Abstract

The 2C, 2C-T and DO-series of designer drugs pose a number of challenges to forensic toxicology laboratories. Although these drugs are seized by law enforcement agencies throughout the United States, they are not readily detected in forensic toxicology laboratories. A systematic evaluation of the cross-reactivity of nine commercial enzyme-linked immunosorbent assays (ELISAs) was conducted using eleven psychedelic amphetamines. Cross-reactivity was measured towards 4-bromo-2,5-dimethoxyphenethylamine (2C-B), 2,5-dimethoxyphenethylamine (2C-H), 2,5-dimethoxy-4-iodophenethylamine (2C-I), 2,5-dimethoxy-4-ethylthiophenethylamine (2C-T-2), 2,5-dimethoxy-4-isopropylthiophenethylamine (2C-T-4), 2,5-dimethoxy-4-propylthiophenethylamine (2C-T-7), 2,5-dimethoxy-4-bromoamphetamine (DOB), 2,5-dimethoxy-4-ethylamphetamine (DOET), 2,5-dimethoxy-4-iodoamphetamine (DOI), 2,5-dimethoxy-4-methylamphetamine (DOM) and 4-methylthioamphetamine (4-MTA). Some cross-reactivity towards 4-MTA was noted, but cross-reactivity towards the ten remaining 2C, 2C-T and DO-series drugs was extremely low (<0.4%), greatly reducing the likelihood of detection during routine screening. As a consequence, laboratories that rely upon immunoassay rather than more broad spectrum chromatographic screening techniques, may fail to detect these powerful psychedelic substances.

A procedure for the simultaneous detection of 2C-B, 2C-H, 2C-I, 2C-T-2, 2C-T-7, DOB, DOET, DOI, DOM and 4-MTA in urine was developed and optimized using gas chromatography/mass spectrometry (GC/MS) and solid phase extraction (SPE). Limits of detection for all analytes were 2 – 10 ng/mL and limits of quantitation were 10 ng/mL or less. Additional reference materials were made available, allowing liquid chromatography-tandem mass spectrometry (LC/MS/MS) to be used for the simultaneous detection of fifteen psychedelic amphetamines in blood and urine. The following additional designer drugs were included in the assay: (2C-T-4, 2,5-dimethoxy-4-chlorophenethylamine (2C-C), 2,5-dimethoxy-4-methylphenethylamine (2C-D), 2,5-dimethoxy-4-ethylphenethylamine (2C-E), and 2,5-dimethoxy-4-chloroamphetamine (DOC). Assay performance was evaluated in terms of analytical recovery, limit of detection, limit of quantitation, precision, accuracy, linearity, matrix effect and interferences. The techniques described allow for the simultaneous detection of fifteen psychostimulants at sub-ng/mL concentrations.

Despite their use among recreational drug users, the prevalence of these drugs in forensic toxicology investigations is not widely reported or understood due to variety of analytical and pharmacological challenges. In a study of 2,021 adjudicated forensic casework specimens, 2,5-dimethoxy-4-iodoamphetamine (DOI) was detected in two cases at a concentrations of 1 and 2 ng/mL in urine. The drug is often sold in the form of LSD-like “blotters”. DOI is a low-dose (1.5-3 mg) psychostimulant that is renowned for its powerful psychedelic effects. This is the first report of DOI to date. The low concentrations and highly sensitive and techniques that are required highlights the significant analytical challenges faced by forensic laboratories when detecting many of the psychedelic amphetamines.

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Executive Summary

The psychedelic amphetamines are an emerging class of designer drugs that are capable of producing a complex array of sought after adrenergic and hallucinogenic effects. Early phenethylamine designer drugs included 3,4-methylenedioxyamphetamine (MDA, "Eve", "Love drug"), 3,4-methylenedioxymethamphetamine (MDMA, "Adam", "Ecstasy") and 3,4-methylenedioxyethylamphetamine (MDEA). MDA and MDMA were synthesized in the early 1900s and by the 1980s, they were classified as Schedule I drugs under the Federal Controlled Substances Act. As existing drugs are recognized and scheduled, new drugs are readily available on the illicit market. This cycle of amphetamine-based designer drug development, use, and legal prohibition, has fueled development of new drugs over the past three decades, and continues today**.**

Many of these drugs are not targeted or identified during routine toxicology testing and as a consequence, they are rarely reported. Designer drugs are often perceived by drug users to be advantageous from both a pharmacological and legal standpoint. Small alterations in structure may produce considerable changes in terms of the perceived effects by the drug user, but may also circumvent existing drug legislation. Demand from recreational drug users, and the clandestine supply and effective “marketing” of designer drugs via the Internet, significantly outpaces the ability of government to regulate, legislate and enforce those actions.

Hallucinogenic phenethylamines were first synthesized by Shulgin (Shulgin and Shulgin, 1991) and later emerged as illicit drugs in Europe and Asia before making an appearance in this country. Although the most widely abused amphetamine in the United States is still *d*-methamphetamine, there is still significant interest in new designer amphetamines (Haroz and Greenberg, 2006). These emerging designer drugs include the dimethoxyphenylethanamine (2C, 2C-T) and dimethoxyphenylpropanamine (DO) series of psychedelics reported in this study. They include 2,5-dimethoxy-4-bromophenethylamine (2C-B), 2,5-dimethoxy-4-chlorophenethylamine (2C-C), 2,5-dimethoxy-4-methylphenethylamine (2C-D), 2,5-dimethoxy-4-ethylphenethylamine (2C-E), 2,5-dimethoxyphenethylamine (2C-H), 2,5-dimethoxy-4-iodophenethylamine (2C-I), 2,5-dimethoxy-4-ethylthiophenethylamine (2C-T-2), 2,5-dimethoxy-4-isopropylthiophenethylamine (2C-T-4), 2,5-dimethoxy-4-propylthiophenethylamine (2C-T-7), 2,5-dimethoxy-4-bromoamphetamine (DOB), 2,5-dimethoxy-4-chloroamphetamine (DOC), 2,5-dimethoxy-4-ethylamphetamine (DOET), 2,5-dimethoxy-4-iodoamphetamine (DOI) and 2,5-

dimethoxy-4-methylamphetamine (DOM). Additionally, 4-methylthioamphetamine (4-MTA) was also included in the study due to its structural similarity, toxicity and reported use.

Despite the fact that state, local and federal law enforcement agencies report seizures of these substances within the United States, forensic toxicology laboratories, crime laboratories and medical examiner's laboratories do not routinely test for many of these designer drugs. Methodology to detect these drugs in biological samples is relatively limited, despite the fact that many of these drugs are powerful psychostimulants with the potential for significant public health, safety and criminal justice consequences. The purpose of this study was to evaluate limitations in existing methodology and develop new procedures for the detection of these drugs using techniques that enjoy widespread acceptance in the forensic toxicology community.

Immunoassay

A systematic evaluation of the cross-reactivity of nine commercial enzyme-linked immunosorbent assays (ELISAs) was undertaken. Cross-reactivity towards the 2C, 2C-T and DO-series of psychedelic amphetamines was negligible (<0.4%). Concentrations as high as 50,000 ng/mL in urine, which greatly exceed those expected in forensic case samples, were not sufficient to produce a positive result. The only substance to produce any measurable cross-reactivity was 4-MTA. Cross-reactivities of 5 and 7% were obtained using four Methamphetamine/MDMA directed assays, 25 and 200% using two amphetamine directed assays. The absence of any measurable cross-reactivity towards the 2C, 2C-T and DO psychedelic phenethylamines makes it harder to detect these drugs during routine screening. As a consequence, laboratories that rely upon immunoassay rather than more broad spectrum chromatographic screening techniques, will most likely fail to detect these powerful psychedelic substances. This is not unexpected because these assays were not ever intended for this purpose. However, it does highlight the importance of using non-immunoassay based broad spectrum screening for some classes of drug, including those described here.

Isolation of Drugs

Solid phase extraction (SPE) was used to develop and optimize a procedure for the extraction of fifteen psychedelic amphetamines from blood and urine. Samples were treated with acidic methanol prior to evaporation to minimize evaporative loss and imprecision. A statistical evaluation of analytes treated with acidic methanol prior to evaporation showed significant improvement compared with those that were not treated (and therefore evaporated in the base, or uncharged form). During the development and optimization procedure for whole blood, a variety of sample preparation techniques were evaluated. Acetonitrile protein precipitation and sample dilution in buffer were optimal. The latter was selected based upon analyte recovery, ease of use and analysis time. Analytical recoveries for all target compounds were 64-93% in urine and 60-91% in blood.

Gas Chromatography/Mass Spectrometry

Gas Chromatography/Mass Spectrometry (GC/MS) is still the most widely used technique in forensic toxicology laboratories. Target drugs in urine were analyzed using selected ion monitoring (SIM) following solid phase extraction. Limits of detection were 2 ng/mL for 4-MTA, DOM, DOB, DOI, DOET, 2-C-B and 2C-I; 5 ng/mL for 2C-T-2 and 2C-T-7; and 10 ng/mL for 2C-H. Limits of quantitation were 2 ng/mL for DOET and DOB; 5 ng/mL for DOM, 2C-B and DOI; and 10 ng/mL for 2C-H, 4-MTA, DOI, 2C-T-2 and 2-C-T-7. Precision evaluated at 50 and 500 ng/mL yielded intra-assay CVs of 0.4-7.9% and accuracy in the range 91-116%, respectively. Inter-assay CVs at 250 ng/mL were 2.5-9.4% for all analytes tested. Calibration curves were linear to 1,500 ng/mL using mescaline-d_9 as the internal standard. No carryover was evident at 5,000 ng/mL (the highest concentration tested) and no interferences were observed from the presence of other structurally related compounds or endogenous bases. It was not necessary to derivatize drugs in order to achieve these low detection limits.

Liquid Chromatography/Tandem Mass Spectrometry

Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS) was successfully used to identify an expanded panel of psychedelic amphetamines in blood and urine. Limits of detection in urine were 0.5 ng/mL (2C-C, 2C-D, 2C-E, 2C-H, 2C-I, 2C-T-2, 2C-T-4, 2C-T-7, 4-MTA, DOB, DOC, DOET, DOI, DOM) and 1 ng/mL (2C-B). The limit of quantitation in urine for

all drugs was 1 ng/mL. Accuracy for controls evaluated at 50 and 250 ng/mL were 96-120% for all target analytes and intra-assay CVs were 0.5-5.6% over the same concentration range. Inter-assay CVs in urine were 2.1-20.8% at 100 ng/mL. No matrix effects were observed and no interferences were present using structurally related compounds, endogenous bases and other common drugs in urine. In blood, limits of detection were 0.5 ng/mL (2C-D, 2C, E, 2C-H, 2C-I, 2C-T-2, 2C-T7, 4-MTA, DOB, DOC, DOET, DOI, DOM) and 1 ng/mL (2C-B, 2C-C, 2C-T-4). Limits of quantitation were 0.5 ng/mL (2C-D, 2C-E, DOI, DOM), 1 ng/mL (2C-B, 2C-C, 2C-H, 2C-I, 4-MTA, DOB, DOC, DOET) and 2 ng/mL (2C-T-2, 2C-T4, 2C-T-7). Whole blood controls at 50 and 250 ng/mL yielded accuracies of 89-112% and intra-assay CVs of 1.1-6.1%. Inter-assay CVs at 100 ng/mL were 2.9-8.2% for all target analytes. Following optimization of the procedure, no matrix effects were present using whole blood. In the interference study however, meperidine was responsible for a small but measurable suppression. As a result, two analytes (2C-T-2 and DOB) provided quantitative results lower than expected and just outside of the acceptable range (± 20%).

In a retrospective study, adjudicated casework samples that were due for destruction were examined to determine the presence of psychedelic amphetamines by LC/MS/MS. Urine samples (N=2,021) from individuals apprehended for suspicion of being under the influence of a controlled substance were provided for this purpose upon Institutional Review Board (IRB) approval by the Protection of Human Subjects Committee of Sam Houston State University. Two of the samples tested contained DOI at concentrations of 1 and 2 ng/mL. This is consistent with the high potency of the DO-series, with recreational doses of DOI reported to be as low as 1.5-3 mg. DOI is renowned for its profound hallucinogenic effects and is frequently encountered on LSD-like blotters. Despite reported seizures of DOI throughout the United States, this is the first report of its use in human subjects to date. The very low concentrations detected highlight the need for very sensitive testing. The psychedelic amphetamines pose a significant analytical challenge to laboratories that perform routine toxicology testing.

Limitations

Despite their use among recreational drug users, the 2C, 2C-T and DO-series of designer drugs continue to pose a number of pharmacological, toxicological and analytical challenges. It is not clear whether these drugs are rarely reported in routine toxicological investigations due to overall low prevalence, or limitations with respect to detectability.

Commercial immunoassays have limited cross-reactivity towards these amphetamine-like drugs. As a consequence, laboratories that rely upon immunoassay, rather than more broad spectrum chromatographic screening techniques may fail to detect them. Highly sensitive and targeted analytical procedures are required for this purpose. Although the metabolic transformation of these drugs has been preliminarily investigated and likely involves a number of common pathways, reference standards are not commercially available. Toxicology laboratories performing routine human performance or postmortem investigations must therefore rely upon detection of the parent drug. Using the approach described here, psychedelic amphetamines were detected at forensically significant concentrations in blood and urine using both GC/MS and LC/MS/MS. Although DOI was detected in adjudicated forensic case samples, very little is understood concerning the stability of this drug class over time, making it difficult to assess prevalence. However, the first report of DOI detection in forensic toxicology casework to date is significant and this finding highlights the difficulties associated with the detection of these emerging drugs of abuse.

Introduction

Statement of the Problem

A number of psychedelic amphetamines that originally emerged as popular drugs of abuse in Europe and Asia, are now routinely seized throughout the United States. These include substances such as 2,5-dimethoxy-4-bromophenethylamine (2C-B), 2,5-dimethoxy-4-chlorophenethylamine (2C-C), 2,5-dimethoxy-4-methylphenethylamine (2C-D), 2,5-dimethoxy-4-ethylphenethylamine (2C-E), 2,5-dimethoxyphenethylamine (2C-H), 2,5-dimethoxy-4-iodophenethylamine (2C-I), 2,5-dimethoxy-4-ethylthiophenethylamine (2C-T-2), 2,5-dimethoxy-4-isopropylthiophenethylamine (2C-T-4), 2,5-dimethoxy-4-propylthiophenethylamine (2C-T-7), 2,5-dimethoxy-4-bromoamphetamine (DOB), 2,5-dimethoxy-4-chloroamphetamine (DOC), 2,5-dimethoxy-4-ethylamphetamine (DOET), 2,5-dimethoxy-4-iodoamphetamine (DOI), 2,5-dimethoxy-4-methylamphetamine (DOM) and 4-methylthioamphetamine (4-MTA). Despite the fact that the Drug Enforcement Administration reports seizures of these substances within the United States, forensic toxicology laboratories, crime laboratories and medical examiner's laboratories do not routinely test for many of these designer drugs. Methodology is relatively limited and there have been very few reports that describe this powerful class of psychostimulants. Previously published methods for the analysis of some of these drugs utilize techniques not widely available in crime laboratories, are not always mindful of forensic issues (such as limited specimen volume, appropriate use of internal standard, scientific validation), or have targeted just one, or a very small number of drugs within the class.

Immunoassays, such as enzyme-linked immunosorbent assay (ELISA) and gas chromatography/mass spectrometry (GC/MS) are among the most widely utilized screening and confirmatory techniques in toxicology testing laboratories. The cross-reactivity of antibodies used in commercial immunoassays for amphetamine-like drugs is largely unknown and must be investigated to determine whether existing commercial immunoassays are effective screening tools for these psychostimulants. Development of extraction protocols and sensitive confirmatory procedures using GC/MS are needed, since it is the most widely used technique. This development must take into consideration existing resources and techniques that are commonly used in the forensic toxicology laboratory so that it can be implemented effectively. Liquid chromatography with tandem mass spectrometry (LC/MS/MS), although not yet as

widespread among routine laboratories, is also a powerful technique for the qualitative and quantitative identification of drugs. In order for forensic toxicology laboratories to effectively detect the psychedelic amphetamines, the limitations of immunoassay screening procedures must be investigated and additional methodology developed. Optimized and scientifically validated confirmatory techniques can then be used to analyze adjudicated forensic casework specimens, to determine the presence of psychostimulants in a population of drug users.

Literature Citations and Review

Designer Drugs

A recent review suggests that more than 100 new psychotropic substances or designer drugs have been introduced of late (Wohlfarth and Weinmann, 2012). Following regulation of conventional counterparts, synthesis of these new derivatives is often a direct attempt to circumvent regulation and criminalization (Poortman and Lock, 1999). The increasing prevalence of designer drugs stems largely from control measures, which serve to schedule or regulate drugs that have the potential for abuse or deleterious effects. Designer drugs are often perceived by drug users to be advantageous from a legal standpoint and may be viewed as having more desirable pharmacologic effects. Small alterations in structure may produce drugs with similar subjective effects, but circumvent existing drug legislation. The innovation of suppliers to create and effectively "market" designer drugs to recreational users via the Internet, significantly outpaces the ability of government to regulate, legislate and enforce those actions.

One of the largest and most important classes of designer drugs are the phenethylamine derivatives. Early examples of these are 3,4-methylenedioxyamphetamine (MDA) and 3,4-methylenedioxymethamphetamine (MDMA), which emerged as popular recreational drugs in the 1960s and 1980s, respectively. Today, many of the phenethyalmine-based designer drugs have become popularized by young recreational drug users, and as a consequence, are of growing concern from a public safety perspective. Although the majority of these designer drugs initially emerged in Europe and Asia, seizures of psychedelic phenethylamines have been reported throughout the United States. Published reports in the Drug Enforcement Administration (DEA) Microgram Bulletin and other intelligence reports indicate that use of the 2C, 2C-T and DO-series of drugs is not geographically isolated (**Table 1**). Although identification of these

substances in seized drugs is somewhat routine, analytical detection in toxicological samples presents a number of challenges and as a consequence, is rarely reported.

Table 1. Reports of psychostimulant seizures throughout the United States.

Date	Drug	Form of Seizure	Location	Reference
Jul-10	2C-B	Tablets	Texas	(DEA, 2010a)
Nov-09	2C-B	Tablets	Tennessee	(DEA, 2009a)
Mar-08	2C-B	Capsules	Pennsylvania	(DEA, 2008d)
Dec-04	2C-B	Clandestine Lab	New York	(DEA, 2004b)
Jul-04	2C-B	Clandestine Lab	South Dakota	(DEA, 2004d)
Jan-04	2C-B	Capsules	Kansas	(DEA, 2004f)
May-01	2C-B	NA	Various locations in the U.S.	(NDIC, 2001)
Nov-05	2C-C	Blotter Paper	Oregon	(DEA, 2005c)
Mar-11	2C-E	NA	Minnesota	(NDIC, 2011)
Jul-08	2C-E	Liquid	Kentucky	(DEA, 2008e)
Mar-08	2C-E	Capsules	Pennsylvania	(DEA, 2008d)
Apr-07	2C-E	Capsule	Oklahoma	(DEA, 2007d)
Apr-05	2C-E	Capsule	Iowa	(DEA, 2005a)
Nov-04	2C-E	Powder	Michigan	(DEA, 2004a)
Nov-04	2C-E	Capsule	Florida	(DEA, 2004a)
Aug-08	2C-I	Tablets	Arkansas	(DEA, 2008a)
Feb-07	2C-I	Blotter Paper	Oregon	(DEA, 2007e)
Sep-06	2C-I	Capsules	Iowa	(DEA, 2006d)
Jan-06	2C-I	Capsules	Florida	(DEA, 2006a)
Jun-04	2C-I	Capsules	Oregon	(DEA, 2004e)
Mar-04	2C-I	Tablets	Spain	(DEA, 2004c)
Jan-04	2C-T-2	Powder	Kansas	(DEA, 2004f)
Apr-09	DOB	Blotter Paper	Kansas	(DEA, 2009b)
Mar-09	DOB	Blotter Paper	Georgia	(DEA, 2009c)
Dec-06	DOB	Blotter Paper	Iowa	(DEA, 2006f)
Nov-06	DOB	Blotter Paper	California	(DEA, 2006c)
Oct-05	DOB	Blotter Paper	Oregon	(DEA, 2005b)
Apr-09	DOC	Blotter Paper	Kansas	(DEA, 2009b)
Mar-09	DOC	Blotter Paper	Georgia	(DEA, 2009c)
Jun-08	DOC	Blotter Paper	Florida	(DEA, 2008c)
Mar-08	DOC	Blotter Paper	Pennsylvania	(DEA, 2008d)
Dec-07	DOC	Blotter Paper, Powder	California	(DEA, 2007f)
Oct-07	DOC	Liquid	New Mexico	(DEA, 2007a)
Apr-07	DOC	Liquid	Florida	(DEA, 2007c)

Nov-06	DOC	Liquid, Powder	Michigan	(DEA, 2006b)
Jun-06	DOC	Blotter Paper	Florida	(DEA, 2006e)
Jun-08	DOI	Blotter Paper	Florida	(DEA, 2008c)
Mar-08	DOI	Blotter Paper	Pennsylvania	(DEA, 2008d)
Sep-07	DOI	Liquid	Wisconsin	(DEA, 2007b)
Nov-06	DOI	Powder	Michigan	(DEA, 2006b)
May-06	DOI	Blotter Paper	Florida	(DEA, 2006g)

Drug Use and Scheduling

At the time of this report, the majority of the drugs under investigation are Schedule I drugs in the Federal Controlled Substances Act (CSA) due to their high potential for abuse and absence of either medical use or accepted safety. However, the majority were not scheduled and were instead considered "drugs or chemicals of concern" by the Drug Enforcement Administration (DEA) until very recently. At the beginning of the study, only DOM, DOET, 2C-B, DOB and 2C-T-7 were federally scheduled drugs. However, in July 2012 the President of the United States signed into U.S. law the Food and Drug Administration Safety and Innovation Act (S.3187), which includes the Synthetic Drug Abuse Prevention Act of 2012. This new legislation updated Schedule I drugs listed in the Controlled Substances Act (21 U.S.C. 812(c)) to include seven additional psychedelic amphetamines included in this study. DOI, DOC and 4-MTA may be regulated by the Federal Analogue Act, which states that any drug substantially similar to a scheduled drug may be treated as though it were scheduled, if intended for human consumption. Scheduling status and common street names for the drugs included in this study are summarized in **Table 2**.

Table 2. Scheduling status and street names of the substances included in this study.

Drug	Federal CSA Schedule	Effective Year	Street Names
2C-B	1	1995	Nexus, 2's; Toonies; Bromo; TC; Spectrum; Venus; Bees; Erox
2C-C	1	2012	-
2C-D	1	2012	LE-25
2C-E	1	2012	Europa
2C-H	1	2012	-
2C-I	1	2012	i
2C-T-2	1	2012	T2
2C-T-4	1	2012	T4
2C-T-7	1	2004	T7; Blue Mystic; Beautiful; Tripstacy; Tweety-Bird Mescaline; Belladona
4-MTA	Not Scheduled[1]	N/A	Flatliner; Golden Eagle
DOB	1	1973	Bob; Dr. Bob; Broloamfetamine
DOC	Not Scheduled[1]	N/A	-
DOET	1	1993	-
DOI	Not Scheduled[1]	N/A	-
DOM	1	1973	STP (Serenity, Tranquility, and Peace)

[1]Possible regulation under the Federal Analogue Act.

