specimens & sample preparation

Four or five human urine specimens for each fentanyl analog from fatal overdose cases were used for metabolite profiling. A corresponding blood sample had always confirmed positive for the respective parent compound in the routine TOF screening [20] without showing presence of other fentanyl analogs. For all cases, the cause of death was ruled to be an overdose with the

respective fentanyl analog, except in four cases, which were ruled intoxication with the fentanyl analog in combination with oxycodone (4-fluoroisobutyrylfentanyl case sample #2), alcohol (4-fluoroisobutyrylfentanyl case sample #4), pregabalin (Furanylfentanyl case sample #1) or fentanyl (Furanylfentanyl case sample #4). Each urine sample was analyzed with and without enzymatic hydrolysis: To cleave phase II conjugates, 100 μ L urine was incubated with 10 μ L β -glucuronidase/sulfatase (4.5 u/mL and 14 U/mL, respectively) in 300 μ L ammonium acetate buffer (pH 5.0) at 40 °C for 2 h. The non-hydrolyzed set of samples was diluted with 310 μ L ammonium acetate buffer only. One μ L of each sample was injected onto the LC-QTOF system.

LC-QTOF analysis

Chromatographic separation was performed on an Agilent 1290 Infinity UHPLC system with an Acquity HSS T3 column (150 mm x 2.1 mm, 1.8 μ m) fitted with an Acquity VanGuard pre-column, both from Waters (Sollentuna, Sweden), at 60 °C. Mobile phases were 10 mM ammonium formate in 0.05% formic acid (A) and 0.05% formic acid in acetonitrile (B) and were run in gradient at a flow rate of 0.5 mL/min starting at 1% B until 0.6 min, ramped to 5% B at 0.7 min, 50% B at 13 min, 95% B at 15 min until 18 min, then ramped down to 1% B at 18.1 min and re-equilibration until 19 min.

Mass spectrometric data was obtained with an Agilent 6550 iFunnel QTOF mass spectrometer with a Dual Agilent Jet Stream electrospray ionization source. Auto MS/MS acquisition was performed in positive ion mode with the following conditions: scan range, 100 – 950 m/z (MS) and 50 – 950 m/z (MS/MS); precursor intensity threshold, 5000 counts; precursor number per cycle, 5; fragmentor voltage, 380 V; collision energy, 3 eV at 0 m/z ramped up by 8 eV per 100 m/z ; gas temperature, 150 °C; nebulizer gas pressure, 50 psig; sheath gas temperature, 375 °C. Automated calibration was in place during data acquisition.

Manual and software-assisted data analysis

Full scan MS and MSMS data sets were analyzed by Agilent MassHunter Qualitative Analysis (version B.07.00) using a PCDL library that contained known biotransformation of fentanyl and fentanyl analogs as well as reactions predicted by MetaSite™ (v.5, Molecular Discovery, Pinner, UK). Searching parameters were as follows: mass error 15 ppm, absolute peak area > 10,000 counts, maximum number of matches 5, chromatogram extraction window 100 ppm. Metabolites were evaluated based on the following criteria: mass error for protonated molecule being less than 5 ppm; consistent isotopic pattern; MSMS product ions consistent

with proposed structure; retention time plausible for the proposed structure; appropriate peak shape; and absence of identical peaks in negative controls.

All in vitro and in vivo samples were also processed with MetaSense™ software (version 1.0, in combination with Spectrus Processor 2016.1.3 and DB Enterprise 2016.1.3, Advanced Chemistry Development, Inc., Toronto, ON, Canada). Processing options were as follows: MS accuracy 0.005 Da; three metabolite generations; minimum metabolite mass 150 Da; human specific and phase II metabolites included; minimum metabolite >0.01% of the parent.

Results

Hydroxydiclofenac was observed in the diclofenac positive control confirming hepatocyte metabolic activity. Any peaks that appeared either in the negative or degradation control were not considered as metabolites.

Metabolic profile of acetylfentanyl

In total, 32 acetylfentanyl metabolites (A1 to A32, in increasing order of retention time, RT) were identified, all of them with mass errors ≤ 4.22 ppm. The parent drug was found in high abundance in all samples. Metabolites were formed via *N*-dealkylation (A3) followed by hydroxylation (A1, A2), monohydroxylation (A24, A25, A28, A30) – preferably at the ethyl linker – followed by glucuronidation (A6, A11, A20) or sulfation (A17, A23), dihydroxylation (A8, A13, A14, A19) followed by glucuronidation (A5, A10) or sulfation (A22), monohydroxylation and carbonylation (A15, A18), dihydrodiol formation (A4, A7), dihydroxylation with methylation at the phenyl ring (A26, A27, A29) followed by glucuronidation (A9, A12) or sulfation (A21) as well as amide hydrolysis (A32) followed by hydroxylation (A16, A31). Retention times were between 2.77 min and 9.09 min, with A32 eluting after the parent (RT 7.92 min). The three sample sets showed different numbers of metabolites with different signal intensities: Specifically, in hepatocytes, seven metabolites were detected (A3, A4, A6, A18, A24, A26 and A30). Based on MS peak areas in the 5 h sample, the nor-metabolite A3 was most abundant followed by the monohydroxylated metabolite A24 and the dihydrodiol metabolite A4. In hydrolyzed urine, 24 metabolites were found showing a wider spectrum of first- and second-generation metabolites and including all hepatocyte metabolites. Ranking MS peak areas, the most dominant metabolite was the hydroxymethoxy metabolite A26, followed by A3 and A24. These findings are in general agreement with Melent'ev et al.'s study [17]. Finally, in non-hydrolyzed urine samples, many additional phase II metabolites were detected, out of 32 metabolites seven were glucuronides and four were sulfates. Generally, hydrolysis led to complete cleavage of the conjugates; exceptions occurred for A17 and A20. **Table 1** lists all 32 metabolites with retention time, accurate mass of the protonated molecule, proposed metabolic transformation, elemental composition, mass error, diagnostic product ions and chromatographic peak areas in hepatocyte, hydrolyzed and non-hydrolyzed urine samples while **Figure 2** shows the proposed metabolic pathway of acetylfentanyl and **Supplementary Figure 1** the MS/MS spectra of selected metabolites and their proposed fragment pattern.

Metabolic profile of acrylfentanyl

For acrylfentanyl, a total of 14 metabolites were identified with mass errors ≤ 4.48 ppm (B1 to B14). The observed biotransformations were *N*-dealkylation (B1), monohydroxylation (B9, B10, B13) followed by glucuronidation (B3), dihydroxylation (B7, B8) followed by glucuronidation (B5), dihydrodiol formation (B2, B6), dihydroxylation with methylation (B11, B12) followed by glucuronidation (B4) or amide hydrolysis (B14). Acrylfentanyl was present in high abundance in all samples. Metabolite retention times were between 5.25 and 9.06 min, the parent eluted at 8.85 min. Preferred molecular site of hydroxylation was the ethylphenyl moiety. In hepatocytes, eight metabolites were observed (B1, B2, B6, B9, B10, B11, B13 and B14), of which the nor-metabolite B1 and the monohydroxylated metabolites B13 and B9 were the most abundant metabolites after 5 h incubation. In hydrolyzed urine samples, twelve metabolites were detected with B1 being the major metabolite followed by B9 and B8. In total, three glucuronides B3, B4 and B5 were identified, which could be successfully hydrolyzed by the enzyme mixture, notably increasing the peak areas of the corresponding aglycones B9, B11 and B8. **Table 2** provides an overview on all acrylfentanyl metabolites, **Figure 3** depicts the proposed metabolic pathway and **Supplementary Figure 2** shows MSMS spectra of metabolites and their proposed fragmentation pattern.

Metabolic profile of 4-fluoro-isobutyrylfentanyl

For 4-fluoro-isobutyrylfentanyl, 17 metabolites were identified (C1 to C17) with mass errors ≤ 5.09 ppm. The metabolites were generated by *N*-dealkylation (C3), hydroxylation (C7, C8, C10, C11, C15, C17) followed by glucuronidation (C5), dihydroxylation (C9), dihydrodiol formation (C4), dihydroxylation with methylation (C12, C13) followed by glucuronidation (C6), amide hydrolysis (C14), oxidative *N*-dealkylation and further reduction of the keto group (C1), carboxylation (C2), and carbonylation (C16). Retention times were between 4.08 and 11.48 min; the hydroxy metabolite C17 eluted after the parent (RT 10.38 min). The parent drug was the most or second most abundant peak in hepatocyte and urine samples. In hepatocytes, nine metabolites were observed (C3, C8, C10, C12, C14-C17), of which the nor-metabolite C3 was the major metabolite in the 5 h sample, followed by the monohydroxylated metabolites C15 and C10. In hydrolyzed urine, eleven metabolites were detected (C1, C2, C3, C4, C7, C9-C13, C15) and although the subset was different from the one in hepatocytes, the major metabolites were almost identical with the only difference that hydroxymethoxy 4-fluoro-isobutyrylfentanyl (C12) was also abundant. In non-hydrolyzed urine, two additional glucuronides C5 and C6 were found. They were completely cleaved by hydrolysis, which was reflected in a significant peak area increase of C15 and C12, respectively. **Table 3** provides an overview on all 4-fluoro-isobutyrylfentanyl metabolites, **Figure 4** depicts the proposed metabolic pathway and **Supplementary Figure 3** shows MSMS spectra of metabolites and their proposed fragmentation patterns.

