



Designer Fentanyls

Drugs that kill and how to detect them

Cyclopropylfentanyl



The *in vitro* metabolism of cyclopropylfentanyl

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Recent communications from the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) have highlighted a growing trend in the seizures and recreational use of fentanyl analogues in certain European countries. As with New Psychoactive Substances (NPS), the fentanyl analogues in circulation are constantly evolving with nine substances reported for the first time in 2016 and ten in 2017. There has been a large increase in availability of these drugs in Europe in the past few years due to their open sale by chemical companies based in China. In Europe they are typically used as 'legal' substitutes for heroin and other illicit opioids.

The fentanyl analogues are a family of highly potent opioid drugs that can rapidly incapacitate by causing central nervous system depression and respiratory depression. Untreated poisoning may rapidly cause death.

As part of LGC's drug testing service out of our Fordham laboratory in the UK, work is performed for forensics

laboratories working for UK coroners. This includes testing for synthetic cannabinoid receptor agonists, other new psychoactive substances (NPS), drugs of abuse and prescription drugs. The technology used is Thermo Scientific™ Orbitrap™-based high-resolution accurate-mass (HRAM) liquid chromatography-mass spectrometry (LCMS) enabling extremely broad analyte coverage at high sensitivity.

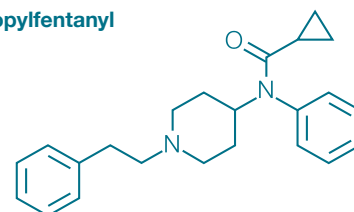
To maintain the efficacy of our drug testing service it is imperative that the metabolic fate of new drug compounds such as the fentanyl analogues is considered. As raw drug material becomes available, either from casework or from purchases, a rapid *in vitro* metabolism technique is employed to generate data for both our HRAM databases and HRAM MS2 library.

In August 2017 cyclopropylfentanyl was identified in a drug seizure in Latvia and was subsequently reported out to European forensic networks. Since then it has been detected in several other European countries including the UK. This compound was submitted for *in vitro* metabolism studies within LGC.

This paper is intended to share knowledge from LGC's laboratories regarding:

1. Analytical methodology enabling the detection of low levels of many drugs including fentanyl analogues
2. Cyclopropylfentanyl *in vitro* metabolism data.

cyclopropylfentanyl



A rapid *in vitro* analysis on cyclopropylfentanyl was performed as follows:

1. Place 240µl 50mM TRIS buffer in a suitable polypropylene tube together with 83.3µl 2.5 mM NADPH cofactor solution.
2. Add 16.67µl of human microsome preparation (Corning Inc. Part no. 452161 – 20-donor pool-mixed gender)
3. Add 4µl of drug solution and vortex mix briefly
4. Incubate at 37C for 3 hours
5. Vortex mix samples briefly and then transfer 160µl to each of 2 Eppendorf tubes.
6. 225µl of ice cold acetonitrile was then added to each Eppendorf tube
7. The Eppendorf tubes were then centrifuged at approximately 11000 rpm for 10 minutes before transferring the supernatants to 5ml glass tubes.
8. The supernatants were then dried, reconstituted in LCMS mobile phase and transferred to suitable vials for analysis

Analytical methodology

Created with contributions from



A 10 μL portion of the prepared *in vitro* sample was injected for analysis onto a Thermo Scientific™ UltiMate™ Closed Sampler XRS Ultra-High Performance Liquid Chromatography (UHPLC) system, interfaced to a Thermo Scientific™ Q Exactive™ Focus hybrid quadrupole Orbitrap™ mass spectrometer, operating in heated positive ion electrospray mode. Chromatographic separation was achieved in 5.0 minutes on a Waters Atlantis T3 HPLC column maintained at 40°C using a gradient consisting of a mixture of 0.1% acetic acid (A) and acetonitrile containing 0.1% acetic acid (B). Using a flow rate of 400 $\mu\text{L}/\text{minute}$, initial solvent conditions were 99% A and 1% B. After 0.3 minutes Solvent B was ramped to 9% over the next 0.9 minutes, to 30% over the next 0.8 minutes, to 43% over the next 0.65 minutes, to 65% over

the next 0.35 minutes and then to 99% after a total run time of 3.4 minutes. The final conditions were held until 4.5 minutes at which point the solvent composition reverted to 1% B. These conditions were held for a further 2 minutes for equilibration prior to the next injection.

