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Structure Elucidation of Urinary Metabolites of Fentanyl and Five Fentanyl Analogs using LC-QTOF-MS, Hepatocyte Incubations and Synthesized Reference Standards

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Abstract

Fentanyl analogs constitute a particularly dangerous group of new psychoactive compounds responsible for many deaths around the world. Little is known about their metabolism, and studies utilizing liquid chromatography–quadrupole time-of-flight mass spectrometry (LC–QTOF-MS) analysis of hepatocyte incubations and/or authentic urine samples do not allow for determination of the exact metabolite structures, especially when it comes to hydroxylated metabolites. In this study, seven motifs (2-, 3-, 4- and β-OH as well as 3,4-diOH, 4-OH-3-OMe and 3-OH-4-OMe) of fentanyl and five fentanyl analogs, acetylfentanyl, acrylfentanyl, cyclopropylfentanyl, isobutyrylfentanyl and 4F-isobutyrylfentanyl were synthesized. The reference standards were analyzed by LC–QTOF-MS, which enabled identification of the major metabolites formed in hepatocyte incubations of the studied fentanyls. By comparison with our previous data sets, major urinary metabolites could tentatively be identified. For all analogs, β-OH, 4-OH and 4-OH-3-OMe were identified after hepatocyte incubation. β-OH was the major hydroxylated metabolite for all studied fentanyls, except for acetylfentanyl where 4-OH was more abundant. However, the ratio 4-OH/β-OH was higher in urine samples than in hepatocyte incubations for all studied fentanyls. Also, 3-OH-4-OMe was not detected in any hepatocyte samples, indicating a clear preference for the 4-OH-3-OMe, which was also found to be more abundant in urine compared to hepatocytes. The patterns appear to be consistent across all studied fentanyls and could serve as a starting point in the development of methods and synthesis of reference standards of novel fentanyl analogs where nothing is known about the metabolism.

Introduction

Fentanyl is a selective μ-opioid agonist first developed by Janssen and is used therapeutically for its analgesic properties (1, 2). The use of fentanyl has expanded to the recreational drug market and poses a serious threat as it can, if overdosed, cause respiratory depression followed by apnea (3). To further complicate the issue, there has been an upsurge in the number of novel fentanyl analogs in recent years (4). These fentanyl analogs constitute a particularly dangerous group of new psychoactive substances (NPS) responsible for numerous deaths globally (5–11). However, estimating the magnitude of the impact of fentanyl analogs on public health is difficult as they are often not included in routine toxicological screening, and some laboratories lack the capability to detect the more uncommon analogs (5, 12).

The prevalence of fatal poisonings associated with fentanyl analogs was reviewed by Kronstrand et al. (13). The analogs in focus of this study were selected in part due to their toxicity and prevalence in Sweden. Acetylfentanyl was associated with 27 deaths in Sweden (6), acrylfentanyl with 43 (7, 14), and cyclopropylfentanyl with 74 (8). While no literature exists for deaths associated with isobutyryl fentanyl in Sweden, the fluorinated analog 4F-isobutyrylfentanyl has been associated with 16 deaths in Sweden alone (9). Apart from Sweden, another 17 deaths were reported in Europe associated with these analogs (6–9).

Studies on the toxicology of fentanyl analogs in particular are generally both scarce and limited (6–11). The investigation of the metabolism of fentanyl analogs plays a crucial part in understanding the toxicokinetics and toxicodynamics as active metabolites can both prolong the effect of the drug and contribute to toxicity (15). Abundant metabolites can also serve as urinary biomarkers in forensic routine analysis to prove drug intake, sometimes extending the window of detection for that analyte (16).

The identification and structural determination of major metabolites are the first steps towards producing the reference standards needed to include them in analytical methods. Traditionally, the structural characterization of metabolites for fentanyl analogs and other NPS has been conducted using LC–QTOF-MS analysis of either incubation with human liver microsomes or hepatocytes, and/or urine samples from forensic toxicology case work (17–28).

Fentanyl metabolism is extensive and fast. Less than 8% of fentanyl is excreted unchanged in urine and feces (28). Most is eliminated in the form of norfentanyl in urine (17). Minor metabolites include hydroxyfentanyl and hydroxynorfentanyl on the propionyl side chain (18) and 4-hydroxyfentanyl on the phenethyl moiety (17). Kanamori et al. (19) identified norfentanyl, two monohydroxylated metabolites on the propionyl moiety (ω and ω-1), 4-OH, β-OH and 4-OH-3-OMe on the phenethyl moiety using hepatocytes.

Regarding fentanyl analogs, the amount of available information regarding their metabolism varies between different analogs. We previously studied acetylfentanyl, acrylfentanyl and 4F-isobutyrylfentanyl using both hepatocytes and authentic urine samples (20).

For acetylfentanyl, the nor-metabolite, a monohydroxy and a hydroxymethoxy metabolite on the phenethyl moiety were identified as major metabolites in both urine and hepatocytes (20). Kanamori et al. (19) found noracetylfentanyl 4-OH, β-OH and 4-OH-3-OMe, as well as one metabolite hydroxylated on the acetyl side chain using hepatocytes. Melent'ev et al. (21) reported the most abundant metabolites in urine to be a hydroxy and a hydroxymethoxy metabolite using GC-MS.

For acrylfentanyl, the nor-metabolite, a monohydroxy, a dihydroxy and a hydroxymethoxy metabolite on the phenethyl moiety were identified as major metabolites in both urine and hepatocytes (20).

Regarding cyclopropylfentanyl, we have previously shown that the nor-metabolite and two metabolites modified on the phenethyl moiety (4-OH-3-OMe and 3,4-diOH) are major metabolites in urine samples (22, 27). The nor-metabolite was also identified in urine by Palaty et al. (23). Furthermore, Cutler and Hudson (24) found the nor-metabolite, two monohydroxylated metabolites and one dihydroxylated metabolite in blood.

To the best of our knowledge, no data have been published on isobutyrylfentanyl metabolites in urine samples. However, two studies on the metabolism of butyrylfentanyl, a closely related analog, have been published. Kanamori et al. (25) reported the nor-metabolite, ω-OH (terminal hydroxylation of the butyl side chain), ω-1-OH and β-OH as major metabolites after hepatocyte incubations. Data from a urine sample were reported by Steuer et al. (26) indicating ω-OH and a metabolite hydroxylated on the phenethyl moiety and a metabolite carboxylated on the butyl sidechain as the major phase I metabolites prior to hydrolysis.

Major metabolites of 4F-isobutyrylfentanyl in urine and after hepatocyte incubation were reported to be the nor-metabolite as well as two monohydroxylated metabolites. In urine, a methylated catechol metabolite was also among the most abundant metabolites (20).

In most of these studies, the major metabolites were identified, but their exact structures could not be determined. This is a result of the interpretation being limited to relying solely on MSMS spectra elucidation, making the differentiation between some metabolite isomers difficult or impossible.

To address this gap in knowledge regarding the metabolism of fentanyl analogs, the aim of this study was to determine the exact structure of major metabolites of fentanyl and five fentanyl analogs, namely acetylfentanyl, acrylfentanyl, cyclopropylfentanyl, isobutyrylfentanyl and 4F-isobutyrylfentanyl (Figure 1). Additionally, metabolite patterns among different analogs were studied and summarized, which could be beneficial in studies of future novel fentanyl analogs. The exact structures of metabolites formed after hepatocyte incubations were determined using in-house synthesized reference standards and LC–QTOF-MS. Based on the above-mentioned metabolism research, (17–27) the phenethyl moiety was chosen as a target of interest. Seven motifs were chosen with modification of the phenethyl moiety: four monohydroxylated metabolites (4-OH, 3-OH, 2-OH and β-OH), the catechol 3,4-dihydroxy (3,4-diOH) and the singly methylated products of the catechol (4-OH-3-OMe and 3-OH-4-OMe). Although urine samples were only analyzed in the case of cyclopropylfentanyl in the present study, major urinary metabolites could be identified through re-analysis of data from earlier studies (20, 22).