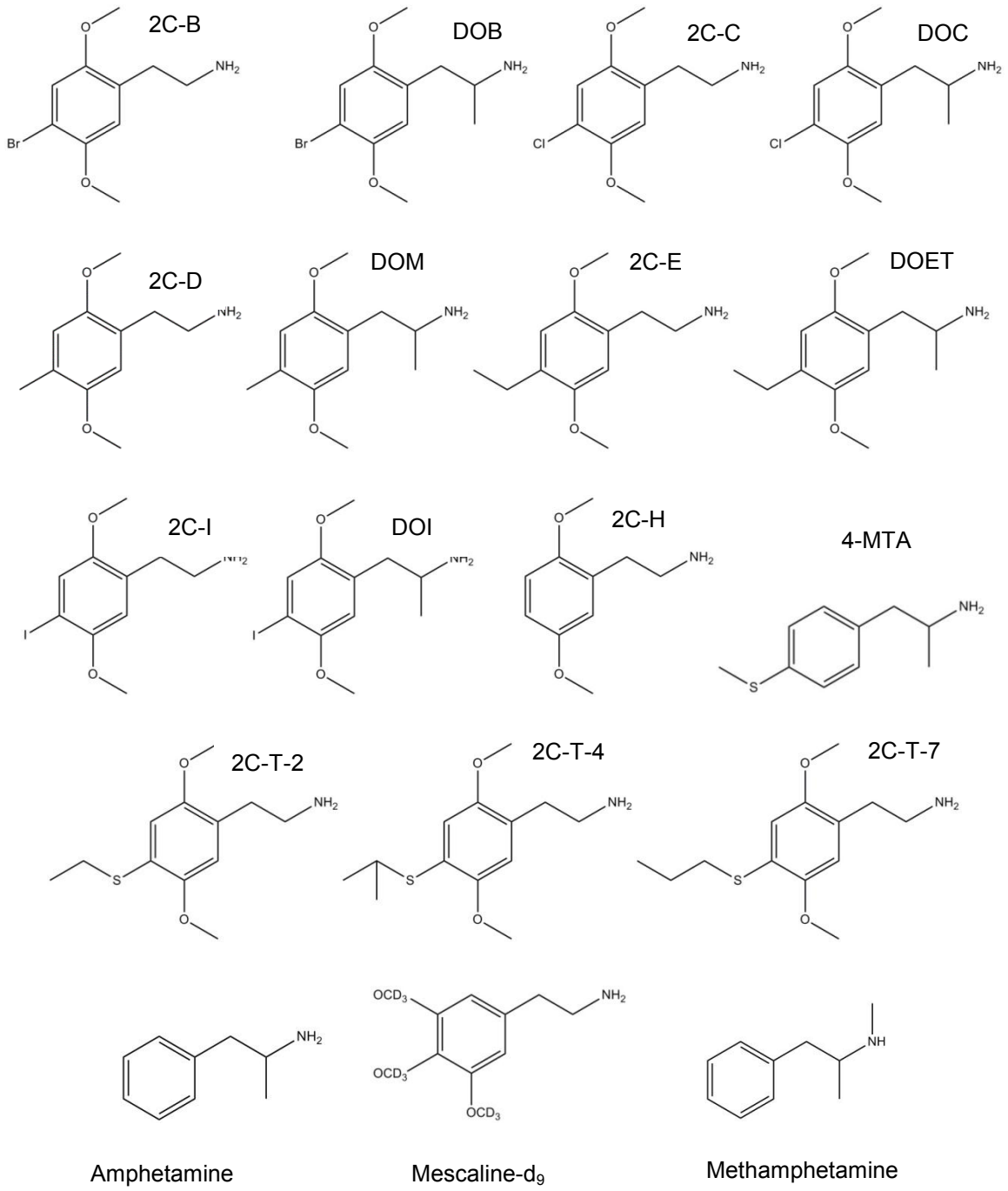
From 2004 through 2010, the DEA published numerous reports of psychostimulant drug seizures throughout the United States (DEA, 2004a, b, c, d, e, f, 2005a, b, c, 2006a, b, d, e, g, 2007a, b, c, d, e, f, g, 2008a, b, c, d, e, 2009a, b, c, 2010a, b). Reports are not geographically isolated; they include Tennessee, Georgia, Arkansas, Kentucky, Florida, Pennsylvania, California, New Mexico, Wisconsin, Oklahoma, Oregon, Iowa, Michigan, New York, South Dakota and Texas. Most of these designer amphetamines are recovered in powder, tablet or blotter form, although some have been encountered as liquids and capsules. There are numerous reports of psychedelic phenethylamines being sold as Ecstasy mimic tablets and "acid" blotter mimics (DEA, 2004a, b, c, d, e, f, 2005a, b, c, 2006a, b, d, e, g, 2007a, b, c, d, e, f, 2008a, b, c, d, e, 2009a, b, c, 2010a, b). LSD-like "blotters" are particularly common for 2C-I, DOB, and DOI. These synthetic drugs are known for their powerful psychedelic effects among recreational drug users and moreover, the difficulty associated with the detection of these

substances in urine is used to promote their use on Internet drug forums.

Chemistry and Effects

Many of the newer drugs were initially developed to harness the psychedelic and introspective potential of certain phenethylamines. Alexander Shulgin synthesized numerous compounds bearing structural or pharmacological similarity to many of the traditional psychedelics. This report focuses on three classes of phenthylamine-type drugs that can produce a combination of hallucinogenic and stimulant effects. The 2C series are dimethoxyphenethylamines, containing two methoxy groups at the 2 and 5 positions of the benzene ring and two carbons ("2C") between the amine and benzene ring (Shulgin and Shulgin, 1991). Some within the 2C series also contain a halogen on the benzene ring at the 4 position; the 2C-T series are dimethoxyphenethylethanamines that typically contain an alkylated thio- group on the 4 position of the ring; the DO-series are dimethoxyphenylpropanamines, containing a methyl on the aminoethyl chain and a halogen or alkyl group on the 4 position of the benzene ring (Shulgin and Shulgin, 1991). The 2-C, 2-C-T and DO-series of drugs share structural features that closely resemble mescaline. Chemical structures of the psychedelic amphetamines described in this study are shown in **Figure 1**.

Figure 1. Structures of the psychedelic amphetamines and the internal standard (mescaline-d_9). Methamphetamine and amphetamine are also shown for comparison purposes.



The 2C, 2C-T and DO-series of designer drugs are not well characterized in humans. There are some notable differences between traditional hallucinogenic amphetamines and these newer alternatives. Despite the structural similarity of many of these synthetic derivatives, minor alterations can have significant impact in terms of potency and onset of action (**Table 3**). The delayed onset of action and increased potency of some drugs within this class relative to MDMA has been noted. This may increase the potential for adverse reactions, toxicity (Soares et al., 2004) or fatal overdose (Curtis et al., 2003). Furthermore, the drugs are sometimes misrepresented, marketed or sold as other substances, which may increase the risk associated with their use and result in unintended consequences.

Several drugs within this class produce a complex array of hallucinogenic and stimulant effects. Users report a variety of sought after effects including psychedelic ideation, a sense of well being, emotional awakening, profound insight, closed and open-eyed visuals, increased appreciation of music, introspection and emphathogenesis. Other effects may include increased blood pressure, blurred vision, dehydration, nausea, vomiting, headache, dilated pupils, muscle tension and tachycardia.

2C-B was first synthesized in the mid-1970s and by 1998, it had become the third most popular designer phenethylamine in England and Wales (after MDMA and MDEA) (Cole et al., 2002). 2C-B was manufactured legally in the 1980's and 1990's by organizations who claimed the drug was a remedy for impotence and frigidity. In 1995, 2C-B became a Schedule I drug in the United States. As with many of these drugs, effects appear to be highly dose dependent (Erowid, 2012; Shulgin and Shulgin, 1991). 2C-I is very similar to 2C-B in terms of pharmacological effect, but it was not scheduled until very recently (2012).

The 2C-T-series designer amphetamines are similar to the 2C-series. 2C-T-4 use produces visual and audio hallucinations (Shulgin and Shulgin, 1991). This is also true of 2C-T-7 (Shulgin and Shulgin, 1991). On the street, 2C-T-7 is referred to as "T7," "Blue Mystic," and "Tripstasy." In a study on stimulus effects it was shown to act more as a hallucinogen such as mescaline rather than a stimulant like MDMA (Khorana et al., 2004). Like other designer drugs, the 2C series are serotonin receptor agonists, and currently they are among the most potent. Like the 2C's, DOB is a serotonin receptor agonist which causes effects more similar to lysergic acid diethylamide (LSD) than amphetamine (Balíková, 2005). According to Shulgin, DOB is more potent than the 2C's and the required dosage is ten-fold lower. Since drug users are not

exactly sure which chemical derivative is present in an illicit drug, accidental overdose from DOB or similar derivatives is a concern. A slower onset of action associated with this drug may also lead an inexperienced user to prematurely ingest more drug to achieve a response. DOET also appears to be more potent than the 2C's, requiring only 2-6 mg doses (Shulgin and Shulgin, 1991). Another very potent designer drug is DOI; the recommended dosage is 1.5-3.0 mg (Shulgin and Shulgin, 1991). One of the older and more historically known designer drugs is DOM. This drug, also referred to as "STP" (Serenity, Tranquility and Peace), was a popular psychedelic drug in the United States in the 1960s. As with the other DO-series drugs, it has high potency and a relatively slow onset of action (Shulgin and Shulgin, 1991). 4-MTA, also called para-methylthioamphetamine (or p-MTA) was first described by Nichols et al. in 1992 (Huang et al., 1992). "Flatliner" is a common street name for this drug which is usually found in tablet form and taken orally (much like the other drugs previously discussed). 4-MTA is also a serotonergic agent, as well as a monoamine oxidase inhibitor (Baselt, 2004). Staack and Maurer report that 4-MTA can induce serotonin syndrome, but unlike other amphetamines is non-neurotoxic to the serotonergic neurons. Even though 4-MTA may not be neurotoxic, several reports of severe poisonings and fatalities have been reported with the use of this drug. 4-MTA has shown a slower onset of effects when compared to MDMA; since it is sometimes sold as ecstasy, there is a higher risk of overdose because the user may take more when the expected effects are not experienced (Staack and Maurer, 2005).

Table 3. Dose and duration of action of 2C, 2C-T and DO-series drugs[2].

Drug	Chemical Name	Dosage (mg)	Duration (h)
2C-B	2,5-dimethoxy-4-bromophenethylamine	12-24	4-8
2C-C	2,5-dimethoxy-4-chlorophenethylamine	20-40	4–8
2C-D	2,5-dimethoxy-4-methylphenethylamine	20-60	4-6
2C-E	2,5-dimethoxy-4-ethylphenethylamine	10-25	8-12
2C-H	2,5-dimethoxyphenethylamine	Unknown	Unknown
2C-I	2,5-dimethoxy-4-iodophenethylamine	14-22	6-10
2C-T-2	2,5-dimethoxy-4-ethylthiophenethylamine	12-25	6-8
2C-T-4	2,5-dimethoxy-4-isopropylthiophenethylamine	8-20	12-18
2C-T-7	2,5-dimethoxy-4-propylthiophenethylamine	10-30	8-15
4-MTA	4-methylthioamphetamine	Unknown	Unknown
DOB	2,5-dimethoxy-4-bromoamphetamine	1-3	18-30
DOC	2,5-dimethoxy-4-chloroamphetamine	1.5-3	12-24

DOET	2,5-dimethoxy-4-ethylamphetamine	2-6	14-20
DOI	2,5-dimethoxy-4-iodoamphetamine	1.5-3	16-30
DOM	2,5-dimethoxy-4-methylamphetamine	3-10	14-20

[2]Due to the lack of scientific pharmacological data, dosage and duration of action are reported from testimonial and nonscientific publications (Erowid, 2012; Shulgin and Shulgin, 1991).

Pharmacology and Toxicology

There is very limited published scientific literature concerning the pharmacology or toxicology of these psychedelic amphetamines. A common route of administration is oral ingestion; insufflation, smoking, and rectal use are not uncommon and intravenous and intramuscular administrations have been reported (Haroz and Greenberg, 2006). Many of the psychedelic phenethylamines derive their effects from their action as 5-HT_2 agonists. The profound hallucinogenic effect associated with several of these drugs is likely due to their strong affinity towards serotonin receptor sites (Fantegrossi et al., 2005; Moya et al., 2007). The differences in their potency and duration of effects come from minor structural differences. The metabolic fate of several of the drugs under investigation have been investigated and reported in animal models. 2C-B is perhaps the most widely studied in rats and mice (Carmo et al., 2004; Kanamori et al., 2002; Kanamori et al., 2003; Rohanová et al., 2008; Theobald et al., 2007). The majority of the drug undergoes a combination of *O*-demethylation, *N*-acetylation, deamination with oxidation to the corresponding acid, or reduction to the alcohol. Transformative pathways for 2C-I (Theobald et al., 2006) and 2C-E (Theobald and Maurer, 2006) are not dissimilar, and in general the *O*-demethylation of methoxy groups, beta-oxidation of the alkyl side chain and oxidative deamination can be likened to mescaline (3,4,5-trimethoxyphenethylamine) (**Figure 1**). In rat studies, the 2CT-series may undergo combinations of *N*-acetylation, *O*-demethylation, sulfoxidation, *S*-dealkylation and *S*-methylation, hydroxylation and demethylation (Lin et al., 2003; Theobald et al., 2005a; Theobald et al., 2005b). A rat study using 2C-T-2 showed that this drug is metabolized primarily through deamination or acylation of the amine group (Chin et al., 2003). DOI has been shown to undergo *O*-demethylation, as well as sulphate and glucuronide conjugation (Ewald et al., 2007). In general the DO- series has been shown to primarily be metabolized via *O*-demethylation as well as hydroxylation (Ewald et al., 2006a; Ewald et al., 2006b; Ewald et al., 2007; Ewald et al., 2008). Metabolism is believed to be catalyzed primarily via CYP2D6 isoenzymes. Although animal pharmacokinetic models are useful, pharmacology in humans has yet to be fully explored. Metabolite drug standards are not commercially available,

posing additional challenges for toxicological detection in the laboratory. An array of phase I and II metabolites have been identified, either tentatively or definitively, but in the absence of commercial reference materials, toxicology laboratories in the United States are unlikely to report them. The absence of metabolite standards means that laboratories must target the parent drug instead. Of the fifteen substances targeted in this study, four of the compounds were still not commercially available (2C-C, 2C-D, 2C-E and DOC) and were instead provided by the DEA Special Testing and Research Laboratory.

The DO-series produce more powerful hallucinogenic effects compared with either the 2C, or 2C-T-series. The methyl group adjacent to the amine is reportedly responsible for the increased potency and duration of action of this group (Maurer, 2010). The DO-series of drugs appear to show affinity for 5-HT_2 receptors and act as agonists and antagonists at different receptor subtypes. The pharmacological activity of the 2C and 2C-T series is attributed to a similar mode of action, although it is less well characterized. Partial agonism of α-adrenergic receptors has been described for 2C-B (Maurer, 2010). The hallucinogenic properties of these drugs is generally attributed to the two carbon spacer that separates the amine form the phenyl ring, methoxy groups at positions 2 and 5, and a hydrophobic substituent in the 4-position (alkyl, thio, alkylthio).

The harm associated with these drugs is not well understood, although it is clear that they are not without risk (Hill and Thomas, 2011). These drugs may be encountered illicitly in a variety of forms, including tablets, capsules, powders (2C and 2C-T series) and LSD-like blotters or liquids for DO-series drugs. Despite the frequency with which they are encountered in controlled substance exhibits, they are infrequently encountered in biological samples submitted for toxicological testing in the United States. Although recreational drug users report a complex array of stimulant and hallucinogenic effects, a relatively small number of fatal and non-fatal reports have been published in the literature (Balíková, 2005; Curtis et al., 2003; Decaestecker et al., 2001; Elliott, 2000; NDIC, 2011). Symptoms associated with these reports include restlessness, vomiting, shaking, sweating, hallucinations, convulsions and coma. The relatively small number of reports is likely due to the challenges associated with their detection in toxicological samples as previously discussed.

Screening of Toxicological Specimens

Not all forensic toxicology laboratories are likely to detect these substances. Common toxicological analysis consists of a presumptive screening test (e.g. immunoassay) and a confirmatory test (e.g. gas chromatography/mass spectrometry or GCMS). In many laboratories, if the immunoassay screening test is negative then additional tests may not be performed. Enzyme-linked immunosorbent assay (ELISA) is a popular screening technique in forensic toxicology laboratories that perform human performance or postmortem investigations because of its heterogeneous nature, making it amenable to a wide variety of toxicological specimens with minimal sample preparation. Commercial ELISAs are widely available for amphetamine-type drugs including methamphetamine, amphetamine and MDMA. The methodology is well established and provides a reliable means by which a laboratory can presumptively identify positive samples for confirmatory testing.

The cross-reactivity of the assay towards other structurally related substances describes in quantitative terms, the ability of the antibody to bind to drugs other than the target analyte. Cross-reactivity is the result of poor antibody specificity. This lack of specificity can be exploited in a beneficial way to develop immunoassays that provide broad spectrum screening, for example when multiple drugs within a class are of interest (e.g. opiates, benzodiazepines). The immunogen structure largely defines antibody specificity. Large carrier proteins must be attached to small drugs to elicit an immune response, and this often results in poor specificity towards the region of the drug where the conjugation took place. For the amphetamine class, immunoassays generally employ antibodies of moderately high specificity (Suttijitpaisal and Ratanabanangkoon, 1992) for good reason. This approach minimizes unwanted cross-reactivity towards over-the-counter drugs (e.g. ephedrine, pseudoephedrine), putrefactive amines in postmortem samples, and other structurally related endogenous substances.

In the absence of immunoassay screening tests specifically directed towards the psychedelic amphetamines, the cross-reactivity of these compounds using commercial immunoassays used in forensic toxicology laboratories must be investigated. A low cross-reactivity towards these substances would indicate that reliance on ELISA, or other immunoassay-based screening methodology, may result in these abused substances not being detected in forensic case samples.

Instrumental Analysis

The DO, 2C and 2C-T series of psychedelic amphetamines are not typically included as part of the regimen of routine toxicology and there is limited data concerning their prevalence in toxicological casework. Existing published instrumental methods are summarized in **Table 4**. Some describe the analysis of non-biological samples (i.e. seized drugs), others use techniques that are not in widespread use in toxicology laboratories (i.e. capillary electrophoresis), and the vast majority target only one, or a limited number of drugs. Gas chromatography/mass spectrometry (GC/MS) is still the most widely used technique for confirmatory toxicology analysis. Most of the published methods to date are limited in their ability to simultaneously target a large number of drugs within the class. Ishida *et al* developed a method for the detection of thirty abused drugs in human urine using GC/MS, including 2C-B, 2C-I, 2C-T-2, 2C-T-7, and 4-MTA (Ishida et al., 2006). However, prior to this study there was no literature that described a comprehensive screening procedure for some of the most common 2C, 2CT and DO-series designer drugs using GC/MS.

Some published methods utilizing GC/MS derivatize these amphetamine-like drugs using acetic anhydride, *n*-butyric anhydride, isobutyric anhydride, heptafluorobutyric anhydride, and pentafluoropropionic anhydride (**Table 4**). Derivatization has many advantages from the standpoint of improved detectability, volatility, specificity and chromatographic separation. However, in this study we found it unnecessary to derivatize the target analytes. Non derivatized drugs can be advantageous if a laboratory is making an identification using a commercial or widely used mass spectral library, particularly if the laboratory conducts full scan screening by GC/MS. The purpose of this study was to establish a simple procedure for the separation and identification of the target drugs, using techniques and instrumentation already widely used in human performance and medical examiner's toxicology laboratories.

Forensic toxicology laboratories in the U.S. do not typically target these recreational drugs during routine testing procedures. Published studies, largely from Europe, have described the use of liquid chromatography methods for the detection of some, but not all of the drugs described here (Apollonio et al., 2007; Bogusz et al., 2000; Decaestecker et al., 2001; Elliott, 2000; Kanai et al., 2008; Kraemer and Maurer, 1998; Peters, 2011; Pichini et al., 2008; Soares

et al., 2004; Vorce and Sklerov, 2004; Wohlfarth et al., 2010). Hyphenated capillary electrophoretic procedures have also been described (Boatto et al., 2005; Nieddu et al., 2007; Tsai et al., 2006), but these techniques enjoy less widespread use in toxicology laboratories. During the final phase of the study, a comprehensive LC/MS/MS procedure was developed for use with blood and urine samples.

Prevalence

Although these drugs are still the subject of ongoing study, it appears clear that their somewhat unique properties are mediated largely by interactions at serotonergic and adrenergic receptors. Many are capable of producing central nervous system effects, euphoria and enhanced visual, auditory, olfactory, or physical sensations similar to LSD; however, reported effects are highly dose dependent (DEA, 2001; Shulgin and Shulgin, 1991). Overdose and death are of concern, and fatal intoxications have been associated with the use of 2C-T-7, 4-MTA, and DOB (Balíková, 2005; Curtis et al., 2003; De Letter et al., 2001; Elliott, 2000; EMCDDA, 2004).

The prevalence of these drugs is poorly understood because of the analytical challenges associated with detection, the low dose of several of the substances, lack of availability of commercial reference materials or standards, limited pharmacological data and lack of commercial availability of metabolites. The majority of reports are associated with fatalities or death investigations, rather than human performance toxicology (i.e. impaired driving). This is not unexpected because although a single dose of a powerful psychostimulant such as DOI (1.5-3 mg) may impair driving, it is very likely to produce a concentration of the parent drug in blood or urine that is undetectable (low ng/mL) during routine toxicological testing. The prevalence of these substances in controlled substance laboratories as seized materials in drug offense investigations is evidence of their use among recreational drug users. However, many have never been reported in toxicological specimens, particularly in the United States.

Table 4. Summary of published instrumental analyses.

Drug(s) of Interest	Matrix	Extraction	Internal Standard	Instrumentation	Derivatization	Reference
4-MTA	NB	None	None	GC/MS, CE/DAD, ATR/FTIR, ^{1}H-NMR	None	(Poortman and Lock, 1999)
4-MTA	Blood Urine	LLE	Fenfluramine; diethylpropion	HPLC/DAD, GC/NPD	None	(Elliott, 2000)
4-MTA	Blood Urine Vitreous Tissue	LLE	Phentermine	LC/MS/MS	None	(Decaestecker et al., 2001)
4-MTA	Blood	LLE	Diphenylamine	GC/MS HPLC/DAD	None	(De Letter et al., 2001)
2C-B	Urine	LLE	None	GC/MS	Isobutyric anhydride	(Kanamori et al., 2002)
2C-T-7	Blood Urine Tissue	LLE	TMA	GC/MS	None	(Curtis et al., 2003)
2C-B	Urine	LLE	2C-T-7	GC/MS	N-butyric anhydride	(Kanamori et al., 2003)
2C-T-2	Urine	LLE	None	GC/MS	None	(Lin et al., 2003)
2C-B, 4-MTA	Urine	SPE	None	HPLC/UV	None	(Soares et al., 2004)
2C-B, 2C-T-7	Blood Urine	SPE	Mescaline-d_9; 5-fluorotryptamine	GC/MS LC/MS	PFPA	(Vorce and Sklerov, 2004)
DOB	Blood Urine	LLE	Brompheniramine	GC/MS	Acetic anhydride	(Balíková, 2005)
2C-B, 2C-I, DOB, DOI, DOM	Urine	SPE	None	CE/MS	None	(Boatto et al., 2005)
4-MTA	Urine	LLE	None	GC/MS	Acetic anhydride	(Ewald et al., 2005)
2C-B, 2C-D, 2C-E, 2C-I, 2C-P, 2C-T-2, 2C-T-7, mescaline	Blood	SPE	AM-d_5; MA-d_5, MDA-d_5; MDMA-d_5; MDEA-d_5;	GC/MS	HFBA	(Habrdova et al., 2005)

			mescaline-d_9			
2C-T-2, 2C-T-7	Urine	LLE	None	GC/MS	Acetic anhydride	(Theobald et al., 2005a; Theobald et al., 2005b)
DOB	Urine	LLE	None	GC/MS	Acetic anhydride	(Ewald et al., 2005)
2C-B, 2C-I, 2C-T-2, 2C-T-7, 4-MTA, Mescaline	Urine	SPE	Medazepam	GC/MS	Acetic anhydride	(Ishida et al., 2006)
2C-I	Urine	LLE	None	GC/MS CE/MS	Acetic anhydride	(Theobald et al., 2006)
2C-B, 2C-I, 2C-T-2, 2C-T-7	Urine	LLE	None	CE/NF, CE/LIF, GC/MS	Fluorescence derivatization	(Tsai et al., 2006)
DOB	NB	None	None	CE/DAD, MS^n, FTIR, GC/MS	None	(da Costa et al., 2007)
DOI	Urine	LLE	None	GC/MS	Acetic anhydride	(Ewald et al., 2007)
DOM, DOET	Urine	SPE	None	CE/MS	None	(Nieddu et al., 2007)
2C-B	Urine	LLE	None	GC/MS	Acetic anhydride	(Theobald et al., 2007)
DOM	Urine	LLE	None	GC/MS	Acetic anhydride	(Ewald et al., 2008)
2C-B	Blood Tissue	SPE	MBDB	GC/MS	Acetic anhydride	(Rohanová et al., 2008)
2C-B, 2C-H, 2C-I, 2C-T-2, 2C-T-7, 4-MTA, DOB, DOET, DOM	Blood	SPE	AM-d_5; MDMA-d_5; MDEA-d_5; cocaine- d_3	LC/MS/MS	None	(Wohlfarth et al., 2010)

NB, non-biological matrix; 2,5-dimethoxy-4-(n)-propylphenethylamine, 2C-P; liquid extraction, LLE; solid phase extraction, SPE; trimethoxyamphetamine, TMA; amphetamine-d_5, AM-d_5; methamphetamine-d_5, MA-d_5; 3,4-methylenedioxyamphetamine-d_5, MDA-d_5; 3,4-methylenedioxymethamphetamine-d_5, MDMA-d_5; 3,4-methylenedioxy-N-ethylamphetamine-d_5, MDEA-d_5; N-methyl-l-(3,4-methylenedioxyphenyl)-2-butanamine, MBDB; gas chromatography/mass spectrometry, GC/MS; capillary electrophoresis/diode-array detection, CE/DAD; attenuated total reflectance/Fourier transform infrared spectroscopy, ATR/FTIR; proton nuclear magnetic resonance, ^{1}H-NMR; high performance liquid chromatography/diode-array detection, HPLC/DAD; gas chromatography/nitrogen-phosphorus detection, GC/NPD; liquid chromatography/tandem mass spectrometry, LC/MS/MS; high performance liquid chromatography/ultraviolet spectrometry, HPLC/UV; liquid chromatography/mass spectrometry, LC/MS; capillary electrophoresis/mass spectrometry, CE/MS; capillary electrophoresis/native fluorescence detection, CE/NF; capillary electrophoresis/light emitting diode (LED)-induced fluorescence detection, CE/LIF; tandem mass spectrometry, MS^n; Fourier transform infrared spectroscopy, FTIR; pentafluoropropioinic anhydride, PFPA; heptafluorobutyric anhydride, HFBA.

