Gas Chromatography Mass Spectrometry (GC-MS) for Identification of Designer Stimulants Including 2C Amines, NBOMe Compounds, and Cathinones in Urine

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Phenethylamine derivatives are being increasingly exploited for recreational use as "designer" stimulants designed to mimic psychostimulant properties of amphetamine or other illicit substances like 3,4-methylenedioxymethamphetamine (MDMA [ecstasy]). Clandestine operations meticulously design phenethylamines so the user can bypass legal action when detected, as many of these are yet to be regulated by government authorities. Substituted phenethylamines or 2C amines, N-methoxybenzyl derivatives of the corresponding 2C amines commonly known as NBOMe compounds, and cathinones are among the most commonly abused phenethylamines. Current FDA-approved assays used in screening for illicit drug use lack the sensitivity needed to detect designer stimulants making it challenging for toxicologists to accurately identify these compounds. Gas chromatography mass spectrometry (GC-MS) is a sensitive method for identifying designer stimulants. This unit describes and compares two qualitative GC-MS methods for identifying 2C amines, NBOMe compounds, and cathinones in urine. © 2017 by John Wiley & Sons, Inc.

Keywords: designer amines • GC-MS • phenethylamines • designer stimulants • designer amphetamines

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INTRODUCTION

During the 1960s and 1970s, several countries, including the United States, witnessed an unprecedented increase in use of drugs of abuse by the general population. These included heroin, cocaine, cannabis, amphetamine, and methamphetamine. The U.S. government developed legislation to restrain the insurgence of drugs of abuse into society. Since 1971, the United States has conducted a nationwide survey (National Survey on Drug Use and Health) which has strongly established that many illegal drug users are either employed full- or part-time. It was proposed that screening individuals at high risk for using drugs of abuse would improve workplace efficiency and safety. Another population in which urine drug screening is useful is patients receiving pharmacotherapy by controlled substances such as opioids and stimulants such as Adderall (amphetamine). In these patients, compliance to appropriate drug is closely monitored to circumvent prescription drug diversion. Due to a widespread need for urine drug tests, several urine drug testing programs have been established and have been used since the 1980s. Urine drug testing is

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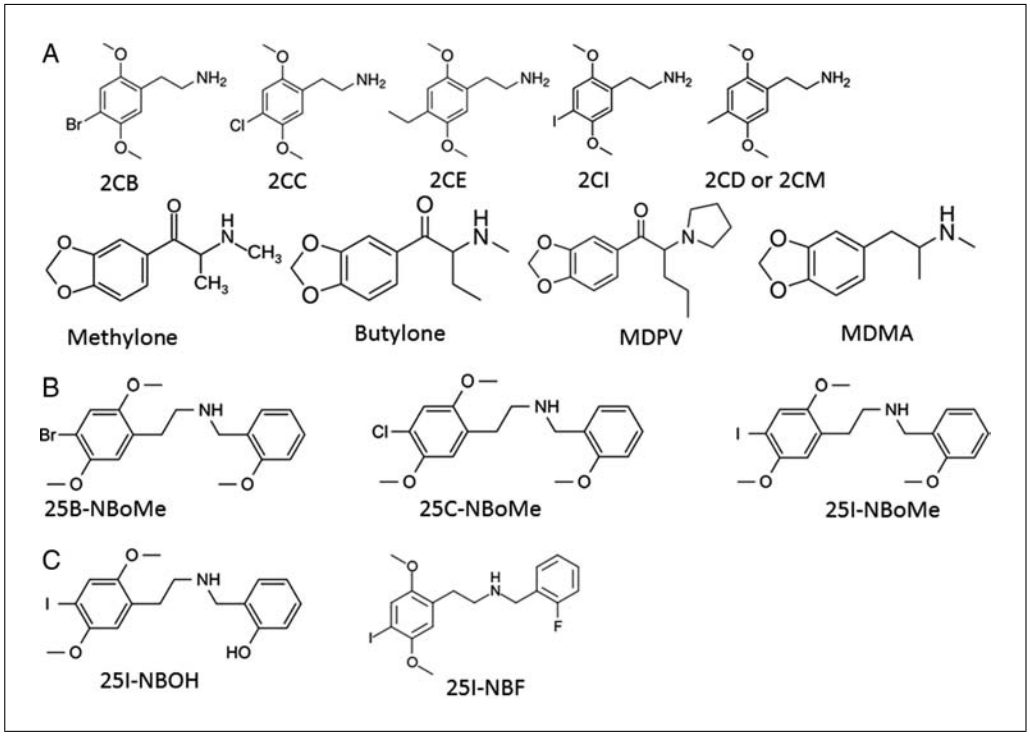

Figure 4.43.1 Chemical structures of commonly abused designer stimulants: (**A**) 2C amines, (**B**) NBOMe compounds, and (**C**) cathinones.

now widely employed for pre-employment screening, monitoring pharmacotherapy compliance, detecting drug use or abuse by military personnel, and rehabilitating prisoners (Moeller, Lee, & Kissack, 2008).

