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N-Ethyl Pentylone (Ephylone) Intoxications: Quantitative Confirmation and Metabolite Identification in Authentic Human Biological Specimens

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Abstract

N-ethyl pentylone (ephylone) has been identified as the most recent novel stimulant to emerge into the arena of evolving novel psychoactive substances (NPS). Due to its novelty, information regarding case reports with associated quantitative confirmations, biotransformation pathways, and identified unique metabolites will assist the scientific community in understanding the implications of the emergence and risks associated with *N*-ethyl pentylone use. Authentic blood specimens ($n = 26$) submitted as part of toxicological death investigations or drugged driving casework tested positive for *N*-ethyl pentylone, and were quantitatively analyzed by liquid chromatography tandem mass spectrometry (LC–MS-MS). *N*-ethyl pentylone concentrations ranged from 12 to 1,200 ng/mL, with mean (±standard deviation) and median concentrations of 313 (±366) and 125 ng/mL, respectively, excluding one case measured at 50,000 ng/mL. *N*-ethyl pentylone was often found in combination with other drugs of abuse and NPS, include a variety of novel opioids including fentanyl analogs. Oral fluid specimens ($n = 5$), collected from recreational drug users at a dance music festival, were quantitatively analyzed using LC–MS-MS. Concentrations ranged from 12.6 to 1,377 ng/mL. Additional analysis was performed to characterize the metabolic profile of *N*-ethyl pentylone using human liver microsomes (HLM), followed by confirmation of the presence of the proposed metabolites in a subset of the blood specimens and oral fluid specimens. Metabolomic analysis was performed using a liquid chromatograph quadrupole time-of-flight mass spectrometer (LC-QTOF), followed by data processing using MetabolitePilot™ software. *In vivo* verification of *in vitro* HLM-generated metabolites resulted in the confirmation of four metabolites. Reduction of the beta-ketone to an alcohol resulted in the most prominent metabolite found in the authentic specimens, and its uniqueness to *N*-ethyl pentylone leads to this metabolite being an appropriate biomarker to determine *N*-ethyl pentylone ingestion. This is the first study to report *N*-ethyl pentylone concentrations and to characterize the metabolic profile of *N*-ethyl pentylone.

Introduction

Since the early 2000s, synthetic cathinones have become popular substances for recreational use due to their psychoactive and euphoric properties. As with all novel psychoactive substances (NPS), the propagation of new compounds has been largely driven by the illegality of previous generations, the ability of clandestine chemists to synthesize new drugs via chemical modifications of a core structure and end user demand. The emergence of *N*-ethyl pentylone on the recreational drug market was reported in drug seizures for the first time in 2016 (1, 2). *N*-ethyl pentylone synthesis dates back to 1969 when it was described alongside the synthesis of other novel stimulants including pentylone, butylone and dibutylone (3). *N*-ethyl pentylone (Figure 1) has differing naming conventions within the scientific and drug-using communities, including beta-keto-ethylbenzodioxolylpentanamine, bk-EBDP, ethyl pentylone, ephylone and NEP. Reference to *N*-ethyl pentylone and threads describing its use and effects have increased substantially over the last year on drug user forums and research chemical vendor websites [www.reddit.com/r/researchchemicals/, www.bluelight.org, etc.].

Identification of *N*-ethyl pentylone in forensic casework was first reported to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) in January 2016 by Slovenia (2). In the USA, the increase in popularity of *N*-ethyl pentylone, among other synthetic cathinones, can be seen by examining data collected by the National Forensic Laboratory Information System (NFLIS) and the Drug Enforcement Administration (DEA). According to the 2016 NFLIS Annual Report, *N*-ethyl pentylone identifications were reported 1,720 times (accounting for 0.50% of phenethylamine reports), making it the third most common synthetic cathinone identified behind dibutylone and ethylone (1, 4). Through the third quarter of 2017, *N*-ethyl pentylone was the number one reported synthetic cathinone accounting for 55%, 50% and 38% of cases reported for the first three quarters, respectively (5–7). These declining percentages in 2017 are likely due to the resurgence in MDMA popularity and recent increase in MDMA seizure as reported by NFLIS, rather than a true decline in popularity.

Based on the increasing prevalence of *N*-ethyl pentylone in seized drug casework, it is likely that increasing reports of adverse events associated with its ingestion will be forthcoming. Several beta-keto-methylenedioxyamphetamines, such as methylone and ethylone, have been implicated as a contributing or sole cause of death (8, 9), but to date there are few reports in the literature involving *N*-ethyl pentylone. The earliest report of an *N*-ethyl pentylone intoxication dates to January 2016 in the USA (10).

Two previously published case reports detail clinical presentation and autopsy findings following *N*-ethyl pentylone ingestion. One case reported a man wandering in a state of agitation, who was flailing and clenching his jaw (10). At the hospital, the subject was hypotensive, experiencing tachycardia and went into cardiac arrest with hyperthermia. Clinical testing revealed extreme acidemia, elevated troponin, rhabdomyolysis, hypoglycemia, liver shock, acute kidney injury, respiratory failure and leukocytosis. Examination at autopsy showed abrasions, pleural and peritoneal effusions, an enlarged heart and left ventricular hypertrophy. *N*-ethyl pentylone was qualitatively confirmed in postmortem blood and the death was ruled accidental due to *N*-ethyl pentylone intoxication.

