Kayla N. Ellefsen, MS

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PROFESSIONAL EXPERIENCE

National Institute on Drug Abuse July 2012-Present

Pre-doctoral Visiting Fellow, Chemistry and Drug Metabolism Section

- Lead associate investigator on IRB and FDA approved study "Pharmacodynamic and pharmacokinetic interactions between intravenous cocaine and acetazolamide or quinine"
- LC-HRMS method development, validation, and confirmation of 28 synthetic cathinones in urine, cocaine and metabolites in breath, cocaine and metabolites in dried blood spots, and MDPV and metabolites in rat plasma**;** LC-MSMS method development, validation, and confirmation of methylone and primary metabolites in rat plasma**;** 2D-GC-MS method development for cocaine and benzoylecgonine in oral fluid and whole blood; evaluation and validation of Randox DOA V immunoassay for detection of synthetic cathinones in urine
- Metabolite identification of synthetic cathinones involving human liver microsome incubations, human hepatocyte incubations, LC-HRMS method development, and metabolite identification software
- Experience with SCIEX 3200 QTrap, SCIEX 5500 QTrap, Thermo Scientific Q Exactive LC-HRMS, Agilent GC-MS; experience writing FDA Investigational New Drug application, standard operating procedures, protocol amendments, and project reports; ordering materials for clinical and research studies

Sam Houston State University Regional Crime Lab January 2011-May 2012

Graduate Assistant

- Method development and validation for target analytes in Controlled Substance Laboratory; updating and maintaining chemical inventory; routine preventative maintenance on Agilent GC-MS; monthly safety testing and practice safety audits; maintaining standard laboratory organization (maintenance and daily quality control logs, sequence logs for laboratory instrumentation, reagent and solutions preparation logs)

Sam Houston State University August 2011-May 2012

Graduate Researcher

- Developed and validated an assay capable of detecting 8 of the most prevalent synthetic cathinones in urine by GC-MS; and evaluated potential of a lesser known derivative to improve analytical characteristics via NIJ/FSF funded grant
- Executed frequent GC-MS troubleshooting and instrument maintenance on Agilent 6890N GC and 5975B MSD

Ameritox Ltd June 2011-August 2011
Intern – Research and Development Department

- Evaluation and validation of Immunalysis Tapentadol Enzyme Immunoassay Kit; experience with Olympus AU640 Enzyme Immunoassay Analyzer, Waters Acquity UPLC-MS/MS, and Thermo HPLC-MS/MS; routine instrument maintenance for UPLC-MS/MS and Thermo HPLC-MS/MS

Laurentian University September 2008-May 2009
Undergraduate Researcher

- Characterized the accuracy and precision of the Intoxilyzer 8000C in a laboratory study involving the measurements of breath alcohol profiles in healthy volunteers and compared venous blood alcohol to breath alcohol concentrations
- Experience with Immunalysis blood alcohol assay kits in conjunction with a Awareness Technologies ChemWell 2910 Automated EIA and Chemistry Analyzer

EDUCATION

University of Maryland Baltimore, *Baltimore, MD* 2012- present
Doctor of Philosophy in Toxicology
GPA: 4.0
Expected Defense Date: April 2016
Advisor: Dr. Marilyn Huestis
Advancing Analytical Methodologies for Classic and Novel Stimulants

Sam Houston State University, *Huntsville, TX* 2010-2012
Masters of Science in Forensic Science
GPA: 3.95
Graduated: May 2012
Advisor: Dr. Sarah Kerrigan
Detection of Beta-Keto Amphetamines in Biological Samples by Gas Chromatography-Mass Spectrometry (NIJ/FSF Funded)
Internship Supervisor: Dr. Jim Bourland/ Ayodele Morris
Evaluation and Validation of the Immunalysis Tapentadol Enzyme Immunoassay

Laurentian University, *Sudbury, ON, Canada* 2005-2009
Honors Bachelor of Science in Forensic Science
Minors: Biology/Chemistry
GPA: 3.5
Graduated: May 2009
Advisor: Dr. James Watterson
Analytical Characterization of Evidentiary Infrared Breath Ethanol Analysis Instrumentation Intended for Field Use in Forensic Applications – The Intoxilyzer® 8000C

PUBLICATIONS

1. **K.N. Ellefsen**, M. Concheiro, and M.A. Huestis. Synthetic Cathinone Pharmacokinetics, Analytical Methods, and Toxicological Findings from Human Performance and Postmortem Cases. *Drug Metabolism Reviews.* 24 March 2016 (Under review).

2. **K.N. Ellefsen**, M. Concheiro, S. Pirard, D.A. Gorelick, and M.A. Huestis. Pharmacodynamic Effects and Relationships to Plasma and Oral Fluid Pharmacokinetics after Intravenous Cocaine Administration. *Drug and Alcohol Dependence.* 13 April 16 (DOI:10.1016/j.drugalcdep.2016.04.004).

3. M.J. Swortwood, **K.N. Ellefsen**, A. Wohlfarth, X. Diao, M. Concheiro-Guisan, R. Kronstrand, M.A. Huestis. First Metabolic Profile of PV8, a Novel Synthetic Cathinone, in Human Hepatocytes and urine by High-Resolution Mass Spectrometry. *Analytical and Bioanalytical Chemistry*. 22 April 2016 (In Press).

4. **K.N. Ellefsen**, M. Concheiro, S. Pirard, D.A. Gorelick, and M.A. Huestis. Cocaine and Benzoylecgonine Oral Fluid On-site Screening and Confirmation. *Drug Testing and Analysis.* 2016; 8(3-4): 296-303 (DOI: 10.1002/dta.1966).

5. **K.N. Ellefsen**, M. Concheiro, S. Pirard, D.A. Gorelick, and M.A. Huestis. Oral Fluid Cocaine and Benzoylecgonine Concentrations Following Controlled Intravenous Cocaine Administration. *Forensic Science International*. 2016; 260: 95-101 (DOI:10.1016/j.forsciint.2016.01.013).

6. M.J. Swortwood, J. Carlier, **K.N. Ellefsen**, A. Wohlfarth, X. Diao, M. Concheiro, R. Kronstrand, and M.A. Huestis. In vitro, in vivo, and in silico Metabolic Profiling of α-pyrrolindinopentiothiophenone, a Novel Stimulant Thiophene Analog. *Bioanalysis*. 2016; 8(1): 65-82.

7. **K.N. Ellefsen**, A. Wohlfarth, M.J. Swortwood, X. Diao, M. Concheiro, and M.A. Huestis. 4-methoxy-α-PVP: *In silico* prediction, metabolic stability, and metabolite identification by human hepatocytes and high resolution-mass spectrometry. *Forensic Toxicology.* 2016; 34(1): 61-75.

8. **K.N. Ellefsen**, J.Luiz da Costa, M. Concheiro, S. Anizan, A.J. Barnes, S. Pirard, D.A. Gorelick, and M.A. Huestis. Cocaine and metabolites concentrations in dried blood spots and venous blood after controlled intravenous administration. *Bioanalysis.* 2015; 7(16): 2041-56.

9. **K.N. Ellefsen**, M. Concheiro, M. Suzuki, K.C. Rice, J.S. Elmore, M.H. Baumann, and M.A. Huestis. Quantification of methylone and metabolites in rat and human plasma by liquid chromatography-tandem mass spectrometry. *Forensic Toxicology.* 2015: 33(2); 202-212.

10. **K.N. Ellefsen,** M. Concheiro, O. Beck, D. Gorelick, S. Pirard, and M.A. Huestis. Quantification of cocaine and metabolites in exhaled breath by liquid chromatography-high-resolution mass spectrometry following controlled administration of intravenous cocaine. *Analytical and Bioanalytical Chemistry*. 2014 Oct; 406(25): 6213-23.

11. **K.N. Ellefsen**, S. Anizan, M.S. Castaneto, N. Desrosiers, T.M. Martin, K.L. Klette, M.A. Huestis. Validation of the Only Commercially Available Immunoassay for Synthetic Cathinones in Urine: Randox Drugs of Abuse V Biochip Array Technology. *Drug Testing and Analysis.* 2014 Jul-Aug: 6(7-8); 728-738.

12. S. Anizan. **K. Ellefsen**, M. Concheiro, K.C Rice, M.H Baumann and M.A. Huestis. Quantification of MDPV and its main metabolites in human and rat plasma by liquid chromatography-high resolution mass spectrometry. *Analytica Chimica Acta.* 2014 May 27;827:54-63.

13. M.S. Castaneto, N. Desrosiers, **K. Ellefsen**, S. Anizan, T.M. Martin, K. Klette and M.A. Huestis. Validation of the Randox Drugs of Abuse V Biochip Array Technology for Detection and Semi-quantification of Synthetic Cannabinoids in Urine. *Bioanalysis.* 2014: 6(21); 2919-2930.

14. **K. Ellefsen** and M.A. Huestis. AM-694 - Emerging Designer Drug Monograph. *SOFT Designer Drug Monographs.* 2014 Jan 26; 1-3. http://www.soft-tox.org/files/designer_drugs/AM-694.pdf.

15. M. Concheiro, S. Anizan, **K. Ellefsen** and M.A. Huestis. Simultaneous quantification of 28 synthetic cathinones and metabolites in urine by liquid chromatography-high resolution mass spectrometry. *Analytical & Bioanalytical Chemistry.* 2013: 405(29); 9437-48.

16. J.H. Watterson and **K.N. Ellefsen**. Examination of Some Performance Characteristics of Breath Alcohol Measurements Obtained with the Intoxilyzer® 8000C Following Social Drinking Conditions. *Journal of Analytical Toxicology*. 2009: 33(8); 514-520.

ABSTRACTS AND ORAL PRESENTATIONS

1. **K.N. Ellefsen**, M. Concheiro, J. Elmore, M. Suzuki, K.C. Rice, M.H. Baumann, and M.A Huestis. Pharmacodynamics and pharmacokinetics of methylone in rats. ORAL PLATFORM. Society of Forensic Toxicologists Annual Conference, Atlanta, October, 2015.

2. M.J. Swortwood, J. Carlier, **K.N. Ellefsen**, A. Wohlfarth, R. Kronstrand, and M.A. Huestis. Metabolic Profiling of α-PVT, a Novel Stimulant Thiophenyl

Analog. ORAL PLATFORM. Society of Forensic Toxicologists Annual Conference, Atlanta, October 2015.

3. **K.N. Ellefsen**, J.Luiz da Costa, M. Concheiro, S. Anizan, A.J. Barnes, S. Pirard, D.A. Gorelick, M.A. Huestis. Cocaine and metabolites concentrations in dried blood spots and venous blood after controlled intravenous administration. ORAL PLATFORM. The International Association of Forensic Toxicologists Annual Conference, Florence, August 2015.

4. M Swortwood, **K. Ellefsen,** A. Wohlfarth, M. Concheiro, and M.A. Huestis. First metabolic profile of PV8, a novel synthetic cathinone, by high-resolution mass spectrometry (HRMS). ORAL PLATFORM. The International Association of Forensic Toxicologists Annual Conference, Florence, August 2015.

5. **K.N. Ellefsen,** M Swortwood, A. Wohlfarth, M. Concheiro, and M.A. Huestis. 4-methoxy-α-PVP: *In silico* metabolite prediction, assessment of metabolic stability with human liver microsomes and metabolite identification after human hepatocyte incubation with high resolution-mass spectrometry. ORAL PLATFORM. American Academy of Forensic Science Annual Conference, Orlando, February 2015.

6. **K.N. Ellefsen,** M. Concheiro, O. Beck, D. Gorelick, S. Pirard, and M.A. Huestis. Quantification of cocaine and metabolites in exhaled breath by liquid chromatography-high-resolution mass spectrometry following controlled administration of intravenous cocaine. ORAL PLATFORM. Society of Forensic Toxicologists Annual Conference, Grand Rapids, October 2014.

7. **K.N. Ellefsen**, S.Anizan, M.S. Castaneto, N.A. Desrosiers, T.M. Martin, K. Klette, and M.A. Huestis. Validation of the Only Commercially Available Immunoassay for Synthetic Cathinones in Urine: Randox Drugs of Abuse V Biochip Array Technology. ORAL PLATFORM. Society of Forensic Toxicologists Annual Conference, Orlando, October 2013.

8. M.S. Castaneto, N.A. Desrosiers, **K. Ellefsen**, S. Anizan, T.M. Martin, K. Klette, and M.A. Huestis. Validation of the Randox Drugs of Abuse V Biochip Array Technology for Detection and Semi-quantification of Synthetic Cannabinoids in Urine. ORAL PLATFORM. Society of Forensic Toxicologists Annual Conference, Orlando, October 2013.

9. S. Anizan, **K. Ellefsen**, M. Concheiro-Guisan, and M. Huestis**.** Development and Validation of a Liquid-Chromatography-High Resolution Mass Spectrometry Method for the Simultaneous Analysis of 28 Cathinones in Urine. ORAL PLATFORM. American Society of Mass Spectrometry Annual Conference, Minneapolis, June 2013.

10. **K. Ellefsen** and S. Kerrigan. Detection of Beta-Keto Amphetamines in Biological Samples by GC-MS. ORAL PLATFORM. Society of Forensic Toxicologists Annual Conference, Boston, July 2012.

11. **K. Ellefsen,** M. Willis, A. Collins, J. Bourland, and R. Backer. Evaluation of the Immunalysis Tapentadol Enzyme Immunoassay Kit. POSTER. American Academy of Forensic Science Annual Conference, Atlanta, February 2012.

12. M. Willis, **K. Ellefsen,** A. Collins, J. Bourland, and R. Backer. Validation and Comparison of Microgenics and Immunalysis Buprenorphine EIA Kits. POSTER. American Academy of Forensic Science Annual Conference, Atlanta, February 2012.

13. **K.N. Ellefsen** and J.H. Watterson. Intra-Individual Variability in Linear Elimination Rates from Breath Alcohol Profiles Following Social Drinking. POSTER. Society of Forensic Toxicologists Annual Conference, Oklahoma City, October 2009.

AWARDS AND HONORS

AAFS Forensic Science Foundation (FSF) Student Scholarship Award	November 2014
SOFT Educational Research Award	October 2014
Randox Toxicology Ltd. Travel Grant	October 2013
Predoctoral Visiting Fellow Award, National Institute on Drug Abuse	2012-present
NIJ Forensic Science Foundation Student Research Grant	2011-2012

PROFESSIONAL ORGANIZATIONS

Society of Forensic Toxicologists (SOFT)
- Student Member (March 2012-present)
- ToxTalk® Publishing Assistant (March 2014- present)

American Academy of Forensic Science (AAFS)
- Toxicology Student Member (September 2010-present)

Abstract

Title of Dissertation: Advancing Analytical Methodologies for Classic and Novel Stimulants

Kayla N. Ellefsen, Doctor of Philosophy, 2016

Dissertation Directed by: Professor Dr. Dr. (h.c.) Marilyn A. Huestis, Retired Chief, Chemistry and Drug Metabolism, National Institute on Drug Abuse (NIDA), National Institutes of Health

Despite decreasing cocaine prevalence in the US, it remains one of the most widely used illicit stimulants worldwide. However, little is known about cocaine and metabolites' disposition in alternative matrices. Additionally, novel psychoactive stimulants, including synthetic cathinones, recently emerged on the drug market with no shortage of challenges for clinical and forensic toxicologists. The primary objective of this research was to advance analysis and interpretation of classic and novel stimulant test results.

We developed and validated methods to identify classical stimulants, e.g. cocaine and its metabolites, in oral fluid (OF), breath and dried blood spots (DBS), to determine whether these alternative matrices are suitable to detect cocaine intake in forensic, clinical and workplace drug testing programs. We demonstrated that OF is a suitable matrix to identify cocaine use following intravenous cocaine. In contrast, although cocaine and metabolites were identified in breath and DBS, further research is necessary

to address limitations of these analyses, such as low prevalence and variability, before routine implementation in drug testing programs. We also evaluated a new OF on-site roadside screening test for cocaine detection and found that the Draeger DrugTest 5000 was a sensitive, specific, and efficient on-site OF screening device for both cocaine and BE with proposed confirmation cutoffs of 8 and 10 µg/L.

Furthermore, we developed and evaluated analytical screening and confirmation methods for novel stimulants, e.g. synthetic cathinones, and human hepatocytes and high resolution-mass spectrometry (HRMS) techniques to elucidate optimal metabolites for identification of a new synthetic cathinone, 4-methoxy-α-PVP. Immunoassays do not readily detect synthetic cathinones, and those specifically developed for cathinone detection exhibited large negative % bias and poor specificity and efficiency. Inclusion of metabolites in confirmatory methods is imperative for quantification and characterization after preclinical *in vivo* drug administration to expand our knowledge of these psychoactive stimulants. Metabolite profiling with human hepatocytes and HRMS identified 4-OH-α-PVP, in addition to the parent compound, as a suitable biomarker. It is critical to determine analytical targets to establish synthetic cathinone intake, link toxicity to causative agents and provide necessary preclinical safety data to support controlled drug administration studies.

Advancing Analytical Methodologies for Classic and Novel Stimulants

by
Kayla N. Ellefsen

Dissertation submitted to the Faculty of the Graduate School of the
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I also need to thank my first mentor, Dr. Sarah Kerrigan. Your continued support and guidance over the past several years has helped shape me into the toxicologist and person I am today. You have been a constant role model and example to me. I would not have embarked on this journey without your encouragement, and without you, I would not be where I am today. Thank you.

To Dr. Barry Levine, I consider it an honor and a privilege to have learned from one of the best in the field. Your expertise and insight has provided me critical knowledge and perspective I will be able to apply throughout my future career. I also know I will always have someone to celebrate, or commiserate, the success and failures of the Baltimore Ravens.

My deepest gratitude also goes to Drs. Kenneth Bauer and Karl Scheidweiler for their time, support, and insightful advice over the years. Their input allowed me to make my research applicable to a broader audience.

To Drs. Katherine Squibb and William Randall, thank you for your constant support and encouragement. You both have always been so kind and helpful. Thank you for teaching me about toxicology and being great program directors. I would also like to thank Linda Horne for everything you have done for me personally, as well as the Toxicology program.

Deepest gratitude is also owed to the laboratory supervisors at the NIDA Chemistry and Drug Metabolism Section, including Drs. Marta Concheiro, Karl Scheidweiler, and Allan Barnes. I have learned immensely about analytical chemistry, method development and validation, critical thinking and manuscript writing over the past several years from you. I thoroughly appreciate the time you took out of your busy schedules to impart this knowledge on me and to make me a better scientist. Thank you also to past and current colleagues, especially Drs. Sebastien Anizan and Ariane Wohlfarth. The support and encouragement you provided me with during the first couple years of my research is greatly appreciated. To Dr. Madeleine Swortwood, Dr. Nathalie Desrosiers, Dr. Sarah Himes-Bartock, Dr. Marisol Castaneto, Dr. Rebecca Hartman, and future Dr. Matthew Newmeyer, thank you for being my sounding board and constant support group. I could

not have survived without you all. And to the little “Black Box”, thank you for helping me churn out data and manuscripts night in and night out.

Last but not least, to my friends and family, you are my biggest cheerleaders. I cannot even begin to thank you for everything you have done for me throughout my lifetime, but especially these past 4 years dealing with all my ups and downs. To my “second family”, The Salamat’s, knowing you guys were always there for me made a world of difference. And finally, to my family: Mom, you are a constant source of inspiration for Kelsy and me. You have instilled in us how to fight and keep persevering, no matter the circumstance. You are always there for me day and night (even for the small stuff). I am so lucky to be your daughter. To my Keisy (you know the voice), thank you for answering when Mom doesn’t pick up. I am so excited we get to switch roles and I get to be your biggest fan. I am so proud of you. You, Mom, and Terry have always stood by my side no matter what the dream I wanted to tackle next was. I look forward to what the future has in store for us! Love you guys!

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List of Abbreviations

α-PBP	-	α-pyrrolidinobutiophenone
α-PPP	-	α-pyrrolidinopropiophenone
α-PVP	-	α-pyrrolidinopentiophenone
2D-GC-MS	-	two-dimensional-gas chromatography-mass spectrometry
3,4-DMMC	-	3,4-dimethylmethcathinone
3-BMC	-	3-bromomethcathinone
3-FMC	-	3-fluoromethcathinone
3-MMC	-	3-methylmethcathinone
4-FMC	-	4-fluoromethcathinone
4-MEC	-	4-methylethcathinone
4-MPBP	-	4-methyl-α-pyrrolidinobutiophenone
4-MPHP	-	4-methyl-α-pyrrolidinohexanophenone
4-MPPP	-	4-methyl-α-pyrrolidinopropiophenone
4-PL	-	4-parameter logistic
4-TFMMC	-	4-trifluoromethylmethcathinone
5-HT	-	serotonin
6-AM	-	6-acetylmorphine
AAPC	-	American Association of Poison Control Centers
AGC	-	automatic gain control
AIF	-	all-ion-fragmentation
AUC	-	area-under-the-curve
BE	-	benzoylecgonine

BSI	-	Bath Salt I
BSII	-	Bath Salt II
CBP	-	Customs and Border Patrol
C_{last}	-	last observed concentration
CL_H	-	human hepatic clearance
CL_{int}	-	intrinsic clearance
$CL_{int,micr}$	-	microsomal intrinsic clearance
C_{max}	-	maximum observed concentration
CSF	-	cerebral spinal fluid
CXP	-	collision exit potential
DA	-	dopamine
DART-MS	-	direct analysis in real time-mass spectrometry
DBS	-	dried blood spot(s)
$ddMS^2$	-	data-dependent MS^2
DEA	-	Drug Enforcement Administration
DFSA	-	drug-facilitated sexual assault
DLLME	-	dispersive liquid-liquid microextraction
DOA-V	-	Drugs of Abuse V
DRE	-	drug recognition examiner
DRUID	-	Driving Under the Influence of Drugs, Alcohol and Medicines
DUID	-	driving under the influence of drugs
ED	-	emergency department
EDTA	-	ethylenediaminetetraacetic acid

EG	-	ecgonine
ELISA	-	enzyme-linked immunosorbent assay
EMCDDA	-	European Monitoring Centre for Drugs and Drug Addiction
EME	-	ecgonine methyl ester
ER	-	extraction ratio
ESI	-	electrospray ionization
FID	-	flame ionization detector
FN	-	false negative
FP	-	false positive
GC-MS	-	gas chromatography-mass spectrometry
GHB	-	γ-hydroxybutyric acid
GI	-	gastric intubation
HCD	-	higher energy collision dissociation
HHMA	-	3,4-dihydroxymethamphetamine
HHMC	-	3,4-dihydroxymethcathinone
HLM	-	human liver microsomes
HMMA	-	4-hydroxy-3-methoxymethamphetamine
HMMC	-	4-hydroxy-3-methoxymethcathinone
HPLC-DAD	-	high performance liquid chromatography-diode array detection
HRMS	-	high resolution-mass spectrometry
IN	-	intranasal
IP	-	intraperitoneal
IS	-	internal standard

IV	-	intravenous
LC	-	liquid chromatography
LC-MS/MS	-	liquid chromatography-tandem mass spectrometry
LLE	-	liquid-liquid extraction
LOD	-	limit of detection
LOQ	-	limit of quantification
MALDI-TOF	-	matrix-assisted laser desorption/ionization-time-of-flight
MDC	-	3,4-methylenedioxycathinone
MDMA	-	3,4-methylenedioxymethamphetamine
MDPBP	-	3,4-methylenedioxy-α-pyrrolidinobutiophenone
MDPPP	-	3,4-methylenedioxy-α-pyrrolidinopropiophenone
MDPV	-	3,4-methylenedioxypyrovalerone
MRM	-	multiple reaction monitoring
MSD	-	mass selective detector
NCE	-	normalized collision energy
NE	-	norepinephrine
NPS	-	novel psychoactive substances
OE	-	Oral-Eze
OF	-	oral fluid
OF/P	-	oral fluid-plasma
PCP	-	phencyclidine
PM	-	postmortem
PMMA	-	para-methoxymethamphetamine

PV9	-	α-pyrrolidinooctanophenone
QC	-	quality control
RT	-	retention time
SAMHSA	-	Substance Abuse and Mental Health Services Administration
SC	-	subcutaneous
SD	-	standard deviation
SIM	-	selected ion monitoring
SM	-	smoked
SMBS	-	sodium metabisulfite
SPE	-	solid phase extraction
SPME	-	solid phase microextraction
SS	-	StatSure
SWGTOX	-	Scientific Working Group for Forensic Toxicology
TDM	-	therapeutic drug monitoring
THC	-	Δ^9-tetrahydrocannabinol
T_{last}	-	time of last observed concentration
T_{max}	-	time to maximum observed concentration
TN	-	true negative
TP	-	true positive
UNODC	-	United Nations Office on Drug Crime
VAS	-	visual analogue scale
vDBS	-	venous dried blood spot(s)
V_{ss}	-	volume of distribution at steady-state

Chapter 1 – Introduction

A. Cocaine Pharmacology and Detection in Alternative Matrices

Cocaine Epidemiology

Driving under the influence of drugs (DUID) is a major worldwide problem. Data from the 2007 United States National Roadside Survey indicated that approximately 11.0% of Friday daytime drivers and 14.4% of weekend nighttime drivers tested positive for drugs [1]. In the European Union, findings from their research project on Driving Under the Influence of Drugs, Alcohol and Medicines (DRUID) found that 1.9% of drivers were positive for illicit drugs [2]. Despite a declining trend in cocaine prevalence among the general population [3], cocaine remains one of the most widely used illicit stimulants in both the United States (1.5 million users) [4] and Europe (3.4 million users) [5]. In the general driving population, cocaine was the second most prevalent illicit drug in the United States (1.4%) and Europe (0.42%), only behind cannabis [5,6].

Subjective and Physiological Effects

Cocaine is highly abused for its pronounced euphoria, heightened stimulation, and elevation in mood; however, there are potential undesirable effects including dysphoria, paranoia, and hallucinations. Cardiovascular effects, tachycardia, arrhythmia and hypertension, also may occur due to cocaine's effects on sodium ion channels. Additional physiological effects include elevated blood pressure, increased respiratory rate, and dilated pupils [7-9].

After cocaine intake, subjects often report an initial "rush" (intense euphoria) lasting only 1-2 min, followed by a prolonged elevated mood, typically only lasting up to 30 min [10,11]. Concurrent with these changes are increases in physiological effects such

as heart rate, blood pressure, respiratory rate and pupil dilation. Subjective and physiological effects are dose related and peak at times of maximum plasma concentrations [7,8,11-13]. These effects rapidly dissipate prior to plasma concentrations returning to baseline, suggesting development of acute tolerance since the magnitude of effects were greater at earlier time points. Acute tolerance to cocaine's subjective and cardiovascular effects was previously reported in humans [7,8,13]. Others previously observed hysteresis with subjective effects (Visual Analogue Scale (VAS) scores) and plasma cocaine concentrations following smoked (SM) and intravenous (IV) cocaine in humans [7,8,11-13] and in rhesus monkeys with discriminative effects (which model subjective effects in humans) and plasma cocaine concentrations following intramuscular cocaine administration [14]. Few studies examined pharmacodynamic effects and correlations of cocaine oral fluid (OF) concentrations [10,15], and none examined concentration-effect curves for subjective measures and OF cocaine concentrations following controlled cocaine administration. Additional research is needed to support OF as an alternative matrix to blood for DUID.

Cocaine Disposition in Blood and Plasma

Cocaine and metabolites' pharmacokinetics are well established in blood and plasma. Previous studies examined cocaine kinetics following controlled IV, SM, intranasal (IN), and oral cocaine administration [7,10,11,13,16-19]. Peak plasma cocaine concentrations are more rapidly achieved following IV and SM cocaine administration compared to oral and IN [7]. Cocaine is rapidly metabolized by butyrylcholinesterase and carboxylesterases to two major inactive metabolites: benzoylecgonine (BE), formed predominantly in the liver, and ecgonine methyl ester (EME), formed in the plasma and

intestinal lining [20,21]. Cocaine also is unstable, hydrolyzing *in vitro* to BE and EME spontaneously (without enzymes) at physiological temperature and pH [22]. Additionally, cocaine is metabolized by *N*-demethylation to norcocaine, an active although minor metabolite in humans [23]. Cocaethylene is another metabolite of cocaine that can be produced when used in combination with ethyl alcohol by the trans-esterification of cocaine with ethyl alcohol *via* liver methylesterases. Cocaethylene also may be present in illicit and pharmaceutical cocaine [24].

Following 25 mg IV cocaine, peak plasma concentrations were reached immediately after dosing (0.04 h) with concentrations ranging from 97-347 µg/L [7]. BE peak concentrations (60.6-165.4 µg/L) were reached 2.0 h post-administration. Reported plasma cocaine half-lives were 37-89 min following controlled IV cocaine administration [7,10,13,17,19], with much longer BE and EME elimination half-lives. Plasma and OF cocaine and metabolite pharmacokinetics and resultant plasma-OF ratios are limited, especially after controlled IV cocaine administration.

Cocaine Disposition in Alternative Matrices

Oral Fluid

The use of alternative matrices, such as OF, for detecting recent drug consumption is increasing due to its non-invasive and observed collection, gender neutral collection, and reduced potential for adulteration, dilution and substitution compared to urine [25-28]. Although plasma provides an estimate of circulating blood concentrations, OF reflects free unbound drug, which may better reflect drug available for binding to the drug's active site or receptor [25,29,30]. Additionally, significant correlations between OF and plasma cocaine concentrations [10,15,31,32] and OF and blood cocaine

concentrations [33] were observed, making OF an attractive matrix for estimating impairment and windows of detection. Previously, cocaine OF concentrations following controlled IV [10,32,34-38], SM [35-38], IN [35,37,38], oral [38,39], and subcutaneous (SC) administration [31] were investigated; however, only three of these studies examined cocaine and metabolite OF pharmacokinetics [31,37,39]. Furthermore, limited studies examined cocaine and metabolite detection windows at different confirmatory cutoff concentrations [31,38], which is critical for establishing confirmatory OF guidelines, and none evaluated commercially available OF collection devices containing stabilizing and elution buffers.

Cocaine detection windows in OF are dependent on factors including analyte, administration route, frequency of drug exposure (repeated administration), cutoffs, and OF collection methods. Additionally, duration of drug detection windows are different depending on the purpose of monitoring. For DUID programs and roadside testing, detection windows should mirror the window of drug impairment; however, for workplace and pain management drug testing, longer detection windows are ideal due to widely separated specimen collections [40]. Cocaine can be detected in OF between 4.1-17.7 h following 25 mg IV, 32 mg IN, 42 mg SM cocaine, and 75 mg/70 kg and 150 mg/70 kg SC cocaine administration, depending upon cutoffs [31,38]; whereas, BE, the primary inactive cocaine metabolite, OF detection rates can range from 5.0-47 h. Following repeated oral cocaine administration (375-2000 mg), OF cocaine and BE were detected up to 21.2 and 50.0 h, respectively [38], indicating extended windows of detection with repeated dosing or bingeing, common with recreational use.

It is unclear if OF collected with various commercially available devices, containing different elution and stabilization buffers, will exhibit similar cocaine concentration time profiles and kinetics over time. The Substance Abuse and Mental Health Services Administration (SAMHSA) and DRUID proposed confirmatory OF cocaine cutoff concentrations of 8 and 10 µg/L, respectively [41,42], and 8 and 10 µg/L for BE. Eight µg/L cocaine in OF also was recommended for suspected drug-impaired driving cases [43] and a 10 µg/L OF cocaine concentration was suggested by the Talloires recommendation [44].

Breath

There is increased interest in utilizing alternative matrices for identifying drug use in DUID, workplace, clinical, and anti-doping programs [26,29,30]. Several studies investigated the potential of exhaled breath as an alternative matrix for detecting recent consumption of drugs of abuse [45-54], including cocaine [51,53,54]. This concept is based on the hypothesis that exogenous substances contaminate the airway lining fluid creating aerosol particles (microparticles) that can be detected in exhaled breath [53]. Exhaled breath contains microparticles that can carry non-volatile compounds, including airway lining fluid, out of the lungs. Formation of these microparticles is believed to occur when the airway opens during inhalation [55]. Therefore, if the lungs are contaminated with exogenous substances, they can become trapped in the airway lining fluid and thus, distribute into the breath upon exhalation.

Three studies documented cocaine in exhaled breath [51,53,54]. Cocaine was detected (80 pg/min) in one patient recovering from acute self-reported cocaine intoxication [51], and in another, cocaine was detected approximately 24 h after drug

intake in 10 patients following acute intoxication [53]. Cocaine breath concentrations were 29-13,000 pg/device (median 60 pg/device); BE was quantified in four cases at 18-560 pg/device (median 43 pg/device). BE was only found when cocaine was detected. In a study examining paired exhaled breath and urine specimens from prison inmates arriving at correctional facilities [54], 22 of 247 specimens were positive for drugs of abuse in both specimens. Only one case detected both cocaine (12 pg/device) and BE (3 pg/device) in breath; however, individual data were not provided to indicate how many urine samples were positive for BE. Currently, there are no controlled cocaine administration studies examining cocaine and its metabolites in exhaled breath.

Dried Blood Spots

Dried blood spot (DBS) sampling refers to a microsampling technique of increasing interest in pharmaceutical, clinical and forensic settings [56]. DBS sampling is not a novel concept, as it was utilized over a century ago to monitor glucose concentrations in rabbits [56] and also proved useful in newborn infant screening programs [57,58]. Recently, the use of alternative matrices like DBS for detecting recent drug consumption is increasing due to easy, less invasive collection (via finger puncture) and increased stability compared to blood [59,60]. Increased analyte stability is especially important when rapid degradation of compounds in blood was documented [61,62]. Several studies demonstrated minimal analyte loss in DBS as compared to other biological matrices, like venous blood, for unstable compounds including benzodiazepines, zopiclone, 6-acetylmorphine (6-AM) and cocaine [60,63,64]. Additionally, DBS concentrations, unlike urine or hair, should reflect current concentrations in blood [65].

There are several published DBS applications for therapeutic drug monitoring (TDM) [66-70], antidoping control [71] and drugs of abuse analysis including cannabinoids [72], opiates [62,73], 3,4-methylenedioxymethamphetamine (MDMA) [74], γ-hydroxybutyric acid (GHB) [75], and cocaine [58,59,60,63,65,71,73,76-78]. Henderson *et al.* described the first qualitative report of drugs of abuse in DBS to determine prenatal cocaine use in mothers, utilizing a modified BE urine radioimmunoassay, and found results correlated well with gas chromatography-mass spectrometry (GC-MS) determinations in venous blood [63]. Since then, multiple liquid chromatography (LC)-mass spectrometry-based methods were published for the detection of cocaine and/or cocaine metabolites (including BE, EME, or cocaethylene) in DBS [56]; however, limited studies examined DBS results with corresponding venous blood concentrations from authentic specimens [58,59,65,73,77,78].

Mercolini *et al.* compared DBS and plasma cocaine, BE, and cocaethylene concentrations from one cocaine user utilizing LC coupled to spectrofluorimetric detection with a 20 µg/L limit of quantification (LOQ) [59]. DBS were collected via finger puncture; 10 µL blood was collected with a micropipette and dispersed onto the DBS card. The authors found that capillary DBS correlated well with venous plasma concentrations after an arbitrary hematocrit factor (1.62 for women and 1.92 for men) was applied. Others also reported good correlations between DBS and venous blood cocaine and metabolite concentrations from limited specimens obtained from polydrug users [78], DUID cases [73], and postmortem (PM) specimens [65]. A limitation of these studies is that the authors did not investigate capillary DBS, as Mercolini *et al.* performed [59]; rather, they dispersed venous blood onto DBS cards. More studies are needed to

demonstrate the equivalence between capillary DBS and venous blood before this alternative matrix can be utilized to determine recent drug consumption.

Roadside Drug Testing

In order to deter drivers from DUID, it is critical for law enforcement to have sensitive, specific, reliable and easy to use devices to detect recent drug use at the roadside. Traditionally, urine is utilized for on-site screening procedures; however, this is difficult and invasive to collect at the roadside, and the presence of drugs and their metabolites in urine does not necessarily reflect recent use [79]. OF offers an advantage over urine and blood as it can be collected non-invasively at the roadside at the time of driving [80,81]. Additionally, previous studies demonstrated correlations between OF cocaine concentrations and pharmacodynamic effects [10,15].

Interest in utilizing OF for DUID cases and inherent advantages over urine led to the production of commercially available OF on-site drug testing devices. Current devices utilize lateral flow immunochromatography, offering presumptive evidence of drug intake [82,83]. If the on-site device screens positive for a drug, a second OF confirmation sample is collected that contains an elution/stabilizing buffer to improve drug recovery and stabilization. Roadside OF testing devices must be reliable and accurate; suggested performance criteria for on-site OF screening devices were 80% for sensitivity, specificity, and efficiency [84]. Several roadside studies, including the 1999 and 2003-2005 European roadside testing assessments (Rosita and Rosita-2) [85,86] and the DRUID project [84] examined various OF on-site testing devices and demonstrated limited sensitivity for all substances tested (amphetamines, cannabis, cocaine, opiates, and benzodiazepines.

The Draeger DrugTest 5000 is an OF on-site screening device capable of detecting cocaine (20 µg/L screening cutoff) and five other drug classes, including opiates, benzodiazepines, Δ^9-tetrahydrocannabinol (THC), amphetamine and methamphetamine. Sensitivities for cocaine detection were 64-67% for 50 µg/L plasma confirmation cutoff [87], 50% for 10 µg/L OF confirmation cutoff [83], 50% for 10 µg/L OF confirmation cutoff [88], 97% for 10 µg/L OF confirmation cutoff [89], 76% for 10 µg/L serum confirmation cutoff [90], and most recently 88.9% for 10 µg/L OF confirmation cutoff [91]. Specificities of the DrugTest 5000 ranged from 60-100% based on cocaine only. Recently, a modified version of the DrugTest 5000 was manufactured replacing the benzodiazepine strip with a new BE strip, with an identical screening cutoff of 20 µg/L and equivalent cross-reactivity to cocaine and BE. To our knowledge, no previous studies evaluated the modified DrugTest 5000 for the detection of cocaine and BE.

B. Synthetic Cathinone Pharmacokinetics, Analytical Methods, and Toxicological Findings from Human Performance and Postmortem Cases

(*As submitted to* Drug Metabolism Reviews, *March 2016*)[1]

Abstract

Synthetic cathinones are commonly abused novel psychoactive substances (NPS). We present a comprehensive systematic review addressing *in vitro* and *in vivo* synthetic cathinone pharmacokinetics, analytical methods for detection and quantification in

[1] Ellefsen K.N. *et al.* Synthetic cathinone pharmacokinetics, analytical methods, and toxicological findings from human performance and postmortem cases. *Drug Metabolism Reviews*. 24 March 2016 (Under review)

biological matrices, and toxicological findings from human performance and PM toxicology cases. Few preclinical administration studies examined synthetic cathinone pharmacokinetic profiles (absorption, distribution, metabolism and excretion), and only one investigated metabolite pharmacokinetics. Synthetic cathinone metabolic profiling studies, primarily with human liver microsomes, elucidated metabolite structures and identified suitable biomarkers in addition to parent compounds. Generally, cathinone derivatives underwent ketone reduction, carbonylation of the pyrrolidine ring, and oxidative reactions, with phase II metabolites also detected. Reliable analytical methods are necessary for cathinone identification in biological matrices to document intake and link adverse events to specific compounds and concentrations. NPS analytical methods are constrained in their ability to detect new emerging synthetic cathinones due to limited commercially available reference standards and continuous development of new analogs. Immunoassay screening methods are especially affected, but also gas-chromatography and liquid-chromatography mass spectrometry confirmation methods. Non-targeted high resolution-mass spectrometry (HRMS) screening methods are advantageous, as they allow for retrospective data analysis and easier addition of new synthetic cathinones to existing methods. Lack of controlled administration studies in humans complicate interpretation of synthetic cathinones in biological matrices, as dosing information is typically unknown. Furthermore, antemortem and PM concentrations often overlap and the presence of other psychoactive substances are typically found in combination with cathinones derivatives, further confounding result interpretation.

Introduction

NPS continue to appear on the drug market in an effort to bypass controlled substances' legislation. These purported "legal highs" pose problems for law enforcement, military and public safety officials, and also toxicologists who must identify an unending variety of new drugs of abuse. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) reported 41 NPS identified for the first time across Europe in 2010, 81 in 2013 and 101 in 2014 [92]. By the end of December 2014, the United Nations Office on Drug Crime (UNODC) early warning advisory reported a total of 541 NPS [93]. There are few pharmacological and toxicological data on NPS, a wide range of compounds including synthetic cathinones and cannabinoids, phenethylamines, tryptamines, piperazines, ketamine, and other plant-based psychoactive substances [94].

Synthetic cathinones were the largest NPS category identified by EMCDDA in 2014 (31/101) [92], with a 60-fold increase in seizures from 2008 to 2013. Synthetic cathinones are sold primarily online and in head shops or convenience stores as "plant food," "bath salts," or "research chemicals," labeled as "not for human consumption" in order to avoid potential regulation [95-97]. These NPS appeared on the US market in 2010 as legal alternatives to MDMA, amphetamine, or cocaine [98]. They are stimulant-like drugs derived from cathinone, the active ingredient of the khat plant *Catha edulis*, often ingested orally or insufflated; however, recent reports showed increased injection administration [99]. In 2010, the American Association of Poison Control Centers (AAPC) reported 304 phone calls regarding "bath salt" consumption, increasing dramatically in 2011 to 6,138 synthetic cathinones calls [100]. As a result of this increased presence, the synthetic cathinones 3,4-methylenedioxypyrovalerone (MDPV), 4-methylmethcathinone (mephedrone), and 3,4-methylenedioxymethcathinone

(methylone) were regulated as Schedule I controlled substances in the US in 2012 and 2013 (methylone) [101,102], following emergency scheduling of these three compounds in 2011. Since initial scheduling, ten additional cathinones were temporarily scheduled as class I drugs: 4-methylethcathinone (4-MEC), 4-methyl-α-pyrrolidinopropiophenone (4-MPPP), α-pyrrolidinopentiophenone (α-PVP), butylone, pentedrone, pentylone, 4-fluoromethcathinone (4-FMC), 3-fluromethcathinone (3-FMC), naphyrone, and α-pyrrolidinobutiophenone (α-PBP) in 2014 [103], and extended for another year in 2016 [104]. Additionally, all 50 states legislatively banned synthetic cathinone derivatives [105]. Despite scheduling, methylone, ethylone and α-PVP remain in the top 25 most frequently identified drugs in the US in 2014 [3].

Little is known about the actual prevalence of synthetic cathinones despite growing media attention and regulation of many cathinone derivatives. Reports of synthetic cathinones from state and local forensic laboratories increased from 142 reports in the first half of 2010 to 7,997 during the first half of 2013 [98], with methylone detected in 65% and α-PVP in 15% of reports in 2013. The Drug Enforcement Administration (DEA) and Customs and Border Patrol (CBP) also detected methylone in just over half (51%) of the reported 518 synthetic cathinone cases from January to June 2013 [98]. By the end of 2014, ethylone (*n*=5,425), methylone (*n*=4,768), α-PVP (*n*=3,905) and MDPV (*n*=409) accounted for 5.3% of total phenethylamine reports from state and local forensic laboratories (methamphetamine comprised 86% of these phenethylamine reports) [106]. Synthetic cathinone abuse is documented across population groups from mid-to-late adolescents to older adults [107]. Kriikku *et al.* reported the prevalence of MDPV among drivers apprehended for DUID in Finland from

August 2009 to August 2010. Of all confirmed DUID cases, 5.7% (*n*=259) tested positive for MDPV [108]. In a recent self-report survey of 2349 students at a large university in the Southeastern United States, 1.1% reported at least one synthetic cathinone intake in their lifetime [109]; likewise, only 1.1% (*n*=94) of high school seniors surveyed between 2012-2013 (*n*=8,604) reported "bath salt" use in the last year, with 33% using on one or two occasions and 18% reporting 40 or more times [110]. In a cohort of injection drug users (*n*=485) in San Diego, California enrolled from June 2012 to September 2013, 7% reported ever using synthetic cathinones in their lifetime [111]. Another US study analyzing 34,561 urine samples for designer stimulants, between February 2011 and January 2013, reported α-PVP (2.5%), MDPV (1.7%), pentedrone (1.2%), and methylone (0.5%) as the most prevalent synthetic cathinones in urine [112]. Although synthetic cathinone prevalence appears to be on the decline, a growing number of countries reported a wider range of emerging NPS [93].

Synthetic cathinones are novel stimulants producing effects similar to methamphetamine, cocaine, or MDMA [113-115]. Symptoms of cathinone ingestion include euphoria [97,114,115], agitation [115-120], combative and violent behavior [116,118,121-123], tachycardia [115,116,118,120], seizures [124], acidosis [120], hallucinations [116,117,122,125], paranoia [116-118,122], psychosis [121,123,126] and in some cases excited delirium [117,120] and even death [119,127-133]. Despite the life-threatening effects of these drugs, few data are available about the pharmacology and the behavioral and physiological mechanisms of action of synthetic cathinones.

Recent preclinical studies demonstrated that synthetic cathinone derivatives interact with monoamine transporters in two ways: monoamine transporter blockers,

similar to cocaine, or monoamine transporter substrates promoting the release of neurotransmitters, like amphetamine and MDMA [134-140]. It was suggested that synthetic cathinones exhibit higher dopaminergic activity compared to their phenethylamine analogs [141], thus producing greater risk of dependence. Simmler *et al.* observed that all cathinones are potent norepinephrine (NE) transporter inhibitors but differ in dopamine (DA) and serotonin (5-HT) transporter inhibition [139]. Baumann *et al.* reported that methylone (a 3, 4-methylenedioxy cathinone derivative) and mephedrone (a ring substituted cathinone) are fully efficacious transporter releasers of DA, NE, and 5-HT, producing *in vivo* neurochemical effects resembling MDMA [135]. 4-MEC was found to be an equipotent inhibitor at all monoamine transporters and a 5-HT releaser similar to MDMA [139]; Saha *et al*. also noted the "hybrid" activity of 4-MEC (inhibiting DA reuptake and releasing 5-HT) [140]. Methedrone, a 4-methoxy-ring substituted cathinone, is a more potent 5-HT transporter inhibitor than DA and released NE and 5-HT similar to para-methoxymethamphetamine (PMMA), its phenethylamine analog [139]. In contrast, α-pyrrolidinophenones MDPV, α-PVP and 4-MPPP act as monoamine transporter blockers increasing extracellular DA [137,140,142-144], and posing a higher risk of addiction [142,144]. In addition, the high lipophilicity of the pyrrolidine ring increases permeability through the blood-brain-barrier, and hence, increased potency and abuse potential [137,145,146].