Metabolic profile of furanylfentanyl

Finally, for furanylfentanyl, 14 metabolites (D1 to D14) were identified with mass errors ≤ 3.11 ppm. With retention times between 3.56 min and 9.05 min, all of them eluted before the parent (9.38 min). Metabolites were formed via *N*-dealkylation (D6), hydroxylation (D11, D13), amide hydrolysis (D14) followed by hydroxylation (D4, D8, D12) with glucuronidation (D3) or sulfation (D5), dihydrodiol formation (D10) followed by hydroxylation (D7) or *N*-dealkylation (D1), oxidative *N*-dealkylation and reduction of the keto group (D2), and furanyl ring opening and carboxylation (D9). In contrast to the other three fentanyl analogs, amide hydrolysis and dihydrodiol formation led to the most abundant phase I metabolites in urine samples and although *N*-dealkylation was the second most common reaction in hepatocytes, the nor-metabolite D6 was only minor in urine. Furanylfentanyl was abundant in hepatocytes over the whole incubation time, but showed less intense signals compared to the other fentanyl analog parents in the five urine samples. In hepatocytes, all metabolites but D3 were found. The amide hydrolysis metabolite D14 was the major metabolite, the nor-metabolite D6 and the dihydrodiol metabolite D10 ranked #2 and #3. In hydrolyzed urine samples, nine metabolites were detected (D1, D2, D4-D8, D10, D14) with D14 being the most abundant peak followed by D10, then D5. In non-hydrolyzed urine samples, one glucuronide (D3) and one sulfate (D5) were found as phase II metabolites. Enzymatic cleavage was successful for the glucuronide resulting in the appearance of D4, but failed for the sulfate. **Table 4** provides an overview on all furanylfentanyl metabolites, **Figure 5** depicts the proposed metabolic pathway and **Supplementary Figure 4** shows MS/MS spectra of metabolites and their proposed fragmentation patterns.

Discussion

Structural elucidation of metabolites

All four fentanyl analogs investigated in this study shared the phenethylpiperidine substructure. Upon collision-induced dissociation, several characteristic fragments can be derived from it, which were used to determine the type of biotransformation and where it could have occurred: If the phenethylpiperidine structure is unchanged, as is the case in all parent molecules and many metabolites, two major signals are usually found in the MSMS spectrum, one at m/z 105.0699, associated with the phenethyl moiety, and one at m/z 188.1434, corresponding to the complete structure. Depending on the compound's peak intensity and preferred fragmentation behavior, also minor fragments at m/z 84.0808 (unchanged piperidine ring) or 132.0808 (phenethyliminomethylion) can be detectable.

Acetylfentanyl metabolites

The MSMS spectrum of acetylfentanyl showed the three aforementioned peaks at m/z 105.0697, 132.0801 and 188.1434. The most abundant metabolite A3 was the nor-metabolite, which is generated by *N*-dealkylation at the piperidine nitrogen resulting in a loss of the phenethyl moiety. The MSMS spectrum showed one intense fragment ion at m/z 84.0805, but lacked m/z 105.0699 and 188.1434. Minor fragment ions were observed at m/z 94.0645 (anilinyli moiety) and 136.0750 (*N*-phenylacetamide moiety). Hydroxylation of A3 yielded A1 and A2 with the major fragment ion at m/z 84.0809 and 84.0803, respectively, suggesting the position of hydroxylation at the acetyl moiety or *N*-phenyl ring.

Monohydroxylated metabolites were identified by the addition of 15.9949 u (+O) to the mass of acetylfentanyl: The most abundant hydroxy metabolite A24 was hydroxylated at the ethyl linker as indicated by m/z 121.0643 (hydroxyphenethyl) and m/z 103.0535, its water loss fragment ion. An aliphatic hydroxy group is likely, because water loss is uncommon at aromatic rings [21]. A25 and A28 were either hydroxylated at the *N*-phenyl ring or the acetyl moiety (m/z 188.1428/188.1430). A30 is generated by hydroxylation at the piperidine ring, which is supported by the fragment ions at m/z 105.0691, 204.1392 (hydroxyphenethylpiperidinyl) and the corresponding water loss peak at m/z 186.1279.

The four dihydroxylated metabolites were identified by a mass shift of 31.9898 u (+2O) compared to the parent. The most dominant dihydroxylated metabolite was A14, which was hydroxylated once at the ethyl linker and once at the adjacent phenyl ring. The fragment ions at m/z 220.1330 and 137.0595 can be associated with dihydroxyphenethylpiperidine and

dihydroxyphenethyl, respectively, while m/z 119.0488 represents the corresponding water loss ion from m/z 137.0595. A8 is suggested to be hydroxylated once on each phenyl ring; the presence of m/z 121.0648 and absence of m/z 103.0542 indicates aromatic hydroxylation and m/z 84.0806 and 204.0378 exclude the possibility of a second hydroxy group at the piperidine ring. A13's MSMS spectrum showed fragment ions at m/z 107.0491 (hydroxytoluene) and m/z 202.1230 (water loss ion from m/z 220.1332 indicating two hydroxy groups at the phenethylpiperidine moiety). A19 is likely hydroxylated twice at the *N*-phenyl ring (m/z 105.0694 and 188.1433) serving as the precursor for A27.

Interestingly, three metabolites generated by dihydroxylation and methylation of one of the hydroxy groups were also observed, one of them (A26) in high abundance: A26 and A29 showed similar characteristic fragment ions. The ions at m/z 151.0758 suggest presence of a hydroxy and a methoxy group at the phenethyl moiety. A loss of methanol will then generate the fragment ion at m/z 119.0490. The methylation reaction is probably catalyzed by catechol-O-methyltransferase, an enzyme that is involved in the metabolism of catecholic neurotransmitters, such as adrenaline, noradrenaline and dopamine [22], and xenobiotics like MDMA, MDEA and MBDB [23]. The literature shows that the hydroxy group in *meta* position is preferred for methylation, suggesting that A26 is methylated there. In contrast, A27 was dihydroxylated and methylated at the *N*-phenyl ring as the phenethyl and piperidinyl moiety remained intact (m/z 188.1426).

Two dihydrodiol metabolites (A4 and A7) are formed via the epoxide-diol-pathway, which has been described for other phenyl-ring-containing drugs as well, such as ethotoin, phenytoin, oxazepam and rofecoxib [24], as well as benzene [25]. A4, the intense isomer, is modified at the ethylphenyl ring. The fragment ion at m/z 222.1489 corresponds to the ethylphenylpiperidine substructure with dihydrodiol; m/z 188.1070 ($\neq m/z$ 188.1434) indicates that the *N*-phenyl ring remained unchanged. As a water loss from one of the dihydrodiol hydroxy groups leads to re-aromatization, we suggest that this reaction is extremely favorable explaining presence of m/z 121.1559, but absence of expected m/z 139.0754. A similar phenomenon was observed for a dihydrodiol metabolite of AM2201 [26]. Notably, the dihydrodiol can be further metabolized by dihydrodiol dehydrogenase yielding a catechol – this reaction could produce the intermediate catechol of the two hydroxy methoxy metabolites A26 and A29. A7, only a minor metabolite, is modified at the *N*-phenyl ring (m/z 105.0699, 188.1434) and is probably the precursor of A27.

Carbonylation, probably at the ethyl linker (m/z 202.1221/202.1215), and hydroxylation, probably at the phenyl or piperidine ring (m/z 164.0707/164.0704 and 218.1134/218.1199) produced two almost equally intense metabolites A15 and A18. The fragment ions at m/z 118.0647/118.0651 and m/z 132.0806 indicate an unchanged *N*-phenyl ring.

A32 is formed by hydrolysis of the amide functional group cleaving off the acetyl moiety and leaving phenethylpiperidine unchanged (m/z 188.1423). Further hydroxylation leads to A16 hydroxylated at *N*-phenyl (m/z 188.1416) and A31 hydroxylated at the ethyl linker (m/z 121.0637, 103.0545).