The first use of amphetamine dates back to 1877, when Romanian chemist Lazar Edeleanau synthesized it from the plant-derived compound ephedrine. In the late 1920s, Smith, Kline, and French (now part of GlaxoSmithKline) used a racemic mixture of amphetamine as a pharmaceutical nasal decongestant due its vasodilatory effects (Nabenhauer, 1933). Later, the discovery of stimulant properties of amphetamine led to its use for treating narcolepsy. It was in the late 1940s and 1950s that amphetamine and its derivatives found a widespread popularity for recreational use especially by the military in World War II. Amphetamine belongs to a broad class of compounds called phenethylamines (PEAs). Several compounds of this class have been synthesized and were shown to have psychostimulant effects in humans. Much of the information known on PEAs has been compiled and made easily available by Alexander Shulgin, a chemist and pharmacologist who compiled and published methods to synthesize PEAs and detailed information on their pharmacological effects in humans in his books PiHKAL (*Phenethylamines I Have Known and Loved*) and TiHKAL (*Tryptamines I Have Known and Loved*) (Baselt, 2004; Kwong, Magnani, Rosano, & Shaw, 2013).

More than 50 years after Shulgin first described the synthetic methods, PEA derivatives that mimic psychostimulant properties of known (e.g., regulated illicit drugs such as 3,4-methylenedioxymethamphetamine [MDMA]) are being exploited for recreational use as "designer" stimulants. Covert operations meticulously design PEAs so the user can bypass legal action when detected, as many of these are yet to be regulated by authorities. Most commonly encountered designer stimulants in a clinical toxicology laboratory include methamphetamine, MDMA, and ephedrine. Recently, several new classes of designer stimulants, including 2C amines, N-methoxybenzyl (NBOMe) compounds, and bath salts, have appeared in public use (Fig. 4.43.1). Adverse effects related to overuse of

amphetamine and related compounds include dependence, headaches, anxiety, agitation, blurred vision, and sleep problems (i.e., insomnia).

The newly emerging designer drugs are developed in clandestine operations, and most compounds may not be available in pure form from street vendors. Additionally, the pharmacokinetics of many new derivatives when present with commonly co-abused drugs like alcohol or other stimulants such as lysergic acid diethylamide (LSD) can be unpredictable leading to adverse events in users. Several fatalities have been reported due to inadvertent overdose with designer stimulants (Giroud et al., 1998; Kanamori et al., 2013; Kanamori, Inoue, Iwata, Ohmae, & Kishi, 2002; Weaver, Hopper, & Gunderson, 2015).

Detection of designer drug use is useful in improving clinical and psychosocial management of overdosed patients and for compliance monitoring. In many institutions, the first step in the urine drug testing process is an immunoassay screen. The immunoassay reagent antibody for detecting compounds in the amphetamine class is commonly selected by assay manufacturers such that the antibody has the highest cross reactivity towards amphetamine and methamphetamine. But commonly used immunoassays lack the sensitivity to detect designer stimulants including 2C amines, NBOMe compounds, and bath salts. Mass spectrometry–based methods, especially gas chromatography mass spectrometry (GC-MS)–based methods, have been routinely used for qualitative and quantitative assessment of previously common and now "designer" PEAs. The protocols presented in this unit describe and compare two qualitative GC-MS methods for identifying 2C amines, NBOMe compounds, and cathinones in urine. We describe the advantages and challenges with each method and include detailed GC-MS characterization of acetate and pentaflouropentanoic acid (PFPA) derivatives of several designer stimulants.

IDENTIFYING DESIGNER STIMULANTS IN URINE USING ORGANIC EXTRACTION FOLLOWED BY GC-MS ANALYSIS

BASIC PROTOCOL 1

When using the protocols described in this unit, it is important to note that Basic Protocol 1 describes a sample extraction and a GC-MS method for detection of designer stimulants including 2C amines, NBOMe compounds, and cathinones in urine as underivatized free bases.