In a second case, a man was taken to hospital following cardiac arrest after being described as uncooperative, agitated, and diaphoretic by responding law enforcement and medical personnel (11). The individual was hypotensive and experiencing tachycardia, with noticeable abrasions, slow pupillary reflex and myoclonus. Clinical testing revealed hyperkalemia, hypoglycemia, rhabdomyolysis, liver failure, renal injury and elevated troponin. Supportive treatment was unsuccessful and the individual expired after four days. Further testing following autopsy qualitatively confirmed the presence of *N*-ethyl pentylone and the death was attributed to the intoxication.

There are no previous quantitative reports of *N*-ethyl pentylone in the scientific literature, and the lack of reference data limits the ability of forensic toxicologists to interpret the significance of *N*-ethyl pentylone findings in biological specimens.

Metabolic studies involving *N*-ethyl pentylone have not been previously reported. Metabolic studies coupled with case reports and quantitative data are important to provide additional information

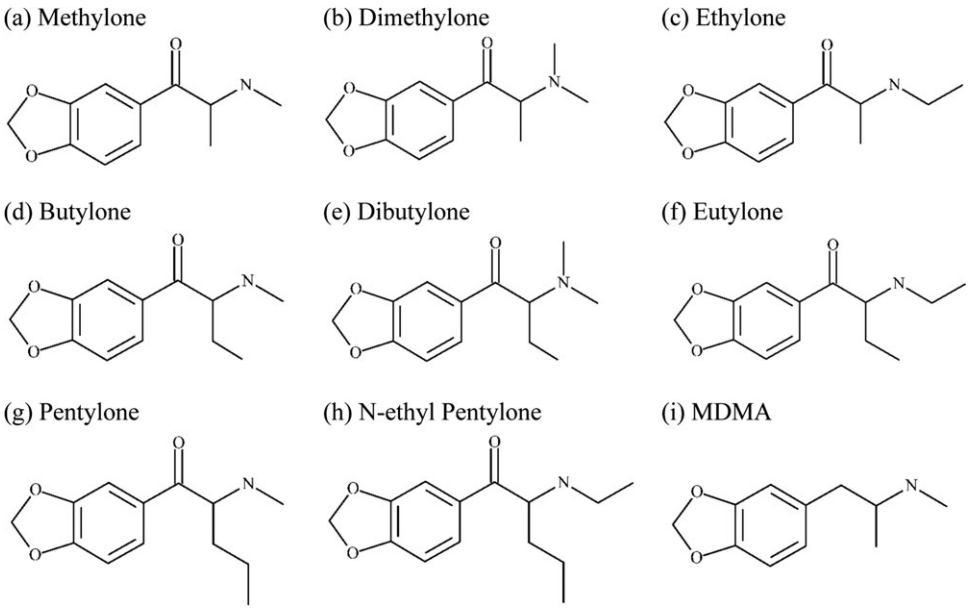

Figure 1. Structures of novel stimulants and MDMA.

that will serve to assist laboratories and toxicologists. This information aids in the understanding of biological processes, prolonging the detection windows, diversifying the ability to identify *N*-ethyl pentylone exposure via additional biomarkers and providing reference data for the interpretation of NPS use. Recent metabolic studies involving the novel stimulants dimethylone (12) and dibutylone (13) have shown the conversion of demethylated species to previously characterized novel stimulants, such as methylone and butylone, respectively. Based on these studies, it was hypothesized that *N*-ethyl pentylone would follow similar metabolic pathways producing common metabolites to other beta-keto-methylenedioxyamphetamines or conversion to previously identified novel stimulants, as well as conversion to unique metabolites of *N*-ethyl pentylone itself. Additional metabolic studies have described the metabolism of butylone, ethylone and pentylone (14–17), and detailed a variety of potential metabolic species including dealkylation, demethylenation and ketone reduction.

To our knowledge, this manuscript reports, for the first time, intoxications involving the novel stimulant *N*-ethyl pentylone including available case history information and quantitative confirmation. Additionally, the *in vitro* metabolism is reported, followed by *in vivo* confirmation using authentic human specimens, which represents the first report of the metabolism and verification of metabolites in man.

Methods

NMS Labs (Willow Grove, PA, USA) received blood specimens (n = 26) collected between June 2016 and July 2017 as part of death investigation and drugged driving toxicology casework that subsequently tested positive for *N*-ethyl pentylone. Initial screening and quantitative confirmation were performed at NMS Labs, while further investigation and metabolite confirmations were performed at the Center for Forensic Science Research and Education (CFSRE, Willow Grove, PA, USA). Personal identification information was removed from any specimen or case history before being provided for further analysis and data compilation during this study. Case histories, when available, were tabulated and are provided in Table I.

As part of a larger Institutional Review Board approved study (Arcadia University, Glenside, PA, USA), oral fluid (OF) specimens were collected (May 2017) using the Quantisal™ oral fluid collection device (Immunalysis, Pomona, CA, USA) from attendees of a dance music festival. All participants that provided OF specimens initially provided informed consent, stating they were over the age of 18, not impaired and understood the purpose of our research study. Initial screening, quantitative analysis and confirmation of metabolites were performed at CFSRE. Reported recreational drug use and demographical information is provided in Table I.

N-ethyl pentylone standard was purchased from Cayman Chemical (Ann Arbor, MI, USA) and prepared at 1 mg/mL in methanol. Analysis of the *N*-ethyl pentylone standard by LC-QTOF resulted in the identification of eutylone at 0.01% peak area, which was confirmed by its fragment ion spectra. Diazepam (1 mg/mL) was purchased from Cerilliant (Round Rock, TX, USA).

Blood confirmation and quantitation

N-ethyl pentylone was initially identified during high resolution broad-based drug screening at NMS Labs using an Agilent 6230 time-of-flight mass spectrometer coupled to an Agilent 1200 series high performance liquid chromatograph (LC-TOF). Positive analyte identification was determined based on mass error (<±30 ppm), retention time error (<±0.03 min), control comparison (in relation to a reporting limit control) and peak shape. Screening results then prompted independent multi-assay quantitative confirmation.