In the non-hydrolyzed samples, several glucuronides (A5, A6, A9, A10, A11, A12, A20) and sulfates (A17, A21, A22, A23) were detected. Phase II metabolites were assigned to their respective phase I metabolites based on shared fragment ions, consistent elution behavior, matching relative intensities and increasing peak areas of the phase I metabolite after hydrolysis. Figure 2 shows the proposed individual assignments. Surprisingly, two glucuronides (A6 and A11) and two sulfates (A17 and A23), all clearly baseline-separated, were identified as possible conjugates of the abundant monohydroxylated metabolite A24. A possible explanation to explain the occurrence of two distinct glucuronide signals for one phase I metabolite is the formation of diastereomers. If the phase I metabolite has a stereogenic center (A24 does), the conjugation with another chiral molecule such as glucuronic acid will lead to diastereomers, which can be separated on non-chiral LC columns [27]. However, this hypothesis does not stand up to the fact that there are also two sulfates formed from A24. Possibly, the broad and intense A24 peak consists of two co-eluting isomers, both hydroxylated at the ethyl linker, but at different positions. The same phenomenon was observed for the dihydroxylated metabolite A14, to which two glucuronides (A5 and A10) could be assigned.

Acrylfentanyl metabolites

As expected, acrylfentanyl fragmented to the two major product ions at m/z 105.0697 and 188.1441. Similarly to acetylfentanyl, acrylfentanyl underwent *N*-dealkylation at the piperidine nitrogen producing the major nor-metabolite B1 (m/z 84.0804 without m/z 105.0699 and 188.1434). Monohydroxylated metabolites were either hydroxylated at the ethyl linker in B9 (m/z 121.0646, 103.0541), the *N*-phenyl ring or acryl moiety in B10 (m/z 105.0696, 188.1370), or the piperidine ring in B13 (m/z 105.0688, 186.1261). One of the two dihydroxy metabolites, B7, was dihydroxylated at the *N*-phenyl ring or the acryl moiety (m/z 105.0695, 188.1441) while the other one, B8, was hydroxylated once each at the ethyl linker and the adjacent phenyl group as indicated by m/z 137.0597 and its water loss fragment at m/z 119.0485. In analogy to acetylfentanyl, we detected two dihydrodiol metabolites (B2, B6); one carrying the two hydroxy groups at the ethylphenyl ring (m/z 121.0648) and the other one on the *N*-phenyl ring (m/z 105.0696, m/z 188.1428). Both dihydroxylated/methylated metabolites B11 and B12 carried the hydroxy and methoxy group at the phenyl ring of the phenethyl moiety (m/z 151.0755 and 151.0736; m/z 119.0493 and 119.0879, the methanol loss fragment ions) and probably originate from B2. In view of strikingly different intensities for both isomers, we suggest that

B11 is methylated at the preferred *meta* position and B12 at the less favored *para* position. Finally, the desacrylated metabolite B14, which is generated by amide hydrolysis, is characterized by the intact phenethylpiperidine moiety (m/z 105.0687, 188.1433) and is, in fact, identical to A32. The three glucuronides that were identified in non-hydrolyzed urine samples were assigned to the following aglycones based on shared fragment ions: B3 is the glucuronide of monohydroxy metabolite B9, B4 the glucuronide of the abundant dihydroxylated/methylated metabolite B11 and B5 the glucuronide of the most intense dihydroxylated metabolite B8.

4-Fluoro-isobutyrylfentanyl metabolites

The MSMS spectrum of 4-fluoro-isobutyrylfentanyl also showed the major fragment ions at m/z 105.0698 and 188.1434. The nor-metabolite C3 was characterized by one single intense product ion at m/z 84.0806. Six monohydroxy metabolites were detected: C7 and C8 had an unchanged phenethylpiperidine moiety (m/z 105.0693 and 105.0698, 188.1407 and 188.1434, respectively); their relatively early retention time suggests aliphatic hydroxylation at the isobutyryl chain rather than aromatic hydroxylation. The abundant metabolite C10 was hydroxylated at the ethyl linker (m/z 103.0543, 121.0645). C11, in contrast, showed an ion at m/z 121.0642, but lacked the water loss fragment at m/z 103.0542 and hence can be hydroxylated at either the ethyl linker or the adjacent phenyl group. C15, one of the major metabolites, is hydroxylated at the piperidine ring (m/z 105.0701, 204.1365). C17 showed product ions at m/z 105.0700 and 164.0869 indicating that phenethyl moiety and *N*-phenyl ring are unchanged and leaving the site of modification to the piperidine ring. Interestingly, C17 eluted after the parent drug, which is rather uncommon for the supposedly more polar hydroxy metabolites. This phenomenon has been frequently observed for *N*-oxides; thus, C17 is probably an *N*-oxide [28-30]. The dihydroxylated metabolite C9 was hydroxylated once each at the ethyl linker and at the adjacent phenyl ring (m/z 119.0482, 137.0590). Only one dihydrodiol metabolite (C4) was identified, which carried the dihydrodiol substructure at the ethylphenyl ring (m/z 121.0639, 234.1250) and likely is the precursor for C12 and C13. Interestingly, the fluoro substituent seems to have blocked dihydrodiol formation at the *N*-phenyl ring. The dihydroxylated/methylated metabolites C12 and C13, similar to acetyl- and acrylfentanyl, were modified at the phenyl ring (m/z 119.0493 and 119.0473, 151.0751 and 151.0744, respectively), with the more abundant isomer C12 proposed to be methylated at *meta* position. Amide hydrolysis generated C14 with phenethylpiperidine moiety intact (m/z 105.0699, 188.1431) and the isobutyryl chain lost. We also observed oxidative *N*-dealkylation with further reduction of the formed keto group, which generated C1. The whole *N*-fluorophenylisobutamide moiety is cleaved off during this process, the phenethyl moiety remains intact (m/z 105.0698). Carboxylation to generate C2 occurred at the isobutyryl chain, the only plausible site (m/z

105.0689, 188.1443). C16's MSMS spectrum displayed a fragment ion at m/z 202.1201 indicating a carbonylated phenethylpiperidine moiety. The carbonyl group is proposed to be next to the piperidine nitrogen, as this would facilitate the formation of m/z 148.0751. Further evidence derives from the absence of m/z 119.0491, which would be expected, if the carbonyl group were on the phenethyl moiety. In the non-hydrolyzed samples, the glucuronides C5 and C6, phase II conjugates of C15 and C12, were detected.

Furanylfentanyl metabolites

While acetyl-, acryl- and 4-fluoro-isobutyrylfentanyl differ only slightly in respect to the substituent at the amide group, furanylfentanyl contains a substantially different structure. Furan is an aromatic, heterocyclic system known to undergo characteristic bioactivation reactions, such as epoxidation and ring scission [31, 32], and the dominance of these reactions significantly affected furanylfentanyl's metabolic profile. Besides, the different structure seemed to favor an otherwise less important reaction, the amide hydrolysis.

Identical to the other analogs, furanylfentanyl generated the common two major fragment ions m/z 105.0699 and 188.1425. Amide hydrolysis produced the most abundant metabolite, D14, which showed an intact phenethylpiperidine moiety (m/z 105.0697, 188.1433) and could be further metabolized by hydroxylation either at the *N*-phenyl to yield D4 (m/z 105.0698, 188.1435), at the ethyl linker to form D8 (m/z 103.0529, 121.0646) or at the piperidine ring to generate D12 (m/z 105.0690, 186.1269). The second most dominant biotransformation was epoxidation of furan followed by hydration, which yielded the dihydrodiol metabolite D10 (m/z 105.0698, 188.1440). Although the MSMS spectrum does not exclude the possibility of dihydrodiol formation at the *N*-phenyl ring, we suggest that the furan ring is the more probable target [31]. Notably, furan epoxidation or ring opening to an α,β -unsaturated carbonyl intermediate can lead to reactive metabolites as shown for other furan-containing compounds like aflatoxin B1, ipomeanol and furosemide, which are known to cause hepatic and renal necrosis [31]. D10 was further hydroxylated at the piperidine ring to form D7 (m/z 103.0562, 121.0649) or *N*-dealkylated to form the nor-metabolite D1 (m/z 84.0789). The nor-metabolite D6, abundant in hepatocytes but minor in human urine, showed the typical fragment ion at m/z 84.0810 and also fragment ions at m/z 188.0694 (resulting from the loss of piperidine) and m/z 95.0128 (further loss of phenylamine). D2 was formed by oxidative *N*-dealkylation and reduction of the keto group retaining the intact phenethyl moiety (m/z 105.0698, 188.1434). D11 and D13 were hydroxylated at the ethyl linker (m/z 103.0533, 121.0649) and the piperidine ring (m/z 105.0685, 186.1274), respectively. Furan ring opening and subsequent carboxylation is proposed to generate D9 with (m/z 105.0693, 188.1448). In non-hydrolyzed samples, we identified D3 and D5, glucuronide (D3) and sulfate conjugate of D4.