Materials

Urine sample
Extraction buffer (see recipe)
Extraction solvent (see recipe)
100 µg/ml dioctylphthalate (see recipe)
Ethyl acetate, LC-MS grade (e.g., Sigma-Aldrich, cat. no. 34972)
GC-MS quality control solution (see recipe)

20 × 125 mm screw-top glass tube
Laboratory rocker
Centrifuge
13 × 100 mm test tubes
Nitrogen evaporator and evaporation tubes
Vortex
250-µl plastic screw-cap vials
Autosampler
GC-MS system (e.g., Agilent GC-7890B and MS-5977A) with capillary and mass ionization detector

Table 4.43.1 GC-MS Parameters Used in Basic Protocols 1 and 2

Property	Parameter
Column	J&W Ultra 2 GC column (12 m, 0.2 mm i.d., 0.3 μm film; Agilent Technologies, cat. no 19091B-101)
Oven temperature	Initial value = 80°C
	Final value = 285°C
Program rate	20°C/min
Carrier gas pressure	Helium 50 kPa
Flow	175 cc/min
Septum purge	2 cc/min
Injection temperature	285°C
Injection type	Splitless
m/z range	50-650 Da
Chromatographic run time	17.5 min

Additional reagents and equipment for GC-MS (see Agilent Operation Manual available at *https://www.agilent.com/cs/library/usermanuals/Public/G3430-90011.pdf*)

1. Transfer ~5 ml urine to a screw-top glass tube. Label with sample identification number.
2. Add 5 ml extraction buffer and 5 ml extraction solvent.
3. Add 50 μl of 100 μg/ml dioctylphthalate, which is used as an internal standard.
4. Tightly close the tube with the screw cap.
5. Mix tube for ~10 min on a laboratory rocker.
6. Centrifuge 5 min at 1650 × *g* (~3000 rpm), room temperature.
7. Transfer upper organic layer to a clean, labeled test tube.
8. Place labeled test tube from step 7 into a nitrogen evaporator at 50°C. Dry with heat and nitrogen.
9. Reconstitute dried extract with 200 μl ethyl acetate and vortex.
10. Transfer 100 μl reconstituted extract into a 250-μl screw-cap vial, and place into the autosampler. Analyze using a GC-MS instrument, and include a quality control solution.

The instrument should be installed as per the manufacturer's instructions. The gas chromatograph parameters used in this protocol are shown in Table 4.43.1.

BASIC PROTOCOL 2

IDENTIFYING DESIGNER STIMULANTS IN URINE USING ORGANIC EXTRACTION FOLLOWED BY DERIVATIZATION AND GC-MS ANALYSIS

Basic Protocol 2 describes a sample extraction and a GC-MS method for detection of designer stimulants including 2C amines, NBOMe compounds, and cathinones in urine as derivatized compounds (i.e., PFPA or acetate derivatives).

Materials

Urine sample
5.0 N aqueous NaOH

Hexane (e.g., Sigma-Aldrich, cat. no. 650420)
Pentaflouropropionic acid anhydride (PFPA; e.g., Sigma-Aldrich, cat. no. 245917)
Pyridine, anhydrous (e.g., Sigma-Aldrich, cat. no. 270970)
Acetic anhydride (e.g., Sigma-Aldrich, cat. no. 45830)
Ethyl acetate, LC-MS grade (e.g., Sigma-Aldrich, cat. no. 34972)
GC-MS quality control solution (see recipe)

13 × 100 mm glass test tubes
Vortex
Centrifuge
50°C water bath
Nitrogen evaporator
Screw-cap vials
Autosampler
GC-MS system (e.g., Agilent GC-7890B and MS-5977A) with capillary and mass ionization detector

Additional reagents and equipment for GC-MS (see Agilent Operation Manual available at *https://www.agilent.com/cs/library/usermanuals/Public/G3430-90011.pdf*)

1. Transfer 1 to 5 ml urine sample into a glass test tube, and add 500 µl of 5.0 N aqueous NaOH and 750 µl hexane. Vortex for 15 sec.
2. Centrifuge 5 min at 1650 × *g* (~3000 rpm), room temperature.
3. Transfer upper organic layer to a glass test tube.
4. Add four drops of PFPA, and thoroughly mix the contents by vortexing. For acetate derivative formation, add two drops of pyridine and two drops of acetic anhydride instead of PFPA.
5. Cap sample tubes, and incubate in 50°C water bath for 15 min.
6. Allow the test tube to cool for a few minutes, add 250 µl of 5.0 N NaOH, and vortex for 30 sec.
7. Centrifuge 5 min at 1650 × *g* (~3000 rpm), room temperature.
8. Carefully transfer top hexane layer to a glass test tube.