Synthetic cathinone identification in biological matrices is essential to document intake in clinical and forensic settings to link toxicity to causative agents and aid in interpretation of NPS results for both human performance and PM cases. This requires laboratories to develop reliable analytical methods (both qualitative and quantitative) and

establish synthetic cathinone detection windows in biological matrices. A challenging issue with synthetic cathinone detection is their apparent instability in biological matrices, especially in blood and urine [147-151]. Additionally, deterrence of synthetic cathinone abuse is often hampered by the lack of a high-throughput immunoassay screen; especially since immunoassay screening methods are limited in their ability to detect new emerging synthetic cathinones. Many GC-MS and liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for synthetic cathinone identification and quantification are increasingly available for detection in a variety of biological matrices; however, with continuous NPS synthesis and marketing, novel cathinones often go undetected. Simultaneous targeted quantitative and non-targeted screening methods by HRMS are advantageous as retrospective data analysis is possible and addition of new synthetic cathinones to existing methods is facilitated. Currently, there are no government mandated screening and confirmation cutoffs for cathinone derivatives in any biological matrix. Commercially available reference standards are necessary to incorporate drugs and metabolites into analytical methods and to support forensic identification. Therefore, *in vitro* and *in vivo* studies to determine the most important analytical targets (parent drug and/or metabolites) for synthetic cathinones in biological specimens are critical.

The scope of this current review is focused on published literature addressing *in vitro* and *in vivo* synthetic cathinone pharmacokinetics, analytical methods for synthetic cathinone detection and quantification in biological matrices, and concentrations detected in both human performance and PM toxicology cases to aid in interpretation of clinical and forensic results.

Methods

We conducted a literature review of 3 electronic databases (PubMed®, Embase™, and Web of Science™) up to July 31, 2015. In addition, we expanded our query employing Google and Google Scholar, and hand searched reference lists of identified articles. Database-specific search strategies with multiple keywords (*eg.* "synthetic cathinones", "cathinone derivatives", and "pyrrolidinophenone derivatives") were employed. Each identified article was categorized into the following topics (based on title and abstract review): epidemiology, legal status, chemistry, animal and human pharmacology (pharmacodynamics and pharmacokinetics), detection and identification, case reports, mechanisms of action, and street use and marketing. Articles before 2010 were included only if the study was not replicated more recently. We identified 460 synthetic cathinone related articles; the review covers pharmacokinetics (45), analytical detection and quantification (68), and toxicological findings in human performance and PM cases (47).

Pharmacokinetics

Limited studies were identified describing *in vitro* and *in vivo* pharmacokinetic profiles of synthetic cathinones. This information is necessary to aid in interpretation of toxicology results, determine analytical targets (parent and/or metabolites), and provide preclinical safety data for future controlled administration studies. Only three preclinical studies were identified examining complete synthetic cathinone pharmacokinetic profiles in rats, including methylone, mephedrone and MDPV. To date, no controlled administration studies investigated synthetic cathinone pharmacokinetic profiles in humans, although one study examined the excretion profile of synthetic cathinones

following self-administration. Several studies (*n*=4) also investigated synthetic cathinone distribution into PM tissues. Additionally, between 2002 and 2015, 37 articles were identified describing synthetic cathinone metabolism including 6 reviews, and 12 *in vitro*, 12 *in vivo* animal and 13 *in vivo* human metabolism studies.

Absorption, Distribution and Excretion

One of the first studies characterizing methylone's pharmacokinetic profile in rats administered 10 mg/kg IV, and 15 and 30 mg/kg oral methylone (doses equivalent to those in humans, 100-300 mg). [152]. For IV administration, maximum methylone plasma concentrations (5,272 µg/L) were reached immediately, with an elimination $t_{1/2}$ of 0.95 h and a 538 mL volume of distribution at steady-state (V_{ss}). Following oral administration, methylone exhibited linear pharmacokinetics, as plasma concentrations were dose-proportionate, with areas-under-the-curve (AUC) of 5740 and 9989 h*µg/L for 15 and 30 mg/kg doses, respectively. Time to maximum concentrations (T_{max}) were reached between 0.5 and 1 h. Steady-state volume of distribution also was high following oral administration (433 mL) suggesting that methylone is extensively distributed into tissues and also rapidly eliminated based on oral $t_{1/2}$ (0.55 h). Methylone bioavailability was 78-89% and approximately 13% protein bound.

Mephedrone pharmacokinetics also were studied in rats following 10 mg/kg IV, and 30 and 60 mg/kg oral mephedrone, with blood collected over 9 h [153]. Maximum mephedrone plasma concentrations (7,221 µg/L) were achieved immediately following administration and were near the LOQ at 4 h. After intravenous administration, mephedrone $t_{1/2}$ was much shorter (0.37 h) than methylone's $t_{1/2}$ (0.95 h) [152], although it was nearly the same following oral mephedrone (0.55 h) [153]. Non-linear increases in

AUC were observed with oral mephedrone administration (30 mg/kg: 295 h*µg/L, 60 mg/kg: 895 h*µg/L), potentially due to saturation of liver metabolic enzymes and pathways consistent with the low bioavailability observed (~10%). Additionally, the authors reported 21.6% protein binding and 1.85 brain-to-plasma concentration ratio indicating that mephedrone readily crosses the blood-brain barrier.

Anizan *et al.* reported the *in vivo* pharmacokinetic profile of MDPV and its metabolites in rats after 0.5, 1 and 2 mg/kg SC administration in rats, with blood collected up to 8 h [154]. This was the first study to also investigate the pharmacokinetics of synthetic cathinone metabolites in addition to the parent compound. The authors reported dose-proportionate increases in maximum concentrations and AUC for MDPV and its two metabolites (4-OH-3-methoxypyrovalerone and 3,4-dihydroxypyrovalerone). The highest AUC was observed for the predominant metabolite, 4-OH-3-methoxypyrovalerone (11,366-47,724 min*µg/L). Peak MDPV plasma concentrations were achieved 0.22-0.31 h with metabolite T_{max} at 3.1-4 h. MDPV half-life (1.3-1.6 h) was longer than that of methylone and mephedrone, as described earlier; MDPV metabolite $t_{1/2}$ could not be calculated due to limited data points.

Excretion of α-PVP and α-PBP was examined in one individual after IV administration of an unknown dose [155]. After hospital admission, urine was collected every morning for a month to examine excretion profiles. The highest detected urinary concentrations were 1,200 and 1,600 µg/L for α-PVP and α-PBP, respectively, at least 32 h after injection. Excretion $t_{1/2}$ was estimated to be 22 (α-PVP) and 11 (α-PBP) h. However, the authors reported that urinary pH is a significant factor in excretion rates for

amphetamine-like substances, as acidified urine facilitates more rapid elimination from the body.

PM distribution of synthetic cathinones, including methylone, MDPV and α-PVP, also was investigated [156-159]. Cawrse *et al.* examined MDPV distribution in blood (central and peripheral), liver, kidney, spleen and bile and reported that methylone does not sequester in a particular tissue type after death; however, there were insufficient data to address PM redistribution. The highest MDPV concentrations after those in urine, were found in the kidneys, with similar liver concentrations, suggesting both are suitable for analyses. Liver-to-blood concentration ratio was 2.7 (range 1.2-4.7). MDPV also showed uniform distribution across multiple tissues [157,158], although tissues responsible for detoxification and excretion (kidney, liver, bile) exhibited higher MDPV concentrations [157]. Interestingly, concentrations varied across different brain locations; cerebellum, medulla and occipital regions were higher than the frontal and lentiform nucleus. The ratio of heart-to-peripheral blood concentrations (1.3) did not indicate PM redistribution [157]. Conversely, Hasegawa *et al.* investigating α-PVP PM distribution, reported that despite lower heart than peripheral blood concentrations, high α-PVP concentrations in heart muscle suggested PM redistribution via diffusion from heart blood to heart muscle [159]. Additionally, the distribution of the α-PVP metabolite, OH-α-PVP, was similar to that of α-PVP. Metabolite concentrations were highest in the liver and kidney, which is expected, as that is likely the main site of α-PVP metabolism.

Metabolism

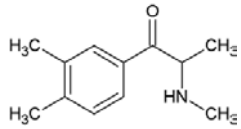
In vitro studies determined metabolite profiles and elucidated new synthetic cathinone structures, while *in vivo* controlled drug administration studies in animals and

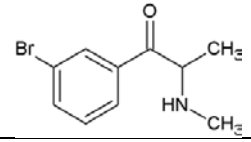
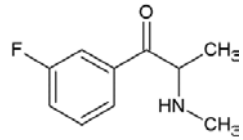
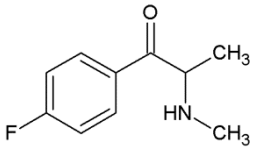
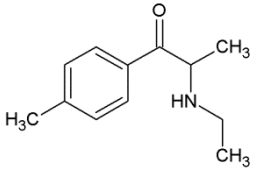
humans characterize pharmacodynamic effects and document authentic metabolites. Human liver microsomes (HLM) incubations were the primary *in vitro* system utilized, although one study utilized rabbit liver slices, one rat liver hepatocytes and only one study utilized human hepatocytes. Compared to other biological systems, like HLM, cytosol and S9 fractions, human hepatocytes are better suited for predicting authentic human metabolites as human phase I and II enzymes, cofactors and membranes more closely mimic the *in vivo* liver environment. Experiments utilizing subcellular fractions (like HLM) may require the addition of cofactors and may not produce metabolites in relevant ratios, as achieved in hepatocytes. With the exception of one study, all *in vivo* animal studies administered synthetic cathinones to rats via gastric intubation (GI) at varying doses to analyze urine and identify biotransformations *in vivo*. *In vivo* human studies generally were self-administration studies with unknown administration route and dose. Urine was the most common matrix for elucidating synthetic cathinone metabolic pathways in humans, although one study reported metabolites in blood. Table 1 contains data on synthetic cathinones biotransformations for a variety of ring and side-chain-, methylenedioxy-, and pyrrolidinyl-substituted cathinones.

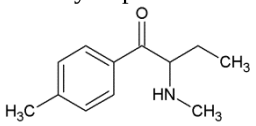
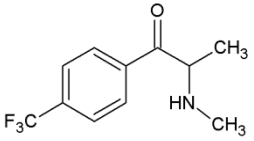
In vitro Studies

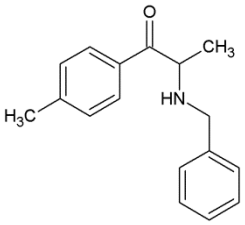
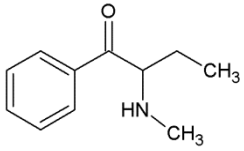
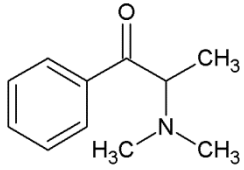
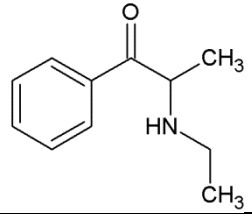
For ring and side-chain substituted cathinones, *N*-dealkylation and ketone reduction were common pathways identified *in vitro*. With the exception of 4-MEC [160,161], *N*-dealkylated alcohol metabolites were reported [162-164]. Ketone reduced diastereisomers also were typically identified *in vitro*. Hydroxylation and carboxylation of the 4′-methyl group also was common for synthetic cathinones with a 4′-methyl substitution [160-165]. Methedrone, a 4-methoxy-substituted cathinone, also underwent

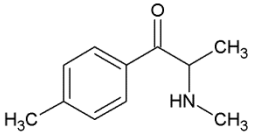
Table 1. In vitro and in vivo synthetic cathinone biotransformations. Isomers not included in total number of metabolites and Phase II metabolites listed in parentheses.

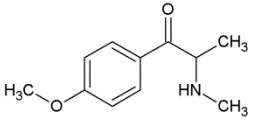
Compounds	Study type	Matrix (species)	Total # Phase I (II)	Biotransformation, Phase I (reaction site)	Major phase I metabolite(s)	Phase II metabolites	Other findings	Reference
Ring or side chain-substituted cathinones								
3,4-DMMC 	*In vivo*	Urine (human)	2	Ketone reduction (β); *N*-demethylation; & combinations	*N*-demethylated alcohol	Not reported	34,561 urine samples estimated metabolic profiles; similar to mephedrone	[112]
	In vivo	Urine (human)	3	Ketone reduction (β); *N*-demethylation; & combinations	β-OH-DMMC (ketone reduction)	Partially conjugated (increased concentrations with hydrolysis)	n=1; 30 mg dose; unknown administration; collected 6 h after intake	[167]
	In vitro	HLM	6	Ketone reduction (β); *N*-demethylation; Hydroxylation (not specified); Carboxylation (4′-methyl); & combinations	Not reported	Not reported	*In silico* software also utilized; *N*-demethylated + hydroxylated metabolite not previously reported	[162]
	In vivo	Urine (human)	6	Same as above	β-OH-DMMC, 3,4-DMC, β-OH-DMC	Not reported	n=2; unknown administration	[162]

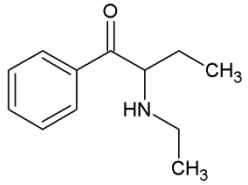
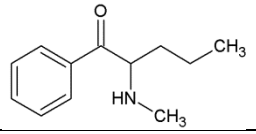
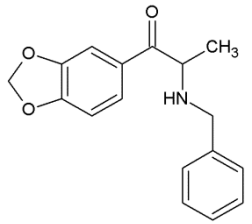
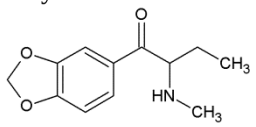
Compounds	Study type	Matrix (species)	Total # Phase I (II)	Biotransformation, Phase I (reaction site)	Major phase I metabolite(s)	Phase II metabolites	Other findings	Reference
3-Bromomethcathinone 	*In vivo*	Urine (rat)	3 (1)	*N*-demethylation; Ketone reduction (β); Hydroxylation (aromatic ring); & combinations	Not reported	Glucuronide	20 mg/kg dose via GI; no parent detected	[168]
3-Fluromethcathinone 	*In vivo*	Urine (rat)	5 (1)	*N*-demethylation; Ketone reduction (β); Hydroxylation (aromatic ring); & combinations	Not reported	Glucuronide	20 mg/kg dose via GI; parent detected	[168]
	In vitro	Liver slices (rabbit)	4	*N*-demethylation; Dehydrogenation; Hydroxylation (aromatic ring); Ketone reduction (β); & combinations	Not reported	Not reported		[163]
4-Fluoromethcathinone 	*In vivo*	Urine (human)	2	Ketone reduction (β); *N*-demethylation; & combinations	*N*-demethylated alcohol	Not reported	Similar profile to mephedrone; parent less abundant	[112]
	In vitro	HLM	2	*N*-demethylation; Hydroxylation (alkyl chain)	Not reported	Not reported		[161]
4-Methylethcathinone 	*In vivo*	Urine (human)	6 (1)	*N*-demethylation; Ketone reduction (β); Hydroxylation (4′-methyl); Carboxylation (4′-methyl); & combinations	Not reported	Glucuronide (hydroxylated metabolite)		[160]

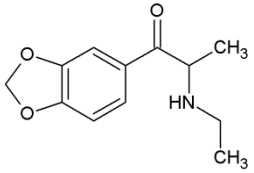
Compounds	Study type	Matrix (species)	Total # Phase I (II)	Biotransformation, Phase I (reaction site)	Major phase I metabolite(s)	Phase II metabolites	Other findings	Reference
4-Methylethcathinone (*continued)*	*In vitro*	HLM	4	*N*-demethylation; Ketone reduction (β); Hydroxylation (4′-methyl); Carboxylation (4′-methyl); & combinations	Not reported	Not reported	Could not confirm *N*-deethyl-dihydro- & 4-carboxy-dihydro-metabolites found *in vivo*	[160]
	In vivo	Urine (human)	2	Ketone reduction (β); *N*-demethylation; & combinations	*N*-dealkylated alcohol	Not reported	Similar profile to mephedrone; parent less abundant	[112]
	In vitro	HLM	3	*N*-demethylation; Hydroxylation (alkyl chain); Ketone reduction (β)	Not reported	Not reported		[161]
4-Methylbuphedrone 	*In vivo*	Urine (human)	2	Ketone reduction (β); *N*-demethylation; & combinations	*N*-dealkylated alcohol	Not reported	Similar profile to mephedrone; parent less abundant	[112]
4-TFMMC 	*In vitro*	Hepatocytes (rat)	7 (4)	*N*-demethylation; Ketone reduction (β); Hydroxylation (aromatic ring); Oxidation (*N*-alkyl chain); Acetylation (primary amine); & combinations	4-trifluoromethyl cathinone; 4-trifluoromethyl ephedrine; 4-trifluoromethyl norephedrine	Glucuronides	$T_{1/2}$ calculated (203.8 min); metabolism significantly different from mephedrone due to trifluoro group	[164]

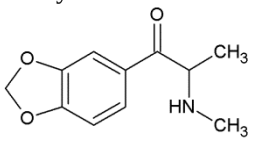
Compounds	Study type	Matrix (species)	Total # Phase I (II)	Biotransformation, Phase I (reaction site)	Major phase I metabolite(s)	Phase II metabolites	Other findings	Reference
Benzedrone 	*In vitro*	HLM	6	*N*-dealkylation; Hydroxylation (benzene, 4′-methyl); Dihydroxylation (benzene, *N*-alkyl chain); Ketone reduction (β); & combinations	Not reported	Not reported		[161]
Buphedrone 	*In vivo*	Urine (human)	2	Ketone reduction (β); *N*-demethylation; & combinations	*N*-dealkylated alcohol	Not reported	Similar profile to mephedrone; parent less abundant	[112]
Dimethylcathinone 	*In vitro*	HLM	2	*N*-demethylation; Ketone reduction (β)	Not reported	Not reported		[161]
Ethcathinone 	*In vivo*	Urine (human)	3	Ketone reduction (β); *N*-dealkylation; Demethylation (*N*-alkyl chain)	*N*-dealkylated alcohol	Not reported	Not reported	[112]

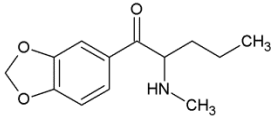
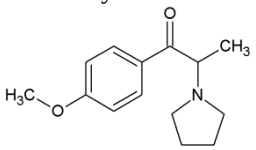
Compounds	Study type	Matrix (species)	Total # Phase I (II)	Biotransformation, Phase I (reaction site)	Major phase I metabolite(s)	Phase II metabolites	Other findings	Reference
Mephedrone 	*In vivo*	Urine (human)	6 (4)	Ketone reduction (β); *N*-demethylation; Hydroxylation (4′-methyl, alkyl chain); Carboxylation (4′-methyl, alkyl chain); & combinations	Not reported	Glucuronides & succinyl conjugate	n=2; 200 mg oral dose; samples collected 4h post-dose; succinyl conjugate not previously reported	[169]
	In vivo	Urine (human)	2	Ketone reduction (β); *N*-demethylation; & combinations	*N*-demethylated alcohol	Not reported	Parent drug less abundant	[112]
	In vitro	HLM	2	*N*-demethylation; Hydroxylation (4′-methyl)	Not reported	Not reported		[165]
	In vivo	Blood (human)	5	Ketone reduction (β); *N*-demethylation; Hydroxylation (4′-methyl); Carboxylation (4′-methyl); & combinations	4′-OH-mephedrone	Not reported	n=4; 4′-carboxy & dihydro-mephedrone not previously reported	[165]

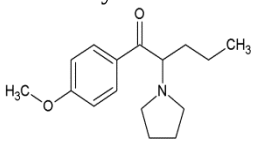
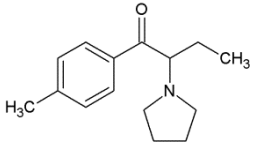
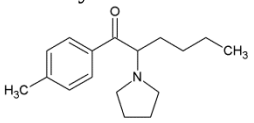
Compounds	Study type	Matrix (species)	Total # Phase I (II)	Biotransformation, Phase I (reaction site)	Major phase I metabolite(s)	Phase II metabolites	Other findings	Reference
Mephedrone *(continued)*	*In vitro*	Hepatocytes (rat)	13(3)	Ketone reduction (β); *N*-demethylation; Hydroxylation (4′-methyl, aromatic ring); Carboxylation (4′-methyl); Acetylation (*N*-alkyl chain, 4′-OH); Oxidation (4′-methyl); & combinations	Normephedrone	Low levels of glucuronides	$T_{1/2}$ calculated (61.9min)	[164]
	In vivo	Urine (rat)	4	Ketone reduction (β); *N*-demethylation; Hydroxylation (4′-methyl); & combinations	Not reported	Increased concentrations of hydroxyl metabolites after hydrolysis	20 mg/kg dose via GI; ketone reduction only observed with *N*-dealkylation	[170]
	In vivo	Urine (human)	5	Same profile as above plus carboxylation (4′-methyl)	4′-carboxy-dihydro mephedrone	Partially excreted as glucuronides &/or sulfates	Patient claimed to take oral mephedrone & butylone powder	[170]
Methedrone 	*In vitro*	HLM & cytosol	5	*O*-demethylation; *N*-demethylation; Ketone reduction (β); Hydroxylation (alkyl chain); & combinations	Not reported	Not reported	3 metabolites reported for 1st time	[166]
	In vitro	HLM	2	*N*-demethylation; *O*-demethylation	Not reported	Not reported		[161]

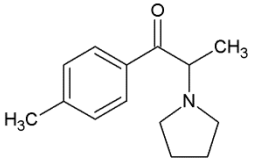
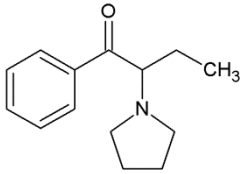
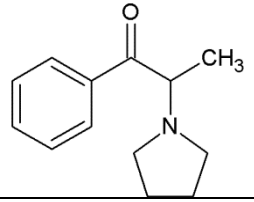
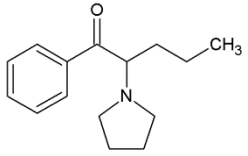
Compounds	Study type	Matrix (species)	Total # Phase I (II)	Biotransformation, Phase I (reaction site)	Major phase I metabolite(s)	Phase II metabolites	Other findings	Reference
N-ethylbuphedrone 	*In vivo*	Urine (human)	2	Ketone reduction (β); *N*-dealkylation; & combinations	*N*-dealkylated alcohol	Not reported	Similar profile to mephedrone; parent less abundant	[112]
Pentedrone 	*In vivo*	Urine (human)	2	Ketone reduction (β); *N*-demethylation; & combinations	*N*-dealkylated alcohol	Not reported	Similar profile to mephedrone; parent less abundant	[112]
Methylenedioxy-substituted cathinones								
BMDP 	*In vitro*	HLM	3	*N*-dealkylation; Ketone reduction (β); Demethylenation	Demethylenated metabolites high intensity	Not reported	Did not add cofactor to observe methylated products	[161]
Butylone 	*In vivo*	Urine (human)	2	Ketone reduction (β); *N*-demethylation; & combinations	Demethylenated metabolite	Not reported	Similar profile to methylone; parent most abundant; methylenedioxy restricts ketone reduction	[112]

Compounds	Study type	Matrix (species)	Total # Phase I (II)	Biotransformation, Phase I (reaction site)	Major phase I metabolite(s)	Phase II metabolites	Other findings	Reference
Butylone *(continued)*	*In vitro*	HLM	3	Demethylenation; *N*-demethylation; Hydroxylation (alkyl chain)	Demethylenated metabolite	Not reported	Did not add cofactor to observe methylated products	[161]
	In vivo	Urine (human)	4 (3)	*N*-demethylation; Ketone reduction (β); Demethylenation; Methylation (3′-OH, 4′-OH); & combinations	4-OH-3-methoxy metabolite	Glucuronides	N=2; route unknown; concentration of hydroxy-methoxy increased after acidic hydrolysis, while only slight increase of unchanged parent & keto-reduced metabolite	[171]
Ethylone 	*In vivo*	Urine (human)	2	Ketone reduction (β); *N*-demethylation; & combinations	Demethylenated metabolite	Not reported	Similar profile to methylone; parent most abundant; methylenedioxy restricts ketone reduction	[112]
	In vitro	HLM	3	Demethylenation; *N*-dealkylation; Hydroxylation (alkyl chain)	Demethylenated metabolite	Not reported	Did not add cofactor to observe methylated products	[161]

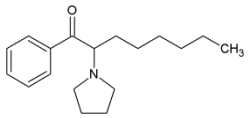
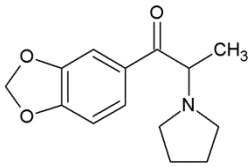
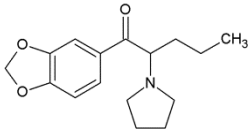
Compounds	Study type	Matrix (species)	Total # Phase I (II)	Biotransformation, Phase I (reaction site)	Major phase I metabolite(s)	Phase II metabolites	Other findings	Reference
Ethylone *(continued)*	*In vivo*	Urine (human)	4 (3)	*N*-dealkylation; Ketone reduction (β); Demethylenation; Methylation (3′-OH, 4′-OH); & combinations	4-OH-3-methoxy metabolite	Glucuronides	n=2; unknown administration; same pathway as butylone	[171]
Methylone 	*In vivo*	Urine (human)	2	Ketone reduction (β); *N*-demethylation; & combinations	Demethylenated metabolite	Not reported	Methylated metabolites not detected; parent most abundant; methylenedioxy restricts ketone reduction	[112]
	In vitro	HLM	4	Ketone reduction (β); *N*-demethylation; Demethylenation; Hydroxylation (*N*)	Dihydro-methcathinone (HHMC)	Not reported	HHMC not previously detected; CYP2D6 primary enzyme	[172]
	In vitro	HLM	3	*N*-demethylation; Demethylenation; Hydroxylation (alkyl chain)	Demethylenated metabolite	Not reported	Did not add cofactor to observe methylated products	[161]
	In vivo	Urine (human)	3	*N*-demethylation; Demethylenation; Methylation (3′-OH or 4′OH); & combinations	4′-OH-3′-methoxy-methcathinone (HMMC)	Glucuronides		[173]

Compounds	Study type	Matrix (species)	Total # Phase I (II)	Biotransformation, Phase I (reaction site)	Major phase I metabolite(s)	Phase II metabolites	Other findings	Reference
Methylone *(continued)*	*In vivo*	Urine (rat)	3	*N*-demethylation; Demethylenation; Methylation (3′-OH or 4′-OH); & combinations	4′-OH-3′-methoxy-methcathinone (HMMC)	Glucuronides; MDC partially conjugated, HMMC mostly conjugated	n=3; 5 mg/kg IP dose	[174]
	In vivo	Urine (human)	3	Same as above	Same as above	Same as above	19 y.o. male oral dose; collected 36h post-dose	[174]
Pentylone 	*In vivo*	Urine (human)	2	Ketone reduction (β); *N*-dealkylation; & combinations	Demethylenated metabolite	Not reported	Similar profile to methylone; parent most abundant; methylenedioxy restricts ketone reduction	[112]
	In vitro	HLM	4	Demethylenation; *N*-dealkylation; Ketone reduction (β); & combinations	Demethylenated metabolites high intensity	Not reported	Did not add cofactor to observe methylated products	[161]
Pyrrolidinyl-substituted cathinones								
4-Methoxy-α-PPP 	*In vivo*	Urine (rat)	7	*O*-demethylation; Hydroxylation (3′); Carbonylation (2″); Oxidative deamination; *O*-methylation (3′); & combinations	4-OH-α-PPP	Hydroxyl groups partially conjugated	20 mg/kg dose via GI	[175]

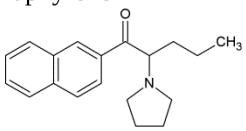
Compounds	Study type	Matrix (species)	Total # Phase I (II)	Biotransformation, Phase I (reaction site)	Major phase I metabolite(s)	Phase II metabolites	Other findings	Reference
4-methoxy-α-PPP *(continued)*	*In vitro*	HLM	1	Not reported	4-OH-α-PPP	Not reported	CYP2C19 catalyzed *O*-demethylation	[175]
4-Methoxy-α-PVP 	*In vitro*	Hepatocytes (human)	9(1)	*O*-demethylation; Ketone reduction (β); *N*-dealkylation; Hydroxylation (2″, pyrrolidine ring); Ring opening; Carbonylation (2″); & combinations	4-OH- α-PVP	Glucuronide	$T_{1/2}$ calculated in HLM (79.7 min); *in silico* software also utilized	[176]
4-Methyl-α-PBP 	*In vivo*	Urine (rat)	7(3)	Hydroxylation (4′-methyl); Carboxylation (4′-methyl); Carbonylation (2″); Ketone reduction (β); Oxidative deamination; & combinations	4-carboxy-α-PBP	Glucuronides	20 mg/kg dose via GI	[177]
4-Methyl-α-PHP 	*In vivo*	Urine (rat)	10	Hydroxylation (4′-methyl, alkyl chain); Carboxylation (4′-methyl); Carbonylation (2″); Ketone reduction (β); & combinations	Not reported	Partial conjugation of carboxy & hydroxyl groups	20 mg/kg dose via GI; dihydro metabolite may be identified in humans; mono-hydroxylations only found in rat liver microsomes	[178]

Compounds	Study type	Matrix (species)	Total # Phase I (II)	Biotransformation, Phase I (reaction site)	Major phase I metabolite(s)	Phase II metabolites	Other findings	Reference
4-Methyl-α-PPP 	*In vivo*	Urine (rat)	5 (3)	Hydroxylation (alkyl chain); Carboxylation (4′-methyl); Carbonylation (2″); Oxidative deamination; & combinations	Not reported	Glucuronides (carboxy groups)	20 mg/kg dose via GI; dihydro metabolite may be identified in humans; mono-hydroxylations only found in rat liver microsomes	[179]
α-PBP 	*In vivo*	Urine (human)	1	Ketone reduction (β) & *N*-dealkylation	Not reported	Not reported	Does not appear to directly reduce ketone, likely due to steric hindrance; parent abundant	[112]
α-PPP 	*In vivo*	Urine (rat)	4	Hydroxylation (4′); Carbonylation (2″); *N*-dealkylation; Ketone reduction (β)	2-oxo-α-PPP	Partially conjugated (hydroxyl groups)	50 mg/kg dose via GI; no hydroxylation of alkyl chain observed	[180]
α-PVP 	*In vitro*	HLM	6(1)	Ketone reduction (β)*;* *N*-dealkylation*;* Ring opening*;* Carbonylation (2″)*;* Hydroxylation (2″, alkyl chain, *N*-alkyl chain)*;* Oxidation (*N*-alkyl chain)*;* & combinations	2″oxo-α-PVP	Glucuronide	Glucuronide not previously identified	[166]

Compounds	Study type	Matrix (species)	Total # Phase I (II)	Biotransformation, Phase I (reaction site)	Major phase I metabolite(s)	Phase II metabolites	Other findings	Reference
α-PVP *(continued)*	*In vivo*	Urine (human)	1	Ketone reduction (β) & *N*-dealkylation	Not reported	Not reported	Similar profile to α-PBP; parent abundant	[112]
	In vivo	Urine (human)	3(1)	Ketone reduction (β); Hydroxylation (2″); Carbonylation (2″)	β-OH-PVP; 2″oxo-α-PVP	Glucuronide (ketone)	n=19; parent detected; interspecies variation accounts for different major metabolites	[181]
	In vivo	Urine (human)	7	Ketone reduction (β); *N*-dealkylation; Hydroxylation (alkyl chain); Carbonylation (2″); Ring opening; Carboxylation (alkyl chain, *N*-alkyl chain); & combinations	β-OH-PVP; 2″oxo-α-PVP; ketone reduction & carbonylation	Not reported	N=8; unknown administration; ketone reduction not previously reported; oxidation of propyl side chain not detected in rats	[162]
	In vitro	HLM	7	Same as above	Not reported	Not reported		[162]
	In vivo	Urine (rat)	10	Hydroxylation (alkyl chain, aromatic ring); *N*-dealkylation; Carbonylation (2″); Ring opening; Carboxylation (*N*-alkyl chain); & combinations	2″oxo-α-PVP; hydroxyl phenyl metabolite	Glucuronides (hydroxylated metabolites)	20 mg/kg dose via GI	[182]

Compounds	Study type	Matrix (species)	Total # Phase I (II)	Biotransformation, Phase I (reaction site)	Major phase I metabolite(s)	Phase II metabolites	Other findings	Reference
PV9 	*In vivo*	Urine (human)	9(2)	Ketone reduction (β); Carbonylation (2″); Hydroxylation (alkyl chain); Oxidation (alkyl chain); Carboxylation (alkyl chain)	Carboxylic acid metabolite	Glucuronides	n=2; Different profile from α-PVP & α-PBP due to alkyl chain length	[183]
MDPPP 	*In vitro*	HLM	1	Demethylenation	3,4-dihydroxy-α-PPP	Not specified	CYP2C19 catalyzed demethylenation	[184]
	In vivo	Urine (rat)	8	Demethylenation; Methylation (3′-OH); Carbonylation (2″); Oxidative deamination; & combinations	4-OH-3-methoxy-α-PPP	Glucuronides (hydroxyl groups partially conjugated)	20 mg/kg dose via GI	[185]
MDPV 	*In vitro*	HLM & cytosol	10(4)	Demethylenation; Hydroxylation (alkyl chain, pyrrolidine ring); Ketone reduction (β); Dehydration (alkyl chain, pyrrolidine ring); *N*-dealkylation; Ring opening; Oxidation (*N*-alkyl chain); & combinations	Catechol pyrovalerone	Glucuronides & sulfates		[166]

Compounds	Study type	Matrix (species)	Total # Phase I (II)	Biotransformation, Phase I (reaction site)	Major phase I metabolite(s)	Phase II metabolites	Other findings	Reference
MDPV *(continued)*	*In vivo*	Urine (human)	-	Not reported	Not reported	Detected in urine with no free excreted metabolites	Similar profile to α-PBP; parent abundant; pyrrolidine ring & methylenedioxy hinder ketone reduction	[112]
	In vivo	Urine (human)	9 (3)	Carbonylation (2″); Demethylenation; Methylation (3′-OH); *N*-dealkylation; Hydroxylation (alkyl chain); & combinations	Demethylenyl-methyl-MDPV	Glucuronides		[186]
	In vivo	Urine (rat)	12 (6)	Carbonylation (2″); Demethylenation; Methylation (3′-OH); Hydroxylation (alkyl chain, aromatic ring); *N*-dealkylation; Ring opening; Carboxylation (*N*-alkyl chain); Oxidation (alkyl chain); & combinations	3′-methylated compound	Glucuronides	Administered via GI; preferably methylated by COMT	[186]
	In vitro	HLM & S9	2	Demethylenation; Methylation (3′-OH); & combinations	Catechol & methyl catechol pyrovalerone	Sulfates & glucuronides	80% MDPV remained unchanged, possibly due to excess drug	[187]

Compounds	Study type	Matrix (species)	Total # Phase I (II)	Biotransformation, Phase I (reaction site)	Major phase I metabolite(s)	Phase II metabolites	Other findings	Reference
Naphyrone 	*In vivo*	Urine (rat)	7(3)	Hydroxylation (naphthyl ring, alkyl chain); Carbonylation (2″); *N*-dealkylation; & combinations	2″oxo-naphyrone	Glucuronides	20 mg/kg dose via GI	[188]
	In vitro	HLM	5	Ketone reduction (β); Hydroxylation (naphthyl ring, alkyl chain); & combinations	Not reported	Not reported	*N*-Dealkylation not detected	[161]

Abbreviations: 3,4-DMC, 3,4-dimethylcathinone; 3,4-DMMC, 3,4-dimethylmethcathinone; 4-TFMMC, 4-trifluoromethylmethcathinone; α-PBP, α-pyrrolidinobutiophenone; α-PHP, α-pyrrolidinohexanophenone; α-PPP, α-pyrrolidinopropiophenone; α-PVP, α-pyrrolidinopentiophenone; BMDP, 3,4-methylenedioxy-*N*-benzylcathinone; COMT, catechol-*O*-methyl transferase; GI, gastric intubation; HHMC, 3,4-dihydroxymethcathinone; HLM, human liver microsomes; IP, intraperitoneal; MDC, 3,4-methylenedioxycathinone; MDPPP, 3,4-methylendioxy-α-pyrrolidinopropiophenone; MDPV, 3,4-methylenedioxypyrovalerone; PV9, α-pyrrolidinooctanophenone; $T_{1/2}$, half-life

O-demethylation [161,166]. Khreit *et al.* was the only study in which $t_{1/2}$ was calculated based on rat hepatocyte incubations for mephedrone and 4-trifluoro-methylmethcathinone (4-TFMMC) [164]. The 4-TFMMC $t_{1/2}$ was significantly longer (203.8 min) compared to mephedrone (61.9 min), likely due to the trifluoro moiety at the 4′ position. These authors also were the first to identify acetylation (*N*-alkyl chain and 4′- OH (mephedrone only)) for these compounds. Glucuronides also were observed, typically bound to hydroxylated metabolites.

Unlike ring and side-chain substituted cathinones, methylenedioxy-cathinones typically underwent demethylenation and *N*-dealkylation *in vitro*, with less ketone reduction [172,161]. Pederson *et al*. reported reduced methylone alcohol [172], not previously observed *in vitro* for methylone, ethylone or butylone [161]. The methylated products of demethylenated metabolites were not identified *in vitro,* as cofactors (*S*-adenosylmethionine) were not added to HLM experiments. In addition, alkyl chain hydroxylation was observed for methylone, ethylone, butylone and pentylone [161,172].

Pyrrolidinyl-substituted cathinones (or α-pyrrolidinophenones) contain a pyrrolidine ring and may also be ring and side-chain- or methylenedioxy-substituted. As such, their *in vitro* metabolism varied from other cathinones. In addition to *N*-demethylation and ketone reduction observed for ring and side-chain substituted cathinones, α-pyrrolidinophenones, like 4-methoxy-α-PVP and α-PVP, also underwent carbonylation to the 2″-oxo metabolite and pyrrolidine ring opening with oxidation reactions [166,176]. Like methedrone, the major metabolic pathway of 4-methoxy-α-PVP also was *O*-demethylation in human hepatocytes [176]. A relatively long $t_{1/2}$ of 79.7 min was determined for 4-methoxy-α-PVP by HLM incubation. Unlike 4-methoxy-α-PVP

and α-PVP, naphyrone did not undergo carbonylation or *N*-dealkylation, although hydroxylations (naphthyl ring and alkyl chain) and ketone reduction were observed [161]. For methylenedioxy-substituted pyrrolidinophenones, carbonylation of the pyrrolidine ring was not identified *in vitro* [166,184,187], although pyrrolidine ring opening with oxidation reactions were reported. The most common biotransformation observed was demethylenation, similar to other methylenedioxy-substituted cathinones. Strano-Rossi *et al*. identified the subsequent methylation of 3′OH following demethylenation for the first time [187]. Eighty percent unchanged parent drug also was detected; however, the authors hypothesized this was due to excess drug in HLM incubations (14.5 µM compared to 1 µM typically employed in HLM experiments).

In vivo Animal Studies

Similar to *in vitro* studies, ring and side-chain substituted cathinones underwent *N*-dealkylation and ketone reduction in rat urine [168,170]. In addition, Meyer *et al*. identified 3′ hydroxylation for halogenated cathinones (3-bromomethcathinone (3-BMC) and 3-FMC) [168]. Mephedrone also exhibited hydroxylation (4′-methyl) in rat urine [170] as cited *in vitro*, but not carboxylation of the 4′-methyl moiety. Following 5 mg/kg intraperitoneal (IP) methylone in rats (n=3), 4-hydroxy-3-methoxymethcathinone (HMMC) was the primary methylone metabolite in rat urine [174], not previously observed *in vitro*. The intermediate demethylenated metabolite and *N*-demethylated metabolite also were reported in rat urine, similar to methylone, butylone, ethylone and pentylone *in vitro*. Increased concentrations following acid and enzymatic (glucuronidase) hydrolysis also were observed for both ring, side-chain and methylenedioxy-substituted cathinones, suggesting conjugation.

For 4′-methyl and 4′-methoxy-substituted pyrrolidinophenones, *N*-dealkylation was observed *in vitro*, but not in rat urine. Similarly, 4′-methoxy-substituted pyrrolidinophenones and 4-MPPP also were not reduced to their corresponding alcohols via ketone reduction potentially due to steric hindrance from the 4′-methyl group [178,179]. Major metabolites identified for 4′-methyl and 4′-methoxy pyrrolidinophenones resulted from carboxylation (4′-methyl) [177,180,189] and *O*-demethylation [179]. Carbonylation of the 2″-oxo metabolite also was reported for all pyrrolidinophenones in rat urine, including methylenedioxy-substituted pyrrolidinophenones, and was the major pathway for α-PPP and α-PVP [180,182]. Interestingly, naphyrone did not undergo carbonylation (2″) *in vitro*; however, it was the main metabolic pathway reported in rat urine [188]. The major metabolic pathway of methylenedioxy-substituted pyrrolidinophenones was demethylenation followed by methylation (3′-OH) [185,186]. Meyer *et al.* predicted that the 3′-OH position was preferentially methylated by COMT [186]. Oxidative deamination also was observed in rat urine for some substituted α-pyrrolidinophenones (4-methoxy-α-PPP, 4-MPPP, 4-methyl-α-PBP (4-MPBP) and 3,4-methylenedioxy-α-pyrrolidinopropiophenone (MDPPP)) [177,179,180,185], unlike in *in vitro* studies. Additionally, partial conjugation of carboxyl and hydroxyl groups was reported.

In vivo Human Studies

Uralets *et al*. analyzed 34,562 urine specimens, submitted for designer drug testing between February 2011 and January 2013, to estimate the range of synthetic cathinones abused in the US and to observe cathinone metabolic profiles at unknown stages of urinary excretion to select metabolites or parent drugs for routine testing [112].

Sixteen synthetic cathinone metabolic profiles were elucidated including 9 ring and side-chain-, 4 methylenedioxy-, and 3 pyrrolidinyl-substituted cathinones. Ring and side-chain substituted cathinones primarily underwent ketone reduction to alcohols (diastereomers) and subsequent *N*-dealkylation to *N*-dealkylated alcohols, with little to no parent compound detected. The same pathway was observed for methylenedioxy-substituted cathinones, although ketone reduction was observed to a much lesser extent, potentially due to steric hindrance from the methylenedioxy group. Ethcathinone also underwent demethylation of the *N*-alkyl chain. Unchanged pyrrolidinophenones were detected in abundance and were not directly metabolized via ketone reduction; only forming *N*-dealkylated alcohols due to pyrrolidine ring hindrance of the ketone group.

In general, β-OH or *N*-dealkylated alcohol metabolites were the most abundant metabolites identified in human urine for ring or side-chain substituted cathinones [112,162,167], similar to *in vitro* and rat urine metabolites. For 4′-methyl substituted cathinones, 4′-methyl hydroxylation and 4′-carboxylation were common biotransformations [160,165,169,170]; 4′-methyl carboxylation had not been previously observed in rat urine [170]. Following 200 mg oral mephedrone administration, *N*-demethylation, ketone reduction, 4′-methyl hydroxylation and carboxylation, in addition to alkyl chain hydroxylation and carboxylation were observed in urine [169]. The succinyl conjugate also was identified for the first time, in addition to glucuronides. The same biotransformations were identified in blood [165] and urine [170] with the exception of hydroxylation and carboxylation of the alkyl chain and succinyl conjugate. The main metabolite identified in blood was 4′-OH mephedrone [165].

Although ketone reduction and subsequent *N*-dealkylation biotransformations were reported for methylenedioxy-substituted cathinones [112,171], the most abundant metabolite identified in human urine resulted from demethylenation with subsequent 3′-OH methylation to produce the 4-hydroxy-3-methoxy metabolites for methylone, butylone and ethylone [112,171,173,174]. Interestingly, Katagi *et al.* [173] and Kamata *et al.* [174] did not detect the ketone reduced alcohol in urine following methylone consumption; however, Uralets *et al*. reported ketone reduction to a lesser extent, due to hindrance caused by the methylenedioxy group [112]. After hydrolysis, increased methylone, butylone and ethylone metabolite concentrations (primarily demethylenated and hydroxy-methoxy metabolites) were observed, suggesting that glucuronidation occurs for these compounds [171,173,174].

Regarding α-pyrrolidinophenones metabolism, Shima *et al.* found in human urine that the two major metabolic pathways of α-PVP were ketone reduction to the corresponding alcohol and carbonylation of the 2″ position of the pyrrolidinophenone ring to produce the 2″-oxo metabolite, in addition to the parent compound which was detected in high abundance [181]. Tyrkko *et al.* while investigating α-PVP phase I metabolites following *in vitro* HLM and in authentic human urine, observed carbonylation (2″) and ketone reduction biotransformations [162]. In contrast, Uralets *et al.* did not directly observe ketone reduction for pyrrolidinophenones; however, no information was available on the time of dosing or when urine specimens were collected [112]. Alpha-pyrrolidinooctanophenone (PV9) exhibited similar metabolism to α-PVP (carbonylation, ketone, reduction, hydroxylation etc.); however, its main metabolite resulted from alkyl chain carboxylation [183]. This difference is likely attributed to

PV9's alkyl chain length. MDPV, a methylenedioxy-substituted pyrrolidinophenone, exhibited the same metabolic pathways in human urine as identified in rat urine: carbonylation, demethylenation, methylation, *N*-dealkylation, and hydroxylations, with the most abundant metabolite being 4-hydroxy-3-methoxypyrovalerone [186], similar to other methylenedioxy-substituted cathinones. Several studies reported conjugation (either glucuronides or sulfates) primarily at the site of hydroxylations and carboxylations of pyrrolidinophenones [112,181,183,186], similar to that reported in animal *in vivo* studies.