Quantitative confirmation of *N*-ethyl pentylone was performed using an in-house fully validated method for the analysis of novel stimulants in blood, including ethylone, butylone, pentylone, dibutylone and *N*-ethyl pentylone. Calibration concentrations ranged from 10 to 1,000 ng/mL, with a reporting limit of 10 ng/mL. Pentylone-D3 was used as the internal standard during analysis. Specimens were solid phase extracted using Agilent Bond Elut Plexa PCX (3 mL, 30 mg) cartridges prior to analysis using a Waters TQD tandem mass spectrometer coupled with a Waters Acquity ultra performance liquid chromatograph (LC–MS-MS). Analytes were ionized via positive electrospray ionization, followed by isolation via multiple reaction monitoring (MRM). Two transitions per analyte (Table II) were used for quantitation and target confirmation, using calculated ion ratios.

Oral fluid confirmation and quantitation

OF samples were first analyzed using high resolution broad-based drug screening at CFSRE using a SCIEX TripleTOF® 5600+ quadrupole time-of-flight mass spectrometer coupled to a Shimadzu Nexera ultra high performance liquid chromatograph (LC-QTOF). Positive analyte identification was determined based on mass error (<10 ppm), retention time error (<0.35 min), isotope difference (<50%), library score (>70, calculated using the "fit" algorithm), signal-to-noise ratio (>10), peak shape and peak area. Screening results then prompted independent quantitative confirmation.

Quantitative confirmation of *N*-ethyl pentylone was performed using an in-house fully validated method for the analysis of novel stimulants in oral fluid, including methylone, dimethylone, ethylone, butylone, dibutylone, pentylone, eutylone, *N*-ethyl pentylone, MDMA and MDA. Calibration concentrations ranged from 4–400 ng/mL and quantitative results were reported as undiluted OF concentrations using a 1:3 dilution factor. Pentylone-D3 was used as the internal standard during analysis. Specimens were liquid–liquid extracted using borax buffer (0.1 M, pH 10.4) and *N*-butyl chloride/ethyl acetate (70:30) prior to analysis using an Agilent 6430 LC–MS-MS. Analytes were ionized via positive electrospray ionization, followed by isolation via MRM. Two transitions per analytes (Table II) were used for quantitation and target confirmation, using calculated ion ratios.

In vitro metabolite analysis

Incubations of *N*-ethyl pentylone with human liver microsomes (HLMs) were conducted according to a previously published protocol (18). A total of six incubations were run on three separate days. All reaction and control mixtures were analyzed using a Sciex TripleTOF® 5600+ QTOF (Framingham, MA, USA) coupled with a Shimadzu (Kyoto, Japan) Nexera XR ultra high performance liquid chromatograph. Mass acquisition was performed using an information dependent acquisition (IDA) mode. Analytes were ionized via positive electrospray and subjected to an accurate mass precursor ion (TOF MS) scan. Subsequently, the 16 most intense precursor ions per cycle were isolated separately in Q1 and fragmented using a collision energy spread (35 ± 15 eV), generating MS-MS fragment ion spectra. Detailed method parameters are described elsewhere (18).

MetabolitePilot™ (SCIEX, Version, 1.5) and PeakView® (SCIEX, Version 2.2) were utilized for the processing of data files produced

Table I. Case information following use of *N*-ethyl pentylone ($n = 31$)

Case	Age	Sex	Matrix	Case history	*N*-ethyl pentylone concentration (ng/mL)	Other findings (ng/mL)
1	–	M	Blood	Death investigation	Positive	Butylone (150), ketamine (870)
2	–	M	Blood	Death investigation, alleged "Molly" use	50,000	Dibutylone (14)
3	–	M	Blood	Unknown	1,200	
4	29	M	Blood	Unknown	1,140	
5	35	M	Blood	Samples collected antemortem (AM) and postmortem (PM), death investigation, homicide	PM: 833 AM: Positive	Butylone, midazolam (8.9), THC (0.78)
6	–	M	Blood	Death investigation	790	Pentylone (96), dibutylone (13), butylone (12)
7	28	M	Blood	Death investigation, suspected drug overdose	600	
8	49	M	Blood	Death investigation, suspected drug overdose, suspected "bath salts" use	550	Dibutylone (10), 4-chloro-alpha-PVP
9	–	M	Blood	Death investigation	540	Dibutylone (11)
10	–	M	Blood	Death investigation, possible drug overdose	430	
11	–	M	Blood	Death investigation	358	
12	–	M	Blood	Death investigation following vehicular crash	210	Pentylone (200)
13	–	F	Blood	Death investigation, homicide	160	
14	31	M	Blood	Death investigation, suicide, gunshot wound	150	
15	33	M	Blood	Unknown	100	Methamphetamine (49), amphetamine (14), lorazepam (40)
16	47	M	Blood	Death investigation, suspected drug overdose	90	Carfentanil (1.3)
17	23	M	Blood	Driving under the influence of drugs	87	
18	53	M	Blood	Death investigation	86	U-47700, U-49900, THFF, Acrylfentanyl (0.4), 4-ANPP
19	40	M	Blood	Driving under the influence of drugs	41	Fentanyl (7), norfentanyl (2.3)
20	–	F	Blood	Death investigation, suspected drug overdose	38	Dibutylone (40), butylone (15.3), FIBF
21	–	M	Blood	Driving under the influence of drugs	34.3	
22	25	M	Blood	Death investigation	24	Alprazolam (28), THC (1.6)
23	36	M	Blood	Driving under the influence of drugs	23	Methamphetamine (55), Amphetamine (<5)
24	32	M	Blood	Driving under the influence of drugs	21	Clonazepam (23)
25	–	M	Blood	Death investigation, suspected drug overdose, found deceased at home, suspected to have snorted cocaine or heroin	18.4	Furanylfentanyl, 4-ANPP, cocaine (130), THC (0.86)
26	–	M	Blood	Death investigation	12	
27	30	M	OF	Recreation drug use ("Molly")	1,377	Eutylone, MDA, Alprazolam
28	20	M	OF	Recreation drug use ("Molly")	132.9	Eutylone, THC (40.5)
29	24	F	OF	Recreation drug use ("Molly")	35.2	Alprazolam
30	21	F	OF	Recreation drug use ("Molly")	31.5	MDA (35.7), THC (2.7)
31	34	F	OF	Recreation drug use ("Molly")	12.6	THC

