Designer Fentanyl
Drugs that kill and how to detect them
Methoxyacetylfentanyl
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 December 2016 methoxyacetylfentanyl was identified in a drug seizure in Slovenia and was subsequently reported out to European forensic networks. Methoxyacetylfentanyl has been submitted for in vitro metabolism studies within LGC Standards.

A rapid in vitro analysis on both substances 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.
3. Add 4µl of drug solution and mix
4. Incubate at 37°C 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.

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

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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 400µl/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 methoxyacetylfentanyl

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

Figure 1 shows accurate mass extracted ion chromatograms for parent methoxyacetylfentanyl, O-desmethyl metabolite, O-desmethyl hydroxy metabolites, nor metabolite and dihydroxy metabolite.

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

**Figure 2**

Full scan HRAM MS2 mass spectrum for m/z 353.2 - methoxyacetylfentanyl

Subsequent re-analysis of the preparation by Orbitrap HRAM LCMS2 using the precursor ions representing parent methoxyacetylfentanyl and the postulated metabolites from the full scan experiment identified one O-desmethyl metabolite, three hydroxylated O-desmethyl metabolites, five hydroxylated metabolites, one dihydroxylated metabolite and one nor-metabolite.

**O-desmethyl metabolite of methoxyacetylfentanyl**

Metabolite M1 is O-demethylated methoxyacetylfentanyl which is one of the expected major metabolites of methoxyacetylfentanyl (Figure 3).
Figure 4 shows the TIC chromatogram constructed from the MS2 data acquired for product ions of m/z 355.2, the precursor ion of monohydroxylated O-demethylated metabolites of methoxyacetylfentanyl.

**Figure 4**
TIC chromatogram from MS2 data for monohydroxylated O-demethylated metabolites of methoxyacetylfentanyl

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**Hydroxy metabolites of O-desmethyl methoxyacetylfentanyl**

Figures 5 through to 7 show the full scan HRAM MS2 mass spectra for monohydroxylated O-demethylated metabolites of methoxyacetylfentanyl, which have been assigned identities of M2 through to M4 based on their chromatographic elution order. Metabolites M2 through to M4 are all O-demethylated with single hydroxylations in different positions.

Metabolite M2 in figure 5 is postulated to be hydroxylated in the benzene ring of the phenethyl moiety.

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**O-desmethyl metabolite of methoxyacetylfentanyl**

**Figure 5**
Full scan HRAM MS2 mass spectrum for m/z 355.2 – hydroxy O-desmethyl methoxyacetylfentanyl (M2)
In figure 6, metabolite M3 is likely to be β hydroxylated in the phenethyl group. The M3 metabolite is postulated to be hydroxylated in the β position of the phenethyl group based on the spectrum retrieved from the mzcloud on line database for β-hydroxyfentanyl.

Metabolite M4 (figure 7) is postulated to be hydroxylated in the N-phenyl portion of the molecule, most likely in the benzene ring.
Hydroxylated metabolites of methoxyacetylfentanyl

The data in figures 8 to 12 shows at least 4 monohydroxylated metabolites (M5-M8) of methoxyacetylfentanyl.

**Figure 8**
TIC chromatogram from MS2 data for monohydroxylated metabolites of methoxyacetylfentanyl.

Metabolite M5 in figure 9 is likely to be a hydroxylation in the benzene ring of the phenethyl moiety.

**Figure 9**
Full scan HRAM MS2 mass spectrum for m/z 369.2 – hydroxy methoxyacetylfentanyl (M5)

Metabolite M6 in figure 10 is likely to be hydroxylated in the beta position of the phenethyl moiety.

**Figure 10**
Full scan HRAM MS2 mass spectrum for m/z 369.2 – hydroxy methoxyacetylfentanyl (M6)
In figure 11, metabolite M7 is postulated to be hydroxylated in the N-phenyl portion of the molecule, most likely in the benzene ring.

**Figure 11**
Full scan MS2 HRAM mass spectrum for m/z 369.2 – hydroxy methoxyacetylfentanyl (M7)

Metabolite M8 in figure 12 is likely to be the N-oxide stereoisomers formed on the piperidine ring structure. As only one peak is seen, it is likely that both are coeluting.

**Figure 12**
Full scan HRAM MS2 mass spectrum for m/z 369.2 – hydroxy methoxyacetylfentanyl (M8)
Dihydroxylated metabolite of methoxyacetylfentanyl

One dihydroxylated metabolite (M9) was seen. Figure 13 shows the TIC chromatogram for the HRAM MS2 data generated for the precursor ion of m/z 385.2. Figure 14 shows the full scan HRAM MS2 mass spectrum for this which is likely to be dihydroxylated in the benzene ring of the phenethyl moiety.

**Figure 13**
TIC chromatogram from MS2 data for dihydroxylated metabolites of methoxyacetylfentanyl

**Figure 14**
Full scan HRAM MS2 mass spectrum for m/z 385.2 – dihydroxy methoxyacetylfentanyl (M9)
As with many fentanyl analogues, dealkylation occurs to give nor metabolites. Figure 15 shows the nor metabolite (M10) of methoxyacetylfentanyl.

**Figure 15**
Full scan HRAM MS2 mass spectrum for m/z 249.2 – nor-methoxyacetylfentanyl (M10)

Figure 16 shows the structures of the postulated metabolites.

**Figure 16**
Structures of postulated metabolites generated by the in vitro metabolism of methoxyacetylfentanyl
The data generated in this *in vitro* study can be used as an aid to detect and tentatively identify methoxyacetylfentanyl and metabolites in biological fluids. Previous comparisons of *in vitro* and *in vivo* metabolism of fentanyls 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/](https://www.mzcloud.org/) is maintained by Thermo Fisher Scientific Inc. and HighChem Ltd.


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