Distinguishing between cyclopropylfentanyl and crotonylfentanyl by methods commonly available in the forensic laboratory

Short title: Identification of cyclopropylfentanyl and crotonylfentanyl

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Abstract

Background: The opioid analgesic fentanyl and its analogues pose a major health concern due to its high potency and increasing number of overdose deaths worldwide. The analogues of fentanyl may differ in potency, toxicity and legal status and it is therefore important to develop analytical methods for correct identification. This can be challenging since many fentanyl analogues are structural isomers. Two fentanyl isomers that have been in the spotlight lately due to difficulties regarding separation and identification are cyclopropylfentanyl and crotonylfentanyl, which have been reported to display nearly identical fragmentation patterns and chromatographic behavior.

Methods: Chromatographic separation of cyclopropylfentanyl and crotonylfentanyl by UHPLC was investigated using three different stationary phases (HSS T3, BEH C18 and Kinetex biphenyl) employing gradient elution with a mobile phase consisting of 10 mM ammonium formate pH 3.1 and MeOH. Detection was performed by MS/MS. In addition, the major metabolites of the two compounds formed upon incubation with human liver microsomes (HLM) were identified by UHPLC-QTOF-MS analysis.

Results: Baseline separation of cyclopropylfentanyl and crotonylfentanyl was achieved on the BEH C_{18} column with retention times of 6.79 and 7.35 min, respectively. The major metabolites of the two analogues formed by HLM differed, with the main biotransformation being N-dealkylation and carboxylation for cyclopropylfentanyl and crotonylfentanyl, respectively. We demonstrated the usefulness of the two approaches by distinguishing between cyclopropylfentanyl and crotonylfentanyl in two authentic post mortem blood samples.

Conclusions: In the present study we successfully demonstrated that cyclopropylfentanyl and crotonylfentanyl can be distinguished by methods commonly available in forensic laboratories.

Keywords: Cyclopropylfentanyl, crotonylfentanyl, Structural isomer separation, Metabolism, UHPLC-MS/MS

INTRODUCTION

Abuse of the opioid analgesic fentanyl and its analogues is increasing at an alarming rate worldwide and poses a major concern due to the high mortality rate associated with these highly potent compounds.[1-3] In the United States alone nearly 30 000 fentanyl-related deaths were reported in 2017.[4] Since fentanyl was first designed in 1960, numerous structural analogues have been synthesized either as pharmaceutical candidates or lately as illicit drugs for recreational use.[5, 6] While fentanyl is estimated to be 100 times more potent than morphine, some of the fentanyl analogues have even higher potency, such as 3-methylfentanyl and carfentanil which are approximately 70 and 100 times more potent than fentanyl, respectively.[3, 5, 7-9] Since these drugs are often sold under the guise of heroin, mixed with heroin or sold as prescription opioids like oxycodone, accidental overdose fatalities are likely to occur.[3, 10]

The fentanyl analogues may vary in potency, toxicity and legal status and it is therefore important to develop analytical methods for precise identification. This can be a challenge since many fentanyl analogues are structural isomers with the same molecular formula. Two structural fentanyl isomers that have been in the spotlight lately due to difficulties regarding separation and identification are cyclopropylfentanyl and crotonylfentanyl,[11-14] which differ in structure from fentanyl by the replacement of the propionamide group with a cyclopropanecarboxamide or 2-butenamide group, respectively (Figure 1).

Several research groups have reported that cyclopropylfentanyl and crotonylfentanyl yield almost identical mass spectra when analyzed with gas chromatography (GC) or liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS).[11-14] Adding to the

challenge, Simons and colleagues stated that the two fentanyl analogues had identical retention times and transition responses when analyzed with GC- or LC-MS/MS and suggested that the substances should be reported as cyclopropylfentanyl/crotonylfentanyl unless an alternative methodology was used for identification.[11] Maher and colleagues reported that the slight difference in retention time accomplished when performing high performance liquid chromatography (HPLC)-MS/MS and ultra high performance liquid chromatography (UHPLC)-quadrupole time of flight mass spectrometry (QTOF-MS) was not sufficient to distinguish the two compounds, however, correct identification was possible by HPLC with diode array UV detection by combination of a small difference in UV maxima and retention time.[14] Millette and coworkers reported separation of the two isomers by GC-MS, but the retention times were very long (~30 min).[13] Cyclopropylfentanyl and crotonylfentanyl have also been identified by non-chromatographic techniques such as attenuated total reflectance Fourier transform infrared spectroscopy or nuclear magnetic resonance spectroscopy,[11, 13, 15, 16] however, these techniques are not available in most forensic laboratories today.

An alternative approach to distinguish between structural isomers in forensic toxicology cases is by identification of unique metabolites of the different compounds. The metabolism of fentanyl analogues closely related to fentanyl (e.g. 4-fluoroisobutyrfentanyl, 3-methylfentanyl and acetylfentanyl) is typically predominated by N-dealkylation, yielding the nor-metabolite.[17] On the other hand, metabolism of fentanyl analogues with a longer acyl chain (e.g. butyrfentanyl) usually takes place at this lipophilic site.[17] Since crotonylfentanyl and cyclopropylfentanyl contain a crotonyl group and a cyclopropyl ring, respectively, it is likely that these two compounds can be distinguished by unique metabolites. The nor-metabolite of

cyclopropylfentanyl has been detected in urine samples[12, 14], while no crotonylfentanyl metabolites have been reported previously, to the best of our knowledge.