Analytical Methods to Identify Synthetic Cathinones in Biological Matrices

We identified 55 articles for synthetic cathinones and metabolite analysis in a variety of biological matrices including blood (*n*=25), plasma (*n*=5), serum (*n*=3), urine (*n*=28), OF (*n*=2), hair (*n*=4), DBS (*n*=1) and other tissues (*n*=4), all published from 2010 to 2015. Method validation data are summarized in Table 2 and Table 3. Seven articles reported immunoassay validation or synthetic cathinone cross-reactivity to immunoassay screening assays. The remaining articles highlighted qualitative (*n*=10) and quantitative (*n*=45) analytical methods utilizing GC-MS, high performance liquid chromatography-diode array detection (HPLC-DAD), LC-MS/MS, LC-HRMS, matrix-assisted laser desorption/ionization- time-of-flight-mass spectrometry (MALDI-TOF), and direct analysis in real time-mass spectrometry (DART-MS). Sample preparation included dilution, dispersive liquid-liquid microextraction (DLLME), liquid-liquid extraction (LLE), protein precipitation, solid phase extraction (SPE), solid phase microextraction (SPME), acid/enzyme hydrolysis (urine and plasma only), washing with or without base digestion (hair only) and derivatization. Method validation parameters included limits of detection (LOD) and LOQ, linearity, assay imprecision and bias, matrix effects,

Table 2. Qualitative analytical methods for the identification of synthetic cathinones in biological matrices.

Analyte(s)	Matrix (volume)	Sample Preparation	Method (run time)	Validation	Study Findings	Application	Reference
143 NPS including 35 cathinones: 2-MMC, 3,4-DMMC, 3-FMC, 3-MMC, 4-BMC, 4-CMC, 4-EMC, 4-FMC, 4-MEC, 4-MPPP, α-PBP, α-PVP, BMDP, benzedrone, buphedrone, bupropion, butylone, dibutylone, diethylpropion, dimethylcathinone, dimethylone, ethcathinone, ethylone, eutylone, MDPBP, MDPPP, MDPV, mephedrone, methcathinone, methedrone, methylone, naphyrone, *N*-ethylbuphedrone, pentedrone, & pentylone	Blood (0.2mL)	Protein precipitation	LC-MS/MS, ESI+ (14 min)	LOD, imprecision, bias, recovery, specificity, stability	MRM; LODs estimated for 104 compounds; LOD=0.01-3.1 µg/L; recovery: 1.8-133%; no interferences; extracts stable under tested conditions	Authentic blood specimens (*n*=1000) from forensic casework 2012-2014; 112 NPS positive cases; 3-MMC (50), α-PVP (23) & pentedrone (16)	[190]
78 NPS including 19 cathinones: α-PVP, 3,4-DMMC, 4-MEC, 4-MPPP, buphedrone, butylone, ethylone, cathinone, 4-CMC, ethcathinone, 4-FMC, MDPV, mephedrone, 3-MMC, methedrone, methylone, naphyrone, pentedrone, & pentylone	Blood (0.5mL)	DLLME	UHPLC-MS/MS, ESI+ (14 min)	In accordance with FDA guidelines, LOD, linearity, ME, recovery, selectivity, stability	MRM; LOD=0.2-2 µg/L; linear from 5-1000 µg/L; recovery 10-99%; no interferences, ME -39% to 10%	Authentic blood specimens (*n*=60) from forensic casework; 1 sample identified 4-MEC	[191]

Analyte(s)	Matrix (volume)	Sample Preparation	Method (run time)	Validation	Study Findings	Application	Reference
13 NPS including 1 cathinone: 4-MEC	Blood (0.5mL)	LLE	LC-QTOF-MS, ESI+	Stability	Primarily a stability study; 4-MEC exhibited 92% loss by day 7 with complete degradation by day 14 in blood stored at room temp, dihydro-MEC degradation product	Authentic blood specimen from PM case; identified 4-MEC, dihydro-MEC, nor-4-MEC, nor-dihydro-MEC & 4-OH MEC	[148]
Mephedrone, & MDPV (calibrators)	Urine (0.5-1mL)	None	Biochip array technology immunoassay	LOD, linearity, imprecision, bias, interference, carryover, cross-reactivity, cutoff optimization	Biochip labeled with 2 antibodies (mephedrone, BSI; MDPV, BSII); performance evaluated with LC-HRMS; 100% sensitivity, 52.1% specificity & 53% efficiency at 5 & 30 µg/L cutoff	Authentic urine specimens (*n*=20017) from random workplace drug testing; 97 presumptive positives identified; only 4 confirmed positive	[192]
96 compounds including 7 cathinones: cathinone, ethylone, mephedrone, methcathinone, methedrone, methylone, & MDPV	Urine (1mL)	Hydrolysis (enzyme), SPE (cation exchange)	UHPLC-MS/MS, ESI+ (23 min)	In accordance with EURCHEM & Trullols; LOD, imprecision, ME, recovery, selectivity, stability	MRM; LOD=10-100 µg/L; ME <35%; recovery 56-106%; stable under tested conditions	Authentic urine samples (*n*=964) from known or suspected drug abusers 2011-2013; methcathinone only cathinone detected; limitation of MRM screen need prior knowledge of analytes	[193]

Analyte(s)	Matrix (volume)	Sample Preparation	Method (run time)	Validation	Study Findings	Application	Reference
26 NPS including 11 cathinones: methcathinone, buphedrone, mephedrone, 3-FMC, 4-FMC, methedrone, methylone, butylone, pentylone, MDPV, & naphyrone	Urine (0.05mL)	Dilution (5-fold)	LC-MS/MS, ESI+ (4 min)	LOD, linearity, ME, selectivity, carryover, stability	SRM; LOD=0.5-50 µg/L; LOQ=0.5-50 µg/L; ME -15.4% to 10%; 3-FMC & 4-FMC decreased to <50% stored at -20°C for 3 months	Authentic urine specimens (*n*=1335) from 12 month period; 87 positive for NPS, MDPV (21 cases)	[151]
8 NPS including 5 cathinones: methylone, methedrone, butylone, mephedrone, & MDPV	Urine (1mL)	Dilution (4-fold)	LC-MS/MS, ESI+ (5 min)	LOD, LOQ, ME, recovery	SRM; LOD=2-3.4 µg/L; LOQ=6.5-11.3 µg/L; all target analytes readily detected at 500µg/L, but 1000 µg/L cutoff selected to mirror AMP	Authentic urine specimens (*n*=2) submitted for mephedrone analysis; detected mephedrone in 1 case, but below cutoff	[194]
64 NPS including 16 cathinones: 3-FMC, 4-MEC, butylone, cathinone, ethcathinone, ethylone, 4-FMC, MDPPP, MDPV, mephedrone, methcathinone, methedrone, methylone, naphyrone, pentylone, & pyrovalerone	DBS (0.01mL)	Methanol extraction	LC-MS/MS, ESI+ (10 min)	LOD, ME, recovery, selectivity, specificity, stability	Scheduled MRM; LOD=1-10 µg/L; no interferences except 4-FMC & 3-FMC (isobaric); stable for at least a week cooled	Authentic DBS specimens (*n*=21) produced from authentic blood specimens; no cathinones detected in DBS	[195]
26 stimulants including 15 cathinones: 3,4-DMMC, 4-MEC, buphedrone, butylone, cathinone, ethcathinone, ethylone, 4-FMC, MDPV, mephedrone, methedrone, methylone, naphyrone, pentedrone, & pentylone	Hair (30 mg)	Wash, incubation, sonication	UHPLC-MS/MS, ESI+ (14 min)	In accordance with FDA guidelines; LOD, LOQ, linearity, imprecision, bias, ME, recovery, specificity, carryover	MRM; LOD=2-20 pg/mL; LOQ=5-20 pg/mL; ME -39 to 11%;	Authentic hair specimens (*n*=50) from forensic casework; 4 tested positive for cathinone (4-MEC & MDPV)	[196]

Analyte(s)	Matrix (volume)	Sample Preparation	Method (run time)	Validation	Study Findings	Application	Reference
14 NPS including 7 cathinones: mephedrone, butylone, cathinone, ethylone, MDPV, methcathinone, & methylone	Hair (20-30 mg)	Wash, ultra-sonication	LC-MS/MS, ESI+ (20 min)	LOD, recovery, stability	MRM; LOD= 10 pg/mg except cathinone (50 pg/mg); performing ultra-sonication 2x improved recoveries by ~50%; mephedrone 5% decrease after 11 month storage (-18°C)	Authentic hair specimens (*n*=325) testing positive for amphetamines 2009-2010; NPS detected in 120 cases, mephedrone detected in 11 cases (3%) & methylone in 1 case	[197]

Abbreviations: 3,4-DMMC, 3,4-Dimethylmethcathinone; 2-MMC, 2-methylmethcathinone; 3-FMC, 3-flurormethcathinone; 3-MMC, 3-methylmethcathinone; 4-BMC, 4-bromomethcathinone; 4-CMC, 4-chloromethcathinone, clephedrone; 4-EMC, 4-ethylmethcathinone; 4-FMC, 4-fluoromethcathinone, flephedrone; 4-MEC, 4-methylethcathinone; 4-MPPP, MPPP, 4-methyl-α-pyrrolidinopropiophenone; α-PBP, α-pyrrolidinobutiophenone; α-PVP, α-pyrrolidinopentiophenone; AMP, amphetamine; BMDP, 3,4-methylenedioxy-*N*-benzylcathinone; BSI, bath salt I; BSII, bath salt II; DBS, dried blood spots; DLLME, dispersive liquid-liquid microextraction; ESI, electrospray ionization; FDA, Food and Drug Administration; HRMS, high resolution-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLE, liquid-liquid extraction; LOD, limit of detection; LOQ, limit of quantification; MDPBP, 3,4-methylenedioxy-α-pyrrolidinobutiophenone; MDPPP, 3,4-methylenedioxy-α-pyrrolidinopropiophenone; MDPV, 3,4-methylenedioxypyrovalerone; ME, matrix effects; MRM, multiple reaction monitoring; NPS, novel psychoactive substances; PM, postmortem; SPE, solid phase extraction; SRM, selective reaction monitoring; TOF, time-of-flight; UHPLC, ultra-high performance liquid chromatography

Table 3. Quantitative analytical methods for the identification of synthetic cathinones in biological matrices.

Analyte(s)	Matrix (volume)	Sample Preparation	Method	Validation	Study Findings	Application	Reference
Ethylone, methylone, mephedrone, & 3 dimethoxyamphetamine analogs	Blood (1 mL)	SPE (clean screen	GC-MS	LOD, LOQ, linearity, imprecision, bias	SIM; LOD=7 µg/L, LOQ=25 µg/L, acceptable bias & imprecision	Authentic blood specimens (*n*=9) from PM cases; 7/9 cases had measureable ethylone concentrations (38-2572 µg/L)	[198]
PVT, F-PVP, 4-MPHP, PV8, PV9, & F-PV9	Blood (0.2 mL)	LLE	MALDI-Q-TOF, positive mode	LOD, LOQ, linearity, imprecision, bias, recovery	MS/MS detection; LOD=1 µg/L, LOQ=2 µg/L, recovery 70-100%	Authentic blood specimens (*n*=4) from PM cases; detected PV9 (163-209 µg/L), PV9 metabolites also identified	[199]
37 NPS including 23 cathinones: cathinone, methcathinone, 4-MEC, 3,4-DMMC, mephedrone, methedrone, buphedrone, pentedrone, 4-FMC, benzedrone, naphyrone, methylone, ethylone, butylone, pentylone, bupropion, pyrovalerone, α-PVP, MDPV, α-PPP, MDPPP, 4-MPBP, & MDPBP	Blood (0.1 mL)	Protein precipitation, LLE	LC-QTOF-MS, ESI+	In accordance with international guidelines; LOD, LOQ, linearity, ME, recovery, selectivity, stability	MS^E acquisition, retrospective analysis possible; LOD=7-70 µg/L, LOQ=50-100 µg/L, recoveries 71-100%, stable under tested conditions except benzedrone at 4°C for a week	Authentic blood specimens from drug intoxications that screened negative; MDPV detected below LOQ	[200]
MDPV	Blood (0.2 mL)	Protein precipitation	LC-MS/MS, ESI+	LOD, LOQ, linearity, imprecision, bias, ME, recovery, specificity	MRM; LOD=0.5 µg/L, LOQ=5 µg/L, recovery 110%, ME <10.5% suppression	Authentic blood specimens from PM (*n*=2) & intoxication (*n*=2) cases; MDPV detected in all cases (17-306 µg/L)	[201]
α-PVP, MDPV, & pyrovalerone	Blood (0.05 mL)	SPME	GC-MS	LOD, LOQ, linearity, imprecision, bias, recovery	SIM; LOD=0.5 µg/L, LOQ=1 µg/L, recovery 3.9-6.1%	Authentic blood specimen (*n*=1) from PM case; detected α-PVP (486 µg/L)	[202]

Analyte(s)	Matrix (volume)	Sample Preparation	Method	Validation	Study Findings	Application	Reference
25 designer cathinones & ephedrines including 19 cathinones: cathinone, methcathinone, methylone, dimethylcathinone, 3-FMC, 4-FMC, ethcathinone, ethylone, methedrone, α-PPP, MDPPP, butylone, diethylcathinone, mephedrone, 4-MEC, pentylone, 3,4-DMMC, MDPV, & pyrovalerone	Blood (0.1 mL)	LLE	LC-MS/MS, ESI+	In accordance with international guidelines; LOD, LOQ, linearity, imprecision, bias, ME, recovery, PE, selectivity, stability	MRM; LOQ=10 µg/L, ME -21 to 2%, recovery 3-94% , hydrophilic cathinones & ephedrines were quite low, but reproducible	No application to authentic specimens	[203]
Methylone, mephedrone, methedrone, & MDPV	Blood (1 mL)	LLE, derivatization	GC-MS	LOD, LOQ, linearity, imprecision, bias, specificity, carryover	SIM; only presented results for methylone; LOD=50 µg/L, LOQ=100 µg/L, no interferences	Authentic blood specimens (*n*=3) from PM cases; methylone detected in all 3 (56-3300 µg/L)	[128]
9 cathinones & 6 related ephedrines including: cathinone, methcathinone, diethylcathinone, ethcathinone, mephedrone, 4-FMC, methedrone, methylone, & butylone	Blood (0.3 mL)	Protein precipitation, ultrafiltration	LC-MS/MS, ESI+	LOD, LOQ, imprecision, bias, ME, recovery, stability	MRM; LOD=0.5-3 µg/L, LOQ=10 µg/L, ME <15% suppression, recovery 87-106%, cathinones unstable in whole blood under neutral conditions, stability improved by acidification	No application to authentic specimens	[204]

Analyte(s)	Matrix (volume)	Sample Preparation	Method	Validation	Study Findings	Application	Reference
Methylone & metabolites (HMMC, MDC, HHMC)	Plasma (0.1 mL)	Hydrolysis (enzyme), protein precipitation, SPE (cation exchange)	LC-MS/MS, ESI+	Accordance with SWGTOX; LOD, LOQ, linearity, imprecision, bias, ME, recovery, selectivity, specificity, stability, cross-validation	MRM; LOQ=0.5 µg/L, ME <11.4%, recovery 89.9-104%, stable under tested conditions most likely due to SMBS & EDTA addition, HHMC considered semi-quantitative only (poor validation results)	Authentic plasma specimens following 6 mg/kg SC methylone administration in rats; methylone & metabolites detected up 480 min post-dose	[205]
MDPV & metabolites (3,4-catechol PV, 4-OH-3-MeO-PV)	Plasma (0.1 mL)	Hydrolysis (enzyme), protein precipitation	LC-HRMS, ESI+	LOD, LOQ, linearity, imprecision, bias, ME, recovery, PE, selectivity, specificity, stability, cross-validation	Targeted MS^2 acquisition; LOD=0.1µg/L, LOQ=0.25 , ME -8 to 12%, recovery 39-83.2%, stable under all tested conditions most likely due to SMBS & EDTA addition	Authentic plasma specimens following 0.5 mg/kg IP & SC MDPV administration in rats; MDPV & metabolites detected 0.9-107.5 µg/L	[206]
11 cathinones including: mephedrone, buphedrone, 4-FMC, 3-FMC, 3-methoxymethcathinone, methedrone, methylone, ethylone, butylone, MDPV, & naphyrone	Plasma (0.5 mL)	LLE	LC-MS/MS, ESI+	Linearity, imprecision, bias, ME, recovery, specificity, stability	MRM; LOD=0.02-0.05 µg/L, LOQ=0.2-1 µg/L, recovery >70%, ME -17.4 to 20.3%, acceptable bias & imprecision	Routinely employed in post-competition samples collected from racehorses in PA to enforce ban	[207]
MDPV	Plasma (1 mL)	LLE	LC-MS/MS, ESI+	LOD, LOQ, linearity, imprecision, bias, ME, recovery, selectivity, dilution, stability	MRM; LOD=0.002 µg/L, LOQ=0.005 µg/L, ME < -5.5%, MDPV stable in plasma & in solution up to 25 days at -25°C	Authentic equine plasma specimen (*n*=1) from racetrack horse; MDPV detected (5.8 µg/L)	[208]

Analyte(s)	Matrix (volume)	Sample Preparation	Method	Validation	Study Findings	Application	Reference
MDPV	Serum (0.2 mL)	SPE (reversed phase)	LC-MS/MS, ESI+	In accordance with GTFCh; LOD, LOQ, imprecision, bias, ME, recovery, selectivity	MRM; LOQ=0.010 µg/L, recovery 67.9-89.8%, poor recovery at low concentrations, ME < 21.5%	Authentic blood specimens (*n*=3000) from suspected DUID; 259/3000 MDPV positive	[108]
32 cathinone derivatives including 9 cathinones: MDPV, mephedrone, cathinone, methcathinone, methedrone, 4-MEC, 4-FMC, methylone, & butylone	Serum, blood (1 mL)	SPE (mixed mode)	LC-MS/MS, ESI+	In accordance with international guidelines; selectivity, LOD, LOQ, linearity, imprecision, bias, ME, recovery, PE, stability	Dynamic MRM; 3 analytes considered semi-quantitative due to validation performance; LOD=0.01-0.1 µg/L, LOQ=1-10 µg/L, recovery ~50%, bias <15% except MDPV, ME 50-168% (cathinone) but reproducible, stable under tested conditions except cathinone	Authentic blood specimens (*n*=2) from PM cases; methylone & MDPV only cathinones detected (63 & 11 µg/L, respectively)	[209]

Analyte(s)	Matrix (volume)	Sample Preparation	Method	Validation	Study Findings	Application	Reference
40 NPS including 28 cathinones & 4 metabolites: 4-FMC, 4-MEC, 4-methoxy-α-PVP, 4-MPBP, α-ethylaminopentiophenone, α-PBP, α-PPP, α-PVP, α-PVT, benzedrone, buphedrone, butylone, cathinone, diethylcathinone, ethylone, MDPBP, MDPPP, MDPV, mephedrone, methcathinone, methedrone, methylone, MPHP, naphyrone, pentedrone, pentylone, PV8, pyrovalerone 4-MEC metabolite, 4-methylephedrine, normephedrone, & buphedrone ephedrine	Urine (0.25 mL)	SPE (cation exchange)	LC-HRMS, ESI+	In accordance with SWGTOX guidelines; LOD, LOQ, linearity, imprecision, bias, ME, recovery, interferences, stability	Full scan DDMS^2 with library matching; LOD=1-5 µg/L, LOQ=2.5-5 µg/L, recovery 87.5-112%, ME - 26.4 to 13.2%, stability of cathinones previously reported by authors	Authentic urine specimens (*n*=64) from stimulant users; cathinones detected in urine with 3/4 metabolites detected, α-PVP most common (23/64), mephedrone (11/64) & normephedrone (12/64) also detected, parent compound detected in high concentrations	[210]
3,4-DMMC, 4-ethylmethcathinone, 2-MMC & 3 metabolites (ketone reduced forms of 3,4-DMMC, pentedrone, & mephedrone)	Urine (0.25mL)	SPME	DART-MS	Detection limits	LOQ=2-200 µg/L, use of SPME increased drug detectability while reducing background noise	Quantified 3,4-DMMC, & β-OH-DMMC (4.4 µg/L)	[211]
39 compounds including 8 cathinones: cathinone, methcathinone, methylone, methedrone, butylone, mephedrone, 4-MEC, & MDPV	Urine (0.2 mL)	LLE	LC-QTOF-MS, ESI+	LOD, LOQ, linearity, bias, ME, recovery, selectivity, stability	Data-dependent acquisition mode with preferred ion list for targeted un-targeted approach; LOD=2-6 µg/L, LOQ=3-17 µg/L, no interferences	Authentic human urine (*n*=1); detected MDPV (350 µg/L) in addition to dihydro-MDPV metabolite (without reference standard)	[212]

Analyte(s)	Matrix (volume)	Sample Preparation	Method	Validation	Study Findings	Application	Reference
24 cathinones & 4 metabolites including: cathinone, methcathinone, 4-FMC, methylone, ethcathinone, α-PPP, buphedrone ephedrine, ethylone, methedrone, buphedrone, normephedrone, diethylcathinone, MDPPP, 4-methylephedrine, butylone, mephedrone, 4-MEC, 4-MEC metabolite, MDPBP, pentedrone, pentylone, 3,4-DMMC, α-PVP, 4-MPBP, MDPV, pyrovalerone, benzedrone, & naphyrone	Urine (0.25mL)	SPE (cation exchange)	LC-HRMS	Linearity, LOD, LOQ, imprecision, bias, ME, recovery, PE, selectivity, specificity, carryover, dilution, stability	Targeted MS^2; LOQ=0.5-1 µg/L, recovery 78.9-117%, ME -29 to 1.5%, most cathinones stable at 4°C for 72 h or after 3 freeze-thaw cycles, but many cathinones unstable at room temperature (*n*=19) with loss up to 67.6%	Authentic urine specimens (*n*=106) from random workplace drug testing; only 4 confirmed positive for cathinones including methylone, α-PVP, pentylone, pentedrone, & pyrovalerone	[150]
36 stimulants including 13 cathinones: dimethylcathinone, buphedrone, ethcathinone, mephedrone, 3-FMC, 4-MEC, methedrone, methylone, ethylone, butylone, benzedrone, MDPBP, & MDPV	Urine (1 mL)	LLE	LC-MS/MS, ESI+	LOQ, linearity, ME, carryover	MRM; LOQ=5 µg/L, ME 0.2-1.23, no carryover at 500 µg/L	Authentic urine specimens from 2010 (*n*=155) & 2011 (*n*=294) from patients at drug treatments centers; 66 & 99 positive specimens, mephedrone widely detected in 2010 (7.6%), & MDPBP in 2011 (16.6%), cathinones detected in 54 cases (25%)	[213]
Mephedrone, & methcathinone	Urine (0.1 mL)	Hydrolysis (enzyme), LLE	HPLC-LC-MS/MS, ESI+	LOD, LOQ, linearity, imprecision, bias, specificity, stability	MRM; LOD=0.17 µg/L, LOQ=0.5 µg/L, acceptable bias & imprecision, no interferences, samples stable over 14 days of freeze-thaw cycles	No application to authentic specimens	[214]

Analyte(s)	Matrix (volume)	Sample Preparation	Method	Validation	Study Findings	Application	Reference
MDPV	Urine (1 mL)	LLE, derivatization	GC-MS	LOQ, linearity, imprecision, bias, recovery	SIM; LOD=10 µg/L, LOQ=20 µg/L, recovery 104%, acceptable bias & imprecision	Authentic urine specimens (*n*=34) from opioid-dependent patients; 9/34 MDPV positives (40-3900 µg/L)	[215]
Methylone & metabolites (HMMC, MDC, 3-OH-4-MeO-MC)	Urine (1 mL)	Hydrolysis (acid & enzyme), LLE, derivatization (GC only)	GC-MS, LC-MS/MS, ESI+	Not specified	Simple, sensitive & reliable analytical method for methylone detection	Authentic urine specimens (*n*=2); detected methylone & metabolites	[173]
Mephedrone, butylone, & methylone	Urine (5 mL)	Hydrolysis (acid), LLE, derivatization	GC-MS	Not specified	Not specified	Authentic human & rat urine; mephedrone, butylone, methylone & metabolites detected in rat urine, methylone & butylone identified in human urine	[216]
MDPV & metabolites	Urine (5 mL)	Hydrolysis (enzyme), LLE, derivatization	GC-MS	Not specified	Not specified	Authentic human urine after unknown ingestion; MDPV detected & most common metabolite was demethylenyl-methyl-MDPV	[186]
Mephedrone, butylone, & methylone	Urine (5 mL)	Hydrolysis (acid), LLE, derivatization	GC-MS	Not specified	Not specified	Authentic human urine collected after oral ingestion; detected mephedrone & butylone in human & rat urine, methylone also detected in rat urine	[170]

Analyte(s)	Matrix (volume)	Sample Preparation	Method	Validation	Study Findings	Application	Reference
MDPV	Urine (2 mL)	Hydrolysis (enzyme), LLE	GC-MS	LOD, LOQ, linearity, imprecision, bias, selectivity	SIM; LOD=2 µg/L, LOQ=10 µg/L, acceptable imprecision & bias	Not application to authentic specimens; unchanged MDPV present in high amounts so MDPV useful for detection in urine	[187]
8 NPS including 5 cathinones: methedrone, methylone, mephedrone, MDPV, & 4-FMC	OF with buffer (0.5 mL)	SPE (cation exchange)	LC-MS/MS, ESI+	LOD, LOQ, linearity, imprecision, bias, ME, selectivity, stability	MRM; LOD=0.025-0.1 µg/L, LOQ=0.2-0.5 µg/L, ME -69.9 to 131%, recovery 79.6-108%, stability < -30.8%	Authentic OF specimens (*n*=10) collected roadside after positive screen; MDPV detected (31.8 µg/L)	[217]
10 cathinones including: mephedrone, MDPV, 4-FMC, methedrone, methylone, butylone, ethylone, α-PVP, methcathinone, & pyrovalerone	OF with buffer (0.4 mL)	SPE (cation exchange)	UHPLC-MS/MS, ESI+	LOQ, imprecision, bias, ME, recovery, selectivity, carryover, stability	MRM; LOQ=1 µg/L, recovery 34.5-90.3%, processed samples stable over 6 h	Authentic OF specimens from patients; 5 specimens positive for cathinone, 3 MDPV, 3 α-PVP	[218]
Mephedrone	Hair (50 mg)	Wash, incubation, sonication, derivatization	GC-MS	LOD, LOQ, linearity, imprecision, bias, recovery, selectivity, specificity, carryover	SIM; LOD=0.08 ng/mg, LOQ=0.2 ng/mg, recovery >76%, acceptable bias & imprecision	Authentic hair specimens (*n*=67); 13 mephedrone positive (0.2-313 ng/mg)	[219]
Mephedrone & metabolites (4-methylephedrine, 4-methylnorephedrine)	Hair (1 mm)	Wash, digestion (NaOH), enzymatic digestion, LLE	LC-MS/MS, ESI+	LOD, LOQ, linearity, imprecision, bias, specificity, selectivity	SRM; LOD=2.5 pg/mg (parent), 5 pg/mg (metabolites), LOQ=5 pg/mg (parent), 10 pg/mg (metabolites), recovery 90.4-110%	Authentic hair specimens (*n*=154) from two higher education institutions; 5/154 mephedrone positive, no metabolites detected	[220]

Analyte(s)	Matrix (volume)	Sample Preparation	Method	Validation	Study Findings	Application	Reference
3,4-DMMC	Blood, urine (0.5 mL)	QuEChERS (blood); hydrolysis, protein precipitation (urine)	LC-MS/MS, ESI+	LOD, LOQ, linearity, imprecision, bias, ME, recovery	MRM; LOD=1.0 µg/L (blood), 1.4 µg/L (urine), LOQ=5 µg/L (blood), 5.4 µg/L (urine), recoveries 85.9-101%,	Authentic blood & urine from PM case thought to involve 3,4-DMMC; 3,4-DMMC identified in blood & urine, also identified metabolites	[221]
4-MEC	Blood, urine (0.2 mL)	LLE	LC-MS/MS, ESI+	LOD, LOQ, linearity, ME, recovery, specificity	MRM; LOD=0.96 µg/L (blood), 0.68 µg/L (urine), LOQ=10 µg/L, recovery 100.5%, ME -17%, no interferences	Authentic blood (*n*=3) & urine (*n*=2) specimens from PM cases; 4-MEC detected in blood in all cases (46-152 µg/L) & detected in urine (122 µg/L)	[222]
Mephedrone & metabolites (4-carboxymephedrone, 4-OH-normephedrone)	Blood, urine (0.2 mL)	Automated SPE, hydrolysis (enzyme; urine only)	UHPLC-TOF-MS, UHPLC-MS/MS, ESI+	Linearity, imprecision, bias, ME, recovery, PE	4-OH-mephedrone, normephedrone qualitative analytes; UHPLC-MS/MS: recovery 80-103%, ME -10 to 17%, , LOQ=1 µg/kg UHPLC-TOF-MS: recovery 77-104%, ME -46 to 10%, LOQ=1 µg/kg	Authentic blood (*n*=4) & urine (*n*=1) from traffic cases; mephedrone detected in all cases in addition to 4-OH-mephedrone, other metabolites qualitatively identified	[165]
Mephedrone	Blood, urine (0.5 mL)	LLE	HPLC-DAD	LOD, LOQ, imprecision, bias	LOD=39 µg/L, LOQ=78 µg/L, acceptable imprecision & bias	Authentic blood & urine from PM cases (*n*=4); mephedrone detected in all cases (0.13-2.24 mg/L) & urine in one (15 mg/100 mL)	[223]

Analyte(s)	Matrix (volume)	Sample Preparation	Method	Validation	Study Findings	Application	Reference
Mephedrone, MDPV, & methylone	Blood, urine (0.5 mL)	LLE	GC-MS	Not reported	Mainly describes clinical effects associated with synthetic cathinones in the US	Authentic specimens from ED (*n*=18) & PM (*n*=1) cases; MDPV detected in blood (13/17) & urine (3/5) from ED cases, MDPV detected in blood & urine in PM case, no mephedrone or methylone detected	[116]
Mephedrone	Blood, urine	Could not be determined	GC-MS	Could not be determined	Article in Hungarian, only abstract available	Authentic specimens (*n*=5386); mephedrone detected in 363 cases (7%)	[224]
Mephedrone	Blood, urine (2 mL)	LLE, derivatization	GC-MS	LOD, LOQ, linearity, imprecision, bias, recovery, selectivity, specificity	SIM; LOD=10 µg/L, LOQ=25 µg/L, recovery >80%, no interferences, acceptable bias & imprecision	Authentic blood & urine specimens from PM case; mephedrone detected in blood & urine (0.5 & 198 mg/L)	[225]
Mephedrone	Blood, urine	SPE, derivatization	GC-MS	Not specified	SIM; LOQ=100 µg/L	Mephedrone detected in blood (*n*=4), urine (*n*=1) & hair (*n*=1)	[226]
4-methoxy-α-PBP	Plasma, urine (0.1 mL)	Protein precipitation	LC-QTOF-MS, ESI+	External calibration; LOD, LOQ, imprecision, bias, recovery	MS^E; LOD=300 µg/L. LOQ=500 µg/L, recovery 88.7-104%, acceptable bias & imprecision	Authentic plasma & urine specimen from PM case; identified 4-methoxy-α-PBP in plasma & urine (9.5, 12 mg/L)	[227]

Analyte(s)	Matrix (volume)	Sample Preparation	Method	Validation	Study Findings	Application	Reference
Mephedrone	Serum, urine	Not reported	GC-MS (qual), LC-MS/MS (quant), ESI+	Not specified	Utilized in-house synthesized mephedrone standard since no commercially available standards	Authentic urine & serum from toxicity case; qual detected mephedrone in urine & serum, quant detected mephedrone in serum (0.15 mg/L)	[228]
Mephedrone	Blood, vitreous humor (0.2 mL)	Protein precipitation	LC-MS/MS, ESI+	LOD, LOQ, linearity, imprecision, bias, ME, recovery, specificity, cross-validation	MRM; LOD=0.08 µg/L, LOQ=1 µg/L, recovery 84.4%, ME <32.3% enhancement, cross-validation <12% bias	Authentic blood & vitreous humour from PM case; mephedrone detected in blood & vitreous (5.5 & 7.1 µg/L, respectively)	[229]
Mephedrone, MDPV, & 2 other NPS	Blood, plasma, urine (0.1 mL)	SPE (clean screen)	UHPLC-MS/MS, ESI+	LOD, LOQ, linearity, ME, stability	MRM; LOQ=5 µg/L, ME <10% suppression; study focuses primarily on stability in different matrices & storage conditions; matrix & storage play large role in stability, mephedrone was the most unstable	No application to authentic specimens	[147]

Analyte(s)	Matrix (volume)	Sample Preparation	Method	Validation	Study Findings	Application	Reference
MDPV, methylone, methedrone, mephedrone, butylone, pyrovalerone, & 3-FMC	Blood, urine, vitreous, CSF, bile, tissue (1 mL)	LLE (alkaline conditions)	LC-MS/MS, ESI+	LOQ, linearity, bias, selectivity, stability	α-PVP & pentylone ions screened; MRM; LOQ=5 µg/L, reference samples within 20%, methylone & methedrone LQC not reproducible in brain & liver but known to be unstable in alkaline conditions	Authentic specimens from 9 human performance & 23 PM cases; SC identified, MDPV primarily in DUID, MDPV & methylone primarily detected PM	[158]
Methylone, mephedrone, & MDPV	Blood, tissue (5 mL)	Chem Elut, LLE	GC-MS	LOD, LOQ, imprecision, bias	SIM; LOQ=25 µg/L, acceptable bias & imprecision, SC cathinone distribution investigated, methylone does not sequester into a specific tissue	Authentic specimens from PM cases (*n*=4); methylone & MDPV identified in variety of matrices	[156]
Mephedrone	Blood, urine, stomach contents (0.25mL)	Protein precipitation	HPLC-MS/MS, ESI+	LOQ, ME, recovery	MRM; LOQ=10 µg/L, recovery 94%, ME < 7% suppression	Authentic specimens from PM case (*n*=1); detected mephedrone in blood (5.1 mg/L), urine (186 mg/L) & stomach contents (1.0 g/L)	[119]

Abbreviations: 3,4-DMMC, 3,4-dimethylmethcathinone; 2-MMC, 2-methylmethcathinone; 3-FMC, 3-fluoromethcathinone; 4-FMC, 4-fluormethcathinone, flephedrone; 4-MEC, 4-methylethcathinone; 4-MPBP, MPBP, 4-methyl-α-pyrrolidinobutiophenone; 4-MPHP, MPHP, 4-methyl-α-pyrrolidinohexanophenone; α-PPP, α-pyrrolidinopropiophenone; α-PVP, α-pyrrolidinopentiophenone; CSF, cerebrospinal fluid; DAD, diode array detection; DART-MS, direct analysis in real time-mass spectrometry; DUID, driving under the influence of drugs; ED, emergency department; EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; F-PVP, 4-fluro-α-pyrrolidinopentiophenone; F-PV9, 4-fluoro-α-pyrrolidinoctanophenone; GC-MS, gas chromatography-mass spectrometry; HHMC, 3,4-dihydroxymethcathinone; HMMC, 4-OH-3-methoxy-methcathinone; HRMS, high resolution-mass spectrometry; HPLC, high performance liquid chromatography; IP, intraperitoneal; LC, liquid chromatography; LLE, liquid-liquid extraction; LOD, limit of detection; LOQ, limit of quantification; LQC, low quality control; MALDI-QTOF, matrix-assisted laser desorption/ionization- time-of-flight-mass spectrometry; MDC, 3,4-methylenedioxycathinone; ME, matrix effect; MDPBP, 3,4-methylenedioxy-α-pyrrolidionobutiophenone; MDPPP, 3,4-methylenedioxy-α-pyrrolidinopropiophenone; MDPV, 3,4-methylenedioxypyrolvalerone; MRM, multiple reaction monitoring; MS/MS, tandem-mass spectrometry; NaOH, sodium hydroxide; OF, oral fluid; PE, process efficiency; PM, postmortem; PV, pyrovalerone; PVT, α-pyrrolidinopentiothiophenone; PV8, α-pyrrolidinoheptanophenone; PV9, α-pyrrolidinoctanophenone; SC, subcutaneous; SIM, selected ion monitoring; SMBS, sodium metabisulfate; SPE, solid phase extraction; SPME, solid phase microextraction; SRM, selective reaction monitoring; SWGTOX, Scientific Working Group for Forensic Toxicology

extraction recovery, process efficiency, selectivity and specificity, carryover, dilution integrity, autosampler and short-term stability under different storage conditions and cross-validation. Cutoff evaluation and cross-reactivity also were included in immunoassay method validation. The following subsections highlight analytical methods and stability studies applicable to clinical and forensic toxicology casework.

Immunoassays and Cross-reactivity

Immunoassay screening methods have limited detection capabilities for new emerging synthetic cathinones, primarily due to the time required to develop antibodies and optimize commercial immunoassays for new compounds. Although synthetic cathinones are chemically related to amphetamines, most amphetamine immunoassays do not detect a wide range of synthetic cathinones in plasma, serum, urine and OF. Swortwood *et al.* investigated NPS cross-reactivity (including 9 cathinones) in serum against 16 commercially available enzyme-linked immunosorbent assay (ELISA) kits [230]. Synthetic cathinone cross-reactivity to amphetamine ELISA kits were <0.5% at concentrations <10,000 µg/L, except for methcathinone (1% at 4,250 µg/L). Positive Immunalysis and Neogen methamphetamine tests (0.25-2% cross-reactivity) occurred with synthetic cathinone concentrations starting at 1,250 µg/L. Greater cross-reactivity (2-25%) was observed for mephedrone, methcathinone, methylone, 4-MEC, 4-FMC, butylone, and methedrone in serum utilizing the OraSure methamphetamine assay (40-450 µg/L).

Cross-reactivity of 94 NPS (including 33 synthetic cathinones) in urine also was evaluated with five immunoassay reagent kits not previously evaluated (CEDIA®Amphetamine/Ecstasy, Syva®EMIT®II Plus Amphetamine, LIN-ZHI

Methamphetamine, Microgenics DRI®MDMA, Microgenics DRI®PCP) [231]. For α-pyrrolidinophenones, the Microgenics PCP assay produced the greatest number of positive results, with concentrations as low as 0.6-65 µg/L; the LIN-ZHI methamphetamine assay was best suited for ring, side-chain and methylenedioxy-substituted cathinones, with positive results produced at concentrations between 11-65 µg/L. Lowest cross-reactivities to synthetic cathinones in urine were observed with the Microgenics MDMA and Syva Plus Amphetamines immunoassay reagent kits. Additionally, some cathinones, including MDPV, produced false-positive results in phencyclidine (PCP) immunoassays in urine, despite their structures being dissimilar [117,232,233]. These authors did not provide potential reasons for the cross-reactivity. Additionally, De Castro *et al.* investigated the synthetic cathinone cross-reactivity in OF against the on-site Draeger DrugTest 5000 [217]. Limited cross-reactivity (0.035-0.35%) for tested cathinones in OF was observed with the methamphetamine test-strip (35 µg/L cutoff). With the continual development of novel cathinone derivatives, it is difficult to predict if emerging compounds will cross-react in existing immunoassays.

Recently, Randox developed a semi-quantitative screening system for synthetic cathinone detection in urine utilizing the Randox Drugs of Abuse V (DOA-V) Biochip Array Technology. To date, this is the only commercially available immunoassay for urinary cathinone detection. Ellefsen *et al.* performed full method validation for both Bath Salt I (BSI), targeting mephedrone/methcathinone, and Bath Salt II (BSII), targeting MDPV/MDPBP and reported large negative % bias [192]. Assay performance in authentic random workplace urine specimens (n=20,017) provided sensitivities ranging from 50-100%; cutoffs were 5 and 30 µg/L for BSI and BSII, respectively. Performance

characteristics improved at higher cutoffs (7 and 40 µg/L); however, there were limited confirmed positive specimens (*n*=4). Additionally, Swortwood *et al.* evaluated synthetic cathinone cross-reactivities in serum with the Randox ELISA kits , the only kit commercially available for synthetic cathinones in blood [234]. Synthetic cathinone cross-reactivities were <0.8% and 0.13% for mephedrone/methcathinone and MDPV, except for butylone which demonstrated 6.4% cross-reactivity with BSII at 156 µg/L.

Qualitative Methods

Several qualitative methods, primarily LC-based (*n*=9), were published between 2011-2015 for the detection of synthetic cathinones and other NPS substances in blood (*n*=3), urine (4), DBS (*n*=1) and hair (*n*=2) (Table 2). Almost exclusively, parent compounds were detected in all matrices from routine forensic casework, PM specimens and workplace drug testing. However, Soh and Elliot detected not only parent 4-MEC, but also 4-MEC metabolites in authentic PM blood with a targeted/nontargeted LC-QTOF-MS screen in blood [148]. Data were acquired in full scan with and without auto MS/MS fragmentation. All remaining qualitative methods, besides the immunoassay validation in urine [192], involved multiple reaction monitoring (MRM) acquisition. A limitation with MRM screening-based methods is that prior knowledge of the compounds is required; with continuous NPS synthesis and limited immunoassays available, high-throughput simultaneous targeted and non-targeted screening methods by LC-HRMS are critical to identify novel stimulants that often go undetected in routine analyses.

Two high-throughput qualitative methods were published for synthetic cathinone and other NPS detection in blood, with 14 min run times [190,191]. The most comprehensive method detected 143 NPS (35 cathinones), with LODs between 0.01-3.1

µg/L [190]. In urine, Al-Saffar *et al.* developed a high-throughput screen (4 min runtime) for 26 NPS (11 cathinones) with detection limits between 0.5-50 µg/L [151]. Tang *et al.* developed a more comprehensive qualitative method in urine (96 compounds; 7 cathinones); however, runtimes were longer (23 min) and LODs higher (10-100 µg/L) [193]. Blood sample preparation typically involved LLE, whereas urinalysis methods employed sample dilution. Enzymatic hydrolysis, prior to SPE, was performed in only one urine method for the detection of 96 compounds, including 7 cathinones [193].

Synthetic cathinone qualitative methods also were developed in alternative biological samples, such as DBS and hair. There is increased interest in detecting drugs of abuse in alternative matrices because these matrices offer several advantages compared to blood and urine. DBS can detect recent drug use with small sample volumes and increased stability compared to blood, whereas hair may be useful for identifying drug intake over time or prolonged abstinence cases. Ambach *et al*. developed a sensitive (1-10 µg/L), comprehensive LC-MS/MS MRM screening method to detect 64 NPS (16 cathinones) in DBS within 10 min; however, no synthetic cathinones were identified in authentic specimens (n=21) [195]. The most comprehensive qualitative LC-MS/MS MRM method for 15 cathinones in hair required an initial wash step and overnight incubation/sonication with formic acid prior to analysis, achieving LODs between 2-20 µg/L [196]. Authentic hair specimens from forensic casework (n=50) were analyzed and 4 tested positive for synthetic cathinones including 4-MEC and MDPV.

Quantitative Methods

The emergence of synthetic cathinones onto the drug market compelled analytical laboratories to develop quantitative analytical methods to confirm these compounds in

biological specimens. We identified targeted quantitative methods developed for synthetic cathinone detection in blood (*n*=8), plasma (*n*=4), serum (*n*=2), urine (*n*=12), OF (*n*=2), hair (*n*=2), and multiple biological matrices (*n*=15) between 2010-2015 (Table 3). These compounds were primarily analyzed by LC-MS/MS (*n*=22), GC-MS (*n*=16), and HRMS/QTOF (*n*=8) methods; over half of GC-based methods were developed between 2010 and 2012 (*n*=14), with LC-based methods typically between 2013-2015 (*n*=19).

Simultaneous targeted quantification and non-targeted screening methods were also developed. These methods quantified known compounds and identified unknown analytes at the same time. This approach is of high interest in NPS analysis, due to the constant appearance of new drugs. We identified five targeted/non-targeted HRMS methods identifying synthetic cathinones and metabolites (both known and unknown) in blood [165,199,200] and urine [165,210,212].

Like qualitative methods, quantitative methods primarily detected parent compounds in all matrices from acute intoxications, DUID, workplace drug testing, drug treatment centers, PM cases, and drug-users. Although some analytical methods did target and quantify synthetic cathinone metabolites (*n*=8), without analytical reference standards and limited knowledge on synthetic cathinone metabolism it is not always plausible to include these compounds in analyses.

Initially, GC-MS and LC-MS/MS methods focused on a single or a few synthetic cathinones in both blood and urine [119,170,187,225,226,228]; however, the capability to identify multiple cathinones became necessary. Ammann *et al.* was one of the first groups to develop an LC-MS/MS MRM confirmation method for multiple cathinones (19

cathinones) in blood, with limited specimen volume (0.1 mL) and sensitive LOQs (10 µg/L) [203]. Additionally, Swortwood *et al.* developed an LC-MS/MS quantitative method for detection of 32 NPS (9 cathinones) in serum with lower LOQs (1-10 µg/L), although specimen volume was greater (1 mL) [209]. This method was successfully applied to PM cases and methylone and MDPV were quantified at 63 and 11 µg/L, respectively. The most comprehensive blood method to date quantified 37 NPS (23 cathinones) by a targeted-non-targeted LC-QTOF-MS method with LOQs and LODs between 50-100 µg/L and 7-70 µg/L, respectively, in only 0.1 mL blood [200]. A lower LOQ was achieved by Minakata *et al.* (2 µg/L) in 0.2 mL specimens; however, the method only targeted 6 cathinones [199]. This targeted/non-targeted MALDI-QTOF method detected PV9 (163-209 µg/L) and a metabolite in authentic PM blood specimens (*n*=4).