   At this step, the interface between layers can be difficult to see.
9. Place the test tube in a water bath at 50°C, and dry the hexane under a gentle stream of nitrogen. Reconstitute the residue in 100 µl ethyl acetate.
10. Pipette the extract into a labeled, screw-cap vial and place into autosampler. Analyze using a GC-MS instrument, and include a quality control solution.

    The instrument should be installed as per the manufacturer's instructions. The details of the parts and parameters for this specific method are shown in the Table 4.43.1.

REAGENTS AND SOLUTIONS

Use sterile double-distilled water or equivalent in all recipes and protocol steps, and use LC-MS–grade solvents. For common stock solutions, see APPENDIX 2A.

Dioctylphthalate, 100 µg/ml

Dissolve 10 mg dioctylphthalate in 100 ml isopropyl alcohol. Store at room temperature for up to 2 years.

Table 4.43.2 Abbreviations, Chemical Names, Molecular Weights, GC Retention Times, and Prominent m/z Peaks Observed in the GC-MS Electron Impact Spectra of 2C Amines[a]

Abbreviation	Chemical name	Molecular weight	Derivative	Retention time (min)	m/z peaks
2CB	2-(4-Bromo-2,5-dimethoxyphenyl) ethanamine	260.13	Underivatized free base	7.85	259, 257, 232[b], 230[b], 215, 213, 121, 91
		406.15	PFPA	8.31	405, 242[b], 229, 201, 148, 119, 91, 77
		302.17	Acetate	9.8	303, 242[b], 229, 148, 105
2CC	2,5-Dimethoxy-4-chlorophenethylamine	215.68	Underivatized free base	7.28	215, 186b, 171, 155, 77,
		361.7	PFPA	7.9	361, 198[b], 185, 155, 119, 105, 91, 77
		257.7	Acetate	8.3	257, 198, 171, 155, 109, 105, 91, 77
2CE	1-(2,5-Dimethoxy-4-ethylphenyl)-2-aminoethane	209.3	Underivatized free base	6.9	209, 180b, 165, 149, 91
		355.3	PFPA	7.49	355, 192, 179[b], 149, 119, 105, 91, 77
		251.3	Acetate	8.81	251, 192[b], 177, 91
2CI	2,5-Dimethoxy-4-iodophenethylamine	307.1	Underivatized free base	8.26	307, 278[b], 263, 247
		453.1	PFPA	8.76	453, 290[b], 277, 247, 148, 119, 105, 91
		349.2	Acetate	10.1	349, 290[b] 275, 148[b]
2CD/2C-M	2,5-Dimethoxy-4-methylphenethylamine	195.13	Underivatized free base	6.6	195, 166[b], 151, 135
		341.3	PFPA	7.22	341, 178[b], 165, 135, 119, 105, 91, 77
		237.3	Acetate	8.56	237, 178[b], 163, 135

[a]Substituted 2,5-dimethoxy phenethylamines.
[b]Represents the base peak in the extracted ion spectrum.
PFPA, pentafluoropentanoic acid.

Extraction buffer

Weigh out 456.5 g $K_2HPO_4 \cdot 3H_2O$. Dissolve in 1 liter deionized water. Adjust the pH to 9.5 with 10 N NaOH. Store at room temperature for up to 1 year.

Extraction solvent: 90:1 (v/v) ethyl acetate:isopropyl alcohol

Mix 900 ml ethyl acetate and 100 ml isopropyl alcohol thoroughly at room temperature. Store at room temperature for up to 1 year.

GC-MS quality control solution

In 100 ml methanol (e.g., Sigma-Aldrich, cat. no. 34966), dissolve 20 mg of each of the following: nicotine, cotinine, caffeine, lidocaine, methadone, imipramine, and dioctylphthalate. Store at 2°C to 8°C for up to 2 years.