In the present study, we have examined two independent approaches to distinguish between cyclopropylfentanyl and crotonylfentanyl by methods commonly available in the forensic laboratory. First, chromatographic separation of the compounds was examined on three different stationary phases using UHPLC-MS/MS. Second, metabolites of cyclopropylfentanyl and crotonylfentanyl formed by human liver microsomes (HLMs) were examined by UHPLC-QTOF-MS analyses to elucidate whether the compounds could be distinguished by unique major metabolites. We also demonstrated the usefulness of the two approaches by distinguishing between cyclopropylfentanyl and crotonylfentanyl in two authentic post mortem blood samples.

MATERIALS AND METHODS

Chemicals and Reagents

Cyclopropylfentanyl hydrochloride and furanylfentanyl-d_5 were acquired from Cayman Chemicals (Ann Arbor, MI, USA). Crotonylfentanyl was acquired from Chiron AS (Trondheim, Norway). Ammoniumformate and formic acid (98%) were acquired from VWR International AS (Oslo, Norway). Chromasolv methanol (MeOH) of LC-MS grade was acquired from Honeywell Riedel-de Haën (Seelze, Germany). Pooled HLMs (XTreme 200 Pool) were acquired from XenoTech (Kansas City, KS, USA). Nicotinamide adenine dinucleotide phosphate (NADPH) regeneration solution A and B were acquired from BD

Biosciences (San Jose, CA, USA). Type 1 water (18.2 MΩ) purified with a Synthesis A 10 milli-Q system from Millipore (Billerica, MA, USA) was employed.

Incubation with Human Liver Microsomes

HLMs from 200 donors of balanced gender (400–500 pmol total CYP450/mg protein) were stored in a 250 mM sucrose solution at -80 °C. HLM incubations were performed in NADPH regeneration solution (final conc. 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM $MgCl_2$) and a final concentration of 1 µM cyclopropylfentanyl or crotonylfentanyl. The reaction was initiated by the addition of HLM (final conc. 2 mg protein/mL) to the tubes (3 tubes pr. time interval), followed by vortexing and incubation at 37 °C in a shaking water bath. The reaction was stopped after 0, 60 and 120 min by addition of formic acid (final conc. 0.1 M) followed by immediate vortexing. The tubes were centrifuged for 10 min at 14 500 g at 4 °C. The supernatants were transferred to autosampler vials placed on ice. Drug degradation control samples were prepared in parallel in the absence of HLM. A negative control sample (HLM incubation without drug) was prepared separately. All samples were analyzed by UHPLC-QTOF-MS.

Forensic Post Mortem Blood Samples

Peripheral blood samples from two autopsy cases with suspected cyclopropylfentanyl or crotonylfentanyl intoxication after forensic routine analyses were examined with approval from the Norwegian Higher Prosecution Authority. The blood samples were taken from the femoral vein at autopsy and stored in 25 mL Steriline® tubes (Cole-Parmer Ltd, Staffordshire, UK) containing 200 mg potassium fluoride as preservative. The blood samples were prepared

by liquid-liquid extraction as described previously[18] and analyzed by qualitative UHPLC-MS/MS and UHPLC-QTOF-MS analysis.

UHPLC-QTOF-MS Analysis

Analyses of HLM samples were performed employing a 1290 Infinity UHPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled to a 6550 iFunnel QTOF (Agilent Technologies) mass spectrometer operating in positive ionization mode. Chromatographic separation was performed on an Acquity HSS T3 column (2.1 x 100 mm, 1.8 µm (Waters (Milford, MA, USA)) fitted with an Acquity HSS T3 VanGuard pre-column (Waters) kept at 65 °C with a mobile phase consisting of 10 mM ammonium formate pH 3.1 (solvent A) and MeOH (solvent B) at a flow rate of 0.5 mL/min. Gradient elution with the following profile was used: 0 min; 2.5 % B, 0-14 min; 2.5-75 % B, 14-17 min; 75-100 % B, 17-19 min; 100 % B, 19-19.01 min; 100-2.5 % B, 19.01-21 min; 2.5 % B. The injection volume was 4 µL and the injection technique was flow through needle with a flexible loop.

QTOF-MS analysis was performed in auto-MS/MS mode (i.e. data dependent acquisition). The mass range was 50 – 1000 *m/z* (MS) with a static exclusion range of 50-122 and 650-1000 *m/z*, acquisition rate was 6 spectra/s, collision energy was 4 eV at 0 *m/z* and increased by 6 eV per 100 *m/z* when searching for parent compounds and unknown metabolites (e.g. in HLM samples and post mortem blood samples). Gas temperature was 150 °C, drying gas flow was 15 L/min, nebulizer pressure was 40 psig, sheath gas temperature and flow were 350 °C and 12 mL/min, respectively, and the fragmentor voltage was 365 V. Nitrogen was used as both desolvation and collision gas.