Key: OF: oral fluid, THFF: tetrahyrofuranylfentanyl, FIBF: fluoroisobutyrylfentanyl, 4-ANPP: 4-anilino-N-phenethylpiperidine, MDA: methylenedioxyamphetamine, THC: tetrahydrocannabinol.

Table II. *N*-ethyl pentylone and pentylone-D3 detection parameters

Analyte	Precursor ion	Source voltage	Quant. ion	CE (eV)	Qual. ion	CE (eV)
Waters TQD (NMS Labs)						
N-ethyl pentylone	250.2	35	202.2	18	100.2	18
Pentylone-D3	239.0	35	191.0	18	221.0	12
Agilent 6430 (CFSRE)						
N-ethyl pentylone	250.0	100	202.2	15	174.1	25
Pentylone-D3	239.0	100	191.2	15	187.1	25

using the above described methods. *In vitro* metabolite identification and determination of proposed structures were made using MetabolitePilot™, a software application available for the extraction of precursor ions consistent with pre-programmed biotransformations and peak-finding strategies. Unidentified peaks were subsequently evaluated using PeakView®, a software application for the manual review of acquired data. All proposed metabolites were tabulated to include information regarding metabolite name, formula, accurate mass and retention time, from which an extracted ion chromatogram (XIC) list was generated. MS-MS spectra of the proposed metabolites were added to an in-house library database for further evaluation and data processing of authentic specimens.

In vivo metabolite confirmation

A subset (Cases 1–6) of the previously mentioned authentic human blood specimens were analyzed for the presence of the proposed *N*-ethyl pentylone metabolites characterized during *in vitro* microsomal incubation. All OF specimens previously mentioned were analyzed for *N*-ethyl pentylone metabolites. Samples were prepared for LC-QTOF analysis (13, 18, 19) and acquired using SWATH® acquisition, a data independent acquisition (DIA) mode (18).

Results and Discussion

Of the individuals (n = 26) in this subset population where blood was collected, 24 were reported as male and 2 were reported as female. The average (±standard deviation) age was 35.4 (±9.3) years, the median age was 33 years and the age range was 23–53 years. States from which the blood specimens were submitted include Florida (n = 8), Illinois (n = 1), Missouri (n = 1), New Jersey (n = 2), New York (n = 4), Pennsylvania (n = 6), Utah (n = 1) and the District of Colombia (n = 2), one state was not specified, illustrating the widespread distribution of *N*-ethyl pentylone cases across the country.

Of the participants (n = 5) providing OF for this study, two reported as male and three reported as female. The average (±standard deviation) age was 25.8 (±6.0) years, the median age was 24 years and the age range was 20–34 years. Interestingly, all five participants indicated recreational drug use of "Molly," a slang term historically used to denote the ingestion of pure MDMA crystals. All OF samples were collected in Florida.

Blood confirmation and quantitation

Quantitative confirmation of *N*-ethyl pentylone and other NPS/drugs of abuse is shown in Table I. Excluding the specimen at 50,000 ng/mL, the average (±standard deviation) *N*-ethyl pentylone blood concentration was 313 (±366) ng/mL, the median was 125 ng/mL and the range was 12–1,200 ng/mL. Pentylone was only detected in two blood specimens, at concentrations of 200 and 96 ng/mL. The blood specimen containing 50,000 ng/mL of *N*-ethyl pentylone was negative for pentylone. As with other NPS (8), *N*-ethyl pentylone was not the only novel stimulant identified in several of the blood specimens; other analytes included MDMA, dibutylone, butylone, pentylone and 4-chloro-alpha-PVP. Interestingly, *N*-ethyl pentylone was found in combination with several novel opioids, including tetrahydrofuranylfentanyl (THFF), fluoroisobutyrylfentanyl (FIBF), carfentanil, furanylfentanyl, acrylfentanyl, U-47700, U-49900 and fentanyl, all of which have also been implicated in fatalities (8, 12, 18–21).

Oral fluid confirmation and quantitation

Quantitative confirmation of *N*-ethyl pentylone and other NPS/drugs of abuse is shown in Table I. The average (±standard deviation) *N*-ethyl pentylone OF concentration was 317.9 (±594.1) ng/mL, the median was 35.9 ng/mL and the range was 12.6–1,377 ng/mL. Pentylone was not detected in any of the OF specimens, but eutylone was detected in two OF specimens. *N*-ethyl pentylone was identified alongside MDA in two of the OF specimens. No other novel simulants were identified in this small subset.

In vitro metabolite analysis

Data processing of the *in vitro* incubation mixtures for *N*-ethyl pentylone resulted in the identification of four metabolites (Figure 2). Accurate mass fragment ions, both common and unique, were examined to determine proposed metabolite structures, as seen in Figure 2. Experimental data based on proposed metabolite identifications was tabulated (Table III) for comparison between and across incubation mixtures.