Data were acquired in full scan mode operating at a mass resolution of 70,000 (FWHM) at m/z 200, across a mass range of 80-550 amu and manually interrogated to locate potential phase I metabolites base on prior knowledge of fentanyl metabolism. A second PRM only method was employed to generate HRAM MS2 data using a stepped HCD setting of 15, 35 and 50 at a mass resolution of 17,500.

Metabolites of cyclopropylfentanyl

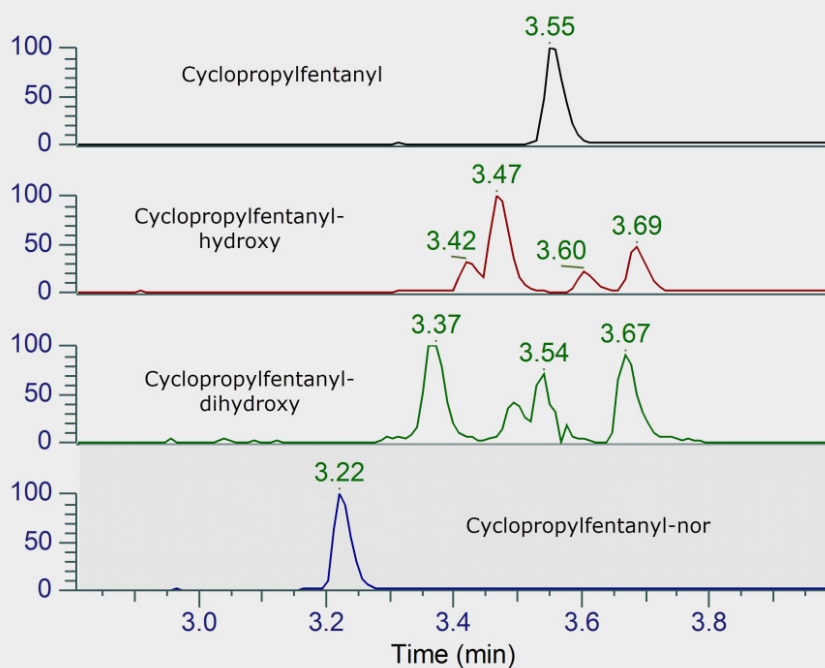
Review of the acquired data for cyclopropylfentanyl revealed that in this particular *in vitro* system, the metabolites were produced predominantly through hydroxylation and dealkylation.

Figure 1 shows accurate mass extracted ion chromatograms for parent cyclopropylfentanyl, hydroxy metabolites, dihydroxy metabolites and a nor metabolite.

Figure 1

Extracted ion chromatograms from full scan data for cyclopropylfentanyl, hydroxy metabolites, dihydroxy metabolites and a nor metabolite.

RT :2.81-3.99



NL: 7.73E9
m/z= 349.2224-349.2324 MS F: FTMS + c ESI
Full ms [80.0000-600.0000] cyclopropylfentanyl

NL: 7.27E8
m/z= 365.2174-365.2274 MS F: FTMS + c ESI
Full ms [80.0000-600.0000] cyclopropylfentanyl