Concheiro *et al.* developed the most extensive LC-HRMS full scan data-dependent MS^2 ($ddMS^2$) method, with library matching, for targeted and non-targeted detection of 40 NPS (28 cathinones and 4 metabolites) in urine [210]. Sensitive detection limits were achieved (LOQs 1-5 µg/L, LODs 1-5 µg/L) in only 0.25 mL urine; recoveries from 87.5-112% also were accomplished with a SPE sample preparation. This method was applied to authentic urine specimens from stimulant users (*n*=64) most commonly detecting α-PVP (23/64), mephedrone (11/64) and normephedrone (12/64) with concentrations ranging from 2.9 to >10,000 µg/L. More sensitive LOQs in urine were only achieved via a targeted-MS^2 LC-HRMS method by the same group [150].

OF is becoming a more common alternative matrix for DUID and workplace drug testing. Two OF LC-MS/MS MRM methods were developed for cathinone detection

[217,218]. De Castro *et al.* achieved sensitive LOQs (0.2-0.5 µg/L) and recoveries between 79.6-108% following SPE of only 0.5 mL OF/buffer mixtures, whereas Amaratunga *et al*. reported a 1 µg/L LOQ and recoveries between 34.5-90.3% in 0.4 mL OF/buffer mixture. Both methods were applied to authentic OF specimens collected with the Quantisal™ collection device; MDPV was detected in both studies.

Several studies investigated synthetic cathinone distribution in a variety of biological specimens [119,156,158]. An LC-MS/MS method for the quantification of 6 cathinones in blood, urine, vitreous humour, cerebral spinal fluid (CSF), bile and other tissues (1 mL) following alkaline LLE with a 5 µg/L LOQ was reported in a human performance and PM distribution study [158]. Validation results demonstrated that methylone and methedrone in brain and liver were not reproducible; however, authors cited cathinone instability in alkaline conditions. Analyses of authentic specimens revealed that MDPV alone, and in combination with methylone, were primarily identified in DUID and PM cases, respectively [158].

Analyte Stability

Often biological specimens cannot be assayed immediately after collection (laboratory workload, instrumentation downtime, shipment delay). This can be problematic, especially for emerging NPS, as stability studies in biological specimens are limited. Synthetic cathinone stability studies were generally performed with fortified matrices rather than authentic blood, plasma, serum, urine, OF and DBS specimens, with varying results.

Johnson and Botch-Jones [147] reported MDPV to be stable in blood at room temperature, 4ºC, and -20ºC for up to 14 days, whereas mephedrone exhibited a 30% loss

after 14 days at 4ºC, and was not detected after 7 days at room temperature. After 14 days at room temperature, Soh and Elliot were unable to detect 4-MEC in blood with a corresponding 54% loss in plasma [148]. Sorensen reported a 30% decrease in cathinone, methcathinone, ethcathinone, mephedrone and 4-FMC concentrations in blood (pH 7.4) after 2 days at 20°C storage; only 10% loss was observed under the same conditions when preserved with NaF/citrate buffer (pH 5.9). This suggested that stability might be improved in acidified specimens [204]. This increased stability under acidic conditions also was reported by Tsujikawa *et al.* [235].

Busardo *et al.* examined the mephedrone stability up to 6 months in both fortified ante-mortem and post-mortem blood at three different temperatures (-20°C, 4°C and 20°C) with and without preservatives (NaF/potassium oxalate; EDTA) [149]. Optimal storage was determined to be -20°C with NaF/potassium oxalate. Mephedrone stability was reduced in PM blood compared to ante-mortem. Ellefsen *et al.* and Anizan *et al.* reported the stability of methylone, MDPV, and their respective metabolites in plasma at room temperature (24 h), 4°C (72 h) and after three freeze-thaw cycles [205,206]. Stability in plasma was hypothesized to be due to SMBS and EDTA use during sample storage and preparation, as they prevented oxidation. Additionally, a number of synthetic cathinones were reported stable in blood [190,200], serum [234] and DBS [195] at varying storage conditions (from 24 h at room temperature to 6 weeks at -20°C).

In urine, mephedrone concentrations decreased by 60% when samples were stored at room temperature for up to 14 days, but were stable at 4ºC over the same time period [147]. MDPV was stable in urine for 14 days at room temperature and 4ºC. Concheiro *et al.* previously documented short-term stability findings during confirmation method

validation and showed that most synthetic cathinones were stable for 72 h at 4ºC, with the exception of benzedrone (27% loss) [150]. However, many synthetic cathinones were not stable for 24 h at room temperature (20% to 67.6% loss). Limited long-term synthetic cathinone urine stability studies are available. Al-Saffar *et al.* examined NPS urine stability up to 3 months at three different temperatures (22°C, 6°C and -20°C) [151] and found cathinones were stable at -20°C for three months (78.3-106% remaining), with the exception of buphedrone, 3-FMC, 4-FMC and pentylone (36.3-62.1% remaining).

In neat OF, cathinone stability ranged from -13.8 to 10.3% at 4°C for 24h and between -7.4 to 7.7% following three freeze-thaw cycles [217]. Cathinones also were stable (-14.8 to 3.8%) in OF/Quantisal buffer mixtures following freeze-thaw cycles [217].

Differences in stability among synthetic cathinones may be attributed to substitutions on the benzene ring, nitrogen, and phenethylamine backbone [204,235]. Enhanced stability of pyrrolidinyl-substituted cathinones, such as MDPV, may be due to steric hindrance from the pyrrolidinyl group adjacent to the carbonyl, as it may block the electrophilic carbon reducing its reactivity. Tsujikawa *et al.* also reported that tertiary amines, such as dimethylcathinone, are more stable [235]. However, the authors hypothesized enhanced stability was attributed to less sensitivity to oxidative deamination, as tertiary amines do not readily undergo oxidative deamination like secondary aliphatic amines. In addition, methylenedioxy-substituted cathinones did not readily reduce to their corresponding alcohols [112,236], potentially due to structural affinity differences to reductive enzymes [236].

Thermal degradation also was reported for synthetic cathinones during GC-MS analysis [237-239]. Kerrigan *et al.* identified *in situ* thermal degradation products for 18 cathinones and suggested that oxidative degradation arises from the loss of two hydrogens thus yielding a prominent iminium base peak with a *m/z* 2 Da lower than the parent compound. Lowering injection temperatures, residence time in the inlet and eliminating active sites during analysis minimized degradation products. Another study reported that split injections and deactivated liners also reduced thermal degradation (even for splitless injections) [238]. In contrast to Kerrigan *et al.*, Tsujikawa found that reducing inlet temperatures (250°C to 200°C) did not prevent α-PVP decomposition and that higher split ratios reduced degradation [238]; however, Kerrigan *et al*. did not specifically investigate α-PVP and examined injection port temperatures between 230°C and 290°C [237]. Special attention to these parameters is warranted to reduce thermal degradation when developing GC-MS detection methods.

Toxicological Findings from Human Performance and Postmortem Cases

Synthetic cathinone identification in biological matrices is essential to aid in interpretation of NPS results for both human performance and PM cases. These substances often pose challenges for forensic and clinical toxicologists, including policy makers, as few data are available. In total, 100 articles were identified highlighting a variety of synthetic cathinone case reports, including information on clinical manifestations, treatment courses, and clinical laboratory results; however, only 47 articles specifically reported synthetic cathinone toxicology results in biological specimens from human performance (n=21) and PM (n=32) cases between 2010 and 2015 (Table 4 and Table 5). Human performance cases included DUID, drug-facilitated

Table 4. Toxicology findings from human performance cases involving synthetic cathinones.

Main Analyte (# of cases)	Toxicology Findings	Type of Case	Other Drugs Detected	Comments	Reference
3-MMC (*n*=1)	Serum: 35.6µg/L	DUID	Methadone 127µg/L, EDDP 6µg/L, Lorazepam 25.4µg/L	Police found suspect sleeping on side of road with damage to driver's side door; reported deficiency in concentration, delayed reaction time, impaired fine motor skills & washed-out pronunciation	[240]
4-FMC (*n*=1)	Serum: 346µg/L Urine: 257µg/L	ED	MDPV in serum & urine (186 & 136µg/L)	Arrived ED with bizarre behavior, suicidal thoughts & hallucinations after insufflating bath salts	[241]
4-MEC (*n*=1)	Maternal hair: 3.9-4.3µg/g Neonatal meconium: 0.7ng/g	Fetal exposure	Methadone & metabolites also measured in maternal & neonatal matrices	Neonatal withdrawal syndrome observed in baby born to chronic 4-MEC abuser; hair concentrations showed exposure before & during pregnancy	[242]
4-MEC (*n*=1)	Blood: 46µg/L	Possession of illicit drugs	Consumed ethanol but concentration unknown	Simultaneous consumption of "bath salts" & ethanol	[222]
4-MPHP (*n*=1)	Serum: 100µg/L	ED	Detected 4-carboxy metabolites in urine; other substances not reported	Admitted agitated to ED, with fractured feet after jumping from a window following insufflation of powder supposed to be cocaine, & rhabdomyolysis	[243]
α-PVP (*n*=1)	Blood: 63µg/L	DUID	Methylone 6.1µg/L & ethylone positive; no other drugs	DRE observed slurred speech, bloodshot watery eyes, dilated pupils, involuntary muscle movements, elevated pulse & BP; admitted taking Kratom, Prozac, saphris, risperidone, depakote, nuvigil, klonopin, also found white powder suspect claimed contained "energy powder, Blast Off, Fusion, Fast Forward, NALT & B-12"	[244]
Butylone (*n*=4)	Blood: 250-910µg/L	ED	Phenazepam & procaine	Phenazepam & procaine also in drug mixture	[245]

Main Analyte (# of cases)	Toxicology Findings	Type of Case	Other Drugs Detected	Comments	Reference
MDPBP (*n*=1)	Only qualitative HRMS urine identification	Alcohol addiction treatment	Not mentioned in report	Stopped ethanol 5 days prior but used "Funky" to combat ethanol withdrawal symptoms	[246]
MDPPP (*n*=1)	Serum: 154µg/L Urine: 5µg/L CSF: 229µg/L	ED	JWH-072 (serum 16µg/L, urine 111µg/L, CSF 19µg/L), cannabicyclohexanol (serum 22µg/L), MDA (serum 11µg/L, urine 1,025µg/L, CSF 11µg/L)	Prolonged psychosis after allegedly smoking "spice"; remained psychotic for almost 1 week	[247]
MDPV (*n*=2)	Serum: 21.3 & 146µg/L	DUID	4-Fluoroamphetamine 89.9µg/L, 2-DPMP 356µg/L, benzedrone <10µg/L; Olanzepine not detected	Admitted injecting bath salts 2 days prior & taking prescribed 20mg olanzapine pills	[240]
MDPV (*n*=2)	Urine: 35 & 55µg/L	ED	Alprazolam 114µg/L, α-OH-alprazolam 104µg/L; alprazolam 10.4µg/L, α-OH-alprazolam 13µg/L, chlordiazepoxide 13µg/L, temazepam 170µg/L, diazepam 13µg/L, nordiazepam 61.5µg/L, oxazepam 115µg/L	Self reported IV MDPV; 15 days after discharge admitted to same ED for agitation, delirium & hallucinations (after MDPV use & pharmaceutical drugs); HRMS revealed MDPV & 6 metabolites	[248]
MDPV (*n*=2)	Blood: 306 & 124µg/L	Narcotics possession; DUID	Diazepam 147µg/L; 4-FMC 346µg/L, THC <1µg/L, THCCOOH 7.9µg/L, JWH-018 5-hydroxyindole 24µg/L	Both admitted to taking "legal highs"	[201]
MDPV (*n*=9)	Blood: 6-368µg/L (median 26.5µg/L)	DUID; domestic violence; drug assault	Benzodiazepines, anti-depressants, opiates, & other stimulants including methylone often detected in combination with MDPV	There was a delay in blood collection for two cases where concentrations were <10µg/L; authors report that blood concentration does not appear to predict impairment vs. fatality	[158]
MDPV (*n*=1)	Urine: 140µg/L	ED	Benzodiazepines administered during medical treatment	Presented severe agitation, hyperthermia, tachycardia progressing to multi-organ failure	[249]

Main Analyte (# of cases)	Toxicology Findings	Type of Case	Other Drugs Detected	Comments	Reference
MDPV (n=259)	Blood: 16-8,400µg/L; (median 240µg/L), 2 outliers 2,400 & 8,400µg/L	DUID	80% cases amphetamine & MDPV, 67% benzodiazepines also present, phenazepam common	Over 1 yr (Aug 2009-2010), 259 positive MDPV cases from apprehended drivers (5.7% of all confirmed DUID cases)	[108]
MDPV (*n*=13)	Blood: 24-241µg/L (mean 58µg/L) Urine: 34-1,386µg/L (median 856µg/L)	Poison Centers	Citalopram, diphenhydramine, hydrocodone, methamphetamine; no methylone or mephedrone detected	Retrospective study examining first 8 mo. of 2 regional poison centers after emergence of bath salts; 236 patients (only 13 had blood concentrations & 4 urine concentrations)	[116]
Mephedrone (*n*=1)	Serum: 412µg/L	DUID	THC 1.8µg/L, 11-OH-THC <0.8µg/L, THCCOOH 11.8µg/L, amphetamine 103µg/L	Admitted taking capsule 1h before traffic control (1h 45min prior to blood draw); police reported delayed reaction time & deranged sensation of time	[240]
Mephedrone (*n*=32)	Blood: 10-740µg/L (median 100µg/L) Mephedrone only (*n*=9): 80-660µg/L (median 160µg/L)	DUID	Benzodiazepines (primarily diazepam), BE, THCCOOH, ethanol, tramadol, BZP, TFMPP, naphyrone, amphetamine	Late 2009-2010, mephedrone 15% of 300 cases; DUID concentrations generally 200-300µg/L	[250]
Mephedrone (*n*=4)	Blood: 1-51µg/kg (median 28.5µg/kg) Urine: 560µg/kg (case 1 only)	DUID	Mephedrone metabolites detected in blood; no other substances mentioned in report	Danish legal limit is 20µg/kg plus 50%; only 1 case above 30µg/kg limit	[165]
Mephedrone (*n*=1)	Serum: 150µg/L	ED	Routine screen of serum & urine did not detect any other substances	Initially ingested 200mg of mephedrone orally with no perceived clinical effects, then injected 3.8g IM; first case of isolated mephedrone toxicity	[228]
Methedrone (*n*=11)	Blood: 0.2-4.8µg/g (median 1.1µg/g)	Suspected petty drug offenses	Methamphetamine & THC		[251]

Main Analyte (# of cases)	Toxicology Findings	Type of Case	Other Drugs Detected	Comments	Reference
Methylone (*n*=45)	Urine: methylone identified in 13% DFSA cases	DFSA	Ethanol, THCCOOH, MDMA, alprazolam & metabolites, methamphetamine, cocaine & metabolites, dextromethorphan; no other cathinones detected	5th most common drug identified in DFSA cases in 2013	[252]
Naphyrone (*n*=1)	Plasma: 30 & 20µg/L (40 & 60h after intake)	ED	Buprenorphine for heroin dependence; urine screen positive for benzodiazepines only	Ingested 100mg naphyrone after requesting MDMA; acute sympathomimetic toxicity with restlessness, anxiety, insomnia & hallucinations for 2 days	[253]

Abbreviations: 3-MMC, 3-methylmethcathinone; 4-FMC, 4-fluoromethcathinone; 4-MEC, 4-methylethcathinone; 4-MPHP, 4-methyl-α-pyrrolidinohexanophenone; α-PVP, α-pyrrolidinopentiophenone; BP, blood pressure; BZP, benzylpiperazine; CSF, cerebral spinal fluid; DFSA, drug-facilitated sexual assault; DRE, drug recognition expert; DUID, driving under the influence of drugs; ED, emergency department; EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; HRMS, high-resolution mass spectrometry; IM, intramuscular; IV, intravenous; MDA, 3,4-methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; MDPBP, 3,4-methylendioxy-α-pyrrolidinbutiophenone; MDPPP, 3,4-methylenedioxy-α-pyrrolidinopropiophenone; MDPV, 3,4-methylenedioxypyrovalerone; ND, not detected; TFMPP, trifluoromethylphenylpiperazine; THC, Δ^9-tetrahydrocannabinol; THCCOOH, 11-nor-9-carboxy-THC

Table 5. Toxicology findings from postmortem cases involving synthetic cathinones.

Main Analyte (# of cases)	Toxicology Findings	Case Findings	Other Drugs Detected	Comments	Reference
3,4-DMMC (*n*=1)	Blood: 3.3mg/L	3,4-DMMC intoxication	None		[131]
3,4-DMMC (*n*=1)	Blood: 27mg/L Urine: 7.6mg/L	Not reported	3,4-DMMC metabolites (2) in blood & urine	LOOP 3 packet found; Suspected rapid death after injection, as blood > urine concentration	[221]
4-methoxy-α-PBP (*n*=1)	Plasma: 9.5mg/L Urine: 12mg/L	Accidental drowning in bathtub	Not mentioned	4 opened Koi, SPACE DRIVE IIIrd, Silver Snow (4-methoxy-α-PBP), PENTDERO2 packages, etizolam & tadalafil also found at scene	[227]
4-MEC (*n*=2)	Blood: 152 & 56µg/L Urine: 122µg/L	Injuries from MVA & multiple drug toxicity	Ethanol 0.12g/dL; PMA 2,347µg/L, PMMA 30µg/L, AMP 378µg/L, MAMP 48µg/L, THC 1.3µg/L, THCCOOH 54.7µg/L	4-MEC not sole cause of crash; PMA intoxication likely COD for case 2	[222]
α-PVP (*n*=1)	Blood: 0.65mg/L (peripheral), 0.44-0.46mg/L (central) Urine: 11.2mg/L Liver: 0.68µg/g Kidney: 1.58µg/g Spleen: 0.32µg/g Brain: 0.52µg/g Stomach contents: 1.0µg/g	Drug toxicity	364µg/L OH-α-PVP peripheral blood & 5.3mg/L urine	Urine >other concentrations; high kidney & urine concentrations suggest rapid excretion	[159]
α-PVP (*n*=1)	Blood: 489µg/L (central)	Drug overdose	Not mentioned	Concentrations 10x higher than clinical cases, but heart blood- PM redistribution may be factor	[202]
Butylone (*n*=1)	Blood: 20mg/L Liver 33µg/g	Cardiac arrest associated with butylone overdose	Not mentioned	Suicide; 10 “Amph-i-bia” tablets ingested	[130]

Main Analyte (# of cases)	Toxicology Findings	Case Findings	Other Drugs Detected	Comments	Reference
Ethylone (*n*=9)	Blood: <25-2,572µg/L (median 1,043µg/L)	COD GSW, drug intoxication (not ethylone), blunt impact (ethylone & ethanol contributed) & hangings	THCCOOH in all cases; ethanol, alprazolam, BE, diphenhydramine, morphine & tramadol	5 cases >400µg/L with ethanol, THCCOOH & doxylamine	[198]
Ethylone (*n*=1)	Blood: 0.39mg/L (peripheral), 0.38mg/L (central) Urine: 20mg/L Vitreous: 0.58mg/L Liver: 1.4µg/g Gastric contents: 12mg	Multiple drug toxicity (ethylone, heroin & alprazolam)	Blood: morphine 50µg/L, alprazolam <50µg/L, THC <1µg/L, THCCOOH 3.6µg/L, naproxen <6µg/L; Urine: 6-AM	Reportedly ingested pills & illicit drugs	[132]
MDPV (*n*=2)	Blood: 38 & 17µg/L	COD MVA & drug overdose	Blood: Buphedrone 127µg/L; Clonazepam & metabolite 1.2 & 96µg/L	Ivory Speed & Exclusive Dust found at car; 2nd case witness testified man took Speedway	[201]
MDPV (*n*=1)	Blood: 1.0mg/L (peripheral), 0.7mg/L (central)	MDPV intoxication	Diphenhydramine 100µg/L, promethazine 200µg/L & nordiazepam 100µg/L	Delusional in ED; agitation, tachycardia, hyperthermia (consistent with excited delirium); screened positive for benzodiazepines (medical intervention) & PCP (false positive)	[254]
MDPV (*n*=16)	Blood: 10-640µg/L (peripheral & central) (median: 91µg/L) MDPV only (*n*=1): Blood: 640µg/L Liver: >4,800ng/g (only specimen submitted for 1 case)	COD included multiple drug toxicity, MDPV intoxication, autoerotic asphyxia, GSWs, MVA, suicide by hanging, natural	Wide range of substances found in combination including benzodiazepines, opiates, anti-depressants, ethanol, THC, other stimulants including cathinones	Additional 7 PM cases detected other cathinones including methylone (*n*=1, 729µg/L), pyrovalerone (*n*=1, 42µg/L), pentylone (*n*=3) & α-PVP (*n*=5); study also examined cathinone distribution in other matrices	[158]

Main Analyte (# of cases)	Toxicology Findings	Case Findings	Other Drugs Detected	Comments	Reference
MDPV (*n*=1)	Blood: 0.44mg/L (peripheral), 0.5mg/L (central) Urine: >5.0mg/L Bile: 0.88mg/L CSF: 0.41mg/L Liver: 0.98µg/g Kidney: 0.84µg/g Lung: 0.60µg/g Muscle:0.56µg/g Brain: 0.42µg/g	MDPV intoxication	Therapeutic lamotrigine <400µg/L, fluoxetine 290µg/L, risperidone 6.8µg/L, benztropine 10µg/L, pseudoephedrine 130µg/L & ibuprofen; methylone also detected in urine	MDPV uniformly distributed among multiple tissues (0.40-0.60mg/L), except kidney, liver, bile higher >0.80mg/L	[157]
MDPV (*n*=2)	Blood: 39 & 130µg/L Urine: 0.76 & 3.8mg/L	Relevant natural causes with MDPV abuse	Metoclopramide 490µg/L; butalbital 5,100µg/L & dextromethorphan 250µg/L	Both cases within recreational range	[255]
MDPV (*n*=1)	Serum: 82µg/L Urine: 670µg/L	MDPV intoxication; cause of death consistent with excited delirium	Trimethoprim 2.2µg/L, ethanol 0.011 & 0.025g/dL (8 & 19h after), salicylate 79mg/L, acetaminophen 2.9mg/L	Developed hyperthermia, rhabdomyolysis, coagulopathy, acidosis, anoxic brain injury; trimethoprim potential adulterant in some bath salts	[129]
MDPV (*n*=1)	Blood: 170µg/L Urine: 1.4mg/L	Not specified	Not mentioned	Retrospective study examining first 8 mo. of 2 regional poison centers after emergence of bath salts	[116]

Main Analyte (# of cases)	Toxicology Findings	Case Findings	Other Drugs Detected	Comments	Reference
Mephedrone (*n*=30)	Blood: 2-3,300µg/L (mean 938µg/L) Mephedrone only: Blood: 190-3,300µg/L (mean 1,745µg/L) Multiple drugs but mephedrone implicated: Blood: 70-2,240µg/L (mean 1,372µg/L) Multiple drugs: Blood: 2-2,000µg/L (mean 518µg/L)	63% accidental poisonings, 10% hangings	Only 4 mephedrone only cases; 87% with other drugs, other substances implicated in 60% of deaths; most commonly found with alcohol, piperazines, cocaine, opiates, AMP, MDMA & benzodiazepines	Mephedrone COD fatalities in individuals ages 16-24 years or if mentioned in coroner's verdict; blood toxicology only available for 17/30	[256]
Mephedrone (*n*=1)	Blood: 5.5mg/L Vitreous: 7.1mg/L	Mephedrone toxicity	None	Mephedrone did not trigger MAMP or AMP ELISA cross-reactivity	[229]
Mephedrone (*n*=12)	Blood: 3-650µg/L (median 80µg/L) Deaths solely attributed to mephedrone: Blood: 2.1 & 1.9mg/L	Suicide hanging (*n*=5), MVA (*n*=1), multiple drug toxicity (*n*=2), accidental drowning (*n*=2)	BZP, TFMPP, Dextromethorphan, THCCOOH, ethanol, diazepam, codeine, MDMA	Late 2009-2010 mephedrone cases (15% of 300 cases); fatality alone likely to occur >2mg/L	[250]
Mephedrone (*n*=1)	Blood: 5.1mg/L (peripheral) Dose remaining in stomach ~ 113mg	Mephedrone overdose	Traces of cocaine, MDMA & oxazepam (7.1, 11, <10µg/L)	Overdose likely due to excited delirium; no definitive COD but likely attributed to fatal mephedrone overdose aggravated by blood loss from multiple wounds	[119]
Mephedrone (*n*=4)	Blood: 980, 2,240, 130 & 240µg/L (peripheral)	Multiple drug toxicity, mephedrone toxicity, blunt force trauma from MVA	Low concentrations paracetamol, 3-TFMPP, diazepam, nordiazepam, olanzepine, chlorpromazine or methadone (300µg/L)	17 to 55 years old	[223]

Main Analyte (# of cases)	Toxicology Findings	Case Findings	Other Drugs Detected	Comments	Reference
Mephedrone (*n*=1)	Blood: 500µg/L Urine: 198mg/L	Multiple drug toxicity	Blood: 60µg/L morphine; Urine: 6-AM, morphine, codeine & doxylamine	Took "black tar" heroin & mephedrone; administered by insufflation, IV & orally prior to injecting heroin; first known death in the US from mephedrone	[225]
Mephedrone (*n*=4)	Blood: 22, 3.3, 5.7 & 1.2mg/L (median 4.5mg/L)	Mephedrone intoxication (cases 1 & 2) & fatal stab wounds (case 4); case 3 undetermined	Blood: <100µg/L diazepam & nordiazepam, 340µg/L AMP; mephedrone only; Urine BE & THCCOOH	Blood & urine Mephedrone 1-3% cross-reactivity with MAMP ELISA assay	[226]
Methedrone (*n*=2)	Blood: 8.4 & 9.6µg/g (peripheral)	Drug toxicity	Diazepam 0.05µg/g, midazolam 0.02µg/g, lidocaine 0.6µg/g	Authors hypothesize narrow therapeutic range; Sweden scheduled methedrone in Dec 2009 due these cases	[251]
Methylone (*n*=1)	Blood: 3.4mg/L (peripheral & central) Vitreous: 4.3mg/L Liver: 11µg/g Gastric contents: 1.7mg	Accidental drowning due to methylone intoxication	No other AMP-like compounds in blood, THC 3.2µg/L, THCCOOH 54.7µg/L	Initially screened positive for MAMP & THC by ELISA	[257]
Methylone (*n*=1)	Blood: 700µg/L	Sudden cardiac death associated with methylone use	None	Collapsed while jogging <2 miles in warm weather (70°F); no significant medical history	[127]
Methylone (*n*=4)	Blood: 60-1,120µg/L (median 429µg/L) (central) In methylone only cases: Blood: 740 & 1,120µg/L Urine: 0.22-38mg/L	Cardiac arrest associated with methylone; GSW to head (suicide & homicide); drowning	MDPV also detected in blood (*n*=2) 30 & 470µg/L; no other drug detected in case 1 or 4; AMP & morphine in other cases	Analysis of several tissues showed methylone does not sequester in particular tissue PM; Average liver: blood ratio 2.68; Urine: chronic use 38mg/L	[156]

Main Analyte (# of cases)	Toxicology Findings	Case Findings	Other Drugs Detected	Comments	Reference
Methylone (*n*=1)	Blood: 272µg/L Liver: 387ng/g	Sudden cardiac death	Not mentioned	Microvascular steatosis in liver suggested possible chronic use; Steatosis of heart muscle, congenital heart disease & bronchial asthma could predispose cardiac death	[258]
Methylone (*n*=3)	Blood: 840, 3,300 & 560µg/L (peripheral) Urine: 0.55-230mg/L	Methylone intoxication	Dextromethorphan; lamotrigine 2.5mg/L	All cases exhibited seizure-like behavior & elevated temps. (104-107°F); methylone >500 µg/L may result in death	[128]
Pentedrone (*n*=1)	Blood: 8.8mg/L Liver: 100µg/g Kidney: 22.1µg/g Brain: 13.2µg/g Stomach contents: 501µg/g	COD drug toxicity from combined use of pentedrone & α-PVP	α-PVP (blood 0.9mg/L, liver 2.6µg/g, kidney 0.46µg/g, brain 0.12µg/g, stomach contents 4.2µg/g; also OH-α-PVP	Investigated distribution of synthetic cathinones in PM samples; α-PVP concentrations higher than previously reported	[259]
PV9 (*n*=1)	Blood: 45.7µg/L (AM), 180µg/L (PM) Urine: 20.3µg/L (AM)	Drug toxicity	Not mentioned	Couple bought "aroma liquid" & bath salt products from drug shop; concentrations AM much lower than PM due to dilution after intensive medical treatment	[133]

Abbreviations: 3,4-DMMC, 3,4-dimethylmethcathinone; 4-MEC, 4-methylethcathinone; 4-methoxy-α-PBP, 4-methoxy-α-pyrrolidinobutiophenone; 6-AM, 6-acetylmorphine; α-PVP, α-pyrrolidinopentiophenone; AMP, amphetamine; AM, antemortem; BE, benzoylecgonine; BZP, benzylpiperazine; COD, cause of death; CSF, cerebral spinal fluid; ED, emergency department; ELISA, enzyme-linked immunosorbent assay; GSW, gunshot wound; MAMP, methamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; MDPV, 3,4-methylenedioxypyrovalerone; MVA, motor vehicle accident; PCP, phencyclidine; PM, postmortem; PMA, para-methoxyamphetamine ; PMMA, para-methoxymethamphetamine; PV9, α-pyrrolidinooctanophenone; TFMPP, trifluoromethylphenylpiperazine; THC, Δ^9-tetrahydrocannabinol; THCCOOH, 11-nor-9-carboxy-THC

assaults, domestic violence, possession of illicit substance(s), poison and treatment centers, emergency department visits (ED) and fetal exposure cases (Table 4). PM cases encompassed drug toxicity and overdoses, and also accidental drowning, gunshot and stab wounds, blunt force trauma from motor vehicle accidents, autoerotic asphyxia, and suicides via hanging, gunshot wounds and drug overdose (Table 5).

A wide range of cathinones including 3,4-dimethylmethcathinone (3,4-DMMC), 3-MMC (3-methylmethcathinone), 4-FMC, 4-MEC, 4-methoxy-α-PBP, 4-methyl-α-pyrrolidinohexanophenone (4-MPHP), α-PVP, butylone, ethylone, 3,4-methylenedioxy-α-pyrrolidinobutiophenone (MDPBP), MDPPP, MDPV, mephedrone, methedrone, methylone, naphyrone, pentedrone and PV9 were identified in matrices including blood (both peripheral and central), plasma, serum, urine, hair, meconium, vitreous humor, CSF, brain, liver and other tissues. The most commonly reported cathinones in human performance and PM cases included MDPV (312 cases, 14 studies) and mephedrone (91 cases, 11 studies). In contrast to human performance cases, methylone also was frequently detected in PM cases (10 cases, 5 studies). Lack of controlled administration studies complicate interpretation of synthetic cathinone results in biological matrices as dose, time of ingestion, administration route, and history of use are often unknown. In addition, antemortem and PM concentrations often overlap, potencies are unknown and other substances are typically found in combination with cathinones derivatives further confounding results (Table 4 and Table 5). Blood, serum and plasma concentrations from human performance cases ranged from 6-8,400 µg/L, with urine concentrations between 5-1,386 µg/L (Table 4) (data did not indicate if urine concentrations were normalized). For PM cases, blood and urine concentrations ranged from 2-27,000 µg/L and 20.3-

198,000 µg/L, respectively (Table 5), with blood concentrations in cathinone only PM intoxication cases between 180-27,000 µg/L. In general, synthetic cathinones were found in combination with ethanol, benzodiazepines, stimulants, cannabis and combinations thereof (Table 4 and Table 5).

Human Performance Cases

One of the first isolated reports of mephedrone toxicity with confirmatory analytical findings involved a 22 year old male who initially ingested 200 mg mephedrone (oral) without any perceived clinical effects, and then proceeded to inject the remaining amount (3.8 g) intramuscularly [228]. He experienced palpitations, "blurred tunnel vision", chest pressure, sweating and presented features of sympathomimetic toxicity at the ED; effects subsided within 4 h after treatment with oral lorazepam. Serum mephedrone concentrations were 150 µg/L with no additional drugs or alcohol detected in serum and urine.

Krikkuu *et al.* examined drug and alcohol findings of MDPV positive cases from drivers suspected of DUID in Finland (2009-2010) to assess the impact of MDPV on driving performance [108], one of the most abused cathinones during this timeframe. Authors reported that MDPV was detected in 5.7% of all confirmed DUID cases (*n*=259 MDPV positive), with blood concentrations varying from 16-8,400 µg/L (highest blood concentration reported in human performance cases) and a 240 µg/L median concentration over a month of cases. Functional impairment was detected in 84% (*n*=208) of cases with observed effects including difficulty in defining time, walking in a straight line, turning around and speech. However, it is difficult to attribute observed behavioral effects to one compound as a high percentage of cases (87%) also contained

other stimulants in high concentrations. Only 8 MDPV-only cases were identified, although the authors hypothesized that the data strongly suggest MDPV is responsible for at least some of the behavioral effects and driving difficulties observed.

Another study examined the prevalence of mephedrone in DUID cases between 2009-2010 [250] in Northern Ireland. Mephedrone was detected in 10.7% of cases (32/300) with blood concentrations ranging from 10-740 µg/L (median 100 µg/L). A more narrow range of mephedrone concentrations (80-660 µg/L) was observed in mephedrone only cases (*n*=9), although a higher median concentration (160 µg/L) was found. Observed symptoms for mephedrone only cases included agitation, hyperactivity, twitchy, slurred speech, glazed eyes and inability to complete field impairment tests. The authors suggested that due to relatively short times between incident and blood collection (1.5-3 h) reported effects are likely a result of mephedrone toxicity; however, caution is warranted in associating concentrations with impairment symptoms as blood collection was delayed.

Alpha-PVP is one of the most frequently identified synthetic cathinones in the US [3] and recently was identified for the first time with methylone and ethylone in a suspected DUID case in Washington [244]. Knoy *et al.* reported blood concentrations in combination with findings from a drug recognition examiner (DRE) officer. The DRE observed slurred speech, bloodshot watery eyes, dilated pupils, slowed reaction to light, involuntary muscle movements and elevated pulse and blood pressure. During standardized field sobriety tests, the suspect presented a fast internal clock during Romberg balance, 7/8 clues for walk-and-turn, 4/4 clues on the one-leg stand and 2/6 clues for horizontal gaze nystagmus. After completion of the examination, the DRE's

opinion was that the suspect was under the influence of central nervous stimulants, specifically Kratom and bath salts. This was supported by the quantification of α-PVP (63 µg/L), methylone (6.1 µg/L) and ethylone (qualitative only) in blood. This study is one of the only synthetic cathinone reports with DRE results and concentrations in biological matrices.

In 2013, methylone was reported as a popular drug in drug-facilitated sexual assaults (DFSA) based on its identification in urine samples [252]. Although quantitative results were not reported, methylone was identified in urine in 13.3% of cases (6/45) without ethanol, GHB, or ketamine (LOD 25 µg/L). Unfortunately, the nature and times of the incidents were largely unknown, including physical symptoms associated with methylone toxicity.

Postmortem Cases

The first reported death in the US attributed to synthetic cathinones was the accidental death of a 22 year old male from mephedrone and heroin intoxication [225]. The decedent was found unresponsive in his living quarters and subsequently taken to the hospital where he later died. Needle marks were found on his lower legs and ankles and investigators discovered he had insufflated, ingested and injected mephedrone in the early morning prior to injecting “black tar” heroin. The decedent’s blood and urine concentrations were 500 and 198,000 µg/L, respectively, with only 60 µg/L morphine in the blood.

Multiple mephedrone fatalities followed this initial mephedrone report, including a study examining 30 PM cases in individuals aged 16-24 years where mephedrone directly impacted cause of death or was mentioned in the coroner’s verdict [256].

Approximately 63% of cases were accidental poisonings and 10% hangings with blood concentrations ranging from 2-3,300 µg/L (mean 938 µg/L). Interestingly, the authors grouped concentrations based on mephedrone only drug detected (*n*=2), only mephedrone implicated in the death but other drugs present (*n*=6) and mephedrone with other drugs and/or alcohol implicated in the cause of death (*n*=9), with mean (range) blood concentrations of 1,745 (190-3,300), 1,372 (70-2,240) and 518 (2-2,000) µg/L, respectively. It should be noted that one case was considered an outlier (22,000 µg/L) and thus excluded from group 2 (multiple drugs but only mephedrone implicated). Based on the results of this study, it appears that mephedrone only fatalities typically exhibited higher mean concentrations; however, ranges demonstrate overlap among the different classifications.

Although mean mephedrone blood concentrations in the previous study were fairly high (938 µg/L) [256], MDPV median blood (central and peripheral) concentrations were lower (91 µg/L) in a study examining synthetic cathinone prevalence and distribution in PM cases [158]. Marinetti *et al.* examined 32 human performance (*n*=9) and fatality (*n*=23) cases containing synthetic cathinones. Of the PM cases, 16 detected MDPV with blood concentrations from 10-640 µg/L; only one fatality detected MDPV without additional substances (640 µg/L). Substances detected with synthetic cathinones included benzodiazepines, opiates, anti-depressants, ethanol, cannabis and other stimulants. MDPV femoral blood concentrations as low as 91 µg/L also were associated with a fatality attributed to MDPV intoxication (lidocaine also detected at 100 µg/L).

In recent years, ethylone emerged as a replacement for methylone and is one of the most frequently identified synthetic cathinones in the US [3]. Lee *et al.* presented 9 PM cases involving ethylone with causes of death ranging from gunshot wounds, multiple drug toxicity, blunt force trauma with contributing ethylone intoxication, hanging, and undetermined causes [198]. Blood concentrations in these fatalities ranged from <25-2,572 µg/L (median 1,043 µg/L). Concurrently identified substances included cannabis, ethanol, alprazolam, benzoylecgonine, diphenhydramine, morphine, tramadol and doxylamine. Two cases did not detect any additional substances in the blood, with ethylone concentrations of 1,837 and 1,617 µg/L, although ethanol and cannabis metabolites were detected in urine. The authors also reported that five cases detected ethylone >400 µg/L with no other drugs found except for ethanol, cannabis metabolites and doxylamine in blood or urine.

Conclusions

The continuous emergence of novel synthetic cathinones poses constant challenges for both clinical and forensic toxicologists. Few data are available on the pharmacology of these substances and with each new compound, modifications to existing analytical methods are needed to detect these NPS in biological matrices and aid in interpretation of results. We reviewed numerous qualitative and quantitative methods to detect synthetic cathinones, and although several screening methods were developed, it would be beneficial to develop more targeted-non-targeted methods utilizing HRMS, as they allow for retrospective data analysis. Another challenging issue with synthetic cathinones is their apparent instability in biological matrices and thermal degradation; special attention to these factors during the analytical process is warranted. In addition,

identification of suitable biomarkers, via *in vitro* studies and the development of commercial reference standards must be addressed in order to document intake of these compounds and link toxicity to the causative agents. It is important to establish the metabolic profiles of these NPS to incorporate cathinone metabolites into quantification methods along with parent compounds and improve result interpretation. Pharmacokinetic studies also are needed to fully characterize synthetic cathinones and their metabolite profiles to help interpret clinical and forensic results including human performance and PM cases. Interpretation of synthetic cathinone results in biological matrices is hindered by the lack of controlled administration studies as dose, time of ingestion, administration route, and potency are typically unknown. In addition, recreational and fatal concentrations often overlap and other substances are typically found in combination with cathinone derivatives further complicating results. It is imperative that laboratories respond rapidly, adapt to changing NPS trends on the drug market, and improve analytical methodologies to improve the detection of these compounds.

Chapter 2 - Pharmacodynamic Effects and Relationships to Plasma and Oral Fluid Pharmacokinetics after Intravenous Cocaine Administration

(*As published in* Drug and Alcohol Dependence, *April 2016*)[2]

Abstract

Background: No controlled cocaine administration data describe cocaine and metabolite disposition in OF collected with commercially-available collection devices, OF-plasma ratios, and pharmacodynamic relationships with plasma and OF cocaine and metabolite concentrations. *Methods:* Eleven healthy, cocaine-using adults received 25 mg IV cocaine. Physiological and subjective effects (VAS), and plasma were collected one hour prior, and up to 21 h post-dose. OF was collected with the Quantisal^{TM} device up to 69 h post-dose. Cocaine, BE and EME were quantified in plasma by LC-MS/MS; cocaine and BE were quantified in OF by two dimensional-gas chromatography-mass spectrometry. *Results:* Increases in heart rate and blood pressure and positive subjective effects occurred within the first 15 min, persisting up to 1 h ("Rush"), with clockwise hysteresis observed for plasma and OF concentrations and some subjective measures. Peak subjective effects ("Rush," "Good drug effect" and "Bad drug effect") occurred prior to peak OF cocaine concentration, whereas observed peak plasma concentrations and subjective measures occurred simultaneously, most likely due to significantly earlier plasma T_{max} compared to OF $T_{max.}$ T_{last} was generally longer in OF (12.5 h cocaine; 33.0 h BE) than plasma (9.5 h cocaine; >21 h BE, cutoffs 1 µg/L); 8 and 10 µg/L OF cocaine confirmatory cutoffs yielded detection times similar to cocaine's impairing effects,

[2] Ellefsen K.N. *et al.* Pharmacodynamic Effects and Relationships to Plasma and Oral Fluid Pharmacokinetics after Intravenous Cocaine Administration. *Drug and Alcohol Dependence*. 13 April 16 (DOI:10.1016/j.drugalcdep.2016.04.004)

suggesting usefulness for DUID testing. *Conclusions:* OF offers advantages as an alternative matrix to blood and plasma for identifying cocaine intake, defining pharmacokinetic parameters at different confirmation cutoffs, and aiding different drug testing programs to best achieve their monitoring goals.

Introduction

There is increasing interest in alternative matrices for drug testing in DUID, workplace, clinical, forensic and anti-doping programs [26,29,30]. OF detects relatively recent drug consumption and provides easy, noninvasive specimen collection. Additionally, there is no need for same-sex collectors and little potential for adulteration [25-28]. Although plasma provides an estimate of circulating blood concentrations, OF reflects free unbound drug, which may better reflect drug available for binding to the drug's active site or receptor [25,29,30].

Cocaine is a psychostimulant abused for its pronounced euphoria and mood elevation, despite cardiotoxicity. Cocaine is rapidly metabolized by butyrylcholinesterase and carboxylesterases to two major inactive metabolites: BE, formed predominantly in the liver, and EME, formed in the plasma and intestinal lining [20,21]. Cocaine also is unstable, hydrolyzing *in vitro* to BE and EME spontaneously (without enzymes) at physiological temperature and pH [22]. Despite declining abuse prevalence in the general population, cocaine remains a widely used illicit drug in Europe (3.4 million users) [93] and the US (1.5 million users) [260]. Cocaine also was the second most prevalent illicit drug (after cannabis) detected in European drivers (0.42%) [2] and in US nighttime drivers (1.4%) [6].

Cocaine's subjective and physiological effects include an initial "rush" (intense euphoria) lasting only 1-2 min, followed by prolonged elevated mood, typically lasting about 30 min [10,11]. Concurrent excitatory physiological effects, such as increased heart rate, blood pressure, respiratory rate and pupil dilation occur [7-9]. Subjective and physiological effects peak at maximum plasma concentrations and are dose-dependent [7,8,11-13]. These effects rapidly dissipate prior to plasma concentrations returning to baseline, suggesting acute tolerance development.

Cocaine and its metabolites' pharmacokinetics are well established in blood and plasma following various administration routes [7,10,11,13,16-19]; however, less is known about cocaine and metabolite disposition in OF, OF-plasma ratios, and correlations of cocaine pharmacodynamic effects with OF and plasma cocaine and metabolite concentrations following controlled cocaine administration. Cocaine and metabolite concentrations following controlled IV [10,32,34-38], SM [35-38], IN [35,37,38], oral [38,39] and SC administration [31] are available; however, only three studies examined cocaine and metabolite OF pharmacokinetics [31,37,39], and none with OF collected with commercially available collection devices containing different elution and stabilization buffers. It is important to characterize OF pharmacokinetics from OF collected with commercially available OF confirmation devices, because recovery of cocaine from the device is improved, and hydrolysis of cocaine is reduced. Use of these collection devices improves the reliability and sensitivity of OF collection and analysis. Furthermore, previous OF pharmacokinetic studies collected OF via stimulated expectoration, which increases bicarbonate in saliva affecting OF pH. The higher pH that occurs with increased bicarbonate reduces the concentration of cocaine in OF and affects

its pharmacokinetics in OF. Furthermore, few evaluated detection windows at different regulatory confirmation cutoffs. Limited studies report correlations between cocaine plasma and OF concentrations and pharmacodynamic effects [10,15], and none report concentration effect curves with subjective measures and OF cocaine concentrations. Significant correlations between OF and plasma [10,15,31,32] and OF and blood [33] cocaine concentrations were reported, suggesting OF might be a good alternative to blood for DUID crash [10,15], home and workplace accident investigations, as well as in clinical toxicology. However, correlations between OF and plasma cocaine metabolite concentrations were not previously investigated following controlled IV cocaine administration. Cocaine contamination of the oral mucosa following smoked crack cocaine or insufflated cocaine is eliminated when cocaine is administered by the IV route.

We investigated plasma and OF cocaine and metabolite concentrations and their relationships to pharmacodynamic effects following controlled IV cocaine administration (25mg). We characterized subjective and physiological effects, evaluated cocaine and metabolite OF pharmacokinetics with OF collected with the Quantisal^{TM} device and determined OF-plasma ratios, correlations and detection windows.

Materials and Methods

Participants

Participants provided written informed consent to participate in this NIDA Institutional Review Board approved study and received comprehensive medical and psychological assessments. Inclusion and exclusion criteria were previously described [261,262]. Briefly, healthy adults ages 18-50 years who smoked or used IV cocaine for at least six months and at least three times per month during the three months prior to

screening participated. Exclusion criteria included physical dependence on any drug (except cocaine, caffeine, or nicotine); clinically significant medical or psychiatric disorder; hypertension or blood pressure readings consistently >140 mm Hg systolic or 90 mm Hg diastolic while at rest; heart rate consistently >90 or <50 bpm while at rest; abnormal 12-lead electrocardiogram (ECG); or interest in drug abuse treatment within three months.