The QTOF data from the HLM samples were analyzed by Masshunter Qualitative Analysis (version B.07.00, Agilent) in combination with a Personal Compound Database Library (PCDL) containing phase I biotransformations predicted by Metabolite Tool 2.0 (Broeckers Solutions, Berlin, Germany) and known metabolic reactions of fentanyl and other fentanyl analogues. The search criteria were as follows: A minimum peak height of 50 000 counts, a maximum mass error of 10 ppm, a maximum of 10 matches per formula and a chromatogram extraction window of maximum 100 ppm. The criteria for metabolite identification were as follows: A symmetrical peak shape, no co-eluting peaks, a retention time fitting the proposed metabolite (compared to the retention time of the parent drug), no presence of identical peaks in the negative control sample, a maximum mass error of 5 ppm for the protonated molecule and a MS/MS fragmentation pattern in accordance with the proposed metabolite structure.

QTOF-data from the two post mortem blood samples were also investigated with respect to metabolites, using Masshunter Qualitative Analysis combined with a PCDL consisting of the metabolites identified in the HLM experiments. The criteria for metabolite identification were a peak height of minimum 10 000 counts, a retention time not deviating more than 0.5 min from the average retention time in the HLM samples, a mass error of the protonated molecule with a maximum of 5 ppm and a fragmentation pattern identical to the metabolites found in the HLM samples.

UHPLC-MS/MS Analysis

Standard solutions of cyclopropylfentanyl and crotonylfentanyl in Type 1 water were analyzed employing an Acquity UPLC™ system (Waters) coupled to a Xevo-TQS triple quadrupole mass spectrometer with an electrospray ionization (ESI) interface (Waters)

operating in positive ionization mode. Chromatographic separation of cyclopropylfentanyl and crotonylfentanyl was investigated using the three following columns: Acquity HSS T3 (2.1 x 100 mm, 1.8 µm, Waters), Acquity BEH C_{18}, (2.1 x 100 mm, 1.7 µm, Waters) and Kinetex biphenyl (2.1 x 100 mm, 1.7 µm, Phenomenex, Verløse, Denmark). The columns were kept at 60 °C with a mobile phase consisting of 10 mM ammonium formate pH 3.1 (solvent A) and MeOH (solvent B) at a flow rate of 0.6 mL/min. The initial testing of the columns was performed using a linear gradient for 10 or 20 min. The 10 min gradient had the following profile: 0-0.5 min; 5 % B, 0.5-10 min; 5-100 % B, 10-11 min; 100 % B, 11-11.1 min; 100-5 % B, 11.1-12 min; 5 % B. The 20 min gradient had the following profile: 0-0.5 min; 5 % B, 0.5-20 min; 5-100 % B, 20-21 min; 100 % B, 21-21.1 min; 100-5 % B, 21.1-22 min; 5 % B. The injection volume was 0.5 µL using a partial loop with the needle overfill injection technique. The method optimized for separation of the two isomers employed a BEH C_{18} column using the chromatographic parameters described for the initial testing with the following 10 min gradient profile: 0-0.5 min; 25 % B, 0.5-8.0 min; 25-30 % B, 8.0-8.1 min; 30-100 % B, 8.1-9 min; 100 % B, 9-9.1 min; 100-25 % B, 9.1-10 min; 25 % B.

Analysis with ESI-MS/MS was performed using either multiple reaction monitoring (MRM) mode or daughter ion scan in the positive mode. The capillary voltage was set to 0.5 kV and the source temperature was 150 °C. Nitrogen gas was used for desolvation, delivered at a temperature of 500 °C and a flow rate of 1000 L/h. Cone gas (nitrogen) and collision gas (argon) flow was 300 L/h and 0.17 mL/min, respectively. Data acquisition and processing were performed using Masslynx™ 4.1 software (Waters). The MRM transitions and MS/MS parameters for the two compounds were identical with the following values: *m/z* 349.2 > 188.2 (Cone voltage 8 V, collision energy 24 eV) and 349.2 > 105.1 (Cone voltage 8 V,

collision energy 40 eV). Daughter ion scans were performed in the *m/z* range of 50-350 with a collision energy of 30 eV and an injection volume of 7.5 µL.

RESULTS AND DISCUSSION

Distinguishing between Cyclopropylfentanyl and Crotonylfentanyl by UHPLC-MS/MS

Structural isomers can be differentiated by MS/MS if the fragmentation patterns of the compounds differ. Daughter ion scans of individual solutions of cyclopropylfentanyl and crotonylfentanyl were therefore performed. The mass spectra of the two fentanyl analogues displayed near identical daughter ions with similar relative abundance after collision-induced dissociation (Figure 2). These findings are in accordance with findings by Maher et al.[14] and Simons et al. [11] and confirm that cyclopropylfentanyl and crotonylfentanyl cannot be distinguished by their fragmentation patterns.

The mass spectra in Figure 2 show that fragmentation of the protonated cyclopropylfentanyl and crotonylfentanyl molecules with *m/z* 349.2274 gave rise to two large peaks, fragment c, corresponding to a cleavage between the phenethylpiperidine moiety and the N-phenyl-cyclopropanecarboxamide/N-phenyl-2-butenamide group and fragment h, which was generated by cleavage between the piperidine ring and the phenethyl moiety. The cleavage between the phenethylpiperidine moiety and the N-phenyl- cyclopropanecarboxamide/N-phenyl-2-butenamide group also generated fragment d. A fragment corresponding to the piperidine ring (fragment i) and fragments corresponding to degradation of the piperidine ring (fragments b, e, f, g) were found in the MS/MS spectrum. Two fragments (fragments a and j)

corresponding to a cleavage between the N-phenyl moiety and the cyclopropanecarboxamide/2-butenamide group were also found.

