Journal of Analytical Toxicology, 2021;45:760–771
doi:https://doi.org/10.1093/jat/bkab053
Advance Access Publication Date: 19 May 2021
Special Issue

OXFORD

Special Issue

Circumstances, Postmortem Findings, Blood Concentrations and Metabolism in a Series of Methoxyacetylfentanyl-Related Deaths

Robert Kronstrand[1,2,*], Anna Åstrand[1,2], Shimpei Watanabe[1,2], Henrik Gréen[1,2] and Svante Vikingsson[1,2,3]

[1]Division of Clinical Chemistry and Pharmacology, Department of Biomedical and Clinical Sciences, Faculty of Medicine and Health and Sciences, Linkoping University, Building 420, 58185 Linköping, Sweden; [2]Department of Forensic Genetics and Forensic Toxicology, National Board of Forensic Medicine, Artillerigatan, 12, 58758 Linköping, Sweden and [3]RTI International, 3040 East Cornwallis Rd, Research Triangle Park, 27709 NC, USA

*Author to whom correspondence should be addressed. Email: robert.kronstrand@liu.se

Abstract

Methoxyacetylfentanyl is one of many fentanyl analogs available as new psychoactive substances. It have been encountered in both the European Union and the United States, and existing literature suggest that methoxyacetylfentanyl is around 3- to 5-fold less potent than fentanyl. The aim of the present work was to combine case information with blood concentrations and abundance of urinary metabolites to investigate the importance of these parameters for toxicological interpretation. Quantification of methoxyacetylfentanyl in femoral blood was performed by LC–MS-MS and urinary metabolites were analyzed by LC–QTOF-MS with and without hydrolysis with β-glucuronidase/arylsulfatase. For confirmation of identified metabolites, methoxyacetylfentanyl was incubated with hepatocytes for up to 5 hours and analyzed with the same method as the urine samples. In eleven postmortem cases (27 to 41 years old and including one female) methoxyacetylfentanyl was reported in femoral blood. The cause of death was intoxication by methoxyacetylfentanyl alone or in combination with other drugs in all but one case, where death was attributed to acute complications of an underlying heart disease but with possible contribution from methoxyacetylfentanyl. In total, 27 urinary metabolites were found, including eight glucuronides. Major biotransformations were O-demethylation, dealkylation to form the nor-metabolite, mono- and dihydroxylations of the phenethyl moiety, as well as combinations thereof. The most abundant metabolites in hydrolyzed urine included O-desmethyl-, O-desmethyl-phenethyl-hydroxy-, O-desmethyl-phenethyl-hydroxymethoxy- and nor-methoxyacetylfentanyl. Differences in the abundance of methoxyacetylfentanyl and its major metabolites could be interpreted to indicate fatal intoxications in abstinent or chronic users. We postulate that urinary concentrations of methoxyacetylfentanyl and two metabolites, in combination with the methoxyacetylfentanyl concentration in femoral blood, might be good indicators of the time between administration and death as well as prior use.

Introduction

Methoxyacetylfentanyl is a fentanyl analog, with the replacement of the propionamide group by a 2-methoxyacetamide group. Methoxyacetylfentanyl is also structurally closely related to ocfentanil having an additional fluorine in the 2-position on the aniline ring structure.

In Europe, methoxyacetylfentanyl became available in the late 2016, and in Sweden, the first methoxyacetylfentanyl-related death occurred in December 2016. Methoxyacetylfentanyl was scheduled

as a hazardous product prohibited to be sold in Sweden on 25 January 2017 and later that year on 19th October, as a narcotic drug. In total, methoxyacetylfentanyl contributed to 11 intoxications in Sweden between 2016 and 2018, and another 10 deaths have been reported from other parts of the European Union (1, 2). From the USA, 24 deaths involving methoxyacetylfentanyl have been reported (3–5).

Methoxyacetylfentanyl has been characterized as a μ-opioid receptor agonist both *in vitro* (6–8) and *in vivo* (2, 9–13). The binding affinity to the μ-opioid receptor was reported to be 17 nM by Hassanien et al. (7) and 0.56 nM by Eshleman (6), ∼11 times and 4.1 times less potent than fentanyl. Using the GTPγS assay, Hassanien et al. reported an EC50 of >500 nM, while Eshleman et al. reported an EC50 of 52 nM being 2.4 times less potent than fentanyl (6, 7). Using a proximity-based assay, Vasudevan et al. reported the recruitment of both mini-G_i and β-arrestin by methoxyacetylfentanyl with EC50 values of 244 and 162 nM, respectively. This indicated methoxyacetylfentanyl to be 3.6- and 8.3-fold less potent than fentanyl with regard to mini-G_i and β-arrestin, respectively (8). The existing *in vitro* literature points toward methoxyacetylfentanyl being less potent than fentanyl.

In the European Monitoring Centre for Drugs and Drug Addiction risk assessment report, an LD50 of 38 mg/kg reported in a mouse model (12) was compared with 11 mg/kg for fentanyl in a similar study, which is 3.4-fold lesser. The ED50 of methoxyacetylfentanyl in a mouse hot plate/tail withdrawal assay have been determined by several groups. Using the peritoneal test, Jílek et al. (12) reported activity but of a lower potency than that of fentanyl. Bagley et al. (9) reported methoxyacetylfentanyl to be 2.9-fold less potent than fentanyl (ED50 0.053 mg/kg). A similar study by Huang et al. (11) reported an ED50 of 0.08 mg/kg, but no fentanyl ED50 values were given. When naltrexone was present, higher concentrations were needed to achieve the effect (10). In the World Health Organization's report (10), further studies were presented showing that methoxyacetylfentanyl could fully substitute morphine in a drug discrimination study. ED50 was calculated as 0.038 mg/kg, which was 4.1-fold higher than that of fentanyl. In summary, there is a reasonable body of evidence suggesting methoxyacetylfentanyl to be less potent, potentially around 3- to 5-fold, compared with fentanyl, also *in vivo*.

As a fentanyl analog less potent than fentanyl, methoxyacetylfentanyl concentrations in postmortem case work would be expected to be higher than those observed for fentanyl. Fogarty et al. (4) reported a mean concentration of 36 ng/mL in 83 cases (median = 6.4 ng/mL, range = 0.06–300), 2.5-fold higher than fentanyl (mean = 14.7 ng/mL, median = 9.4 ng/mL). Similar results were also reported by Beck et al. (3) with a mean of 110 ng/mL from 10 cases (median = 14 ng/mL, range = 5–449). Mardal et al. (1) reported three cases with a mean concentration of 29 ng/g (range = 22–41 ng/g). In these datasets, the mean concentrations are affected by cases with very high concentrations, and the observed median concentrations are lower and more similar to those observed for fentanyl. The significance of opioid concentrations' differences in case work suffers from limitations caused by tolerance, a factor generally unknown. In addition, postmortem concentrations changes can further obscure potency differences between opioids seen in both *in vitro* and *in vivo* experimental settings. Thus, even in the living extraordinary concentrations can be found. Muller et al. (14) reported an intoxication case where a patient survived despite serum concentrations of 40 ng/mL methoxyacetylfentanyl as well as 76 ng/mL cyclopropylfentanyl. The initially unconscious patient had stable pulmonary condition, and no specific treatment was given at the emergency room.