Drug Administration

(-) Cocaine hydrochloride (25mg) obtained from Mallinckrodt (St. Louis, MO) was dissolved in 2mL 0.9% sterile saline. Participants resided on a secure research unit (13 days and 12 nights), and were administered three 25 mg IV cocaine bolus (10 sec) doses on study Days 1, 5 and 10. A cocaine dose (25mg) lower than what is typically self-administered in the community was selected to ensure measureable subjective and cardiovascular effects without producing clinically significant adverse effects; a short injection (10sec) duration was utilized to closely mirror cocaine injection in the community. Cocaine was administered alone on Day 1, in combination with oral acetazolamide on Day 5 (after daily acetazolamide on Days 2-4) and with oral quinine on Day 10 (after daily quinine on Days 6-9). The primary study aims evaluated potential pharmacodynamic and pharmacokinetic interactions between cocaine and acetazolamide and quinine, as they were being considered as medication adherence markers in cocaine use disorder pharmacotherapy studies (data not yet published).

Plasma and Oral Fluid Specimen Collection

Peripheral venous blood was collected via indwelling catheter into grey-top tubes, placed on ice (maximum 2 h), centrifuged at 3,000 rpm for 10 min and plasma decanted

into 3.6 mL Nunc CryoTubes (Nalgene, Penfield, NY) for storage at -20ºC prior to analysis. Specimens were collected at -1 h prior, and 0.017, 0.083, 0.17, 0.25, 0.33, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6.5, 9.5, 12.5 and 21 h after cocaine administration.

OF (1 ± 0.1 mL) was collected with the Quantisal™ device (Immunalysis, Pomona, CA, USA) until the volume-adequacy indicator turned blue or 5min had elapsed, whichever occurred first, due to the tight study timeline. Collection pads were placed in collection tubes containing elution/stabilization buffer and stored up to 12 h at 4°C to allow analyte elution; serum separators facilitated decanting into a 3.6 mL Nunc CryoTube. Specimens were stored refrigerated at 4°C prior to analysis. OF specimens were collected -1 h prior, and 0.17, 0.5, 1, 1.5, 2, 3, 4, 6.5, 9.5, 12.5, and 21 h post-administration. Additional collections occurred at 33 h (Day 1), 45 and 69 h (Days 1 and 5) and 28 h (Day 10).

Cocaine, BE and EME Quantification in Plasma

Cocaine (1 g/L), EME (1 g/L) and internal standard (IS) d_3-cocaine (100 mg/L), d_3-EME (1 g/L) in acetonitrile and BE (1 g/L), d_3-BE (1 g/L), d_8-BE (100 mg/L) in methanol were obtained from Cerilliant (Round Rock, TX, USA). Calibrators were prepared from 1-1000 µg/L and quality controls (QC) at 3, 50 and 800 µg/L in 0.1 mL plasma. Briefly, 10µL IS (500 ug/L d_3-cocaine, d_3-BE, and d_3-EME) were added to 0.1 mL human plasma with 0.4 mL water, prior to extraction on Waters OASIS 3CC (60 mg) SPE cartridges (Milford, MA) preconditioned with methanol (Fisher Scientific, Fair Lawn, NJ) + 0.06% trifluroacetic acid (Spectrum, New Brunswick, NJ) and water. Columns were washed (water) before elution (1 mL methanol + 0.06% trifluroacetic acid) and eluents (200 µL) transferred to autosampler vials. Extracts were analyzed for

cocaine, BE, and EME by LC-MS/MS on a Shimadzu LC-20 AD XR LC system coupled with a Sciex API 5000 detector. An Allure PFP Propyl column (4.6 x75 mm, 5 µm) with isocratic acetonitrile:water:formic acid (90:10:0.5, v/v/v) + 5 mM ammonium formic acid mobile phase and a 1 mL/min flow rate at 30°C were utilized. Electrospray ionization (ESI) in positive mode was utilized for mass spectrometric detection. MRM transitions were: cocaine 304→182 *m/z*, BE 290→168 *m/z*, EME 200→182 *m/z*, d_3-cocaine 307→185 *m/z*, d_3-BE 293→171 *m/z*, and d_3-EME 203→185 *m/z*.

Plasma methods were validated according to Food and Drug Administration Bioanalytical Method Validation Guidance for Industry [263]. Calibrators were linear from 1-1000 µg/L with $1/x^2$ weighting for cocaine, BE, and EME and with residuals <15%. LOQs were 1 µg/L. Bias was ± 5.7%, with within- and between-run imprecision <12.1% and <10.0%, respectfully, at all three QC concentrations for all analytes evaluated in six replicates over four days (*n*=24). Cocaine, BE, and EME extraction efficiencies were 91.4, 94.8, and 91.5%, respectively. Analytes were considered stable under evaluated conditions (% difference < ±21.5%).

Cocaine and Benzoylecgonine Quantification in Oral Fluid

OF cocaine and BE quantification was performed according to a previously validated (Scientific Working Group for Forensic Toxicology recommendations (SWGTOX) [264]) OF two-dimensional-gas chromatography-mass spectrometry (2D-GC-MS) analytical method [265], with modifications to calibrator and QC preparation only. Calibrators and QCs (3, 25, and 75 µg/L) were prepared in 1 mL OF (0.25 mL blank OF and 0.75 mL Quantisal buffer) solutions. Calibrators were linear from 1-100 µg/L with $1/x^2$ weighting for cocaine and BE with coefficients (R^2) above 0.9911 ±

0.0027 (*n*=5) and residuals <15%. LOD for cocaine and BE were 1 and 0.5 µg/L, respectively. Bias was within ±14.8% with within- and between-run imprecision <5.9% and <7.0%, at all QC concentrations evaluated in four replicates over five days (*n*=20). Specimens exceeding 100 µg/L were diluted with OF and buffer (4-fold), re-extracted and analyzed. OF-plasma (OF/P) ratios for cocaine and BE were determined in simultaneously collected specimens.

Physiological and Subjective Effects

Heart rate, respiration rate and systolic and diastolic blood pressure were monitored at -0.5, -0.25, 0.083, 0.17, 0.25, 0.5, 0.75, 1, 2 and 3 h relative to dosing. Subjective effects were assessed with 100-mm VAS "Rush," "Good drug effect," "Bad drug effect," "Crave cocaine," "Want cocaine," "Like cocaine," "Strong effect," "High," "Elated," "Stimulated," "Suspicious," "Anxious" and "Tired" at -0.25, 0.25, 0.5, 0.75, 1, 2 and 3 h relative to dosing. Additionally, "Rush," "Good drug effect" and "Bad drug effect" were measured at 30 sec and then every min for 15min after cocaine.

Statistical and Pharmacokinetic Analyses

All statistical analyses were performed with SPSS version 20 for Windows (SPSS, Chicago, IL) and GraphPad Prism Version 5.02 (GraphPad Software Inc, La Jolla, CA). General linear models repeated-measures ANOVA evaluated changes in participants' VAS scores and physiological measures over time, with Greenhouse-Geisser corrections for sphericity violations (identified with Mauchly's test) and Bonferroni post-hoc testing determining differences between specific time points. Pharmacokinetic parameters were evaluated with non-parametric *t* tests because visual data inspection and D'Agostino-Pearson normality test (omnibus K2) indicated non-normal data distribution.

Comparisons were considered significant if P <0.05. Noncompartmental analysis was performed with Phoenix WinNonlin® 6.3 for Windows (Pharsight, Mountain View, CA). Maximum concentration (C_{max}), T_{max}, AUC from 0-21 (plasma) and 0-69 h (OF) post-dose, half-life ($t_{1/2}$), T_{last}, and last observed concentration (C_{last}) were calculated. At least five data points on the descending linear limb of the concentration-time curve were employed to calculate $t_{1/2}$. For statistical purposes, concentrations less than the LOQ were set to 0. Cocaine and BE OF to plasma concentration correlations were performed using Spearman's rho correlation (r) and least-squares regression analysis in Prism.

Results

Participants

Participant demographic characteristics and self-reported cocaine histories are detailed in Table 8 (Supplemental); nine completed all dosing sessions. Only plasma data from Day 1 (cocaine alone) IV cocaine administration were evaluated to eliminate potential acetazolamide or quinine pharmacokinetic interactions. Two scheduled blood specimens (Participant B at 0.75 and 1.5 h) were not collected due to complications with his IV catheter; all OF data were available except for Participant I's Quantisal OF specimens.

Cocaine and Metabolite Concentrations in Plasma

Of 168 plasma specimens quantified for cocaine, BE and EME from 0-21 h post-administration, 28, 4 and 7 specimens were <1 µg/L, respectively. Median (interquartile range) plasma cocaine and metabolite concentration-time curves are illustrated in Figure 1 and Figure 5 (Supplemental), with pharmacokinetic parameters summarized in Table 6. Median (range) baseline (-1 h) plasma cocaine concentrations were 0.7 (<LOQ-2.0) µg/L,

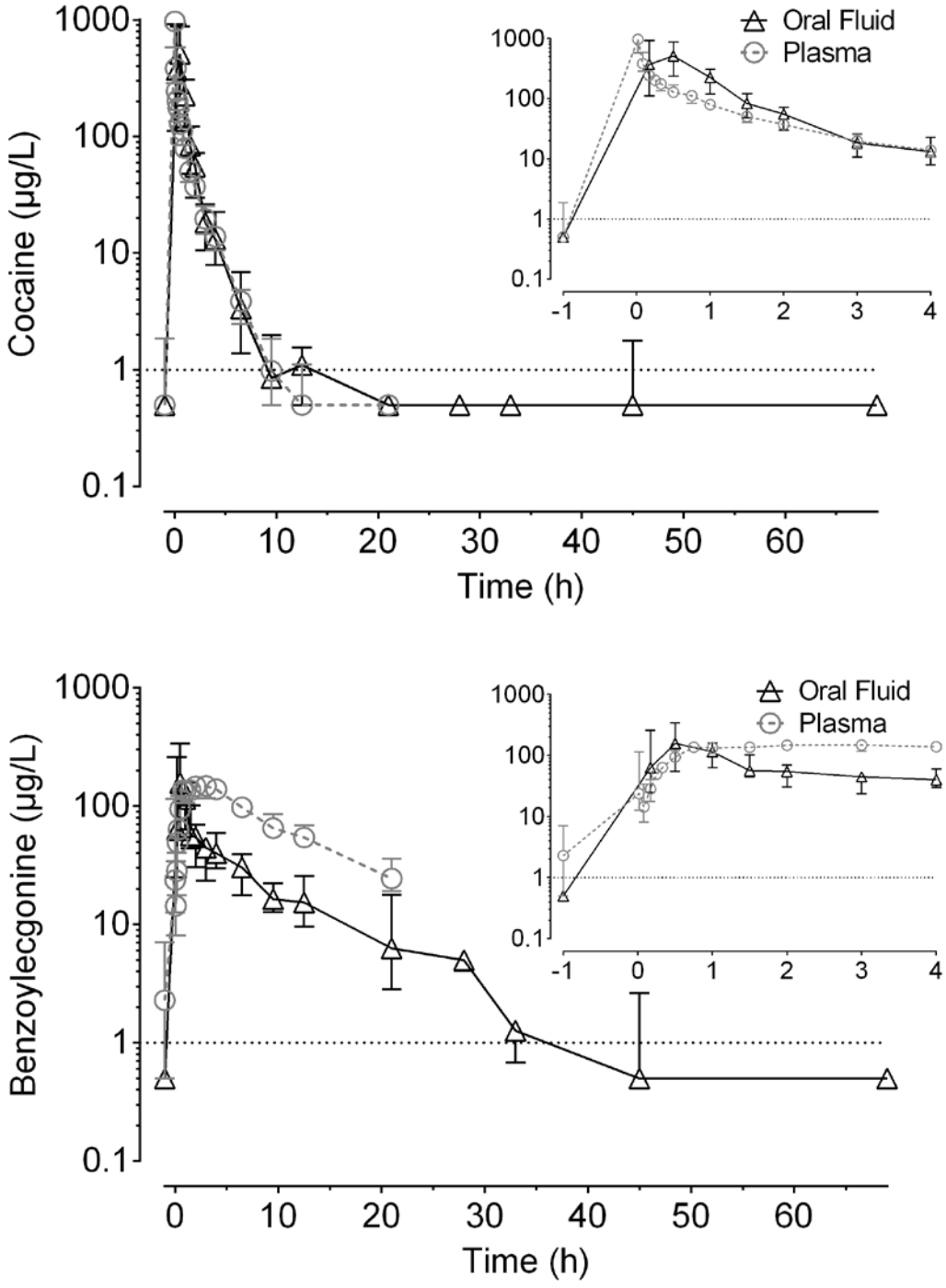


Figure 1. Median (interquartile range) concentration-time curves for cocaine and benzoylecgonine in oral fluid and plasma from 10 cocaine-using adults after 25 mg intravenous cocaine through 69 h (21 h for plasma). Inserts represent time course from-1 to 4 h post-administration. Dotted lines represent the 1 µg/L analytical limit of quantification (LOQ). Concentrations <LOQ are represented as ½ LOQ. Note y-scales are logarithmic.

Table 6. Median (range) pharmacokinetic parameters for cocaine, benzoylecgonine (BE), and ecgonine methyl ester (EME) in plasma and oral fluid following 25 mg intravenous cocaine administration in 10 cocaine-using adults.

	Cocaine	Benzoylecgonine	Ecgonine Methyl Ester
Plasma			
C_{max} (µg/L)	969 (356-14,700)*†	165 (96.2-720)*‡	22.5 (13.5-106)†‡
T_{max} (h)	0.017$^{*\dagger\|\|}$	2.0 (0.017-4.0)$^{*\|\|}$	1.5 (0.017-4.0)†
$t_{1/2}$ (h)	1.5 (1.0-2.5)*†	7.9 (5.8-8.8)*	6.8 (4.8-8.6)†
$AUC_{0\text{-}21h}$ (h x µg/L)	345 (238-937)*†	1,544 (1,014-2,196)$^{*\ddagger\|\|}$	200 (102-294)†‡
T_{last} (h)	9.5 (6.5-12.5)*†	>21*	>21 (12.5-21)†
C_{last} (µg/L)	1.6 (1.1-4.7)*†	24.5 (16.1-43.4)*‡	3.7 (1.0-5.2)†‡
Oral Fluid			
C_{max} (µg/L)	660 (72.0-1,764)*	157 (59.6-645)*	-
T_{max} (h)	0.50 (0.17-1.0)$^{\|\|}$	0.50 (0.017-1.0)$^{\|\|}$	-
$t_{1/2}$ (h)	1.4 (0.79-2.2)*	7.3(4.9-11.5)*	-
$AUC_{0\text{-}69h}$ (h x µg/L)	582 (106-1,521)	632 (476-1,207)	-
$AUC_{0\text{-}21h}$ (h x µg/L)	-	610 (469-953)$^{\|\|}$	-
T_{last} (h)	12.5 (6.5-45)*	33.0 (21-69)*	-
C_{last} (µg/L)	1.5 (1.0-20.5)	2.5 (1.2-18.2)	-

C_{max} maximum concentration detected, T_{max} time of maximum concentration, $T_{1/2}$ terminal half-life, AUC area under the curve, C_{last} last detected concentration, T_{last} time of last detected concentration

Significant difference P<0.05: *cocaine versus BE; †cocaine versus EME; ‡BE versus EME; $^{\|\|}$plasma versus oral fluid. No statistical comparison between plasma and oral fluid BE T_{last} and C_{last} were performed due to differences in timecourse and last positive specimen between matrices; comparisons were made for BE AUC from 0-21h only.

peaking in the first sample (0.017 h) at 969 (356-14,700) µg/L, and decreasing to 244 (107-412) µg/L at 0.17 h, 129 (99.3-194) µg/L at 0.5h and 3.9 (1.2-5.8) µg/L 6.5 h post-dose. By 21 h, all participants' plasma cocaine concentrations were ≤ LOQ.

Median BE and EME C_{max} were approximately 17% and 2% of median cocaine C_{max}, respectively (Table 6). Cocaine time to maximum concentration (T_{max}) was significantly shorter (0.017 h) than for BE (2.0 h) and EME (1.5 h), as was cocaine $T_{1/2}$ (1.5, 7.9, 6.8 h, respectively) (Table 6). Cocaine was detected up to 12.5 h post-dose and its metabolites up to 21 h at the method LOQ (1 µg/L). BE $AUC_{0\text{-}21h}$ was significantly greater than cocaine or EME.

Cocaine and Metabolite Concentrations in Oral Fluid

In total, 142 OF specimens were analyzed for cocaine and BE from 0-69 h post-dose. Median cocaine OF concentrations were higher than BE's for approximately 2 h (Figure 1). Median cocaine and BE OF concentrations were 372 (56-1,693) µg/L and 57.5 (8.5-645) µg/L at 0.17 h, 514 (51.8-1,764) µg/L and 157 (31.8-395) µg/L at 0.5 h, and 3.4 (1.2-12.2) µg/L and 30.3 (14.3-87.2) µg/L at 6.5 h, respectively. Most participants' cocaine concentrations were <LOQ at 21 h, while BE remained detectable for 45-69 h. High inter-subject variability was observed for cocaine and metabolite OF concentrations.

OF cocaine concentrations increased rapidly with median (range) C_{max} of 660 (72.0-1,764) µg/L and T_{max} 0.5 (0.17-1) h (Table 6). Median BE C_{max} was approximately 23.8% of cocaine's C_{max} and occurred at 0.5 h. BE T_{last} (33.0 h) was significantly longer than cocaine's T_{last} 12.5 h at the method LOQ (1 µg/L). Cocaine's $t_{1/2}$ (1.4 h) was significantly shorter than BE's (7.3 h).

Cocaine and BE Detection Rates and Detection Times in Oral Fluid

Cocaine and BE detection rates from 0.17-69 h at the LOQ, SAMHSA (8 µg/L) and DRUID (10 µg/L) confirmation cutoffs are highlighted in Table 7. Cocaine and BE detection rates were lower at SAMHSA and DRUID confirmatory cutoffs compared to the LOQ. In general, detection times were longer at the LOQ (cocaine 21 h; BE 69 h) compared to SAMHSA and DRUID (cocaine 6.5 h; BE 21 h) confirmation cutoffs.

Comparison of Cocaine and BE Pharmacokinetics in Oral Fluid and Plasma

Cocaine T_{max} was significantly longer in OF (0.5 h) compared to plasma (0.017 h) ($P < 0.0001$), whereas BE's T_{max} was significantly shorter in OF (0.5 h) compared to

plasma (2.0 h) (P <0.042) (Table 6). No significant cocaine and BE $t_{1/2}$ differences were observed between matrices. Unlike C_{max}, BE exhibited significantly higher $AUC_{0\text{-}21h}$ in plasma than OF (P <0.0001), when considering an equivalent 21 h monitoring period. Statistical comparisons between plasma and OF BE T_{last} and C_{last} could not be performed, as plasma BE concentrations were still positive in plasma at the last collection (21 h); OF was analyzed up to 69 h. No significant differences in cocaine T_{last} and C_{last} were observed between matrices.

Table 7. Detection rates for cocaine and benzoylecgonine (BE) in oral fluid (OF) collected with Quantisal devices up to 69 h from 10 cocaine-using adults after 25 mg intravenous cocaine. Detection rates were calculated with the assay limit of quantification (LOQ)

Time (hours)	*n*	Cocaine Detection Rates (%)			BE Detection Rates (%)		
		LOQ (≥1µg/L)	SAMHSA (≥8 µg/L)	DRUID (≥10 µg/L)	LOQ (≥1 µg/L)	SAMHSA (≥8µg/L)	DRUID (≥10 µg/L)
-1	10	0	0	0	20	0	0
0.17	10	100	100	100	100	100	90
0.5	10	100	100	100	100	100	100
1	10	100	100	100	100	100	100
1.5	10	100	100	100	100	100	100
2	10	100	100	100	100	100	100
3	10	100	90	80	100	100	100
4	10	100	80	60	100	100	100
6.5	10	100	10	10	100	100	100
9.5	10	50	0	0	100	100	100
12.5	10	60	0	0	100	100	80
21	10	10	0	0	100	40	40
28	2	0	0	0	100	0	0
33	4	0	0	0	100	0	0
45	8	25	12.5	12.5	37.5	0	0
69	8	0	0	0	12.5	0	0

Comparison of Cocaine and BE Pharmacokinetics in Oral Fluid and Plasma

Cocaine T_{max} was significantly longer in OF (0.5 h) compared to plasma (0.017 h) ($P < 0.0001$), whereas BE's T_{max} was significantly shorter in OF (0.5 h) compared to plasma (2.0 h) ($P < 0.042$) (Table 6). No significant cocaine and BE $t_{1/2}$ differences were observed between matrices. Unlike C_{max}, BE exhibited significantly higher $AUC_{0\text{-}21h}$ in plasma than OF ($P < 0.0001$), when considering an equivalent 21 h monitoring period. Statistical comparisons between plasma and OF BE T_{last} and C_{last} could not be performed, as plasma BE concentrations were still positive in plasma at the last collection (21 h); OF was analyzed up to 69 h. No significant differences in cocaine T_{last} and C_{last} were observed between matrices.

Oral Fluid-Plasma Ratios and Correlations

Median OF/P ratios were highly variable between participants (especially at early time points) over time (0.17-21 h) (Figure 2). Median cocaine OF/P ratios were ≥1 from 0.17-2 h and approximately 1 from 3-12.5 h. BE OF/P also were ≥1 initially (0.17-1 h), but then decreased to <1 until 21h. OF and plasma cocaine and BE concentrations were significantly correlated ($P < 0.0001$) from 0.17 to 21h (r=0.94 and 0.55, respectively) with low R^2 values (0.45, 0.008, respectively) (Figure 6 (Supplemental)). Large concentration variabilities precluded calculating one concentration from the other.

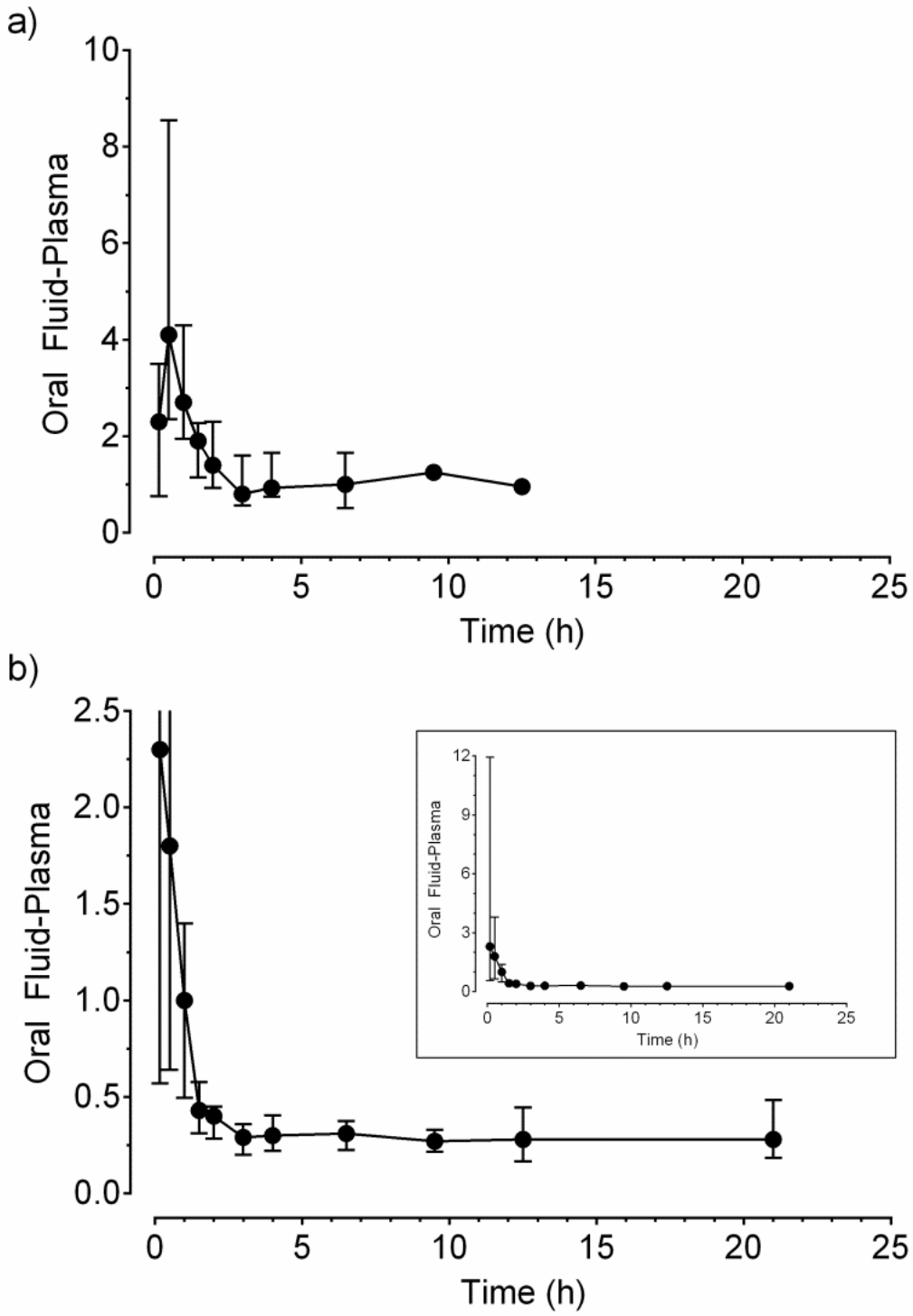


Figure 2. Median (interquartile range) oral fluid-plasma ratios for a) cocaine and b) benzoylecgonine from 0.17 to 21 h after 25 mg intravenous cocaine administration in 9 cocaine-using adults. Only paired positive specimens were included. Note y-scales vary by magnitude.

Physiological Effects

Systolic blood pressure significantly increased from baseline [F(8,72)=8.68, *P* <0.001] 11.4% at 5 min (*P* <0.009), 10.6% at 10min (*P* <0.002) and 8.6% at 15 min (*P* <0.021); heart rate significantly increased 22.2% at 5 min (*P* <0.001) and 19.7% at 10 min (*P* <0.004), but decreased significantly at 1h (*P*<0.48) and 2h (*P*<0.047) [F(2.9,26.1)=12.21, *P* <0.001] (Figure 7 (Supplemental)). There were no significant changes in diastolic blood pressure or respiration (Figure 7 (Supplemental)).

Subjective Effects

IV cocaine significantly increased "Rush" [F(2.77, 22.18)=23.41, *P* <0.001], "Good drug effect" [F(2.71, 21.69)=22.77, *P* <0.001], "Stimulated" [F(1.16, 9.31)=6.87, *P*=0.024], "High" [F(1.13, 9.06)=12.68, *P*=0.005], "Want cocaine" [F(1.58, 12.63)=4.24 , *P*=0.047], and "Strong effect" [F(1.04, 8.28)=5.94, *P*=0.039] (Figure 8 (Supplemental)). Significant increases occurred within 15 min and persisted up to 1 h ("Rush"). There were no significant changes in remaining VAS parameters (Figure 9 (Supplemental)).

Median plasma and OF cocaine concentration-effect curves for 13 VAS scales are shown in Figure 3 and Figure 4, respectively. Peak VAS scores for "Rush", "Good drug effect" and "Bad drug effect" and peak observed cocaine concentrations occurred at/or before 0.017 h post-dose, and at/or prior to 0.25 h for all other VAS parameters, except for "Like cocaine" and "Tired" which demonstrated little change. Clockwise hysteresis was observed for "Want cocaine," "Crave cocaine," "Stimulated," "High," "Anxious" and "Strong effect" (Figure 3). Only "Rush," "Good drug effect," "Bad drug effect," "Suspicious" and "Strong effect" exhibited statistically significant correlations with cocaine plasma concentrations (*r*=0.98, 0.94, 0.90, 0.86 and 0.82, *P* <0.05, respectively).

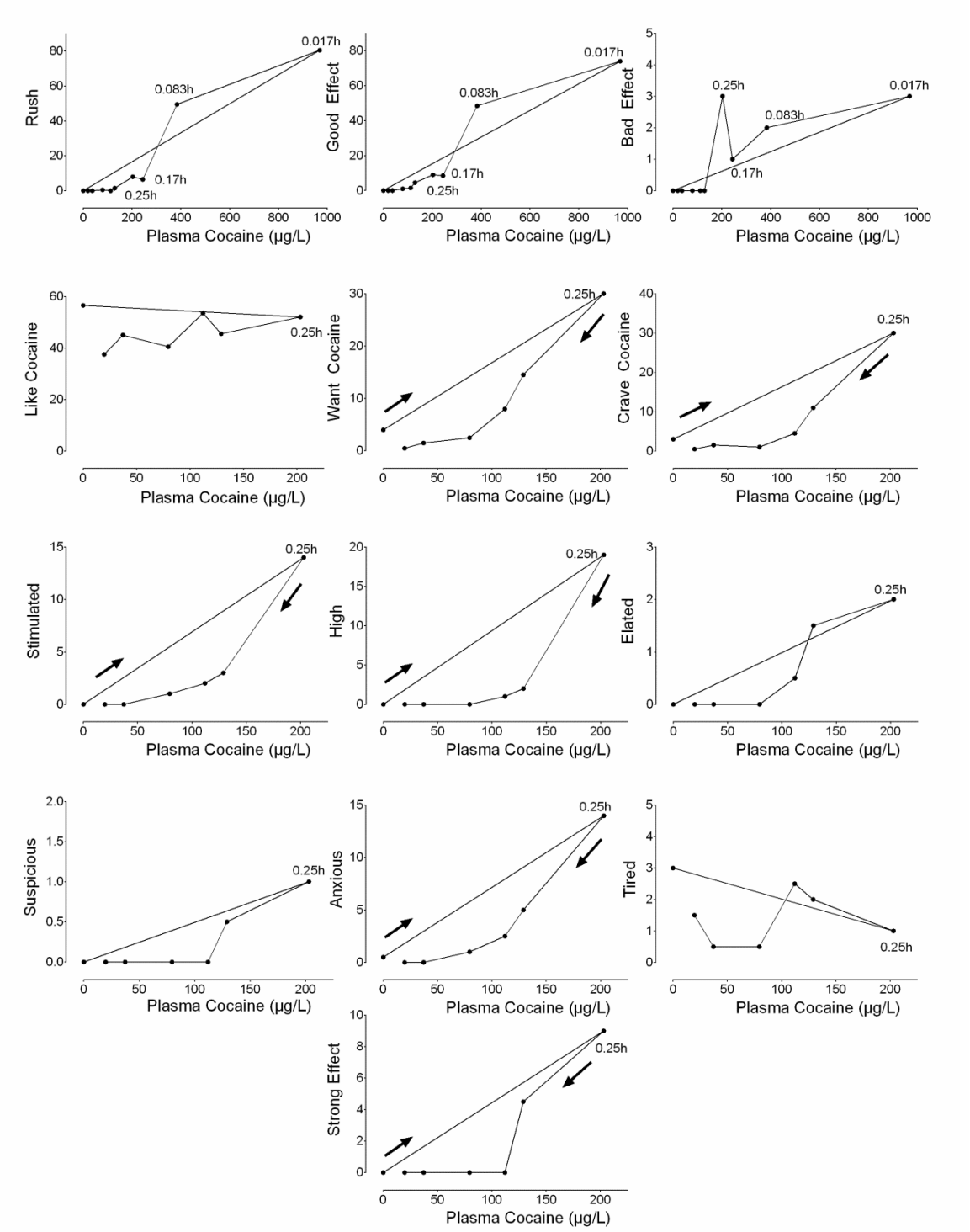


Figure 3. Relationship between subjective effects (median visual-analogue scale (VAS) scores) and median plasma cocaine concentrations in 10 cocaine-using adults from -1 to 3 h after 25 mg intravenous cocaine administration. Only paired specimens were included. Arrows represent hysteresis curve progression over time.

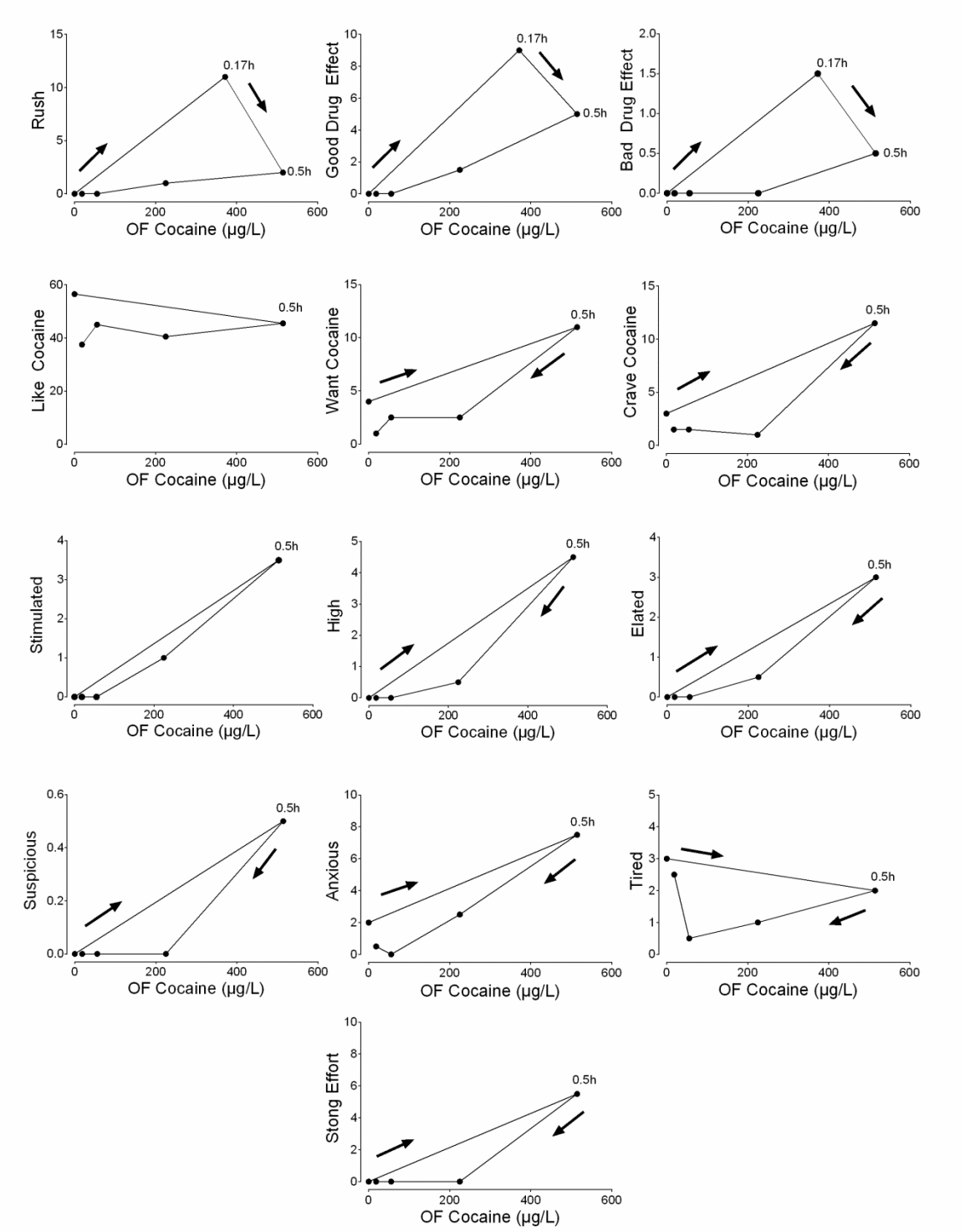


Figure 4. Relationship between subjective effects (median visual-analogue scale (VAS) scores) and median oral fluid (OF) cocaine concentrations in 10 cocaine-using adults from -1 to 3 h after 25 mg intravenous cocaine administration. Only paired specimens were included. Arrows represent hysteresis curve progression over time.

For OF, peak subjective effects for “Rush,” “Good drug effect,” and “Bad drug effect” occurred prior to (0.17 h) peak cocaine OF concentration (0.5 h) (Figure 4). For remaining VAS parameters, peak subjective effects and peak cocaine OF concentrations occurred simultaneously (0.5 h); however, data were not collected at 0.17 h for these VAS parameters. Clockwise hysteresis was observed with all VAS measures except “Like cocaine” and “Stimulated.” Only “Rush,” “Good drug effect,” “Stimulated,” “High,” and “Strong effect” exhibited statistically significantly correlations with cocaine OF concentrations (r=0.43, 0.44, 0.30, 0.31 and 0.29, P <0.05, respectively).

Discussion

IV cocaine administration produced the expected increases in heart rate, blood pressure, and subjective ratings within the first 15 minutes, persisting up to 1 h (“Rush”). Of the six subjective measures exhibiting significant increases, only “Rush,” “Good drug effect” and “Strong effect” were strongly correlated with plasma cocaine concentrations. These three effects, in addition to “Stimulated” and “High,” had weak-moderate correlations with OF concentrations. Peak subjective effects generally occurred at observed peak cocaine plasma concentrations. Similar to previous reports [7,8,11-13], rapid decreases in effects occurred while plasma cocaine concentrations were still elevated; these prolonged cocaine concentrations with decreasing subjective effects combined with observed clockwise hysteresis suggests development of acute tolerance. Louizos *et al.* also suggested that tolerance is one mechanism that may explain the observed clockwise hysteresis, where reduced pharmacological effects are observed at the same cocaine concentrations that earlier produced stronger effects [266]. Acute tolerance to cocaine’s subjective and cardiovascular effects was previously reported in

humans [7,8,13]. Others previously observed hysteresis with subjective effects (VAS scores) and plasma cocaine concentrations following SM and IV cocaine in humans [7,8,11-13] and in rhesus monkeys with discriminative effects (which model subjective effects in humans) and plasma cocaine concentrations following intramuscular cocaine administration [14].

We are not aware of any previous presentation of concentration-effect curves for subjective measures and OF cocaine concentrations following controlled cocaine administration. In contrast to plasma, "Rush," "Good drug effect" and "Bad drug effect" peak effects were observed (0.17 h) prior to maximum OF concentrations (0.5 h). This is expected as cocaine OF T_{max} was significantly longer than plasma T_{max}. The first measurement (0.5 h) for other subjective effects was delayed; thus, this difference could not be detected. Following 100 mg SM cocaine, maximum behavioral responses also occurred prior to plasma T_{max}, while cocaine plasma concentrations were increasing [267]. This suggests that the effect of cocaine concentration on acute subjective effects is less on the descending limb of the hysteresis curve, potentially due to acute tolerance development.

We observed significant correlations between cocaine (r=0.94, P <0.001) and, for the first time, BE (r=0.55, P <0.001) OF and plasma concentrations following IV cocaine, consistent with previous studies finding significant correlations ($r \geq 0.94$, r=0.89) between OF and plasma cocaine concentrations following 15 and 40 mg IV cocaine [10,32]. Significant correlations between OF and plasma metabolite concentrations were observed following 75 and 150 mg/75 kg SC cocaine [31]. However, high inter-subject variability in OF concentrations and OF/P were observed, making prediction of plasma

cocaine concentrations from OF concentrations inaccurate (Figure 1, Figure 2; Figure 6 (Supplemental)). Similar findings were noted in other studies [268,31].

Prior studies reported cocaine OF/P ratios typically ≥1, with the reverse shown for its metabolite BE [31,36,37,39]. This is due to cocaine becoming ion trapped in OF, as OF pH is typically lower than neutral blood pH. Consistent with previous reports, cocaine OF/P ratios were typically ≥1 from 0.17-12.5 h, and cocaine OF AUC (582 h x µg/L) tended to be greater than cocaine plasma AUC (345 h x µg/L). Cone *et al.* also reported higher cocaine OF AUC compared to plasma following 25 mg IV cocaine [37]. Significant increases in BE plasma AUC were observed compared to OF AUC, consistent with previously reported BE OF/P ratios (<1). BE OF/P ratios were generally ≥1 (like cocaine) from 0.17-1 h post-cocaine, despite increased plasma $AUC_{0\text{-}21h}$, before decreasing to <1 (21 h). However, BE OF T_{max} also occurred significantly earlier (0.5 h) than plasma T_{max} (2 h) in this study, much earlier than after 75 and 150 mg/70 kg subcutaneous cocaine administration (OF T_{max}: 2 h, plasma T_{max}: 4 h) [31]. BE OF concentrations were less than BE plasma concentrations ~1h post-dose.

Cocaine, BE and EME plasma T_{max} and BE OF T_{max} after IV cocaine administration is shorter than following SC dosing [31]. A contributor to the observed BE OF T_{max} difference is that OF in the SC study was collected via citric-acid stimulated expectoration (known to increase bicarbonate excretion and affect cocaine and BE ion trapping); potentially increasing the time required to reach C_{max} following SC administration.

Previous SC [31], SM [37] and IN [36,37] cocaine administration studies found significantly prolonged cocaine half-lives in plasma compared to OF. In contrast, we

found no significant differences between cocaine and BE half-lives in OF compared to plasma, similar to a study with 25 mg IV cocaine [37]. IV cocaine is immediately available in the blood to diffuse into OF, which could account for the lack of difference in plasma vs. OF $t_{1/2}$. In contrast, other administration routes result in delayed blood cocaine availability. Additionally, EME plasma $t_{1/2}$ (6.8 h) following IV cocaine was similar to its $t_{1/2}$ following SC cocaine administration (4.5-6.7 h) [31]. Although there were no significant differences in cocaine and BE OF and plasma $t_{1/2}$, we did observe a trend that cocaine OF T_{last} was longer than in plasma; plasma and OF BE T_{last} could not be statistically compared, as BE plasma concentrations were still positive at the last measurement (21 h).

Cocaine OF detection times (6.5 h at SAHMSA and DRUID cutoffs) from the Quantisal collection device after IV cocaine were similar to those reported with Oral-Eze and StatSure devices (4-6.5 h) containing stabilizing/ elution buffers at 8 and 10 µg/L confirmation cutoffs following IV cocaine [265]. Longer detection windows were achieved for BE (21 h) in this study compared to IV, IN, and SM cocaine administration (5.0-8.7 h) at 8 µg/L cutoffs [39]; however, those were collected via citric acid stimulated expectoration with no stabilizers or elution buffer. Monitoring cocaine and BE at the LOQ (1 µg/L) had longer detection windows (cocaine: 21 h, BE: >69 h) compared to SAMHSA and DRUID confirmation cutoffs; similar to what was reported with Oral-Eze and StatSure collection devices following IV cocaine [265]. Confirming for cocaine and/or BE compared to BE only at 8 and 10 µg/L, yielded one extra participant positive at 45 h (12.5% detection rate). Besides this one sample, detection rates and windows remained unchanged, unlike a prior study which found increased detection rates and

windows with cocaine and/or BE confirmation compared to BE alone [31]. These differences could be due to stabilization and elution buffers present in OF collected with the Quantisal device compared to expectoration.

We documented clockwise hysteresis for the relationship between plasma and OF concentrations and subjective measures (Figure 3, Figure 4). Peak subjective effects (for some measures) occurred prior to peak OF cocaine concentrations, whereas they occurred simultaneously with observed peak plasma concentrations and subjective measures, most likely due to significantly earlier plasma T_{max} compared to OF $T_{max.}$ Additionally, we reported significant correlations between cocaine and, for the first time, BE plasma and OF concentrations following IV cocaine. SAMHSA and DRUID OF cocaine cutoffs yielded OF detection windows similar to the time course of cocaine's impairing effects, making them useful for roadside DUID and other home and workplace accident investigations. These data advance our understanding of OF as an alternative matrix to blood and plasma for identifying cocaine intake, defining pharmacokinetic parameters at different confirmation cutoffs, and aiding different drug testing programs in the selection of confirmation analytes and cutoffs to best achieve their monitoring goals.

Table 8. (Supplemental) Demographic characteristics and self-reported cocaine use histories for 10 cocaine-using adults.