Table 4.43.3 Abbreviations, Chemical Names, Molecular Weights, GC Retention Times, and Prominent m/z Peaks Observed in the GC-MS Electron Impact Spectra of NBOMe Compounds[a]

Abbreviation	Chemical name	Molecular weight	Derivative	Retention time (min)	MS peaks
25I-NBOMe	4-Iodo-2,5-dimethoxy-N-(2-methoxybenzyl)-phenethylamine	427.3	Underivatized free base	12.11	427, 150, 121[b], 91
		573.3	PFPA	11.94	150, 121, 91
		469.3	Acetate	13.24	469, 150, 121[b], 91
25B-NBOMe	4-Bromo-2,5-dimethoxy-N-(2-methoxybenzyl)-phenethylamine	380.3	Underivatized free base	11.65	230, 199, 150, 121[b], 91, 77
		526.3	PFPA	11.55	242, 227, 201, 121[b], 91
		422.3	Acetate	12.89	421, 242, 192, 150, 121[b], 91
25C-NBOMe	4-Chloro-2,5-dimethoxy-N-(2-methoxybenzyl)-phenethylamine	335.8	Underivatized free base	6.9	335, 150, 121[b], 91, 77
		481.8	PFPA	11.31	481, 198[b], 185, 121, 91
		377.9	Acetate	11.21	377, 198, 150, 121[b], 91
25I-NBOH	2-((2-(4-iodo-2,5-dimethoxy phenyl)ethylamino)methyl)-phenol	413.25	Underivatized free base	12.49	307, 278[b], 263, 247, 232, 180, 121, 105, 91
		559.3	PFPA	11.75	290[b], 276, 253, 148, 119, 105
		455.3	Acetate	13.75	497, 290[b], 178, 149, 136, 107
25I-NBF	2-(4-Iodo-2,5-dimethoxyphenyl)-N-[(2-fluorophenyl)methyl] ethanamine	415.24	Underivatized free base	8.54	278, 247, 232, 138, 109[b], 91
		561.3	PFPA	11.32	290[b], 275, 247, 148, 109, 91
		457.3	Acetate	12.47	457, 290[b], 277, 247, 138, 109, 91

[a]Substituted 2,5-dimethoxy phenethylamines.
[b]Represents the base peak in the extracted ion spectrum.
PFPA, pentafluoropentanoic acid.

COMMENTARY

Background Information

The prevalence of designer drug use is difficult to measure, but self-reported use among club goers has been reported to be up to 41%. In the United Kingdom, 4% to 10% of high school and college students reported having used cathinones (Weaver et al., 2015). Due to its widespread use and the lack of analytical sensitivity with immunoassay screens to detect designer drugs, it is important to develop mass spectrometry–based methods for identification of commonly abused designer drugs. We have described two methods for identification of designer stimulants in this unit. While the methods themselves are not novel, GC-MS spectra of acetate and PFP derivatives of designer stimulants has not been previously published.

Gas chromatography mass spectrometry (GC-MS) is the method of choice for confirming the presence of several illicit substances in urine because of the high degree

Table 4.43.4 Abbreviations, Chemical Names, Molecular Weights, GC Retention Times, and Prominent m/z Peaks Observed in the GC-MS Electron Impact Spectra of Cathinones

Abbreviation	Chemical name	Molecular weight	Derivative	Retention time (min)	m/z peaks
Methylone	3,4-Methylenedioxy-N-methylcathinone	207.2	Underivatized free base	7.17	149, 121, 58[a]
		353.3	PFPA	7.59	353, 204, 160, 149[a], 121, 91
		249.3	Acetate	8.95	249, 149, 121, 100, 91, 58[a]
Butylone	3,4-Methylenedioxy-N-ethylcathinone	221.2	Underivatized free base	7.52	149, 121, 72[a]
		367.3	PFPA	7.95	367, 218, 160, 149[a], 121, 91
		263.3	Acetate	9.22	263, 149, 72[a]
MDPV	Methylenedioxypyrovalerone	275.4	Underivatized free base	6.9	207, 149, 126[a]
MDMA	3,4-Methylenedioxymethamphetamine	193.2	Underivatized free base	6.25	135, 85, 71, 58b
		339.3	PFPA	7.30	339, 204b, 162, 135,119
		235.3	Acetate	8.47	162, 135, 100, 77, 58[a]

[a]Represents the base peak in the extracted ion spectrum.
PFPA, pentafluoropentanoic acid.

of specificity and sensitivity offered by this methodology. GC-MS based methods have also been used to detect designer stimulants in blood, saliva, and hair (Saito et al., 2011). GC-MS, while a sensitive and specific technique, is not without challenges. It requires organic extraction of urine or serum samples into an appropriate solvent. Optimization of the extraction method to detect low levels of several designer drugs with a wide range of physiochemical properties is required and can be laborious and time consuming. Additionally, previously unidentified compounds may not be identified easily. The extraction process of GC-MS screens commonly used in clinical laboratories may or may not allow a sensitive detection of several designer stimulants depending on the sample preparation method and/or the designer stimulant in question. The methods described in this unit can be used to quantitate designer stimulants in urine provided the appropriate calibrators are prepared and the method is evaluated for validation parameters including recovery, linearity, specificity, limit of detection, precision, and accuracy.