N-ethyl pentylone was found to undergo reduction of the beta-ketone to an alcohol (hydrogenation), producing M1, 1-(1,3-benzodioxol-5-yl)−2-(ethylamino)pentan-1-ol (Figure 2). M1 exhibited a

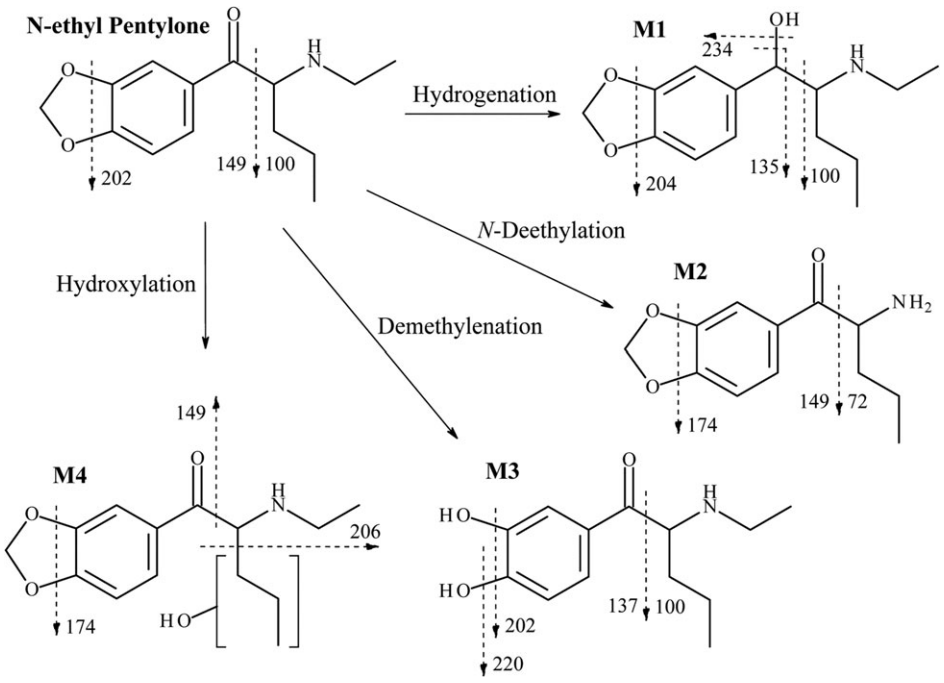

Figure 2. Metabolic pathway of *N*-ethyl pentylone (dashed lines indicate fragmentation).

Table III. Identification of *N*-ethyl pentylone metabolites using HLMs

ID	Biotranformation	Formula	$[M + H]^+$ (Da) Theoretical	$[M + H]^+$ (Da) Experimental	Mass error (ppm)	Average retention time (min)	Average peak area	Accurate fragment mass (Da)
Parent	*N*-ethyl pentylone	C14H19NO3	250.1438	250.1445	3.1	5.27	5.99E+07	232.1333 202.1214 189.0778 149.0229 135.0436 100.1127
M1	Hydrogenation	C14H21NO3	252.1594	252.1595	0.3	5.20	6.12E+05	234.1485 204.1278 191.0939 176.0693 159.0802 135.0435 100.1109
M2	Deethylation	C12H15NO3	222.1125	222.1125	0.1	4.97	1.24E+06	204.1016 174.0910 149.0226 146.0958 135.0436 117.0575 72.0822
M3	Demethylenation	C13H19NO3	238.1438	238.1446	3.6	3.80	8.33E+06	220.1330 202.1224 177.0781 162.0456 137.0232 123.0442 100.1126
M4	Hydroxylation	C14H19NO4	266.1387	266.1388	0.3	4.29	9.95E+04	248.1280 230.1197 206.0796 176.0695 149.0636 135.0437

protonated ion of 252.1595 Da at a retention time of 5.20 min (Table III). The most notable fragment ion, 234.1485 Da, differed from that of *N*-ethyl pentylone, 149.0229 Da, accounted for by the reduction of the ketone and absence of the formation of the acylium ion (Figure 3). This metabolite is unique to *N*-ethyl pentylone. Isomeric species to *N*-ethyl pentylone could metabolize to an isomer of M1, which should be considered as new novel stimulants emerge.

N-deethylation of *N*-ethyl pentylone resulted in the formation of M2, 2-amino-1-(1,3-benzodioxol-5-yl)pentan-1-one (Figure 2), not to be confused with pentylone. This is a common metabolite of both *N*-ethyl pentylone and pentylone. M2 exhibited protonated ion of 222.1125 Da at a retention time of 4.97 min (Table III). The most notable fragment ion, 174.0910 Da, differed from that of *N*-ethyl pentylone, 202.1214 Da, accounted for by the loss of the ethyl group. This metabolite is an isomer of ethylone, butylone and dimethylone, therefore identification could be complicated following ingestion of multiple novel stimulants.

Demethylenation of *N*-ethyl pentylone resulted in the formation of M3, 1-(3,4-dihydroxyphenyl)−2-(ethylamino)pentan-1-one (Figure 2). M3 exhibited a protonated ion of 238.1446 Da at a retention time of 3.80 min (Table III). The most notable fragment ion, 137.0232 Da, differed from that of *N*-ethyl pentylone, 149.0229 Da, due to loss of the carbon atom associated with the methylenedioxy-bridge and conversion to the dihydroxyl species (Figure 3). Like M1, M2 was determined to be unique to *N*-ethyl pentylone.