The nearly identical fragmentation patterns of cyclopropylfentanyl and crotonylfentanyl make chromatographic separation necessary in order to distinguish between the compounds by UHPLC-MS/MS. However, recent studies have reported that separation of the two structural isomers by LC is challenging.[11, 14] We examined chromatographic separation of cyclopropylfentanyl and crotonylfentanyl using three different UHPLC stationary phases; High strength silica (HSS) T3 C_{18}, BEH bridged ethylsiloxane/silica hybrid (BEH) C_{18} and biphenyl (Figure 3A). The latter column is employed in our laboratory for separation of 26 fentanyl analogues, including several isomers.[18] Unexpectedly, the biphenyl column did not achieve chromatographic separation of cyclopropylfentanyl and crotonylfentanyl, possibly due to similar polarity and lack of electron rich double bonds in the functional groups which would enable additional π-π system interactions with the biphenyl phase.

The two C_{18} columns gave a noticeable separation when employing a 10 or 20 min linear gradient. The isomer separation achieved on the C_{18} columns might be explained by the linear 2-butene group of crotonylfentanyl being more available to the long C_{18} chains of the stationary phase compared to the more rigid cyclopropyl ring of cyclopropylfentanyl. An optimized 10 min gradient profile for cyclopropylfentanyl and crotonylfentanyl was developed employing the BEH C_{18} column (Figure 3B) which achieved baseline separation of the two isomers with retention times of 6.8 and 7.4 min, respectively.

Identification of Metabolites after Incubation with HLMs

In order to investigate whether cyclopropylfentanyl and crotonylfentanyl could be distinguished by unique metabolites, we examined the major metabolites of the isomers formed by incubation with HLMs. Because the objective of the present study was to distinguish between cyclopropylfentanyl and crotonylfentanyl, we did not perform a comprehensive metabolism study, but selected up to four phase I metabolites which displayed the highest peak intensities in the HLM samples after 60 min incubation and remained at high intensities 60 min later. Fragmentation patterns for the metabolites were compared to the fragmentation patterns for the parent compounds (Figure 2) to assign the suggested metabolite structures. The identified crotonylfentanyl and cyclopropyl metabolites are listed in Table 1 and the proposed structures are shown in Figure 4.

Cyclopropylfentanyl Metabolites

The cyclopropylfentanyl metabolite with the highest abundance was metabolite A1, which corresponds to the nor-metabolite, cyclopropylnorfentanyl. A1 was generated by N-dealkylation at the piperidine ring, resulting in the loss of the phenethyl group. This is indicated by the base peak being fragment i, and the absence of the characteristic fragments c and h in the MS/MS spectra of A1. The spectrum also showed a fragment at *m/z* 177.1382, which corresponds to the N-dealkylated fragment a. Fragments d and j were present as well, which further indicates that A1 is cyclopropylnorfentanyl. Cyclopropylnorfentanyl has previously been detected in patient urine samples[12] and post mortem urine samples.[14]

The biotransformation leading to A2, the second most abundant metabolite, was identified as a hydroxylation because 15.9949 u (mass of O) was added to the mass of cyclopropylfentanyl.

The base peak of A2 was 204.1382, indicating that the hydroxylation takes place on the phenethylpiperidine moiety (fragment c + O). The presence of *m/z* 121.0647, corresponding to fragment h + O, further narrows down the possible position to either the ethyl linker or the phenyl. This assumption is supported by the presence of fragment i. As no fragments suggested the presence of a hydroxylated phenyl ring (93.034) or a hydroxylated ethyl linker (91.0548), the exact location of the hydroxylation remains uncertain. Watanabe et al.[19], argued that hydroxylation taking place at the ethyl linker often produces a H_2O loss fragment ion (*m/z* 103.0548) from *m/z* 121.0647, whereas this fragment is not observed when hydroxylation takes place at the phenyl ring since H_2O loss is uncommon at aromatic rings. The absence of this H_2O loss fragment in the MS/MS spectra of A2 therefore indicates aromatic hydroxylation.

The third most abundant metabolite, A3, was also generated by hydroxylation (+O) at the phenethylpiperidine moiety as indicated by the base peak at *m/z* 204.1379. Hydroxylation is further supported by the presence of the fragment at *m/z* 347.2112, which can be generated by H_2O loss from the hydroxylated parent ion. The hydroxylation resulting in A3 is believed to take place at the piperidine ring as fragment f indicates an unchanged phenethyl moiety. The fragment at *m/z* 186.1277 is probably generated by H_2O elimination from the hydroxylated phenetylpiperidine moiety, which can further yield the fragment at *m/z* 174.1279 after degradation of the piperidine ring as described previously by Steuer et al.[20]

The fourth metabolite, A4, was generated by formation of a dihydrodiol from a C-C double bond, as the mass difference compared to cyclopropylfentanyl corresponds to addition of two O (31.9898 u) and two H (2.0156 u) atoms. The MS/MS spectra of A4 had a base peak of *m/z*