Rationale for the Research

The psychedelic phenethylamines described in this study are a series of psychoactive derivatives that produce sought after effects for recreational drug users. Many of these synthetic psychotropics were introduced as recreational drugs in an attempt to bypass controlled substance legislation in the United States. Hallucinogenic phenethylamines were first synthesized by Shulgin (Shulgin and Shulgin, 1991) and later emerged as illicit drugs in Europe and Asia before making an appearance in this country. Although the most widely abused amphetamine in the United States is *d*-methamphetamine, there is still significant interest in new designer amphetamines as the drug scene continues to evolve (Haroz and Greenberg, 2006). These emerging designer drugs include the dimethoxyphenylethanamine (2C, 2C-T) and dimethoxyphenylpropanamine (DO) series of psychedelics, which include 2,5-dimethoxy-4-bromophenethylamine (2C-B), 2,5-dimethoxy-4-chlorophenethylamine (2C-C), 2,5-dimethoxy-4-methylphenethylamine (2C-D), 2,5-dimethoxy-4-ethylphenethylamine (2C-E), 2,5-dimethoxyphenethylamine (2C-H), 2,5-dimethoxy-4-iodophenethylamine (2C-I), 2,5-dimethoxy-4-ethylthiophenethylamine (2C-T-2), 2,5-dimethoxy-4-isopropylthiophenethylamine (2C-T-4), 2,5-dimethoxy-4-propylthiophenethylamine (2C-T-7), 2,5-dimethoxy-4-bromoamphetamine (DOB), 2,5-dimethoxy-4-chloroamphetamine (DOC), 2,5-dimethoxy-4-ethylamphetamine (DOET), 2,5-dimethoxy-4-iodoamphetamine (DOI) and 2,5-dimethoxy-4-methylamphetamine (DOM). 4-Methylthioamphetamine (4-MTA) was also included in the study due to its structural similarity, toxicity and reported use. During the initial phase of the study, eleven psychedelic amphetamines were available for investigation. Additional derivatives were made available from the Drug Enforcement Agency (DEA) Special Testing and Research Laboratory (Dulles, VA). As a consequence, it was possible to include an expanded panel of 15 target analytes in all subsequent work involving confirmatory assays by LC/MS/MS and the analysis of adjudicated forensic case samples.

Methods

Reagents and Chemicals

Mescaline-d_9 (internal standard), N-methyl-l-(3,4-methylenedioxyphenyl)-2-butanamine-d_5 (MBDB-d_5) and 3,4-methylenedioxymethamphetamine–d_5 (MDMA-d_5) were obtained from Cerriliant (Round Rock, TX). The hydrochloride salts 2C-B, 4-MTA, DOET, DOM, 2C-T-2, 2C-T-4, 2C-T-7, 2C-H, 2C-I, and DOB were obtained from Lipomed (Cambridge, MA) in 1 mg/mL solutions. The hydrochloride salt of DOI was obtained as a powder from Sigma-Aldrich (St. Louis, MO). The Drug Enforcement Agency (DEA) Special Testing and Research Laboratory (Dulles, VA) provided solid reference standards for 2C-C, 2C-D, 2C-E and DOC in the latter part of the study. Methanolic stock solutions for all target analytes were routinely prepared at 1 mg/mL (free base) and stored at -10^{o}C prior to use. Diluted working standards used for the preparation of all calibrators and controls were stored under similar conditions. Calibrators were prepared at the appropriate concentration by fortification of drug-free matrix with methanolic working standards.

For the interference study, methanolic standards for amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyethylamphetamine (MDEA), ephedrine, pseudoephedrine, phentermine, phenylpropanolamine, alprazolam, amitriptyline, cocaine, codeine, dextromethorphan, diazepam, hydrocodone, ketamine, meperidine, methadone, nordiazepam, oxycodone, phencyclidine (PCP), tramadol, and zolpidem were obtained from Cerilliant (Round Rock, TX). For the purpose of the cross-reactivity study alone, pure isomers of *d*-amphetamine and *d*-methamphetamine were purchased from Cerilliant. Standards of phenethylamine, putrescine, tryptamine, tyramine were obtained from Sigma Aldrich (St. Louis, MO) and N-methyl-l-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB) was obtained from Lipomed (Cambridge, MA).

Acetic acid, acetonitrile, ethyl acetate, hexane, isopropanol, methanol and methylene chloride were obtained from Mallinkrodt-Baker (Hazelwood, MO). Concentrated ammonium hydroxide and sodium fluoride were obtained from Fisher Scientific (Pittsburgh, PA). Ammonium acetate, ethanol, 1,1,1,3,3,3-hexamethyldisilazane, sodium tungstate dihydrate,

trichloroacetic acid, zinc sulfate heptahydrate and monobasic sodium phosphate were purchased from Sigma-Aldrich (St. Louis, MO). Dibasic sodium phosphate heptahydrate was obtained from VWR International (West Chester, PA). Concentrated hydrochloric acid was obtained from EMD Chemical (Gibbstown, NJ). A Millipore Milli-Q (Billerica, MA) was used to purify all deionized water (DIW) used in this study. All reagents were of ACS or HPLC grade. Acidic methanol consisted of 1% (v/v) concentrated hydrochloric acid in methanol. Drug-free urine preserved with 1% (w/v) sodium fluoride was obtained from human volunteers. Drug-free bovine blood preserved with 0.2% (w/v) potassium oxalate and 1% (w/v) sodium fluoride was purchased from (Quad Five Materials, Ryegate, MT).

Phosphate buffer at a concentration of 0.1M (pH 6) was prepared by adding 0.1 M dibasic sodium phosphate to 0.1 M monobasic sodium phosphate until a pH of 6.0 was reached. Acidic methanol (1%) was prepared by adding concentrated hydrochloric acid to methanol in a ratio of 1:99 (v/v). A 10% sodium tungstate precipitant solution (w/v) and a 10% trichloroacetic acid precipitant solution (w/v) were prepared by making 10:90 solutions in DIW for each. A 0.2 M zinc sulfate precipitant solution was prepared by diluting to 0.2 M in DIW. This solution was then used to prepare a 20:80 solution in methanol. A 10% zinc sulfate solution (w/v) was prepared by preparing a 10:90 solution in DIW. This solution was then used to prepare a 50:50 solution (w/v) in ethanol. Elution solvent was prepared by making a 95:5 methylene chloride: isopropanol solution (v/v). Concentrated ammonium hydroxide solution was added prior to use to achieve a final concentration of 2% (v/v).

Glassware was silanized using Napco Vacuum Oven (Model 6504/6506). The oven was heated to 160°C and a pressure of 15 psi. A gas tight syringe was used to add 100 µL of 1,1,1,3,3,3-hexamethyldisilazane into the injector valve of the oven. After the glassware was silanized for one hour, it was cooled to room temperature before use.

Immunoassay Kits

Nine immunoassay kits were obtained from five established commercial vendors: Immunalysis Amphetamine ELISA, catalog no. 209-0192 and Methamphetamine ELISA, catalog no. 211-0192 (Pomona, CA); International Diagnostic Systems Corp (IDS) MDMA/Methamphetamine One-Step ELISA, catalog no. MA-96 (St. Joseph, MI); Neogen

Amphetamine Forensic (RTU) ELISA Kit, catalog no. 130819, and Methamphetamine/MDMA Forensic (RTU) ELISA Kit, Catalog no. 130919 (Lexington, KY); OraSure Technologies Inc. Micro-Plate Amphetamine Specific EIA, catalog no. 1103ET and Micro-Plate Methamphetamine EIA, catalog no. 1104ET (Bethlehem, PA); Venture Labs Inc. Methamphetamine Assay, catalog no. SA008K and MDMA Assay, catalog no. SA018K (Redwood City, CA). Each kit consisted of 96-well microtiter plates coated with antibody directed towards the target drug(s), enzyme conjugate (3,3'5,5'-tetramethylbenzidine or TMB substrate solution), and stop solution (dilute acid).

GC/MS Instrumentation

GC/MS analysis was performed using an Agilent HP 5975 MSD/6890 GC (Santa Clara, CA) with a DB-5MS (30 m x 0.25 mm x 0.25 µm) capillary column purchased from VWR (West Chester, PA). The injector and interface were both set at 280°C. Injections (2 µL) were made in split mode with a 5:1 split ratio. Ethyl acetate was used as the wash solvent, with a total of six pre-and post injection syringe washes between samples. The oven temperature was held at 130°C for 0.50 min, ramped to 170°C at a rate of 15°C/min with a hold time of 1 min, ramped to 180°C at a rate of 5°C/min with a hold time of 9 min, ramped to 200°C at a rate of 15°C/min and then ramped to 290°C at a rate of 30°C/min with a final hold time of 1 min. The total run time was 20.0 min. Helium was used as the carrier gas at a flow rate of 1.3 mL/min. The MS was operated in the electron impact (EI) ionization mode. The ion source and quadrupole were set at 230°C and 150°C, respectively. Data was acquired using selected ion monitoring (SIM) using quantitation and qualifier ions shown in **Table 5**. Biological extracts were reconstituted in 20 µL of ethyl acetate, and transferred to autosampler vials for GC/MS analysis.

Table 5. Chemical and mass spectral data for target analytes. Quantitation ions are shown in bold and ion ratios for qualifier ions are shown in parentheses.

Drug	Formula	Molecular Weight	Ions *m/z* (Ion Ratio)	Base Peak
2C-H	$C_{10}H_{15}NO_2$	181	**152.1**, 181.1 (19), 137.1 (53)	152
4-MTA	$C_{10}H_{15}NS$	181	**138.0**, 122.0 (31), 44.0 (497)	44
DOM	$C_{12}H_{19}NO_2$	209	**166.1**, 151.1 (35), 44.1 (158)	166

DOET	$C_{13}H_{21}NO_2$	223	**180.1**, 165.1 (32), 91.1 (11)	180
2C-B	$C_{10}H_{14}BrNO_2$	261	**232.0**, 261.0 (11), 216.9 (24)	232
DOB	$C_{11}H_{16}BrNO_2$	274	**232.0**, 216.9 (17), 77.1 (44)	44
2C-I	$C_{10}H_{14}INO_2$	307	**278.0**, 307.0 (14), 262.9 (19)	278
DOI	$C_{11}H_{16}INO_2$	321	**278.0**, 262.9 (12), 77.1 (19)	44
2C-T-2	$C_{12}H_{19}NO_2S$	241	**212.1**, 241.1 (29), 183.1 (39)	212
2C-T-7	$C_{13}H_{21}NO_2S$	255	**226.1**, 255.1 (41), 183.0 (60)	226
Mescaline-d_9, IS	$C_{11}H_8D_9NO_3$	220	**191.0**, 220.0 (22), 173.0 (53)	191

LC/MS/MS Instrumentation

Separation was achieved using a Shimadzu high-performance liquid chromatography (HPLC) system (Columbia, MD) with a Phenomenex Luna 5µm C18 Colum (100 x 2.0 mm) (Torrance, CA) equipped with a guard column (4.0 x 2.0 mm). An API 3200 tandem mass spectrometer from AB Sciex (Foster City, CA) and Analyst 1.4.2 software from Applied Biosystems were used for detection. Gradient elution was necessary for separation of the target analytes. Mobile phase A consisted of 50mM ammonium acetate in DIW/methanol (95:5). Mobile phase B consisted of 50mM ammonium acetate in a mixture of acetonitrile/DIW (90:10). A flow rate of 0.4 mL/min was used in accordance with the following gradient profile: 20% mobile phase B for 0-1 min, increased to 65% by 4 min, held at 65% until 4.5 min, then decreased to 20% by 6 min. Positive electrospray ionization (ESI) and multiple reaction monitoring (MRM) were used throughout. Acquisition parameters and optimized conditions are summarized in **Table 6**. Reconstituted extracts were injected (30 µL) onto the LC/MS/MS using a Shimadzu Sil-20A HT autosampler equipped with two LC-20AT pumps.

Table 6: LC/MS/MS acquisition parameters and optimized conditions. The MRM transitions used for quantitation are shown in bold.

Drug	**m/z Transition**	**Abundance**	**DP**	**EP**	**CEP**	**CE**	**CXP**
mescaline-d_9	**221 → 204**	100%	26	10	16	25	4

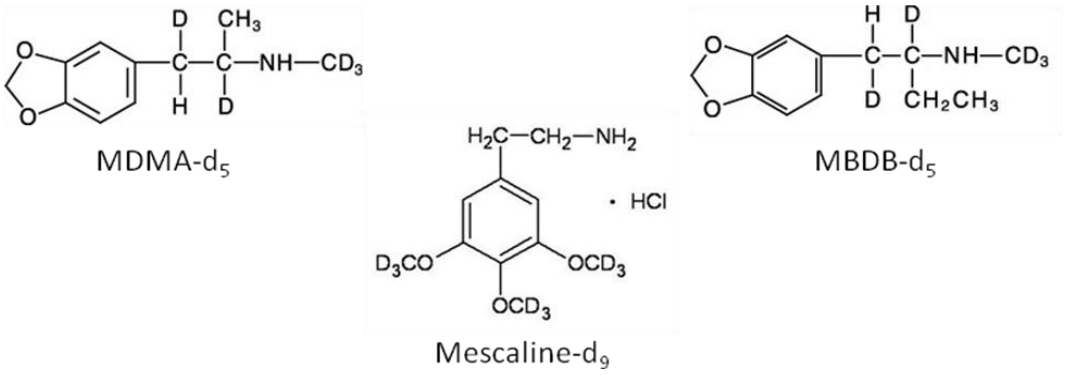
	221 → 178	29%	26	10	16	25	4
2C-H	182 → 165	100%	26	4	16	13	4
	182 → 150	133%	26	4	16	27	4
4-MTA	182 → 165	224%	26	3	16	13	4
	182 → 117	100%	26	3	16	27	4
2C-D	**196 → 179**	100%	21	6.5	15	13	4
	196 → 164	50%	21	6.5	15	25	4
2C-C	**216 → 199**	100%	26	4.5	15	15	4
	216 → 184	53%	26	4.5	15	29	4
2C-B	**262 → 245**	100%	26	3	14	15	6
	262 → 230	49%	26	3	14	27	4
DOM	**210 → 193**	100%	21	4	16	13	4
	210 → 178	38%	21	4	16	27	4
DOC	**230 → 213**	100%	26	7.5	16	13	4
	230 → 185	71%	26	7.5	16	21	4
DOB	**276 → 259**	100%	27	10	18	17	5
	276 → 231	47%	27	10	18	25	5
2C-T-2	**242 → 225**	100%	26	4	18	15	4
	242 → 134	12%	26	4	18	37	4
2C-I	**308 → 291**	100%	26	4.5	20	17	6
	308 → 276	50%	26	4.5	20	25	6
2C-E	**210 → 193**	100%	26	6	16	15	4
	210 → 178	41%	26	6	16	31	4
DOI	**322 → 305**	100%	36	8	18	17	6
	322 → 105	26%	36	8	18	57	4
DOET	**224 → 207**	100%	26	3.5	18	15	4
	224 → 179	37%	26	3.5	18	25	4
2C-T-4	**256 → 239**	100%	26	3.5	14	15	4
	256 → 197	77%	26	3.5	14	27	4
2C-T-7	256 → 239	603%	26	3.5	14	15	4
	256 → 197	100%	26	3.5	14	27	4

Declustering potential (DP); Entrance potential (EP); Cell entrance potential (CEP); Collision energy (CE); Cell exit potential (CXP).

Sample Preparation and Extraction

Methanolic working standards were used to prepare all calibrators and controls. Silanized glassware was used throughout. In the absence of deuterated analogs for each of the target drugs, a number of alternatives were evaluated. These included MDMA-d_5, MBDB-d_5 and mescaline-d_9 (**Figure 2**). From a quantitative standpoint, mescaline-d_9 yielded the most promising results during method development. This was not unexpected based upon its structural similarity (trimethoxy-derivative) to the target compounds (dimethoxy-derivatives). Internal standards used in other published methods are summarized in **Table 4**. In several instances, internal standards are not used, or bear very little structural similarity to the target analytes.

Figure 2. Structures of internal standards considered for quantitative purposes.



Urine

Drugs were extracted from urine by solid phase extraction (SPE) using PolyChrom Clin II mixed-mode polymeric columns from SPEware (Baldwin Park, CA). All extractions were performed using 2 mL of urine. Following the addition of mescaline–d_9 internal standard to achieve a final concentration of 100 ng/mL, 2 mL of pH 6.0 phosphate buffer was added, samples were vortex mixed and transferred to SPE columns. Samples were drawn through the

column under gravity, or sufficient vacuum to maintain continuous flow. Columns were then washed with 1 mL of deionized water, 1 mL of 1M acetic acid and then dried at full vacuum for 5 minutes. Columns were washed with hexane (1 mL), ethyl acetate (1 mL), and methanol (1 mL) in a successive fashion. Target analytes were eluted with 1 mL methylene chloride:isopropanol (95:5, v/v) containing 2% concentrated ammonium hydroxide. Acidic methanol (30 µL) was added to each sample prior to evaporation at 50°C using a TurboVap® II (Caliper Life Sciences, Hopkinton, MA). Extracts were reconstituted in 50 µL of mobile phase A and transferred to autosampler vials for LC/MS/MS analysis. For GC/MS analysis, extracts were reconstituted in 20 µL of ethyl acetate.

Analytical recovery was evaluated to determine the extraction efficiency of the SPE method. Additionally, the effect of "salting out" prior to evaporation was investigated to determine if sample losses could occur due to the volatility of some target drugs in the base (uncharged) form. Acidic methanol was used for this purpose as described above. Analytical recovery was determined by fortifying drug-free urine with target analytes at 100 ng/mL (N=5). The analytical recovery was estimated by comparison of the relative peak areas of target analytes. The extract containing internal standard (IS) alone was fortified with target drugs immediately after the extraction, prior to the evaporation step. Samples were reconstituted and analyzed by LC/MS/MS. The analytical recovery (extraction efficiency) was calculated from the relative peak area (drug/IS) of extracted and non-extracted samples.

Blood

Drugs were extracted from whole blood by solid phase extraction (SPE) using a modification of the procedure developed for urine. All extractions were performed using 1 mL of whole blood. Following the addition of mescaline–d_9 internal standard to achieve a final concentration of 100 ng/mL, calibrators and quality controls were fortified with the appropriate volume of working standard. A variety of sample pretreatment steps and protein precipitants were evaluated, including acetonitrile, methanol, 10% sodium tungstate, 20:80 0.2 M zinc sulfate/methanol, 10% trichloroacetic acid (TCAA), acetone and 50% ethanol in 10% zinc sulfate. Simple dilution in phosphate buffer (0.1 M, pH 6) was also evaluated. During protein precipitation, 2 mL of the appropriate protein precipitation agent was added to the blood with vortex mixing. Acetonitrile was stored in the freezer prior to use. Samples were then centrifuged for 10 minutes at 8,000 rpm. Supernatants were diluted in pH 6.0 phosphate buffer (4 mL) and

added to Cerex PolyChrom Clin II solid-phase extraction (SPE) columns from SPEware (Baldwin Park, CA). The SPE columns were attached to Teflon needle inserts and then a vacuum manifold, both from J.T. Baker (Phillipsburg, VA). Samples were drawn through the columns under gravity or with mild vacuum, as needed. The columns were washed with 1 mL of DIW and then 1 mL of 1 M acetic acid. Columns were dried for 5 minutes at full vacuum and then washed consecutively with 1 mL each of hexane, ethyl acetate and methanol. The designer amphetamines were then eluted with 1 mL of 2% concentrated ammonium hydroxide in 95:5 (v/v) methylene chloride: isopropanol. Acidic methanol (30 µL) was added to each extract after the elution step. The extracts were then evaporated using a Caliper Life Sciences TurboVap® II evaporator (Hopkinton, MA). Dried extracts were reconstituted in 50 µL of mobile phase A and 20 µL of the sample was injected onto the LC/MS/MS.

Immunoassay Cross-Reactivity

Pooled drug-free urine preserved with 1% sodium fluoride was used for the preparation of urine immunoassay calibrators and controls. Drug-free urine was fortified with each of the drugs of interest over a wide range of concentration. Each assay was performed in accordance with the manufacturer's specifications, as depicted in **Table 7.** However, to ensure consistency between assays, all urine calibrators and controls were subjected to the same pre-dilution. All urine samples were diluted 1:20 prior to analysis. Dilution buffer (OraSure Forensic Diluent) was used unless the kit contained its own assay-specific diluent. The dilution factor selected was a compromise, but was most representative of manufacturer recommendations among all of the assays. One limitation of this approach is that the dilution factor selected for this study may not be optimal for every assay, but in order to compare cross-reactivity and dose-response between assays, it was necessary to have some uniformity in the experimental design. ELISAs were performed manually using an 8-channel pipette, Biotek ELx50/8 Microplate Strip Washer (Winooski, VT) and a Dynex Technologies Opsys MR Plate Reader at 450-630 nm (Chantilly, VA). Incubation times and volumes of enzyme conjugate, substrate solution and stop reagent were conducted in accordance with the manufacturer's guidelines (**Table 7**).

Table 7. Experimental conditions for ELISA. Sample volume was uniform for all assays. All other conditions reflect the recommendations of the manufacturer.

Assay	Sample Volume[1] (µL)	Conjugate Volume (µL)	Incubation Time (min)	Wash[2]	Substrate Volume (µL)	Incubation Time (min)	Stop Reagent Volume (µL)
Immunalysis Amphetamine	50	100	60	6 x DIW	100	30	100
Immunalysis Methamphetamine	50	100	60	6 x DIW	100	30	100
IDS Methamphetamine/MDMA	50	100	30	3 x DIW	100	30	100
Neogen Amphetamine	50	100	45	3 x Wash Buffer	100	30	100
Neogen MDMA Methamphetamine	50	100	45	3 x Wash Buffer	100	30	100
OraSure Amphetamine	50	100	30	6 x DIW	100	30	100
OraSure Methamphetamine	50	100	30	6 x DIW	100	30	100
Venture Labs Methamphetamine	50	75	30	10 x Tap water & 1 x Rinse Solution	100	15	50
Venture Labs MDMA	50	75	30	10 x Tap water & 1x Rinse Solution	100	15	50

[1]Sample volume reflects the volume of diluted urine (1:20)
[2]Wash buffers and/or rinse solution provided by the manufacturer.
DIW, Deionized water.

Dose-response curves were generated for each assay. In addition to the drugs of interest (2C-B, 2C-H, 2C-I, 2C-T-2, 2C-T-4, 2C-T-7, (±)-DOB, (±)-DOET, (±)-DOM, (±) DOI and (±)-4-MTA), drug-free urine was also fortified with (+)-amphetamine, (+)-methamphetamine and (±)-MDMA (target drugs for ELISA kits) for comparison purposes. Percent binding was calculated using equation 1, where A was the absorbance of the sample and A_0 was the absorbance of the drug-free urine. Cross-reactivity was calculated using equation 2, whereby C_{200} was the concentration of the drug necessary to produce an absorbance reading equivalent to 200 ng/mL of target drug for each assay (specifically (+)-methamphetamine for methamphetamine assays or (+)-amphetamine for amphetamine assays). For the purpose of this study, 200 ng/mL was chosen as the arbitrary positive cutoff concentration. Although laboratories conducting human performance and postmortem investigations determine and use their own cutoffs, 200 ng/mL was chosen because it is consistent with recommended cutoffs in urine for amphetamines in impaired driving casework (Farrell et al., 2007). One should recognize 200 ng/mL may not correspond with the manufacturer's recommended cutoff concentration. However, in order to compare cross-reactivities uniformly between assays, it was necessary to establish a fixed concentration of target analyte so that the concentration of designer drug necessary to produce a presumptive positive result, could be determined.

$$\%\ Binding = \frac{A}{A_0}\ X\ 100 \qquad \text{Equation 1}$$

$$\%\ Cross\ Reactivity = \frac{200}{C_{200}}\ X\ 100 \qquad \text{Equation 2}$$

Assay Performance by GC/MS

Although quantitative analyses are not routinely performed on urine samples, the procedure was evaluated qualitatively and quantitatively in order to determine overall assay performance. All method development, optimization and validation was performed in urine. The limit of detection (LOD) was defined as the lowest concentration of analyte that met the following criteria: signal-to-noise (S/N) ratio of at least 3:1 for the total ion chromatogram; ion ratios for both qualifiers within acceptable ranges (± 20%); and a retention time within 2% of the expected value. The limit of quantitation (LOQ) was defined as the lowest concentration of analyte that met the following criteria: S/N ratio of at least 10:1 for the total ion chromatogram; ion ratios for both qualifiers within acceptable ranges (± 20%); the retention time within 2% of the expected value; and a calculated concentration within 20% of the expected value. The LOD and LOQ were assessed using urine fortified with target drugs. For the purpose of the LOQ, the urine calibrators and controls were prepared using independently prepared stock solutions.