Methods

Cryopreserved hepatocytes were used to produce metabolites of fentanyl and five fentanyl analogs (acetyl-, acryl-, cyclopropyl-, isobutyryl- and 4F-isobutyrylfentanyl) by incubation for 5 h. Targeted metabolites in the hepatocyte incubations were analyzed using LC–QTOF-MS and compared to analytical data of in-house synthesized reference standards of potential metabolites. Additionally, the data from the hepatocyte incubations were

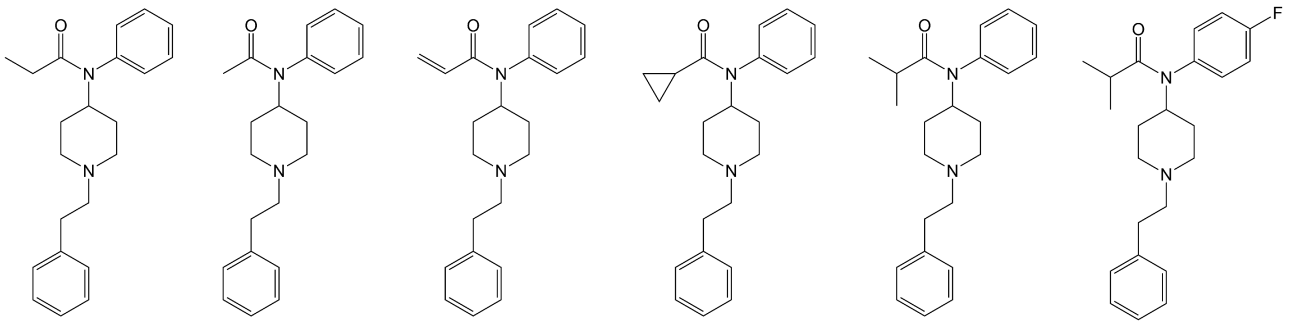

Figure 1. Structures of fentanyls. From left to right: fentanyl, acetylfentanyl, acrylfentanyl, cyclopropylfentanyl, isobutyrylfentanyl and 4F-isobutyrylfentanyl.

matched to data from hepatocyte incubations in earlier studies allowing for exact identification of urinary metabolites in those studies.

Metabolite generation using hepatocytes and sample preparation

In order to generate metabolites, the drugs were incubated in triplicate (except isobutyrylfentanyl $n = 2$) using human hepatocytes for a period of 5 h making use of a slightly modified setup of the procedure used by Watanabe et al. (20). The cryopreserved hepatocytes were thawed at 37°C and added into inVitro Gro HT thawing medium (48 mL). The hepatocytes were pelleted using centrifugation at 100 g for 5 min at room temperature before the supernatant was aspirated. Subsequently, the pellet was re-suspended in Williams E medium (50 mL, supplemented with L-glutamine, 2 mM and HEPES, 20 mM). The suspension was centrifuged at 60 g for 5 min at room temperature, and the resulting supernatant was aspirated. The pellet was re-suspended in Williams E medium (2 mL). The cell concentration of the suspension was determined by the Trypan blue exclusion method and adjusted to 2×10^6 cells/mL. To a 96-well plate, aliquots of the cell suspension (50 µL) were added together with drug solutions in medium (50 µL, 10 µM) resulting in a final substrate mixture (100 µL, 5 µM, 1×10^6 cells/mL). Organic solvent content was ≤0.2%. The substrate mixtures were incubated for 5 h at 37°C. The reactions were quenched by the addition of ice-cold acetonitrile (100 µL) prior to the storage of the 96-well plate in the freezer (−20°C, ≥10 min) to ensure complete protein precipitation. The plate was then centrifuged (15 min, 1100 g, 4°C), and 100 µL aliquots of the supernatant were transferred to an injection plate.

In addition to the samples, sets of controls were produced: a positive control containing diclofenac in duplicate, a negative control without drug in duplicate, a degradation control without hepatocytes and a 0 h sample where the cell suspension was added to the acetonitrile and drug solution.

Reference standard synthesis and sample preparation

Based on preliminary data from urinary analysis and previously published hepatocyte metabolites (17–27), seven motifs were selected for synthesis across the six different fentanyls: four motifs monohydroxylated on the phenethyl moiety (4-OH, 3-OH, 2-OH and β-OH), the catechol 3,4-dihydroxyphenethyl and the two different O-methylation products of the catechol. The nor-metabolites were synthesized but not purified as they can be unambiguously identified by their accurate masses and MS-MS spectra.

TLC was performed using 0.25 mm precoated silica-gel plates (Merck 60 F_{254}), detection by UV-abs at 254 nm. Flash chromatography was performed using the following silica gel: high purity grade (Merck Grade 9385), pore size 60 Å and 230–240 mesh particle size. Analytical liquid chromatography was performed on a Waters system equipped with a Waters 1525 gradient pump, 2998 photodiode array detector, 2424 evaporative light scattering detector, SQD 2 Mass Detector and an Xbridge® C18 column (4.6 × 50 mm, 3.5 µm). Flow rate 1.5 mL/min. A binary linear gradient of A/B 80:20 → A/B 0:100 over 4 min followed by a hold time of 2 min was used. The mobile phases were comprised of A (95:5 H_2O/acetonitrile, 10 mM ammonium acetate) and B (90:10 acetonitrile/H_2O, 10 mM ammonium acetate). Preparative liquid chromatography was performed on a Waters system equipped with a 2535 quaternary gradient pump, 2998 Photodiode Array Detector, 2424 Evaporative Light Scattering Detector, SQD 2 Mass Detector and an xselect® phenyl-hexyl column (19 × 250 mm, 5.0 µm). A flow rate of 25 mL/min was used. A binary linear gradient of A/B 80:20 → A/B 0:100 over 8 min followed by a hold time of 4 min was used. The mobile phases were comprised of A (95:5 H_2O/acetonitrile, 10 mM ammonium acetate) and B (90:10 acetonitrile/H_2O, 10 mM ammonium\uline{a} acetate).

^1H, ^{13}C-NMR spectra were recorded on a Varian Mercury 300 MHz instrument (25°C in $CDCl_3$ or CD_3OD).

A general synthetic route was developed to produce the metabolites of interest (Figure 2). As a first step, the amine of 4-piperidone was boc-protected using di-*tert*-butyl decarbonate and NaOH in a solvent mixture comprising water:tetrahydrofuran (1:1). The resulting carbamate underwent reductive amination by the addition of either aniline or 4-fluoroaniline, acetic acid and $Na(CH_3COO)_3BH$ in dichloromethane to form *tert*-butyl 4-anilinopiperidine-1-carboxylate. Subsequently, the free amine was acylated using the corresponding acyl chloride together with *N*,*N*-diisopropylethylamine in dichloromethane to create the nor-metabolite of the different analogs. These compounds were used as scaffolds for the synthesis of the seven different motifs of fentanyl and five of its analogs. N-alkylation using the corresponding bromide with Cs_2CO_3 in acetonitrile was used as part of the final steps. The bromides were either purchased or synthesized in-house. The last steps in the synthetic route diverged depending on the motif. One synthesis route for a bromide was reduction followed by an Appel reaction prior to reaction with the different nor-metabolites (**A5-F5** and **A6-F6**). Another route was modification after N-alkylation either by reduction using $NaBH_4$ (**A4-F4**) or by debromination using BBr_3 under N_2(g) (**A2-3-F2-3**) (Figure 2).