PPT	Race	Sex	Age (years)	Weight (lbs)	Days used in 14 days prior to screening (SM or IV)	Mean use during 3 months prior to screening (days/time period) (SM or IV)	Age first use (SM or IV) (years)	Duration of lifetime cocaine use (years)
B	AA	M	38.1	202	11 SM	5/week	32	6.1
C	1+	M	42.4	162	5 IV	3/week	32	10.4
					0 SM	3-4/month	21	21.4
D	W	M	35.2	165	3 SM	1/week	25	10.2
E	AA	M	45.3	206	0 IV	N/A	34	11.3
					5 SM	4/week	18	27.3
F	W	M	44.0	197	5 SM	Daily	18	26
G	W	M	42.5	180	6 IV	3/week	28	14.5
					7 SM	2/week	31	11.5
H	AA	M	43.7	150	14 SM	Daily	21	22.7
I	AA	M	49.9	175	10 SM	4-5/week	25	24.9
J	AA	M	46.5	176	1 SM	3/week	15	31.5
K	W	M	39.2	175	0 IV	N/A	35	4.2
					0 SM	Daily	11	28.2
M	AA	F	36.6	140	0 SM	2/week	20	16.6
Median	-	-	42.5	175	5	-	25	16.6

PPT participant, SM smoked, IV intravenous, AA African-American, 1+ more than one race, W white, M male; F female

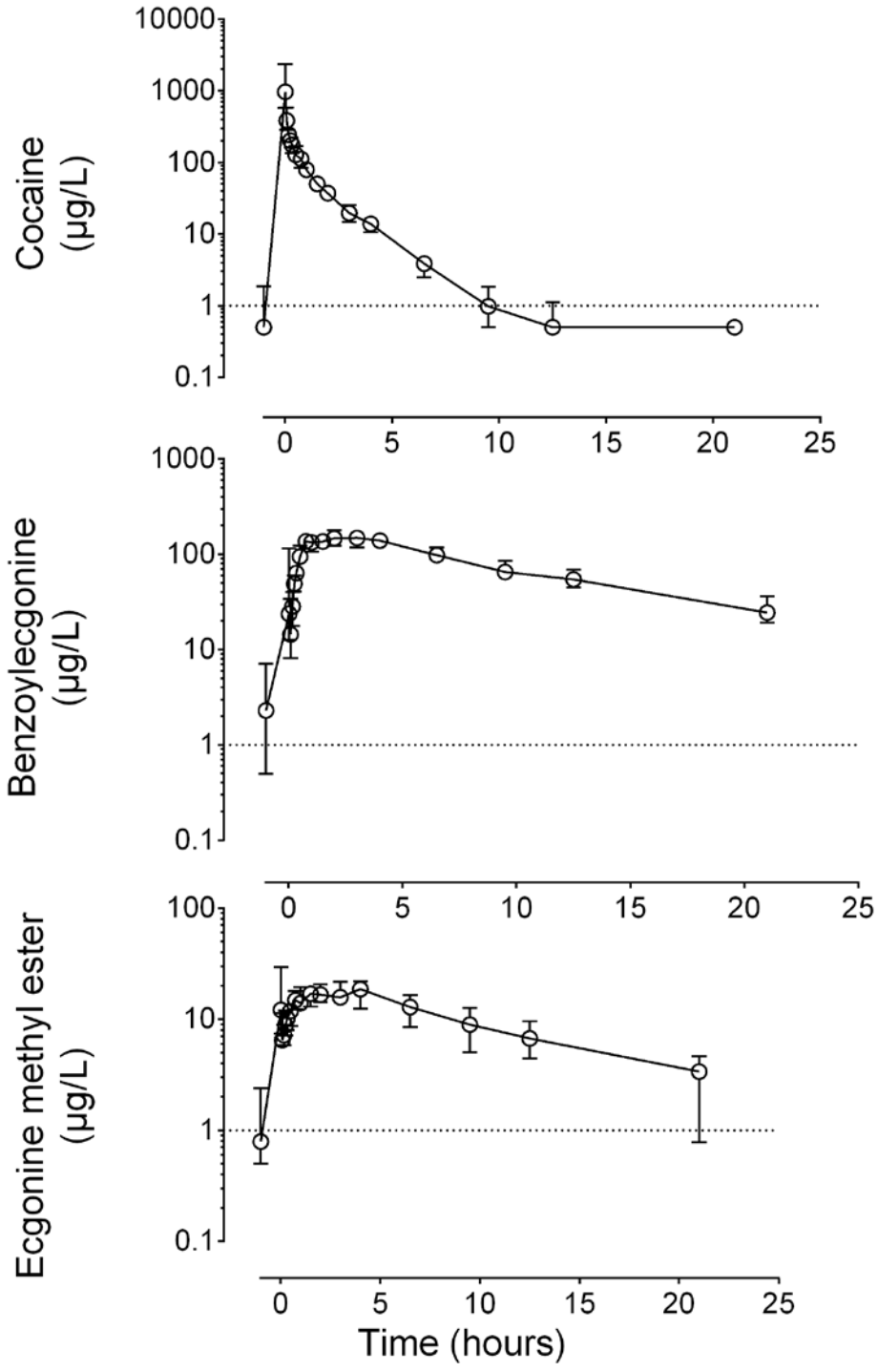


Figure 5. (Supplemental) Median (interquartile) range concentration-time curves for cocaine, benzoylecgonine (BE) and ecgonine methyl ester (EME) in plasma in 10 cocaine-using adults up to 21h after 25mg intravenous (IV) cocaine. Dotted lines represent the 1µg/L analytical limit of quantification (LOQ). Concentrations <LOQ are represented as ½ LOQ. Note y-scales are logarithmic.

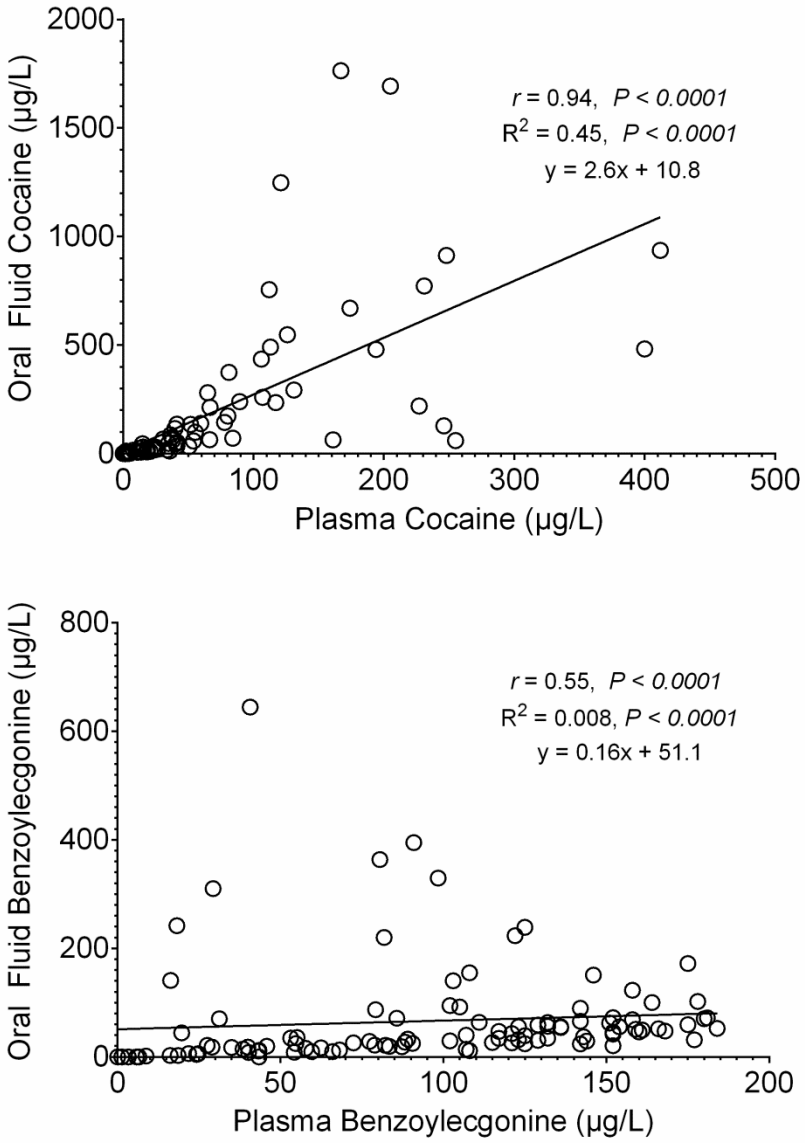


Figure 6. (Supplemental) Correlation of oral fluid (OF) with plasma cocaine (n = 75) and benzoylecgonine (n = 98) concentrations between 0.17 and 21 h after 25 mg intravenous cocaine administration in 9 cocaine-using adults. Regression was calculated by least-squares analysis. Only paired positive paired OF and plasma specimens were included.

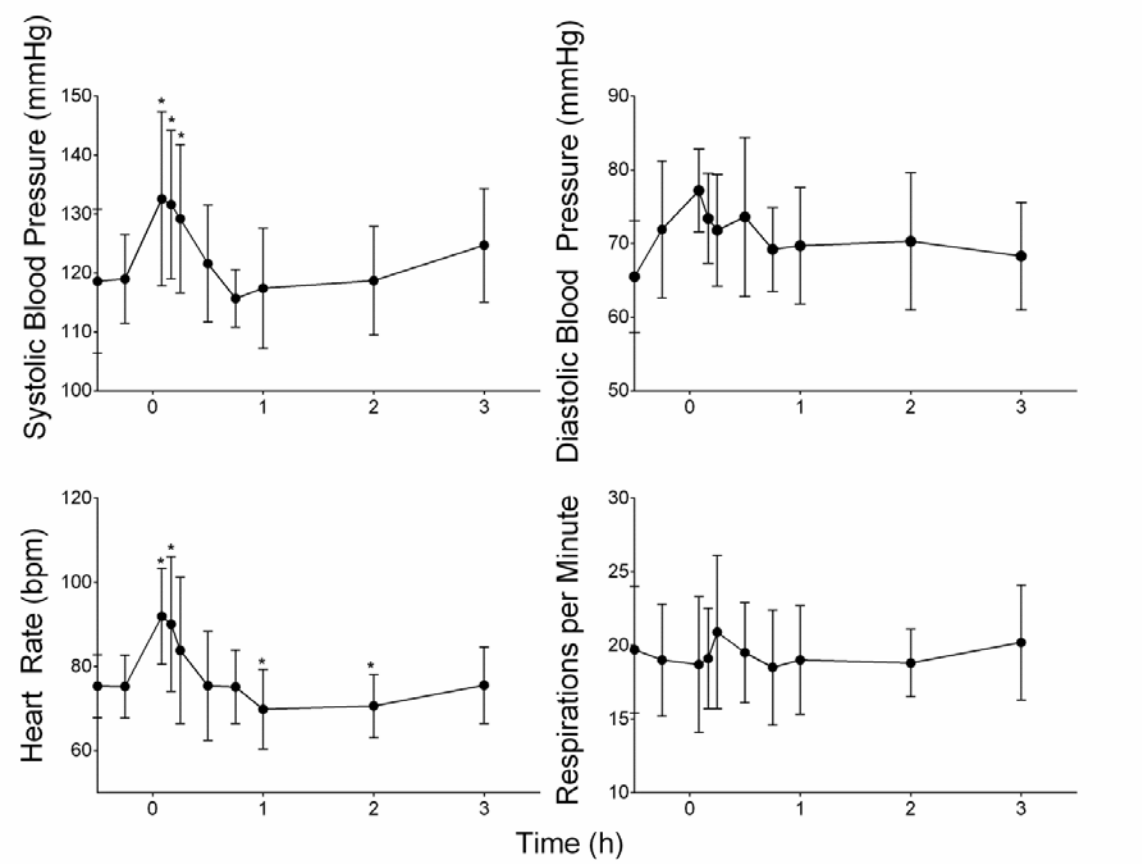


Figure 7. (Supplemental) Mean (standard deviation) physiological measures for 10 cocaine-using adults following controlled cocaine administration (25 mg intravenous). *P <0.05 versus baseline (-0.25 h) physiological measures.

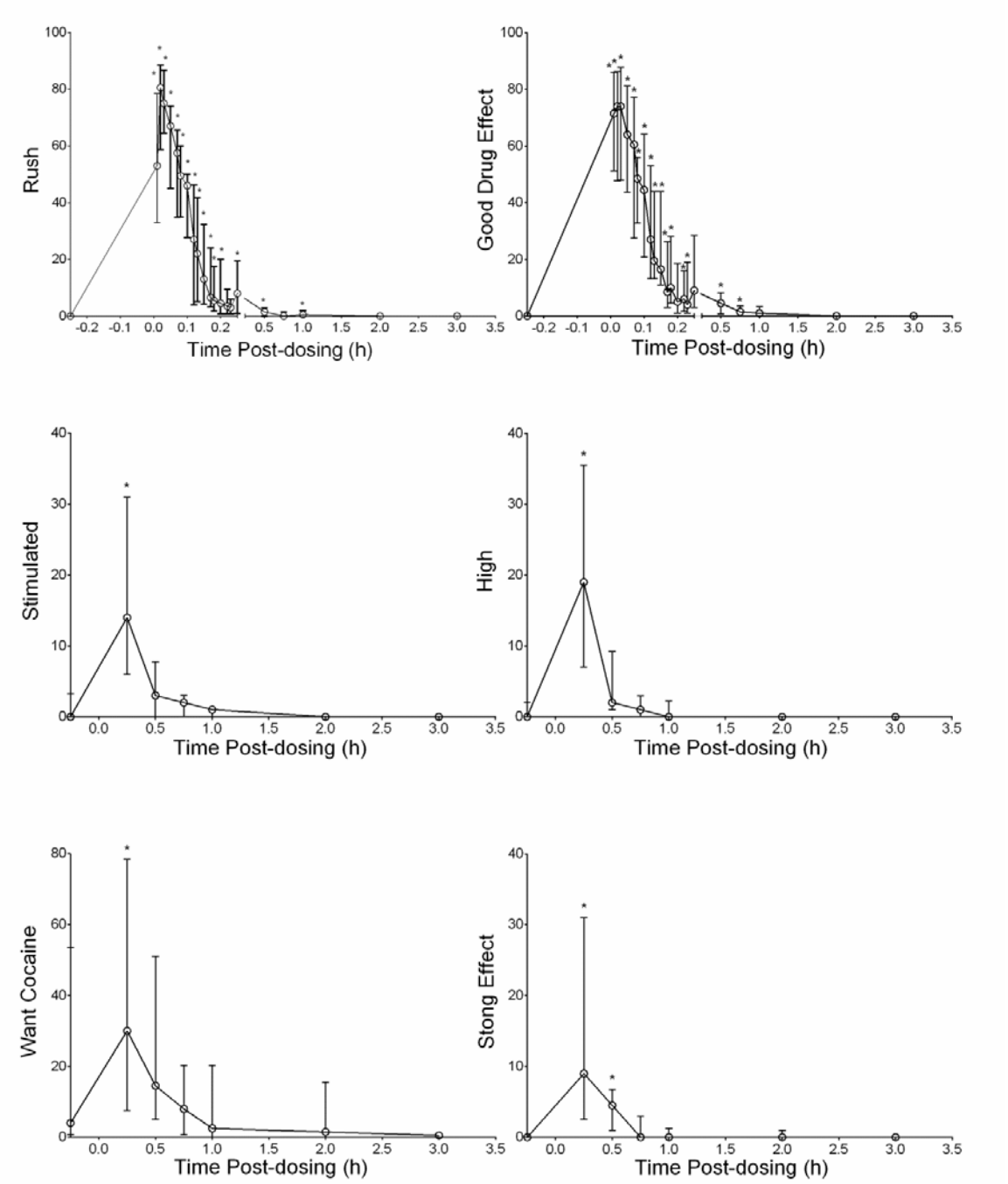


Figure 8. (Supplemental) Median (interquartile range) subjective effects (visual-analogue scales (VAS)) versus time in 10 cocaine-using adults after 25 mg intravenous cocaine administration. Note axis scales vary by measure; all VAS were out of 100; only statistically significant (P <0.05) subjective measures are presented. *P <0.05 versus baseline (-0.25 h) subjective measures.

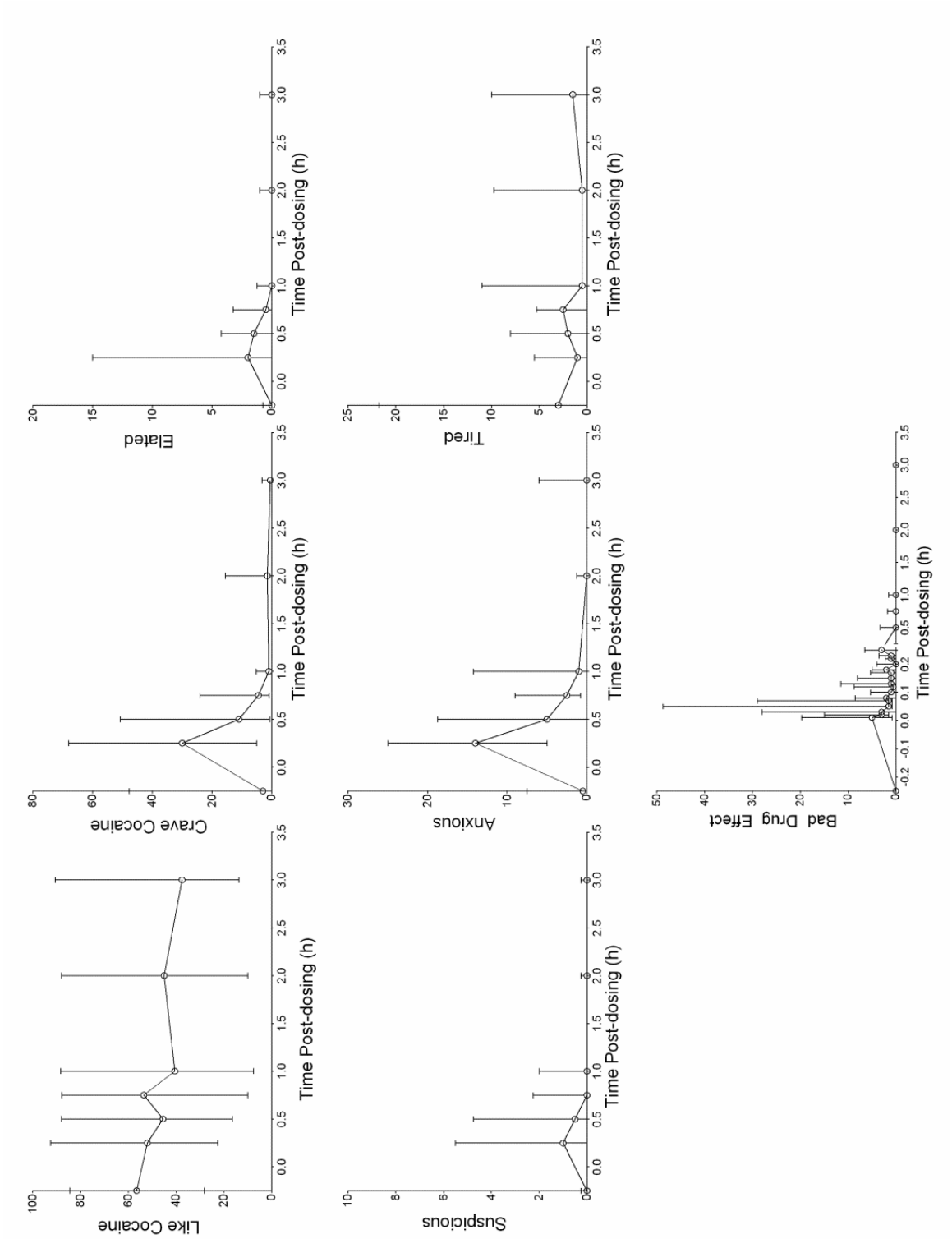


Figure 9. (Supplemental) Median (interquartile range) subjective effects (visual-analogue scales (VAS)) versus time in 10 cocaine-using adults after 25 mg intravenous cocaine administration. Note axis scales vary by measure; all VAS were out of 100; statistical tests did not yield statistically significant results (P <0.05) from baseline (-0.25 h) subjective measures.

Chapter 3 - Oral Fluid Cocaine and Benzoylecgonine Concentrations Following Controlled Intravenous Cocaine Administration

(*As published in* Forensic Science International, *January 2016*)[3]

Abstract

Limited OF pharmacokinetic data collected with commercially available collection devices after controlled cocaine administration hinder OF result interpretations. Ten cocaine-using adults provided OF, collected with Oral-Eze® (OE) and StatSure Saliva Sampler™ (SS) devices, an hour prior to and up to 69 h after 25 mg IV cocaine administration. Cocaine and BE were quantified by a validated 2D-GC-MS method. Large inter-subject variability was observed. Cocaine was detected in OF in the first 0.17 h sample after IV administration, with much more rapid elimination than BE. OE observed C_{max} median (range) concentrations were 932 (394-1,574) µg/L for cocaine and 248 (96.9-953) µg/L for BE. SS observed cocaine and BE C_{max} median (range) concentrations trended lower at 732 (83.3-1,892) µg/L and 360 (77.2-836) µg/L, respectively. OE and SS cocaine OF detection times were 12.5 and 6.5 h and for BE 30.5 and 28.0 h, respectively at 1 µg/L. There were no significant pharmacokinetic differences between OE and SS OF collection devices, except cocaine half-life was significantly shorter in SS OF specimens. This difference could be attributed to differences in stabilizing buffers present in OF collection devices, which may affect cocaine stability in OF specimens, or decreased recovery from collection pads. Both OE and SS OF collection devices were effective in monitoring cocaine and metabolite concentrations

[3] Ellefsen K.N. *et al.* Oral Fluid Cocaine and Benzoylecgonine Concentrations Following Controlled Intravenous Cocaine Administration. *Forensic Science International.* 2016; 260:95-101.

with similar detection windows. Furthermore, we demonstrated that different confirmatory OF cutoffs can be selected to produce shorter or longer cocaine and metabolite detection windows to address specific needs of clinical and forensic drug testing programs.

Introduction

Despite declining cocaine use prevalence in the general population, cocaine remains a widely used illicit drug in Europe (3.4 million users) [5] and the United States (1.5 million users) [4], behind only cannabis and methamphetamine [3]. Cocaine was the second most prevalent illicit drug (after cannabis) in the general driving population (0.42%), in Europe, [2] and the second most prevalent drug in nighttime drivers (1.4%) in the United States [6].

Alternative matrices, such as OF, are increasingly employed for detecting recent drug consumption due to non-invasive and observed collection, no need for same-sex collectors, and little potential for adulteration, dilution and substitution compared to urine [25-28]. OF cocaine concentrations were significantly correlated to those in plasma [10,15,31,32] and blood [33], making OF an attractive matrix for estimating cocaine-associated impairment and windows of detection. However, studies have demonstrated large inter- and intra-subject variability in OF to plasma ratios precluding calculating one concentration from the other [31,268]. Detection windows vary based on dose, analyte, administration route, drug intake frequency, cutoffs, and OF collection methods. Ideal drug detection windows differ depending upon the monitoring purpose. For DUID programs detection windows should mirror the period of impairing effects; however, for

workplace and pain management drug testing, longer detection windows are ideal due to widely separated specimen collections [40].

Cocaine pharmacokinetics are well studied in blood, plasma, and urine after various administration routes, but less is known about cocaine disposition in OF following controlled drug administration. Previous studies investigated OF cocaine and metabolite concentrations following controlled IV [10,32,34-38], SM [35-38], IN [35,37,38], oral [38,39], and SC administration [31]; however, only three studies examined cocaine and metabolite OF pharmacokinetics [31,37,39], and none evaluated commercially available OF collection devices containing stabilizing and elution buffers. Furthermore, cocaine and metabolite detection windows are needed for recently published US SAMHSA National Laboratory Certification Program Mandatory Guidelines for federally-mandated oral fluid testing [41] and established DRUID OF guidelines [269].

Cocaine was previously quantified in OF collected via citric-acid stimulated expectoration between 4.1-17.7 h following 25 mg IV, 32 mg IN, 42 mg SM, and 75 mg/70 kg and 150 mg/70 kg SC cocaine administration [31,38]. For BE, the primary inactive cocaine metabolite, OF detection times ranged from 5.0-47 h. Following repeated oral cocaine administration (375-2000 mg), OF cocaine and BE were identified up to 21.2 and 50.0 h, respectively [38], indicating extended windows of detection with repeated dosing. It is unclear if OF collected with various commercially available devices, containing different elution and stabilization buffers, will exhibit similar cocaine concentration time profiles and kinetics over time. SAMHSA and DRUID proposed confirmatory OF cocaine cutoff concentrations of 8 and 10 µg/L, respectively [41,269],

and 8 and 10 µg/L for BE. Eight µg/L also was recommended for suspected drug-impaired driving cases [43], and a 10 µg/L OF cocaine concentration was suggested by the Talloires recommendations [44].

The aims of this study were to characterize for the first time cocaine and BE OF pharmacokinetics collected with the OE and SS devices (two of the most commonly used commercial devices) following controlled IV cocaine administration. Additionally, we investigated detection windows with different OF collection devices at the recommended SAMHSA and DRUID OF testing guidelines.

Materials and Methods

Participants

Participants provided written informed consent for this NIDA Institutional Review Board and Food and Drug Administration-approved study. Eligibility criteria included healthy adults ages 18-50 years who smoked or used IV cocaine for at least six months and at least three times per month during the three months prior to screening. Exclusion criteria included pregnant or nursing women; current physical dependence on any drug other than cocaine, caffeine, or nicotine; current clinically significant medical or psychiatric disorder; hemoglobin less than 12.5 g/dL or blood donation within eight weeks of study entry; current hypertension or blood pressure readings consistently above 140 mm Hg systolic or 90 mm Hg diastolic while at rest; heart rate consistently above 90 or below 50 bpm while at rest; abnormal 12-lead ECG; history of clinically significant adverse reaction to cocaine, acetazolamide, or quinine; or interest in drug abuse treatment within three months of study screening.

Drug Administration

Participants resided on a secure research unit for 13 days and 12 nights, and received a single dose of 25 mg IV cocaine through a peripheral venous catheter on three separate days (Days 1, 5, and 10). Cocaine was administered alone on Day 1, in combination with oral acetazolamide on Day 5, and with oral quinine on Day 10. The study's primary aims were to evaluate potential pharmacodynamic and pharmacokinetic interactions between cocaine and acetazolamide and quinine, as they are being considered as medication compliance markers for cocaine use disorder treatment pharmacotherapies, similar to a study performed with oxycodone and quinine by Babalonis *et al* [270].

Oral Fluid Specimen Collection

OF was collected with either the Oral-Eze® (Quest Diagnostics) or StatSure Saliva Sampler™ (StatSure Diagnostic Systems) devices by placing the absorptive pad under the tongue until the volume-adequacy indicator turned blue, indicating 1 mL OF was collected, or 5 min had elapsed, whichever occurred first. OF was collected with a single device type prior to and following each IV cocaine dose in each participant. All participants had OF collected with each device type. OE collection tubes contained 2 mL stabilizing buffer, yielding a 3-fold OF dilution, whereas SS tubes contained 1 mL buffer for a 2-fold dilution. Following manufacturer's recommendations, the pad was removed and placed in the stabilizing buffer and left at room temperature (OE) or 4°C (SS) for ≥ 12 h for drug elution. Serum separators depressed into the collection tube were utilized to facilitate decanting into a 3.6 mL Nunc® CryoTube®, with samples stored refrigerated at 4° C prior to analysis. The majority of OF specimens were analyzed within 4 months, although all specimen analysis was complete within 8 months. No oral intake or smoking

was permitted 10 min before OF collection at -1.0, 0.17, 0.5, 1, 1.5, 2, 3, 4, 6.5, 9.5, 12.5 and 21 h from dosing. Additional collections were obtained at 28 (Day 10 only), 33 (Day 1 only), 45 and 69 h (Days 1 and 5) post-administration.

Quantification of Cocaine and Benzoylecgonine in Oral Fluid

Cocaine and BE OF were quantified with modifications of a previously validated venous blood analytical method [261]. Calibrators were prepared at drug concentrations from 1-100 µg/L in 0.75 mL (OE: 0.25 mL blank OF + 0.5 mL OE stabilizing buffer) or 0.5 mL (SS: 0.25 mL blank OF + 0.25 mL SS buffer) solutions at the same dilutions as authentic specimens, therefore, accounting for the dilution factor. QCs were prepared at 3, 25, and 75 µg/L in the same manner as calibrators, although from different standard ampoules. Briefly, 25 µL IS (250 µg/L D_3-cocaine and D_8-BE) were added to either 0.75 or 0.5 mL OF with 2 mL phosphate buffer (pH 6). After vortexing and centrifugation, the filtrate was decanted onto preconditioned UCT Clean Screen DAU 200 mg 10 mL SPE cartridges. Columns were washed (water, 0.1 M hydrochloric acid, methanol), dried for 20 min, and eluted into conical glass centrifuge tubes with dichloromethane:isopropanol (80:20, v/v prepared for 100 mL) mixed with 2 mL ammonium hydroxide. After evaporating to dryness, samples were derivatized with 20 µL ethyl acetate:MTBSTFA + 1% t-BDMS (50:50 v/v) for 40 min at 70°C. The derivatized extracts (2 µL) were analyzed by an electron impact two-dimensional (2D) GC-MS method [261] with oven temperature program modifications for a total run time of 18.05 min (Table 12 (Supplemental)). Instrument parameters were described in detail previously [261] and are outlined in Table 12 (Supplemental). The upper limit of linearity was 100 µg/L;

specimens exceeding this limit were diluted with oral fluid and buffer mixture (2:1, v/v; 1:1 v/v), re-extracted and analyzed.

Method Validation

OF methods were validated in accordance with the SWGTOX recommendations [264]. Linearity from 1-100 µg/L with $1/x^2$ weighting was achieved for cocaine and BE in OF (OE and SS) with calibration curve (n=5) coefficients (R^2) above 0.9916 ± 0.0040 and residuals <15%. LODs for cocaine and BE were 1 and 0.5 µg/L, respectively, with a 1 µg/L LOQ for each analyte for OE and SS assays, respectively. Bias and imprecision were evaluated in four replicates over five days (n = 20) at each QC concentration with a One-way Analysis of Variation approach to calculate combined within- and between-run imprecision. Bias was ±9.2% and 14% for cocaine and BE, respectively. OE within- and between-run imprecision was <3.9% and <4.3%, whereas SS exhibited <3.4% (within-run) and <5.7% (between-run) imprecision for cocaine and BE. Cocaine OE and SS extraction efficiencies were 93.1-95.5% and 96.4-97.2%, respectively, with BE results 70.9-73.5% and 79.7-82.7%, respectively. Analytes were stable in the autosampler for 72 h at low and high QC concentrations with percent differences between −3.9% and 1.7% (n=4). Short-term stability was evaluated at room temperature for 24 h and at 4°C for 72 h. Analytes were considered stable under evaluated conditions (% difference < ±16.7%).

Statistical and Pharmacokinetic Analyses

Visual inspection of data and evaluation by D'Agostino-Pearson normality test (omnibus K2) indicated non-normal data distribution. Therefore, statistical comparisons were conducted with non-parametric tests (Mann-Whitney or Wilcoxon signed-rank) using GraphPad Prism Version 5.02 (GraphPad Software Inc, La Jolla, CA).

Comparisons were considered significant if $P < 0.05$. Pharmacokinetic analyses were performed utilizing noncompartmental analysis with Phoenix WinNonlin® 6.4 for Windows (Pharsight, Mountain View, CA). C_{max}, T_{max}, AUC from 0 to 69 h post-dose (AUC_{0-69}), $t_{1/2}$, T_{last}, and C_{last} were calculated. At least five data points on the descending linear limb of the time-concentration curve were employed to calculate $t_{1/2}$. For statistical purposes, concentrations less than the LOQ were set to 0.

Results

Participants

Participant demographics and self-reported cocaine histories are detailed in Table 9. Ten subjects (9 men, 1 woman; 5 black, 4 white, 1 > one race) aged 35-50 participated in the study, with nine completing all dosing sessions. One participant was medically discharged prior to the third cocaine dose (Day 10) due to elevated heart rate (Participant I). Frequency of cocaine use (SM or IV) among participants ranged from daily to once per week, with a median duration of cocaine use of 16.6 years (range: 4.2-31.5 years).

Oral Fluid Results

In total, 139 and 141 OF specimens were collected with the OE and SS collection devices from 1 h prior to 69 h post-administration and analyzed for cocaine and BE. Median (interquartile range) OF cocaine and BE concentration time courses are illustrated in Figure 10 for OE and SS collection devices. Cocaine exhibited higher concentrations than BE for approximately 2 h for both OE and SS. OE cocaine and BE median (range) OF concentrations immediately following administration (0.17 h) were 673 (76.5-1,574) µg/L and 145 (0-953) µg/L, respectively. Concentrations of cocaine and BE 2 h post-dose were 43.8 (19.3-194) µg/L and 80.6 (30-150) µg/L, respectively. Most

participants' cocaine concentrations were below the method LOQ at 9.5 h, while BE was detectable up to 33 h. Median (range) SS cocaine and BE concentrations immediately after dosing (329 (26.4-1,892) µg/L and 172 (10.9-836) µg/L) trended lower than OE OF specimens, and fell to 70.4 (15.7-115) µg/L and 79.9 (42-271) µg/L at 2 h. In contrast to OE OF specimens, most participants' cocaine concentrations were below the LOQ at 6.5 h, whereas BE concentrations remained detectable for 28 h with the exception of a few specimens at 45 h (4/7 OF specimens). There was high inter-subject variability in cocaine and metabolite concentrations in OF from both collection devices.

Table 9. Demographics and self-reported cocaine histories for 10 cocaine users. Participant I was discharged prior to the third cocaine dose, however, all Oral-Eze and StatSure oral fluid specimens were collected prior to discharge.

PPT	Race	Sex	Age (yrs)	Weight (lbs)	Days used in past 14 (SM or IV)	Mean use (SM or IV)	Age first use (SM or IV) (years)	Lifetime cocaine use (years)
B	AA	M	38.1	202	11 SM	5/week	32	6.1
C	1+	M	42.4	162	5 IV	3/week	32	10.4
					0 SM	3-4/month	21	21.4
D	W	M	35.2	165	3 SM	1/week	25	10.2
F	W	M	44.0	197	5 SM	Daily	18	26
G	W	M	42.5	180	6 IV	3/week	28	14.5
					7 SM	2/week	31	11.5
H	AA	M	43.7	150	14 SM	Daily	21	22.7
I	AA	M	49.9	175	10 SM	4-5/week	25	24.9
J	AA	M	46.5	176	1 SM	3/week	15	31.5
K	W	M	39.2	175	0 IV	N/A	35	4.2
					0 SM	Daily	11	28.2
M	AA	F	36.6	140	0 SM	2/week	20	16.6

PPT participant, SM smoked, IV intravenous, AA African-American, 1+ more than one race, W white, M male

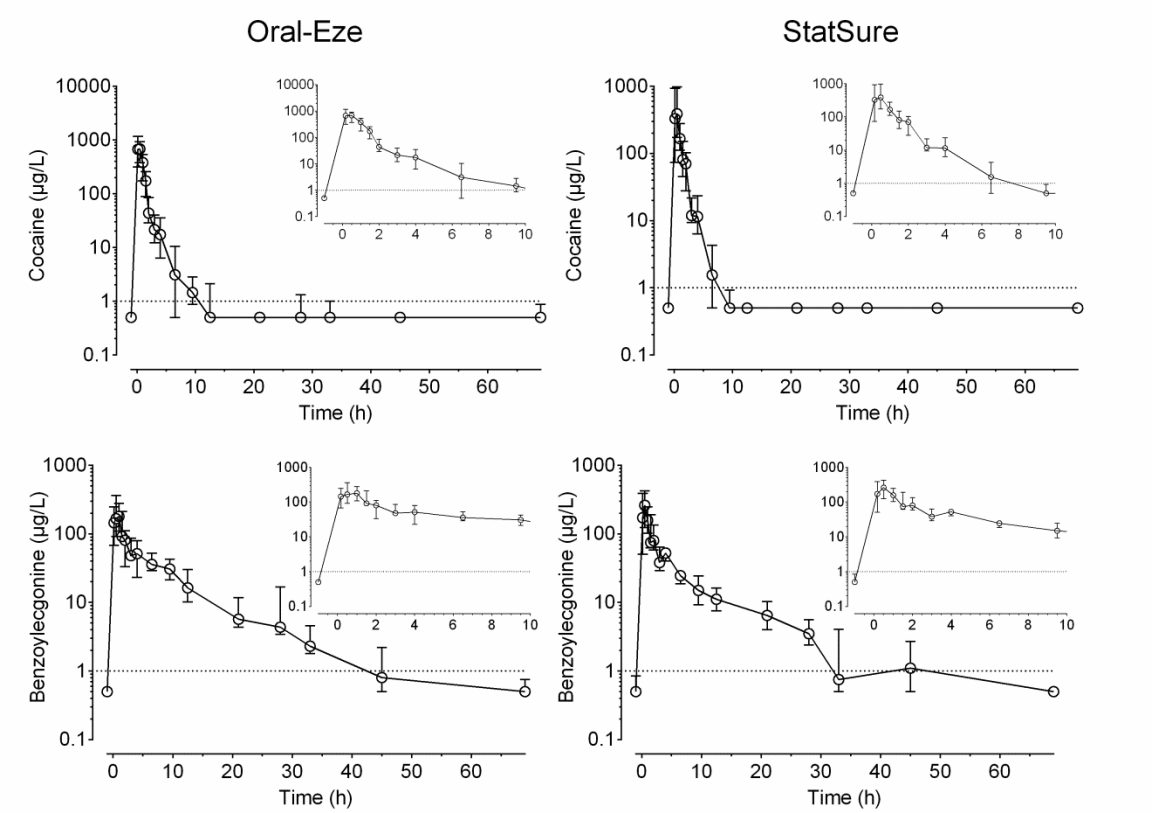


Figure 10. Median (interquartile range) cocaine and benzoylecgonine oral fluid concentration-time curves up to 69 h from 10 participants after 25 mg intravenous cocaine collected with Oral-Eze and StatSure oral fluid collection devices. Inserts represent time course from 0.17 to 10 h post-administration. Dotted lines represent the 1 µg/L analytical limit of quantification (LOQ). Concentrations <LOQ are represented as ½ LOQ.

Detection Rates of Oral Fluid Cocaine and Benzoylecgonine

Cocaine and BE detection rates 0.17-69 h after IV cocaine are in Table 10. Cocaine and BE initially appeared in OF as early as 0.17 h, the first specimen collected after administration, in both collection devices, with the exception of one participant where BE was not detected until 0.5 h. In total there were 2, 32 and 88 OE and 0, 41, and 81 SS OF cocaine only, BE only, and cocaine and BE positive specimens, respectively.

Table 10. Detection rates for cocaine (COC), benzoylecgonine (BE), and COC and/or BE in oral fluid collected with Oral-Eze (OE) and StatSure (SS) collection devices up to 69 h from 10 participants after 25 mg intravenous cocaine. Detection rates (%) were calculated at the assay limit of quantification (1 µg/L). Note that not all oral fluid collection time points were *n*= 10 as different cocaine dosing days had different oral fluid collection times beyond 21 h.

Time (h)	Oral-Eze Detection Rates (%)				StatSure Detection Rates (%)			
	n	COC	BE	COC and/or BE	*n*	COC	BE	COC and/or BE
-1	10	0	10	10	10	10	20	20
0.17	10	100	90	100	10	100	100	100
0.5	10	100	100	100	10	100	100	100
1	10	100	100	100	10	100	100	100
1.5	10	100	100	100	10	100	100	100
2	10	100	100	100	10	100	100	100
3	10	100	100	100	10	100	100	100
4	10	90	90	90	10	100	100	100
6.5	10	60	100	100	10	60	100	100
9.5	10	80	100	100	10	20	100	100
12.5	10	30	100	100	10	10	100	100
21	10	10	100	100	10	0	100	100
28	4	25	100	100	3	0	100	100
33	3	33.3	100	100	4	0	50	50.0
45	6	0.0	50	50	7	14.3	57.1	57.1
69	6	16.7	16.7	33.3	7	0	14.3	14.3

Figure 11 depicts detection rates for up to 69 h after IV cocaine based on the LOQ, SAMHSA (8 µg/L) and DRUID (10 µg/L) OF cutoffs. Typically, OE OF detection times for cocaine and BE were up to 6.5 and 28 h (SAMHSA and DRUID cutoffs). At the LOQ, increased detection rates for cocaine and BE occurred with detection times up to 21 and 69 h, respectively. SS last positive times were typically shorter than for OE with cocaine detected up to 4 h and BE 21 h (SAMHSA and DRUID cutoffs) and 12.5 and 69 h post-administration (LOQ cutoffs), respectively. Detection times were extended (21-69 h) when either cocaine and/or BE was tested as compared to cocaine testing only (4-21 h). Cocaine was always present in combination with BE, with the exception two OE

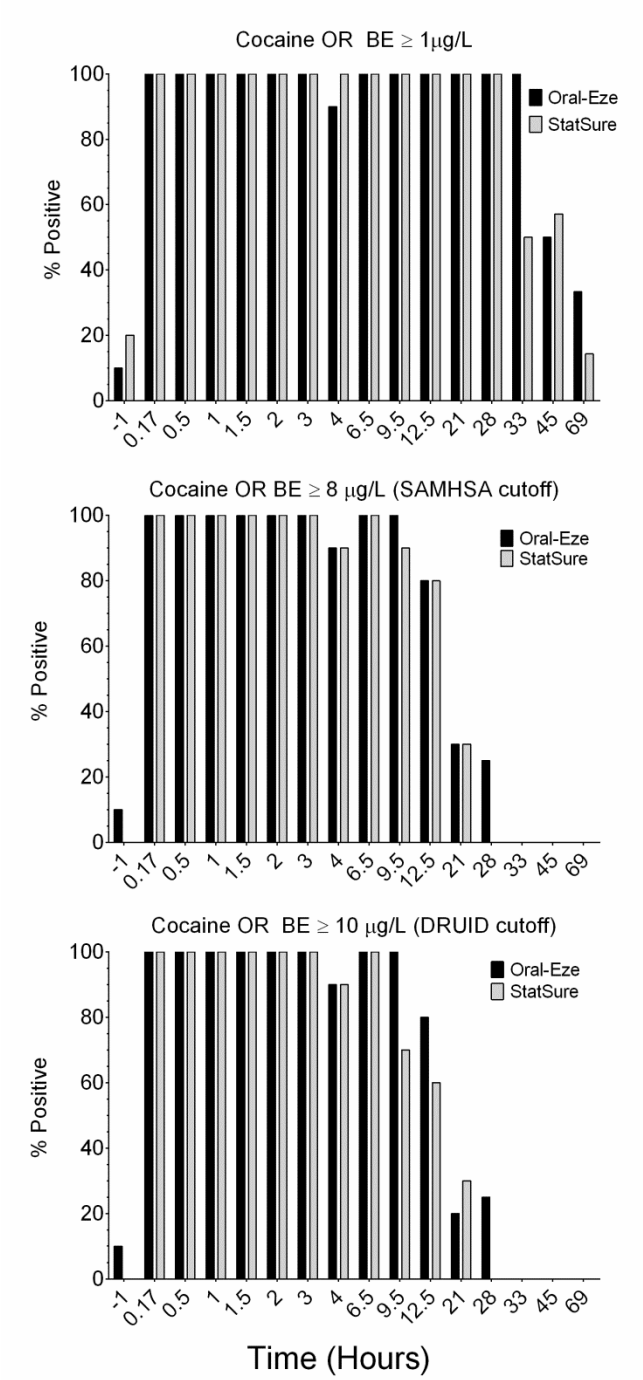


Figure 11. Percentages of positive specimens in oral fluid (OF) at different confirmatory cutoffs for 10 participants after 25 mg controlled intravenous cocaine administration. Cutoffs evaluated were: cocaine ≥ 1 µg/L, benzoylecgonine (BE) ≥ 1 µg/L, cocaine ≥ 8 µg/L (SAMHSA), BE ≥ 8 µg/L (SAMHSA), cocaine ≥ 10 µg/L (DRUID), and BE ≥ 10 µg/L (DRUID). Note that not all oral fluid collection time points were *n*= 10 as different cocaine dosing days had different oral fluid collection times beyond 21 h: four Oral-Eze (OE) and three StatSure (SS) OF specimens were collected at 28 h; three OE and four SS OF specimens at 33 h; and six OE and seven SS OF specimens at 45 and 69 h.

specimens at 0.17 and 69 h where only cocaine was quantified at the LOQ, thus increasing % positives from 90% to 100% at 0.17 h and 16.7% to 33.3% at 69 h, and overall from 86.3% to 87.8% (Table 10). Although there was a small increase in OE % positives at 0.17 and 69 h compared to BE testing only, detection windows were unchanged. SS detection rates with either cocaine and/or BE were identical to BE only testing.

Pharmacokinetics of Cocaine and Benzoylecgonine in Oral Fluid

Table 11 presents the median (range) cocaine and BE pharmacokinetic parameters in OF collected via OE and SS. After IV cocaine, OF cocaine concentrations increased rapidly for OE and SS devices with median (range) C_{max} concentrations of 932 (394-1,574) and 732 (83.3-1,892) µg/L with corresponding T_{max} of 0.34 and 0.17 h, respectively. One participant had cocaine T_{max} at 1 h corresponding to C_{max} of 737 µg/L (OE). Median OE and SS BE C_{max} were approximately 26.6% and 49.2% of median cocaine C_{max} with T_{max} observed at 0.5 h. Time to cocaine C_{max} peaked significantly earlier than BE for OE collections only, with cocaine T_{last} as late as 12.5 h post-dose, and BE up to 30.5 h. SS cocaine (6.5 h) and BE (28 h) tended to be shorter than OE T_{last}. Although significant differences in T_{last} were observed between the two analytes, T_{last} were not significantly different between the two collection devices. No significant differences were observed in AUC between analytes and collection methods. Cocaine half-lives (1.3 and 0.89 h) were significantly shorter than BE (6.9 and 6.6 h) for both OE and SS, indicating faster elimination than its primary metabolite.

Table 11. Median (range) pharmacokinetic parameters for cocaine and benzoylecgonine (BE) in oral fluid (N = 10 participants) following 25 mg intravenous (IV) cocaine administration collected with Oral-Eze (OE) and StatSure (SS) collection devices up to 69 h.

	Oral-Eze		StatSure	
	Cocaine	BE	Cocaine	BE
C_{max} (µg/L)	932 (394-1,574)*	248 (96.9-953)*	732 (83.3-1,892)	360 (77.2-836)
T_{max} (h)	0.34 (0.17-1)*	0.5 (0.17-1)*	0.17 (0.17-0.5)	0.5 (0.17-1)
$t_{1/2}$ (h)	1.3 (0.6-2.2)*†	6.9 (4.4-12.1)*	0.89 (0.57-1.4)§†	6.6 (4.1-14.1)§
$AUC_{0\text{-}69h}$ (h x µg/L)	802 (314-1,447)	814 (425-1,801)	467 (174-1,378)	670 (390-1,298)
T_{last} (h)	12.5 (4-69)*	30.5 (21-69)*	6.5 (4-45)§	28 (21-69)§
C_{last} (µg/L)	1.9 (1.0-35.1)	2.8 (1.1-20.8)	5.3 (1.0-26.6)	3.5 (1-11.4)

C_{max} maximum concentration detected, T_{max} time of maximum concentration, $t_{1/2}$ terminal half-life, AUC area under the curve, C_{last} last detected concentration, T_{last} time of last detected concentration

Significant difference $P < 0.05$: *OE cocaine versus BE; §SS cocaine versus BE; †OE versus SS cocaine; ‡OE versus SS BE

Comparison of Oral Fluid Collection Methods

No statistically significant differences were observed between OE and SS OF cocaine or BE pharmacokinetics, with the exception of cocaine $t_{1/2}$ (Table 11). OF collected with OE exhibited significantly longer $t_{1/2}$ (1.3 h) than SS (0.89 h) (Figure 10). Detection times were longer in OE than in SS-collected specimens (Table 10). This could be attributed to the OE elution buffer that may have stabilized cocaine and BE better or improved recovery from the collection device more efficiently than the SS buffer.