Accuracy and intra-assay precision were assessed by replicate analysis (N=4) of drug-free urine fortified with target drugs at 50 and 500 ng/mL. Inter-assay precision was also evaluated at 250 ng/mL (N=4). Linear regression analysis was used to determine the limit of linearity of the assay. The limit of linearity was evaluated by adding successively higher calibrators to a calibration curve, and monitoring the change in gradient and correlation coefficient. Linearity was maintained if the R^2 value was at least 0.99, if the gradient changed by 10% or less and the back-calculated concentration of the calibrator was within 20% of the expected value. Carryover was evaluated using drug-free matrix injected immediately after extracts containing high concentrations of target drugs.

Interferences were evaluated using a number of structurally related substances, endogenous bases and other common drugs. For quantitative purposes, an interference was defined as a substance that caused the calculated concentrations of a target drug to deviate from the expected value by more than ± 20%. The potential interference of other abused amphetamine-like drugs was investigated. Negative and positive (250 ng/mL) controls were assayed in the presence of 1 mg/L (1000 ng/mL) of amphetamine-like drugs (amphetamine, methamphetamine, MDA, MDMA, MDEA, MBDB, ephedrine, pseudoephedrine, phentermine, phenylpropanolamine); endogenous bases (phenethylamine, putrescine, tryptamine, tyramine);

and common basic drugs, including dextromethorphan, zolpidem, ketamine, diphenhydramine, cocaine, amitriptyline, diazepam, nordiazepam, oxycodone, hydrocodone, alprazolam, phencyclidine (PCP), methadone, tramadol and codeine.

Assay Performance by LC/MS/MS

The limit of quantitation (LOQ) and limit of detection (LOD) were determined empirically by analyzing successively lower concentrations. Drug free blood or urine was fortified with target analyte to determine the lowest concentration that met the following criteria. For LOD, relative retention time within 2% of the expected value; signal-to-noise ratio of at least 3:1; ion ratios within 25% of expected value. For LOQ, relative retention time within 2% of the expected value; signal-to-noise ratio of at least 10:1; ion ratios within 25% of expected value and calculated concentrations of independently fortified controls within 20% of the expected values. Intra-assay precision and accuracy were evaluated at 50 and 250 ng/mL by replicate analysis (N=4). Inter-assay precision was evaluated at 100 ng/mL in urine and blood over 4 and 7 days, respectively. Due to the anticipated low concentration of target analytes expected in actual casework, accuracy was further evaluated at much lower concentrations (between 0.5 to 5 ng/mL).

Linearity was evaluated using regression analysis. The limit of linearity was evaluated by adding successively higher calibrators to a calibration curve, and monitoring the change in gradient and correlation coefficient. Linearity was evaluated up to a concentration of 2000 ng/mL and was maintained if the R^2 value was at least 0.99, if the gradient changed by 10% or less and the back-calculated concentration of the calibrator was within 20% of the expected value. Matrix effect was evaluated qualitatively and quantitatively using two well-established approaches: post-column infusion and post-extraction addition. During method development and during the preliminary stages of validation, ion suppression or enhancement was evaluated using a T-connector to allow post-column infusion of drugs. Target drugs were infused post-column, while drug-free urine extracts from different individuals (N=20) were injected on the LC/MS/MS. This approach allowed potential matrix interferences to be identified qualitatively over the entire chromatographic run. Urine samples varied in age from 1 to three years and were stored refrigerated prior to use. Matrix effect was also assessed quantitatively using post-extraction addition. Target analytes were fortified into drug-free urine extracts from different volunteers (N=20). The transition ion abundance for analytes fortified into matrix (*A*) was compared quantitatively with target analytes fortified into non-matrix, specifically mobile phase

(*B*). The percent matrix effect (ME) was calculated numerically using *% ME = (A-B)/B*. Matrix effects using whole blood were assessed in a similar fashion. Whole blood samples of variable age and quality (up to 3 years) were used (N=20), containing either sodium fluoride/potassium oxalate i.e. grey top tubes or EDTA i.e. lavender top tubes, commonly encountered in forensic toxicology laboratories.

Potential interferences from other amphetamine-like drugs, endogenous bases and common basic drugs were also investigated. Interferences were evaluated qualitatively and quantitatively using both negative and positive controls. Drug-free and positive controls containing 0 and 100 ng/mL of each target analyte, respectively were fortified with potential interferences at a concentration of 1000 ng/mL. The common amphetamine-like drugs included in the study were amphetamine, methamphetamine, MDA, MDMA, MDEA, MBDB, ephedrine, pseudoephedrine, and phentermine. Endogenous bases included phenethylamine, putrescine, tryptamine, and tyramine. Finally, the following commonly encountered drugs were also investigated: alprazolam, amitriptyline, cocaine, codeine, dextromethorphan, diazepam, hydrocodone, ketamine, meperidine, methadone, nordiazepam, oxycodone, phencyclidine, tramadol and zolpidem.

All LC/MS/MS method development, optimization and validation was performed using blood and urine. Additionally, at the time of the LC/MS/MS study, additional drugs that were not commercially available were made available by the DEA for research purposes. This allowed a total of fifteen target analytes to be determined in an expanded assay.

Casework Samples

The presence of 4-MTA, 2C, 2C-T and DO-series drugs was determined in a population of adjudicated casework samples that were due for destruction. Urine samples (N=2,021) obtained from individuals apprehended for suspicion of being under the influence of a controlled substance (California Health and Safety Code Section 11550) were provided by the California Department of Justice Toxicology Laboratory, Bureau of Forensic Services (Sacramento, CA). The study was subject to Institutional Review Board (IRB) approval by the Protection of Human Subjects Committee of Sam Houston State University.

Results and Discussion

Immunoassay

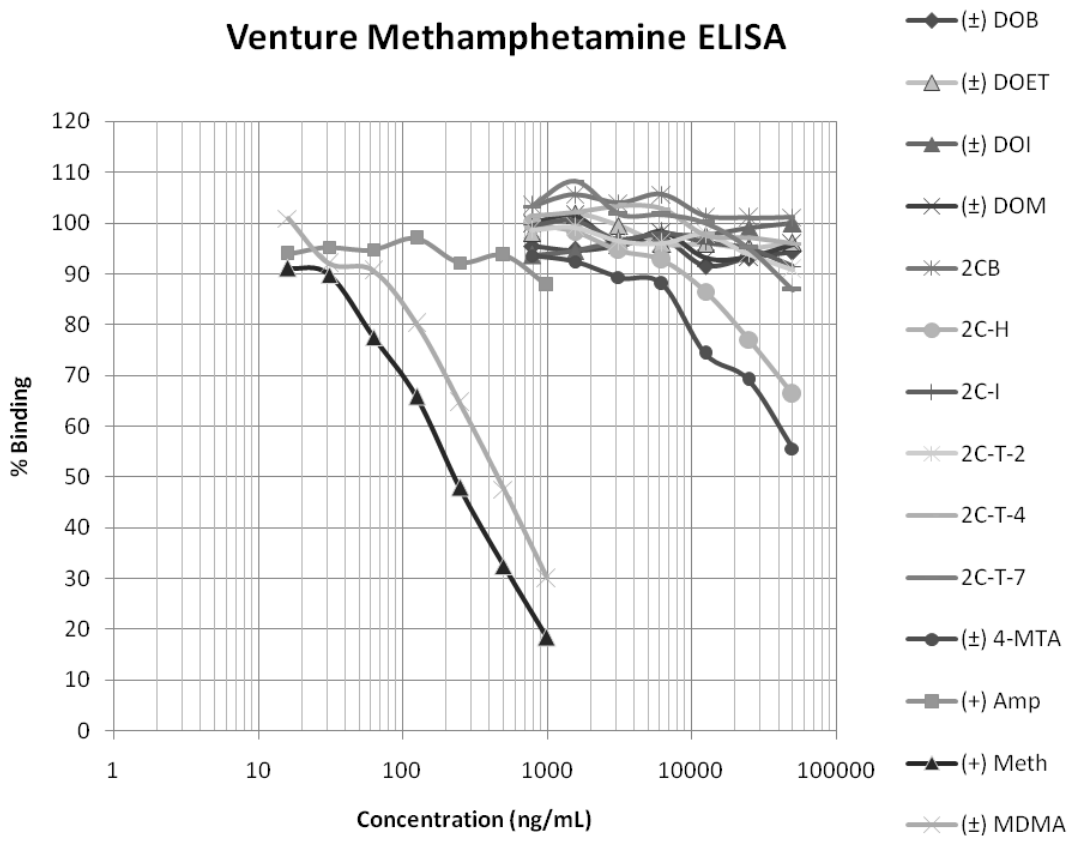
Cross-reactivity data calculated from dose-response curves for each ELISA are summarized in **Tables 8 and 9** for methamphetamine and amphetamine assays, respectively. Two representative data sets are depicted in **Figure 3**. Dose-response curves for an ELISA directed towards methamphetamine (**Figure 3A,** Venture Methamphetamine), and amphetamine (**Figure 3B,** Immunalysis Amphetamine) are shown. Cross-reactivities were <0.4% for all ten 2C, 2C-T and DO-series drugs using commercial amphetamine, methamphetamine or MDMA assays. Concentrations of 50,000 ng/mL were not sufficient to produce a positive response. This clearly demonstrates that although these kits are extremely effective for the target drugs for which they were intended (methamphetamine, MDMA, amphetamine), they cannot be used to reliably identify these designer drugs, even at concentrations that would greatly exceed those expected in case samples.

Clinical data is sparse, but in one reported overdose attributed to 2C-T-7, urinary concentrations of the drug were 1,120 ng/mL (Curtis et al., 2003), well below the threshold necessary to produce a positive immunoassay result. In another report of a 40-year old male suffering from acute psychosis caused by 2C-T4 (Miyajima et al., 2008) a point of care immunoassay (Triage®) failed to produce a positive result. Since many of the DO-series of drugs are generally administered in much lower doses (1-3 mg) compared with the 2C or 2C-T-series, which are more on the order of 10-30 mg (Shulgin and Shulgin, 1991), one would expect even lower urinary drug concentrations following consumption. An earlier report described the problems associated with the detection of amphetamine analogs (including DOB and DOM) by fluorescence polarization immunoassay due to very low cross-reactivity (Cody and Schwarzhoff, 1993). There is limited data on urinary drug concentration following the use of 2C, 2C-T or DO-series drugs in humans. However, animal studies have shown that these drugs are extensively metabolized in rats via hydroxylation, oxidation, deamination, reduction, *O*-demethylation, acetylation, *S*-depropylation and conjugation (Apollonio et al., 2007; Ewald et al., 2006b; Ewald et al., 2007; Ewald et al., 2005; Ewald et al., 2008; Kanamori et al., 2002; Kanamori et al., 2003; Lin et al., 2003; Rohanová et al., 2008; Theobald et al., 2005a; Theobald et al.; Theobald et al., 2006; Theobald et al., 2005b). The low cross-reactivity of the parent drug in an immunoassay is

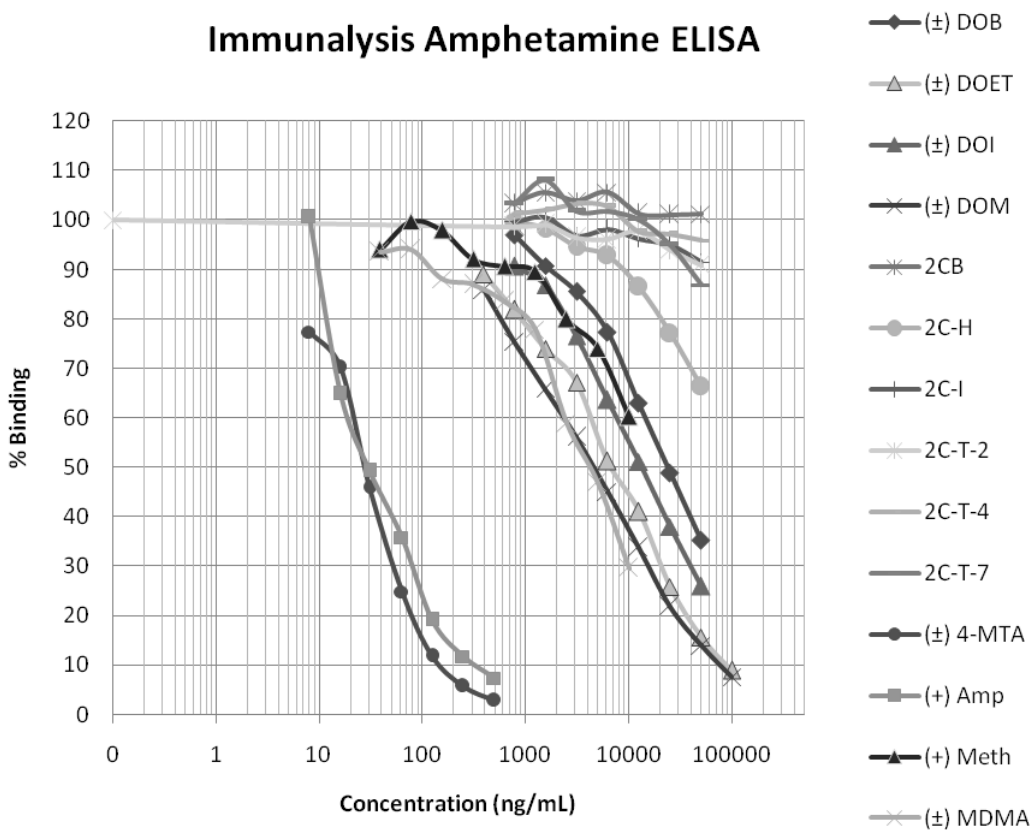
compounded by the unknown cross-reactivities of the metabolites, and the absence of commercial reference standards for these substances.

Figure 3. Representative dose-response curves showing cross-reactivity of a commercial methamphetamine ELISA (A) and an amphetamine ELISA (B) towards drugs of interest. Cross-reactivity was calculated from the response equivalent to 200 ng/mL of target analyte (either *d*-amphetamine or *d*-methamphetamine) in addition to the EC50 (the effective concentration for 50% binding). Data for all nine commercial immunoassays are summarized in **Tables 8 and 9**.

A



B



4-Methylthioamphetamine (4-MTA) was the only substance to cross-react to any significant extent. Commercial methamphetamine assays showed negligible or low cross-reactivities of 5% (OraSure, IDS and Neogen) and 7% (Immunalysis). ELISAs directed towards amphetamine were more reactive towards 4-MTA, providing cross-reactivities of 25% (OraSure) and 200% (Immunalysis). This was not unexpected, given the structural similarity of 4-MTA to the target analyte (**Figure 1**) and the absence of dimethoxy substituents at positions 2 and 5, which differentiates it from all the other substances under investigation. Our results are consistent with an earlier publication using the Bio-Quant Direct ELISA, which reported 4-MTA cross-reactivities of 5% and 280% using their methamphetamine and amphetamine assays, respectively (Apollonio et al., 2007). The decreased antibody specificity with respect to this region of the molecule, suggests that the immunogen used to develop antibodies used in both the Immunalysis and Bio-Quant assays was conjugated in the 4-position.

Limitations

In order to compare assays side-by-side, it should be noted that the sample dilution (1:20) and volume was uniform in all assays, and this may not have been optimal for every kit. Dose-response curves using the target analyte for the kit demonstrated that all assays performed well for their intended purpose, and allowed cross-reactivities to be measured relative to the target analyte (methamphetamine or amphetamine). None of the immunoassays evaluated were developed with the intent or purpose of detecting designer drugs such as those described here, so any reactivity towards these drugs would have been serendipitous, rather than by design. Since cross-reactivity depends on the specificity and properties of the antibody reagent that is used in the kit, this data may be variable over time, particularly if the vendor replaces antibody reagents. Finally, the absence of commercial standards for the many metabolites of these drugs, prevents the cross-reactivity of the metabolites from being determined. Until these are available, analytical methodology in forensic case samples will focus principally on detection of the parent drug in biological matrices.

Table 8. Methamphetamine/MDMA assay cross-reactivity towards designer drugs.

Drug	Immunalysis Methamphetamine			OraSure Methamphetamine			Venture Labs Methamphetamine		
	C_{200}	Cross-Reactivity (%)	EC 50 (ng/mL)	C_{200}	Cross-Reactivity (%)	EC 50 (ng/mL)	C_{200}	Cross-Reactivity (%)	EC 50 (ng/mL)
(+)-AMP									
(+)-METH	**200**	**100**	**10**	**200**	**100**	**180**	**200**	**100**	**350**
(±)-MDMA									
(±)-4-MTA	3000	7	500	4000	5	3800	>50,000	<0.4	>50,000
(±)-DOB	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
(±)-DOET	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
(±)-DOI	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
(±)-DOM	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
2C-B	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
2C-H	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
2C-I	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
2C-T-2	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
2C-T-4	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
2C-T-7	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
Drug	**IDS Methamphetamine/MDMA**			**Neogen Methamphetamine/MDMA**			**Venture Labs MDMA**		
	C_{200}	Cross-Reactivity (%)	EC 50 (ng/mL)	C_{200}	Cross-Reactivity (%)	EC 50 (ng/mL)	C_{200}	Cross-Reactivity (%)	EC 50 (ng/mL)
(+)-AMP									
(+)-METH	**200**	**100**	**15**	**200**	**100**	**125**			
(±)-MDMA							**200**	**100**	**270**
(±)-4-MTA	4000	5	500	4,000	5	2,000	>50,000	<0.4	>50,000
(±)-DOB	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
(±)-DOET	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
(±)-DOI	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
(±)-DOM	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
2C-B	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
2C-H	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
2C-I	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
2C-T-2	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
2C-T-4	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
2C-T-7	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000

C_{200} – Concentration of drug that produces an absorbance reading equivalent to 200 ng/mL of target analyte (methamphetamine).

Table 9. Amphetamine assay cross-reactivity towards designer drugs.

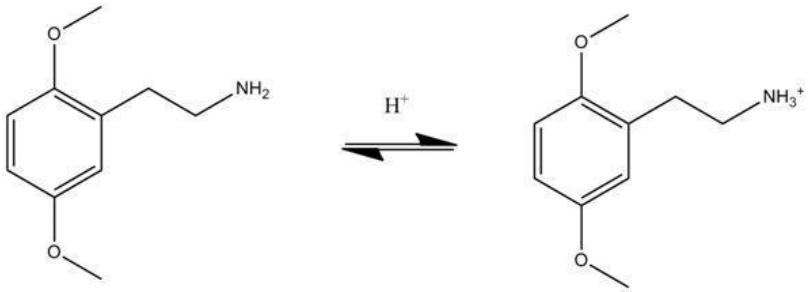
Drug	Neogen Amphetamine			Immunalysis Amphetamine			OraSure Amphetamine		
	C_{200}	Cross-Reactivity (%)	EC 50 (ng/mL)	C_{200}	Cross-Reactivity (%)	EC 50 (ng/mL)	C_{200}	Cross-Reactivity (%)	EC 50 (ng/mL)
(+)-AMP	**200**	**100**	**80**	**200**	**100**	**25**	**200**	**100**	**170**
(+)-METH									
(±)-MDMA									
(±)-4-MTA	>50,000	<0.4	>50,000	100	200	30	800	25	700
(±)-DOB	40,000	0.5	>50,000	>50,000	<0.4	25,000	>50,000	<0.4	>50,000
(±)-DOET	>50,000	<0.4	>50,000	50,000	0.4	7,000	>50,000	<0.4	>50,000
(±)-DOI	50,000	0.4	>50,000	>50,000	<0.4	12,500	>50,000	<0.4	>50,000
(±)-DOM	>50,000	<0.4	>50,000	50,000	0.4	5,000	>50,000	<0.4	>50,000
2C-B	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
2C-H	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
2C-I	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
2C-T-2	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
2C-T-4	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000
2C-T-7	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000	>50,000	<0.4	>50,000

C_{200} – Concentration of drug that produces an absorbance reading equivalent to 200 ng/mL of target analyte (amphetamine).

Sample Preparation and Extraction

Each of the psychedelic amphetamines included in this study can exist in an uncharged or charged form as shown in **Figure 4**. This must be considered when determining optimum conditions for extraction. Using fully optimized conditions, analytical recoveries for each of the target drugs (N=5) were 64-93% in urine and 60-91% in whole blood (**Table 10**). During the method development stage it was necessary to increase the polarity of the elution solvent in order to optimize extraction efficiency. An elution solvent consisting of 2% ammonium hydroxide in 95:5 (v/v) methylene chloride:isopropyl alcohol was selected for this purpose. The influence of evaporative losses was also evaluated. Overall, the addition of acidic methanol to extracts prior to evaporation significantly improved the recovery of target analytes (**Figure 5**). The abundance of drugs evaporated in the base form (uncharged) were significantly lower (19-75%), relative to those treated with acidic methanol prior to evaporation (**Table 11**). T-tests (p=0.05) were used to show that these results were significant. Not surprisingly, the evaporative losses also introduced significant imprecision, with CVs in the range 12-31% compared to 2-9% when acidic methanol was used. Consequently, all extracts were "salted out" using acidic methanol prior to evaporation for the remainder of the study. The susceptibility to evaporative losses (particularly the marked decrease with 4-MTA) is not unexpected given the similarity of the target analytes to other amphetamines that are known to be volatile. Evaporative losses might have been minimized using a lower temperature (<50^{o}C), but since this method was being developed to screen a large number of samples in a high throughput setting, the addition of acidic methanol was preferable to extending assay time.

Figure 4. Base and salt form of a representative designer amphetamine, 2C-H.



Base form (uncharged) Salt form (charged)

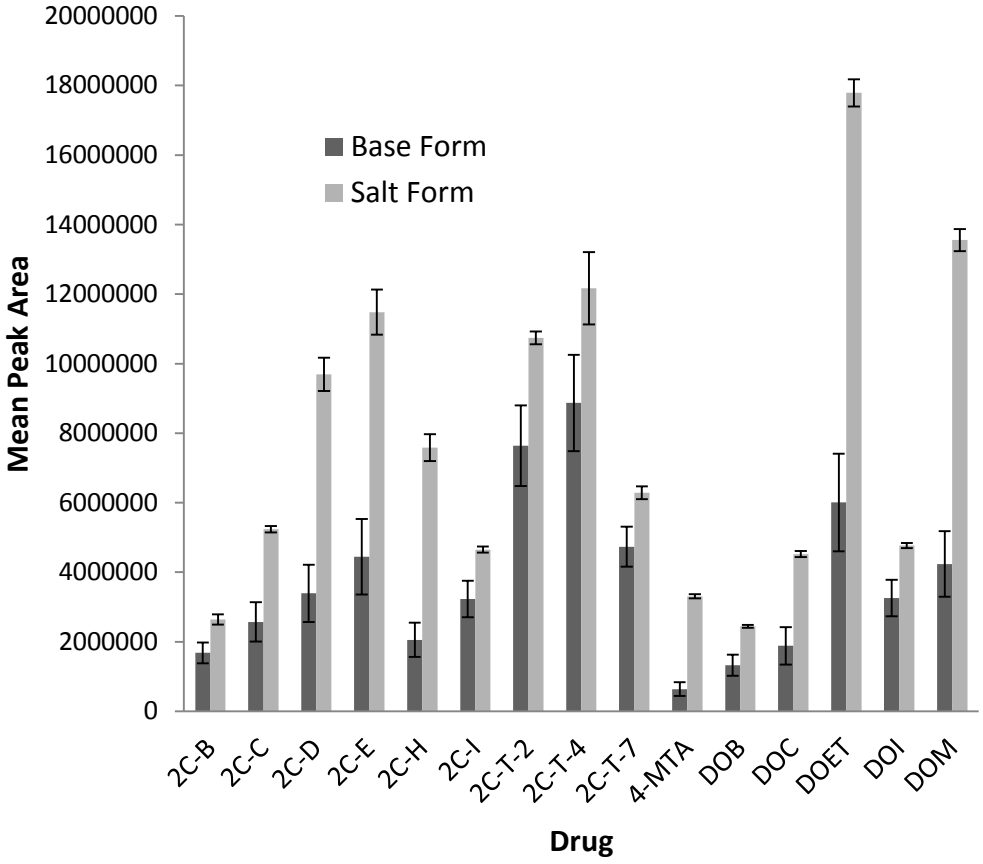
Table 10. Analytical recovery of target analytes from urine samples.