Stock solutions of the synthesized compounds (1 mg/mL) were prepared in methanol. The stock solutions were diluted using a 50/50

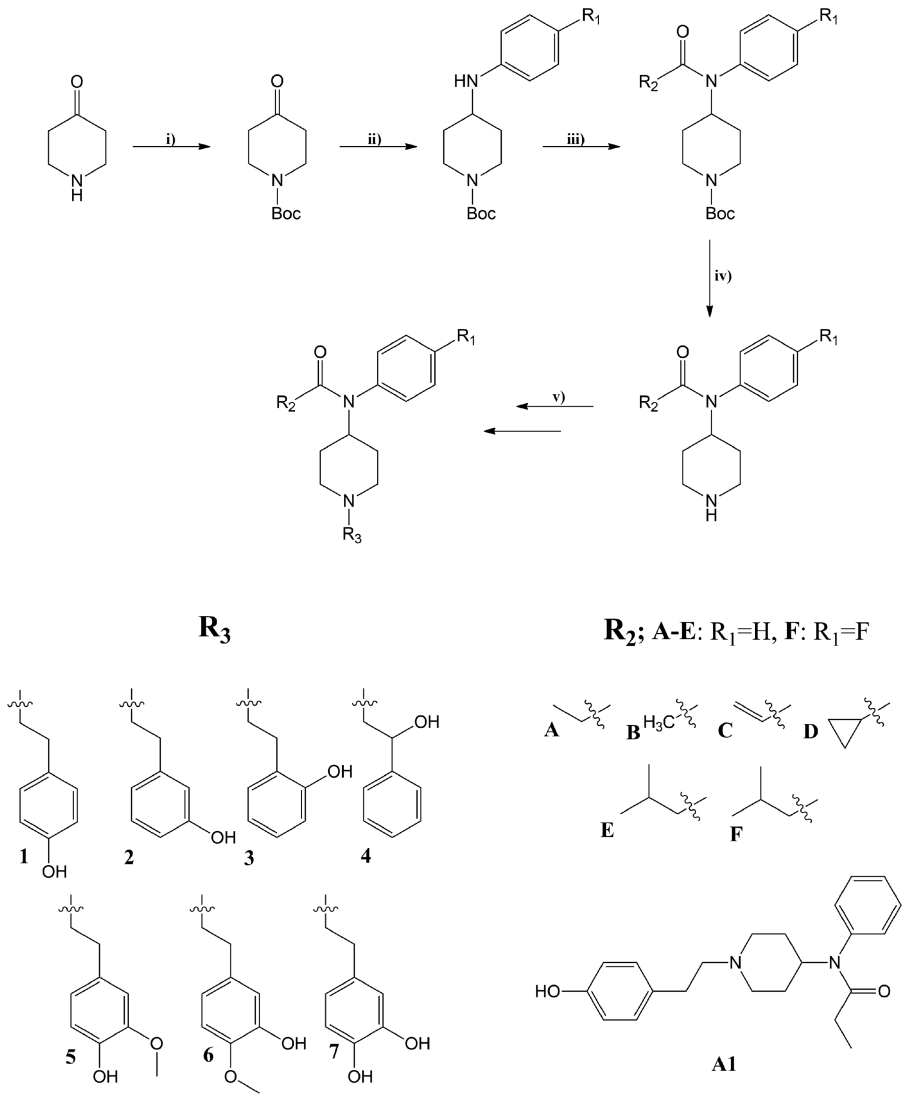

Figure 2. General synthetic route. (i) Boc_2O, sodium hydroxide, water:tetrahydrofuran (1:1), rt, 72 h; (ii) aniline or 4-fluoroaniline, acetic acid, $Na(OAc)_3BH$, dichloromethane, rt, 16 h; (iii) corresponding acyl chloride, *N*,*N*-diisopropylethylamine, dichloromethane, rt, 16 h; (iv) dichloromethane:trifluoroacetic acid (5:1), rt, 1 h; (v) corresponding bromide, Cs_2CO_3, acetonitrile, 60°C, 16 h. The letters A–F correspond to the different fentanyl analogs: fentanyl (A), acetylfentanyl (B), acrylfentanyl (C), cyclopropylfentanyl (D), isobutyrylfentanyl (E) and 4-fluoroisobutyrylfentanyl (F). The numbers 1–7 correspond to the different motifs: 4-OH (1), 3-OH (2), 2-OH (3), β-OH (4), 4-OH-3-OMe (5), 3-OH-4-OMe (6) and 3,4-DiOH (7). Thus, 4-OH fentanyl is denoted as A1.

mixture of LC–QTOF mobile phases A and B (see details below) to prepare the samples (100 ng/mL).

LC–QTOF-MS analysis

The chromatographic system included an Agilent 1290 Infinity ultrahigh-performance liquid chromatography system (Kista, Sweden) and an Agilent 6550 iFunnel QTOF. Five-microliter injections of samples and reference standards were separated using an Acquity HSS T3 column (150 × 2.1 mm, 1.8 µm) (Waters, Sollentuna, Sweden) fitted with an Acquity VanGuard precolumn at 60°C. A binary mobile phase system comprising 0.05% formic acid and 10 mM ammonium formate in water (A) and 0.05% formic acid in acetonitrile (B) was delivered at 0.5 mL/min. For fentanyl, acetylfentanyl, acrylfentanyl and 4F-isobutyrylfentanyl, the gradient began with a hold of 1% B for 0.6 min, followed by a first ramp to 5% B at 0.7 min, followed by a second ramp to 40% B at 13 min, followed by a third ramp to 95% B at 15 min, which was penultimately held until 18 min before going back to 1% B for re-equilibration until 19 min. For isobutyrylfentanyl and cyclopropylfentanyl, the gradient began with a hold of 1% B for 0.6 min, followed by a first ramp up to 25% B at 0.7 min, followed by a second ramp to 65% B at 13 min, followed by a third ramp to 95% B at 15 min, which was penultimately held until 18 min before going back to 1% B for re-equilibration until 19 min. The gradients were chosen with the aim to elute the parent compound between 8 and 13 min.

The QTOF was run in positive electrospray ionization mode (gas temperature 150°C, gas flow 18 L/min, nebulizer 50 psig, sheath gas temperature 375°C, sheath gas flow 11 L/min). Mass spectrometric data were acquired using Data Dependent Auto MS-MS (fragmentor voltage 380 V, collision energy 3 eV at 0 m/z ramped up by 8 eV per 100 m/z, scan rate 6 spectra/s (MS) and 10 spectra/s (MS-MS),

scan range 100–950 m/z (MS) and 50–950 m/z (MS/MS), precursor intensity threshold 5000 counts, precursor number per cycle 5 within 200–800 m/z).

Data analysis

Data analysis of the LC–QTOF-MS data was performed using MassHunter Qualitative Analysis. A library containing the molecular formulae of the studied motifs (monohydroxylated, dihydroxylated and hydroxymethoxylated) and nor-metabolites was used to identify peaks of interest.

Analytes were identified and matched between samples based on the chromatographic peak shape (visual assessment), accurate mass (±5 ppm unless saturated), retention times and MS-MS spectra (fragment mass and relative intensity). Furthermore, peaks also identified in the 0 h samples, and/or the degradation controls were disregarded.

For cyclopropylfentanyl, reference standards were included in the same run as the urine samples (22), but as the reference standards were not available at the time of analysis, this was not true for acetyl-, acryl- and 4F-isobutyrylfentanyl (20). The urine samples were not rerun in the current data set as they were either discarded or stored for several years. Instead, metabolite matching was based on reanalysis of the old data sets. Peaks were matched between the data sets based on their accurate mass, MS-MS spectra, abundance in hepatocyte samples and a linear regression analysis of the retention times. Three motifs (3-OH, 4-OH-3-OMe and 3,4-diOH) were almost exclusively observed in the urine samples and therefore matched to the reference standards without considering hepatocyte abundance.

Prior to 25 May 2018, the use of case work data and samples within the National Board of Forensic Medicine was not regulated, and therefore, no ethics approval was necessary. After that date, all research is approved by the regional ethics committee in Linköping (Approval Number 2018-186/31).

Materials

Reference standards of acetylfentanyl, acrylfentanyl, cyclopropylfentanyl, isobutyrylfentanyl and 4F-isobutyrylfentanyl were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Fentanyl was obtained from Cerilliant (Round Rock, TX, USA). Cryopreserved human hepatocytes (LiverPool, 10 donor pool) and inVitro Gro HT thawing medium were acquired from Bioreclamation IVT (Brussels, Belgium). L-glutamine, HEPES buffer and Williams E medium were procured from ThermoFisher Scientific. Diclofenac was purchased from Sigma-Aldrich (Stockholm, Sweden).