To ensure that the most suitable target analytes are used when screening for drugs, it is important to understand the metabolism. Metabolism can also be used to understand the time frame from intake to death (or other events such as an involuntary intake or an accident) (15–18). Especially parent drug/metabolite ratios of other opioids have been used to estimate the time course until death (15, 17, 18). It is not unreasonable to think this might also be true for fentanyl analogs. The metabolism of methoxyacetylfentanyl has been studied both using *in vitro* models (1, 19–21) and in authentic case samples (1). Wilde reported that O-demethylation was a characteristic biotransformation after incubation with human liver microsomes. N-dealkylation, hydroxylation, amide hydrolysis and N-oxidation was also reported (21). A similar study was carried out by Hudson and Cutler reporting *O*-desmethyl methoxyacetylfentanyl, normethoxyacetylfentanyl and β-hydroxy methoxyacetylfentanyl as the major metabolites after incubation with human liver microsomes (19). Using human cryopreserved hepatocytes, Mardal et al. (1) reported an O-methylated metabolite as the most abundant metabolite alongside 4-aminophenyl-1-phenethylpiperidine (4-ANPP, despropionylfentanyl) formed by amide hydrolysis. The latter was further hydroxylated on the phenyl ring forming a third major metabolite. Nordmeier et al. (20) qualitatively reported metabolites in rat urine and after incubation with human pooled tissue homogenate (phS9 fraction) that could potentially be identical to the major metabolites reported by Mardal et al. with the exception of 4-ANPP in rat urine. Mardal et al. also reported abundance of methoxyacetylfentanyl and metabolites in various matrices in three postmortem cases, including urine. In all three cases, the parent compound was the most abundant peak in urine, and 4-ANPP was among the three most abundant metabolites, and in two of three cases, that was also true for *O*-desmethyl methoxyacetylfentanyl. In the third case, the hydroxylated ANPP identified after hepatocyte incubation was the third most abundant peak while not detected at all in one of the other cases. Other metabolites identified in all three cases included normethoxyacetylfentanyl, a phenethyl hydroxylated metabolite and a combination of amide hydrolysis and phenethyl hydroxylation (hydroxy-4-ANPP) (1).

Using the comparatively large number of cases identified in Sweden, the aim of the present work was to combine case information with blood concentrations and abundance of urinary metabolites to investigate the importance of these parameters for toxicological interpretation.

Material and Methods

In this study, case circumstances are presented together with blood concentrations of methoxyacetylfentanyl obtained using a liquid chromatography–tandem mass spectrometric (LC–MS-MS) method alongside urinary metabolite data obtained using liquid chromatography–quadrupole time of flight–mass spectrometry (LC–QTOF–MS).

Methoxyacetylfentanyl was obtained from Cayman Chemical (Ann Arbor, MI), and 4-ANPP was obtained from Toronto Research Chemicals (North York, Canada). The internal standard fentanyl-d_5 was obtained from Cerilliant (Round Rock, TX). For LC–MS-MS quantification, LC–MS grade acetonitrile, formic acid and methanol were obtained from Fisher Scientific (Gothenburg, Sweden) and ammonium formate from Fluka (Sigma-Aldrich, Stockholm, Sweden). For sample preparation, gradient grade

acetonitrile, methanol and formic acid p.a. (98%) from Merck (Darmstadt, Germany), 95% ethanol from Kemetyl (Haninge, Sweden) and β-glucuronidase/arylsulfatase (*Helix pomatia*) from Roche (Mannheim, Germany) were used.

Cases

The study was approved by the regional ethics committee in Linkoping (Dnr: 2018-186-31) and included all autopsy cases with a reported blood concentration of methoxyacetylfentanyl ($n = 11$). Postmortem urine samples for metabolite identification were retrieved from 9 of the 11 autopsy cases.

LC–MS-MS quantification in blood

Quantification of methoxyacetylfentanyl in femoral blood was performed on an LC-30AD liquid chromatography system (Shimadzu Scientific Instruments, Kyoto, Japan) equipped with a Triple Quad 4500 mass spectrometer and a Turbo V interface (AB SCIEX Instruments, Concord, Ontario). Mobile phases A (0.05% formic acid in 10 mM ammonium formate) and B (0.05% formic acid in methanol) were used at a flow rate of 0.8 mL/min; the linear gradient was from 2% B to 100% B in 3.0 min. A Waters Acquity BEH Phenyl column (2.1 × 50 mm, 1.7 µm, 60°C) was used. Electrospray in positive mode was used for ionization. Data acquisition with two transitions for methoxyacetylfentanyl with *m/z* 353.20/188.10 as quantifier and *m/z* 353.20/105.10 as qualifier was used with fentanyl-d_5 as internal standard (*m/z* 342.0/188.0). A 0.5 g aliquot of blood was fortified with 25 µL of internal standard (1.0 µg/mL) and precipitated with 0.75 mL of acetonitrile:ethanol (90:10) with the addition of 0.075% formic acid. After centrifugation for 10 min, 100 µL supernatant was transferred to an injection vial and 2 µL injected onto the column.

Method validation was performed according to Peters et al. (22) as described for rare analytes and included selectivity, calibration model, accuracy, precision, lower limit of quantitation (LLOQ) and matrix effects. Selectivity was investigated by analyzing five different sources of blank matrix showing no interfering peaks. Calibration model was investigated using triplicates at six levels from 2 to 400 ng/g, and a calibration range from 2 to 200 ng/g was established with calibrator accuracy within 1% and coefficients of variation between 2% and 8%. Accuracy and imprecision under repeatability conditions ($n = 5$) were investigated at three levels, 10, 30 and 150 ng/g. The coefficient of variation (CV%) ranged between 1% and 3% with accuracies of 101%, 104% and 96%, respectively. LLOQ was investigated with control samples at low concentrations and showed satisfactory results at 2 ng/g with an accuracy of 97% and a CV% of 4%. Since the sample preparation consisted of a protein precipitation only, process efficiency could be investigated by comparing analyte areas from five negative autopsy cases fortified with methoxyacetylfentanyl at 10 ng/g with areas from the analyte in neat precipitation media at the same concentration taking into account an 80% water content of the whole blood. The experiments showed a process efficiency of 113% for methoxyacetylfentanyl and 111% for the internal standard suggesting some ion enhancement. The area CV% was <5% for both the autopsy cases and the neat standards.