189.1386 (Table 1) which corresponds to a N-phenyl-pyridine ring fragment generated by cleavage between the two carbon atoms of the phenethyl linker and between the cyclopropanecarboxamide and N-phenyl ring. The presence of a fragment with *m/z* 222.1482 (fragment c + 2OH) indicates that the dihydrodiol formation occurs at the phenyl ring of the phenethyl moiety, although the exact position of the dihydrodiol is unknown. The fragment at *m/z* 204.1385 corresponds to H_2O loss from one of the dihydrodiol hydroxyl groups. Watanabe et al.[19] proposed that such H_2O loss can lead to re-aromatization to a hydroxylated phenyl ring, which could explain the presence of *m/z* 121.0648 corresponding to the hydroxylated phenethyl fragment (fragment h + OH).

Crotonylfentanyl Metabolites

The most abundant metabolite of crotonylfentanyl, B1, can be generated by carboxylation, with the mass difference compared with crotonylfentanyl corresponding to the addition of two O (31.9898 u) atoms and subtraction of two H (2.0156 u) atoms. The base peak at fragment c and the presence of fragment h in the spectra indicate that the carboxylation occurs at the 2-butenamide group. This is also supported by the presence of a fragment at *m/z* 258.1126, which corresponds to the carboxylated fragment b, and the presence of fragment e.

The second most abundant metabolite, B2, is suggested to be generated by formation of a dihydrodiol from an alkene, with the addition of two O (31.9898 u) and two H (2.0156 u) atoms to the mass of crotonylfentanyl. The base peak corresponding to fragment c and the presence of fragment h suggest that the dihydrodiol formation does not take place on the phenethylpiperidine moiety. This is also supported by the presence of the fragment at *m/z* at 262.1439, which corresponds to fragment b + 2OH. The presence of fragment a indicates that

the dihydrodiol formation takes place at the 2-butenamide group, while the fragment at *m/z* 85.0282 could be a result of H_2O loss from the 2,3-dihydroxybutanamide group (*m/z* 103.0395, not found).

The third most abundant metabolite, B3, has a mass change corresponding to the addition of two O (31.9898 u) atoms, indicating a dihydroxylation of crotonylfentanyl. The base peak at *m/z* 204.1379 (fragment c + O) indicates that one hydroxylation occurred on the phenethylpiperidine moiety. The presence of fragment f further narrows down the options to the piperidine ring. As mentioned previously, the fragment at *m/z* 186.1266 can be produced by elimination of H_2O from the hydroxylated piperidine ring, which can yield the fragment at *m/z* 174.1272 by degradation of the ring. The second hydroxylation is suggested to take place at 2-butenamide as indicated by *m/z* 85.0281 (fragment j + O). The fragment at *m/z* 363.2051 implies elimination of H_2O from the parent ion. Metabolite A3, which is also believed to have a hydroxy group at the piperidine ring, shows a similar H_2O loss during fragmentation.

Distinguishing Between Cyclopropylfentanyl and Crotonylfentanyl by Their Major Metabolites

The biotransformations of cyclopropylfentanyl and crotonylfentanyl differ regarding their most abundant metabolites as observed after incubation with HLM (Table 1 and Figure 4). The metabolism of cyclopropylfentanyl appears to be similar to the metabolism of fentanyl, which is dominated by the formation of the nor-metabolite by N-dealkylation.[17] Cyclopropyl groups are known to be more stable toward hydrogen atom abstraction than other alkyl groups, which could be the reason why metabolism is not favored at this location.[21] As expected, crotonylfentanyl seems to be metabolized in a manner similar to fentanyl analogues with longer acyl chains (e.g. butyrfentanyl) with biotransformation at this lipophilic site

instead of N-dealkylation.[17] We cross-checked the metabolic profiles of cyclopropylfentanyl and crotonylfentanyl for presence of the main metabolites of the other analogue. A mass corresponding to the most abundant metabolite of cyclopropylfentanyl, the nor-metabolite (A1, saturated signal), was found with the same fragmentation pattern and retention time in the crotonylfentanyl samples, but at very small amounts (peak height of ~0.7-1x10^4 counts). No mass corresponding to A2 was found, while isomers of A3 and A4 were detected in the crotonylfentanyl HLM samples and isomers corresponding to B1, B2 and B3 were detected in the cyclopropylfentanyl HLM samples. However, since none of the isomers detected shared a common fragmentation pattern or retention time with the cyclopropylfentanyl or crotonylfentanyl metabolites, the isomers could easily be distinguished. Provided that a similar metabolic profile is generated in HLM compared to the human body, we suggest that cyclopropylfentanyl and crotonylfentanyl can be distinguished by identification of their main metabolites.