Critical Parameters

Basic Protocol 1 describes a simple and efficient method for identification of designer stimulants. Basic Protocol 1 uses a buffer at pH 9.5, which is high enough to convert most designer stimulants to free-base form making them more amenable to organic extraction. However, underivatized amine groups do not have optimal chromatographic properties and may lead to poor GC peak shape. Amphetamine and its related compounds are volatile, and the organic extract of samples containing low levels of designer stimulants may need to be dried under nitrogen at room temperature to avoid loss of compound during the drying step (Basic Protocol 1, step 8).

However, once the compounds are converted to the corresponding acetate or pentaflouropentanoic acid (PFPA) derivative as described in Basic Protocol 2, evaporating the extract in a water bath will not cause compound loss. The GC-MS method used in both protocols is a non-targeted screening approach that uses a splitless injection allowing for a greater amount of injection volume to be transferred onto the GC column. Depending on the instrument sensitivity and background signal

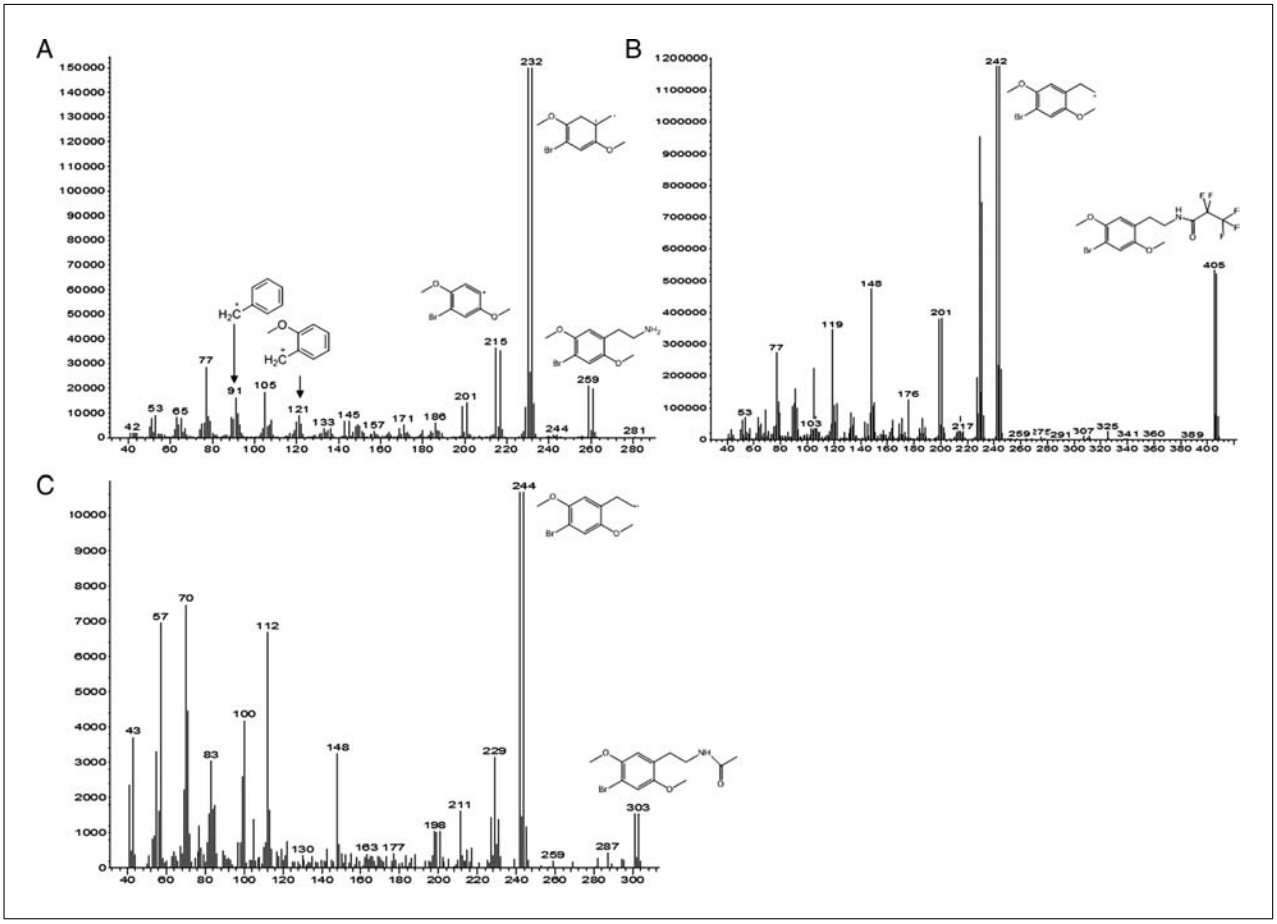

Figure 4.43.2 Extracted ion chromatogram of 2CB reference material (**A**) underivatized as a free base, (**B**) underivatized as a pentaflouropentanoic acid (PFPA) ester, and (**C**) as an acetate derivative.

to noise ratio, this may be modified to extend column life.

When using the protocols described in this unit, it is important to note that Basic Protocol 1 is a suitable method when concentrations of suspected compounds are present in high concentrations (>500 ng/ml) or for compounds with low water solubility. Basic Protocol 2 offers greater