LC–MS grade acetonitrile, water and formic acid used for the LC–QTOF-MS were obtained from Fisher Scientific (Gothenburg, Sweden), whereas ammonium formate (Fluka) was acquired from Sigma-Aldrich (Stockholm, Sweden).

Chemicals and solvents used in the synthesis of the reference standards were obtained from Sigma-Aldrich (Stockholm, Sweden) apart from 4-piperidone monohydrate hydrochloride, which was purchased from Merck (Hohenbrunn, Germany).

Results

Synthesized reference standards of fentanyl (**A**), acetylfentanyl (**B**), acrylfentanyl (**C**), cyclopropylfentanyl (**D**), isobutyrylfentanyl (**E**) and 4F-isobutyrylfentanyl (**F**) metabolites with seven different motifs (**1**–**7**) were cross-checked with metabolites produced by hepatocytes using LC–QTOF-MS. In total, 20 out of the 42 synthesized reference standards were identified in the hepatocyte incubations using accurate mass, retention time and MS-MS spectra as tools for identification (Table I). The abundance of different metabolites in hepatocyte incubations and urine samples is shown in Table II. Data from urine samples were adopted from Watanabe et al. (20) and Vikingsson et al. (22). The acetyl-, acryl-, cyclopropyl- and 4F-isobutyrylfentanyl urinary metabolites were identified based on relative retention time, abundance, diagnostic ions and accurate mass.

Retention time order and regression analysis

The order of elution of metabolites with different motifs was consistent across the six fentanyls (Table I). The first motif to elute was always the catechol, 3,4-diOH (**7**), followed by 4-OH (**1**) and 3-OH (**2**). Thereafter, the methylated catechols eluted, among which 4-OH-3-OMe (**5**) eluted before 3-OH-4-OMe (**6**). The last metabolites to elute were β-OH (**4**) followed by 2-OH (**3**).

Retention time regression was conducted for acetyl, acryl and 4F-isobutyryl fentanyl using a linear model including the retention times for the motifs observed after hepatocyte incubation as well as the nor-metabolites (n = 12). The observed error between predicted and observed retention times in the Watanabe data set for all motifs (n = 19) ranged from −0.05 to 0.05 min. Given the observed separation of metabolite analogs, this indicates a low risk of incorrect metabolite identification in the urine samples due to retention time shift. This is further corroborated by the agreement between the data identified by this procedure and the findings of cyclopropylfentanyl by Vikingsson et al. (22) where the urine samples were analyzed together with the reference standards.

Fentanyl

Out of the monohydroxylated metabolites, 4-OH (**A1**) and β-OH (**A4**) were identified in hepatocytes, while 3-OH (**A2**) and 2-OH (**A3**) were not (Figure 3). The methylated catechol 4-OH-3-OMe (**A5**) was identified in hepatocytes while its isomer 3-OH-4-OMe (**A6**) was not. Lastly, the catechol 3,4-diOH (**A7**) was not present in the hepatocyte incubations. Two additional monohydroxylated metabolites, 2 and 44% of the peak area of β-OH (**A4**), were observed at 7.14 and 7.46 min, respectively (Figure 3). Both MS-MS spectra contained fragments m/z 188 and 105 indicating that these metabolites could be the ω-OH and the ω-1-OH as reported by Kanamori et al. (19).

Acetylfentanyl

For acetylfentanyl, the metabolites positively found in hepatocytes were the monohydroxylated metabolites 4-OH (**B1**), 3-OH (**B2**) and β-OH (**B4**) (Figure 3) as well as the methylated catechol 4-OH-3-OMe (**B5**) and the catechol 3,4-diOH (**B7**). Out of the seven synthesized metabolites, only 2-OH (**B3**) and 3-OH-4-OMe (**B6**) were not identified in hepatocyte incubations.

Acrylfentanyl

Two monohydroxylated metabolites (4-OH (**C1**) and β-OH (**C4**)) were identified in hepatocyte incubations (Figure 3). Additionally, one methylated catechol in 4-OH-3-OMe (**C5**) was also positively confirmed in the hepatocyte incubations. The two remaining monohydroxylated metabolites 3-OH (**C2**) and 2-OH (**C3**), the methylated catechol 3-OH-4-OMe (**C6**) and the catechol 3,4-diOH (**C7**) were not positively matched upon comparison with the metabolically produced metabolites using hepatocyte incubations.

Table I. Summary of the Synthesized Reference Standards along with their Chemical Formulae, Retention Times (RT) in the LC–QTOF-MS Analysis and Mass-to-Charge Ratios (*m/z*)

Compound	Chemical formula	RT (min)	*m/z*
Fentanyl Nor	**C14 H20 N2 O**	**5.83**	**233.1662**
Fentanyl 4-OH (A1)	**C22 H28 N2 O2**	**7.80**	**353.2231**
Fentanyl 3-OH (A2)	C22 H28 N2 O2	8.06	353.2230
Fentanyl 2-OH (A3)	C22 H28 N2 O2	8.66	353.2233
Fentanyl β-OH (A4)	**C22 H28 N2 O2**	**8.48**	**353.2224**
Fentanyl 4-OH-3-OMe (A5)	**C23 H30 N2 O3**	**8.06**	**383.2333**
Fentanyl 4-OMe-3OH (A6)	C23 H30 N2 O3	8.30	383.2331
Fentanyl 3,4-DiOH (A7)	C22 H28 N2 O3	7.24	369.2176
Acetylfentanyl Nor	**C13 H18 N2 O**	**4.36**	**219.1501**
Acetylfentanyl 4-OH (B1)	**C21 H26 N2 O2**	**6.45**	**339.2070**
Acetylfentanyl 3-OH (B2)	**C21 H26 N2 O2**	**6.76**	**339.2068**
Acetylfentanyl 2-OH (B3)	C21 H26 N2 O2	7.41	339.2075
Acetylfentanyl β-OH (B4)	**C21 H26 N2 O2**	**7.20**	**339.2072**
Acetylfentanyl 4-OH-3-OMe (B5)	**C22 H28 N2 O3**	**6.76**	**369.2176**
Acetylfentanyl 3-OH-4-OMe (B6)	C22 H28 N2 O3	7.04	369.2171
Acetylfentanyl 3,4-DiOH (B7)	**C21 H26 N2 O3**	**5.89**	**355.2018**
Acrylfentanyl Nor	**C14 H18 N2 O**	**5.48**	**231.1505**
Acrylfentanyl 4-OH (C1)	**C22 H26 N2 O2**	**7.51**	**351.2071**
Acrylfentanyl 3-OH (C2)	C22 H26 N2 O2	7.77	351.2071
Acrylfentanyl 2-OH (C3)	C22 H26 N2 O2	8.41	351.2073
Acrylfentanyl β-OH (C4)	**C22 H26 N2 O2**	**8.15**	**351.2065**
Acrylfentanyl 4-OH-3-OMe (C5)	**C23 H28 N2 O3**	**7.78**	**381.2190**
Acrylfentanyl 3-OH-4-OMe (C6)	C23 H28 N2 O3	8.04	381.2193
Acrylfentanyl 3,4-DiOH (C7)	C22 H26 N2 O3	6.93	367.2016
Cyclopropylfentanyl Nor	**C15 H20 N2 O**	**8.11**	**245.1679**
Cyclopropylfentanyl 4-OH (D1)	**C23 H28 N2 O2**	**10.32**	**365.2227**
Cyclopropylfentanyl 3-OH (D2)	C23 H28 N2 O2	10.59	365.2225
Cyclopropylfentanyl 2-OH (D3)	C23 H28 N2 O2	11.28	365.2225
Cyclopropylfentanyl β-OH (D4)	**C23 H28 N2 O2**	**11.07**	**365.2227**
Cyclopropylfentanyl 4-OH-3-OMe (D5)	**C24 H30 N2 O3**	**10.61**	**395.2330**
Cyclopropylfentanyl 3-OH-4-OMe (D6)	C24 H30 N2 O3	10.87	395.2331
Cyclopropylfentanyl 3,4-DiOH (D7)	C23 H28 N2 O3	9.70	381.2174
Isobutyrylfentanyl Nor	**C15 H22 N2 O**	**8.86**[a]	**247.1842**[a]
Isobuturylfentanyl 4-OH (E1)	**C23 H30 N2 O2**	**10.93**	**367.2387**
Isobuturylfentanyl 3-OH (E2)	C23 H30 N2 O2	11.18	367.2381
Isobuturylfentanyl 2-OH (E3)	C23 H30 N2 O2	11.84	367.2377
Isobuturylfentanyl β-OH (E4)	**C23 H30 N2 O2**	**11.64**	**367.2387**
Isobuturylfentanyl 4-OH-3-OMe (E5)	**C24 H32 N2 O3**	**11.20**	**397.2495**
Isobuturylfentanyl 3-OH-4-OMe (E6)	C24 H32 N2 O3	11.43	397.2487
Isobuturylfentanyl 3,4-DiOH (E7)	C23 H30 N2 O3	10.31	383.2328
4F-Isobuturylfentanyl Nor	**C15 H21 F N2 O**	**7.49**	**265.1727**
4F-Isobuturylfentanyl 4-OH (F1)	**C23 H29 F N2 O2**	**9.21**	**385.2285**
4F-Isobuturylfentanyl 3-OH (F2)	C23 H29 F N2 O2	9.44	385.2285
4F-Isobuturylfentanyl 2-OH (F3)	C23 H29 F N2 O2	10.01	385.2283
4F-Isobuturylfentanyl β-OH (F4)	**C23 H29 F N2 O2**	**9.85**	**385.2283**
4F-Isobuturylfentanyl 4-OH-3-OMe (F5)	**C24 H31 F N2 O3**	**9.44**	**415.2393**
4F-Isobuturylfentanyl 3-OH-4-OMe (F6)	C24 H31 F N2 O3	9.63	415.2386
4F-Isobuturylfentanyl 3,4-DiOH (F7)	C23 H29 F N2 O3	8.69	401.2232