LC–QTOF-MS identification of metabolites

Urinary metabolites were analyzed by LC–QTOF-MS (Agilent 1290/6550, Kista, Sweden) as described previously (23). Briefly, 100 µL of urine was diluted 1:4 in 1 M sodium acetate buffer pH 5 and 10 µL β-glucuronidase/arylsulfatase (*H. Pomatia*, Roche). After incubation (40°C, 2 h), 1 µL of urine was injected for analysis. Non-hydrolyzed samples were diluted using the same buffer and a negative urine sample for a healthy volunteer was included as a control. For metabolite separation, a 19-min gradient on an Acquity HSS T3 column (150 × 2.1 mm, 1.8 µm, Waters), using 0.1% formic acid in water (A) and acetonitrile (B) as mobile phases, was selected. After a 0.7-min hold at 1% B, a linear gradient reaching 40% B at 13 min was used for elution. To wash the column, the amount of acetonitrile was increased to 95% B at 15 min and held until 18 min, followed by re-equilibration at 1% B.

Hepatocyte incubation of methoxyacetylfentanyl

For confirmation of identified metabolites, methoxyacetylfentanyl was incubated with hepatocytes (5 µM, 1 million cells/mL) as described previously (24). Duplicate incubations for 0, 1, 3 and 5 h were analyzed with the same method as the urine samples. Retention times over multiple runs were correlated using linear regression.

Results

Autopsy cases

The 11 deceased were aged from 27 to 41 years (mean 32.5); one was female, and 10 were male (Table I). The manner of death was accidental in seven cases, natural disease in one and undetermined in three cases. The cause of death was intoxication by methoxyacetylfentanyl alone ($n = 4$) or in combination with other drugs in all but one case, where death was attributed to acute complications of an underlying heart disease but with possible contribution from methoxyacetylfentanyl. Significant postmortem findings that pointed toward opiate toxicity were lung congestion and lung and brain edema with a mean combined lung weight of 1,469 g. Three subjects also presented with froth in the airways. Atherosclerosis of varying degrees were pathologies found that might have contributed to the death. At least eight of the decedents had a history of drug abuse, and all but two were found dead indoors. One subject was found alive, but with respiratory and cardiac arrest. The patient presented to the hospital with RLS8 and a methoxyacetylfentanyl concentration of 41 ng/g. During hospitalization, the patient never regained consciousness and also developed acute kidney injury.

The methoxyacetylfentanyl concentrations in femoral blood ranged between 18 and 140 ng/g with a mean of 47 ng/g and a median of 34 ng/g. Polydrug use was confirmed from toxicological analyses in all cases, but no other non-prescription opioids were present. The prescription benzodiazepines alprazolam and clonazepam as well as the non-prescription norfludiazepam and etizolam were considered contributing to death. In addition, pregabalin, tramadol and alimemazine were among listed contributing medications. At least three different routes of administration were suggested from the death scene findings: injection, oral administration of tablets and snorting.

Methoxyacetylfentanyl metabolites

In total, 27 different urinary metabolites were identified, including eight glucuronides, as seen in Table II and Figure 1. The most abundant metabolite was *O*-desmethyl methoxyacetylfentanyl (M22), and most other metabolites were also detected with and without the methyl group, contributing to the large number of identified metabolites. Other major biotransformations included dealkylation to form the nor-metabolite, mono- and dihydroxylations of the phenethyl

Table I. Demographics, Postmortem Findings, Contributing Pathology and Toxicology, Cause and Manner of Death in the 11 Cases

Case	Age	BMI	Circumstances	Lungs (g)	Brain (g)	Contributing pathology	MeAcF (ng/g)	Contributing toxicology	CoD	MoD
1	33	22.7	Found dead at assisted living. Known history of drug abuse	1240	1600	none	52	Pregabalin (9.2 g/g blood)	Mixed intox	U
2	38	33.0	Found dead at home	1310	1545	Atheroschlerosis	18	Tramadol (0.06 μg/g), oxycodone (0.03 μg/g), norfludiazepam (1.0 μg/g)	Mixed intox	A
3	41	25.4	Found unresponsive at home. Treated 3 days at intensive care. History of drug abuse	1899	1578	Pneumonia	41[a]	Norfludiazepam[a] (0.82 μg/g)	Mixed intox	U
4	28	18.8	Found dead at home	1206	1591	Heart disease. Previous heart infarcts.	25	None	Acute heart complications	D
5	34	27.8	After an evening out, found severely intoxicated and later found unresponsive at home. History of drug abuse	1245	1384	Fatty liver	21	Norfludiazepam (0.06 μg/g) and ethanol (0.26 g/dL)	Mixed intox	A
6	28	25.2	Found dead at home. History of drug abuse	960	1307	None	76	None	Intox MeACF	U
7	27	32.0	Found unresponsive at friend's apartment. Froth in airways. Resuscitation but unsuccessful. History of drug abuse	1761	1740	Atheroschlerosis	140	None	Intox MeACF	A
8	29	28.0	Found dead at home. Froth in the airways. History of drug abuse	1275	1620	Atheroschlerosis	31	Tramadol (0.44 μg/g), etizolam (not quantified), alimemazine (0.09 μg/g)	Mixed intox	A
9	35	25.1	Found dead at home. History of drug abuse	1274	1498	None	37	None	Intox MeACF	A
10	35	28.9	Found unresponsive outdoors. Pronounced dead at hospital. History of drug abuse	1984	1570	Atheroschlerosis	17	Clonazepam (0.01 μg/g), alprazolam (0.007 μg/g), pregabalin (11 μg/g)	Mixed intox	A
11	30	27.5	Found dead at home. Froth in airways	2009	1475	None	51	None	Intox MeACF	A

[a] Antemortem blood sample obtained at hospital.
BMI = body mass index, CoD = cause of detah, MoD = manner of death, A = accident, D = disease, U = uncertain, MeACF = methoxyacetylfentanyl.