Drug	Recovery from Urine Mean ± SD (N=5)	Recovery from Blood Mean ± SD (N=5)
2C-B	87 ± 4 %	72 ± 5 %
2C-C	89 ± 4 %	73 ± 4 %
2C-D	86 ± 4 %	66 ± 3 %
2C-E	80 ± 4 %	73 ± 6 %
2C-H	80 ± 5 %	60 ± 4 %
2C-I	87 ± 5 %	72 ± 4 %
2C-T-2	64 ± 4 %	75 ± 6 %
2C-T-4	75 ± 5 %	74 ± 3 %
2C-T-7	66 ± 3 %	78 ± 6 %
4-MTA	75 ± 3 %	91 ± 8%
DOB	92 ± 6%	83 ± 5%
DOC	93 ± 3 %	85 ± 6%
DOET	90 ± 2 %	81 ± 3%
DOI	86 ± 1 %	81 ± 6 %
DOM	84 ± 4 %	86 ± 4 %

Table 11. Effect of pH on evaporative losses.

Drug	Salt Form		Base Form	
	Mean Relative Peak Area	%CV (N=10)	Mean Relative Peak Area	CV (N=10)
2C-B	100	6%	64	18%
2C-C	100	2%	49	22%
2C-D	100	5%	35	24%
2C-E	100	6%	39	24%
2C-H	100	5%	27	24%
2C-I	100	2%	69	16%
2C-T-2	100	2%	71	15%
2C-T-4	100	9%	73	16%
2C-T-7	100	3%	75	12%
4-MTA	100	2%	19	31%
DOB	100	2%	54	23%
DOC	100	2%	42	29%
DOET	100	2%	34	23%
DOI	100	2%	68	16%
DOM	100	2%	31	22%

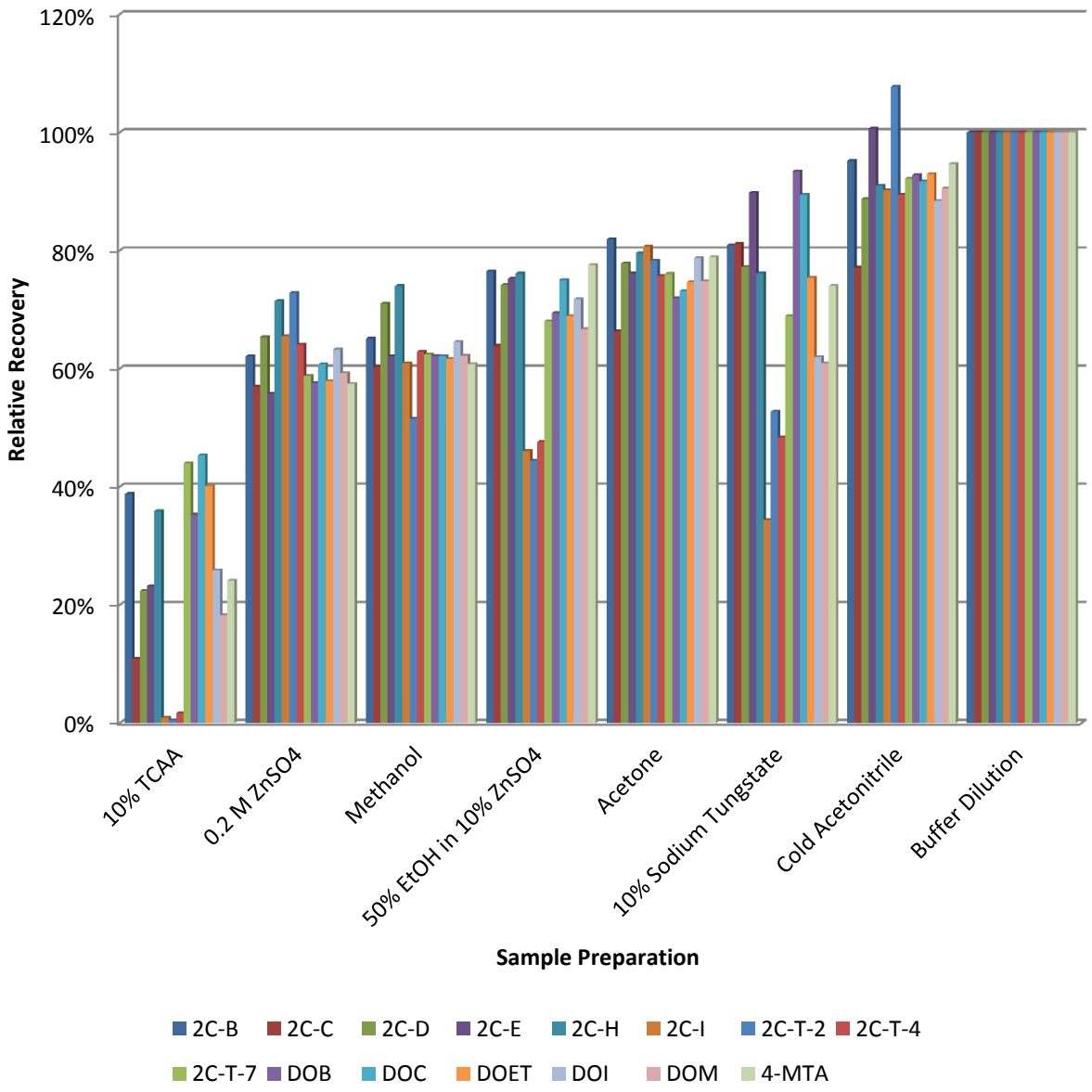
Figure 5. Effect of "salting out" target drugs with acidic methanol prior to evaporation. Data shows the mean peak area (N=10) and 1 SD (error bars).



Optimization for Blood

In order to optimize the procedure for whole blood, eight sample preparation techniques were evaluated. Simple dilution with 0.1 M phosphate buffer (pH 6) was compared with protein precipitation using cold acetonitrile, methanol, 0.2 M zinc sulfate with methanol, 10% sodium tungstate, 10% trichloroacetic acid (TCAA), acetone, and 50% ethanol in 0.2 M zinc sulfate. For clarity, drug recoveries relative to phosphate buffer dilution are summarized in **Figure 6**. Sample preparation was also evaluated qualitatively in terms of cleanliness of the extract, coextractive peaks and interferences.

Figure 6. Comparison of sample preparation for whole blood.



Dilution in phosphate buffer and acetonitrile were the most promising and were further investigated using replicate analysis (N=5) at 100 ng/mL. Analytical recovery and a preliminary assessment of matrix effect were evaluated. Both sample preparation techniques provided acceptable results in terms of analytical recovery (**Figure 7**) and matrix effect (**Table 12**). Five whole blood samples were used for the preliminary assessment of matrix effect using the post extraction addition technique described earlier. Both sample preparation techniques were highly

comparable, but dilution in buffer was selected as the method of choice based on recovery, ease of use and analysis time. Sonication and centrifugation at different speeds (4,000 – 8,000 rpm) were also investigated during method development, but had little benefit. Optimum sample preparation for whole blood involved a simple dilution of 1mL blood with 2 mL 0.1M pH 6.0 phosphate buffer. Diluted blood samples were added to SPE columns and extracted as described earlier.

Figure 7. Analytical recovery of target drugs from whole blood using acetonitrile precipitation and dilution in phosphate buffer. Error bars represent 1SD of replicate measurements (N=5).

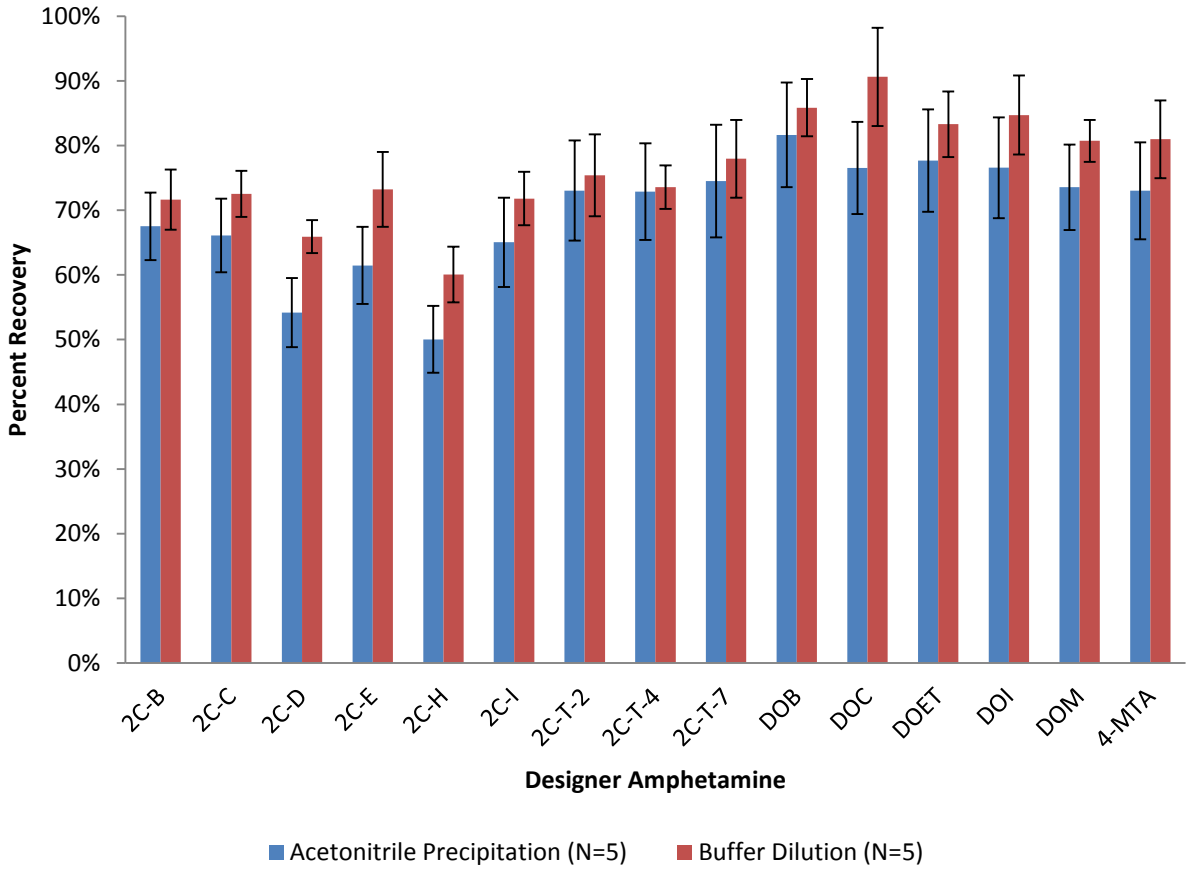


Table 12. Preliminary evaluation of matrix effect using post-extraction addition. Data represents the mean of replicate measurements (N=5).

Drug	Acetonitrile Precipitation Matrix Effect (%)	Buffer Dilution Matrix Effect (%)
2C-B	1%	1%
2C-C	0%	-4%
2C-D	0%	-2%
2C-E	0%	-4%
2C-H	-1%	-6%
2C-I	-1%	-4%
2C-T-2	-4%	-9%
2C-T-4	-3%	-5%
2C-T-7	-13%	-18%
4-MTA	1%	0%
DOB	2%	-12%
DOC	4%	2%
DOET	4%	2%
DOI	-1%	-4%
DOM	3%	3%

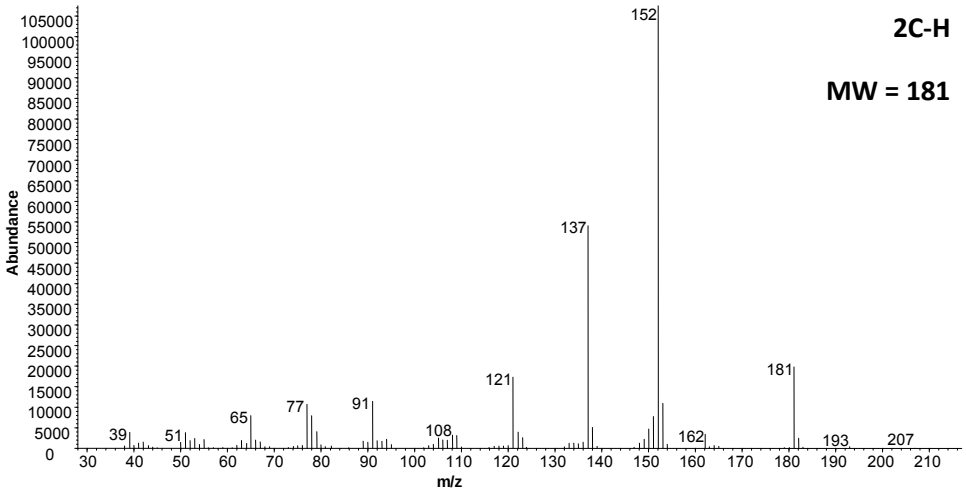
GC/MS

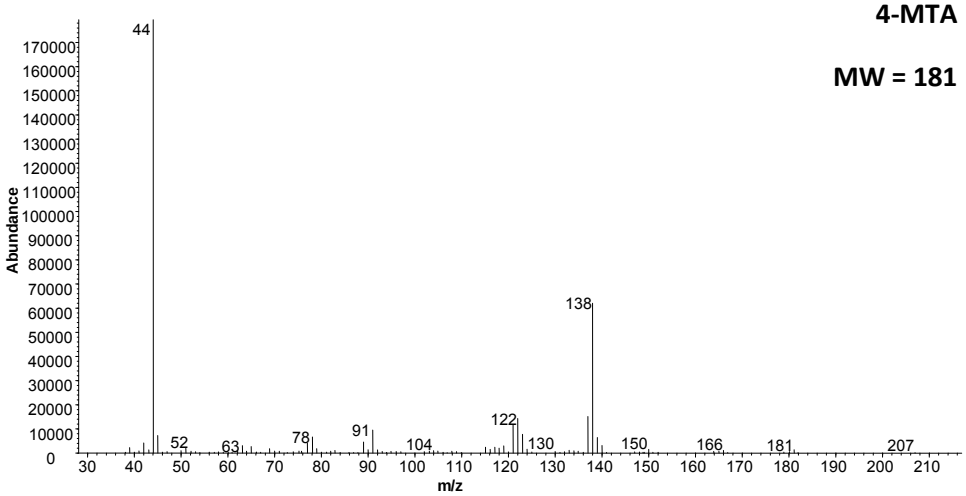
Mass Spectrometric Identification and Chromatographic Separation

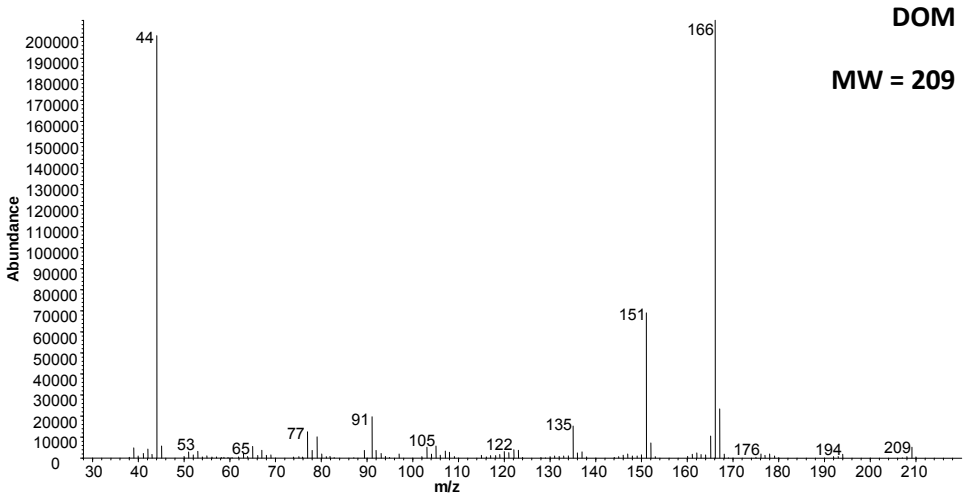
Full scan mass spectra for the target drugs are shown in **Figure 8**. Full scan spectra were used to identify ions for selected ion monitoring based upon specificity and sensitivity. Chemical derivatization was briefly explored, but found to be unnecessary. The most challenging drugs to separate and identify were the following pairs of structurally related compounds: 2C-B and DOB; 2C-I and DOI. Despite the structural similarity, chromatographic and spectroscopic resolution was achieved for all ten target drugs following optimization of flow rates, inlet conditions and oven temperatures. One limitation of the GC/MS assay was the inability to separate 2C-T2 and 2C-T4 chromatographically. Due to the mass spectral similarity and presence of identical characteristic ions, it was not possible to analyze these drugs

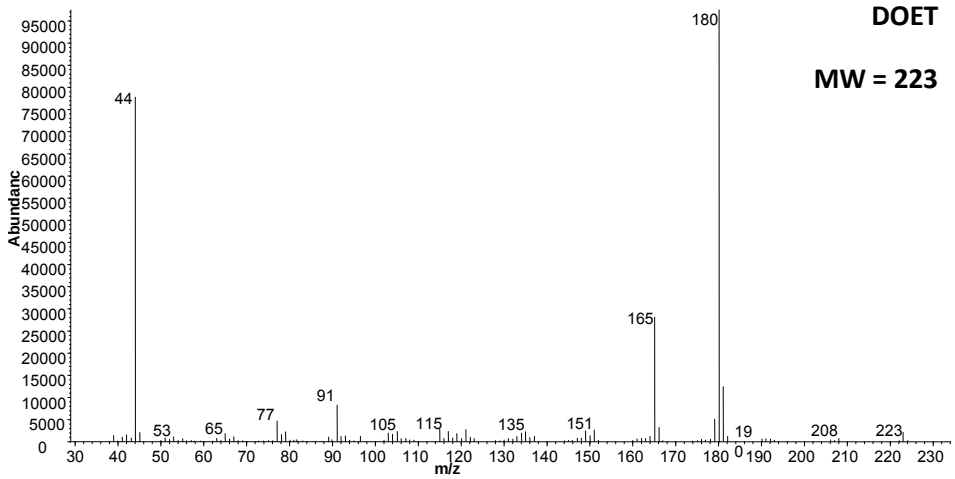
simultaneously. As a result, 2C-T-2 was selected as the preferred analyte due to its more widespread reported use relative to 2C-T-4 (DEA, 2004f). Representative urine extracts containing 10 and 100 ng/mL of each drug are depicted in **Figure 9** and extracted ion chromatograms are shown in **Figure 10**.

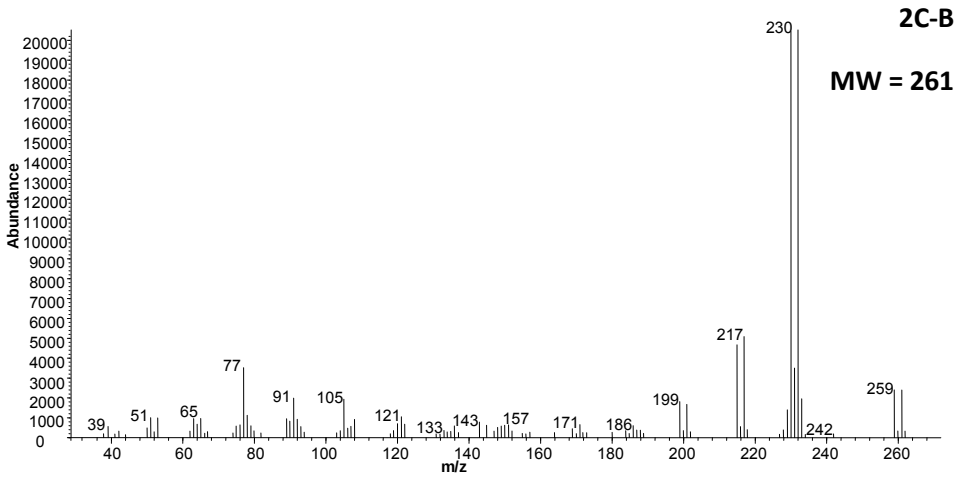
Figure 8. Full scan EI mass spectra for target analytes by GC/MS.





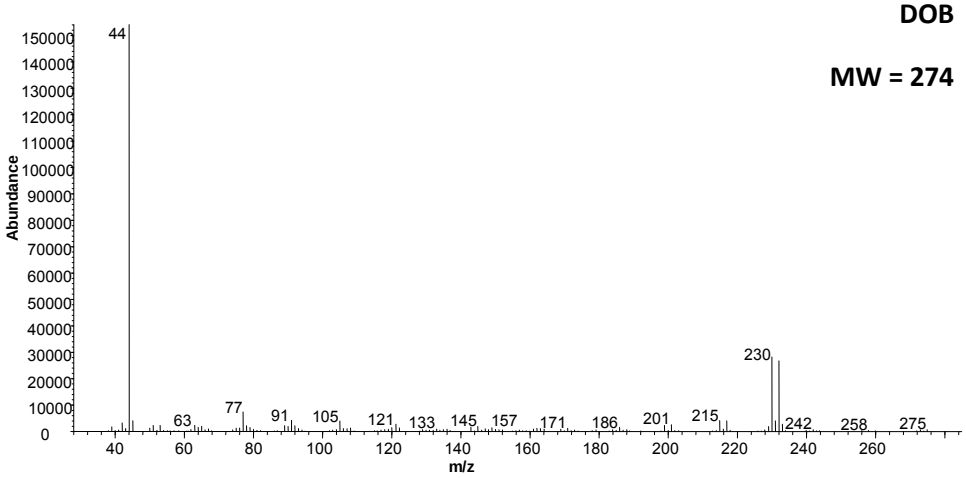






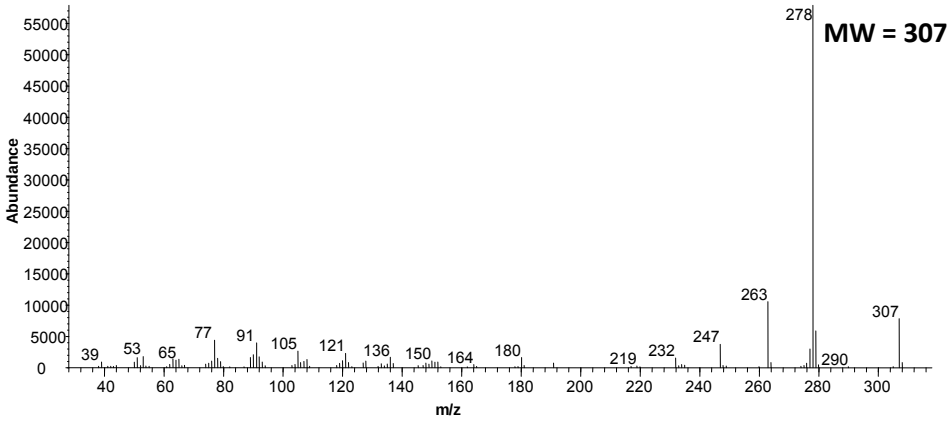
DOB

MW = 274



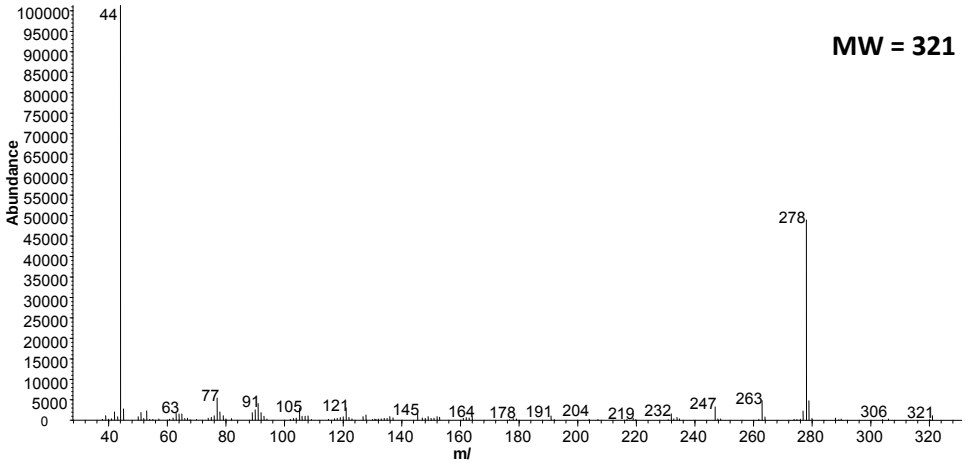
2C-I

MW = 307



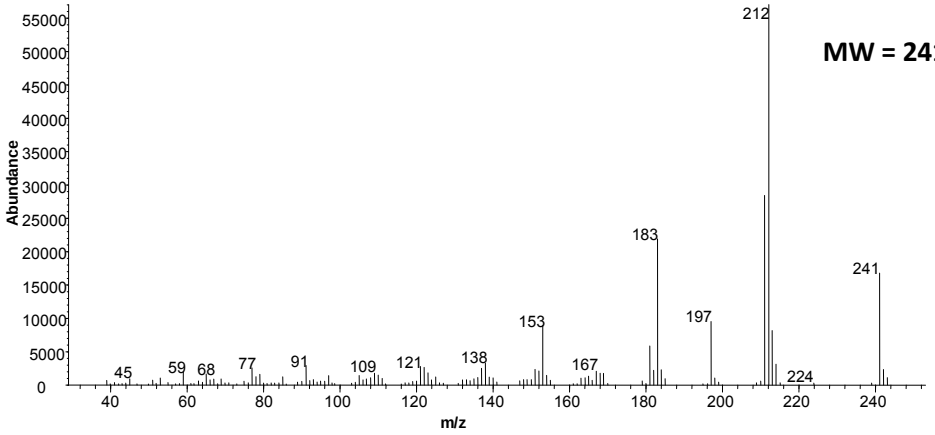
DOI

MW = 321



2C-T-2

MW = 241



2C-T-7

MW = 255

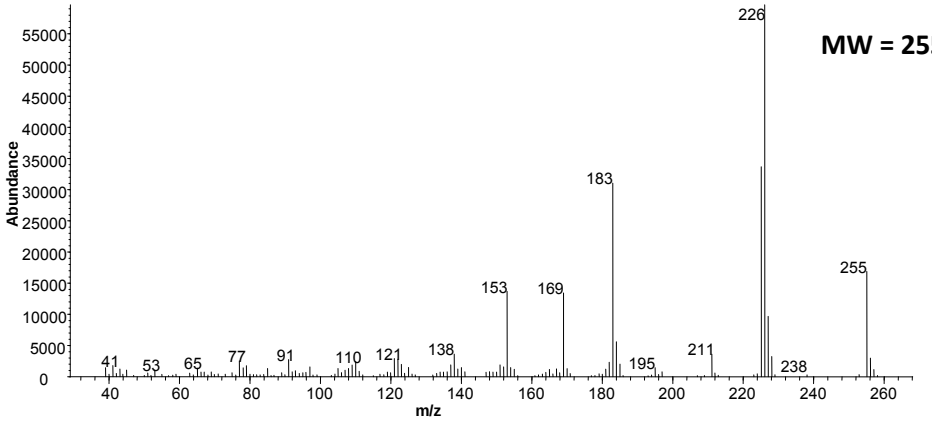


Figure 9. Total ion chromatograms of target drugs in urine at 10 ng/mL (A) and 100 ng/mL (B). All extracts contain 250 ng/mL of mescaline-d_9 (internal standard).