How well did hepatocyte study results correlate with the findings in human urine samples?

In general, major hepatocyte metabolites were in good agreement with major urine metabolites adding to the finding of previous studies that human metabolism can be well-predicted by *in vitro* hepatocyte experiments [18, 27, 33]. The same biotransformations were observed and for each drug two out of the three major urine metabolites also ranked among the top three metabolites in hepatocytes (three out of four for 4-fluoro-isobutyrylfentanyl). Many deviations between hepatocytes and urine metabolites can be explained by the different time allowed for metabolism, which leads to fewer metabolites in general and a lower prevalence of 2nd or 3rd generation metabolites in hepatocytes. Another possible reason is the enrichment of polar and conjugated metabolites in urine. Different genotypes and phenotypes of the drug-metabolizing enzymes could be another factor for different metabolic profiles. However, such variance was expected to be reduced, at least partially, in the hepatocytes incubation since a pool from 10 donors was used in this study. For acetylfentanyl, the three most abundant urinary metabolites (#1 hydroxymethoxy metabolite A26, #2 hydroxy metabolite A24, #3 nor-metabolite A3) were also generated by hepatocytes and ranked #4, #2, #1 in terms of abundance in the 5h sample. Similarly, for acrylfentanyl, three out of the four most abundant urinary metabolites (#1 nor-metabolite B1, #2 hydroxy metabolite B9, #3 dihydroxy metabolite B8 and #4 hydroxymethoxy metabolite B11) were also observed in hepatocytes ranking #1, #3 and #8. B8 was not detected, probably due to the relatively short incubation time. For the same reason, i.e. increased prevalence of 2nd and 3rd generation metabolites in urine, the second most abundant metabolite in hepatocytes B13 only ranked #7 in the case samples. For 4-fluoro-isobutyrylfentanyl, the three most abundant metabolites (#1 nor-metabolite C3, #2 monohydroxy metabolites C10 and C15) were identical for hepatocytes and urine samples. The hydroxymethoxy metabolite C12, #3 in urine samples, ranked #8 in hepatocytes. For furanylfentanyl, the two major urinary metabolites (#1 hydrolyzed metabolite D14, #2 dihydrodiol metabolite D10) were detected after hepatocyte incubation ranking #1 and #3. However, the third most abundant metabolite in urine, D7, only ranked #10 and would have never been predicted as an abundant *in vivo* metabolite. In fact, the hepatocyte experiment also suggested high prevalence for the nor-metabolite D6 (#2 in hepatocytes), which strikingly mismatched the actual findings in the urine samples, illustrating the need to analyze *in vivo* human samples. Most likely, the nor-metabolite was further metabolized *in vivo*: We checked the data for 2nd generation nor-metabolites and found intense signals for a potential nordesfuranyl metabolite at 0.92 min, albeit without MSMS spectral information. Steuer et al. observed a similar phenomenon with butyrylfentanyl, where the nor-metabolite was major in human liver microsome incubations but insignificant in a postmortem human urine and blood sample [34].

It should be noted that phase II conjugation can affect the availability of the phase I metabolite biomarkers. Particularly in acetylfentanyl urine samples, a several-fold increase (up to 50x) in phase I metabolite peak area was not uncommon after hydrolysis. Ten out of 32 metabolites were conjugates. While major metabolites, e.g. A24, A26 and A14, could be both glucuronidated and sulfated, less abundant metabolites, e.g. A29 and A30, were only glucuronidated. Phase II conjugation seemed to play a smaller role in acrylfentanyl, 4-fluoro-isobutyrylfentanyl and furanylfentanyl metabolism, as we identified only 3, 2 and 2 phase II metabolites, respectively. Nevertheless, a hydrolysis step should always be included in the sample preparation.

Which metabolites are suitable analytical targets for urine analysis?

The studied fentanyl analogs were generally highly abundant in urine indicating that parent drugs are one of the most suitable targets nonetheless. However, all urine samples were from fatal overdose cases; consequently, parent drugs might be less abundant in non-overdose samples. As the window of detection of metabolites is usually longer than for the parent drugs and as the additional presence of metabolites increases the plausibility of the results, it is useful to target not only one, but two or three compounds including metabolites.

Target metabolites should generally be abundant, specific of the parent drug, and prevalent in most, if not all, case samples. As routine analysis usually involves a hydrolysis step and as reference standards usually become available earlier, phase I metabolites are preferred. It can be useful to target not only one, but two or three metabolites in addition to the parent drug. With this in mind, the nor-metabolite, although commonly abundant and prevalent, is a less suitable target because it has lost substantial parts of the molecule and consequently can be formed from similar drugs. The same is true for metabolites generated by oxidative *N*-dealkylation and amide hydrolysis. Both are reactions that cleave off the specific moiety to distinguish between different analogs. As a result of that, in our study metabolites were shared across the different metabolic profiles and none of them represents a suitable analytical target: hydrolyzed metabolites (A32 = B14 = D14, RT 9.07 min), hydrolyzed/hydroxylated metabolites (A16 = D4, RT 5.70 min as well as A31 = D8, RT 7.22 min) and the metabolites generated by oxidative *N*-dealkylation and reduction (C1 = D2, RT 4.06 min).

Based on the aforementioned criteria, we suggest the following analytical targets: the hydroxymethoxy metabolite A26 and the monohydroxylated metabolite A24 for acetylfentanyl; the monohydroxy metabolite B9 and the dihydroxy metabolite B8 for acrylfentanyl and the monohydroxy metabolites C15 and C10 and the hydroxymethoxy metabolite C12 for 4-fluoro-isobutyrylfentanyl. Regarding furanylfentanyl, none of the metabolites satisfied all criteria. The

dihydrodiol metabolite D10 was not prevalent in all tested samples, but due to its abundance and specificity it should be considered the best target. In the absence of D10, e.g. in urine sample #1 and #3, the amide hydrolysis metabolite D14 may be targeted, although it does not prove furanylfentanyl consumption beyond doubt. As mentioned above, the abundance of major metabolites can be affected by different phenotypes of the metabolizing enzymes due to different genotypes, but also co-administered drugs (34).

Is the metabolism of the new fentanyl analogs consistent with previous findings?

In general, metabolism of fentanyl analogs follows similar pathways. Three of our four analogs, acetylfentanyl, acrylfentanyl and 4-fluoro-isobutyrylfentanyl, produced large amounts of the nor-metabolite, a hydroxyethyl metabolite and a hydroxymethoxy metabolite. This is consistent with previous findings that the nor-metabolite is a major metabolite of fentanyl [11] and other fentanyl analogs [13, 14] and that hydroxy metabolites are commonly observed for fentanyl and analogs [11, 13, 14, 34]. Notably, the hydroxymethoxy metabolite has not been reported for fentanyl itself, but was observed for other analogs, e.g. 3-methylfentanyl, isofentanyl [14] and butyrylfentanyl [34]. The discrepancy might indicate that this metabolite was not searched for fentanyl in the previous studies.

In contrast, furanylfentanyl's major metabolites were generated by amide hydrolysis with/without hydroxylation and dihydrodiol formation while the nor-metabolite was not even detected in most of the urine samples. The aromatic furan ring, which has different reactivity from the side chains in the other three analogs, is considered to be the reason for the preference of other biotransformations. Another fentanyl analog with different major metabolites is butyrylfentanyl, which is mainly metabolized *in vivo* by hydroxylation and carboxylation on the butyryl side chain [34]. Surprisingly, this side chain, which is only one carbon atom longer than fentanyl's, seems to have changed the metabolic profile.

Conclusion

Given the great number of possible structural variations and the current phenomenon of constantly emerging new psychoactive substances, more 'new' fentanyl analogs are likely to appear. Taken together our results, we consider it legitimate to – initially – predict metabolites of newly emerged fentanyl analogs based on current knowledge about the metabolism of known fentanyl analogs. Likely, the nor-metabolite, one or several hydroxy metabolites and/or a hydroxymethoxy metabolite will be prevalent. However, as some analogs can show a significantly different metabolism, we consider it crucial to confirm the predictions with comprehensive metabolite identification studies, if possible with both *in vitro* and *in vivo* studies.

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Conflicts of interest

None.

Legends to Figures

Fig. 1: Structures of fentanyl and analogs that have approved medicinal use, others that have been abused for several decades and those that appeared recently on the illicit drug market.

Fig. 2: Proposed metabolic pathway of acetylfentanyl combining both human hepatocyte and human urine metabolites. Markush structures are used where the exact position of functional groups cannot be determined. Enclosed metabolites are the major metabolites detected in hydrolyzed human urine samples, metabolite in italics were only found in either the *in vitro* or *in vivo* experiment

Fig. 3: Proposed metabolic pathway of acrylfentanyl combining both human hepatocyte and human urine metabolites. Markush structures are used where the exact position of functional groups cannot be determined. Enclosed metabolites are the major metabolites detected in hydrolyzed human urine samples, metabolite in italics were only found in either the *in vitro* or *in vivo* experiment.