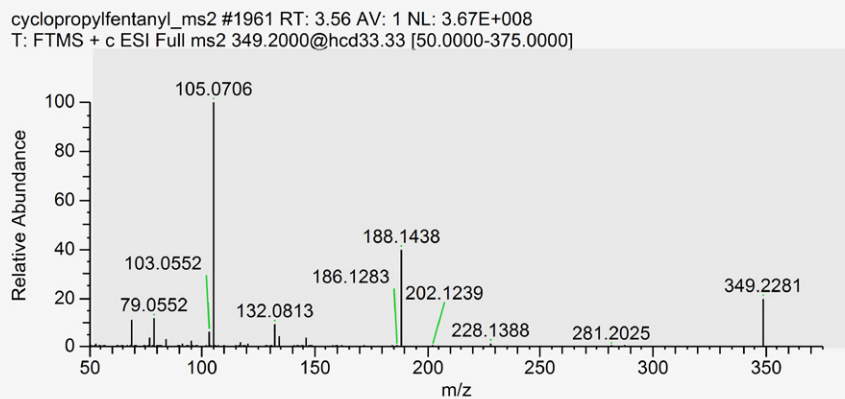
NL: 1.05E7
m/z= 381.2123-381.2223 MS F: FTMS + c ESI
Full ms [80.0000-600.0000] cyclopropylfentanyl

NL: 4.21E9
m/z= 245.1598-245.1698 MS F: FTMS + c ESI
Full ms [80.0000-600.0000] cyclopropylfentanyl

The full scan HRAM MS2 mass spectrum for cyclopropylfentanyl is shown in figure 2.

Figure 2

Full scan HRAM MS2 mass spectrum for m/z 349.2281 - cyclopropylfentanyl



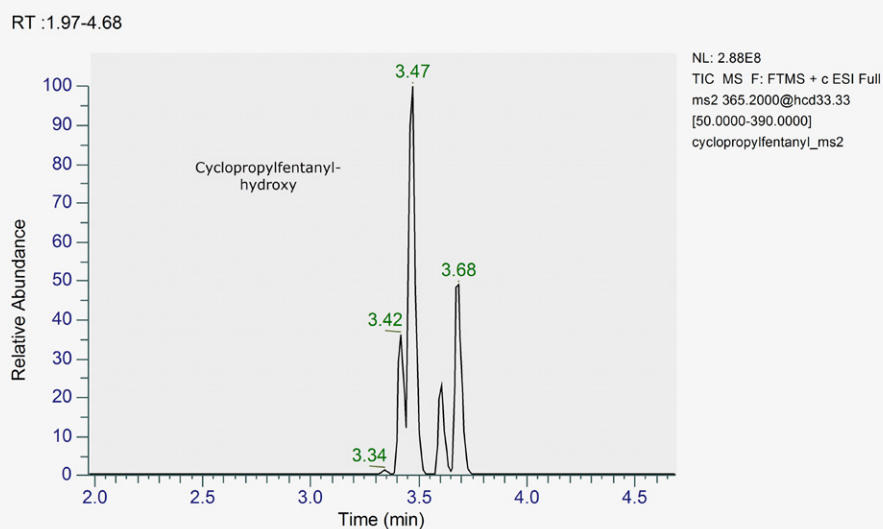
Subsequent re-analysis of the *in vitro* preparation by Orbitrap HRAM LCMS2 using the precursor ions representing parent cyclopropylfentanyl and the postulated metabolites from the full scan experiment identified five hydroxylated metabolites and four dihydroxylated metabolites in addition to the nor metabolite.

Monohydroxylated metabolites of cyclopropylfentanyl

A total of five monohydroxylated metabolites were seen. Figures 4 through to 8 present the full scan HRAM MS2 mass spectra for these and they have been assigned identities of M1 through to M5 based on their chromatographic elution order.