Distinguishing Between Cyclopropylfentanyl and Crotonylfentanyl in Post Mortem Blood Samples

Two forensic post mortem blood samples from autopsy cases with suspected cyclopropylfentanyl or crotonylfentanyl intoxication after forensic routine analyses were examined using the developed UHPLC-MS/MS method to correctly identify which compound was present. In both samples cyclopropylfentanyl was identified (data not shown) based on retention time. Furthermore, the samples were analyzed with UHPLC-QTOF-MS and a metabolite search was conducted (Table 1). In both samples the cyclopropylfentanyl metabolites A1, A2, A3 and A4 were identified with fragmentation patterns and retention time matching the HLM findings, confirming the presence of cyclopropylfentanyl in the blood

samples. The relative abundance of the cyclopropylfentanyl metabolites in the blood samples were A1>A2>A4>A3, as compared to A1>A2>A3>A4 in the HLM samples, indicating that in vitro metabolism in HLM reflects the biotransformation pattern in the human body.

CONCLUSION

In the present study, we demonstrated two different approaches for distinguishing between cyclopropylfentanyl and crotonylfentanyl using methods commonly available in the forensic laboratory. First, we achieved baseline separation of the two compounds by reversed phase UHPLC-MS/MS using a BEH C_{18} column and gradient elution. Second, we identified the most abundant cyclopropylfentanyl and crotonylfentanyl metabolites formed by HLMs using UHPLC-QTOF-MS and found that the two analogues produced unique major metabolites. Knowledge of the main metabolites of these compounds can be useful to distinguish between the isomers, especially in cases where a chromatographic system with a BEH C_{18} column or reference standards are not available. The two approaches were successfully applied by distinguishing between cyclopropylfentanyl and crotonylfentanyl in authentic post mortem blood samples.

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Figure legends

Figure 1: Structural formulas, chemical formulas and mass to charge ratio (*m/z*) of fentanyl, cyclopropylfentanyl and crotonylfentanyl.

Figure 2: Daughter ion scans of cyclopropylfentanyl and crotonylfentanyl (2 µM, individually prepared in water) in the *m/z* range of 50-350 with proposed fragmentation pattern. For simplicity, protons are not indicated on metabolite fragment formulas.

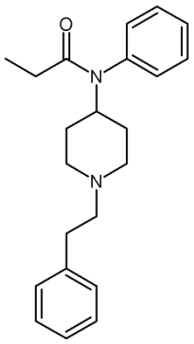
Figure 3: A: Total ion chromatograms of cyclopropylfentanyl (purple) and crotonylfentanyl (black) (0.5 µM, prepared in water) separated on three different columns by a 10 and 20 min linear gradient (5-100 % MeOH). A mobile phase consisting of 10 mM ammonium formate pH 3.1 (A) and MeOH (B) with a flow rate of 0.6 mL/min and a column temperature of 60 °C was used. B: Total ion chromatogram of cyclopropylfentanyl and crotonylfentanyl separated on a BEH C_{18} column with an optimized gradient profile (0-0.5 min; 25 % B, 0.5-8.0 min; 25-30 % B, 8.0-8.1 min; 30-100 % B, 8.1-9 min; 100 % B, 9.0-9.1 min; 100-25 % B, 9.1-10 min; 25 % B).

Figure 4: Proposed structures of cyclopropylfentanyl metabolites (A1-A4) and crotonylfentanyl metabolites (B1-B3). Markush bonds in red color indicate possible locations for a hydroxy group. The location of the two double bonds on the phenyl ring of the phenethyl moiety of A4 is unknown and randomly depicted in the figure.

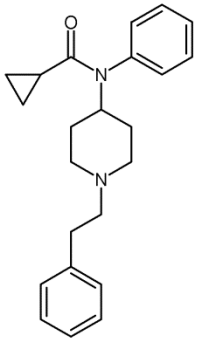
Table 1: Proposed main metabolites of cyclopropylfentanyl (A1-A4) and crotonylfentanyl (B1-B3) in HLM and human blood samples with biotransformation, retention time (T_R), molecular formula, accurate mass of protonated molecule, mass error, and MS/MS product ions.

							Peak heights in HLM[a]			Peak heights in blood samples		
Drug	ID	Biotransformation	T_R (min)	Molecular formula	$[M+H]^{+}$ [b]	Mass error (ppm)[c]	0 min	60 min	120 min	Sample 1	Sample 2	MS/MS product ions[d]
Cyclopropylfentanyl	A1[e]	N-dealkylation	6.50	$C_{15}H_{20}N_2O$	245.1648	-1.31	N.D.	4.0×10^6	4.0×10^6	8.4×10^5	1.9×10^6	177.1382, 162.0910, ***84.0810***, **69.0333**
	A2	Hydroxylation	7.68	$C_{23}H_{28}N_2O_2$	365.2224	-1.08	N.D.	1.0×10^6	5.1×10^5	3.8×10^5	2.8×10^5	***204.1382***, **121.0647**, 84.0807, 69.0331
	A3	Hydroxylation	8.42	$C_{23}H_{28}N_2O_2$	365.2224	1.49	N.D.	5.9×10^5	2.5×10^5	2.5×10^5	2.4×10^5	347.2112, ***204.1379***, **186.1277**, 174.1279, 134.0960
	A4	Dihydrodiol formation	6.62	$C_{23}H_{30}N_2O_3$	383.2329	1.42	N.D.	9.0×10^4	7.7×10^4	3.4×10^5	5.2×10^5	222.1482, 204.1385, ***189.1386***, **146.0961**, 121.0648
Crotonylfentanyl	B1	Carboxylation	6.72	$C_{23}H_{26}N_2O_3$	379.2016	0.92	N.D.	6.1×10^5	7.8×10^5	N.D.	N.D.	258.1126, ***188.1430,*** 146.0959, 132.0797, **105.0697**
	B2	Alkene to dihydrodiol	6.08	$C_{23}H_{30}N_2O_3$	383.2329	1.28	N.D.	3.5×10^5	3.1×10^5	N.D.	N.D.	281.2006, 262.1439, ***188.1435***, 146.0962, **105.0701**, 85.0282
	B3	Di-hydroxylation	5.98	$C_{23}H_{28}N_2O_3$	381.2173	-3.69	N.D.	1.4×10^5	9.5×10^4	N.D.	N.D.	363.2051, ***204.1379,*** **186.1266**, 174.1272, 134.0964, 85.0281