sensitivity for detection of 2C amines and some NBOMe compounds compared to Basic Protocol 1. Even though GC-MS-based methods are sensitive and specific for identifying designer stimulants, some members of the 2C amine series may not be detected using Basic Protocol 1.

Troubleshooting

The GC-MS instrument must be tuned appropriately at recommended time intervals or after any run failure. Quality control material should be analyzed before every analytical run, and appropriate GC retention times should be tracked to ensure long-term system stability and robust GC-column performance. The GC-MS septa must be replaced at appropriate intervals to ensure extraneous peaks are not observed. When the GC columns are trimmed for maintenance, the chromatographic retention times may change. Concurrent use of Rf value for compound relative to the internal standard for identification is recommended.

Another major challenge in identification of designer stimulants is the lack of reference spectra for parent compounds and their derivatives. GC-MS analysis using Basic Protocol 1 alone may not be able to distinguish between regioisomers (as free bases), and reference spectra for PFPA or acetate derivatives using Basic Protocol 2 may have to be generated with reference materials to correctly identify the suspected compound in the urine sample. When analyzing consecutive samples containing designer stimulants, it may be necessary to inject a blank (ethyl acetate) sample to rule out carryover issues. Injector carryover issues may be resolved by adding extra pre- and postinjection needle wash steps.

Anticipated Results

Basic Protocol 1 detects designer stimulants as free bases (underivatized compounds) and may be used as a screening method, while