Discussion

OF drug testing for DUID, workplace, drug treatment and pain management monitoring is increasing due to its inherent advantages over other biological matrices. We present the first cocaine and BE OF data collected with commercially available devices after controlled IV cocaine administration. OE and SS collection devices contain stabilizing and elution buffers that preserve labile analytes like cocaine and improve drug recovery from the collection pad. Knowing detection rates and windows and

pharmacokinetic parameters for cocaine markers in these OF collection devices is important for valid interpretation of results.

Similar cocaine and BE concentration-time courses were observed for OF collected with OE and SS collection devices (Figure 10). Peak cocaine concentrations occurred 0.17-1 h after dosing, with BE C_{max} slightly later (0.17 and 1.5 h). There were significant differences in cocaine and BE C_{max} and T_{max} in OF specimens collected with the OE device, potentially due to buffer pH and composition differences. Although cocaine T_{max} in this IV study was consistent with previous findings after SC administration [31], median BE T_{max} occurred much earlier: 0.5 h versus 2-2.1 h, respectively. However, the previous SC cocaine study collected OF via citric-acid stimulated expectoration. This could contribute to the observed T_{max} difference, as stimulation increases bicarbonate excretion, increasing OF pH and thus affecting cocaine and BE ion trapping into OF, thereby potentially increasing the time required to reach C_{max}. Additionally, IV administration delivers cocaine more rapidly to the bloodstream than does SC administration, presumably contributing to an earlier T_{max}.

Cocaine was quickly eliminated from OF, with median half-lives of 1.3 (0.6-2.2) and 0.89 (0.57-1.4) h in OE and SS OF specimens, respectively (Table 11). Our results are comparable to mean cocaine $t_{1/2}$ after IV 0.58-2.45 [10,34,36,37], SM (0.86-2.63 h) [36,37], IN (1.61) [37], and oral (1.2 h) [39] dosing, but shorter than the median $t_{/2}$ after SC cocaine administration (2.6-3 h) [31]. This could be due to differences in administration routes, as IV cocaine should peak earlier and be eliminated more rapidly than SC administration. Additionally, differences in $t_{1/2}$ are potentially due to the OF collection method used in previous studies, as citric-acid stimulated expectoration alters

cocaine and BE ion trapping into OF (as explained above), and does not benefit from stabilization of analytes in the elution buffer. After self-reported cocaine use in the community, mean ± SEM cocaine $t_{1/2}$ 7.9 ±3.1 h, was much longer than observed in our study, potentially due to higher doses and bioaccumulation following repeated cocaine intake [271]. BE remained in the OF for a significantly longer time than cocaine with $t_{1/2}$ of 6.9 (4.4-12.1) and 6.6 (4.1-14.1) h for OE and SS, respectively (Table 11). This is consistent with reports after SC administration (7.4-9.1 h) [31] and self-reported use (9.2 h) [271], and slightly longer than previous IV (2.3-6.5 h) [34,37], SM 3.5 h, and IN 4.7 h $t_{1/2}$ [37]. However, previous studies demonstrated large variability in half-lives based on a limited number of participants ($n \leq 7$) that makes direct comparisons difficult between studies.

This is the first study of which we are aware that compared cocaine and BE pharmacokinetics in two OF collection devices containing different stabilizing and elution buffers. Although both devices were effective in monitoring cocaine and BE disposition in OF, SS OF concentrations trended lower and detection times were slightly shorter than those for OE. Significant differences in pharmacokinetics parameters were only observed for cocaine $t_{1/2}$; SS exhibited significantly shorter cocaine $t_{1/2}$ (Table 11), with cocaine eliminated more quickly (Figure 10) than in OE OF specimens. These observed differences could be due to differences in analyte recovery from the absorbent pad, and/or buffer pH and composition in the OF collection devices. There were no differences in time of analysis between the two collection devices for each participant; time of analysis should not be a contributing factor for the small differences observed between collection devices.

OE and SS devices performed comparably during method validation with acceptable extraction efficiencies and short term stability results, making these factors an unlikely source of variation. OE and SS extraction efficiencies ranged from 79.7-97.2% and 70.9-95.5% with short term stability results within ±9.6% and ±16.7% of target, respectively. In a prior study examining nine different OF collection devices, including SS, cocaine recoveries of fortified specimens were 85.4% and stability was within ±17.4% of target up to 28 days stored at -18°C [272]. Although we did not observe issues in cocaine recovery and short term analyte stability in fortified OF samples during method development, it is possible that recovery from the absorbent pads and stability in authentic specimens might account for significant differences observed in cocaine $t_{1/2}$ between the two devices and lower SS concentrations observed. OE devices may provide improved cocaine stabilization for specimens. Recovery and stability in fortified authentic or synthetic OF may not be the same as for authentic OF [273,274]; however, this has not yet been evaluated for cocaine and its metabolites. Therefore, differences observed between OE and SS OF collection devices, although minor, could be attributed to variations in stabilizing buffers present in the devices or decreased recovery from the collection pad.

Cocaine OF detection times (4-6.5 h at SAMHSA and DRUID cutoffs) from two commercially available collection devices following controlled IV cocaine administration are presented for the first time. Detection windows were similar to those reported in OF following 25 mg IV, 32 mg IN, and 42 mg SM cocaine (4.1-6.3 h) [38] and after 75 mg/70 kg and 150 mg/70 kg SC (8-9.8 h) [31] when OF was collected up to 12 and 48 h after dosing. BE detection times reported in this study were consistent with those after SC

administration (28-32 h) with up to 48 h collection [31], but longer than after IV, IN, and SM cocaine administration (5.0-8.7 h) [38] with up to 12 h collection. Longer detection windows were achieved in our study with a 1 µg/L LOQ (cocaine 12.5-21 h; BE 69 h) compared to SC administration at a higher assay LOQ (2.5 µg/L) [31], especially for BE (cocaine 11.5-17.7 h; BE 32-47 h), with longer OF collection up to 69 h. After SC cocaine administration detection rates and windows were increased with the application of cocaine and/or BE confirmation cutoffs compared to BE alone [31]; however, this was not observed in our IV cocaine administration study. Although detection windows were not widened in our study, small increases in OE % positives were observed at 0.17 and 69 h when evaluating cocaine and/or BE compared to BE testing alone. These findings coincide with the earlier observed BE T_{max} reported in our study compared to SC cocaine administration. It also is important to note that these differences could be due to stabilization and elution buffers present in the OF collection devices utilized in our study that may have stabilized cocaine, compared to OF specimens obtained via citric-acid stimulated expectoration following SC administration that had no stabilizers or elution buffer.

Our results highlight that cocaine and BE confirmatory cutoffs can be selected to meet different drug testing needs. SAMHSA (8 µg/L) and DRUID (10 µg/L) OF cocaine cutoffs yield detection times similar to the time course of cocaine's impairing effects, making them useful for roadside testing. In contrast, the application of a low cutoff (LOQ) extended OF detection times, which could be beneficial for pain management drug testing programs. Additionally, including cocaine in the confirmation did not widen detection windows compared to BE only testing, as reported previously, due to BE being

present in the OF immediately after controlled IV cocaine administration and perhaps to the presence of stabilizing and elution buffers in the collection devices.

Table 12. (Supplemental) Gas chromatography, mass spectrometry (MS), Deans switch, flame ionization (FID) and mass selective (MSD) detectors and back inlet (cold trap) method parameters for the detection and quantification of cocaine and benzoylecgonine (BE) in oral fluid.

Electron Ionization Parameters			
Deans switch		Back Inlet	
FID restrictor L.	212.7 cm	Initial T	250ºC
FID restrictor i.d.	0.180 mm	Initial time	2.5 min
Aux 3 pressure	12.9 psi	Ramp #1	800ºC/min
Front inlet		Final T; time	100ºC; 5.3 min
Flow mode	Constant pressure	Ramp #2	800ºC/min
Pressure	21.85 psi	Final T; time	250ºC; 0 min
Total flow	55.3 mL/min	Oven	
Inlet T	250ºC	Initial oven T	150ºC
Injection mode	Splitless	Initial oven hold	0.5 min
Injection volume	2 µL	Ramp #1	30ºC/min
Liner type	Single-taper	Final T; time	290ºC; 0.5 min
FID and MSD		Ramp #2	75ºC/min
FID T	250ºC	Final T; time	180ºC; 1.5 min
Hydrogen flow (OFF)	40 mL/min	Ramp #3	30ºC/min
Air flow (OFF)	450 mL/min	Final T; time	290ºC/min; 1 min
MSD reagent gas	Vacuum	Post Ramp	40ºC/min
MSD transfer line T	280ºC	Post T	320ºC; 4 min
MS source T	230ºC	Post time	4 min
MS quad T	150ºC	Column 1 pressure	21.85 psi
EM offset (total)	506 (2612)	Column 2 pressure	12.9 psi

Chapter 4 – Cocaine and Benzoylecgonine Oral Fluid On-Site Screening and Confirmation

(*As published in* Drug Testing and Analysis, *February 2016*)[4]

Abstract

Accurate on-site devices to screen for drug intake are critical for establishing that an individual is DUID; however, on-site OF cocaine device performance is variable. We evaluated performance of a newly developed BE test-strip for the Draeger® DrugTest 5000 device (20 µg/L cutoff) with equivalent cross reactivity for cocaine and BE. Ten cocaine users provided OF, collected with the Draeger cassette and Oral-Eze® and StatSure Saliva Sampler™ devices, up to 69 h following 25 mg intravenous cocaine administration. All screening results were confirmed by a validated 2D-GC-MS method for cocaine and/or BE. Cocaine test-strip median T_{last} for screening only results was 6.5 h, and 6.5 h with OE and 4 h for SS OF confirmation for cocaine and/or BE at 1, 8 and 10 µg/L; sensitivity, specificity and efficiency ranged from 85.5-100% and 83.3-100% for cocaine only confirmation at 8 and 10 µg/L. For the BE test-strip, median T_{last} was 12.5 h for screening only and confirmation for cocaine and/or BE at all three cutoffs; sensitivity, specificity and efficiency ranged from 85.5-97.5% and 78.4-97.4% with cocaine and/or BE confirmation at 8 and 10 µg/L cutoffs, respectively. The Draeger cocaine test-strip with cocaine only confirmation offers a useful option for monitoring DUID; additionally the BE test-strip with cocaine and/or BE confirmation increases the length of detection of cocaine intake for DUID, workplace drug testing, drug court, parole, pain management,

[4] Ellefsen K.N. *et al.* Cocaine and Benzoylecgonine Oral Fluid On-Site Screening and Confirmation. *Drug Testing and Analysis*. 2016; 8(3-4): 296-303.

drug treatment programs and both the acute cocaine intoxication and cocaine crash/fatigue phase of DUID.

Introduction

DUID is a major worldwide problem. In the United States, approximately 11.0% of Friday daytime and 14.4% of weekend nighttime drivers tested positive for drugs [1]; in Europe 1.9% were positive for illicit drugs [84]. Despite declining cocaine prevalence in the US population, there are approximately 1.5 million current cocaine users [3,4,260], with increasing use in Europe (approximately 3.4 million users) [5]. Cocaine was the second most prevalent illicit drug (after cannabis) in the general driving population in the United States (1.4%) and Europe (0.42%) [5,6].

A deterrent to DUID is sensitive, specific, reliable and easy identification of recent drug use at the roadside. Traditionally, urine is utilized for on-site screening procedures, but urine is invasive to collect roadside, and urinary drugs and metabolites do not necessarily reflect recent use [79]. Interest in OF as an alternative matrix for DUID cases is increasing due to its easy, non-invasive collection and reduced adulteration potential [25-28]. OF also can be rapidly collected at the roadside close to the time of driving [80,81].

Current OF on-site drug testing devices utilize lateral flow immunochromatography, offering presumptive evidence of drug intake [82,83]. If the on-site device screens positive for a drug, a second OF confirmation sample is collected that contains an elution/stabilizing buffer to improve drug recovery and stabilization. Roadside OF testing devices must be sensitive, specific, and accurate; suggested performance criteria for on-site OF screening devices were 80% for sensitivity,

specificity, and efficiency [2]. The 1999 and 2003-2005 European roadside testing assessments (Rosita and Rosita-2) [86,275,276] and the DRUID projects [2] demonstrated limited OF on-site testing device sensitivity for all substances tested (amphetamines, cannabis, cocaine, opiates, and benzodiazepines).

Cocaine on-site OF screening device sensitivities varied from 11.0-97.0% [83,87-90,277-279], due to different analyte recoveries from collection pads, stabilizer buffers, cutoffs and antibodies. The Draeger DrugTest® 5000 (DrugTest 5000) OF on-site screening device (20 µg/L cocaine cutoff) detected cocaine use in OF from drugged drivers or drug treatment patients with reported sensitivities of 64-67% compared to a 50 µg/L plasma confirmation cutoff [87], 50% for 10 µg/L OF cutoff [83,88], 97% for 10 µg/L OF cutoff [89], 76% for 10 µg/L serum cutoff [90], and 88.9% for 10 µg/L OF cutoff [91]. DrugTest 5000 specificities for cocaine in OF ranged from 60-100%.

Draeger recently developed a new benzoylecgonine (BE) test-strip with a 20 µg/L cutoff and equivalent cross-reactivity to cocaine and BE. The aims of this study were to evaluate for the first time OF detection windows and performance characteristics of the new BE test-strip and compare performance with the original platform for cocaine detection.

Materials and Methods

Participants

Participants provided written informed consent for this NIDA Institutional Review Board approved study and received a comprehensive medical and psychological assessment. Eligibility criteria included healthy adults ages 18-50 years who smoked or used IV cocaine for at least six months and at least three times per month during the three

months prior to screening. Exclusion criteria included pregnant or nursing women; current physical dependence on any drug other than cocaine, caffeine, or nicotine; current clinically significant medical or psychiatric disorder; hemoglobin less than 12.5 g/dL or blood donation within eight weeks; current hypertension or blood pressure consistently above 140 mm Hg systolic or 90 mm Hg diastolic while at rest; heart rate consistently above 90 or below 50 bpm while at rest; abnormal 12-lead ECG; history of clinically significant adverse reaction to cocaine, acetazolamide, or quinine; or interest in drug abuse treatment within three months of study screening. Participants also were questioned prior to admission to verify that they had not taken any prescription or over-the-counter medication, grapefruit juice, or herbal supplement within the prior 14 days, to ensure pharmacokinetic validity. If they indicated that they had, they were not allowed to begin the study

Study Design

Participants resided on a secure research unit for 13 days and 12 nights, and received a single 25 mg IV cocaine dose on Days 1, 5 and 10. Cocaine was administered alone on Day 1, in combination with oral acetazolamide on Day 5 (after daily acetazolamide doses on Days 2-5), and with oral quinine on Day 10 (after daily quinine doses on Days 7-10). The primary study aim was to evaluate potential pharmacodynamic and pharmacokinetic interactions between cocaine and acetazolamide or quinine, as these drugs were under consideration as compliance markers for cocaine use disorder pharmacotherapies. This provided the opportunity to evaluate performance of the on-site Draeger® DrugTest 5000 device (Draeger Safety Diagnostics, Lübeck, Germany) original cocaine and new BE test-strips against OF collected with either the Oral-Eze®

(Quest Diagnostics, Madison, NJ) or StatSure Saliva Sampler™ (StatSure Diagnostic Systems, Brookline, MA) device for two-dimensional-gas chromatography-mass spectrometry (2D-GC-MS) confirmation of cocaine and BE.

OF Sample Collection

OF samples were collected -1, 0.17, 0.5, 1, 1.5, 2, 3, 4, 6.5, 9.5, 12.5 and 21 h after cocaine administration. Additional collections were obtained at 28 (Day 10 only), 33 (Day 1 only), 45 and 69 h (Days 1 and 5) post-administration. OF was first collected with the Draeger device and screened for cocaine intake on the on-site DrugTest 5000 within 15 min. OF was then collected with either the OE or SS device and OF-buffer mixtures were stored at 4°C before 2D-GC-MS analysis for cocaine and BE.

The DrugTest 5000 cassette is equipped with a polymeric non-compressible pad for OF collection. Participants were instructed to continuously move the collector around their mouths until the indicator turned blue, or until 5 min elapsed. Test cassettes turned blue when 270 µL ±15% OF was collected. For the OE or SS device, OF was collected by placing the absorptive pad under the tongue until the volume-adequacy indicator turned blue, indicating 1 mL OF was collected, or 5 min had elapsed, whichever occurred first. There was a tight time line for the study that necessitated collection within 5 min. No fluids, food or smoking were permitted for 10 min before collection.

OF Analysis

The DrugTest 5000 includes an analyzer, test cassette and a buffer cartridge for determining presence of cocaine, opiates, benzodiazepines, cannabinoids, amphetamines or methamphetamine in OF. The manufacturer reported cross-reactivity with the original cocaine antibody as 100% for cocaine, 28.6% for BE, 5% for norcocaine, 4% for

cocaethylene and <0.2% for EME. We also evaluated the new BE test-strip with a 20 µg/L cutoff and 100% cross-reactivity to cocaine and BE. A "Positive/Negative" or "Invalid" (if improper lateral flow was detected) response occurs following lateral flow immunochromatography. Cocaine and/or BE confirmation was performed by a fully validated 2D-GC-MS method [265], in accordance with the SWGTOX recommendations [264]. Linear ranges were 1-100 µg/L. Analytical bias ranged from -9.2 and 14.0% and within- and between-run imprecision <5.7%. Cocaine Oral-Eze and StatSure extraction efficiencies were 93.1-95.5% and 96.4-97.2%, respectively, and BE 70.9-73.5% and 79.7-82.7%, respectively. Short-term stability was evaluated at room temperature for 24 h and at 4°C for 72 h. Analytes were considered stable under evaluated conditions (% difference <16.7%).

Data Analysis

For cocaine and BE test-strip performance evaluation, qualitative DrugTest 5000 results were compared to quantitative OE and SS OF results. True positive (TP) samples screened and confirmed positive; true negative (TN) samples were negative in both assays. False positive (FP) samples screened positive but cocaine and BE were not present at the specified confirmation cutoffs; false negative (FN) samples screened negative but confirmed positive for cocaine or BE. Performance parameters were calculated as: sensitivity = 100x(TP/TP+FN); specificity = 100x(TN/TN+FP); and efficiency = 100x(TP+TN/Total results). Sensitivity, specificity, and efficiency were calculated at the DrugTest 5000 screening cutoff of 20 µg/L and Oral-Eze and StatSure OF GC-MS confirmation cutoffs of 1 (limit of quantification (LOQ)), 8 (SAMHSA), 10 (DRUID), and 20 µg/L (DrugTest 5000) for cocaine, BE, or cocaine and/or BE.

Additionally, detection rates and windows were evaluated with screening results compared to cocaine and/or BE results at various confirmation cutoffs.

Results

Participants

Participant demographics and self-reported cocaine histories are detailed in Table 13. Ten subjects (9 men, 1 woman; 5 African-Americans, 4 Caucasians, 1 >one race) aged 35-50 participated in the study, with all completing OF collection sessions. Median length of self-reported cocaine use was 18.0 (4.2-31.5) years, with a frequency of daily to once per week. A total of 280 cocaine and 224 BE screening tests were performed. One hundred thirty-nine OF Draeger-Oral-Eze and 141 OF Draeger-StatSure cocaine test-strip sample pairs, and 111 OF Draeger-Oral-Eze and 113 OF Draeger-StatSure BE test-strip sample pairs were obtained. Two participant's (K and M) OF was not evaluated with the new BE test-strip due to limited supplies; instead their OF was only evaluated with the original cocaine test-strip.

DrugTest 5000 Cocaine and BE Test-strip OF Screening Detection Rates and Times with a 20 µg/L Cutoff

Participants resided on the secure clinical unit throughout the study and did not test positive for any other drugs besides cocaine and BE (including amphetamines, cannabis, or opiates) with the DrugTest 5000. Based on the 20 µg/L DrugTest 5000 cocaine-screening cutoff, 53.6% of OF samples were positive up to 69 h after IV administration of 25 mg cocaine. In contrast, for the new BE test-strip (20 µg/L cutoff), 70.1% were positive. No cocaine test-strip tests were "Invalid"; only one (0.45%) new

Table 13. Demographics and self-reported cocaine histories for 10 cocaine users. Participant I was discharged prior to the third cocaine dose; however, all Draeger DrugTest 5000, Oral-Eze and StatSure oral fluid samples were collected prior to discharge.

PPT	Race	Sex	Age (yrs)	Weight (lbs)	Days used in 14 days prior to screening (SM or IV)	Mean use during 3 months prior to screening (days/time period) (SM or IV)	Age first use (SM or IV) (yrs)	Lifetime cocaine use (yrs)
B	AA	M	38.1	202	11 SM	5/week	32	6.1
C	1+	M	42.4	162	5 IV	3/week	32	10.4
					0 SM	3-4/month	21	21.4
D	W	M	35.2	165	3 SM	1/week	25	10.2
F	W	M	44.0	197	5 SM	Daily	18	26
G	W	M	42.5	180	6 IV	3/week	28	14.5
					7 SM	2/week	31	11.5
H	AA	M	43.7	150	14 SM	Daily	21	22.7
I	AA	M	49.9	175	10 SM	4-5/week	25	24.9
J	AA	M	46.5	176	1 SM	3/week	15	31.5
K	W	M	39.2	175	0 IV	N/A	35	4.2
					0 SM	Daily	11	28.2
M	AA	F	36.6	140	0 SM	2/week	20	16.6

PPT participant, SM smoked, IV intravenous, AA African-American, 1+ more than one race, W Caucasian, M male, N/A not assessed

BE test-strip gave an “Invalid” result. Of the positive DrugTest 5000 OF samples, 39 screened positive with the BE but not the cocaine test-strip, while only five OF samples positive with the cocaine test-strip were negative with the BE test-strip. There were no patterns identified to explain the cocaine test-strip results, while the BE positive results were clearly related to BE’s longer detection time. Draeger DrugTest 5000 detection rates for cocaine and BE at the 20 µg/L screening cutoff are presented in Figure 12. All 0.17 h (first sample after IV dose) OF samples (n=10) screened positive with both cocaine and BE test-strips. Median (range) T_{last} was 6.5 h (4-12.5) for the cocaine test-strip screen and 12.5 h (12.5-21) for the new BE test-strip screen at the 20 µg/L screening cutoffs (Table 14).

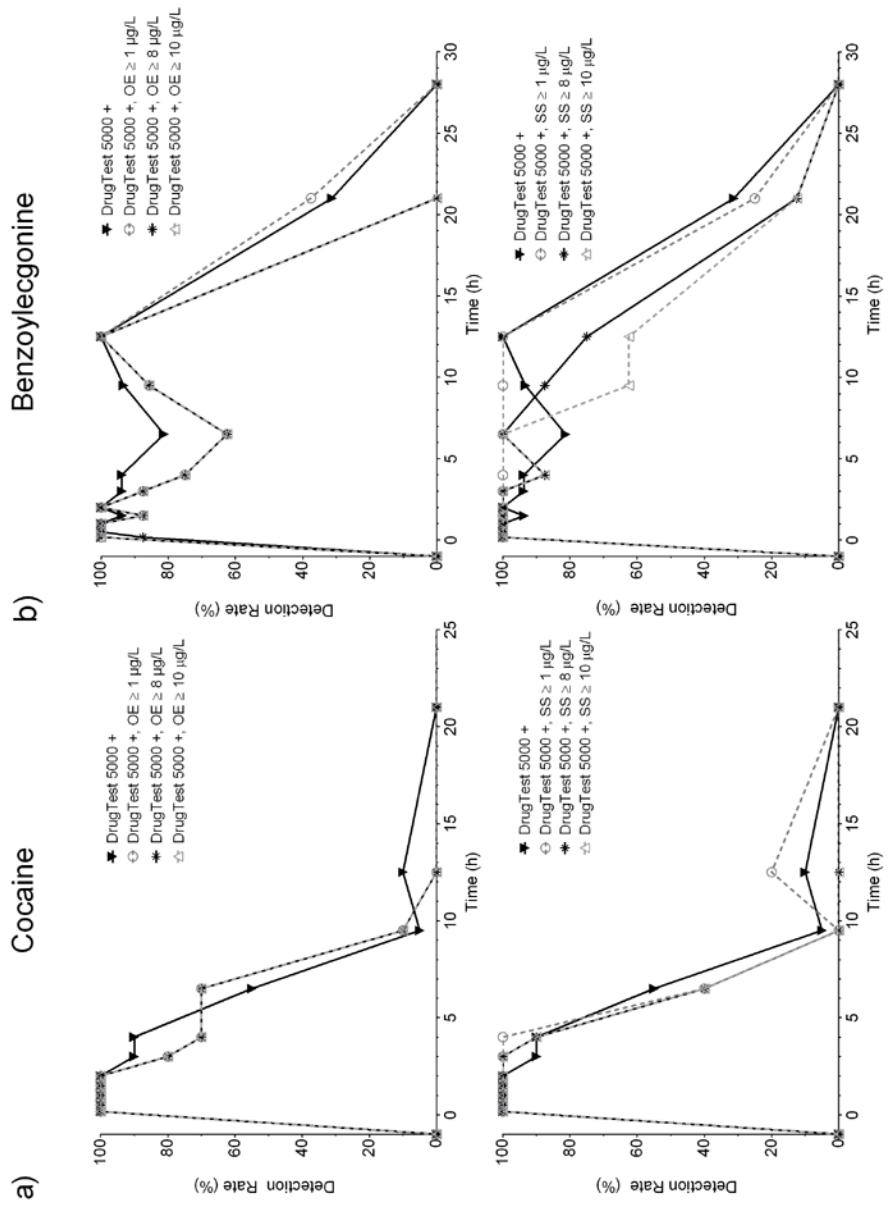


Figure 12.Oral fluid (OF) detection rates after controlled 25 mg intravenous cocaine administration in samples collected with the DrugTest 5000 on-site screening test and Oral-Eze (OE) and StatSure (SS) confirmation devices over time (69 h post-administration) in 10 participants. A) Positive cocaine DrugTest 5000, positive cocaine DrugTest 5000 with cocaine and/or BE OF confirmation ≥1 µg/L (analytical LOQ), 8 µg/L Substance Abuse and Mental Health Services Administration (SAMHSA), and 10 µg/L Driving Under the Influence of Drugs, Alcohol and Medicines (DRUID) for OE and SS. B) Positive benzoylecgonine (BE) DrugTest 5000, positive BE DrugTest 5000 with cocaine and/or BE OF confirmation ≥ the LOQ, SAMHSA, and DRUID cutoffs for OE and SS. Participants n = 10 cocaine; n = 8 BE.

Table 14. Median (range) times of last (Tlast) DrugTest 5000 test for screen only or confirmed for cocaine and/or benzoylecgonine (BE) at ≥1 µg/L (limit of quantification), 8 µg/L (Substance Abuse and Mental Health Services Administration, SAMHSA), or 10 µg/L (Driving Under the Influence of Drugs, Alcohol and Medicines, DRUID) oral fluid confirmation cutoffs following 25 mg intravenous cocaine administration to 10 participants.

Cutoffs	Cocaine Test-strip T_{last} (h)		Benzoylecgonine Test-strip T_{last} (h)	
DrugTest 5000 screen only	6.5 (4-12.5)		12.5 (12.5-21)	
	Oral-Eze	StatSure	Oral-Eze	StatSure
Coc AND/OR BE ≥ 1 µg/L	6.5 (4-9.5)	4 (4-12.5)	12.5 (12.5-21)	12.5 (12.5-21)
Coc AND/OR BE ≥ 8 µg/L	6.5 (4-9.5)	4 (4-6.5)	12.5	12.5 (9.5-21)
Coc AND/OR BE ≥ 10 µg/L	6.5 (4-9.5)	4 (4-6.5)	12.5	12.5 (6.5-21)

Detection Rates and Times for Cocaine and BE Test-strip OF Screens with 2D-GC-MS Oral-Eze and StatSure OF Confirmations

Detection rates and times for the DrugTest 5000 cocaine and BE test-strip results confirmed for cocaine and/or BE in OF collected with the OE or SS OF devices at different confirmation cutoffs from 10 participants for up to 69 h after controlled IV cocaine administration are presented in Figure 12. Only one OE OF sample was confirmed positive for cocaine only, while exhibiting positive cocaine and BE test-strip screening results (participant H: 0.17 h). A cocaine positive test-strip screen and cocaine and/or BE above the LOQ resulted in 52.5% (73/139) and 53.9% (76/141) Draeger-Oral-Eze and Draeger-StatSure positive OF pairs (Table 15 and Table 16). For a BE test-strip positive screen and cocaine and/or BE ≥1 µg/L confirmation, 66.7% (74/111) and 72.6% (82/113) Draeger-Oral-Eze and Draeger-StatSure OF pairs were positive, respectively (Table 15 and Table 16). Cocaine OF times of last positive test above the LOQ were 9.5 (OE) and 12.5 h (SS), whereas BE OF detection windows were longer (21 h) for both collection devices (Figure 12). Detection times for both cocaine (OE 9.5 h, SS 6.5 h) and BE (OE 12.5 h, SS 21 h) were generally shorter at the higher SAMHSA and DRUID confirmation cutoffs compared to those at the LOQ.

Table 15. Performance characteristics for the DrugTest 5000 with 20 µg/L cocaine and benzoylecgonine (BE) oral fluid (OF) screening cutoffs with different confirmation cutoffs (n = 139 Cocaine; n = 111 BE) up to 69 h following 25 mg intravenous cocaine administration. OF confirmation samples were collected with Oral-Eze devices.

Cutoff	TP	TN	FP	FN	Sensitivity (%)	Specificity (%)	Efficiency (%)
Cocaine Test-strip + Cocaine ≥ 1 µg/L	72	47	2	18	80.0	95.9	85.6
Cocaine Test-strip + Cocaine ≥ 8 µg/L	65	60	9	5	92.9	87.0	89.9
Cocaine Test-strip + Cocaine ≥ 10 µg/L	64	61	10	4	94.1	85.9	89.9
Cocaine Test-strip + Cocaine ≥ 20 µg/L	56	63	18	2	96.6	77.8	85.6
Cocaine Test-strip + BE ≥ 1 µg/L	72	17	2	48	60.0	89.5	64.0
Cocaine Test-strip + BE ≥ 8 µg/L	72	36	2	29	71.3	94.7	77.7
Cocaine Test-strip + BE ≥ 10 µg/L	72	37	2	28	72.0	94.9	78.4
Cocaine Test-strip + BE ≥ 20 µg/L	70	45	4	20	77.8	91.8	82.7
Cocaine Test-strip + Cocaine and/or BE ≥ 1 µg/L	73	16	1	49	59.8	94.1	64.0
Cocaine Test-strip + Cocaine and/or BE ≥ 8 µg/L	73	36	1	29	71.6	97.3	78.4
Cocaine Test-strip + Cocaine and/or BE ≥ 10 µg/L	73	37	1	28	72.3	97.4	79.1
Cocaine Test-strip + Cocaine and/or BE ≥ 20 µg/L	71	45	3	20	78.0	93.8	83.5
BE Test-strip + BE ≥ 1 µg/L	73	13	2	23	76.0	86.7	77.5
BE Test-strip + BE ≥ 8 µg/L	70	24	5	12	85.4	82.8	84.7
BE Test-strip + BE ≥ 10 µg/L	70	25	5	11	86.4	83.3	85.6
BE Test-strip + BE ≥ 20 µg/L	63	27	12	9	87.5	69.2	81.1
BE Test-strip + Cocaine ≥ 1 µg/L	64	27	11	9	87.7	71.1	82.0
BE Test-strip + Cocaine ≥ 8 µg/L	51	32	24	4	92.7	57.1	74.8
BE Test-strip + Cocaine ≥ 10 µg/L	50	33	25	3	94.3	56.9	74.8
BE Test-strip + Cocaine ≥ 20 µg/L	45	35	30	1	97.8	53.8	72.1
BE Test-strip + Cocaine and/or BE ≥ 1 µg/L	74	12	1	24	75.5	92.3	77.5
BE Test-strip + Cocaine and/or BE ≥ 8 µg/L	71	24	4	12	85.5	85.7	85.6
BE Test-strip + Cocaine and/or BE ≥ 10 µg/L	71	25	4	11	86.6	86.2	86.5
BE Test-strip + Cocaine and/or BE ≥ 20 µg/L	64	27	11	9	87.7	71.1	82.0
Cocaine and/or BE Test-strip + Cocaine and/or BE ≥ 1 µg/L	79	12	1	20	79.8	92.3	82.0
Cocaine and/or BE Test-strip + Cocaine and/or BE ≥ 8 µg/L	76	24	4	8	90.5	85.7	90.1
Cocaine and/or BE Test-strip + Cocaine and/or BE ≥ 10 µg/L	76	25	4	7	91.6	86.2	91.0
Cocaine and/or BE Test-strip + Cocaine and/or BE ≥ 20 µg/L	69	27	11	5	93.2	71.1	86.5

Table 16. Performance characteristics for the DrugTest 5000 with 20 µg/L cocaine and benzoylecgonine (BE) oral fluid (OF) screening cutoffs with different confirmation cutoffs (n = 141 Cocaine; n = 113 BE) up to 69 h following 25 mg intravenous cocaine administration. OF confirmation samples were collected with StatSure devices.

Cutoff	TP	TN	FP	FN	Sensitivity (%)	Specificity (%)	Efficiency (%)
Cocaine DrugTest + Cocaine ≥ 1 µg/L	74	58	2	7	91.4	96.7	93.6
Cocaine DrugTest + Cocaine ≥ 8 µg/L	65	65	11	0	100	85.5	92.2
Cocaine DrugTest + Cocaine ≥ 10 µg/L	63	65	13	0	100	83.3	90.8
Cocaine DrugTest + Cocaine ≥ 20 µg/L	54	65	22	0	100	74.7	84.4
Cocaine DrugTest + BE ≥ 1 µg/L	76	20	0	45	62.8	100	68.1
Cocaine DrugTest + BE ≥ 8 µg/L	74	40	2	25	74.7	95.3	80.9
Cocaine DrugTest + BE ≥ 10 µg/L	73	43	3	22	76.8	93.5	82.3
Cocaine DrugTest + BE ≥ 20 µg/L	70	58	6	7	90.9	90.6	90.8
Cocaine DrugTest + Cocaine and/or BE ≥ 1 µg/L	76	20	0	45	62.8	100	68.1
Cocaine DrugTest + Cocaine and/or BE ≥ 8 µg/L	74	40	2	25	74.7	95.2	80.9
Cocaine DrugTest + Cocaine and/or BE ≥ 10 µg/L	73	43	3	22	76.8	93.5	82.3
Cocaine DrugTest + Cocaine and/or BE ≥ 20 µg/L	72	58	4	7	91.1	93.5	92.2
BE DrugTest + BE ≥ 1 µg/L	82	15	0	16	83.7	100	85.8
BE DrugTest + BE ≥ 8 µg/L	78	29	4	2	97.5	87.9	94.7
BE DrugTest + BE ≥ 10 µg/L	74	29	8	2	97.4	78.4	91.2
BE DrugTest + BE ≥ 20 µg/L	62	31	20	0	100	60.8	82.3
BE DrugTest + Cocaine ≥ 1 µg/L	64	29	18	2	97.0	61.7	83.2
BE DrugTest + Cocaine ≥ 8 µg/L	51	31	31	0	100	50.0	72.6
BE DrugTest + Cocaine ≥ 10 µg/L	49	31	33	0	100	48.4	70.8
BE DrugTest + Cocaine ≥ 20 µg/L	42	31	40	0	100	43.7	64.6
BE DrugTest + Cocaine and/or BE ≥ 1 µg/L	82	15	0	16	83.7	100	85.8
BE DrugTest + Cocaine and/or BE ≥ 8 µg/L	78	29	4	2	97.5	87.9	94.7
BE DrugTest + Cocaine and/or BE ≥ 10 µg/L	74	29	8	2	97.4	78.4	91.2
BE DrugTest + Cocaine and/or BE ≥ 20 µg/L	64	31	18	0	100	63.3	84.1
Cocaine and/or BE DrugTest + Cocaine and/or BE ≥ 1 µg/L	82	15	0	16	83.7	100	85.8
Cocaine and/or BE DrugTest + Cocaine and/or BE ≥ 8 µg/L	78	29	4	2	97.5	87.9	94.7
Cocaine and/or BE DrugTest + Cocaine and/or BE ≥ 10 µg/L	74	29	8	2	97.4	78.4	91.2
Cocaine and/or BE DrugTest + Cocaine and/or BE ≥ 20 µg/L	64	31	18	0	100	63.3	84.1

Cocaine was detected longer in Draeger-Oral-Eze OF pairs than Draeger-StatSure OF pairs; median cocaine T_{last} were 6.5 and 4 h, respectively, at all confirmation cutoffs (LOQ, SAMHSA and DRUID) (Table 14). BE median (range) T_{last} were 12.5 h (6.5-21) for both OF confirmation devices and at all confirmation cutoffs.

Performance Comparison of the DrugTest 5000 Cocaine Test-strip and BE Test-strip Assays with OF Confirmation collected by the Oral-Eze and StatSure Devices and Evaluated by 2D-GC-MS

Table 15 and Table 16 present Draeger cocaine and BE test-strip performance characteristics and confirmation by 2D-GC/MS with different OF collection devices, confirmation analytes and cutoff results over the 69 h monitoring period. The cocaine test-strip screen and cocaine only confirmation sensitivities, specificities and efficiencies at ≥8 and ≥10 µg/L cutoffs were always ≥87.0 and ≥85.9% (OE device), respectively, and ≥85.5 and ≥83.3% (SS device), respectively, but the total number of TP results was low, as there were many TN results the study (Table 15 and Table 16). For the cocaine test-strip screen and ≥8 and ≥10 µg/L cocaine and/or BE confirmation, performance characteristics were ≥71.3 and ≥72.0% (OE), respectively, and ≥74.7 and ≥76.8%, (SS), respectively (Table 15 and Table 16). The new BE test-strip performed better than the cocaine test-strip at ≥8 and ≥10 µg/L BE only confirmation cutoffs with performance characteristics ≥82.8 and ≥83.3% (OE), respectively, and ≥87.9 and ≥78.4% (SS), respectively (Table 15 and Table 16). When comparing a positive BE test-strip screen with cocaine and/or BE confirmation at ≥8 and ≥10 µg/L cutoffs, performance was ≥85.5 and ≥86.2% (OE), respectively, and ≥87.9 and ≥78.4% (SS), respectively. Confirming cocaine and/or BE at ≥8 or ≥10 µg/L with either a cocaine and/or BE positive test-strip resulted in sensitivities, specificities, and efficiencies ≥85.7% and ≥86.2% (OE),

respectively (Table 15); SS performance for these combinations was the same as for a positive BE screen and BE confirmation only (≥87.9 and ≥78.4%, Table 16).

Median (range) times after cocaine dosing and median (range) concentrations of all FP and FN samples are presented in Table 17 for cocaine and BE test-strip screening results at different confirmation cutoffs. Cocaine DrugTest 5000 FP results (*n*=47) had cocaine concentrations from <LOQ-9.6 µg/L and BE concentrations <LOQ-88.0 µg/L and occurred from 3-12.5 h. BE FP results (*n*=34) occurred from 0.17-21 h with BE concentrations from <LOQ-9.6 µg/L and cocaine concentrations <LOQ-76.5 µg/L. More variability was observed for DrugTest 5000 FN samples. Cocaine FN (*n*=24) concentrations ranged from 1.0-44.0 µg/L occurring between -1-69 h and BE concentrations <LOQ-87.0 µg/L. BE FN samples (*n*=66) had BE concentrations from 1.0-106 µg/L and cocaine concentrations <LOQ-35.8 µg/L with FN occurring from -1-45h.

Table 17. Median (range) concentrations and times of false positive (FP) and false negative (FN) DrugTest 5000 cocaine and benzoylecgonine (BE) test-strips for identifying cocaine markers in oral fluid (OF) samples. OF samples were confirmed for cocaine only or BE only ≥ the limit of quantification (LOQ)(1 µg/L); Substance Abuse and Mental Health Services Administration (SAMHSA) (8 µg/L); and Driving Under the Influence of Drugs, Alcohol and Medicines (DRUID) (10 µg/L) confirmation cutoffs following controlled intravenous cocaine administration up to 69 h post-dosing. OF confirmation samples were collected with Oral-Eze (OE) and StatSure (SS) devices.

DrugTest 5000			OE ≥ 1	SS ≥ 1	OE ≥ 8	SS ≥ 8	OE ≥ 10	SS ≥ 10
Cocaine test-strip	FP	Cocaine (µg/L)	<LOQ	<LOQ	2.8 (<LOQ-7.7)	4.7 (<LOQ-6.5)	3.6 (<LOQ-8.3)	5.8 (<LOQ-9.6)
		BE (µg/L)	14.7 (<LOQ-29.4)	14.3 (8.0-20.6)	29.4 (<LOQ-74.6)	29.9 (6.0-63.1)	29.9 (<LOQ-74.6)	29.9 (6.0-88.0)
		Time (h)	5.3 (4-6.5)	9.5 (6.5-12.5)	6.5 (3-9.5)	6.5 (3-12.5)	6.5 (3-9.5)	4 (3-12.5)
		n	2	2	9	11	10	13
	FN	Cocaine (µg/L)	2.0 (1.0-44.0)	1.6 (1.1-4.2)	16.1 (8.7-44.0)	-	26.2 (12.3-44.0)	-
		BE (µg/L)	24.7 (<LOQ-87.0)	17.7 (4.4-36.5)	40.8 (12.2-87.0)	-	41.7 (36.0-87.0)	-
		Time (h)	9.5 (3-69)	6.5 (-1-45)	4 (3-12.5)	-	3.5 (3-4)	-
		n	18	7	5	0	4	0
BE test-strip	FP	BE (µg/L)	<LOQ	-	4.2 (<LOQ-6.2)	6.6 (5.7-7.9)	4.2 (<LOQ-6.2)	8.0 (5.7-9.6)
		Cocaine (µg/L)	38.3 (<LOQ-76.5)	-	<LOQ (<LOQ-76.5)	0.5 (<LOQ-5.8)	<LOQ (<LOQ-76.5)	<LOQ (<LOQ-5.8)
		Time (h)	2.1 (0.17-4)	-	21 (0.17-21)	11 (4-21)	21 (0.17-21)	11 (4-21)
		n	2	0	5	4	5	8
	FN	BE (µg/L)	9.9 (1.1-106)	3.9 (1.0-11.4)	31.9 (9.9-106)	10.7 (10.0-11.4)	34.3 (16.4-106)	10.7 (10.0-11.4)
		Cocaine (µg/L)	<LOQ (<LOQ-35.8)	<LOQ (<LOQ-1.5)	1.4 (<LOQ-35.8)	<LOQ	1.6 (<LOQ-35.8)	<LOQ
		Time (h)	21 (-1-45)	24.5 (-1-45)	6.5 (-1-28)	21	6.5 (-1-28)	21
		n	23	16	12	2	11	2

Discussion

To our knowledge, these are the first data identifying cocaine and BE OF detection rates and windows of detection following controlled cocaine administration for the Draeger DrugTest 5000 original OF cocaine test-strip and the new BE test-strip, with performance characteristics obtained with different OF cocaine markers and confirmation cutoffs. Cocaine was administered by the IV route to avoid contamination of the OF mucosa that occurs following smoked or insufflated cocaine. All participants screened cocaine and BE positive up to 2 h following cocaine administration, with the exception of one participant who screened positive for cocaine only at 1.5 h.

Cocaine test-strip screening only detection rates were similar to both Draeger-Oral-Eze and Draeger-StatSure OF paired samples confirmed at the LOQ (Figure 12). Detection rates for a positive cocaine test-strip and cocaine and/or BE confirmation at the SAMHSA 8 µg/L confirmation cutoff were the same as detection rates at the 10 µg/L DRUID cutoff. As expected, higher detection rates were observed for the BE test-strip with cocaine and/or BE confirmation at different cutoffs for both confirmation devices compared to the cocaine test-strip. Additionally, detection rates for both the cocaine and BE test-strips were slightly higher for OF samples collected with SS compared to OE devices (Figure 12).

Times of last positive result were dependent on screening test-strip, confirmation analyte(s) and cutoff, and OF confirmation collection device. Screening only detection windows were longer with the BE test-strip compared to the cocaine test-strip (Figure 12). Confirming with cocaine and/or BE at all cutoffs with a positive cocaine test-strip produced identical T_{last} (6.5 h) as cocaine screening results only, except for Draeger-

StatSure OF pairs (4 h). This difference could be due to variations in buffer composition and analyte recovery from collection pads. No differences between Draeger-Oral-Eze and Draeger-StatSure OF BE T_{last} (21 h) were observed with the BE test-strip at all cutoffs analyzed. However, different administration routes, dose, and dosing frequency may produce different detection windows.

DrugTest 5000 cocaine and BE screening results were evaluated against Oral-Eze and StatSure OF confirmation data to determine TP, TN, FP, FN, sensitivity, specificity, and efficiency at different confirmation analytes and cutoffs (Table 15 and Table 16). As expected, more FP occurred with higher confirmation cutoffs and FN substantially decreased contributing to improved sensitivities. It is possible cross-reactivity with other drugs and cocaine metabolites could contribute to FP results, but this is unlikely, as participants resided on a secure research unit throughout the study with no access to other drugs. More FN samples were observed with the new BE test-strip compared to the cocaine test-strip, especially for samples confirmed with Oral-Eze that had longer detection times. In addition, the BE screen has a higher screening cutoff than the confirmation cutoffs (8 and 10 µg/L), likely contributing to FN results. FP and FN samples typically had concentrations at or near confirmation cutoffs (Table 17).