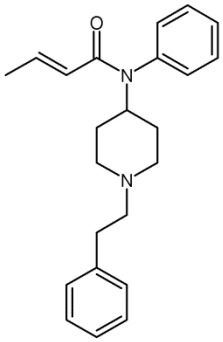
[a]Average peak height for each time point (n=3), [b]Average *m/z* of the molecular ions detected in the 60 min and 120 min HLM samples combined (n=6) [c]Average mass error obtained for all the analyzed HLM samples, [d]Average of the MS/MS product ions detected in the 60 min and 120 min HLM samples combined (n=6), with base peaks (bold italics) and second most abundant peaks (bold) highlighted, [e]Signal intensity in HLM samples at 60 and 120 min were saturated.



Fentanyl
$C_{22}H_{28}N_2O$
m/z: 336.2202

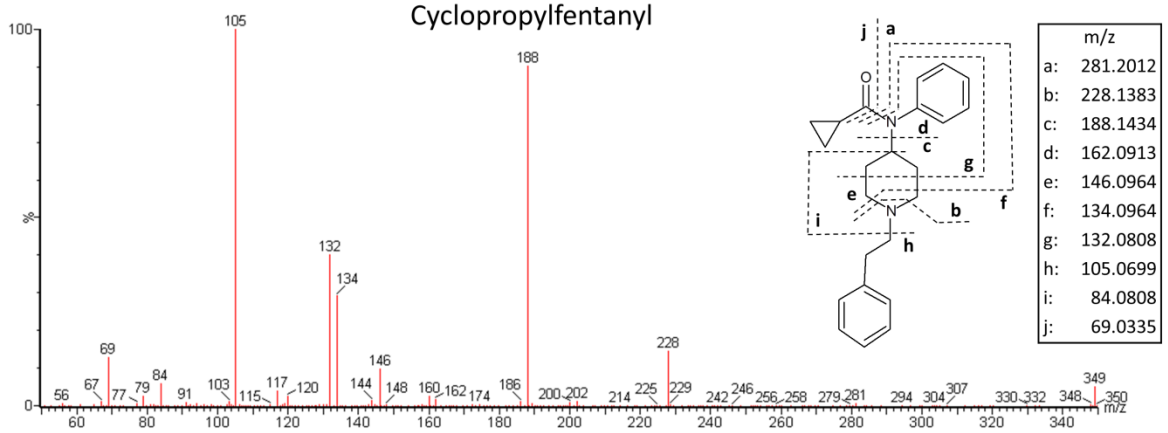


Cyclopropylfentanyl
$C_{23}H_{28}N_2O$
m/z: 348.2202



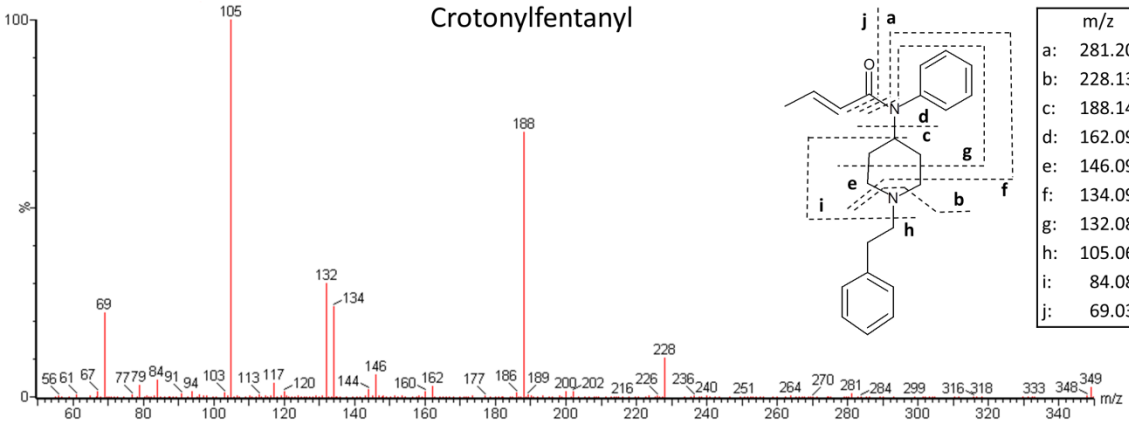
Crotonylfentanyl
$C_{23}H_{28}N_2O$
m/z: 348.2202