Hydroxylation of *N*-ethyl pentylone resulted in the formation of M4, 1-(1,3-benzodioxol-5-yl)–2-(ethylamino)-hydroxy-pentan-1-one (Figure 2). M4 exhibited a protonated ion of 266.1388 Da at a retention time of 4.29 min (Table III). Point of hydroxylation along the alkyl chain for this metabolite could not be determined using this methodology, therefore the absolute positioning on that chain of the hydroxyl group is unknown.

As previously mentioned, eutylone was identified as a contaminant in the standard reference material for *N*-ethyl pentylone. Eutylone was also identified in the HLM incubation samples during this study, however, its presence cannot be ruled out as a metabolite. Eutylone is an isomer of pentylone and dibutylone and was analytically confirmed in this study using its standard reference material, monitoring retention time and MS-MS fragmentation pattern (Figure 4). The presence of eutylone is noteworthy because of potential complications arising from its detection in casework.

In vivo metabolite confirmation

All four metabolites of *N*-ethyl pentylone, and eutylone, were identified (Table IV) in the authentic blood and OF specimens previously described. *N*-ethyl pentylone was identified in all specimens analyzed during this study. The ratio of metabolite to parent, *N*-ethyl pentylone, was significantly small for M2–M4 eluding to their

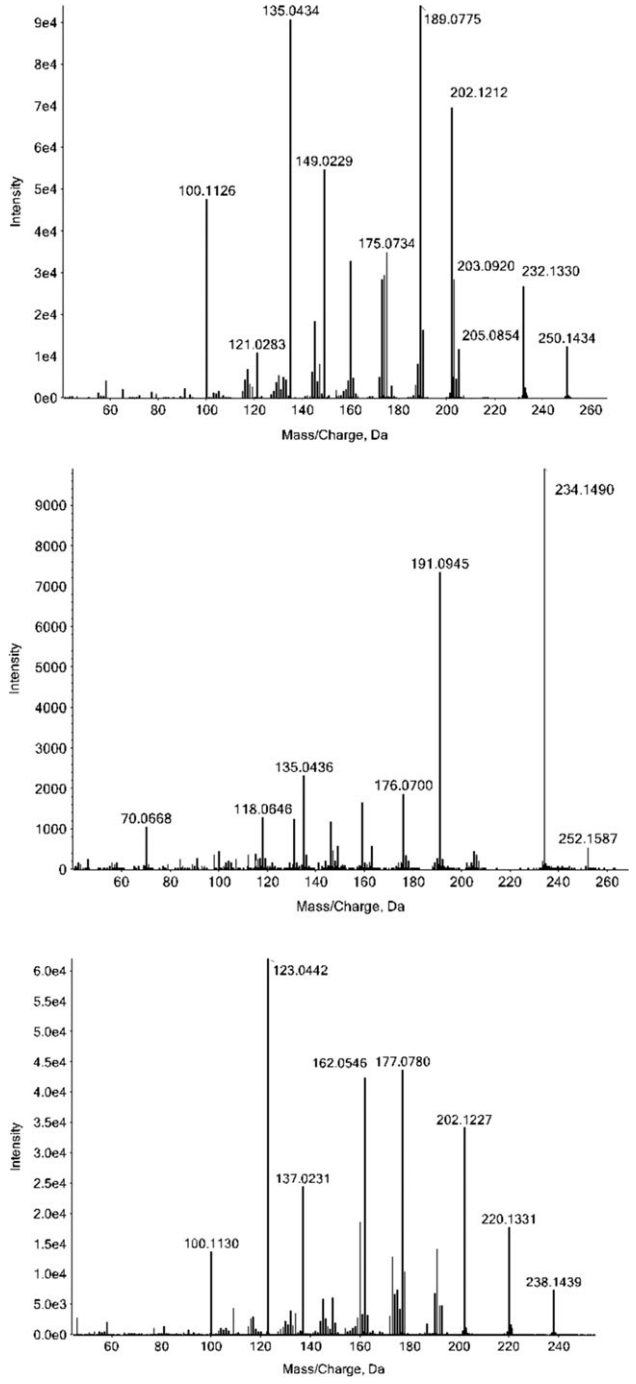

Figure 3. MS-MS spectra of parent *N*-ethyl pentylone (top), M1 (middle) and M3 (bottom).

presence as minor metabolites. M1, reduction of the beta-ketone, was identified as the most prominent blood and OF metabolite.

Synthetic variations of novel stimulants have resulted in the identification of several isomeric species. While eutylone was confirmed in four blood specimens and two OF specimens through our routine broad-based drug screening procedure, its isomeric nature to dibutylone and pentylone required additional analyst review. Eutylone and dibutylone have very similar retention times using the previously described LC-QTOF method and similar but not identical fragmentation; therefore, routine screening tentatively identified dibutylone and eutylone in the specimens. MS-MS fragment data was used to confirm the identity as eutylone.

Conclusions

Human exposure to *N*-ethyl pentylone has increased over the last several months following its earliest known emergence in January 2016. After the initial identification of *N*-ethyl pentylone on the NPS drug market, it has become important for laboratories to develop quantitative methods for the identification of *N*-ethyl pentylone in biological specimens. As methods have come available, *N*-ethyl pentylone has been identified in clinical intoxications, death investigations and drugged driving casework.