Fig. 4: Proposed metabolic pathway of 4-fluoro-isobutyrylfentanyl combining both human hepatocyte and human urine metabolites. Markush structures are used where the exact position of functional groups cannot be determined. Enclosed metabolites are the major metabolites detected in hydrolyzed human urine samples, metabolite in italics were only found in either the *in vitro* or *in vivo* experiment.

Fig. 5: Proposed metabolic pathway of furanylfentanyl combining both human hepatocyte and human urine metabolites. Markush structures are used where the exact position of functional groups cannot be determined. Enclosed metabolites are the major metabolites detected in hydrolyzed human urine samples, metabolite in italics were only found in either the *in vitro* or *in vivo* experiment.

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Table 1: Acetylfentanyl metabolites with proposed biotransformation, retention time, elemental composition, accurate mass of protonated molecule, mass error of proposed metabolite, MS peak areas in hepatocyte samples (0h, 1h, 3h and 5h) and in five urine samples (hydrolyzed and non-hydrolyzed), and diagnostic product ions (masses and mass errors taken from sample #3, and #5 in case of saturation).

ID	Biotransformation	RT (min)	Elemental composition	m/z	mass error (ppm)	Peak areas in the hepatocyte samples				Peak areas in the urine samples					Diagnostic ions	
						0h	1h	3h	5h	#1	#2	#3	#4	#5		
										HYD NON-HYD	HYD NON-HYD	HYD NON-HYD	HYD NON-HYD	HYD NON-HYD		
Blood acetylfentanyl concentration (in µg/g)											0.77	0.17	0.18	0.04	0.02	
A1	N-dealkylation + hydroxylation	2.77	C ₁₃ H ₁₈ N ₂ O ₂	235.1441	0.08	ND	ND	ND	ND	1.0E+06	ND	1.9E+05	ND	ND	84.0809	
											ND	ND	1.7E+05	ND	ND	
A2	N-dealkylation + hydroxylation	2.93	C ₁₃ H ₁₈ N ₂ O ₂	235.1444	2.24	ND	ND	ND	ND	3.6E+05	ND	1.1E+05	ND	ND	84.0803	
											2.7E+05	ND	6.9E+04	ND	ND	
A3	N-dealkylation (yielding nor- metabolite)	4.19	C ₁₃ H ₁₈ N ₂ O	219.1490	-0.89	4.6E+05	2.1E+06	4.4E+06	5.6E+06	2.5E+07	2.9E+06	1.8E+07	2.3E+05	2.3E+06	84.0805, 94.0645, 136.0750	
											2.4E+07	3.0E+06	1.7E+07	2.3E+05	2.4E+06	
A4	Dihydrodiol formation (ethylphenyl)	4.66	C ₂₁ H ₂₈ N ₂ O ₃	357.2195	4.22	ND	7.1E+05	1.2E+06	1.7E+06	1.7E+07	4.6E+05	1.0E+07	5.1E+04	3.7E+05	121.0644, 146.0962, 158.0952, 188.1071, 204.1374, 222.1462	
											1.6E+07	4.4E+05	9.5E+06	ND	3.2E+05	
A5	Dihydroxylation + Glucuronidation	4.74	C ₂₇ H ₃₄ N ₂ O ₉	531.2339	0.15	ND	ND	ND	ND	ND	ND	ND	ND	ND	91.0532, 119.0475, 137.0595, 220.1317, 355.2043	
											1.8E+06	ND	3.6E+05	ND	3.4E+04	

A6	Hydroxylation + Glucuronidation	4.79	C ₂₇ H ₃₄ N ₂ O ₈	515.2380	-2.04	ND	ND	ND	1.0E+04	2.9E+06	ND	2.2E+04	ND	ND	84.0808, 121.0641, 204.1379
										2.8E+07	7.4E+05	1.7E+07	7.1E+04	1.3E+06	
A7	Dihydrodiol formation (<i>N</i> - phenyl)	4.85	C ₂₁ H ₂₈ N ₂ O ₃	357.2170	-0.94	ND	ND	ND	ND	1.8E+06	ND	3.5E+05	ND	ND	105.0697, 188.1436
										2.1E+06	ND	3.9E+05	ND	ND	
A8	Dihydroxylation	4.92	C ₂₁ H ₂₆ N ₂ O ₃	355.2013	-3.27	ND	ND	ND	ND	8.6E+05	ND	1.9E+05	ND	4.1E+04	84.0806, 121.0648, 204.0378
										1.9E+05	ND	5.1E+04	ND	ND	
A9	Dihydroxylation + Methylation + Glucuronidation	4.97	C ₂₈ H ₃₆ N ₂ O ₉	545.2526	3.87	ND	ND	ND	ND	ND	ND	ND	ND	ND	84.0808, 119.0486, 151.0749, 234.1486
										2.6E+07	5.6E+05	1.5E+07	1.7E+05	1.8E+06	
A10	Dihydroxylation + Glucuronidation	5.10	C ₂₇ H ₃₄ N ₂ O ₉	531.2333	-0.4	ND	ND	ND	ND	ND	ND	ND	ND	ND	84.0802, 119.0473, 137.0586, 220.1319, 355.1995
										3.7E+06	ND	1.1E+06	ND	8.6E+04	
A11	Hydroxylation + Glucuronidation	5.17	C ₂₇ H ₃₄ N ₂ O ₈	515.2383	-0.65	ND	ND	ND	ND	ND	ND	ND	ND	ND	121.0644, 204.1379, 339.2073
										4.0E+06	6.7E+04	8.9E+05	ND	3.6E+04	
A12	Dihydroxylation + Methylation + Glucuronidation	5.55	C ₂₈ H ₃₆ N ₂ O ₉	545.249	-0.52	ND	ND	ND	ND	ND	ND	ND	ND	ND	119.0487, 151.0752, 192.0989, 234.1476
										2.4E+06	1.6E+04	5.9E+05	ND	5.6E+04	
A13	Dihydroxylation	5.60	C ₂₁ H ₂₆ N ₂ O ₃	355.2018	0.46	ND	ND	ND	ND	1.2E+06	ND	2.7E+05	ND	ND	107.0491, 121.0630, 188.1064,

															202.1230, 337.1857
										6.6E+05	ND	9.7E+04	ND	ND	
A14	Dihydroxylation	5.72	C ₂₁ H ₂₆ N ₂ O ₃	355.2012	-1.12	ND	ND	ND	ND	2.1E+07	6.5E+05	1.6E+07	1.5E+05	2.5E+06	91.0539, 119.0488, 137.0595, 220.1330
										5.3E+06	1.4E+05	7.4E+06	ND	2.5E+05	
A15	Carbonylation + Hydroxylation	5.74	C ₂₁ H ₂₄ N ₂ O ₃	353.1854	-1.11	ND	ND	ND	ND	7.3E+06	3.6E+04	9.8E+05	ND	1.9E+05	118.0647 132.0806, 164.0705, 202.1221, 218.1167
										8.1E+06	4.2E+04	1.1E+06	ND	2.4E+05	
A16	Hydroxylation + Amide hydrolysis	5.75	C ₁₉ H ₂₄ N ₂ O	297.1959	-1.05	ND	ND	ND	ND	ND	ND	3.8E+05	ND	5.1E+04	105.0689, 188.1416
										ND	ND	ND	ND	ND	
A17	Hydroxylation + Sulfation	5.87	C ₂₁ H ₂₆ N ₂ O ₅ S	419.1634	-0.78	ND	ND	ND	ND	1.2E+06	ND	2.5E+05	ND	ND	103.0530, 121.0650, 204.1380
										1.2E+06	ND	2.5E+05	ND	ND	
A18	Carbonylation + Hydroxylation	5.88	C ₂₁ H ₂₄ N ₂ O ₃	353.1852	-1.94	ND	2.4E+04	2.8E+04	2.5E+04	7.1E+06	2.3E+04	8.8E+05	ND	1.9E+05	118.0651, 132.0806, 164.0704, 202.1215
										8.0E+06	2.5E+04	1.1E+06	ND	2.1E+05	
A19	Dihydroxylation	5.89	C ₂₁ H ₂₆ N ₂ O ₃	355.2010	-2.56	ND	ND	ND	ND	3.2E+06	ND	1.3E+06	ND	5.5E+04	91.0540, 105.0694, 188.1433
										6.4E+05	ND	3.0E+05	ND	ND	
A20	Hydroxylation + Glucuronidation	5.90	C ₂₇ H ₃₄ N ₂ O ₈	515.2380	-1.85	ND	ND	ND	ND	7.4E+05	ND	7.9E+04	ND	ND	186.1280, 321.1947
										1.1E+06	ND	7.9E+04	ND	ND	
A21	Dihydroxylation + Methylation + Sulfation	5.93	C ₂₂ H ₂₈ N ₂ O ₆ S	449.1741	0.14	ND	ND	ND	ND	ND	ND	ND	ND	ND	84.0806, 119.0483,