Figure 3

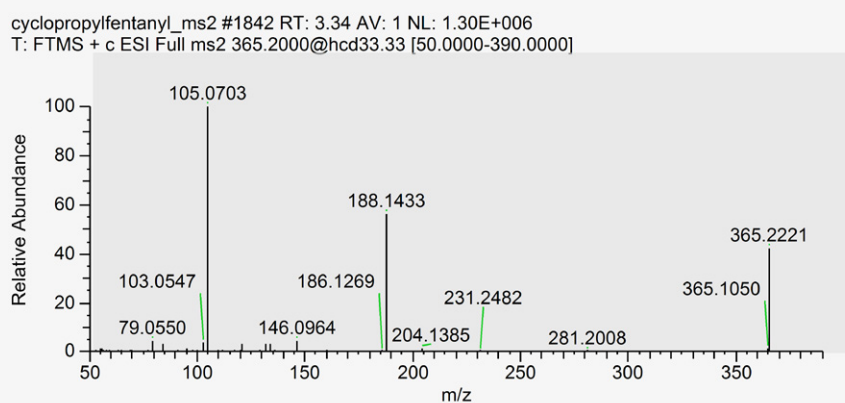
TIC chromatogram from MS2 data for monohydroxylated metabolites of cyclopropylfentanyl



Metabolite M1 is postulated to be hydroxylated in the N-phenylcyclopropanecarboxamide portion of the molecule, most likely in the benzene ring.

Figure 4

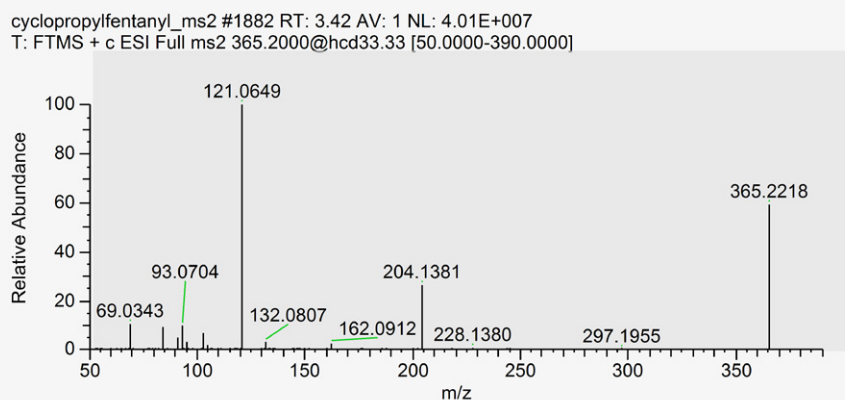
Full scan MS2 HRAM mass spectrum for m/z 365.2221 – hydroxy cyclopropylfentanyl (M1)



Metabolite M2 is likely to be hydroxylated in the benzene ring of the phenethyl group.

Figure 5

Full scan MS2 HRAM mass spectrum for m/z 365.2221 – hydroxy cyclopropylfentanyl (M2)

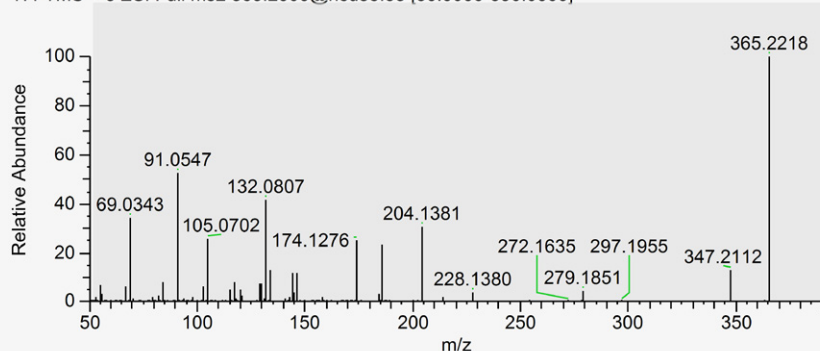


The M3 metabolite is postulated to be hydroxylated in the β position of the phenethyl group based on the spectrum retrieved from the mzcloud¹ on line database for β -hydroxyfentanyl.