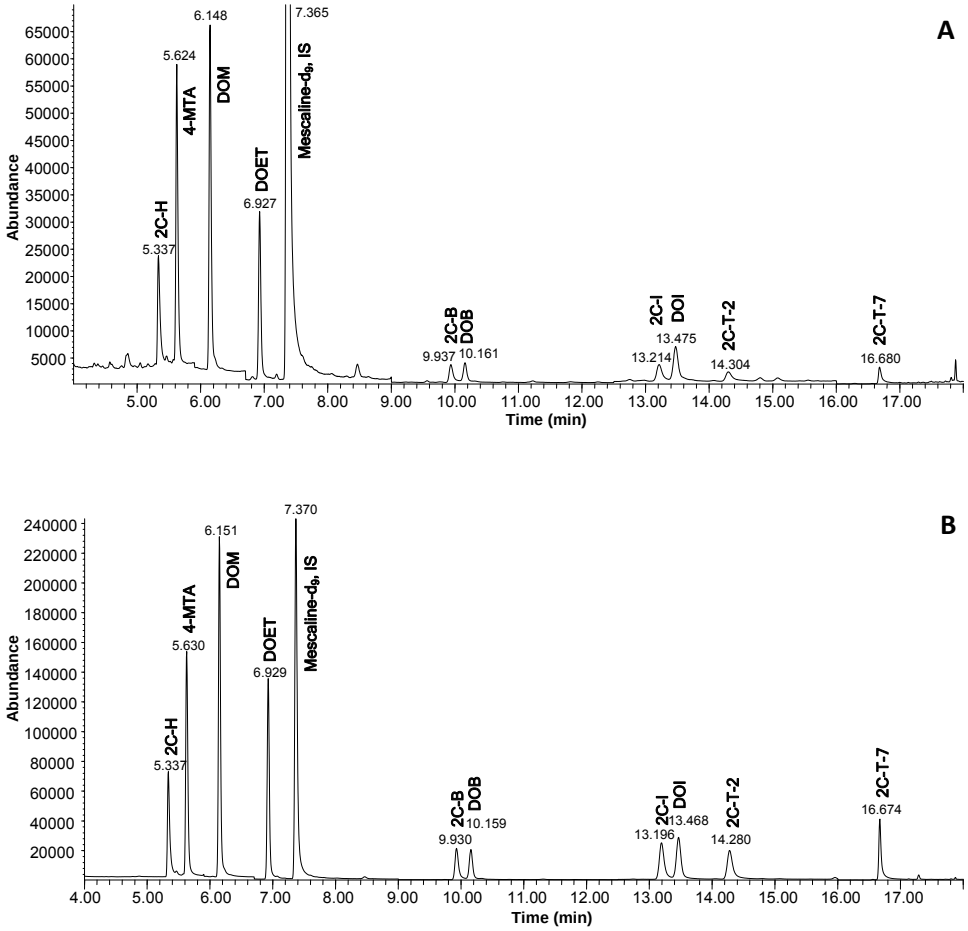
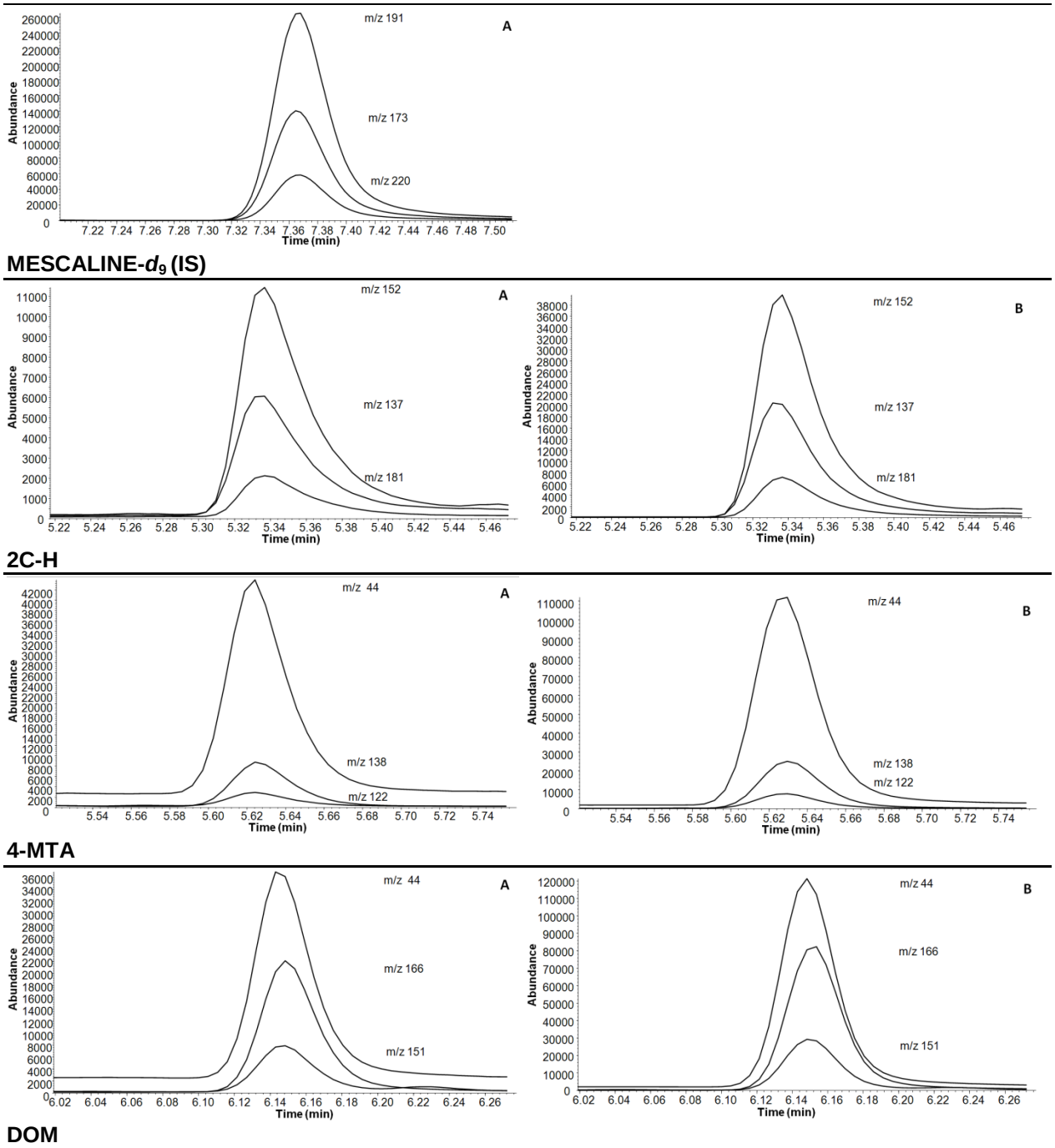
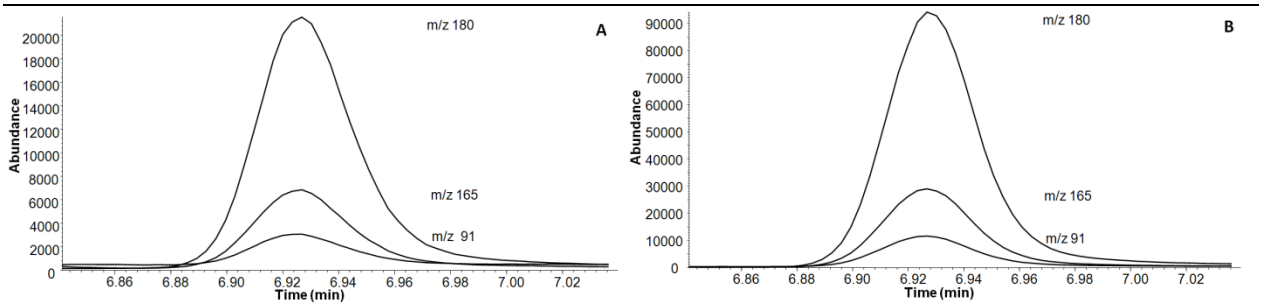
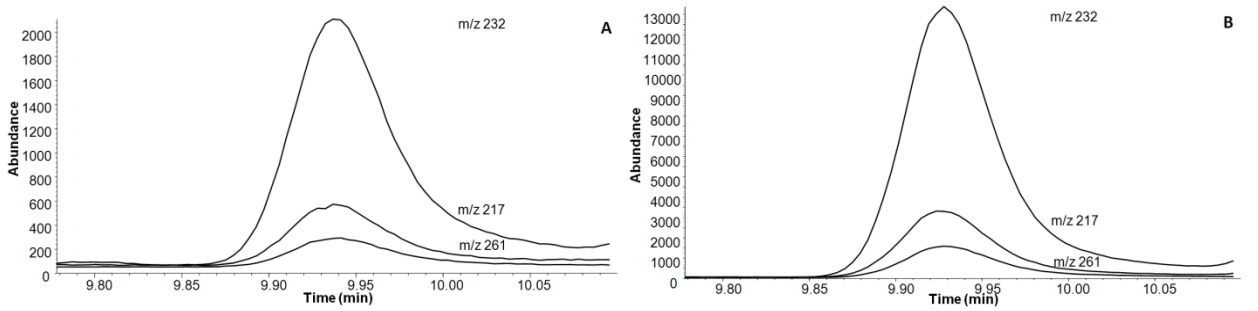


Figure 10. Extracted ion chromatograms for target analytes and internal standard in urine at 10 ng/mL (A) and 100 ng/mL (B). Internal standard (mescaline-d_9) was present at 250 ng/mL.

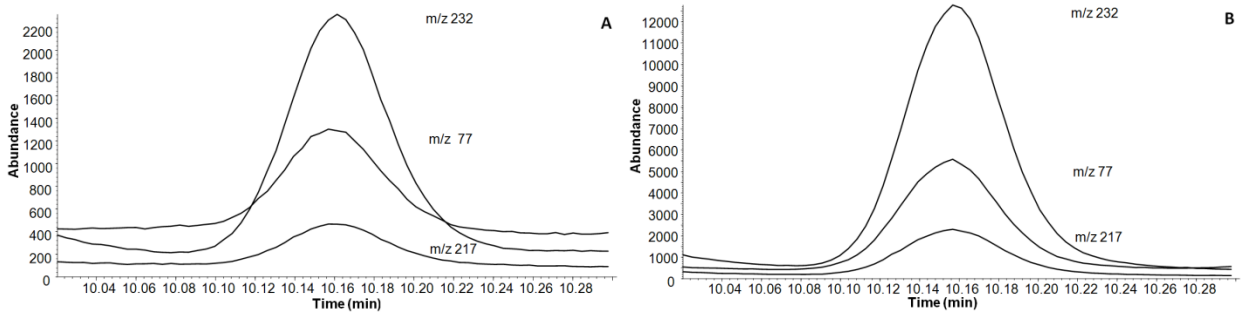




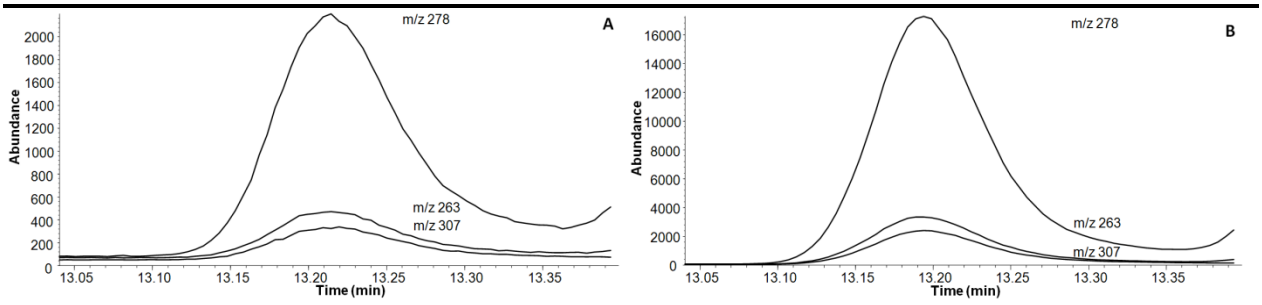
DOET



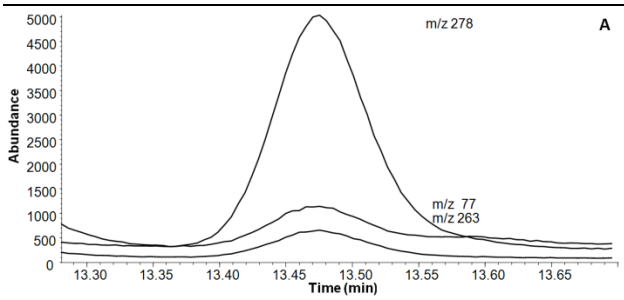
2C-B

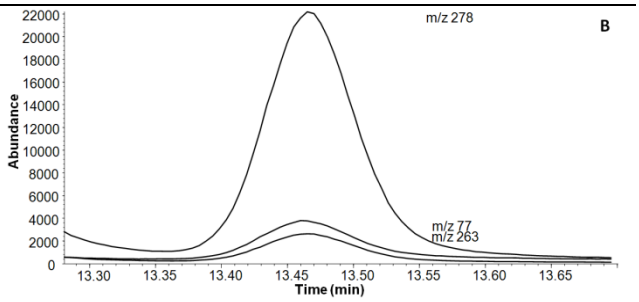


DOB

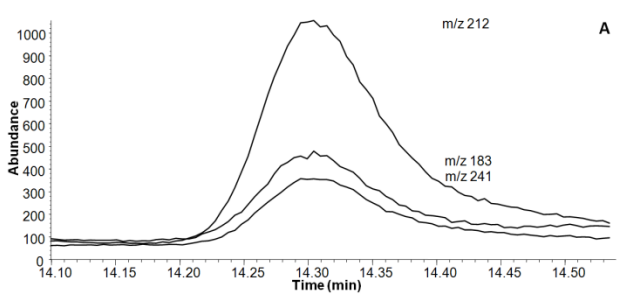


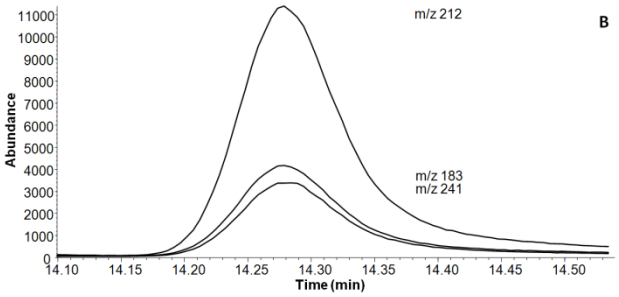
2C-I



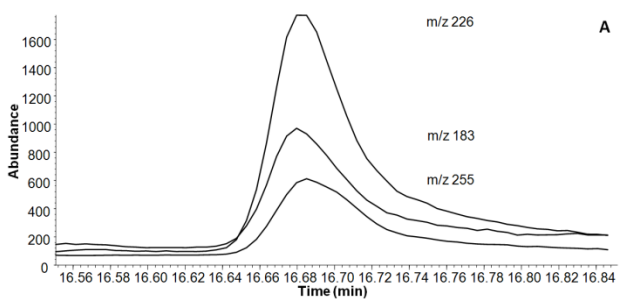


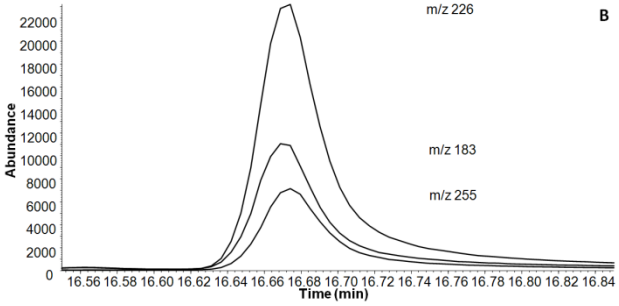
DOI





2C-T-2





2C-T-7

Limits of Detection, Quantitation and Linearity

Limits of detection ranged from 2 - 10 ng/mL and limits of quantitation (LOQ) were 10 ng/mL or less for all analytes (**Table 13**). Ion ratio acceptance was the most common liming factor when evaluating either LOD or LOQ. Calculated concentrations for controls run at the quantitation limits are also shown in **Table 14.** The corresponding signal to noise ratios for the total ion chromatogram (TIC) and acquired ions are depicted in **Table 15**. Low limits of detection are preferable for this class of drug because of the limited pharmacological data in humans and the absence of metabolites from commercial sources.

Table 13. Limit of detection (LOD), limit of quantitation (LOQ), limit of linearity (LOL) and correlation coefficients (R^2).

Drug	Retention Time (min)[1]	LOD (ng/mL)	LOQ (ng/mL)	LOL (ng/mL)	R^2
2C-H	5.3	10	10	1,500	0.997
4-MTA	5.6	2	10	1,500	0.994
DOM	6.1	2	5	1,500	0.995
DOET	6.9	2	2	1,500	0.993
Mescaline-d_9	7.3	-	-	-	-
2C-B	9.9	2	5	1,500	0.997
DOB	10.1	2	2	1,500	0.992
2C-I	13.1	2	5	1,500	0.990
DOI	13.4	2	10	1,500	0.988
2C-T-2	14.2	5	10	1,500	0.990
2C-T-7	16.7	5	10	1,500	0.993

[1]Absolute retention time variable with column length.

Table 14. Calculated concentration at the LOQ.

Drug	LOQ (ng/mL)	Calculated Concentration at the LOQ (ng/mL)	Accuracy (%)
2C-H	10	11.1	111
4-MTA	10	9.9	99
DOM	5	5.8	116
DOET	2	2.1	110
2C-B	5	4.8	96
DOB	2	1.9	95
2C-I	5	5.1	102
DOI	10	10.8	108
2C-T-2	10	8.6	86
2C-T-7	10	8.1	81

Table 15. Signal to noise (S/N) ratios and calculated concentrations at the limit of quantitation. S/N ratios were evaluated for the total ion chromatogram (TIC) and for each acquired ion.

Drug	m/z	S/N Ratio
2C-H	152	79:1
	181	110:1
	137	35:1
	TIC	**10:1**
4-MTA	138	103:1
	122	13:1
	44	20:1
	TIC	**31:1**
DOM	166	570:1
	151	151:1
	44	20:1
	TIC	**34:1**
DOET	180	430:1
	165	103:1
	91	18:1
	TIC	**119:1**
2C-B	232	212:1
	261	122:1
	217	24:1
	TIC	**52:1**
DOB	232	398:1
	217	19:1
	77	18:1
	TIC	**63:1**
2C-I	278	100:1
	307	84:1
	263	16:1
	TIC	**32:1**
DOI	278	184:1
	263	21:1
	77	15:1
	TIC	**39:1**
2C-T-2	212	67:1
	241	76:1
	183	10:1
	TIC	**10:1**
2C-T-7	226	49:1
	255	46:1
	183	10:1
	TIC	**17:1**

Precision and Accuracy

Precision and accuracy data are summarized in **Table 16**. Accuracy was 91-116% and 98-109% at 50 and 500 ng/mL. Corresponding intra-assay CVs were 0.9-6.5% and 0.4-5.6%, respectively. Inter-assay CVs for all analytes at 250 ng/mL in urine were in the range 2.5-9.4%. No carryover was evident following injection of an extract containing 5000 ng/mL of target drugs. During method development, a comparison of silanized and non-silanized glassware indicated the former to be preferable. This suggests that some of the methoxylated species may have a tendency to adsorb to the surface of glass.

Table 16. Intra-assay precision and accuracy at 50 ng/mL (A) and 500 ng/mL (B). Inter-assay precision at 250 ng/mL (C).

(A)

Drug	Calculated Concentration (ng/mL) Mean ± SD (N=4)	Accuracy (%)	%CV (N=4)
2C-B	47.4 ± 0.4	95	0.9
2C-H	46.2 ± 0.5	93	1.2
2C-I	47.7 ± 0.5	95	1.1
2C-T-2	48.0 ± 3.1	96	6.5
2C-T-7	57.8 ± 0.9	116	1.6
4-MTA	50.8 ± 4.0	102	7.9
DOB	46.3 ± 1.5	93	3.1
DOET	49.2 ± 1.3	98	2.5
DOI	45.4 ± 1.3	91	2.9
DOM	50.4 ± 1.3	101	2.6

(B)

Drug	Calculated Concentration (ng/mL) Mean ± SD (N=4)	Accuracy (%)	%CV (N=4)
2C-B	524.2 ± 2.3	105	0.4
2C-H	494.2 ± 20.3	99	4.1
2C-I	522.5 ± 8.4	105	1.6
2C-T-2	518.4 ± 15.7	104	3.0
2C-T-7	545.4 ± 30.7	109	5.6
4-MTA	488.3 ± 26.5	98	5.4
DOB	502.1 ± 7.4	100	1.5
DOET	498.7 ± 9.7	100	1.9
DOI	508.6 ± 5.3	102	1.0
DOM	499.4 ± 14.5	100	2.9

(C)

Drug	Calculated Concentration (ng/mL) Mean ± SD (N=4)	Accuracy (%)	Inter-assay CV (%) (N=4)
2C-B	247.0 ± 7.1	99	2.9
2C-H	235.2 ± 10.2	94	4.3
2C-I	265.1 ± 17.8	106	6.7
2C-T-2	246.9 ± 7.7	99	3.1
2C-T-7	261.9 ± 23.3	105	8.9
4-MTA	249.5 ± 6.4	100	2.5
DOB	269.6 ± 19.1	108	7.1
DOET	255.8 ± 13.8	102	5.4
DOI	279.5 ± 26.2	112	9.4
DOM	246.9 ± 8.2	99	3.3

Interferences

Interferences were evaluated qualitatively and quantitatively using negative and positive controls fortified with potential interferents. None of the amphetamine-like drugs (amphetamine, methamphetamine, MDA, MDMA, MDEA, MBDB, ephedrine, pseudoephedrine, phentermine, and phenylpropanolamine) or endogenous bases (phenethylamine, putrescine, tryptamine, and tyramine) interfered with the assay. With the exception of MDMA, all amphetamine-like and endogenous bases eluted prior to data acquisition (solvent delay 5 mins). Negative controls remained blank and quantitative controls containing target drugs at 250 ng/mL produced calculated concentrations within 82-104% of expected values for stimulants, and 81-116% for endogenous amines. In the presence of other common drugs, both 2C-I and 2C-T-7 quantitated just outside of the acceptable (± 20%) range (79% for both). Although this appeared marginal, negative controls were always drug-free, indicating the absence of interfering ions from these species. Although the quantitative discrepancy was very small, it was reproducible. There was no obvious source of the possible interference because none of the common drugs coeluted with the target analytes with the exception of 2C-I and phencyclidine at relative retention times of 1.80 and 1.79, respectively (**Table 17**).