Metabolites in bold identified in hepatocytes. Retention times and *m/z* values from reference materials except for normetabolites where it is the average after hepatocyte incubation ($n = 3$). RT, retention time.
[a] $n = 2$.

Cyclopropylfentanyl

For cyclopropylfentanyl, the monohydroxylated metabolites 4-OH (**D1**) and β-OH (**D4**) (Figure 3) and the methylated catechol 4-OH-3-OMe (**D5**) were successfully identified in hepatocyte incubations. The remaining metabolites, 3-OH (**D2**), 2-OH (**D3**), 3-OH-4-OMe (**D6**) and the catechol 3,4-diOH (**D7**), were not present in the hepatocyte incubations.

Isobutyrylfentanyl

Two out of the four monohydroxylated metabolites, 4-OH (**E1**) and β-OH (**E4**), were identified in hepatocyte incubations (Figure 3).

Table II. Metabolite Abundances Normalized to the Most Abundant Monohydroxylated Metabolite

Hepatocyte metabolite abundance (average, n = 3)	Fentanyl	Acetyl	Acryl	Cyclopropyl	Isobutyryl[a]	FIBF
Nor	486%	215%	877%	906%	355%	366%
4-OH	43%	100%	82%	33%	7%	7%
3-OH	ND	3%	ND	ND	ND	ND
2-OH	ND	ND	ND	ND	ND	ND
β-OH	100%	33%	100%	100%	100%	100%
4-OH-3-OMe	3%	12%	5%	2%	1%	1%
3-OH-4-OMe	ND	ND	ND	ND	ND	ND
3,4-DiOH	ND	1%	ND	ND	ND	ND

Urinary metabolite abundance (median of all samples detected)	Acetyl	Acryl	Cyclopropyl	FIBF
Nor	86%	182%	357%	1632%
4-OH	100%	100%	100%	100%
3-OH	ND	ND	ND	8%
2-OH	ND	ND	ND	ND
β-OH	13%	6%	24%	94%
4-OH-3-OMe	100%	42%	52%	104%
3-OH-4-OMe	33%	1%	4%	5%
3,4-DiOH	68%	43%	37%	24%

Urinary data adopted from Watanabe et al. (20) and Vikingsson et al. (22). Data normalized to the most abundant monohydroxylated metabolite.

[a] n = 2.

One of the methylated catechols, 4-OH-3-OMe (**E5**), was also present. 3-OH (**E2**), 2-OH (**E3**), 3-OH-4-OMe (**E6**) and the catechol 3,4-diOH (**E7**) were not found in the hepatocyte incubation of isobutyrylfentanyl.

4F-isobutyrylfentanyl

The positively identified metabolites in hepatocyte incubations comprised two monohydroxylated metabolites (4-OH (**F1**) and β-OH (**F4**)) (Figure 3) and one methylated catechol in 4-OH-3-OMe (**F5**). The other analyzed metabolites, 3-OH (**F2**), 2-OH (**F3**), 3-OH-4-OMe (**F6**) and the catechol 3,4-diOH (**F7**), were not identified in hepatocytes incubations.

Discussion

In this study, we confirmed the exact structures of major hydroxylated, catechol and methylated catechol metabolites in hepatocyte incubations. We could also identify metabolites in authentic urine samples by reanalysis of previous data sets.

Metabolites identified after hepatocyte incubations

The metabolite patterns of the six fentanyls are in general similar. Metabolites with the same motifs 4-OH (**1**), β-OH (**4**) and 4-OH-3-OMe (**5**) were identified in hepatocyte incubations across the studied fentanyls. Furthermore, the motifs 3-OH (**2**), 2-OH (**3**), 3-OH-4-OMe (**6**) and 3,4-diOH (**7**) were not found in hepatocytes incubated with any of the fentanyls with the exception of acetylfentanyl where trace amounts of 3-OH (**B2**) and 3,4-diOH (**B7**) were identified (Figure 3).

This suggests that the 4- and β-positions are highly selective for monohydroxylation, which is believed to be mediated by cytochrome P450 (29).

These motifs are potentially active; β-hydroxyfentanyl was placed under international control in 1990 (30), β-hydroxy-3-methylfentanyl (ohmefentanyl) is a potent analog (31) and 4-hydroxyfentanyl has been shown to have an activity between morphine and pethidine (32).

The selectivity towards 4-OH-3-OMe (**5**) over 3-OH-4-OMe (**6**) can be explained by this metabolite being a product of methylation of the catechol 3,4-diOH (**7**) facilitated by catechol-*O*-methyltransferase (COMT) since this enzyme has been shown to favor the *meta* position (33).

Not only the same motifs, but also the relative abundance between the motifs was similar (Figure 4 and Table II). β-OH (**4**) was the most abundant of the metabolites monohydroxylated on the phenethyl moiety for five out of the six fentanyls, ranging from 70 to 94% of the combined hydroxy metabolite peak area, the remainder being filled by 4-OH (**1**). That said, the most abundant metabolites for most fentanyl analogs both after hepatocyte incubation and in urine samples were their respective nor-metabolites (Table II). This has also been well established in the literature (17, 19–20, 22–23, 25, 27).

Interestingly, the metabolite pattern of acetylfentanyl appears different from the others. 4-OH (**B1, 100%**) is the major monohydroxylated metabolite instead of β-OH (**B4, 33%**). In addition, small peaks corresponding to 3-OH (**B2, 3%**) and 3,4-diOH (**B7, 1%**) were detected.

Looking closer at Table II, there seems to be a pattern that the relative amount of 4-OH (**1**) decreases with increasing length of the amide side chain. Similarly, the relative abundance of the nor-metabolite seems to be higher for acryl- and cyclopropylfentanyl which are the only fentanyls with unsaturated or cyclic amide side chains.