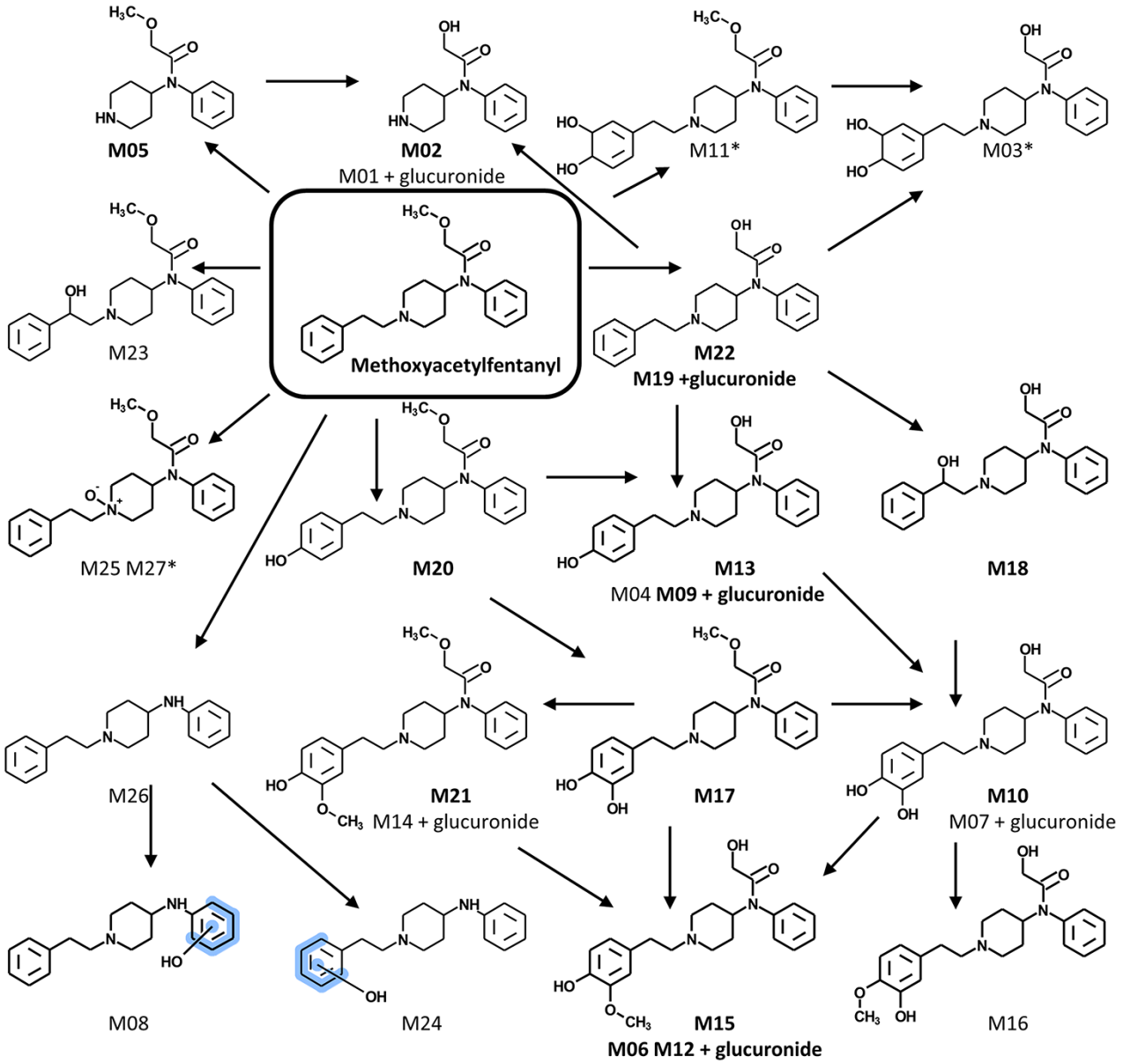

Figure 1. Detected metabolites of methoxyacetylfentanyl. Major metabolites in bold. Some structural isomers deducted from previous work on other fentanyl analogs. Shaded areas show possible placement of the hydroxy group.

moiety (the latter with subsequent methylation). Examples of chromatograms from a case and from a hepatocyte incubation are shown in Figure 2. The metabolite results from the cases were grouped into rapid and delayed deaths based on the analytical findings and described in Table III.

Discussion

In this study, blood concentrations are combined with urinary metabolites and case information to provide a comprehensive dataset. This allowed us to explore the utility of drug concentrations as well as metabolite information in the context of methoxyacetylfentanyl overdoses. The mean and median blood concentrations in this study were 47 and 34 ng/g, respectively. Mean concentrations between 29 ng/g and 110 ng/mL and median concentrations of 6.4 and 14 ng/mL have been reported by others (1, 3, 4). Four cases were signed out as intoxications caused by methoxyacetylfentanyl alone. These cases had methoxyacetylfentanyl concentrations between 37 and 140 ng/g in femoral blood, in the high end of the 11 cases. In the majority of cases, the combined lung weights were consistent with opioid intoxication.

Identification of methoxyacetylfentanyl metabolites

Methoxyacetylfentanyl was identified using a reference standard. The spectrum was dominated by *m/z* 105 corresponding to the phenethyl moiety and *m/z* 188 corresponding to the phenethyl moiety as well as the piperidine ring. The spectrum as well as those of all included metabolites can be found in Supplemental Figure S1. Assigning the exact positional isomer based on MS spectra alone

Table II. Metabolite Identification in Hepatocytes and Authentic Urine Samples

							Area case samples (10^3)								Area hepatocyte samples (10^3)					
		Avg RT	Formula	Avg *m/z*	%	5	7	9	8	10	2	4	11	3		0h_1	1h_1	3h_1	5h_1	Diagnostic
#	Name	(min)	Exact mass	ME (ppm)	conj		Top: hydrolyzed, bottom: non-hydrolyzed								Deg	0h_2	1h_2	3h_2	5h_2	product ions
M01	Nordesmethyl glucuronide	2.95	C19 H26 N2 O8	411.1752							24									84
			410.1689	−3.1							49	73	51							
M02	Nordesmethyl	3.44	C13 H18 N2 O2	235.1439	44			54	73	26	285	471	590	129			309	717	1,438	84
			234.1368	−1.3				44	59	37	203	322	255	51			294	695	1,644	
M03	Phenethyl dihydrodiol desmethyl	4.41	C21 H28 N2 O4	373.2118	35						116	214	75				51	108	222	84, 121, 204
			372.2049	−1.2							75	160	28				53	117	278	222, 247
M04	4-Phenethyl-hydroxy desmethyl glucuronide	5.00	C27 H34 N2 O9	531.2321								21								84, 121, 204
			530.2264	−3.1							75	117								355
M05	Nor	5.06	C14 H20 N2 O2	249.1594	8			27	1,072	614		134	1,343		174	447	3,054	4,460	6,109	84
			248.1525	−0.6				27	1,070	591		128	1,238			209	2,911	4,445	6,311	
M06	4-Phenethyl-hydroxy, 3-phenethyl-methoxy desmethyl glucuronide	5.28	C28 H36 N2 O10	561.2443								32								84, 151, 234
			560.237	−0.1							78	488								
M07	3,4-Phenethyl-dihydroxy desmethyl glucuronide	5.37	C27 H34 N2 O10	547.2280																84, 137, 220
			546.2213	−1.4							45	106								
M08	Phenyl-hydroxy-4-ANPP	5.43	C19 H24 N2 O	297.1953	≥59							28	60					42	123	105, 188
			296.1889	−3.5														36	132	
M09	4-Phenethyl-hydroxy desmethyl glucuronide	5.51	C27 H34 N2 O9	531.2331							48	58								84, 121, 204
			530.2264	−1.3							275	451	48							
M10	3,4-Phenethyl-dihydroxy desmethyl	5.61	C21 H26 N2 O4	371.1958	≥98			60			235	1′002	173							84, 119, 137
			370.1893	−2.6																220

Abundance of methoxyacetylfentanyl metabolites. Ions 132, 146, 158 and 174 not included as diagnostic ions due to limited value in structure elucidation. RT, avg m/z and ME calculated from urine samples, except for M23 where they were calculated from hepatocyte samples (RT was corrected based on regression). The % conj was calculated as signal increase after hydrolysis compared to total signal. In samples where no signal was observed in non-hydrolyzed samples, lowest degree of conjugation was calculated based on a 20 k area threshold. RT, retention time; ME average mass error compared to exact mass.