Figure 6

Full scan MS2 HRAM mass spectrum for m/z 365.2221 – hydroxy cyclopropylfentanyl (M3)

cyclopropylfentanyl_ms2 #1912 RT: 3.47 AV: 1 NL: 5.37E+007
T: FTMS + c ESI Full ms2 365.2000@hcd33.33 [50.0000-390.0000]



Metabolites M4 and M5 are likely to be N-oxide stereoisomers formed on the piperidine ring structure.

Figure 7

Full scan MS2 HRAM mass spectrum for m/z 365.2221 – hydroxy cyclopropylfentanyl (M4)

cyclopropylfentanyl_ms2 #1987 RT: 3.60 AV: 1 NL: 1.60E+007
T: FTMS + c ESI Full ms2 365.2000@hcd33.33 [50.0000-390.0000]

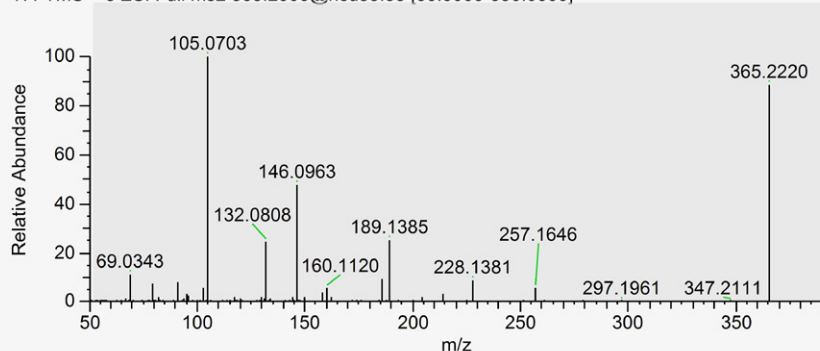
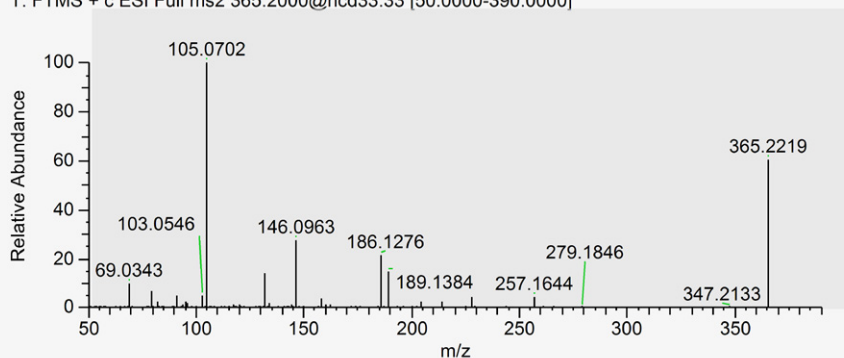


Figure 8

Full scan MS2 HRAM mass spectrum for m/z 365.2221 – hydroxy cyclopropylfentanyl (M5)

cyclopropylfentanyl_ms2 #2032 RT: 3.68 AV: 1 NL: 4.28E+007
T: FTMS + c ESI Full ms2 365.2000@hcd33.33 [50.0000-390.0000]



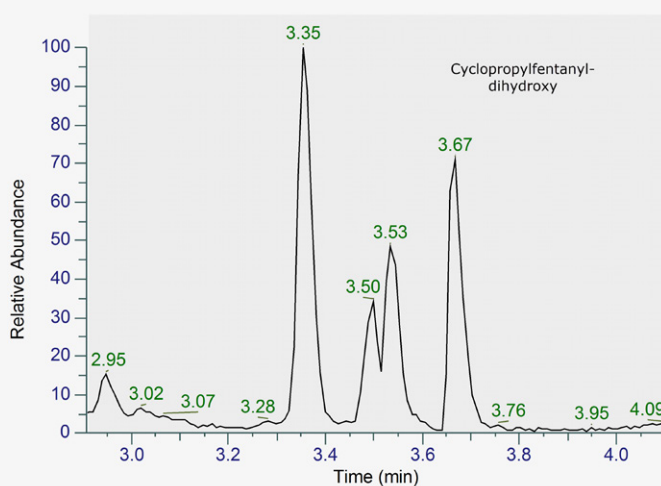
Dihydroxylated metabolites of cyclopropylfentanyl