Metabolites identified in urine samples

The urine samples apart from those of cyclopropylfentanyl could not be analyzed at the same time as the reference standards. This resulted in small differences in retention time (0.14–0.29 min), which could be

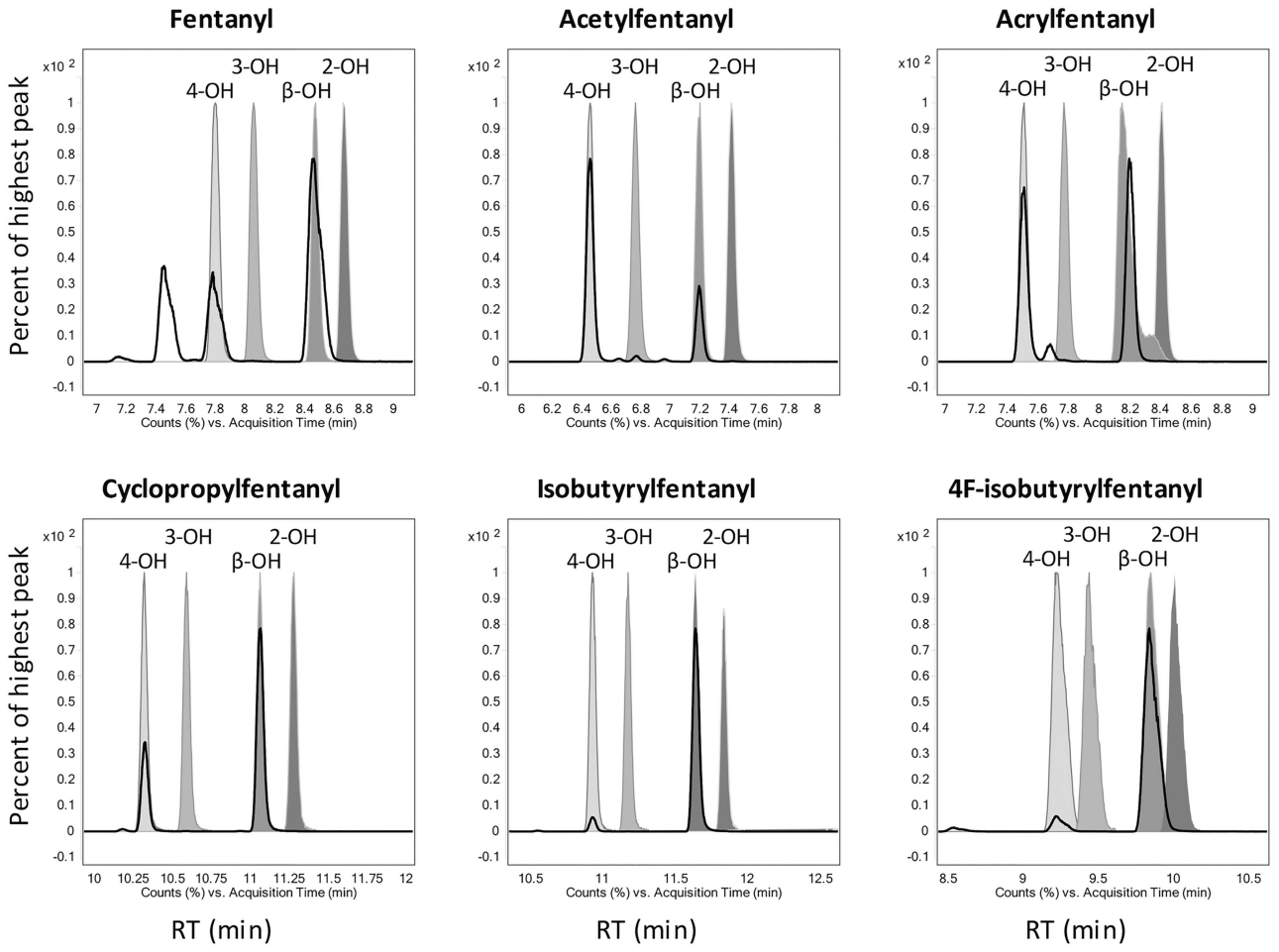

Figure 3. Chromatograms of monohydroxylated metabolites on the phenethyl moiety (1–4) across six fentanyls (A–F). The peaks of the synthesized reference standards (blue traces) are compared with the metabolites produced by hepatocytes (black trace).

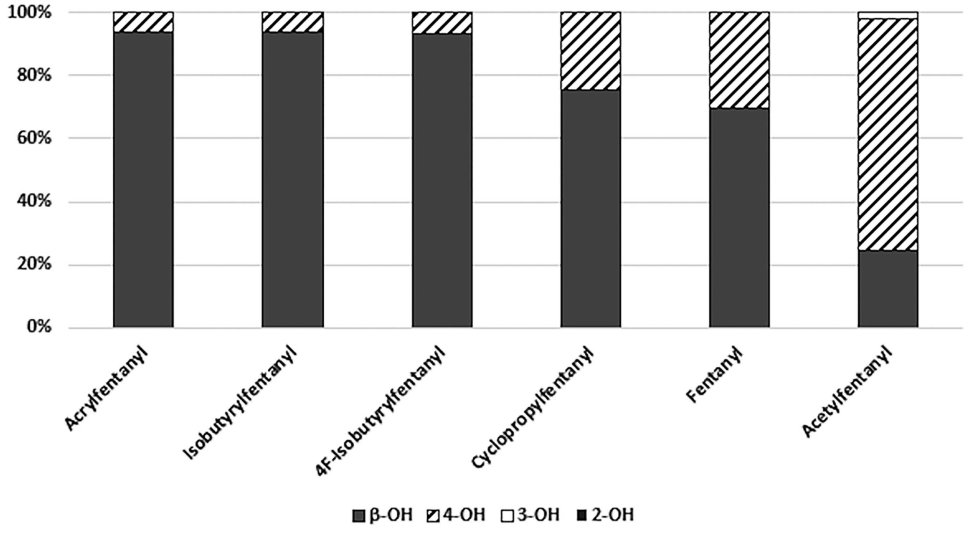

Figure 4. Distribution of monohydroxylated metabolites on the phenethyl moiety in percent. The relative abundance of the four motifs: β-OH (blue trace), 4-OH (dashed trace), 3-OH (white trace) and 2-OH (black trace) across the six fentanyls are visualized in the diagram showcasing a propensity towards β-OH and less so with 4-OH but an almost complete lack of 3-OH and 2-OH.

observed between the current and earlier data sets for acetyl-, acryl- and 4F-isobutyryl-fentanyl (20), most likely caused by differences in the chromatographic properties of the columns used. Even though the columns are supposed to be identical, this phenomenon is common. Although this limits the strength of our identifications, as described previously, both the retention time regression analysis and the agreement with cyclopropylfentanyl data indicate that our identifications are correct.

In addition to the motifs identified after hepatocyte incubations, 3-OH-4-OMe (**6**) and 3,4-diOH (**7**) were identified in urine samples for all analogs. For 4F-isobutyrylfentanyl, 3-OH (**F2**) was also identified but not in urine samples associated with any other analog. There is a clear pattern that while β-OH (**4**) is the most abundant hydroxylated metabolite in hepatocyte samples, 4-OH (**1**) is more abundant in the urine samples (Table II). This illustrates the limitations of the hepatocyte model system. Similarly, 4-OH-3-OMe (**5**) and 3,4-diOH (**7**) appear to be more abundant metabolites in the urine samples than in the hepatocyte samples (Table II).

Investigating the reasons behind the differences between hepatocytes and authentic urine samples is beyond the scope of this study. However, several different factors could have affected the differences as well as the variability between different samples, including accumulation effects during urine production, time between drug intake and sampling, concomitant drug use, as well as genetic variants in drug metabolizing enzymes such as CYP3A4 and CYP3A5.

Agreement with previous studies

In general, the results of the present study are in good agreement with earlier works on fentanyls using hepatocyte incubations. For fentanyl, the two monohydroxylated metabolites 4-OH (**A1, 43%**) and β-OH (**A4, 100%**), as well as 4-OH-3-OMe (**A5, 3%**), which were identified in the hepatocyte incubations are in line with the findings of Kanamori et al. (19).