(Continued)

Table II. Metabolite Identification in Hepatocytes and Authentic Urine Samples

							Area case samples (10^3)								Area hepatocyte samples (10^3)					
		Avg RT	Formula	Avg *m/z*	%	5	7	9	8	10	2	4	11	3		0h_1	1h_1	3h_1	5h_1	Diagnostic
#	Name	(min)	Exact mass	ME (ppm)	conj		Top: hydrolyzed, bottom: non-hydrolyzed								Deg	0h_2	1h_2	3h_2	5h_2	product ions
M11	Phenethyl dihydrodiol	5.67	C22 H30 N2 O4	387.2272	38							61	29				79	104	172	84, 121, 204
			386.2206	−1.7								56					82	120	193	222
M12	4-Phenethyl-hydroxy, 3-phenethyl-methoxy desmethyl glucuronide	5.88	C28 H36 N2 O10	561.2432							25	75								84, 151, 234
			560.237	−3.1							96	541	23							
M13	4-Phenethyl-hydroxy desmethyl	6.19	C21 H26 N2 O3	355.2011	81		32	284	46		1,076	4,335	976	71			248	336	786	84, 121, 204
			354.1943	−2.2				152			97	1,180	116	22			319	501	931	
M14	4-Phenethyl-hydroxy, 3-phenethyl-methoxy glucuronide	6.38	C29 H38 N2 O10	575.2587																84, 119, 151
			574.2526	−1.7							46	90							27	234, 399
M15	4-Phenethyl-hydroxy, 3-phenethyl-methoxy desmethyl	6.58	C22 H28 N2 O4	385.2128	78			380			1,404	7,603	440	65				64	181	84, 119, 151
			384.2049	1.4				334			48	1,993	30	25			27	112	216	234
M16	3-Phenethyl-hydroxy, 4-phenethyl-methoxy desmethyl	6.79	C22 H28 N2 O4	385.2112	≥56						57		47							119, 151, 234
			384.2049	−3.6																
M17	3,4-Phenethyl-dihydroxy	6.94	C22 H28 N2 O4	385.2098	25	36		47			366	506		59						84, 137, 220
			384.2049	−7.8		32	30	30	33		276									
M18	β-Phenethyl-hydroxy desmethyl	6.98	C21 H26 N2 O3	355.2016	77			28			123	233	204	29			36	73	215	84, 91, 105
			354.1943	−0.8								59	26	22			53	123	226	186, 204

Abundance of methoxyacetylfentanyl metabolites. Ions 132, 146, 158 and 174 not included as diagnostic ions due to limited value in structure elucidation. RT, avg m/z and ME calculated from urine samples, except for M23 where they were calculated from hepatocyte samples (RT was corrected based on regression). The % conj was calculated as signal increase after hydrolysis compared to total signal. In samples where no signal was observed in non-hydrolyzed samples, lowest degree of conjugation was calculated based on a 20 k area threshold. RT, retention time; ME average mass error compared to exact mass.

(Continued)

Table II. Metabolite Identification in Hepatocytes and Authentic Urine Samples

							Area case samples (10^3)								Area hepatocyte samples (10^3)					
		Avg RT	Formula	Avg *m/z*	%	5	7	9	8	10	2	4	11	3		0h_1	1h_1	3h_1	5h_1	Diagnostic
#	Name	(min)	Exact mass	ME (ppm)	conj		Top: hydrolyzed, bottom: non-hydrolyzed								Deg	0h_2	1h_2	3h_2	5h_2	product ions
M19	Desmethyl glucuronide	7.24	C27 H34 N2 O8	515.2382							357	306					361	1,636	4,310	105, 188, 339
			514.2315	−1.3			21		277	109	2,521	2,759	2,314	39			312	1,472	4,928	
M20	4-Phenethyl-hydroxy	7.36	C22 H28 N2 O3	369.2166	66				84	30	119	142	309				415	393	804	84, 121, 204
			368.21	−1.6					45	29	31	66	95				570	614	810	
M21	4-Phenethyl-hydroxy,3-phenethyl-methoxy	7.68	C23 H30 N2 O4	399.2273	88			35			226	599	123					39	91	84, 119, 151
			398.2206	−1.6				31				88					26	76	87	234
M22	Desmethyl	8.01	C21 H26 N2 O2	339.2074	78		266	107	3,265	1,208	9,324	10,550	10,628	523			8,241	7,413	8,122	105, 188
			338.1994	1.3			43	72	730	146	809	4,033	1,584	332			9,982	10,103	7,942	
M23	β-Phenethyl-hydroxy	8.10	C22 H28 N2 O3	369.2176	9				70	68	47	29	107				619	723	1,685	91, 105, 186
			368.21	0.6					68	81	48	26	93				874	1,247	1,639	204
M24	Phenethyl-hydroxy-4-ANPP	8.11	C19 H24 N2 O	297.1958	≥5								26					22	58	84, 121, 204
			296.1889	−1.9														31	62	
P	Parent	9.04	C22 H28 N2 O2	353.2231	2	371	255	350	8,640	2,421	4,977	4,814	3,090			25,356	20,283	14,526	12,095	105, 188
			352.2151	1.6		349	233	358	8,523	2,536	4,802	4,993	2,862			19,392	23,899	18,572	10,830	
M25	N-oxide	9.84	C22 H28 N2 O3	369.2187	1						29					128	169	154	243	105, 146, 186
			368.21	4.0							29					151	241	199	241	
M26	4-ANPP	10.12	C19 H24 N2	281.2003	14				141		35	76	54	21		37	253	144	65	105, 188
			280.1939	−3.1					139		33	52	52	23			370	253	57	
M27	N-oxide	10.23	C22 H28 N2 O3	369.2168													78	116	214	105, 186
			368.21	−1.6													75	114	253	

Abundance of methoxyacetylfentanyl metabolites. Ions 132, 146, 158 and 174 not included as diagnostic ions due to limited value in structure elucidation. RT, avg *m/z* and ME calculated from urine samples, except for M23 where they were calculated from hepatocyte samples (RT was corrected based on regression). The % conj was calculated as signal increase after hydrolysis compared to total signal. In samples where no signal was observed in non-hydrolyzed samples, lowest degree of conjugation was calculated based on a 20 k area threshold. RT, retention time; ME average mass error compared to exact mass.