Cyclopropylfentanyl



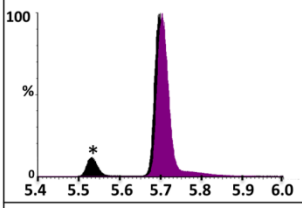
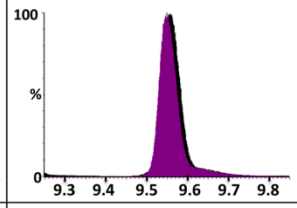
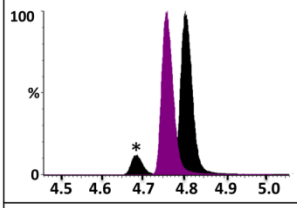
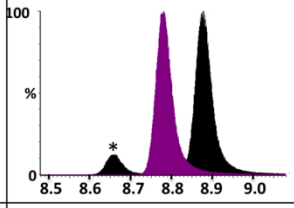
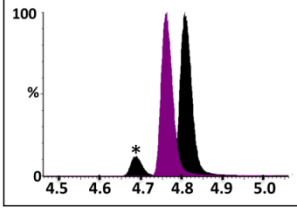
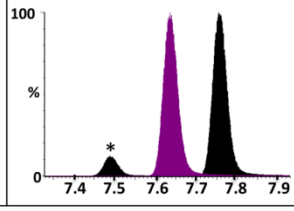
	m/z
a:	281.2012
b:	228.1383
c:	188.1434
d:	162.0913
e:	146.0964
f:	134.0964
g:	132.0808
h:	105.0699
i:	84.0808
j:	69.0335

Crotonylfentanyl

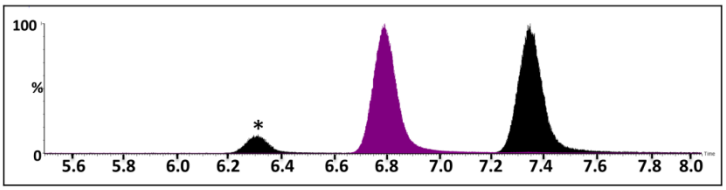


	m/z
a:	281.2012
b:	228.1383
c:	188.1434
d:	162.0913
e:	146.0964
f:	134.0964
g:	132.0808
h:	105.0699
i:	84.0808
j:	69.0335

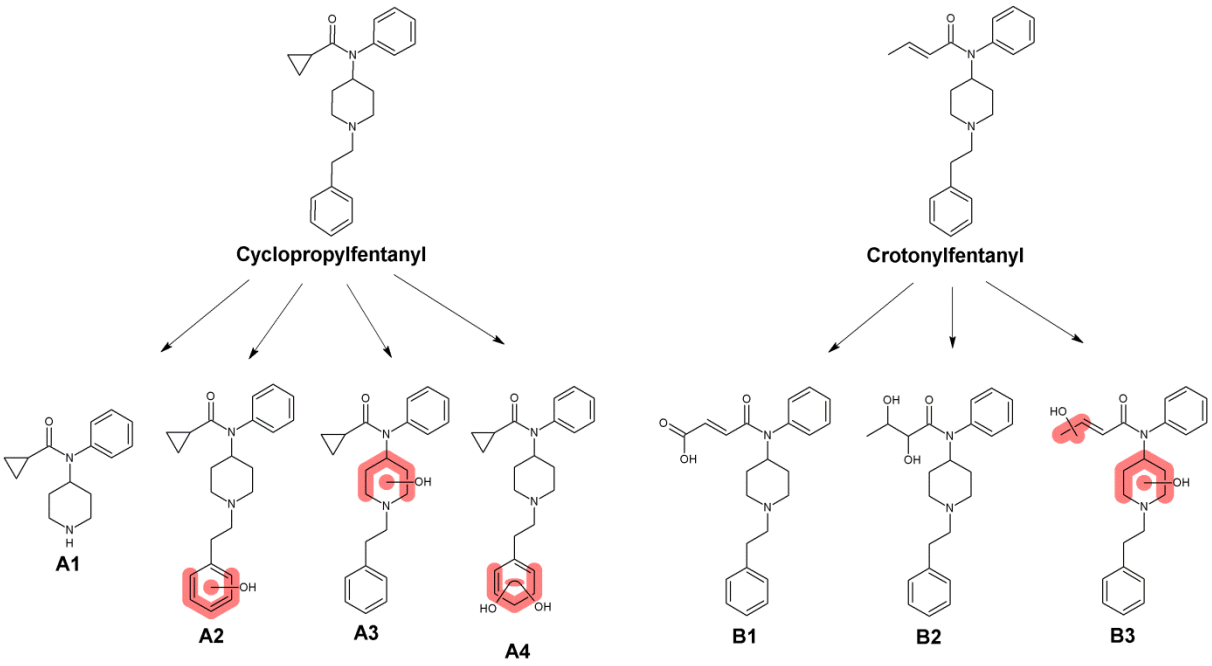
A

Linear 10 min gradient	Linear 20 min gradient	Column
		Kinetex biphenyl
		HSS T3 C_{18}
		BEH C_{18}

B



*** Impurity from the crotonylfentanyl standard**


Cyclopropylfentanyl
Crotonylfentanyl
A1
A2
A3
A4
B1
B2
B3