															151.0748, 234.1476
										6.0E+06	4.5E+04	8.6E+05	ND	1.2E+05	
A22	Dihydroxylation + Sulfation	6.12	C ₂₁ H ₂₆ N ₂ O ₆ S	435.1582	-0.21	ND	ND	ND	ND	ND	ND	ND	ND	ND	84.0809, 137.0581, 220.1336
										7.7E+05	ND	1.3E+05	ND	5.0E+04	
A23	Hydroxylation + sulfation	6.26	C ₂₁ H ₂₆ N ₂ O ₅ S	419.1629	-1.41	ND	ND	ND	ND	ND	ND	ND	ND	ND	84.0805, 121.0636, 186.9208, 204.1377, 339.2067
										2.5E+05	ND	1.0E+05	ND	ND	
A24	Hydroxylation	6.30	C ₂₁ H ₂₆ N ₂ O ₂	339.2071	0.56	ND	1.4E+06	2.5E+06	2.9E+06	2.4E+07	4.1E+06	2.1E+07	2.2E+05	6.2E+06	84.0806, 103.0535, 121.0643, 204.1385
										1.9E+07	8.5E+05	1.4E+07	ND	1.3E+06	
A25	Hydroxylation	6.46	C ₂₁ H ₂₆ N ₂ O ₂	339.2063	-1.05	ND	ND	ND	ND	5.7E+06	3.2E+04	8.4E+05	ND	ND	105.0696, 188.1428
										2.4E+06	ND	3.1E+05	ND	ND	
A26	Dihydroxylation + Methylation	6.60	C ₂₂ H ₂₈ N ₂ O ₃	369.2189	3.1	ND	1.1E+05	1.5E+05	1.7E+05	2.4E+07	3.9E+06	2.1E+07	8.6E+05	8.6E+06	91.0542, 119.0490, 151.0758, 234.1487
										1.5E+07	4.3E+05	1.1E+07	9.5E+04	1.1E+06	
A27	Dihydroxylation + Methylation	6.72	C ₂₂ H ₂₈ N ₂ O ₃	369.2168	-1.28	ND	ND	ND	ND	2.5E+06	ND	4.4E+05	ND	4.8E+04	84.0807, 105.0698, 188.1426
										2.6E+05	ND	5.8E+04	ND	ND	
A28	Hydroxylation	6.74	C ₂₁ H ₂₆ N ₂ O ₂	339.2063	-0.81	ND	ND	ND	ND	4.9E+06	3.5E+04	7.7E+05	ND	ND	105.0697, 188.1430
										9.2E+05	ND	ND	ND	ND	
A29	Dihydroxylation + Methylation	6.87	C ₂₂ H ₂₈ N ₂ O ₃	369.2183	1.91	ND	ND	ND	ND	1.2E+07	2.2E+05	7.9E+06	7.2E+04	7.9E+05	91.0540, 119.0488,

															151.0750, 234.1477
										2.8E+05	ND	1.5E+05	ND	ND	
A30	Hydroxylation	7.00	C ₂₁ H ₂₆ N ₂ O ₂	339.2063	-1.21	ND	2.9E+05	5.9E+05	7.3E+05	1.2E+07	1.1E+05	2.7E+06	ND	ND	91.0537, 105.0691, 132.0806, 186.1278, 204.1392
										1.1E+07	9.8E+04	1.5E+06	ND	ND	
A31	Hydroxylation + Amide hydrolysis	7.24	C ₁₉ H ₂₄ N ₂ O	297.1954	-3.92	ND	ND	ND	ND	9.8E+04	ND	6.9E+04	ND	4.7E+04	103.0545, 121.0637
										8.5E+04	ND	4.6E+04	ND	3.8E+04	
P	Acetylfentanyl	7.92	C ₂₁ H ₂₆ N ₂ O	323.2120	0.49	2.5E+07	2.4E+07	2.2E+07	2.1E+07	2.5E+07	1.7E+07	2.1E+07	6.7E+05	4.3E+06	105.0697, 132.0801, 188.1434
										2.6E+07	1.7E+07	2.1E+07	7.3E+05	4.8E+06	
A32	Amide hydrolysis	9.09	C ₁₉ H ₂₄ N ₂	281.2009	-1.19	ND	ND	ND	ND	3.8E+05	ND	2.7E+06	ND	4.9E+05	105.0696, 134.0955, 188.1423
										4.0E+04	ND	2.9E+06	ND	5.5E+05	

HYD: hydrolyzed; NON-HYD: non-hydrolyzed; ND: Not detected; RT: retention time, P: parent compound

Table 2: Acrylfentanyl metabolites with proposed biotransformation, retention time, elemental composition, accurate mass of protonated molecule, mass error of proposed metabolite, MS peak areas in hepatocyte samples (0h, 1h, 3h and 5h) and in five urine samples (hydrolyzed and non-hydrolyzed), and diagnostic product ions (masses and mass errors generally taken from sample #3, except for B12 (#2), B1 and B14 (#5) and B10 (hepatocytes).

ID	Biotransformation	RT (min)	Elemental composition	m/z	mass error (ppm)	Peak areas in the hepatocyte samples				Peak areas in the urine samples					Diagnostic ions
						0h	1h	3h	5h	#1 HYD NON-HYD	#2 HYD NON-HYD	#3 HYD NON-HYD	#4 HYD NON-HYD	#5 HYD NON-HYD	
Blood acrylfentanyl concentration (in ng/g)										0.06	1.20	0.10	0.05	0.11	
B1	N-Dealkylation (yielding nor- metabolite)	5.25	C ₁₄ H ₁₈ N ₂ O	231.1503	4.48	5.0E+04	3.5E+06	6.5E+06	8.2E+06	2.0E+06	3.2E+05	1.3E+07	2.1E+06	5.1E+06	84.0804
B2	Dihydrodiol formation (at ethylphenyl)	5.65	C ₂₂ H ₂₈ N ₂ O ₃	369.2172	-0.09	ND	1.3E+05	2.2E+05	2.6E+05	1.5E+06	2.1E+05	1.1E+07	1.2E+06	3.4E+06	
										7.2E+05	3.5E+05	2.4E+06	1.2E+05	6.2E+05	121.0648, 146.0966, 158.0957, 200.1067
B3	Hydroxylation (at ethyl) + Glucuronidation	5.78	C ₂₈ H ₃₄ N ₂ O ₈	527.2381	-0.93	ND	ND	ND	ND	3.4E+05	ND	4.9E+05	3.7E+04	1.3E+05	
										ND	ND	3.4E+05	ND	ND	103.0546, 121.0646, 204.1361, 351.2007
B4	Dihydroxylation + Methylation + Glucuronidation	5.92	C ₂₉ H ₃₆ N ₂ O ₉	557.2497	0.97	ND	ND	ND	ND	1.3E+05	1.3E+05	7.9E+05	2.4E+04	3.0E+05	
										ND	ND	2.1E+05	ND	ND	91.0559, 151.0762, 234.1470
B5	Dihydroxylation (at ethylphenyl) + Glucuronidation	6.01	C ₂₈ H ₃₄ N ₂ O ₉	543.2338	0.29	ND	ND	ND	ND	6.5E+04	1.2E+05	4.5E+05	ND	9.6E+04	
										ND	ND	ND	ND	ND	84.0813, 137.0599, 220.1343
										1.9E+04	ND	1.4E+05	ND	3.7E+04	