Table 17. Relative retention times of target analytes and other drugs (in order of elution). All measurements are made relative to the internal standard, mescaline-d_9.

Drug	Relative Retention Time (Min)
MDMA	0.71
2C-H	0.72
4-MTA	0.76
MDEA	0.78
DOM	0.83
MBDB	0.86
DOET	0.94
Mescaline-d_9	**1.00**
Tryptamine	1.13
2C-B	1.35
DOB	1.38
Ketamine	1.60
Diphenhydramine	1.66
PCP	1.79
2C-I	1.80
DOI	1.84
2C-T-2	1.95
Tramadol	2.07
2C-T-7	2.23
Methadone	2.41
Dextromethorphan	2.42
Amitriptyline	2.47
Cocaine	2.47
Codeine	2.62
Diazepam	2.66
Hydrocodone	2.67
Nordiazepam	2.72
Oxycodone	2.73
Zolpidem	2.98
Alprazolam	2.82

LC/MS/MS

Separation and Optimization

Initial optimization of target analytes by positive electrospray ionization was achieved by post column infusion and final acquisition parameters were described earlier (**Table 6**). During the initial development and optimization of chromatographic separation, optimization of the

gradient elution profile was necessary to reduce ion suppression of the internal standard (mescaline-d_9).

Detection and Quantitation

With the exception of 2C-B, the limit of detection in urine was 0.5 ng/mL for all analytes (**Table 18**). The limiting factor was ion ratio stability at sub-ng/mL concentrations, rather than signal to noise ratio or retention time. However, an evaluation of ion ratio stability over a wide range of concentrations (0.5 – 50 ng/mL) indicated highly favorable results (± 25%) and the reproducibility of ion ratios (%CV) was <10%. 2C-B was the only exception, with a limit of detection of 1 ng/mL. For all target analytes except 2C-B, the criteria for the LOQ were also met at 0.5 ng/mL in urine (1 ng/mL for 2C-B). However, the limit of quantitation was arbitrarily set at 1 ng/mL in urine for all target analytes. Signal to noise ratios and accuracy for all target drugs at the LOQ are summarized in **Table 18**. MRM transitions for a representative group of target analytes from the 2C, 2C-T, DO and 4-MTA are shown in **Figure 11.** The limit of linearity was reached at 500 ng/mL and calibration curves were routinely run in the 0 – 300 ng/mL range. In this range, R^2 values of at least 0.99 were achieved for all drugs.

Following optimization of sample preparation for whole blood samples, limits of detection and quantitation in blood were comparable to urine (**Table 18**). With the exception of 2C-B, 2C-C and 2C-T-4, all designer amphetamines had a limit of detection of 0.5 ng/mL; 1.0 ng/mL for 2C-B, 2C-C and 2C-T-4. Limits of quantitation for all drugs were in the range 0.5 – 2.0 ng/mL. Low limits of quantitation and detection are important because of the low dosage of many of these drugs, particularly the DO-series (**Table 3**). MRM transitions for all fifteen target analytes in blood at 2 ng/mL are depicted in **Figure 12.**

Table 18. Limits of detection and quantitation in urine and blood.

Urine						
Drug	LOD		LOQ			
	LOD (ng/mL)	S/N Ratio	LOQ (ng/mL)	S/N Ratio	Calculated Concentration (ng/mL)	Accuracy (%)
2C-B	1.0	20:1	1.0	43:1	0.98	98
2C-C	0.5	115:1	1.0	282:1	0.97	97
2C-D	0.5	246:1	1.0	444:1	0.99	99
2C-E	0.5	256:1	1.0	464:1	1.06	106
2C-H	0.5	138:1	1.0	268:1	0.96	96
2C-I	0.5	55:1	1.0	187:1	0.94	94
2C-T-2	0.5	221:1	1.0	557:1	1.00	100
2C-T-4	0.5	67:1	1.0	164:1	1.10	110
2C-T-7	0.5	25:1	1.0	66:1	1.00	100
4-MTA	0.5	179:1	1.0	356:1	1.04	104
DOB	0.5	37:1	1.0	64:1	1.01	101
DOC	0.5	113:1	1.0	193:1	0.97	97
DOET	0.5	639:1	1.0	1290:1	1.02	102
DOI	0.5	17:1	1.0	27:1	1.07	107
DOM	0.5	463:1	1.0	719:1	1.06	106
Blood						
Drug	LOD		LOQ			
	LOD (ng/mL)	S/N Ratio	LOQ (ng/mL)	S/N Ratio	Calculated Concentration (ng/mL)	Accuracy (%)
2C-B	1.0	80:1	1.0	80:1	1.2	119
2C-C	1.0	44:1	1.0	44:1	1.2	118
2C-D	0.5	86:1	0.5	86:1	0.5	95
2C-E	0.5	154:1	0.5	154:1	0.5	99
2C-H	0.5	44:1	1.0	113:1	0.9	87
2C-I	0.5	35:1	1.0	69:1	1.2	116
2C-T-2	0.5	86:1	2.0	221:1	2.3	118
2C-T-4	1.0	37:1	2.0	79:1	2.4	112
2C-T-7	0.5	46:1	2.0	132:1	2.2	100
4-MTA	0.5	54:1	1.0	119:1	1.2	119
DOB	0.5	57:1	1.0	67:1	1.2	115
DOC	0.5	37:1	1.0	70:1	1.2	115
DOET	0.5	211:1	1.0	478:1	1.2	115
DOI	0.5	13:1	0.5	13:1	0.4	83
DOM	0.5	157:1	0.5	157:1	0.5	104

Figure 11. MRM transitions of representative drugs at the LOD in urine: 2C-B, 4-MTA, DOI and 2C-T-7.

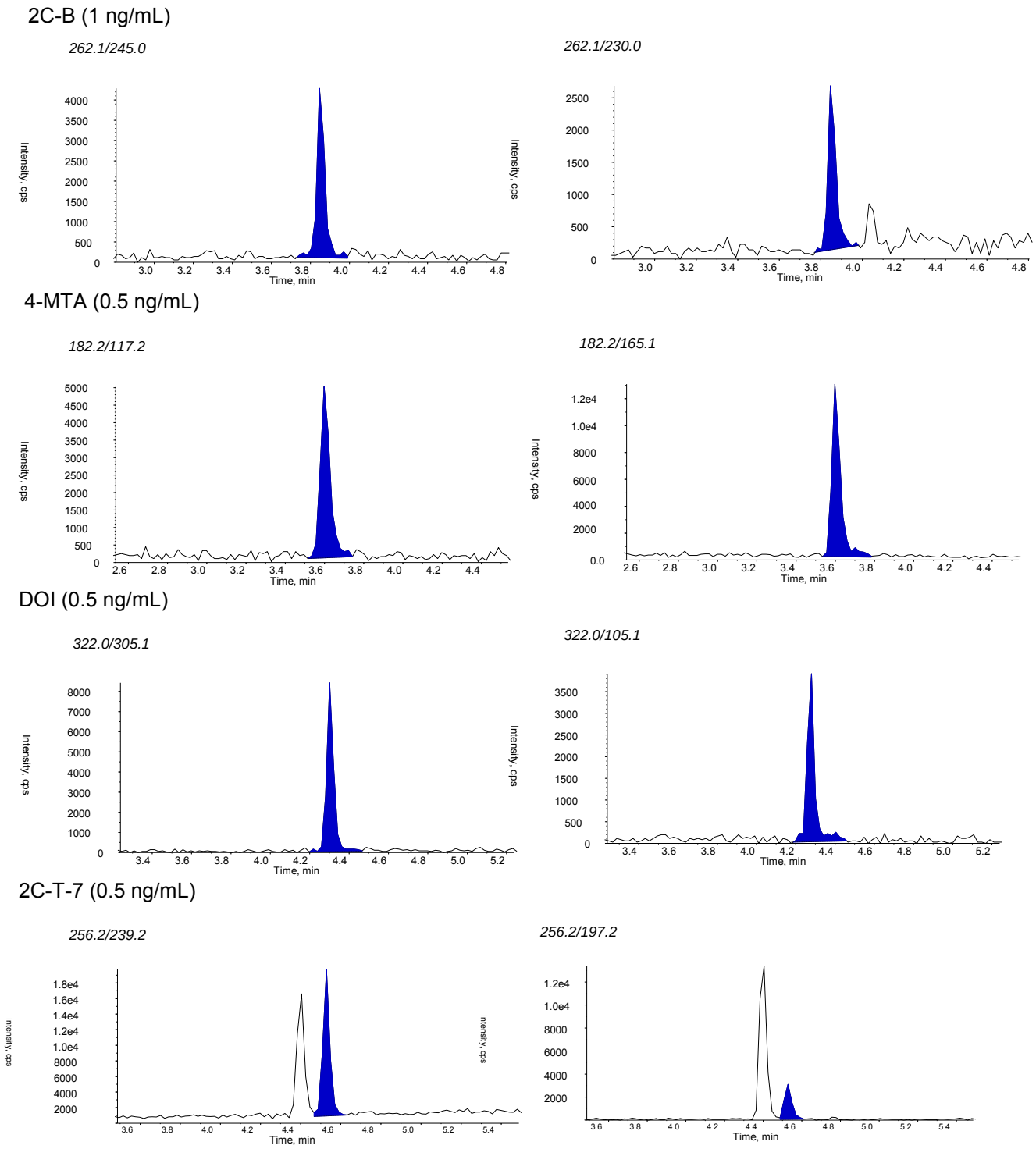
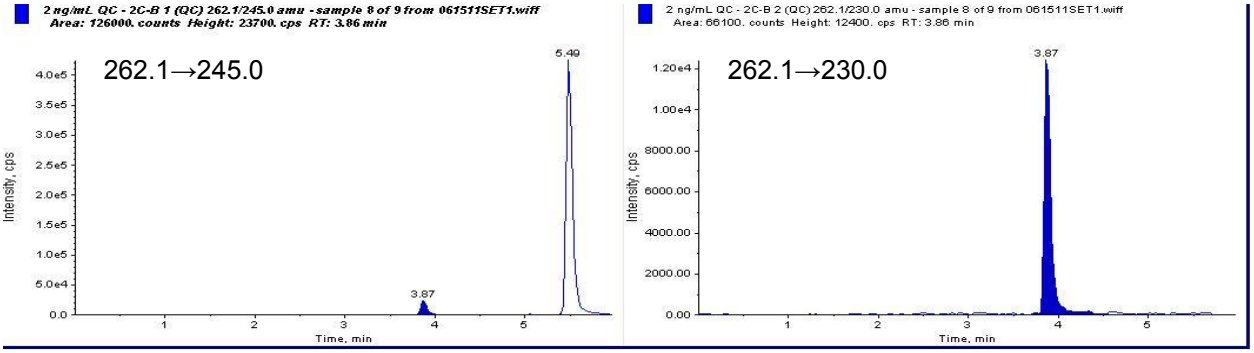
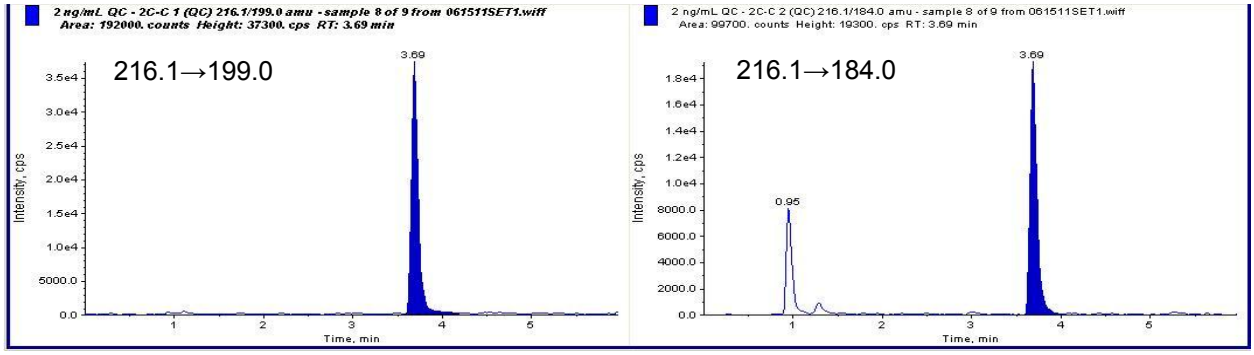


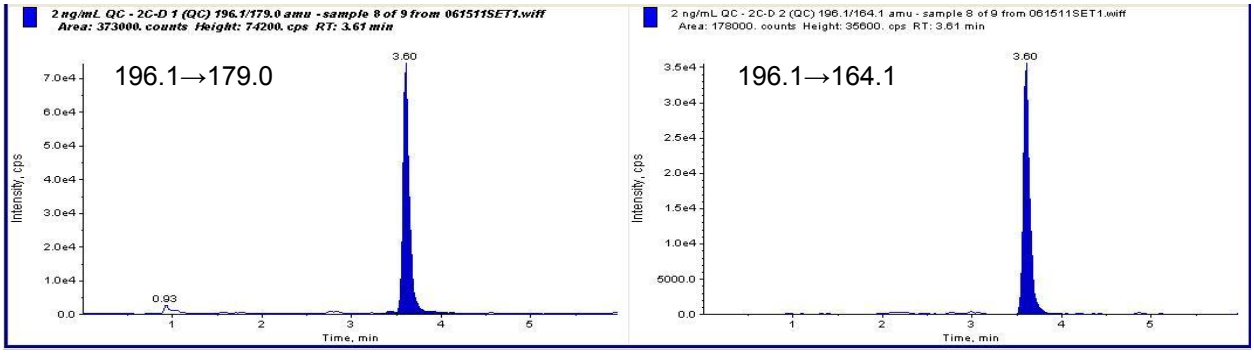
Figure 12. MRM transitions of all fifteen target analytes in blood at 2 ng/mL.