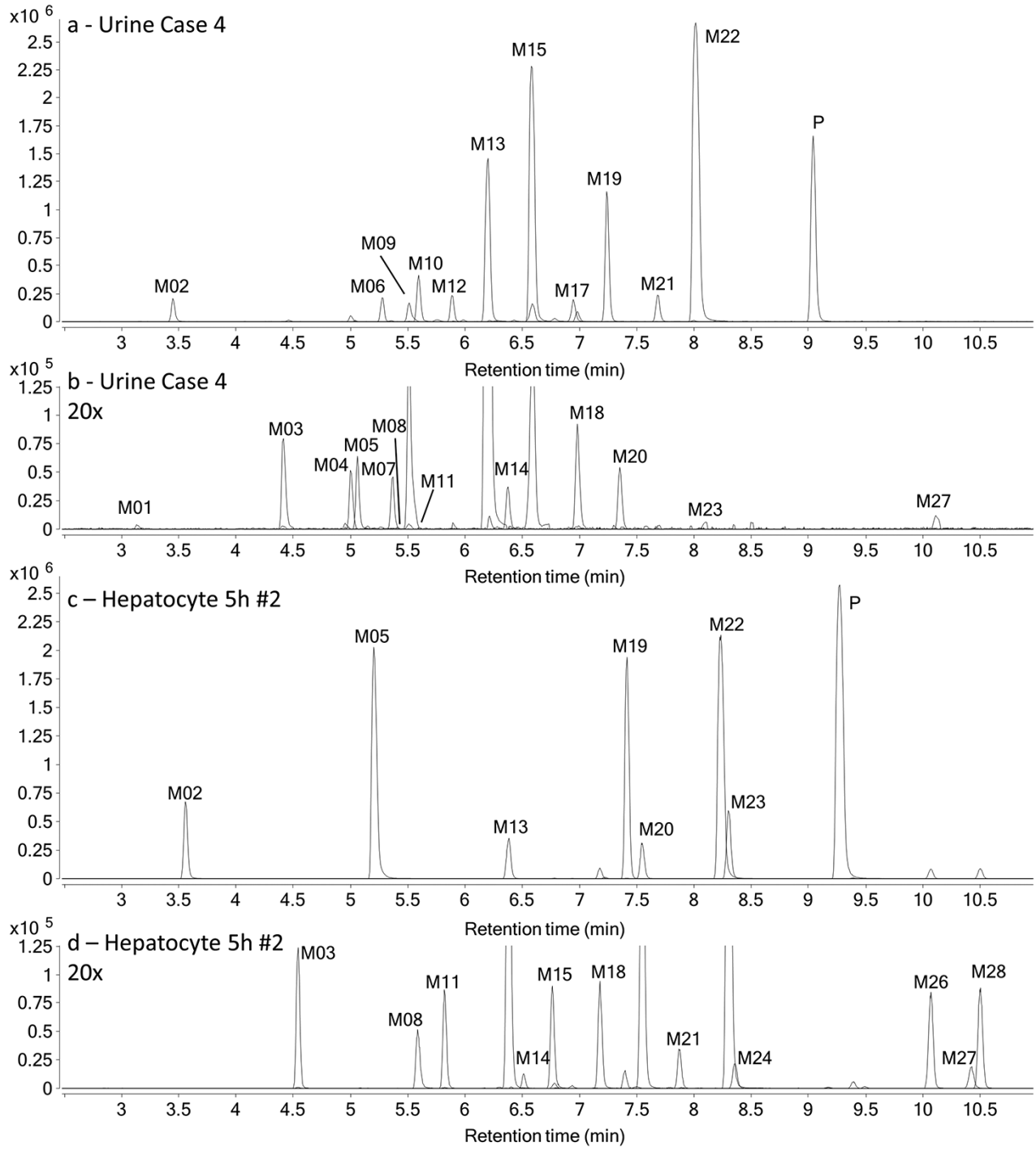

Figure 2. Chromatogram of methoxyacetylfentanyl metabolites. Overlaid extracted ion chromatograms of methoxyacetylfentanyl metabolites in urine sample from Case #4 (a), including 20× magnification of minor metabolites (b). Peaks from hydrolyzed sample except glucuronide metabolites. Metabolites identified in replicate 2 after 5 h of hepatocyte incubation (c), including 20× magnification of minor metabolites (d).

without access to synthesized reference materials is often impossible. This is also true for most metabolites of methoxyacetylfentanyl. However, methoxyacetylfentanyl is structurally similar to acetyl-, acryl-, cyclopropyl- and 4-fluoro-isobutyrylfentanyl previously studied by our group using reference materials synthesized in-house and behaved very similar when comparing positional preference on the phenethyl moiety during metabolism (25). Assuming the same is true for methoxyacetylfentanyl, we tentatively assigned positional isomers on the phenethyl ring for many metabolites, but the reader should bear in mind that this was not confirmed. The MS-MS spectrum of the demethylated metabolite M22 was also dominated by *m/z* 105 and 188, indicating unmodified phenethyl moiety and piperidine ring. The corresponding glucuronide (M19) contained the same product ions as M22 (*m/z* 105 and 188) as well as *m/z*

Table III. Cases Grouped Based on Analytical Findings

Group	Rapid death	Delayed death abstinent	Delayed death chronic	Hospital
Cases	5, 7, 9	8, 10	2, 4, 11	3
Parent blood (ng/mL)	21–140	17–31	18–51	N/A
Parent urine (area 10^3)	255–371	2,400–8,600	3,100–5,000	<20
M22 (area 10^3)	<20–970	1,200–4,700	9,300–10,600	520
M19 (area 10^3)	<20–21	110–280	2,300–2,800	39
Area ratio parent/M22	0.96 –> 1.8	2.0–2.6	0.29–0.53	<0.04
Area ratio M19/M22	ND, 0.078, <0.19	0.085–0.090	0.22–0.27	0.08

339, corresponding to the protonated molecular ion of M19. The spectrum of normethoxyacetylfentanyl (M05) as well as the corresponding demethylated metabolite (M02) are dominated by the product ion *m/z* 84. The same is true for its glucuronide (M01).

Hydroxylated and dihydroxylated metabolites

Four metabolites with the formula C22H28N2O3 corresponding to monohydroxylation were identified. The MS spectrum of M20 was dominated by *m/z* 121 and 204, indicating hydroxylation on the phenethyl moiety. Similar spectra were also observed for M13, the demethylated analog of M20. It is postulated that M20 and M13 are hydroxylated in the 4-phenethyl position based on the known preference for this configuration as shown by Wallgren et al. (25). Two monohydroxylated and demethylated glucuronides (M04 and M09) were identified. It is likely that these are both glucuronides of M13 with the glucuronide attached either to the exposed primary alcohol of the hydroxyacetyl group or the 4-phenethyl-hydroxy group. The spectra of M23 and M18 are very different from those of M20 and M13, respectively. Major ions include *m/z* 91 tropylium ion formed from a phenyl ring, 204 (hydroxylated phenethyl including piperidine), 186 (water loss from 204) and 105, indicating an unmodified phenethyl moiety. This odd pattern actually suggests M23 and M18 to be β-phenethyl-hydroxyls as similar patterns have been observed for the β-phenethyl-hydroxy metabolites of several other fentanyl analogs (25). The product ion *m/z* 174 indicates a cleavage in the middle of the piperidine ring, while several possibilities exist for *m/z* 132. The spectra of M25 and M27 contain *m/z* 105 and 186 (water loss from *m/z* 204), which are consistent with a metabolite hydroxylated on the piperidine ring. However, retention times after the parent indicate that they might be N-oxide metabolites. The spectra also contain fragments *m/z* 146 and/or 158, which most likely represent cleavages across the piperidine ring although it is difficult to assign the exact structure of these ions. M17 was identified as dihydroxylated on the phenethyl moiety based on product ions *m/z* 84 (piperidine), 137 (dihydroxylated phenethyl) and 220 (dihydroxylated phenethyl including piperidine). The same product ions were also identified for the demethylated analog M10 and its corresponding glucuronide M7. The positional isomers are tentatively assigned as 3,4-catechol metabolites based on the previous research (23, 25, 26). As water loss from aromatic rings is uncommon, it is interesting that a small product ion with *m/z* 119, matching a water loss from *m/z* 137, was observed for M10.