B6	Dihydrodiol formation (<i>N</i> -phenyl ring)	6.01	C ₂₂ H ₂₈ N ₂ O ₃	369.2175	0.55	ND	1.9E+05	4.8E+05	7.3E+05	3.5E+05	7.4E+05	3.0E+06	7.4E+05	2.6E+06	105.0696, 188.1428
										2.3E+05	ND	1.3E+06	2.9E+05	1.1E+06	
B7	Dihydroxylation (at <i>N</i> -phenyl or acryl moiety)	6.53	C ₂₂ H ₂₆ N ₂ O ₃	367.2022	1.6	ND	ND	ND	ND	ND	ND	1.7E+05	ND	ND	105.0695, 188.1441
										ND	ND	ND	ND	ND	
B8	Dihydroxylation (at phenethyl moiety)	6.72	C ₂₂ H ₂₆ N ₂ O ₃	367.2028	2.54	ND	ND	ND	ND	8.1E+05	3.3E+05	5.6E+06	1.3E+05	1.5E+06	84.0807, 91.0541, 119.0485, 137.0597, 220.1330
										7.8E+04	6.8E+04	9.9E+05	ND	5.9E+04	
B9	Hydroxylation (at ethyl)	7.28	C ₂₂ H ₂₆ N ₂ O ₂	351.2075	1.8	ND	4.2E+05	6.6E+05	8.6E+05	1.1E+06	1.3E+06	5.4E+06	3.8E+05	3.5E+06	103.0541, 121.0646, 204.1384
										1.1E+05	1.5E+05	3.3E+05	4.9E+04	2.6E+05	
B10	Hydroxylation (at <i>N</i> -phenyl or acryl moiety)	7.47	C ₂₂ H ₂₆ N ₂ O ₂	351.2072	1.13	ND	1.6E+05	1.8E+05	1.9E+05	ND	ND	ND	ND	ND	105.0696, 188.1370
										ND	ND	ND	ND	ND	
B11	Dihydroxylation + Methylation (both at phenyl moiety)	7.54	C ₂₃ H ₂₈ N ₂ O ₃	381.2174	0.33	ND	1.7E+04	2.6E+04	3.3E+04	4.6E+05	1.0E+06	2.9E+06	1.1E+05	8.1E+05	91.0542, 119.0493, 151.0755
										ND	7.0E+04	1.0E+05	ND	ND	
B12	Dihydroxylation + Methylation (both at phenyl moiety)	7.8	C ₂₃ H ₂₈ N ₂ O ₃	381.2179	1.3	ND	ND	ND	ND	ND	7.2E+04	7.9E+04	ND	2.7E+04	91.0532, 119.0879, 151.0736
										ND	ND	ND	ND	ND	
B13	Hydroxylation (at piperidiny)	7.94	C ₂₂ H ₂₆ N ₂ O ₂	351.2071	1.18	ND	3.9E+05	7.5E+05	9.8E+05	ND	2.8E+04	3.5E+05	8.1E+04	2.1E+05	91.0533, 105.0688, 186.1282
										ND	ND	1.3E+05	ND	7.4E+04	
P	Acrylfentanyl	8.85	C ₂₂ H ₂₆ N ₂ O	335.213	2.99	2.6E+07	2.4E+07	2.3E+07	2.1E+07	8.8E+05	1.3E+06	6.6E+06	2.7E+06	7.1E+06	105.0697, 188.1441

										7.0E+05	9.2E+05	5.4E+06	1.7E+06	5.3E+06	
B14	Amide hydrolysis	9.06	C ₁₉ H ₂₄ N ₂	281.2015	1.09	ND	3.1E+04	6.4E+04	7.2E+04	ND	1.6E+04	ND	ND	9.8E+04	105.0687, 188.1433
										ND	ND	2.2E+04	ND	5.6E+04	

HYD: hydrolyzed; NON-HYD: non-hydrolyzed; ND: Not detected; RT: retention time, P: parent compound

Table 3: 4-Fluoro-isobutyrylfentanyl metabolites with proposed biotransformation, retention time, elemental composition, accurate mass of protonated molecule, mass error of proposed metabolite, MS peak areas in hepatocyte samples (0h, 1h, 3h and 5h) and in five urine samples (hydrolyzed and non-hydrolyzed), and diagnostic product ions (masses and mass errors generally taken from sample #3, except for C2 (#1) and C8, C14, C16 and C17 (hepatocytes)).

ID	Biotransformation	RT	Elemental composition	m/z	mass error (ppm)	Peak areas in hepatocytes				Peak areas in urine samples				Diagnostic ions
						0h	1h	3h	5h	#1	#2	#3	#4	
										HYD	HYD	HYD	HYD	
										NON-HYD	NON-HYD	NON-HYD	NON-HYD	
	Blood 4-fluoro-isobutyrylfentanyl concentration (in ng/g)									78	3.2	102	46	
C1	Oxidative N-dealkylation + reduction	4.08	C ₁₃ H ₁₉ NO	206.1543	-0.57	ND	ND	ND	ND	1.2E+06	ND	2.3E+04	ND	105.0698
										1.1E+06	ND	2.2E+04	ND	
C2	Carboxylation	6.64	C ₂₃ H ₂₇ FN ₂ O ₃	399.2101	5.09	ND	ND	ND	ND	1.3E+05	ND	ND	ND	105.0689, 188.1443
										ND	ND	ND	ND	
C3	N-dealkylation	7.23	C ₁₅ H ₂₁ FN ₂ O	265.1710	-0.38	4.9E+04	5.0E+06	8.1E+06	9.2E+06	1.5E+07	3.8E+06	1.2E+07	3.0E+06	84.0806
										1.5E+07	3.8E+06	1.2E+07	3.3E+06	
C4	Dihydrodiol formation (at ethylphenyl)	7.46	C ₂₃ H ₃₁ FN ₂ O ₃	403.2387	-0.51	ND	4.6E+04	8.0E+04	9.8E+04	2.3E+05	2.9E+04	6.9E+05	1.6E+05	121.0639, 150.0701, 164.0874, 207.1286, 234.1250
										2.3E+05	3.0E+04	7.6E+05	1.9E+05	
C5	Hydroxylation + glucuronidation	7.51	C ₂₉ H ₃₇ FN ₂ O ₈	561.2607	-0.11	ND	ND	ND	ND	ND	ND	ND	ND	103.0512, 121.0644, 204.1406
										4.2E+04	1.2E+04	1.7E+05	1.5E+04	
C6	Di-hydroxylation & methylation & glucuronidation	7.6	C ₃₀ H ₃₉ FN ₂ O ₉	591.2714	-0.28	ND	ND	ND	ND	ND	ND	ND	ND	84.0808, 119.0486, 151.0747, 234.1486, 415.2304
										2.9E+04	2.8E+04	2.0E+05	1.2E+04	

C7	Hydroxylation	7.97	C ₂₃ H ₂₉ FN ₂ O ₂	385.2283	-1.02	ND	ND	ND	ND	7.1E+04	ND	1.0E+05	1.9E+04	105.0693, 188.1407
										5.7E+04	ND	5.9E+04	ND	
C8	Hydroxylation	8.28	C ₂₃ H ₂₉ FN ₂ O ₂	385.2294	1.67	ND	7.9E+04	1.5E+05	2.0E+05	ND	ND	ND	ND	105.0698, 188.1434
										ND	ND	ND	ND	
C9	Dihydroxylation	8.44	C ₂₃ H ₂₉ FN ₂ O ₃	401.2231	-0.66	ND	ND	ND	ND	9.1E+04	3.7E+04	1.5E+06	5.0E+04	84.0805, 91.0534, 119.0484, 137.0590, 220.1323
										ND	ND	6.9E+04	1.9E+04	
C10	Hydroxylation	8.95	C ₂₃ H ₂₉ FN ₂ O ₂	385.2284	-0.65	ND	1.7E+05	2.9E+05	4.0E+05	1.0E+06	1.9E+05	3.1E+06	1.7E+05	103.0543, 121.0645, 204.1368
										3.7E+05	4.2E+04	6.3E+05	6.2E+04	
C11	Hydroxylation	9.15	C ₂₃ H ₂₉ FN ₂ O ₂	385.2282	-0.40	ND	ND	ND	ND	8.1E+04	ND	9.2E+04	2.5E+04	84.0804, 121.0642, 204.1368
										ND	ND	ND	ND	
C12	Di-hydroxylation & methylation	9.17	C ₂₄ H ₃₁ FN ₂ O ₃	415.2395	0.62	ND	1.8E+04	3.1E+04	5.3E+04	5.6E+05	4.6E+05	3.5E+06	1.6E+05	84.0810, 91.0537, 119.0493, 151.0751, 234.1474
										8.3E+04	3.3E+04	3.6E+05	3.7E+04	
C13	Di-hydroxylation & methylation	9.37	C ₂₄ H ₃₁ FN ₂ O ₄	415.2393	0.34	ND	ND	ND	ND	ND	ND	7.3E+04	1.3E+04	84.0801, 91.0523, 119.0473, 151.0744, 234.1421
										ND	ND	ND	ND	
C14	Amide hydrolysis	9.40	C ₁₉ H ₂₃ FN ₂	299.1923	1.71	ND	2.7E+04	3.4E+04	8.9E+04	ND	ND	ND	ND	105.0699, 132.0808, 164.0870, 188.1431