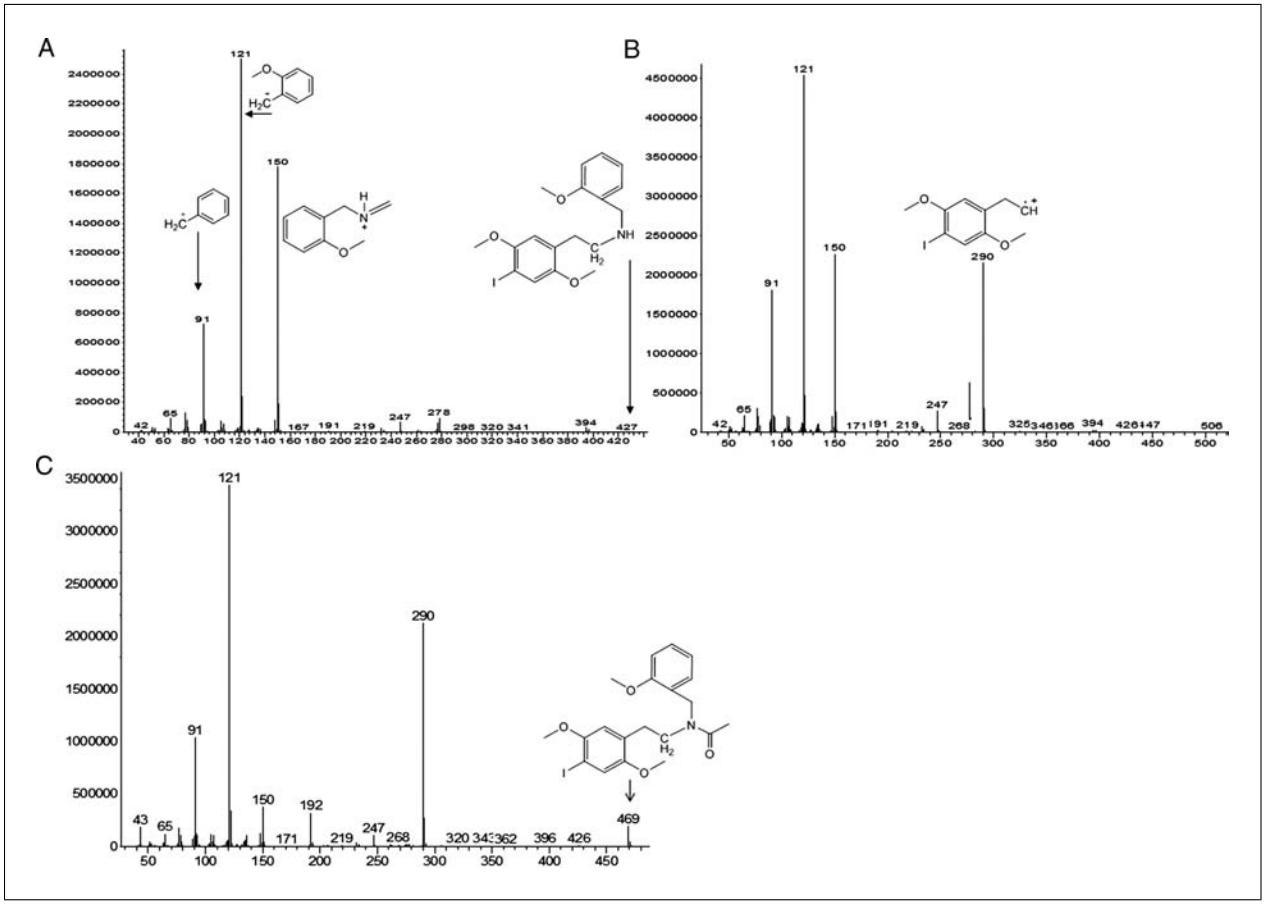

Figure 4.43.3 Extracted ion chromatogram of 25I-NBOMe reference material (**A**) underivatized as a free base, (**B**) underivatized as a pentaflouropentanoic acid (PFPA) ester, and (**C**) as an acetate derivative.

Basic Protocol 2 can be used to confirm the presence of the designer stimulant. Basic Protocol 2 offers improved sensitivity of detection since extraction at a higher pH and PFPA or acetate derivatization improves chromatographic retention and MS identification. Since the PFPA and acetate derivatives of many designer stimulants have not yet been included in publicly available GC-MS spectral libraries, the user will have to generate these reference spectra using commercially available material.

The retention times of free-base forms generally tend to be lower (8 to 10 min) compared to derivatized forms. The acetate derivatives of most compounds have a longer retention time compared to the corresponding PFPA derivatives. The expected m/z values for some designer stimulants and their PFPA and acetate derivatives are shown in Tables 4.43.2, 4.43.3, and 4.43.4. GC-MS spectra generated using electron impact ionization for 2C amines, NBOMe compounds, and cathinones lack a prominent molecular ion peak. This can make an accurate identification of certain regioisomers difficult (Maher, Awad, DeRuiter, & Clark, 2012).

An extracted ion chromatogram of 2CB reference material underivatized as a free base, as a PFPA ester, and as an acetate derivative is shown in Figure 4.43.2. The predicted structure of ionized fragments is also shown. The PFPA and acetate derivatives yield many common m/z fragments for 2C amines and NBOMe compounds (Fig. 4.43.3). Identification of tertiary amines in the cathinone class (e.g., methylenedioxypyrovalerone [MDPV]) is particularly difficult using electron impact ionization GC-MS spectrum, as it has only one prominent m/z peak (m/z = 126 in case of MDPV). Since formation of PFPA or acetate derivatives is not possible for tertiary amines, analysis by a softer ionization method such as electrospray ionization may be needed to identify these class of compounds.

Time Considerations

Identification of designer stimulants by GC-MS can be time consuming. Once the GC-MS method for quality control samples has

been optimized and the appropriate reference compounds library updated with the reference spectra, the user should allow ~1 to 2 hr of processing time for successfully extracting and analyzing urine samples by the methods described. The sample extraction steps described in Basic Protocol 2 are more time consuming compared to Basic Protocol 1 due to derivatization steps. The GC run time is 17.5 min per sample (included in the 1 to 2 hr time estimate). The time needed for data analysis is dependent upon operator experience with identification of compound peaks and availability of reference spectra in the GC-MS compound library.

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