Other metabolites

No less than six methylated catechol metabolites were identified for methoxyacetylfentanyl (M15, M16, M21 and the glucuronides M06, M12 and M14), all of which were modified on the phenethyl moiety and identified by product ions *m/z* 84, 119 (not in M06 and M12), 151 and 234. Most likely, these represent the catechol-*O*-methyltransferase methylation of a catechol intermediate (27). Similar to the monohydroxylated metabolites discussed above, the most abundant analogs were assigned as 4-phenethyl-hydroxy,3-phenethyl-methoxy (M21) and correspondingly as 4-phenethyl-hydroxy,3-phenethyl-methoxy desmethyl (M15), based on the work by Wallgren et al. (25). Two glucuronides (M06 and M12) were assumed to both be 4-phenethyl-hydroxy,3-phenethyl-methoxy, as M15, but with the glucuronide either at the primary alcohol of the hydroxyacetyl group or the 4-phenethyl-hydroxy group. The minor hydroxy, methoxy metabolite (M16) was assumed to be 3-phenethyl-hydroxy,4-phenethyl-methoxy desmethyl metabolite. Interestingly, two metabolites corresponding to 4-phenethyl-hydroxy,3-phenethyl-methoxy desmethyl glucuronide (M06 and M12) were identified.

Despropionylfentanyl was formed by the loss of the methoxyacetyl group and confirmed using a reference material as 4-ANPP (M26). The spectra contained *m/z* 105 and 188. Two hydroxylated metabolites (M08 and M24) were also identified. The MS spectra of M08 also contained product ions *m/z* 105 and 188, indicating hydroxylation on the phenyl ring, while the spectra of M24 instead contained product ions *m/z* 121 and 204 indicating phenethyl ring hydroxylation. The dihydrodiol (M11) and dihydrodiol desmethyl (M03) metabolites were identified by the product ions *m/z* 84, 121 (water loss from 139), 204 (water loss from 222) and 222, indicating the phenethyl moiety as the site of the dihydrodiol. The exact location of the dihydrodiol could not be determined, but it appears likely that it is in the same position in both M03 and M11.

Comparison with previous publications

In general, our data are in agreement with metabolites reported by other studies. Comparisons are limited by the fact that all studies used different chromatographic methods producing different retention times. Comparisons were based on accurate mass, relative retention, relative peak area and, when relevant, mass spectra. When looking at the five most abundant metabolites in our urine samples and hepatocytes (six metabolites in total), Nordmeier et al. reported all of them in both rat urine and after s9 incubation with the exception of the desmethyl hydroxy-methoxy metabolite (M15), which was only found in rat urine (20). Interestingly, this metabolite was not reported by either Mardal et al. or Hudson and Cutler, nor did they report nordesmethyl methoxyacetylfentanyl (M02) or, in the case of Hudson and Cutler, the desmethyl glucuronide (M19) (1, 19). However, Mardal et al. reported 4-ANPP (M26) to be one of the most abundant metabolites in both urine samples and after hepatocyte incubation, while in the present study, it was not detected after hepatocyte incubation and only at modest levels in the urine samples

(1). A similar difference was observed for hydroxylated 4-ANPP (M08). As Mardal et al. used a seized material for their hepatocyte incubations and 4-ANPP is a known precursor to fentanyl analogs, it is possible that those metabolites were caused by impurities in the material used.

Interpretation of urinary metabolite profiles

When looking at the abundances of the parent compound methoxyacetylfentanyl, M22 (*O*-desmethyl) and M19 (glucuronide of M22) in urine, four distinct groups were observed as shown in Table III. In three cases (#5, #7 and #9), the abundances of the parent methoxyacetylfentanyl, M19 and M22 were all low (<380 k, <21 k and <970 k counts, respectively), which in combination with a substantial concentration in femoral blood (21–140 ng/g) may indicate an acute intake while abstinent and a "rapid" death. In two of those cases (#7 and #9), a syringe was found at the scene, suggesting intravenous administration and a rapid onset.

In Cases #8 and #10, the abundances of methoxyacetylfentanyl (>2,400 k) and M22 (>1,200 k) were high, while the abundance of the phase II metabolite M19 was low (<280 k). This could indicate an acute intake while abstinent but a more delayed death where phase I metabolite M22 was formed and only some phase II metabolite M19 was produced. Femoral blood concentrations were 17 and 31 ng/g. In hepatocytes, M22 reached a plateau after 1 h, while M19 was still increasing at 5 h of incubation.

In Cases #2, #4 and #11, the abundances of methoxyacetylfentanyl (>3,000 k) as well as both M19 (>2,300 k) and M22 (>9,300 k) were high, which could be indicative of an acute intake in a chronic user. Femoral blood concentrations were 18–51 ng/g.

In Case #3, the subject was treated in the hospital for 3 days before pronounced deceased. The concentration of methoxyacetylfentanyl in hospital blood was 41 ng/mL. While the femoral concentration in blood postmortem is unknown, methoxyacetylfentanyl was not detected in postmortem urine, and urinary metabolite areas were low, showing the patient to be in the late elimination phase.

Conclusions

Based on the hepatocyte experiments and authentic cases in our study, the major urinary metabolites of methoxyacetylfentanyl were identified. Differences in the abundance of methoxyacetylfentanyl and its major metabolites were found useful to indicate fatal intoxications in abstinent or chronic users. We postulate that urinary concentrations of methoxyacetylfentanyl, desmethyl methoxyacetylfentanyl (M22) and the corresponding glucuronide metabolite (M19), in combination with the methoxyacetylfentanyl concentration in femoral blood, might be good indicators of the time between administration and death as well as prior use. Although the published data suggest that methoxyacetylfentanyl is less potent than fentanyl, we conclude that users are at a high risk of accidental fatal intoxication.

Supplementary data

Supplementary data is available at *Journal of Analytical Toxicology* online.

Funding

This study was supported by Strategiområdet Forensiska Vetenskaper (Strategic Research Area Forensic Sciences) at Linköping University, Sweden, for the project 2017-10.