A total of four dihydroxylated metabolites were seen. Figure 9 shows the TIC chromatogram for the HRAM MS2 data generated for the precursor ion of m/z 381.2. Figures 10 through to 13 present the full scan HRAM MS2 mass spectra for these.

Figure 9

TIC chromatogram from MS2 data for dihydroxylated metabolites of cyclopropylfentanyl

RT :2.91-4.10



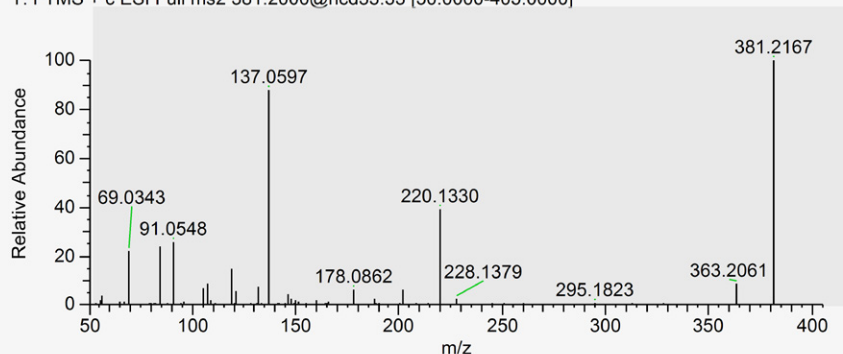
NL: 5.07E6
TIC MS F: FTMS + c ESI Full
ms2 381.2000@hcd33.33
[50.0000-405.0000]
cyclopropylfentanyl_ms2

Dihydroxy metabolite M6 is postulated to be dihydroxylation of the benzene ring of the phenethyl group.

Figure 10

Full scan MS2 HRAM mass spectrum for m/z 381.2167 – dihydroxy cyclopropylfentanyl (M6)

cyclopropylfentanyl_ms2 #1853 RT: 3.36 AV: 1 NL: 1.08E+006
T: FTMS + c ESI Full ms2 381.2000@hcd33.33 [50.0000-405.0000]

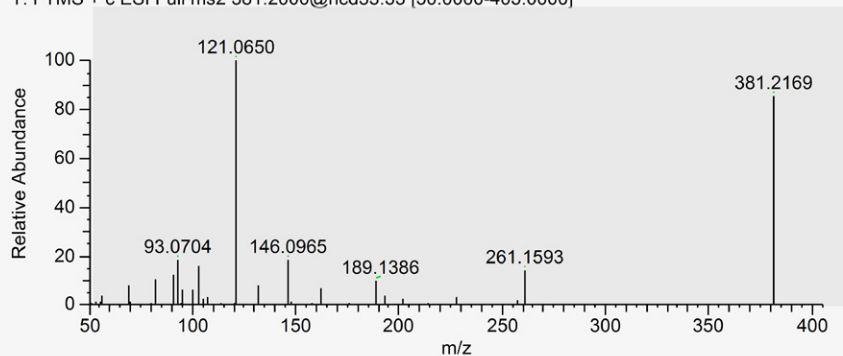


Dihydroxy metabolite M7 is believed to be the N-oxide with additional hydroxylation in the benzene ring of the phenethyl portion of the molecule. On the basis of M4 and M5, one would expect to see both isomers here. As only one is seen, it is possible that both are co-eluting.