Through analysis of blood specimens during this study, *N*-ethyl pentylone was quantitatively confirmed with concentrations ranging from 12 to 1,200 ng/mL, with an additional case at 50,000 ng/mL, demonstrating the wide concentration range associated with this novel stimulant. Specimens collected as part of death investigation cases (n = 17) ranged in concentration from 12 to 790 ng/mL, excluding the specimen at 50,000 ng/mL, with mean (±standard deviation) and median of 305 (±282) ng/mL and 185 ng/mL, respectively. Specimens collected as part of drugged driving cases (n = 5), ranged in concentration from 21 to 87 ng/mL, with mean (±standard deviation) and median of 41 (±26) ng/mL and 34 ng/mL, respectively. In general, *N*-ethyl pentylone was found in combination with other NPS and drugs of abuse, including a variety of novel opioids.

Between July 2017 and December 2017, NMS Labs reported an additional 52 cases positive for *N*-ethyl pentylone (mean + standard deviation: 323 ± 382 ng/mL, median: 160 ng/mL, range: 10–1,700 ng/mL) (22), consistent with the concentrations identified in the cases described above.

N-ethyl pentylone was quantitatively confirmed in OF specimens collected from dance music festival attendees with concentrations ranging from 12.6 to 1,377 ng/mL, again demonstrating the wide concentration range associated with this novel stimulant. All positive cases of *N*-ethyl pentylone use were correlated with user reported ingestion of "Molly". In these specimens, *N*-ethyl pentylone was not found in conjunction with other novel stimulants, other than eutylone, but this information is limited due to the small subset (n = 5).

In total, four metabolites were identified and confirmed during the metabolic profiling of *N*-ethyl pentylone. Reduction of the beta-ketone (M1) was identified as the most abundant metabolite during microsomal incubations and analysis of authentic human specimens, and therefore, serves as an appropriate biomarker to assist in the identification of *N*-ethyl pentylone use. Standard reference materials for all four metabolites (M1–M4) are currently unavailable.

As novel stimulants continue to emerge, specifically the beta-keto-methylenedioxyamphetamines, the use of accurate mass spectral data can assist toxicologists and laboratory personnel in their identification. As seen among previously characterized novel stimulants, the presence of the 149 Da fragment, using positive electrospray ionization mass spectrometry techniques, is very common for drugs with this core structure. This fragment correlates to the acylium ion produced when the amino/alkyl backbone is removed and is often the most intense fragment. Since most of the structurally related novel simulants described above retain the 1,3-benzodioxole-5-carbonyl moiety, its use in identification of newly emerging substances could be highly beneficial. Additionally, this fragment has been shown to be useful in the identification of metabolites.

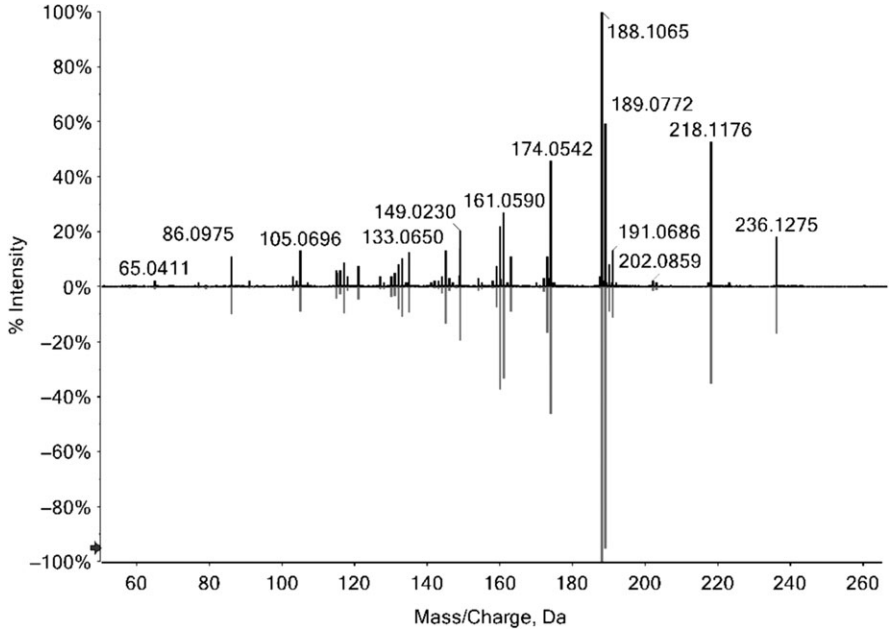

Figure 4. MS-MS mirror spectral comparison for eutylone (top) and reference standard (bottom).

Table IV. *N*-ethyl pentylone and metabolites identified in authentic blood specimens (% BasePeak)

Case #	Matrix	*N*-ethyl pentylone (%)	M1 (%)	M2 (%)	M3 (%)	M4 (%)	(Eutylone) (%)
1	Blood	100	67	2	LR	ND	LR
4	Blood	100	6	0.3	0.4	0.1	0.3
5 AM	Blood	100	23	0.2	ND	ND	ND
5 PM	Blood	100	3	LR	ND	ND	ND
11	Blood	100	8	ND	ND	ND	ND
21	Blood	100	11	1	LR	0.2	0.3
25	Blood	100	27	2	ND	0.6	1
27	OF	100	17	4	0.3	0.2	0.1
28	OF	100	5	0.4	ND	ND	ND
29	OF	100	6	1	ND	ND	ND
30	OF	100	6	3	ND	ND	ND
31	OF	100	10	ND	ND	ND	ND

AM: antemortem, PM: postmortem, ND: not detected, LR: low response.

This work provides comprehensive insight into the detection and toxicological significance of *N*-ethyl pentylone, providing information regarding biotransformation, unique metabolites and reference data from authentic biological specimens obtained during forensic investigations. These data can be used by forensic toxicologists to improve understanding of this novel substance, which continues to increase in popularity.