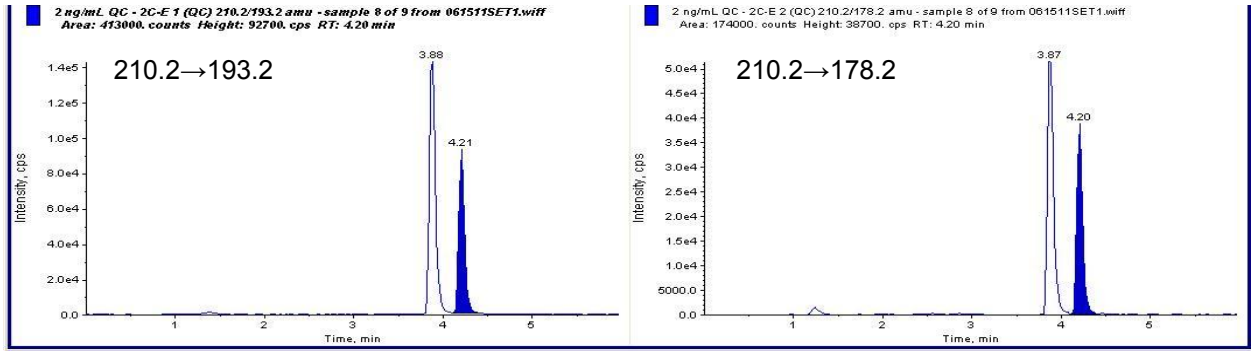
2C-B



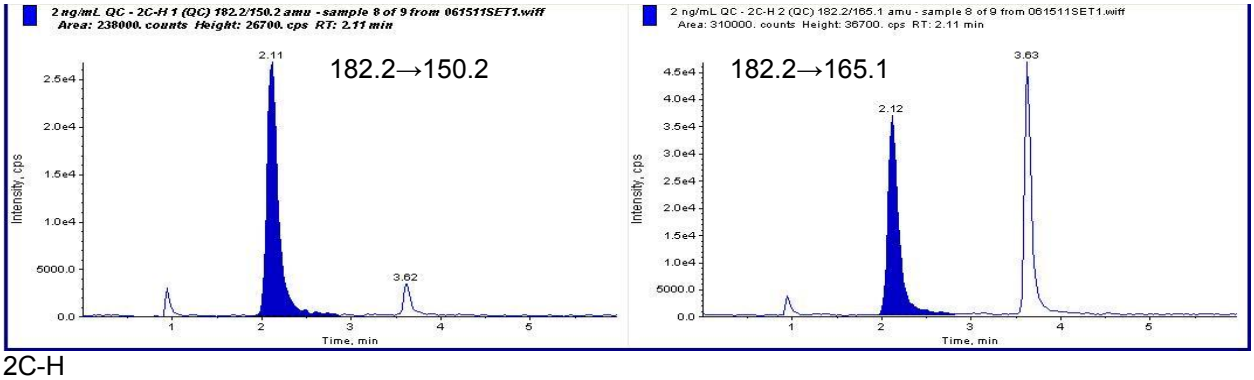
2C-C



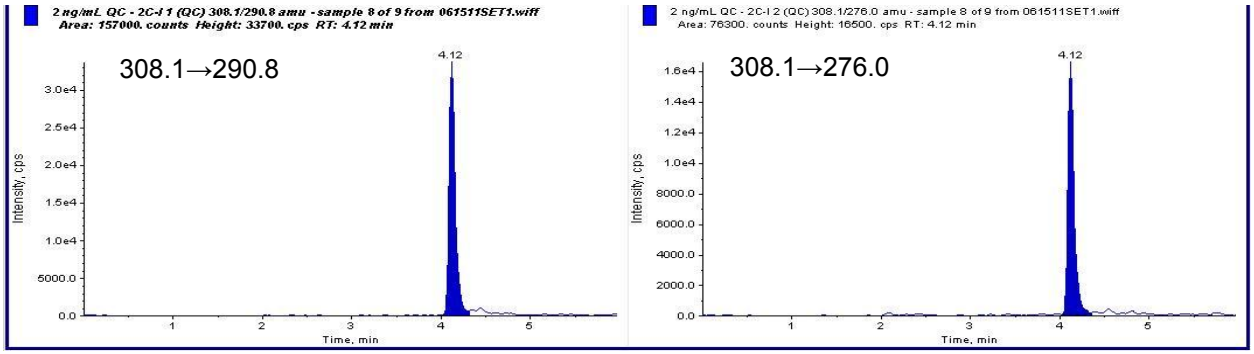
2C-D



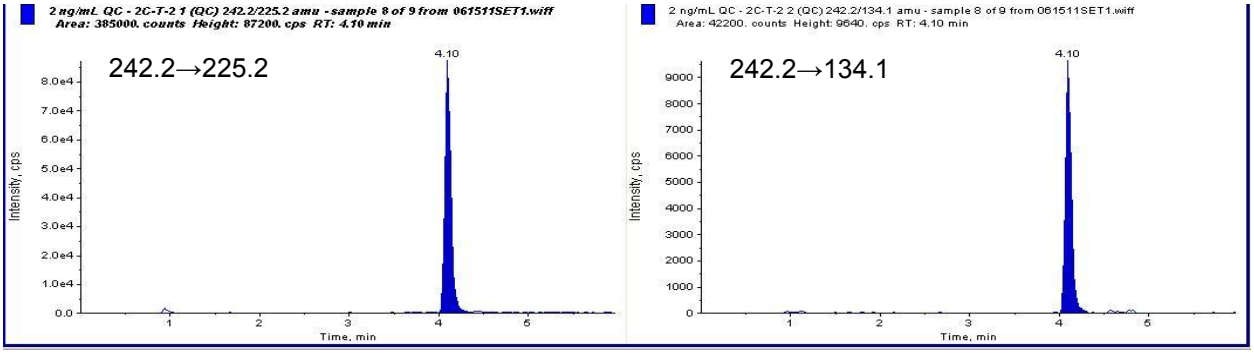
2C-E



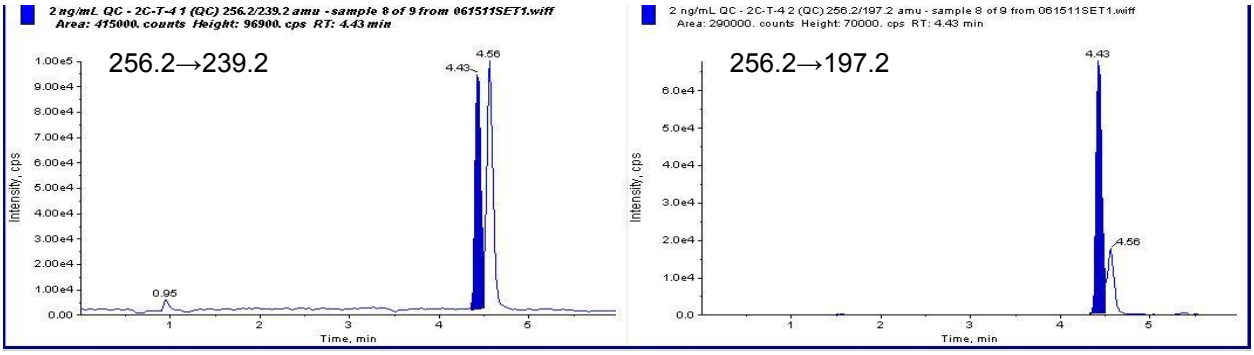
2C-H



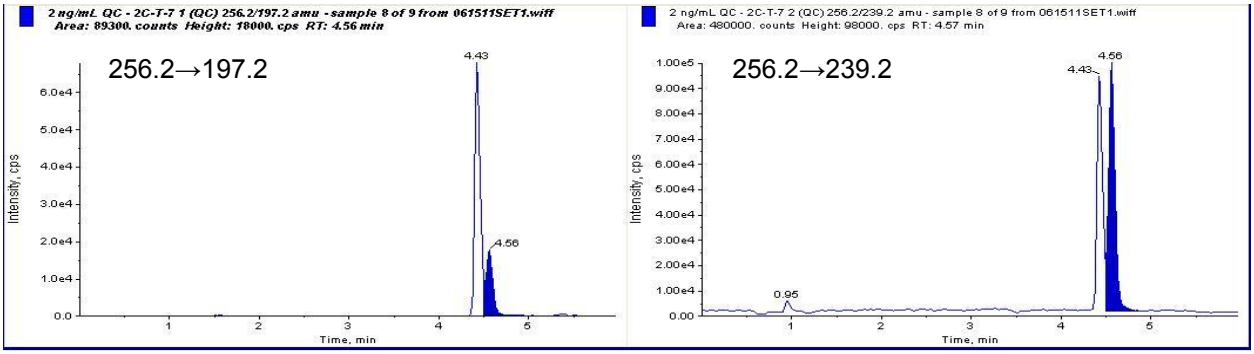
2C-I



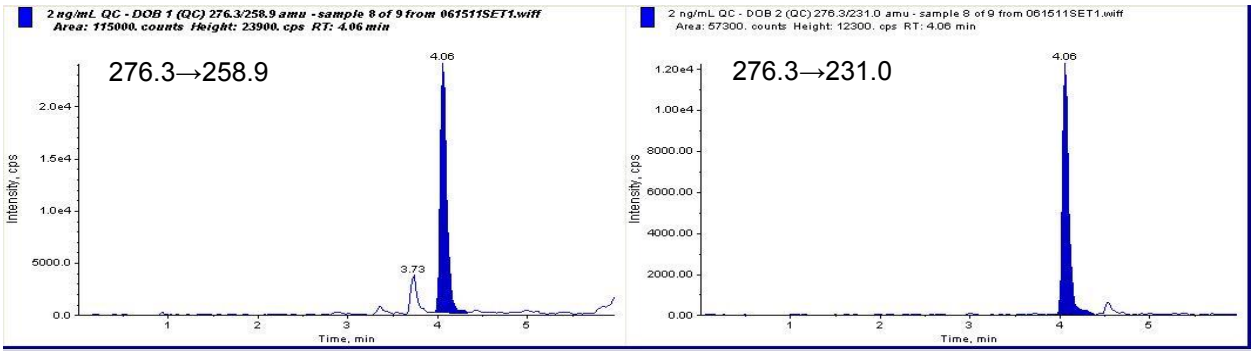
2C-T-2



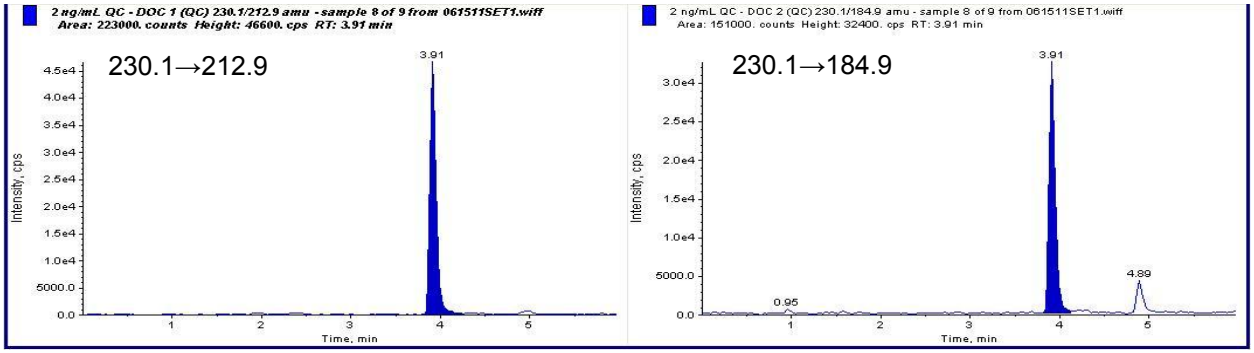
2C-T-4



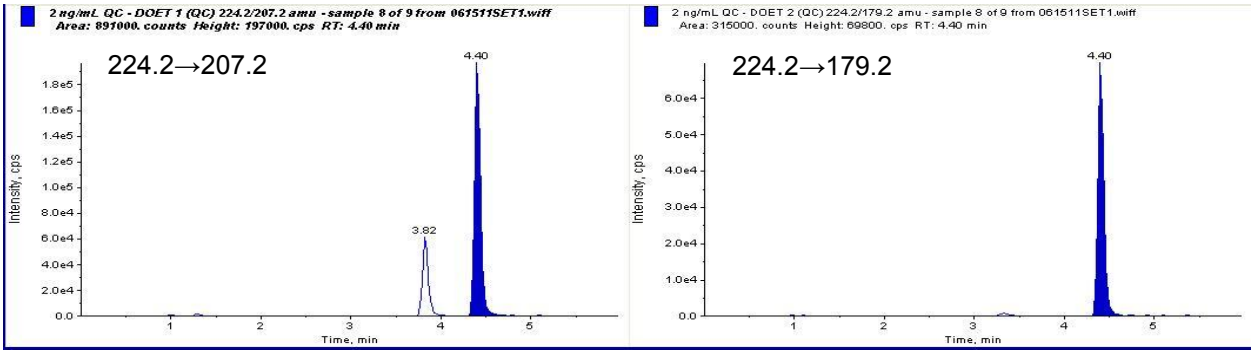
2C-T-7



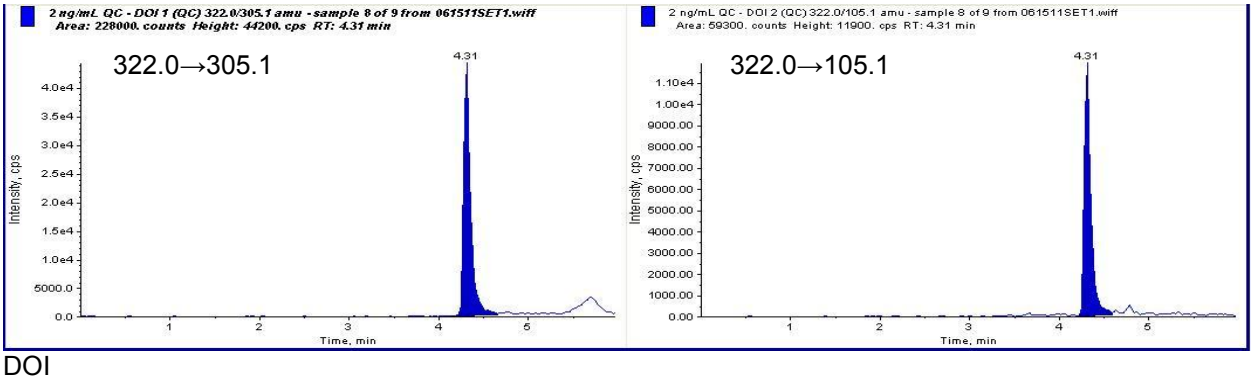
DOB



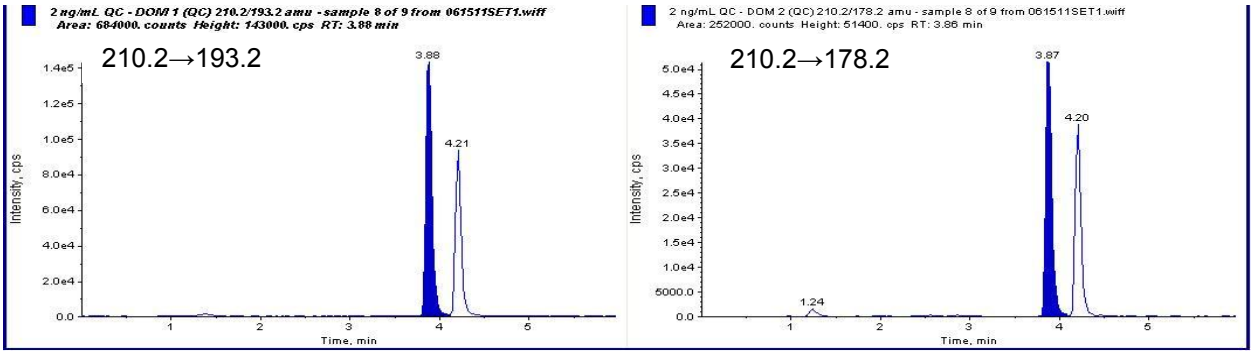
DOC



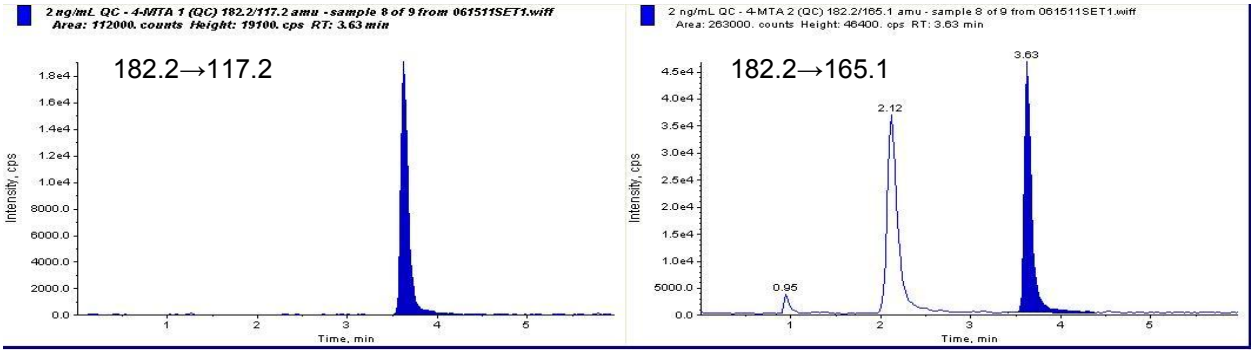
DOET



DOI



DOM



4-MTA

Precision and Accuracy

Mean accuracies for controls evaluated at 50 and 250 ng/mL were 96-120% for all target analytes in urine and 89-112% in blood. Intra-assay precision over the same range yielded CVs between 0.5 - 5.6% in urine and 1-6% in blood (**Table 19**). Inter-assay precision in blood and urine produced CVs of 2.1-20.8% (N=4) and 2.9-8.2% (N=7), respectively for urine and blood. Quantitative performance at much lower concentrations was also evaluated as part of limit of quantitation.

Table 19. Intra- (A) and inter-assay (B) precision and accuracy in blood and urine.

(A)

Urine						
Drug	50 ng/mL (N=4)			250 ng/mL (N=4)		
	Mean ± SD (ng/mL)	Accuracy (%)	CV (%)	Mean ± SD (ng/mL)	Accuracy (%)	CV (%)
2C-B	55 ± 1	111	1.6	250 ± 5	100	2.0
2C-C	55 ± 2	109	4.2	245 ± 4	98	1.7
2C-D	56 ± 3	111	4.7	244 ± 8	98	3.4
2C-E	58 ± 3	116	5.6	252 ± 6	101	2.3
2C-H	53 ± 3	106	5.3	250 ± 3	100	1.3
2C-I	54 ± 2	109	2.7	249 ± 8	99	3.1
2C-T-2	56 ± 1	113	1.6	251 ± 6	101	2.6
2C-T-4	56 ± 2	112	2.7	243 ± 11	97	4.4
2C-T-7	56 ± 3	111	5.6	249 ± 12	100	4.8
4-MTA	56 ± 1	112	2.0	252 ± 8	101	3.3
DOB	57 ± 2	115	3.0	253 ± 3	101	1.3
DOC	58 ± 1	116	1.1	251 ± 9	100	3.4
DOET	60 ± 2	120	3.6	242 ± 5	97	2.0
DOI	54 ± 0	108	0.5	246 ± 6	98	2.5
DOM	60 ± 2	120	3.3	241 ± 14	96	5.7
Blood						
Drug	50 ng/mL (N=4)			250 ng/mL (N=4)		
	Mean ± SD (ng/mL)	Accuracy (%)	CV (%)	Mean ± SD (ng/mL)	Accuracy (%)	CV (%)
2C-B	55 ± 2	110	3.2	222 ± 9	89	4.2
2C-C	52 ± 2	105	4.4	226 ± 10	90	4.3
2C-D	56 ± 2	112	3.4	223 ± 4	89	1.8
2C-E	54 ± 3	109	6.1	223 ± 10	89	4.6
2C-H	52 ± 1	103	2.5	236 ± 13	94	5.5
2C-I	49 ± 3	99	6.4	230 ± 15	92	6.3
2C-T-2	53 ± 2	107	3.1	222 ± 9	89	4.1
2C-T-4	52 ± 1	104	2.8	238 ± 14	95	6.0
2C-T-7	53 ± 1	105	2.3	225 ± 10	90	4.4
4-MTA	55 ± 3	110	5.2	235 ± 8	94	3.4
DOB	52 ± 2	103	3.4	246 ± 12	98	5.0
DOC	56 ± 1	112	1.1	236 ± 7	94	2.9
DOET	51 ± 2	103	2.9	223 ± 6	89	2.9
DOI	51 ± 1	103	2.5	246 ± 10	99	4.2
DOM	53 ± 1	106	1.2	224 ± 6	89	2.6

(B)

Drug	**Urine 100 ng/mL** (N=4)		**Blood 100 ng/mL** (N=7)	
	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
2C-B	107	2.1	109	2.9
2C-C	101	6.1	107	5.8
2C-D	106	18.7	112	5.0
2C-E	109	11.1	108	8.2
2C-H	103	8.5	105	7.5
2C-I	102	5.6	107	6.5
2C-T-2	94	11.7	106	6.4
2C-T-4	97	13.4	100	5.4
2C-T-7	105	20.8	108	8.1
4-MTA	86	16.4	107	6.4
DOB	94	10.9	100	4.6
DOC	103	4.9	108	8.1
DOET	114	8.3	112	5.1
DOI	95	9.9	104	7.7
DOM	113	8.9	111	6.9

Matrix Effects

Matrix effects were evaluated using a combination of post-column infusion and post-extraction addition techniques. A preliminary evaluation of matrix effect during method development revealed significant ion suppression of mescaline-d_9 internal standard. This was addressed early-on by modifying the gradient elution program (from 25% mobile phase B initially, to 20%). This slight shift in retention time was sufficient to prevent suppression of mescaline-d_9. This highlights the value of post-column infusion during method development, as this approach allows the influence of the matrix on ionization to be evaluated over the entire chromatographic run. Following method development and optimization, a more rigorous statistical approach to matrix effect was taken during the final validation of the assay using post-extraction addition. These results are summarized in **Table 20** as the mean percent matrix effect for all twenty drug free blood and urine samples that were evaluated. No matrix effects greater than 30% were measured in any of the samples tested.

Table 20. Determination of matrix effect in using post-extraction addition (N=20).

Drug	Urine		Blood	
	Matrix Effect	CV (%) N=20	Matrix Effect	CV (%) N=20
2C-B	-11%	6.2	-15%	5.3
2C-C	-3%	7.1	-15%	4.4
2C-D	-10%	7.8	-16%	3.9
2C-E	-7%	5.9	-17%	3.9
2C-H	-6%	6.9	-17%	4.1
2C-I	-10%	7.5	-14%	8.1
2C-T-2	-11%	8.0	-6%	7.8
2C-T-4	-7%	7.8	-9%	10.3
2C-T-7	-18%	9.0	-12%	6.9
4-MTA	-14%	7.5	-16%	5.2
DOB	-1%	8.2	-17%	5.0
DOC	-5%	6.0	-21%	4.2
DOET	-8%	5.9	-16%	3.9
DOI	-7%	7.9	-16%	7.7
DOM	-10%	5.5	-18%	3.6

Interferences

No interferences were present for any of the common amphetamines, endogenous bases or common drugs investigated in urine. None of the twenty nine interferences evaluated produced either a qualitative or quantitative interference for the negative control (0 ng/mL) or positive control (100 ng/mL) when evaluated independently. All quantitative values for the positive control were within 20% of expected, and no false positives were obtained for the negative controls. However, when the positive control (100 ng/mL) was evaluated with a mixture containing all of the interfering substances (1000 ng/mL), suppression of 2C-T-2 (4.12 min) and DOB (4.10 min) was evident. Although this might be attributed to meperidine (4.05 min), the substance that eluted with closest proximity, no interference was present when samples were evaluated with meperidine alone (**Table 21**). The excess quantity of so many drugs in the interference mix may overload the source and decrease the efficiency of ionization, but the presence of such a large number of drugs in an actual sample appears most unlikely. However,

it does highlight a limitation of ultra sensitive LC/MS/MS techniques, whereby ionization is somewhat capacity limited.

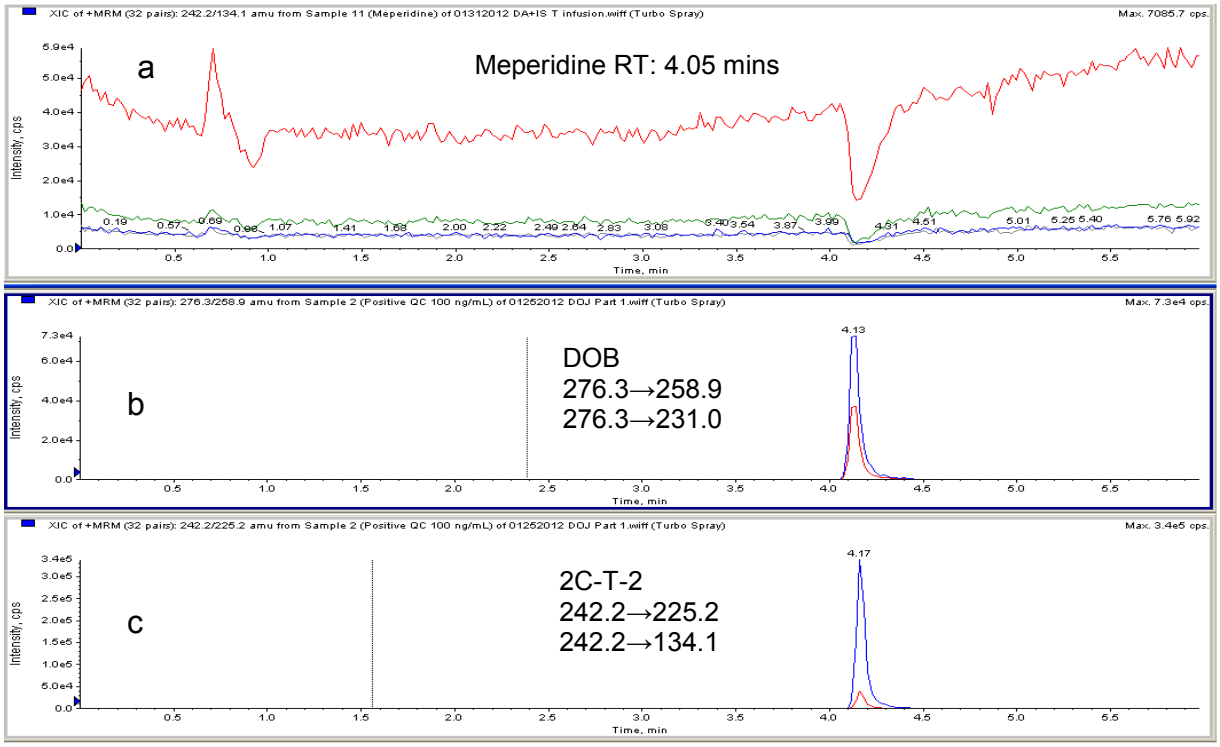
In the blood however, meperidine was responsible for a slight suppression of 2C-T-2 and DOB. None of the substances evaluated produced an interference in any of the negative controls, but positive quantitative controls for 2C-T-2 and DOB were outside of acceptable ranges, with accuracies of 76% and 67%, respectively for the 100 ng/mL control. The retention time for meperidine was within 0.03 and 0.05 minutes of DOB and 2C-T-2 **(Table 21**). Post column infusion of the target analytes and internal standard with injection of a meperidine standard was used to evaluate suppression over the entire run, and this is shown in **Figure 13**. Meperidine was the only substance to produce an interference in the blood assay. Although quantitative determination of DOB or 2C-T-2 would not be possible in the presence of meperidine, qualitative identification was still possible.

Table 21. Retention times for target analytes and compounds evaluated during the interference study (listed in retention time order).

Drug	RT (min)
Tyramine	0.69
Putrescine	0.71
Pseudoephedrine	1.30
Ephedrine	1.37
Codeine	1.49
Oxycodone	1.70
Tryptamine	1.78
Amphetamine	1.83
MDA	1.85
Phenethylamine	2.00
Methamphetamine	2.04
2C-H	2.09
MDMA	2.18
Phentermine	2.23
Hydrocodone	2.31
MDEA	2.73
Tramadol	3.44
2C-D	3.63
4-MTA	3.65

2C-C	3.71
2C-B	3.88
DOM	3.88
DOC	3.93
Meperidine	4.05
MBDB	4.06
DOB	4.06
2C-T-2	4.10
2C-I	4.12
2C-E	4.20
Ketamine	4.24
DOI	4.31
Cocaine	4.39
DOET	4.40
2C-T-7	4.42
2C-T-4	4.56
Dextromethorphan	4.58
Zolpidem	5.00
Alprazolam	5.05
PCP	5.23
Methadone	5.35
Amitriptyline	5.36
Nordiazepam	5.47
Diazepam	6.02

Figure 13. Post-column infusion of target analytes and internal standards, with injection of meperidine (a); DOB transitions (b) and 2C-T-2 transitions (c) showing retention time proximity are shown for comparison.



Limitations

Pharmacological and toxicological data for many of these drugs are still somewhat limited. However, animal, and to a lesser extent human studies for select drugs within the class, suggest a number of common metabolic pathways. The DO-series of drugs may undergo hydroxylation of the 4 methyl, followed by conjugation or oxidation to the corresponding acid, deamination (to a ketone), reduction to an alcohol, O-demethylation, or combinations of these pathways (Ewald et al., 2006b; Ewald et al., 2007; Ewald et al., 2008). In a somewhat similar fashion, proposed pathways for the 2C-series include O-demethylation, deamination, alcohol formation, acid formation, reduction and acetylation (Kanamori et al., 2002; Kanamori et al., 2003; Rohanová et al., 2008; Theobald et al., 2007; Theobald et al., 2006). Sulfur-containing drugs in the 2C-T series likely undergo similar transformations, in addition to *S*-depropylation followed by methylation of the resulting thiol (Lin et al., 2003; Theobald et al., 2005a; Theobald et al., 2005b). Conjugation (glucuronidation and sulfation) takes place and several metabolic studies employ a deconjugation step prior to the identification of proposed metabolites.

A significant limitation however, is the absence of commercial standards for these metabolites. From a practical standpoint this limits most laboratories to the identification of the parent drug alone. Although concentrations of 2C-T-7 in heart blood and urine were 57 ng/mL and 1,120 ng/mL following a fatality (Curtis et al., 2003), concentrations in recreational drug users are not well established. DOB concentrations in serum following a fatal overdose were particularly low (19 ng/mL) (Balíková, 2005), but this is perhaps not surprising considering the very low dose (1-3 mg) of this drug (**Table 3**). Authors in the study tentatively identified urinary metabolites in addition to DOB, but were unable to identify them due to the absence of a commercial standard.

Casework Samples

Only 2 of the 2,021 urine samples tested contained any of the target analytes. Both samples in question contained DOI at concentrations of 1 and 2 ng/mL in urine. A search of the literature revealed no other reports of DOI in human subjects to date. The samples tested were adjudicated urine specimens that were due for destruction and subsequently, several years old. Specimens were routinely stored at room temperature following release of the report and adjudication of the case. Although samples were stored refrigerated prior to testing in this study, very little is known of the stability of these drugs. This is a significant limitation. The two samples tested contained DOI at very low concentrations, consistent with the high potency of the DO-series. Recreational doses of DOI are very low (1.5-3 mg). Frequently encountered on LSD-like blotters, DOI is renowned for its profound hallucinogenic effects and is sometimes referred to as “synthetic acid”. Although the limitations regarding storage and stability should be considered, it is clear that many of these substances will pose an analytical challenge to many laboratories that perform routine toxicology testing.

Conclusions

Discussion of Findings

The purpose of the study was to investigate and improve detection methods for 2C, 2C-T and DO-series psychedelic amphetamines in toxicological samples. These drugs pose a number of challenges to forensic toxicology laboratories. Although they are seized by law enforcement agencies throughout the United States, they are not readily detected in forensic toxicology laboratories. It is not clear whether these drugs are rarely encountered due to overall low prevalence, or limitations with respect to detectability.

Initially, commercial ELISAs in widespread among forensic toxicology laboratories were investigated. This was the first systematic investigation of cross-reactivity towards this class of designer drug. Commercial immunoassays have limited cross-reactivity towards these amphetamine-like drugs. As a consequence, laboratories that rely upon immunoassay, rather than more broad spectrum chromatographic screening techniques may fail to detect these and other similar substances. This highlights the importance of using non-immunoassay-based screening techniques (such as GC/MS) as a complementary screening tool for human performance and postmortem investigations.

In this study a basic solid phase extraction procedure was developed to isolate drugs of interest. Although the metabolic transformation of these drugs has been preliminarily investigated and likely involves a number of common pathways, commercial standards are not readily available. Toxicology laboratories performing routine human performance or postmortem investigations must therefore rely upon detection of the parent drug. Using the approach described here, psychedelic amphetamines were detected at forensically significant concentrations in blood and urine using both GC/MS and LC/MS/MS. In a retrospective analysis of adjudicated casework, the presence of DOI in two human subjects was confirmed at concentrations of 1 and 2 ng/mL, respectively. This is the first report of DOI detection in forensic toxicology casework to date and this finding highlights the difficulties associated with the detection of these emerging drugs of abuse in routine testing.

Implications for Policy and Practice

The DO, 2C and 2CT-series of psychedelic amphetamines are an emerging class of designer drugs capable of producing a complex array of hallucinogenic and adrenergic effects. The unique pharmacological effects of the drugs has fueled their popularity, yet detection in toxicological samples is rarely reported. Recreational drug users are often aware of the limitations of analytical testing and several of the drugs are specifically marketed and promoted on the Internet for this purpose. Forensic toxicology laboratories that perform routine testing struggle to stay abreast of new drug use and trends. Development and validation of new procedures is labor intensive and often a secondary concern to the processing of routine casework and backlogs. In this study, techniques and instrumentation that is in widespread use in forensic toxicology laboratories was used to develop a simple and effective procedure for the detection of psychostimulants in toxicological samples. Should laboratories choose to implement these procedures into their repertoire of testing, these substances would be identified with greater frequency. This would not only contribute to our scientific understanding of their pharmacological and toxicological effects in humans, but also help understand implications of their use from both a public safety and criminal justice perspective.

Implications for Further Research

In order for forensic toxicology laboratories to stay abreast of new drug trends, continued research is needed. The ability to synthesize new analogs and bypass controlled substance legislation makes this an ongoing task. During the course of this work, additional classes of designer drugs have emerged. These include the synthetic cathinones, a structurally related class of amphetamine-like drugs that are capable of producing both stimulant and psychomimetic effects. There has been widespread activity at the State and Federal level in terms of regulation and control measures, most notably the Synthetic Drug Abuse Prevention Act of 2012, which went into effect in July 2012. While regulation and control measures are critical from a public safety standpoint, forensic laboratories must also have the necessary resources and access to scientific information, reference materials and research to detect their use.

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