Figure 11

Full scan MS2 HRAM mass spectrum for m/z 381.2167 – dihydroxy cyclopropylfentanyl (M7)

cyclopropylfentanyl_ms2 #1928 RT: 3.50 AV: 1 NL: 4.73E+005
T: FTMS + c ESI Full ms2 381.2000@hcd33.33 [50.0000-405.0000]



Dihydroxy metabolites M8 and M9 are postulated to be both N-oxide isomers M4 and M5 with additional hydroxylation in the β position

Figure 12

Full scan MS2 HRAM mass spectrum for m/z 381.2167 – dihydroxy cyclopropylfentanyl (M8)

cyclopropylfentanyl_ms2 #1953 RT: 3.54 AV: 1 NL: 6.30E+005
T: FTMS + c ESI Full ms2 381.2000@hcd33.33 [50.0000-405.0000]

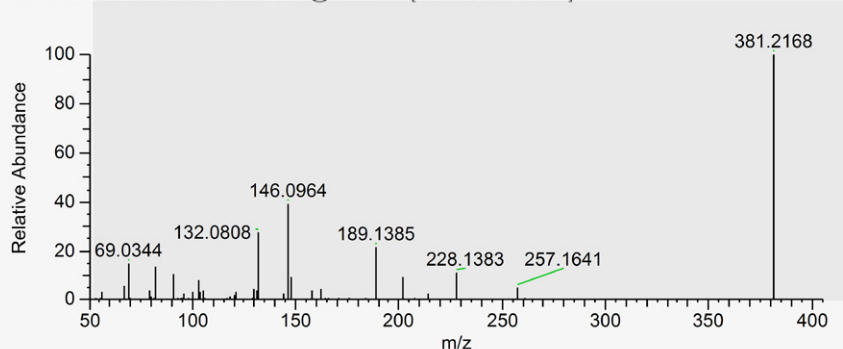
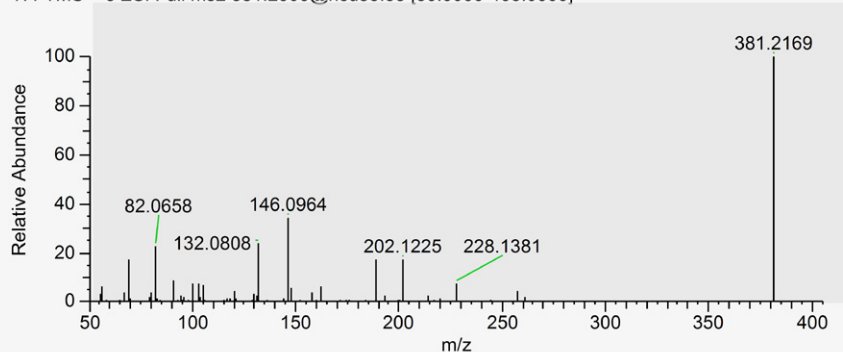


Figure 13

Full scan MS2 HRAM mass spectrum for m/z 381.2167 – dihydroxy cyclopropylfentanyl (M9)

cyclopropylfentanyl_ms2 #2023 RT: 3.67 AV: 1 NL: 9.78E+005
T: FTMS + c ESI Full ms2 381.2000@hcd33.33 [50.0000-405.0000]



As with many fentanyl analogues, dealkylation occurs to give nor metabolites. Metabolite M10 is the nor metabolite which is formed through the loss of the phenethyl moiety.

Figure 14

Full scan MS2 HRAM mass spectrum for m/z 245.1647 – norcyclopropylfentanyl (M10)

cyclopropylfentanyl_ms2 #1774 RT: 3.22 AV: 1 NL: 1.65E+008
T: FTMS + c ESI Full ms2 245.2000@hcd33.33 [50.0000-270.0000]