The presence of acetylfentanyl metabolites 4-OH (**B1, 100%**), β-OH (**B4, 33%**) and 4-OH-3-OMe (**B5, 12%**) after hepatocyte incubation could be confirmed, which was previously reported by Kanamori et al. (19) as well as Watanabe et al. (20). Moreover, 3-OH (**B2, 3%**) was also identified in the hepatocyte incubations, which has not been reported previously.

Two monohydroxylated metabolites of acrylfentanyl, 4-OH (**C1, 82%**) and β-OH (**C4, 100%**) as well as the methylated catechol metabolite 4-OH-3-OMe (**C5, 5%**) were identified in the hepatocyte incubations. These metabolites were also reported by Watanabe et al. (20).

Regarding cyclopropylfentanyl, two monohydroxylated metabolites, 4-OH (**D1, 33%**) and β-OH (**D4, 100%**), as well as the methylated catechol metabolite 4-OH-3-OMe (**D5, 2%**) were detected in the hepatocyte experiments. These results are in good agreement with those reported previously *in vitro* by Åstrand et al. (27) using hepatocytes and by Cutler and Hudson (24) using liver microsomes.

Not many studies have been carried out on the metabolism of isobutyrylfentanyl. However, given its structural similarities with 4F-isobutyrylfentanyl, it was expected to produce a similar metabolic pattern. The presence of isobutyrylfentanyl metabolites, 4-OH (**E1, 7%**), β-OH (**E4, 100%**) and 4-OH-3-OMe (**E5, 1%**) could be confirmed after hepatocyte incubation. For 4F-isobutyrylfentanyl, 4-OH (**F1, 7%**), β-OH (**F4, 100%**) and 4-OH-3-OMe (**F5, 1%**) can be matched with metabolites C10, C15 and C12 as reported by Watanabe et al. (20), although the structures of C10 and C15 had been proposed to be hydroxylated at the ethyl linker and the piperidine ring, respectively. The discrepancies between the structures suggested by Watanabe et al. (20) and the structures of this study can be explained by the difficulties in interpreting mass spectra of complex molecules. For instance, the hydroxy group of C10 was assigned to the ethyl linker given the presence of fragments with *m/z* 103.0543 and 121.0645, suggesting a loss of water which is more common on aliphatic hydroxy groups than aromatic ones (34). However, in this scenario, it did not lead to the correct structure, 4-OH (**F1**), further highlighting the importance of reference standards in determining the exact structures.

In addition, some studies on the metabolism of butyrylfentanyl, which has a similar structure to isobutyrylfentanyl, have been reported in the literature. Kanamori et al. (25) reported that the two major hydroxylated metabolites after hepatocyte incubation were hydroxylated on the two terminal positions of the butyryl side chain, and similar metabolites were reported by Steuer et al. (26), after human liver microsome incubation, although less abundant than β-OH. This is in contrast to our results of isobutyrylfentanyl where the hydroxylations happen mainly on the phenethyl moiety.

Impact for forensic toxicology

Taken together, the results of this study indicate that in addition to the well-known nor-metabolites, 4-OH (**1**) and 4-OH-3-OMe (**5**) are important motifs for urinary fentanyl metabolites. This study provides synthesis routes for these metabolites allowing for the manufacture of reference standards to aid in method development.

Exact structure elucidation of metabolites is also important as the metabolites might have similar effects and activities as the parent compounds. Of special interest here are the 4-OH (**1**) and β-OH (**4**) motifs which are major metabolites shown to be active for some analogs (see above). Knowing their exact structures and having access to the reference standards allow for studies on the effects of these metabolites and might serve as a stepping-stone to a better understanding of their toxicology.

Furthermore, as the results show patterns in metabolism across different fentanyls these results can serve as a starting point for predicting metabolites of novel fentanyl analogs as well as increasing the rate and reducing the work needed to provide reference standards. It is important to keep in mind that previous metabolism studies have shown distinctly different metabolite patterns for some fentanyl analogs, such as furanylfentanyl, which is primarily metabolized on the furan ring (20).

As fentanyl analogs are frequently encountered at low concentrations, metabolites might be important for detection of these drugs, especially using immunoassays and in urine. Even though immunoassays for fentanyl in general do not cross react with nor-metabolites there is some evidence that monohydroxylated metabolites interact with fentanyl immunoassays (35).

Finally, the results illustrate systematic differences between metabolite abundance in urine samples and after hepatocyte incubation. The potential reasons for this are beyond the scope of this study but being aware of these differences will help forensic toxicologists to estimate the metabolism when relying mainly on hepatocyte data.

Conclusions

By adding synthesis of reference standards to the combination of LC–QTOF-MS analysis and drug incubations with hepatocytes, the exact structures of 20 metabolites across six different fentanyls were

determined. Additionally, reference standards have the added benefit of safeguarding against the assignment of potentially erroneous structures based on mass spectral interpretation alone.

Similar metabolite profiles were observed for all six fentanyls. Major metabolites of the studied fentanyls were 4-OH (**1**), β-OH (**4**) and 4-OH-3-OMe (**5**) together with the nor-metabolite. They were consistently found after hepatocyte incubation with all six fentanyls and it is likely that other fentanyls show similar metabolite profiles.

In general, similar metabolites were identified after hepatocyte incubation as well as in the urine samples. However, when comparing the hepatocyte sample data with the authentic urine data reported earlier by Watanabe et al. (18) and Vikingsson et al. (20) the ratios of the 4-OH/β-OH abundances were always higher in the urine samples.

Based on our results, 4-OH (**1**), and 4-OH-3-OMe (**5**) are suggested as starting points for synthesizing reference standards for major urinary metabolites of novel fentanyl analogs.

Authorship contributions

J.W., S.V., R.K., H.G., X.W., P.K. and J.D. conceived and designed the research. J.W., S.V., A.Å. and S.W. performed the hepatocyte experiments. J.W., T.R., E.N. and X.W. synthesized the reference standards. J.W. and S.V. analyzed and interpreted the data. J.W. and S.V. drafted the manuscript. All authors contributed to the manuscript. All authors approved the final version of the manuscript.