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References

1. U.S. Department of Justice Drug Enforcement Administration. (2016) National Forensic Laboratory Identification System 2016 Annual Report. https://www.nflis.deadiversion.usdoj.gov/DesktopModules/ReportDownloads/Reports/NFLIS2016AR.pdf (6 November 2017).
2. European Monitoring Centre for Drugs and Drug Addiction. (2016) EMCDDA–Europol 2016 Annual Report on the implementation of Council Decision 2005/387/JHA. http://www.emcdda.europa.eu/system/files/publications/4724/TDAN17001ENN_PDFWEB.pdf (13 October 2017).
3. Koppe, H., Ludwig, G., Zeile, K. Aryl-alpha-Aminoketone Derivatives. https://ewsd.wiv-isp.be/Publications%20on%20new%20psychoactive%20substances/bk-MBDB/GB1085135A_butylon-analog.pdf (30 June 2017).
4. Drug Enforcement Administration: Special Testing and Research Laboratory. (2017) Emerging Threat Report: Annual 2016.

5. Drug Enforcement Administration. (2014) Emerging Threat Report First Quarter 2017. https://ndews.umd.edu/sites/ndews.umd.edu/files/emerging-threat-report-2017-quarter-1.pdf (7 November 2017).
6. Drug Enforcement Administration. (2017) Emerging Threat Report Second Quarter 2017. https://ndews.umd.edu/sites/ndews.umd.edu/files/emerging-threat-report-2017-quarter-2.pdf (7 November 2017).
7. Drug Enforcement Administration. (2017) Emerging Threat Report Third Quarter 2017. https://ndews.umd.edu/sites/ndews.umd.edu/files/emerging-threat-report-2017-quarter3.pdf (7 November 2017).
8. Logan, B.K., Mohr, A.L.A., Friscia, M., Krotulski, A.J., Papsun, D.M., Kacinko, S.L., *et al*. (2017) Reports of adverse events associated with use of novel psychoactive substances, 2013–2016: a review. *Journal of Analytical Toxicology*, **41**, 573–610.
9. Elliott, S., Evans, J. (2014) A 3-year review of new psychoactive substances in casework. *Forensic Science International*, **243**, 55–60.
10. Thirakul, P., Hair, S.L., Bergen, L.K., Pearson, M.J. (2017) Clinical presentation, autopsy results and toxicology findings in an acute N-ethylpentylone fatality. *Journal of Analytical Toxicology*, **41**, 342–346.
11. Ikeji, C. (2017) Maryland ACP Winning Abstracts 2017. *Journal of Community Hospital Internal Medicine Perspectives*, **7**, 140–143.
12. Mohr, A.L.A., Friscia, M., Logan, B.K. (2016) Identification and Prevalence Determination of Novel Recreational Drugs and Discovery of Their Metabolites in Blood, Urine and Oral Fluid. U.S. Department of Justice. https://www.ncjrs.gov/pdffiles1/nij/grants/250338.pdf (4 October 2017).
13. Krotulski, A.J., Mohr, A.L.A., Papsun, D.M., Logan, B.K. (2018) Dibutylone (bk-DMBDB): intoxications, quantitative confirmations, and metabolism in authentic biological specimens. *Journal of Analytical Toxicology*. [Epub ahead of print].
14. Meyer, M.R., Wilhelm, J., Peters, F.T., Maurer, H.H. (2010) Beta-keto amphetamines: studies on the metabolism of the designer drug mephedrone and toxicological detection of mephedrone, butylone, and methylone in urine using gas chromatography? Mass spectrometry. *Analytical and Bioanalytical Chemistry*, **397**, 1225–1233.
15. Zaitsu, K., Katagi, M., Kamata, H.T., Kamata, T., Shima, N., Miki, A., *et al*. (2009) Determination of the metabolites of the new designer drugs bk-MBDB and bk-MDEA in human urine. *Forensic Science International*, **188**, 131–139.
16. Uralets, V., Rana, S., Morgan, S., Ross, W. (2014) Testing for designer stimulants: metabolic profiles of 16 synthetic cathinones excreted free in human urine. *Journal of Analytical Toxicology*, **38**, 233–241.
17. Mueller, D.M., Rentsch, K.M. (2012) Generation of metabolites by an automated online metabolism method using human liver microsomes with subsequent identification by LC-MS(n), and metabolism of 11 cathinones. *Analytical and Bioanalytical Chemistry*, **402**, 2141–2151.
18. Krotulski, A.J., Mohr, A.L.A., Papsun, D.M., Logan, B.K. (2018) Metabolism of novel opioid agonists U-47700 and U-49900 using human liver microsomes with confirmation in authentic urine specimens from drug users. *Drug Testing and Analysis*, **10**, 127–136.
19. Krotulski, A.J., Papsun, D., Friscia, M., Swartz, J.L., Holsey, B.D., Logan, B. K. (2018) Fatality Following Ingestion of Tetrahydrofuranylfentanyl, U-49900 and Methoxy-Phencyclidine. *Journal of Analytical Toxicology*, **42**, e27–e32.
20. Papsun, D., Isenschmid, D., Logan, B.K. Observed Carfentanil Concentrations in 355 Blood Specimens from Forensic Investigations. *Journal of Analytical Toxicology*. doi:10.1093/jat/bkx068.
21. Butler, D.C., Shanks, K., Behonick, G.S., Smith, D., Presnell, S.E., Tormos, L.M. (2017) Three Cases of Fatal Acrylfentanyl Toxicity in the United States and a Review of Literature. *Journal of Analytical Toxicology*. doi:10.1093/jat/bkx083.
22. Papsun, D.M. (2017) *N*-ethyl pentylone concentrations. (21 December 2017).