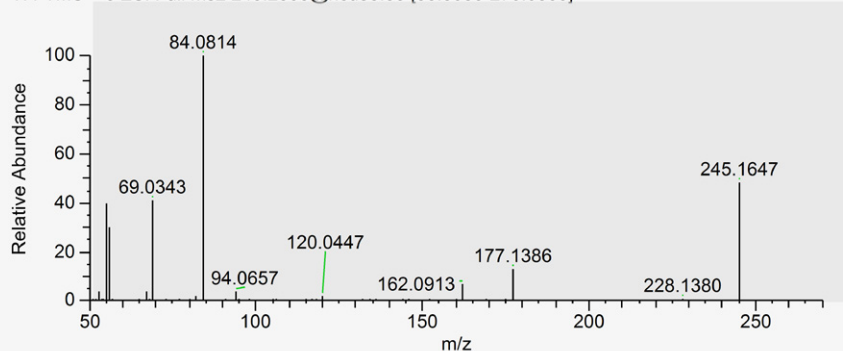
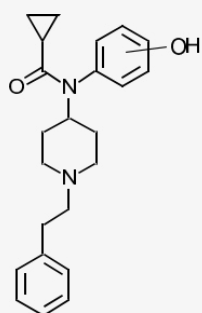
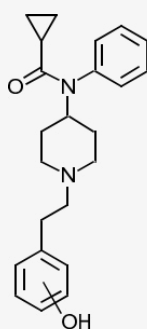


Figure 15 shows the structures of the postulated metabolites

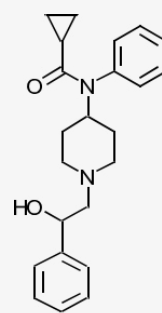
Figure 15
Structures of
postulated metabolites
generated by the *in vitro* metabolism of
cyclopropylfentanyl



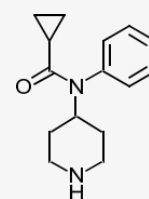
M1



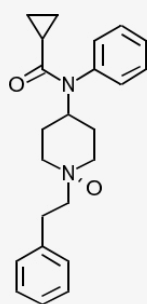
M2



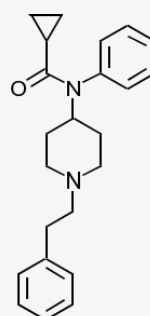
M3



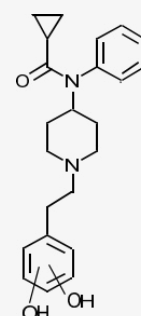
M10



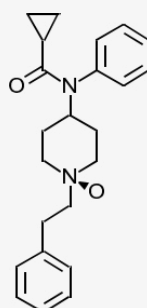
M4



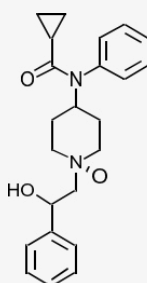
cyclopropylfentanyl



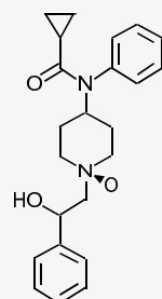
M6



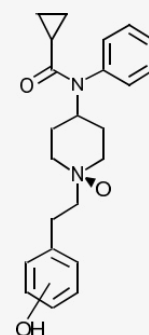
M5



M8



M9



M7

Summary

The data generated in this *in vitro* study can be used as an aid to detect and tentatively identify cyclopropylfentanyl and metabolites in biological fluids. Previous comparisons of *in vitro* and *in vivo* metabolism of fentanyl suggests that the N-oxide metabolites may be unique to the *in vitro* model², but other hydroxylations and dealkylation are common to both.

References

1. The mzCloud mass spectral database, accessible at. <https://www.mzcloud.org/> is maintained by Thermo Fisher Scientific Inc. and HighChem Ltd.
2. 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). Steuer AE, Williner E, Staeheli SN, Kraemer T. 7, s.l. : Wiley, 2017, Drug Testing and Analysis, Vol. 9, pp. 1085-1092.

LGC offers an extensive range of fentanyl reference materials including precursors and metabolites. To find exactly what you need visit **[lgcstandards.com](https://www.lgcstandards.com)**