Data Availability

The data underlying this article will be shared on reasonable request to the corresponding author.

References

1. Mardal, M., Johansen, S.S., Davidsen, A.B., Telving, R., Jornil, J.R., Dalsgaard, P.W., et al. (2018) Postmortem analysis of three methoxyacetylfentanyl-related deaths in Denmark and in vitro metabolite profiling in pooled human hepatocytes. *Forensic Science International*, **290**, 310–317.
2. EMCDDA. (2018) Report on the risk assessment of 2-methoxy-N-phenyl-N-[1-(2-phenylethyl)piperidin-4-yl]acetamide in the framework of the council decision on new psychoactive substances. European Monitoring Centre for Drugs and Drug Addiction.
3. Beck, R.C., Kloda, S., Whiddon, J., Dye, D.W., Robinson, C.A. Jefferson County fentalogues: a 6 month review. SOFT-TIAFT Meeting: Boca Raton, FL, 2017.
4. Fogarty, M.F., Papsun, D.M., Logan, B.K. (2018) Analysis of fentanyl and 18 novel fentanyl analogs and metabolites by LC-MS-MS, and report of fatalities associated with methoxyacetylfentanyl and cyclopropylfentanyl. *Journal of Analytical Toxicology*, **42**, 592–604.
5. Smith, A., Kinkaid, D. (2017) Fentanyl and designer opioid-related deaths in Allegheny County. *Toxtalk*, **41**, 6–8.
6. Eshleman, A.J., Nagarajan, S., Wolfrum, K.M., Reed, J.F., Nilsen, A., Torralva, R., et al. (2020) Affinity, potency, efficacy, selectivity, and molecular modeling of substituted fentanyls at opioid receptors. *Biochemical Pharmacology*, **182**, 114293.
7. Hassanien, S.H., Bassman, J.R., Perrien Naccarato, C.M., Twarozynski, J.J., Traynor, J.R., Iula, D.M., et al. (2020) In vitro pharmacology of fentanyl analogs at the human mu opioid receptor and their spectroscopic analysis. *Drug Testing and Analysis*, **12**, 1212–1221.
8. Vasudevan, L., Vandeputte, M., Deventer, M., Wouters, E., Cannaert, A., Stove, C.P. (2020) Assessment of structure-activity relationships and biased agonism at the Mu opioid receptor of novel synthetic opioids using a novel, stable bio-assay platform. *Biochemical Pharmacology*, **177**, 113910.
9. Bagley, J.R., Kudzma, L.V., Lalinde, N.L., Colapret, J.A., Huang, B.S., Lin, B.S., et al. (1991) Evolution of the 4-anilidopiperidine class of opioid analgesics. *Medicinal Research Reviews*, **11**, 403–436.
10. WHO. (2018) Critical review report: methoxyacetyl fentanyl. World Health Organization.
11. Huang, B.-S., Terrell, R.C., Deutsche, K.H., Kudzma, L.V., Lalinde, N.L. (1986) N-aryl-N-(4-piperidinyl)amides and pharmaceutical compositions and method Employing such compounds. 4,584,303 Patent. United States.
12. Jílek, J., Rajšner, M., Valenta, V., Borovička, M., Holubek, J., Ryska, M., et al. (1990) Synthesis of piperidine derivatives as potential analgesic agents. *Collection of Czechoslovak Chemical Communications*, **55**, 1828–1853.
13. Jílek, J., Protiva, M., Metyš, J. (1992) Preparation of substituted N-[1-(2-phenylethyl)-4-piperidinyl]acetanilides and their maleates as analgesics. CS276281 Patent. Czechoslovakia.
14. Muller, D., Neurath, H., Neukamm, M.A., Wilde, M., Despicht, C., Blaschke, S., et al. (2019) New synthetic opioid cyclopropylfentanyl together with other novel synthetic opioids in respiratory insufficient comatose patients detected by toxicological analysis. *Clinical Toxicology (Phila)*, **57**, 806–812.
15. Darke, S., Duflou, J. (2016) The toxicology of heroin-related death: estimating survival times. *Addiction*, **111**, 1607–1613.
16. Kronstrand, R., Nystrom, I., Andersson, M., Gunnarsson, L., Hagg, S., Josefsson, M., et al. (2008) Urinary detection times and metabolite/parent compound ratios after a single dose of buprenorphine. *Journal of Analytical Toxicology*, **32**, 586–593.

17. Skopp, G., Lutz, R., Ganssmann, B., Mattern, R., Aderjan, R. (1996) Postmortem distribution pattern of morphine and morphine glucuronides in heroin overdose. *International Journal of Legal Medicine*, **109**, 118–124.
18. Thaulow, C.H., Oiestad, A.M.L., Rogde, S., Andersen, J.M., Hoiseth, G., Handal, M., et al. (2018) Can measurements of heroin metabolites in post-mortem matrices other than peripheral blood indicate if death was rapid or delayed? *Forensic Science International*, **290**, 121–128.
19. Designer Fentanyls. (2018) *Drugs That Kill and How to Detect Them. The in Vitro Metabolism of Methoxyacetylfentanyl*. LGC. https://hybris-static-assets-production.s3-eu-west-1.amazonaws.com/sys-master/images/hce/hbc/9616887349278/Designer%20Fentanyls%20-%20Drugs%20that%20kill%20and%20how%20to%20detect%20them%20-%20Methoxyacetylfentanyl.pdf (Accessed Apr 19, 2021).
20. Nordmeier, F., Richter, L.H.J., Schmidt, P.H., Schaefer, N., Meyer, M.R. (2019) Studies on the in vitro and in vivo metabolism of the synthetic opioids U-51754, U-47931E, and methoxyacetylfentanyl using hyphenated high-resolution mass spectrometry. *Scientific Reports*, **9**, 13774.
21. Wilde, M., Angerer, V., Huppertz, L.M., Moosmann, B., Auwärter, V. Characterization of the new synthetic fentanyl derivatives 4-chloroisobutyrfentanyl, 4-methoxybutyrfentanyl, benzodioxolfentanyl, cyclopentylfentanyl, methoxyacetylfentanyl, and tetrahydrofuranfentanyl and identification of their in vitro phase I main metabolites. SOFT-TIAFT Meeting: Boca Raton, FL, 2017.
22. Peters, F.T., Drummer, O.H., Musshoff, F. (2007) Validation of new methods. *Forensic Science International*, **165**, 216–224.
23. 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.
24. 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.
25. Wallgren, J., Vikingsson, S., Rautio, T., Nasr, E., Åstrand, A., Watanabe, S., et al. (2021) Structure elucidation of urinary metabolites of fentanyl and five fentanyl analogs using LC-QTOF-MS, hepatocyte incubations and synthesized reference standards. *Journal of Analytical Toxicology*, **44**, 993–1003.
26. 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.
27. Guldberg, H.C., Marsden, C.A. (1975) Catechol-O-methyl transferase: pharmacological aspects and physiological role. *Pharmacological Reviews*, **27**, 135–206.