										ND	ND	ND	ND	
C15	Hydroxylation	9.56	C ₂₃ H ₂₉ FN ₂ O ₂	385.2280	-1.04	ND	1.7E+06	2.7E+06	3.8E+06	4.0E+06	1.8E+05	1.2E+06	1.6E+05	91.0547, 105.0701, 132.0810, 150.0709, 174.1281, 186.1275, 204.1365
										1.9E+06	1.2E+05	7.9E+05	7.7E+04	
C16	Carbonylation	10.16	C ₂₃ H ₂₇ FN ₂ O ₂	383.2125	-1.12	ND	2.1E+04	2.5E+04	3.3E+04	ND	ND	ND	ND	91.0532, 148.0752, 174.0921, 202.1201
										ND	ND	ND	ND	
P	4-fluoro- isobutyrylfentanyl	10.38	C ₂₃ H ₂₉ FN ₂ O	369.2343	1.10	2.6E+07	2.4E+07	2.2E+07	2.2E+07	1.2E+07	3.6E+05	6.8E+06	1.9E+06	105.0698, 188.1434
										1.2E+07	3.8E+05	7.0E+06	2.2E+06	
C17	Hydroxylation	11.48	C ₂₃ H ₂₉ FN ₂ O ₂	385.2296	2.43	ND	2.0E+05	3.1E+05	3.1E+05	ND	ND	ND	ND	105.0700, 164.0869
										ND	ND	ND	ND	

HYD: hydrolyzed; NON-HYD: non-hydrolyzed; ND: Not detected; RT: retention time, P: parent compound

Table 4: Furanylfentanyl metabolites with proposed biotransformation, retention time, elemental composition, accurate mass of protonated molecule, mass error of proposed metabolite, MS peak areas in hepatocyte samples (0h, 1h, 3h and 5h) and in five urine samples (hydrolyzed and non-hydrolyzed), and diagnostic product ions (masses and mass errors generally taken from sample #5, except for D6, D9, D11, D12 and D13 (hepatocytes)).

ID	Biotransformation	RT (min)	Elemental composition	m/z	mass error (ppm)	Peak areas in hepatocytes				Peak areas in urine samples				Diagnostic ions
						0h	1h	3h	5h	#1	#2	#3	#4*	
										HYD NON-HYD	HYD NON-HYD	HYD NON-HYD	HYD NON-HYD	
	Blood furanylfentanyl concentration (in ng/g)									0.90	0.41	1.10	0.38	
D1	N-dealkylation + Dihydrodiol formation	3.56	C ₁₆ H ₂₀ N ₂ O ₄	305.15	-0.98	ND	ND	2.3E+04	3.2E+04	ND	ND	9.6E+04	1.1E+05	84.0789, 146.0595
										ND	ND	7.1E+04	9.5E+04	
D2	Oxidative N- dealkylation + reduction	4.05	C ₁₃ H ₁₉ NO	206.155	3.11	ND	1.5E+04	2.2E+04	2.3E+04	ND	1.9E+04	7.2E+05	1.5E+05	105.0703, 188.1438
										ND	ND	5.0E+05	9.5E+04	
D3	Amide hydrolysis + Hydroxylation (N- phenyl) + Glucuronidation	4.42	C ₂₅ H ₃₂ N ₂ O ₇	473.229	1.25	ND	ND	ND	ND	ND	ND	ND	ND	105.0699, 188.1405
										ND	ND	6.5E+04	2.0E+05	
D4	Amide hydrolysis + Hydroxylation (N- phenyl)	5.65	C ₁₉ H ₂₄ N ₂ O	297.196	0.99	ND	1.4E+05	9.8E+05	2.4E+06	ND	2.9E+04	5.9E+04	3.9E+05	105.0698, 188.1435
										ND	ND	ND	ND	
D5	Amide hydrolysis + Hydroxylation (N- phenyl) + sulfation	5.77	C ₁₉ H ₂₄ N ₂ O ₄ S	377.153	-0.05	ND	ND	ND	1.2E+04	ND	1.6E+05	6.2E+05	1.3E+06	105.0698, 188.1438
										ND	1.4E+05	5.3E+05	1.1E+06	
D6	N-dealkylation (yielding nor- metabolite)	5.79	C ₁₆ H ₁₈ N ₂ O ₂	271.144	0.52	ND	2.8E+06	4.9E+06	4.0E+06	ND	ND	3.5E+04	ND	84.0810, 95.0128, 188.0694
										ND	ND	2.6E+04	ND	

D7	Dihydrodiol formation + Hydroxylation (ethyl)	5.82	C ₂₄ H ₂₈ N ₂ O ₅	425.207	0.82	ND	1.9E+04	3.8E+04	2.7E+04	ND	2.3E+05	1.6E+05	4.6E+05	103.0562, 121.0649
										ND	1.4E+05	7.9E+04	2.8E+05	
D8	Amide hydrolysis + Hydroxylation (ethyl)	7.19	C ₁₉ H ₂₄ N ₂ O	297.196	-0.03	ND	6.5E+04	2.0E+05	2.6E+05	ND	4.1E+04	1.2E+05	2.6E+05	103.0529, 121.0646
										ND	ND	7.4E+04	1.6E+05	
D9	Furan ring opening + Carboxylation	7.38	C ₂₄ H ₂₈ N ₂ O ₃	393.2175	0.25	ND	ND	ND	3.0E+04	ND	ND	ND	ND	105.0693, 188.1448
										ND	ND	ND	ND	
D10	Dihydrodiol formation	7.5	C ₂₄ H ₂₈ N ₂ O ₄	409.212	-0.34	ND	1.8E+06	3.5E+06	2.9E+06	ND	4.8E+05	2.3E+05	1.3E+06	105.0698, 188.1440
										ND	3.0E+05	1.8E+05	9.5E+05	
D11	Hydroxylation (ethyl)	7.86	C ₂₄ H ₂₆ N ₂ O ₃	391.2018	0.99	ND	4.6E+04	3.7E+04	1.4E+04	ND	ND	ND	ND	121.0649
										ND	ND	ND	ND	
D12	Amide hydrolysis + Hydroxylation (piperidine)	7.99	C ₁₉ H ₂₄ N ₂ O	297.1963	0.96	ND	3.3E+05	1.1E+06	1.3E+06	ND	ND	ND	ND	91.0539, 105.0690, 132.0818
										ND	ND	ND	ND	
D13	Hydroxylation (probably at piperidine)	8.52	C ₂₄ H ₂₆ N ₂ O ₃	391.2024	1.65	ND	4.1E+05	4.3E+05	1.8E+05	ND	ND	ND	ND	91.0538, 132.0792
										ND	ND	ND	ND	
D14	Amide hydrolysis	9.05	C ₁₉ H ₂₄ N ₂	281.202	1.22	2.0E+04	1.0E+07	1.7E+07	1.8E+07	5.9E+04	4.9E+05	5.4E+05	3.8E+06	105.0697, 188.1433
										ND	3.5E+05	3.8E+05	2.9E+06	
P	Furanylfentanyl	9.38	C ₂₄ H ₂₆ N ₂ O ₂	375.207	0.62	2.5E+07	2.1E+07	1.6E+07	1.0E+07	1.6E+04	3.8E+05	6.7E+04	2.2E+06	105.0699, 188.1433
										1.5E+04	2.9E+05	1.8E+05	1.6E+06	

HYD: hydrolyzed; NON-HYD: non-hydrolyzed; ND: Not detected; RT: retention time, P: parent compound

* The corresponding blood sample of urine #4 contained 1.2 ng/g fentanyl

Figures

Fig. 1

Approved medications	'Old' analogs	'New' analogs
<p>fentanyl</p>	<p>3-methylfentanyl</p>	<p>acetylfentanyl</p>	<p>furanylfentanyl</p>
<p>alfentanil</p>	<p>β-hydroxyfentanyl</p>	<p>acrylfentanyl</p>	<p>4F-isobutyrylfentanyl</p>
<p>sufentanil</p>	<p>α-methylfentanyl</p>	<p>butyrylfentanyl*</p>	<p>β-hydroxythiofentanyl</p>
<p>remifentanyl</p>	<p>isofentanyl</p>	<p>isobutyrylfentanyl*</p>	<p>ocfentanyl</p>
<p>carfentanyl (for veterinary use)</p>		<p>* Different butyrylfentanyl and isobutyrylfentanyl analogs with fluoro, chloro or methoxy substituents in 4-position have been detected as well.</p>	

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Supplementary figures

Supplementary Fig. 1: MSMS spectra and proposed fragmentation pattern of acetylfentanyl and its major metabolites in hydrolyzed human urine samples.

Supplementary Fig. 2: MSMS spectra and proposed fragmentation pattern of acrylfentanyl and its major metabolites in hydrolyzed human urine samples.

Supplementary Fig. 3: MSMS spectra and proposed fragmentation pattern of 4-fluoroisobutyrylfentanyl and its major metabolites in hydrolyzed human urine samples.

Supplementary Fig. 4: MSMS spectra and proposed fragmentation pattern of furanylfentanyl and its major metabolites in hydrolyzed human urine samples.