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References

1. Janssen, P.A. (1962) A review of the chemical features associated with strong morphine-like activity. *British Journal of Anaesthesia*, 34, 260–268. doi: 10.1093/bja/34.4.260.
2. Chen, J.-C., Smith, E., Cahill, M., Cohen, R., Fishman, J. (1993) The opioid receptor binding of dezocine, morphine, fentanyl, butorphanol and nalbuphine. *Life Sciences*, 52, 389–396. doi: 10.1016/0024-3205(93)90152-S.
3. Nelson, L., Schwaner, R. (2009) Transdermal fentanyl: pharmacology and toxicology. *Journal of Medical Toxicology*, 5, 230–241. doi: 10.1007/BF03178274.
4. World Drug Report (2018) *United Nations Publication, Sales No. E.18.XI.9*.
5. O'Donnell, J.K., Halpin, J., Mattson, C.L., Goldberger, B.A., Gladden, R.M. (2017) Deaths involving fentanyl, fentanyl analogs, and U-4770—10 states, July–December 2016. *MMWR. Morbidity and Mortality Weekly Report*, 66, 1197–1202. doi: 10.15585/mmwr.mm6643e1.
6. European Monitoring Centre for Drugs and Drug Addiction. (2016) *EMCDDA–Europol Joint Report on a new psychoactive substance: N-phenyl-N-[1-(2-phenylethyl)piperidin-4-yl] acetamide (acetylfentanyl), Joint Reports*. Publications Office of the European Union: Luxembourg.
7. European Monitoring Centre for Drugs and Drug Addiction. (2017) *Report on the risk assessment of N-(1-phenethylpiperidin-4-yl)-N-phenylacrylamide (acryloylfentanyl) in the framework of the Council Decision on new psychoactive substances. Risk Assessments*. Publications Office of the European Union: Luxembourg.
8. European Monitoring Centre for Drugs and Drug Addiction. (2018) *Report on the risk assessment of N-phenyl-N-[1-(2-phenylethyl)piperidin-4-yl]cyclopropanecarboxamide (cyclopropylfentanyl) in the framework of the Council Decision on new psychoactive substances. Risk Assessments*. Publications Office of the European Union: Luxembourg.
9. European Monitoring Centre for Drugs and Drug Addiction. (2018) *Report on the risk assessment of N-(4-fluorophenyl)-2-methyl-N-[1-(2-phenylethyl)piperidin-4-yl]propanamide (4-fluoroisobutyrylfentanyl 4F-iBF), in the framework of the Council Decision on new psychoactive substances. Risk Assessments*. Publications Office of the European Union: Luxembourg.
10. European Monitoring Centre for Drugs and Drug Addiction. (2018) *Report on the risk assessment of N-phenyl-N-[1-(2-phenylethyl)piperidin-4-yl] acetamide in the framework of the Council Decision on new psychoactive substances. Risk Assessments*. Publications Office of the European Union: Luxembourg.
11. European Monitoring Centre for Drugs and Drug Addiction. (2018) *Report on the risk assessment of methyl 1-(2-phenylethyl)-4-[phenyl(propanoyl)amino]piperidine-4-carboxylate (carfentanil) in the framework of the Council Decision on new psychoactive substances. Risk Assessments*. Publications Office of the European Union: Luxembourg.
12. European Monitoring Centre for Drugs and Drug Addiction. (2019) *European Drug Report 2019: Trends and Developments*. Publications Office of the European Union: Luxembourg.
13. Kronstrand, R., Guerrieri, D., Vikingsson, S., Wohlfarth, A., Green, H. (2018) Fatal poisonings associated with new psychoactive substances. *Handbook of Experimental Pharmacology*, 252, 495–541. doi: 10.1007/164_2018_110.
14. Guerrieri, D., Rapp, E., Roman, M., Thelander, G., Kronstrand, R. (2017) Acrylfentanyl: another new psychoactive drug with fatal consequences. *Forensic Science International*, 277, e21–e29.
15. Obach, R.S. (2013) Pharmacologically active drug metabolites: impact on drug discovery and pharmacotherapy. *Pharmacological Reviews*, 65, 578–640. doi: 10.1124/pr.111.005439.
16. Depriest, A., Heltsley, R., Black, D.L., Cawthon, B., Robert, T., Moser, F., et al. (2010) Urine drug testing of chronic pain patients. III. Normetabolites as biomarkers of synthetic opioid use. *Journal of Analytical Toxicology*, 34, 444–449.
17. Goromaru, T., Kawauchi, T., Katashima, M., Matsuura, H., Yoshimura, N., Sameshima, T., et al. (1986) Urinary excretion of fentanyl and its metabolites at high-dose infusion. *Masui. The Japanese Journal of Anesthesiology*, 35, 35–39.
18. Goromaru, T., Matsuura, H., Yoshimura, N., Miyawaki, T., Sameshima, T., Miyao, J., et al. (1984) Identification and quantitative determination of fentanyl metabolites in patients by gas chromatography–mass spectrometry. *Anesthesiology*, 61, 73–77.
19. Kanamori, T., Iwata, Y.T., Segawa, H., Yamamuro, T., Kuwayama, K., Tsujikawa, K., et al. (2018) Metabolism of fentanyl and acetylfentanyl in human-induced pluripotent stem cell-derived hepatocytes. *Biological and Pharmaceutical Bulletin*, 41, 106–114. doi: 10.1248/bpb.b17-00709.
20. Watanabe, S., Vikingsson, S., Roman, M., Green, H., Kronstrand, R., Wohlfarth, A. (2017) In vitro and in vivo metabolite identification studies for the new synthetic opioids acetylfentanyl, acrylfentanyl, furanylfentanyl, and 4-fluoro-isobutyrylfentanyl. *The AAPS Journal*, 19, 1102–1122. doi: 10.1208/s12248-017-0070-z.
21. Melent'ev, A.B., Kataev, S.S., Dvorskaya, O.N. (2015) Identification and analytical properties of acetyl fentanyl metabolites. *Journal of Analytical Chemistry*, 70, 240–248. doi: 10.1134/S1061934815020124.
22. Vikingsson, S., Rautio, T., Wallgren, J., Åstrand, A., Watanabe, S., Dahlén, J., et al. (2019) LC–QTOF-MS identification of major urinary cyclopropylfentanyl metabolites using synthesized standards. *Journal of Analytical Toxicology*, 43, 607–614. doi: 10.1093/jat/bkz057.
23. Palaty, J., Konforte, D., Karakosta, T., Wong, E., Stefan, C. (2018) Rapid identification of cyclopropyl fentanyl/crotonyl fentanyl in clinical urine specimens: a case study of clinical laboratory collaboration in Canada. *Clinical Biochemistry*, 53, 164–167. doi: 10.1016/j.clinbiochem.2018.01.013.
24. Cutler, C., Hudson, S. (2019) In vitro metabolism of the novel synthetic opioid agonist cyclopropylfentanyl and subsequent confirmation in

authentic human samples using liquid chromatography-high resolution mass spectrometry. *Drug Testing and Analysis*, 11, 1134–1143. doi: 10.1002/dta.2611.

25. Kanamori, T., Iwata, Y.T., Segawa, H., Yamamuro, T., Kuwayama, K., Tsujikawa, K., et al. (2019) Metabolism of butyrylfentanyl in fresh human hepatocytes: chemical synthesis of authentic metabolite standards for definitive identification. *Biological and Pharmaceutical Bulletin*, 42, 623–630.
26. Steuer, A., Willnier, E., Staeheli, S., Kraemer, T. (2017) Studies on the metabolism of the fentanyl-derived designer drug butyrfentanyl in human in vitro liver preparations and authentic human samples using liquid chromatography-high resolution mass spectrometry (LC-HRMS). *Drug Testing and Analysis*, 9, 1085–1092. doi: 10.1002/dta.2111.
27. Åstrand, A., Töreskog, A., Watanabe, S., Kronstrand, R., Gréen, H., Vikingsson, S. (2019) Correlations between metabolism and structural elements of the alicyclic fentanyl analogs cyclopropyl fentanyl, cyclobutyl fentanyl, cyclopentyl fentanyl, cyclohexyl fentanyl and 2,2,3,3-tetramethylcyclopropyl fentanyl studied by human hepatocytes and LC-QTOF-MS. *Archives of Toxicology*, 93, 95–106. doi: 10.1007/s00204-018-2330-9.
28. McClain, D.A., Hug, C.C., Jr. (1980) Intravenous fentanyl kinetics. *Clinical Pharmacology and Therapeutics*, 28, 106–114. doi: 10.1038/clpt.1980.138.
29. Labroo, R., Paine, M., Thummel, K., Kharasch, E. (1997) Fentanyl metabolism by human hepatic and intestinal cytochrome P450 3A4: implications for interindividual variability in disposition, efficacy, and drug interactions. *Drug Metabolism and Disposition*, 25, 1072–1080.
30. UNODC. (2017) *Global SMART Update Volume 17*.
31. Wang, Z., Zhu, Y., Jin, W., Chen, X., Chen, J., Ji, R., et al. (1995) Stereoisomers of *N*-[1-(2-hydroxy-2-phenethyl)-3-methyl-4-piperidyl]-*N*-phenylpropanamide: synthesis, stereochemistry, analgesic activity, and opioid receptor binding characteristics. *Journal of Medicinal Chemistry*, 38, 3652–3659.
32. Schneider, E., Brune, K. (1986) Opioid activity and distribution of fentanyl metabolites. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 334, 267–274.
33. Guldberg, H., Marsden, C. (1975) Catechol-O-methyl transferase: pharmacological aspects and physiological role. *Pharmacological Reviews*, 27, 135–206.
34. Holpacek, M., Jirasko, R., Lisa, M. (2010) Basic rules for the interpretation of atmospheric pressure ionization mass spectra of small molecules. *Journal of Chromatography A*, 1217, 3908–3921. doi: 10.1016/j.chroma.2010.02.049.
35. Guerrieri, D., Kjellqvist, F., Kronstrand, R., Green, H. (2019) Validation and cross-reactivity data for fentanyl analogs with the immunalysis fentanyl ELISA. *Journal of Analytical Toxicology*, 43, 18–24.