Performance characteristics of the DrugTest 5000 cocaine test-strip with cocaine only confirmation at 10 µg/L were consistent with previous reports [89,91]; Strano-Rossi *et al.* reported sensitivity, specificity, and efficiency of 82%, 100%, and 97% for the cocaine test-strip in randomly stopped drivers (n=403) with a 10 µg/L cocaine OF confirmation cutoff [89]. Logan *et al.* reported comparable performance in individuals under arrest for driving under the influence of cocaine (88.9%, 100%, and 98.9%) (n=91),

screened with the cocaine test-strip and confirmed for cocaine and metabolites ≥10 µg/L [91]. In our study, we reported greater sensitivities (94.1-100%) than what were previously reported with a positive cocaine test-strip and cocaine only confirmation at ≥10 µg/L; however, specificity (83.3-85.9%) and efficiency (89.9-90.8%) were lower. One possible explanation for these differences could be our longer monitoring windows yielding a larger number of TN. Our sensitivities were higher compared to Blencowe *et al.*[83] and Vanstechelman *et al.* [88], who reported only 50% sensitivity in drug treatment and addiction center samples screened with the cocaine test-strip and confirmed for cocaine and/or BE at 10 µg/L. Vanstechelman *et al.* hypothesized that their high FN rate could be explained by confirmatory cocaine concentrations below the screening cutoff, whereas Blencowe *et al.* suggested poor sensitivity due to few positive cases.

Screening with the cocaine test-strip and confirming for BE or cocaine and/or BE at 8 and 10 µg/L for both devices, yielded increased specificity over cocaine confirmation only; however, sensitivity and efficiency were typically not above the DRUID recommended 80% performance criteria (sensitivity 71.3-76.8%, efficiency: 77.7-82.3%). This could be attributed to limited BE cross-reactivity with the cocaine strip; BE was <70 µg/L at the time of the last cocaine positive screen. BE also was confirmed at ≥8 and ≥10 µg/L increasing FN results and decreasing sensitivity. BE is a valuable analyte to document cocaine intake, and increase detection rates and windows compared to cocaine only.

Drug testing programs have different sensitivity, specificity, and detection rate and window requirements for selecting an on-site OF screening device. Based on our data, monitoring programs can select the cocaine or BE screening test-strip and

confirmation analyte and cutoff to meet their testing requirements. A positive cocaine test-strip and cocaine only confirmation at SAMHSA or DRUID cutoffs yielded performance characteristics ≥83.3%, with detection times mirroring cocaine's acute impairing effects. This combination of screening and confirmation results may be optimal for DUID testing, where documenting impairment at the time of driving with an active cocaine marker is critical. Additionally, when positive roadside screening tests result in administrative sanctions, like driving bans [87,279], more weight is placed on reducing FP rates and increasing specificity of the on-site device. For workplace, drug court, parole or pain management drug testing, screening and confirmation should accurately detect cocaine or BE for the longest timeframe possible. Based on our results, we suggest screening with the new BE test-strip with cocaine and BE confirmation (SAMHSA and DRUID cutoffs), as performance characteristics were ≥85.5% with the OE confirmation device, and ≥78.4% for the Draeger-StatSure device, and had the longest median detection times and rates. Additionally, this combination of screening and confirmation tests may be useful for cocaine crash/fatigue phase of DUID. Laboratories also may choose to screen with the BE-test strip only and confirm for BE only as this combination only missed one additional TP for OE samples and no additional TP for SS samples compared to cocaine and/or BE confirmation. Additionally, for drug treatment programs with 2-3 samples per week, and the need to identify drug relapse between OF collections, screening for BE and confirmation with BE also appears suitable. Screening with the cocaine and BE test-strips and confirming for cocaine and/or BE, although expensive, additionally could be considered when identifying all TP is important; an additional six TP were identified for Draeger-Oral-Eze OF pairs.

These are the first data to demonstrate that the DrugTest 5000 is a sensitive, specific, and efficient on-site OF screening device for both cocaine and BE following controlled administration of IV cocaine at proposed SAMHSA and DRUID confirmation cutoffs. The BE test-strip had longer detection windows compared to the cocaine test-strip. Additionally, we showed how different cutoffs and confirmation analytes influence performance characteristics, detection rates and windows of detection. Based on the results of this study, the cocaine DrugTest 5000 with cocaine only OF confirmation is a suitable option for monitoring the acute cocaine intoxication phase of DUID, whereas the BE DrugTest 5000 with a cocaine and/or BE confirmation is better suited for workplace drug testing, drug court, parole, pain management, drug treatment monitoring, and both the acute cocaine intoxication and cocaine crash/fatigue phase of DUID. Additional research is needed for other routes of administration and cocaine doses.

Chapter 5 - Quantification of Cocaine and Metabolites in Exhaled Breath by Liquid Chromatography High Resolution-Mass Spectrometry following Controlled Administration of Intravenous Cocaine

(*As published in* Analytical and Bioanalytical Chemistry, *October 2014*)[5]

Abstract

Breath has been investigated as an alternative matrix for detecting recent cocaine intake; however, there are no controlled cocaine administration studies investigated the drug's disposition into breath. Breath was collected from 10 healthy adult cocaine users by breathing into the SensAbues device for 3 min before and up to 22 h following 25 mg IV cocaine dosing on days 1, 5 and 10, and assayed with a validated LC-HRMS method to quantify breath cocaine, BE, EME, and norcocaine. The assay was linear from 25-1,000 pg/filter, extraction efficiencies were 83.6-126%, intra- and inter-assay imprecision <10.6% and bias between -8.5-16.8%. No endogenous or exogenous interferences were observed of more than 75 tested. Analytes were generally stable under short-term storage conditions. Ion suppression was less than 46%. Of breath specimens collected after controlled cocaine administration, 2.6% were positive for cocaine (26.1-66 pg/filter, 1-9.5 h), 0.72% BE (83.3-151 pg/filter, 6.5-12.5 h), and 0.72% EME (50-69.1 pg/filter, 6.5-12.5 h); norcocaine was not detected. Methanolic extraction of the devices themselves, after filters were removed, yielded 19.2% positive cocaine tests (25.2-36.4 pg/device, 10min-22 h) and 4.3% positive BE tests (26.4-93.7 pg/device, 10min-22 h), explaining

[5] Ellefsen K.N. *et al.* Quantification of cocaine and metabolites in exhaled breath by liquid chromatography high resolution-mass spectrometry following controlled administration of intravenous cocaine. *Analytical and Bioanalytical Chemistry*. 2014 Oct; 406(25): 6213-23.

differences between the two extraction techniques. These results suggest that the device reflects drug in oral fluid as well as lung microparticles, while the filter reflects only drug-laden microparticles. A sensitive and specific method for cocaine, BE, EME, and norcocaine quantification in breath was developed and validated. Cocaine in breath identifies recent cocaine ingestion, but its absence does not preclude recent use.

Introduction

Cocaine is the second most frequently identified illicit drug in the United States, after cannabis, and is the most widely used illicit stimulant in Europe [280,281]. In 2012, there were approximately two million current cocaine users in the US and Europe [260,281].

Cocaine is rapidly metabolized, primarily in the liver, by hydrolysis of the two ester linkages [23]. Its two major inactive metabolites are BE and EME. Additionally, cocaine is metabolized via *N*-demethylation by cytochrome P450 enzymes to produce a minor active metabolite, norcocaine [282,283].

There is an increased interest in utilizing alternative matrices, other than blood and urine, for identifying drug use in DUID, workplace, clinical, and anti-doping programs [26,29,30]. Studies focused on OF as a viable alternative matrix due to its non-invasive, observed collection, reduced potential for adulteration, and general identification of recent use [25,28,31,284]. However, OF has several disadvantages. Sample volume is small, especially after intake of sympathomimetic drugs, and interpretation of results can be difficult due to oral mucosa contamination, yielding a poor correlation of OF/plasma concentrations early in the time course. Lately, several studies investigated the potential of exhaled breath as an alternative matrix for detecting recent

consumption of drugs of abuse [45-54], including cocaine [51,53,54]. This concept is based on the hypothesis that exogenous substances contaminate the airway lining fluid creating aerosol particles (microparticles) that can be detected in exhaled breath [53]. Exhaled breath contains microparticles that can carry non volatile compounds, including airway lining fluid, out of the lungs. Formation of these microparticles is believed to occur when the airway opens during inhalation [55]. Therefore, if the lungs are contaminated with exogenous substances they can become trapped in the airway lining fluid and thus, distribute into the breath upon exhalation.

Three previous studies documented cocaine in exhaled breath [51,53,54]. Cocaine was detected (80 pg/min) in one patient recovering from acute self-reported cocaine intoxication [51]. Another study detected cocaine in 10 patients following acute intoxication, approximately 24 h after drug intake [53]. Cocaine breath concentrations ranged from 29-13,000 pg/device (median 60 pg/device); BE was quantified in four of these cases with concentrations of 18-560 pg/device (median 43 pg/device). BE was only found when cocaine was detected. Two of the positive breath cases did not have detectable plasma or urine concentrations, or self-reported cocaine use. More recently, a study examined exhaled breath in parallel with urine specimens from prison inmates following arrival at correctional facilities [54]. Only 22 of 247 specimens were positive for drugs of abuse in urine and breath. Cocaine (12 pg/device) and BE (3 pg/device) were detected in breath in only one of 247 cases where breath and urine specimens were available; however, individual data were not provided in the article to state how many urine samples were positive for BE.

Currently, there are no controlled cocaine administration studies examining cocaine and its metabolites in exhaled breath. This investigation evaluated pharmacodynamic and pharmacokinetic interactions between controlled IV cocaine administration and two potential medication adherence markers, acetazolamide and quinine. The SensAbues breath device collected cocaine and metabolites in breath, and their concentrations were quantified in a new validated LC-HRMS method.

Materials and Methods

Participants

Ten healthy volunteers ages 18-50 years who smoked or used IV cocaine for at least six months and at least three times per month during the three months prior to screening, were eligible. Participants are screened prior to enrollment in the study by recruitment staff. Volunteers receive extensive psychological and psychosocial testing including a Structured Clinical Interview for DSM-IV, drug use survey, and Addiction Severity Index. In addition, participants also complete comprehensive medical history and physical examinations to determine their eligibility for the study based on our inclusion and exclusion criteria. Exclusion criteria included pregnant or nursing women; current physical dependence on any drug other than cocaine, caffeine, or nicotine; current clinically significant medical or psychiatric disorder; current hypertension or blood pressure readings consistently above 140 mm Hg systolic or 90 mm Hg diastolic while at rest; heart rate consistently above 90 bpm or below 50 bpm while at rest; abnormal ECG; history of clinically significant adverse reaction to cocaine; or interest in drug abuse treatment, within three months of study screening. Subjects provided written informed

consent to participate in this NIDA Institutional Review Board approved study. Participants resided on a secure research unit for 13 days and 12 nights.

Cocaine Administration and Breath Collection

Participants were administered a single 25 mg IV cocaine dose through a peripheral venous catheter on three separate days during the study (Day 1, 5 and 10). Cocaine was administered alone on Day 1, with oral acetazolamide on Day 5 and with oral quinine on Day 10. Breath specimens were collected over 3 min with the SensAbues (SensAbues AB, Huddinge, Sweden) device an hour prior to IV cocaine, and 10 min, and 0.5, 1, 1.5, 2, 3, 4, 6.5, 9.5, 12.5, and 21 h (Day 1 and 10) or 22 h (Day 5) post administration. The SensAbues device consists of a mouthpiece, collection chamber and polymeric filter [51]. Participants were asked to breathe normally during sampling, inhaling through their nose and exhaling through the mouthpiece. After 3 min, the mouthpiece was discarded as per manufacturer's instructions and both ends of the collection chamber were sealed and stored at -20ºC within 2 h. New devices were used for each time point. No oral intake or smoking was allowed 10 min prior to collection. Devices are designed to protect against oral fluid contamination with barrier ledges inside the mouthpiece [51].

Chemicals and Materials

Cocaine, EME, and norcocaine HCl standards in acetonitrile and BE standard in methanol (1 mg/mL) were purchased from Cerilliant (Round Rock, TX). IS, cocaine-d3 (1 mg/mL), EME-d3 (100 ug/mL), and norcocaine-d3 HCl (100 ug/mL) in acetonitrile and BE-d8 (1 mg/mL) in methanol also were obtained from Cerilliant. SPE was performed with UCT Clean Screen cartridges (United Chemical Technologies, Inc., PA).

Formic acid, methanol and water were from Fisher Scientific (Fair Lawn, NJ). Ammonium formate and ammonium hydroxide 28-30% were from JT Baker (Phillipsburg, NJ). All solvents employed in the extraction were HPLC grade and in the chromatographic system, LC-MS grade. Water was purified-in-house by an ELGA Purelab Ultra Analytic purifier (Siemens Water Technologies, Lowell, MA).

Instrumentation

LC-high resolution MS was performed on a Thermo Scientific Ultimate 3000 RSLCnano system coupled to a Thermo Scientific QExactive Mass Spectrometer (Thermo Scientific, Fremont, CA). The Ultimate 3000 RSLCnano system consisted of a degasser, a tertiary loading pump, a binary eluting pump, a column oven and an RS Autosampler. Filter extraction was performed on a horizontal shaker from Eberbach© (Ann Arbor, MI) and SPE using a negative pressure manifold. Evaporation under compressed air was completed using a TurboVap LV® evaporator from Zymark (Hopkinton, MA).

Preparation of Standard Solutions

Methanolic working solutions at 0.5-20 µg/L were prepared yielding 25, 50, 100, 250, 500, and 1000 pg/filter calibrator concentrations when blank filters were fortified with 50 µL of working solution. QC working solutions were prepared from different ampoules, to produce 75, 375, and 750 pg/filter QC samples. An IS solution containing 2 µg/L of cocaine-d3, EME-d3, BE-d8, and norcocaine-d3 was prepared in methanol.

Specimen Procedure

Sample preparation was modeled after a previously described method [52] with modifications. Blank filters were fortified with their respective calibrator and QC concentrations. In authentic specimens, the filter was removed from the collection chamber. Calibrator, QC, and specimen filters were transferred with sterile tweezers to 20 mL amber-glass screw-top vials, and fortified with 50 µL IS solution. Six milliliters of 1% formic acid in water was added to each vial prior to shaking on a horizontal shaker at 260 oscillations/min. After shaking, vials were allowed to sit for approximately 5 min before transferring 3 mL to a 10 mL plastic Sarstedt tube. An additional 3 mL of 1% formic acid in water was added to the filters, vials shaken for 5 min, and 3 mL removed to plastic tubes. This procedure was repeated a total of three times (9 mL total). Other solvents were evaluated during method development, including methanol, and 0.2 M sodium acetate buffer; however, water with 1% formic acid yielded the best recoveries from the filter, with less interference/background noise.

Supernatants were loaded onto UCT Clean Screen cartridges preconditioned with methanol (3 mL) and water (3 mL). Columns were washed with 2% formic acid in water (3 mL) and 2% formic acid in methanol (3 mL) before drying columns under nitrogen using negative pressure at 10 psi for 20 min. Elution was performed with 3 mL methanol:ammonium hydroxide (95:5, v/v). Eluates were evaporated to dryness under compressed air at 40ºC. Samples were reconstituted in 200 µL mobile phase A (1 mM ammonium formate with 0.01% formic acid in water), vortexed briefly, and centrifuged at 4,000 x g for 3 min.

Additionally, a methanolic extraction of devices (without filters) was performed, in the same manner as Beck *et al.* [53]. The breath device was reassembled following

removal of the filter and 5 mL methanol directly added to the device. The device was shaken to ensure methanol extraction of any remaining drug from the device. The methanol was eluted into a plastic Sarstedt tube, and 50 µL IS (2 µg/L) and 20 µL 10% formic acid in methanol were added. Eluates were evaporated to dryness under compressed air at 40ºC, reconstituted in 200 µL mobile phase A, vortexed briefly, and centrifuged at 4,000 x g for 3 min.

Liquid Chromatography-Tandem Mass Spectrometry

Chromatographic separation was achieved at 30ºC with a Phenomenex Synergi Polar-RP 100 x 2 mm 2.5 µm column and identically packed defender guard cartridges (10 x 20 mm 2.5 µm). Gradient elution with 1 mM ammonium formate and 0.01% formic acid (pH~3.24) (solvent A) and methanol (solvent B) at 0.3 mL/min flow rate was performed. Initial composition (5% B) was maintained for 1.5 min, increased from 5 to 95% over 5.5 min, held for 1.5 min, returned to initial conditions over 30 sec, and held for 1.5 min for a total run time of 10.5 min. Injection volume was 10 µL.

Targeted-MS/MS scans were acquired at 35,000 resolution (full width at half maximum at *m/z* 200) with a heated electrospray ionization source (HESI-II) and operated in positive ionization mode. Automatic Gain Control (AGC) target was set at 5 x 10^5 and maximum injection time at 100 ms. Precursor ions were selected in the quadrupole with a 3 *m/z* window and subsequently fragmented in the higher energy collision dissociation (HCD) cell. A full scan of fragmented ions was performed, and two specific product ions utilized for data analysis with a mass tolerance of 5 ppm (Table 18). The spray voltage was 3 kV, capillary temperature 350^{0}C, heater temperature 425^{0}C, S-lens RF level 50, sheath gas flow rate 50, auxiliary gas flow rate 13 and sweep gas 3

(manufacturer's units). Nitrogen was used for spray stabilization, for collision-induced dissociation experiments in the HCD cell, and as the damping gas in the C-trap. The instrument was calibrated in the positive and negative mode every 25 h. Thermo TraceFinder Clinical Research 3.1 software was used for data collection and processing.

Table 18. Liquid chromatography high resolution mass spectrometry parameters and retention times (RT) for cocaine, benzoylecgonine (BE), ecgonine methyl ester (EME), and norcocaine. Quantitative ions are italicized.

Analyte	Precursor ion (m/z)	Product ions (m/z)	NCE (%)	RT (min)
EME	200.1	*182.1174*, 82.0656	60	1.6
EME-d_3	203.1	*185.1363*, 85.0844	60	1.6
BE-d_8	298.1	*171.1205*, 110.0651	50	6.4
BE	290.1	*168.1017*, 105.0338	45	6.4
Cocaine	304.1	*182.1174*, 82.0656	50	7.7
Cocaine-d_3	307.2	*185.1363*, 85.0844	50	7.7
Norcocaine-d_3	293.1	*171.1205*, 136.0756	20	7.9
Norcocaine	290.1	*136.0756*, 168.1017	45	7.9

NCE normalized collision energy

The following criteria identified compounds: retention time (RT) within ±0.2 min of average calibrator RT, presence of 2 product ions, and ion ratio of the quantifying ion/qualifier ion within ±20% of the average of all calibrators.

Method Validation

Method validation was performed on the breath filter extraction procedure, as the methanolic extraction of the breath devices (without the filters) was solely to determine oral fluid drug contamination of the device. Validation parameters included linearity, LOD, LOQ, bias and imprecision, ionization suppression/enhancement, extraction efficiency, process efficiency, interference studies, carryover, and autosampler and short-term stability studies. Linearity (R^2) was evaluated by least squares regression with ≥6 nonzero calibrators on 5 days. Acceptable linearity was achieved when $R^2 \geq 0.99$ and

calibrators quantified within ±20%. LOD and LOQ were determined with decreasing concentrations of drug-fortified filters; LOD was the lowest concentration with acceptable chromatography, signal/noise ratio ≥3, with analytes identified according to previously described criteria. LOQ was the lowest concentrations that met LOD criteria and a signal/noise ratio of at least 10, and bias and imprecision within ±20%. LOD and LOQ were evaluated in triplicate on three different days (n=9).

Assay bias and imprecision were determined at three concentrations (low, medium and high QCs) in triplicate over five days (n=15). Bias was evaluated for each concentration as 100 x [(group mean observed concentration – known concentration)/ known concentration]. Acceptable bias was ±20% of target. Precision was expressed as coefficient of variation (%CV) and determined by the one-way ANOVA approach to calculate combined within- and between-run imprecision, as described in the SWGTOX method validation guidelines [264]. Acceptable precision was <20 %CV.

Ion suppression/enhancement, extraction efficiency, and process efficiency for each analyte was measured at low and high QC concentrations as described by Matuszewski *et al.* [285]. Ion suppression/enhancement was assessed by comparing analyte peak areas of neat samples (n=6) (Set 1) to peak areas of ten different blank samples fortified with analyte and internal standard after extraction (Set 2). Extraction efficiency was examined by comparing analyte peak areas of five different samples fortified at low and high concentrations with internal standard before extraction (Set 3), to peak areas of Set 2. Process efficiency examined the overall effect of extraction efficiency and ion suppression/enhancement on quantification of analytes.

Endogenous and exogenous interferences were evaluated. Interferences from endogenous matrix components were investigated by analyzing breath samples from ten individuals without the addition of internal standard. Exogenous interferences including opiates, stimulants, benzodiazepines, cannabinoids, and synthetic cathinones (Table 23 (Supplemental)) were analyzed by fortifying neat samples with interferences equivalent to 1,000 pg/filter. Interferences were considered insignificant if analytes of interest were <LOD. Lack of carryover was demonstrated by injecting triplicate internal standard-fortified blank samples after a sample fortified at 2,500 pg/filter. Carryover was considered negligible if the measured concentration was <LOD.

Autosampler stability was investigated by reinjecting low and high QC samples stored 48 h at 4ºC on the autosampler (*n*=3) and calculating results against the original calibration curve. In addition, short-term stability was evaluated with breath filters fortified at low and high QC concentrations and stored in 20 mL amber glass vials for 24 h at room temperature (*n*=3), 72 h at 4ºC (*n*=3) and -20ºC (*n*=3), and after three freeze-thaw cycles (*n*=3). IS was added to each sample just prior to extraction and processed as described. Stability was considered acceptable if QC samples quantified within ±20% of freshly prepared QC samples (*n=3*).

Results

Method Performance

A linear range from 25 to 1,000 pg/filter with 1/x weighting was achieved for all four analytes. 1/x weighting was selected for this method as correlation coefficients were acceptable and the data demonstrated homoscedasticity, or random distribution of the individual residuals around the zero line, suggesting that this linear model was optimal in

comparison to other models. Calibration curves from five separate days yielded determination coefficients (R^2) above 0.99 ± 0.003 with residuals within ±20%. LOD was 15 pg/filter for all analytes, except EME (25 pg/filter), and LOQ was 25 pg/filter for all compounds. Although EME demonstrated a signal-to-noise ratio greater than 3, the ion ratios and peak shapes were not acceptable at 15 pg/filter; however, at the LOQ (25 pg/filter) the LOD acceptance criteria were achieved, in addition to the acceptance criteria for establishing the LOQ. Figure **13** illustrates a chromatogram of a breath sample fortified at the LOQ for all analytes.

Bias and imprecision results are presented in Table 19. All analytes were <10.6% for within- and between-run imprecision (%CV), and bias ranged from -8.5-16.8%. Extraction efficiencies were 83.6-125.5%, and process efficiencies 52.7-78.8% (Table 19). Ion suppression was less than -25%, except for EME and cocaine (high concentration), which exhibited suppression up to -46%. Despite observed ion suppression, variation among 10 different breath sources was <15% for EME; however, %RSD for the high cocaine concentration was 21% (Table 19).

There were no endogenous interferences in 10 different breath samples. Addition of 1,000 pg/filter potentially interfering drugs and metabolites to neat samples did not produce any interfering peaks meeting the identification criteria. No carryover was observed for BE and EME in internal standard-fortified blank samples after injection of a 2,500 pg/filter (2.5xULOQ) sample. For cocaine and norcocaine, carryover was observed at 2500 pg/filter, but not at the ULOQ (1000 pg/filter). In addition, as an additional precaution, two mobile phase injections were performed after the highest calibrator and QC. Analytes were stable in the autosampler for 48 h at low and high QC concentrations

(*n*=3), with % differences between -7.4 to -0.11%. When stored at room temperature for 24 h, at 4ºC for 72 h, and at -20ºC for 72 h all compounds were stable (-19.3 to 15.0%), except for cocaine at room temperature (-20.9%). Cocaine and metabolites also were stable after three freeze-thaw cycles (-10.2 to 9.4%). Data are shown in Table 20.

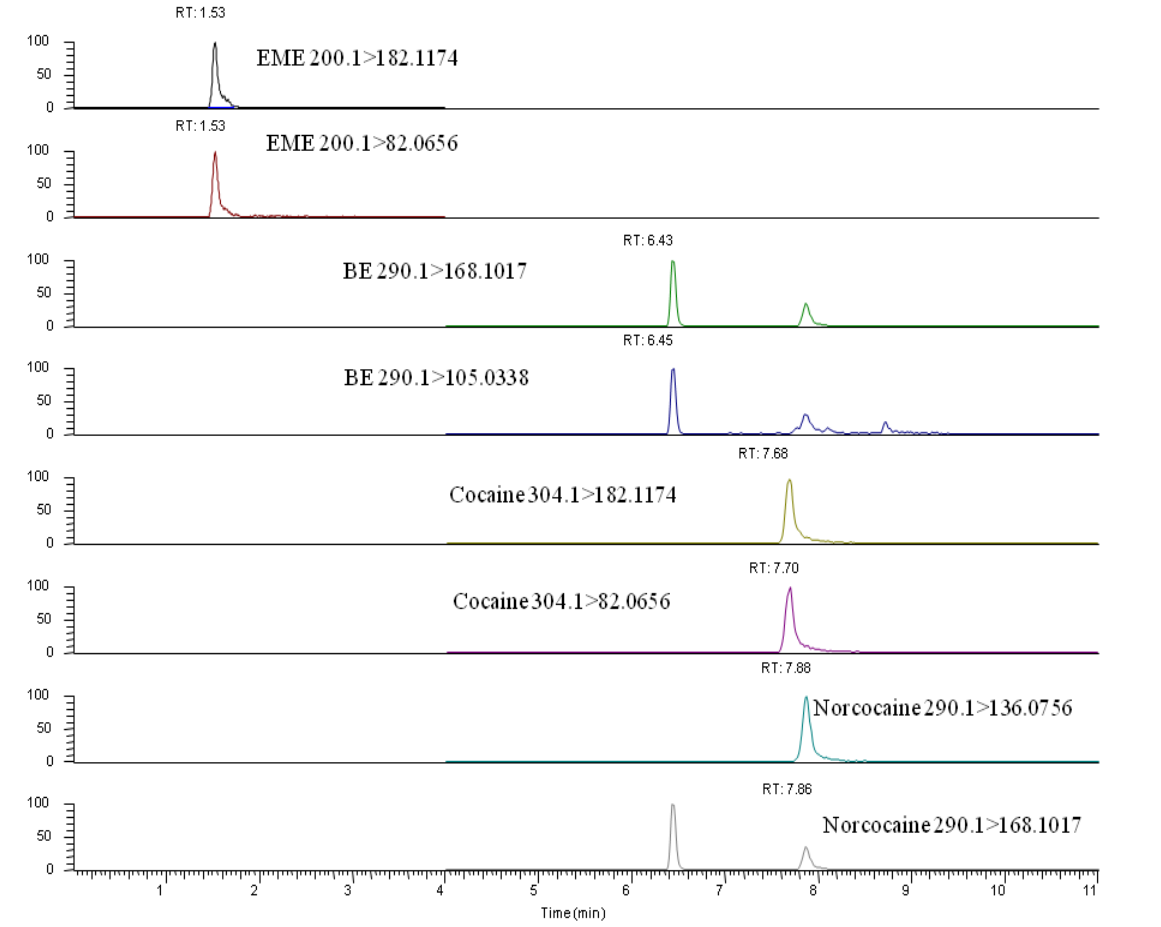


Figure 13. Liquid chromatography tandem mass spectrometry chromatogram of a breath sample fortified at the limits of quantification (25 pg/filter) for cocaine, benzoylecgonine (BE), ecgonine methyl ester (EME) and norcocaine.

Table 19. Within-run imprecision, between-run imprecision, and %bias for cocaine, benzoylecgonine (BE), ecgonine methyl ester (EME), and norcocaine in breath at low (L, 75 pg/filter), medium (M, 375 pg/filter), high (H, 750 pg/filter) concentrations and efficiency, process efficiency, and ion suppression/enhancement for cocaine, BE, EME, and norcocaine in breath at low and high concentrations.

Analyte	Within-run imprecision (*n*=15, %CV)			Between-run imprecision (*n*=15, %CV)			%Bias (*n*=15)			Extraction efficiency (%)(*n*=5)		Process efficiency (%) (*n*=5)		Ion suppression/ enhancement (%) (*n*=10) (%RSD)	
	L	M	H	L	M	H	L	M	H	L	H	L	H	L	H
Cocaine	8.3	7.7	9.9	7.9	10.6	10.3	-7.4	-8.5	-2.9	89.0	90.0	69.5	63.2	-21.9 (9.6)	-29.8 (21)
BE	3.1	4.7	6.4	2.9	5.8	7.7	16.8	-0.02	3.8	80.4	82.5	65.0	69.9	-19.1 (3.4)	-15.3 (5.8)
EME	4.6	8.0	6.5	7.6	8.7	9.1	-3.0	-0.03	-0.40	125.5	97.8	78.8	52.7	-37.2 (5.5)	-46.1 (13)
Norcocaine	6.7	7.0	7.6	6.9	8.8	9.3	1.5	-3.8	-1.2	83.6	85.7	63.2	64.3	-24.3 (9.3)	-25.0 (19)

Table 20.Short term stability data (%difference) of cocaine, benzoylecgonine (BE), ecgonine methyl ester (EME), and norcocaine in breath at low (75 pg/filter) and high (750 pg/filter) concentrations after storage at 4ºC 48 h autosampler storage, at room temperature 48 h, at 4ºC 72 h, at -20ºC 72 h and after 3 freeze-thaw cycles (over 72 h).

Stability Conditions	Cocaine		BE		EME		Norcocaine	
	Low	High	Low	High	Low	High	Low	High
48 h 4ºC autosampler (*n*=3)	-3.4	-4.3	-0.11	-7.1	-0.90	-7.4	-2.8	-0.23
24 h room temperature (*n*=3)	-19.3	-20.9	-4.3	-17.1	-12.1	-13.5	-14.2	-13.5
72 h 4ºC (*n*=3)	-6.1	-6.7	15	-0.67	-10.4	-4.2	0.25	-0.27
72 h -20ºC (*n*=3)	-8.5	-12.2	-0.10	-4.1	-15.4	-8.0	-1.8	-3.2
3 Freeze-thaw cycles (*n*=3)	4.8	-8.2	4.2	-0.92	-6.5	-10.2	0.12	9.4

Breath Cocaine and Metabolites

Breath specimens from 10 participants ages 35-50 years (Table 21) were analyzed (A-J) (Table 22). Two participants were medically discharged prior to the second cocaine dose on Day 5, and one prior to the third cocaine dose (Day 10). Only 6/228 specimens from three participants were positive for cocaine, with concentrations from 26.1-66 pg/filter (median 34.0 pg/filter). No specimens were positive for any cocaine marker in the first breath specimens at 10 and 30 min after IV cocaine administrations. The majority of positive cocaine specimens were collected 1-2 h post-dosing; however, two specimens were positive at 4 and 9.5 h. Figure 14a illustrates an LC-MS/MS chromatogram of a positive cocaine breath specimen (Participant D). Only two specimens from one participant (J) were positive for BE and EME 6.5 and 12.5 h post-dosing with concentrations of 83.3 and 151 pg/filter and 50 and 69.1 pg/filter, respectively. Norcocaine was not detected in any specimen.

Based on the low prevalence of positive specimens observed in breath, methanolic extraction of the device was performed after the breath filter was removed utilizing the

same extraction technique as Beck *et al.* [53]. Methanolic extraction of the devices yielded 32/182 positive cocaine tests (10 min to 22 h post-dosing), with semi-quantitative concentrations from 25.2-364 pg/device (median 53.4 pg/device). Unlike for the filters, BE was detected in 10/230 devices, with semi-quantitative concentrations between 26.4-93.7 pg/device (median 39 pg/device). In nine of ten BE-positive devices, cocaine also was present at higher concentrations. Figure 14b illustrates the LC-MS/MS chromatogram of a device positive for both cocaine and BE (Participant D). EME and norcocaine were not detected in any device.

Table 21. Demographic and self-reported cocaine histories for 10 cocaine users.

PPT	Race	Sex	Age (years)	Weight (lbs)	Days used in past 14 (SM or IV)	Mean use (SM or IV)	Age first use (SM or IV) (years)
A	AA	M	44.0	176	4 SM	2-3/week	38
B	AA	M	38.1	202	11 SM	5/week	32
C	More than 1	M	42.4	162	5 IV 0 SM	3/week 3-4/month	32 21
D	W	M	35.2	165	3 SM	1/week	25
E	AA	M	45.3	206	0 IV 5 SM	N/A 4/week	34 18
F	W	M	44.0	197	5 SM	Daily	18
G	W	M	42.5	180	6 IV 7 SM	3/week 2/week	28 31
H	AA	M	43.7	150	14 SM	Daily	21
I	AA	M	49.9	175	10 SM	4-5/week	25
J	AA	M	46.5	176	1 SM	3/week	15

PPT participant, *SM* smoked, *IV* intravenous, *AA* African-American, *W* White, *M* male

Table 22. Breath cocaine, benzoylecgonine (BE), and ecgonine methyl ester (EME) concentrations (pg/filter) in filters and cocaine and BE concentrations (pg/device) in breath collection devices (without the filters) from individuals following 25 mg IV cocaine.

Participant[a,b]	Filter Cocaine Concentrations (pg/filter)										
	Time after IV cocaine administration (h)[c]										
	10min	0.5	1	1.5	2	3	4	6.5	9.5	12.5	21/22[d]
D_1[e]	-[f]	-	35.2		26.1	-	-	-	-	-	-
D_2	-	-	-	-	-	-	-	-	33.2	-	-
F_1	-	-	-	34.8	-	-	-	-	-	-	-
H_1	-	-	-	66.0	-	-	27.3	-	-	-	-
Participant	Filter BE Concentrations (pg/filter)										
	Time after IV cocaine administration (h)										
	10min	0.5	1	1.5	2	3	4	6.5	9.5	12.5	21/22
J_2	-	-	-	-	-	-	-	151	-	83.3	-
Participant	Filter EME Concentrations (pg/filter)										
	Time after IV cocaine administration (h)										
	10min	0.5	1	1.5	2	3	4	6.5	9.5	12.5	21/22
J_2	-	-	-	-	-	-	-	50.0	-	69.1	-
Participant	Device Cocaine Concentrations (pg/device)										
	Time after IV cocaine administration (h)										
	10min	0.5	1	1.5	2	3	4	6.5	9.5	12.5	21/22
B_2	-	-	-	-	-	-	-	-	-	-	28.8
D_1	-	79.6	-	-	-	-	-	29.8	-	-	-
D_2	-	-	-	108	25.9	157	364	73.2	178	-	184
D_3	63.3	35.3	53.3	68.3	37.9	-	-	148	-	-	-
F_1	-	86.6	114	42.6	107	29.0	25.2	29.2	52.2	205	42.6
F_2	110	-	-	-	-	-	-	-	47.3	48.9	-
F_3	-	-	-	-	-	47.2	-	-	-	-	-
H_1	-	-	-	-	-	25.6	-	-	28.2	-	-
J_1	-	-	-	-	60.1	-	-	-	-	-	-
J_3	-	-	53.4	-	-	-	-	-	287	-	-
Participant	Device BE Concentrations (pg/device)										
	Time after IV cocaine administration (h)										
	10min	0.5	1	1.5	2	3	4	6.5	9.5	12.5	21/22
D_2	-	-	-	37.5	-	-	39.7	-	50.7	-	93.7
D_3	38.3	-	-	52.4	-	-	-	47.6	-	-	-
F_2	27.4	-	-	-	-	-	-	-	-	-	-
J_2	-	-	-	-	-	26.4	-	-	-	-	-
J_3	-	-	-	-	-	-	-	-	30.9	-	-

[a]All breath filters collected an hour prior to IV cocaine administration were <limit of quantitation (LOQ)
[b]Only results from participants with positive results are presented; 10 participants specimens were analyzed
[c]All specimens were collected within ±15 min of scheduled collection times
[d]Breath specimens collected at 21 h on Days 1 and 10, and at 22 h on Day 5
[e]$_1$ Represents participant results from Day 1, $_2$ represents participant results from Day 5, and $_3$ represents participant results from Day 10
[f] - , <LOQ

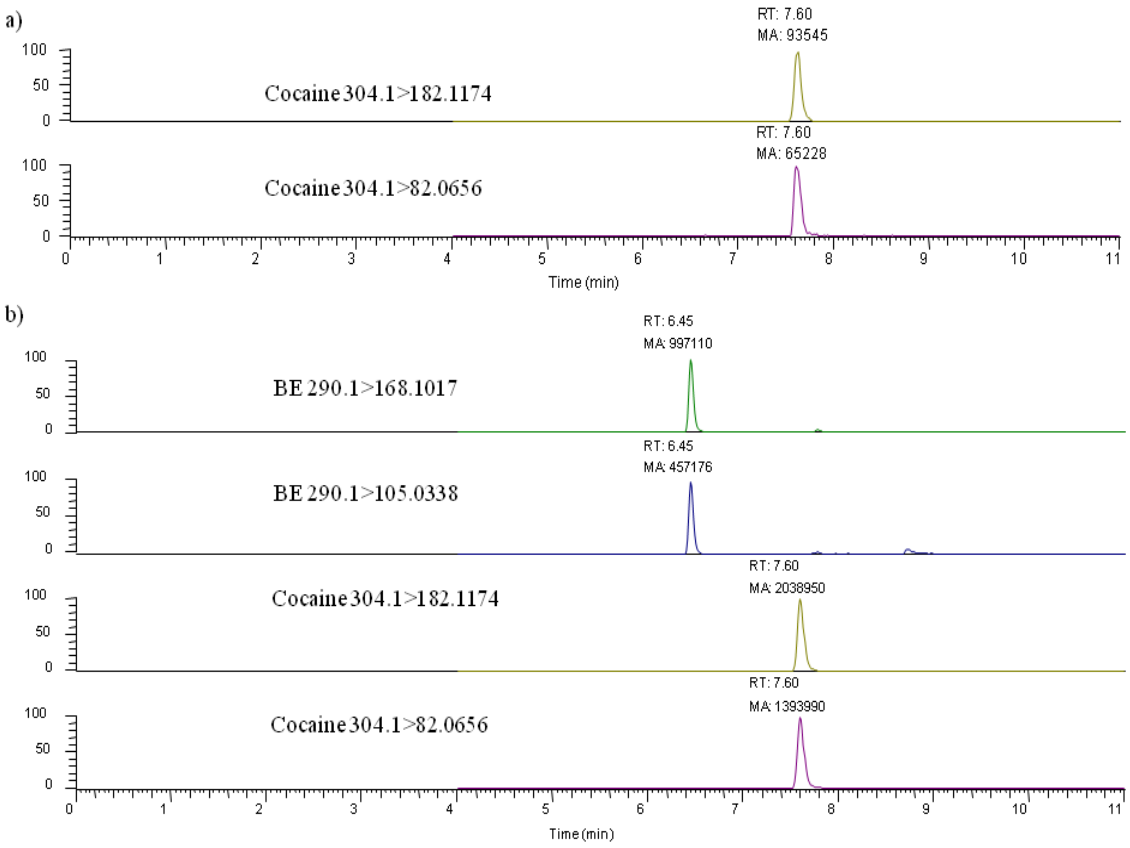


Figure 14.Liquid chromatography tandem mass spectrometry chromatogram of a) positive cocaine breath specimen (Participant D) with 33.2 pg cocaine/filter and b) cocaine 177.8 pg/device and benzoylecgonine (BE) 50.7 pg/device, 9.5 h after 25 mg IV cocaine.

Discussion

A fully validated analytical method for the quantification of cocaine, BE, EME, and norcocaine in exhaled breath was developed. There are no previous cocaine breath quantification methods including EME and norcocaine. Two previous studies reported validation results for cocaine and BE only [51,53]. LOQs for cocaine and BE in these investigations were 0.6 and 0.1 pg on column [51] and 6 pg/filter [53], estimated from diluted calibrator extracts with criteria of signal-to-noise ≥10 and concentrations within

±20% of target. In the present method, with an empirical determination of lower and lower analyte concentrations required to meet identification and quantitative criteria, the LOQs for all four analytes were 25 pg/filter. Based on concentrations reported by Beck *et al.*, 25 pg/filter should be adequate to detect cocaine and BE in authentic cases [53]; however, in previous studies the extraction was of the entire device and not just the filter. This sensitivity following our controlled cocaine administration study identified cocaine in breath in only a few subject's samples. Additional research is necessary to study correlations between cocaine dose, route of administration and breath concentrations.

Sample preparation for this method required two steps: first, extraction of analytes from the breath filter, followed by SPE. Filters were removed from the SensAbues breath collection devices prior to extracting the analytes with water and 1% formic acid. Following extraction from the filters, a simple solid phase extraction procedure was performed. The combination of analyte extraction from the filters and solid phase extraction reduced matrix effects compared to filter extraction alone. Recently, published breath methods extracted analytes from the filter by directly adding methanol to the intact SensAbues breath collection devices, with no subsequent solid phase extraction [53,54]. This likely resulted in higher analyte concentrations due to contribution from drug in oral fluid and also greater ion suppression.

The QExactive mass spectrometer was utilized for this method for its high resolution and high mass accuracy for the two product ions employed for identification. This increased specificity and sensitivity by improving signal-to-noise. Typical breath cocaine concentrations are in the pg/filter range, which is why increased sensitivity was an important feature of this method, enabling cocaine and metabolites quantification in

exhaled breath. No endogenous or exogenous interferences of structurally similar compounds were observed. However, BE and norcocaine have identical molecular weights and common fragmentation. As such, HRMS identification was not sufficient to differentiate these two compounds; chromatographic separation was necessary for accurate identification.

Validation results for cocaine and its metabolites were acceptable. Extraction efficiencies were 80-126% for all four analytes, and ion suppression less than 25% except EME (-37% and -46%), and the high cocaine concentration (-30%). Although ion suppression was high for EME, its %RSD was acceptable (<15%) demonstrating little variation among the 10 different matrix sources. Despite the high %RSD (21%) for the high cocaine concentration, cocaine QC concentrations were quantified within acceptable %bias and precision. Additionally, inclusion of matched deuterated IS compensated for these observed matrix effects. Previous analytical breath cocaine methods did not quantify matrix effects in breath [51,53,54]; however, THC and its metabolites produced breath matrix effects ranging from 12% to -35% [52], similar to what was observed in this study. Process efficiencies were low (53-79%) for all four analytes; this could be explained by loss of analyte during the extraction procedure and the ion suppression observed in this method. Despite these lower process efficiencies and ion suppression, a sensitive LOQ of 25 pg/filter was achieved.

To minimize possible carryover, additional mobile phases were analyzed after the highest calibrator (1,000 pg/filter) and high QC (750 pg/filter). However, in most reported cases, cocaine breath concentrations are below 80 pg/device [51,53,54]; therefore, cocaine and norcocaine carryover should not be an issue. Additionally, breath

specimens were stable under all tested conditions, with the exception of cocaine after 24 h at room temperature. Despite this observation, this was not problematic as collected breath specimens were stored at -20ºC within two hours of collection. Based on this investigation, breath specimens should be stored refrigerated or frozen immediately following collection in order to limit analyte loss.

Exhaled breath is commonly utilized and accepted for the detection of alcohol. Its ease and accessibility in collection make it a more desirable matrix for roadside collection than blood. Other matrices, like OF, offer the same ease in collection as breath; however, dry mouth, especially after stimulant abuse, can produce low volume OF collections. Exhaled breath contains microparticles that carry non-volatile substances out of the lungs including components of the airway lining fluid and proteins of the surfactant phase [286,287]. Recent studies hypothesized that exogenous substances, including drugs of abuse, can contaminate the airway lining and therefore, become detectable in exhaled breath [45-54].

Previous studies demonstrated that cocaine is detected in exhaled breath; however, this study is the first to investigate cocaine in exhaled breath after controlled cocaine administration. We found few specimens (2.6%) positive for cocaine in breath following 25 mg IV cocaine, with even fewer (0.7%) containing BE and EME. Cocaine was typically observed in breath for up to 2 h, with some up to 9.5 h. BE and EME were observed 6.5 and 12.5 h post-dosing with concentrations ranging from 83.3-151 pg/filter and 50-69.1 pg/filter, respectively. This is the first report of EME in exhaled breath. Cocaine concentrations in our study ranged from 26.1-66 pg/filter, much lower than those previously reported. A probable explanation for this difference could be that prior studies

extracted analytes from the filter by directly adding methanol to the intact SensAbues breath collection device, collecting drug in OF on the device, rather than solely that on the filter [53,54]. Our study removed the filters from the devices prior to extraction, thereby, reducing the possibility of contamination from the device itself.

To further investigate this hypothesis, we performed methanolic extractions in the same manner as Beck *et al.* [53] on the breath collection devices, after the filters were removed, and identified the presence of cocaine and its metabolites in the devices themselves. Cocaine was detected in the devices for a longer duration (up to 22 h), more frequently (19.2%), at higher concentrations (25.2-363 pg/device), and with a median concentration (53.4 pg/device) that more closely resembled previous studies [53]. BE also was detected in 4.3% of devices with concentrations from 26.4-93.7 pg/device. The increased presence of cocaine and BE in devices as compared to filters suggests that the devices reflect OF contamination as well as lung microparticles, while the filters, which are designed to protect against OF contamination, reflect only drug-laden microparticles.

The median concentrations found in the devices after performing a methanolic extraction without the filter were comparable to what was observed in previous studies (cocaine median 60 pg/device; BE median 43 pg/device) [53]. The higher prevalence and concentrations observed in the devices as compared to the filters (Table 22) highlights the fact that the extraction procedure is critical in determining concentrations of cocaine and its metabolites in exhaled breath, and not contaminated OF.

Although the detection rates in the breath filters were low, this study quantified cocaine and its metabolites (BE and EME) in breath following controlled IV cocaine administration. The route of administration and low dose (25 mg) impacted the presented

results. SM or IN cocaine administration could yield higher results in exhaled breath than after IV cocaine administration, and methods of cocaine extraction also affect measureable cocaine markers. Additionally, a higher dosage could also increase the detection rates. Previous studies examining cocaine in exhaled breath were not controlled, thus, route of administration and dosage information were unknown [51,53,54], making direct comparisons to this current study difficult. Additional controlled administration studies examining different routes of administration and dosages utilizing the presented extraction technique are needed to determine if exhaled breath can be utilized as a reliable alternative sampling matrix for identifying recent cocaine intake.

Conclusion

We developed and validated a sensitive and specific method for cocaine, BE, EME, and norcocaine quantification in exhaled breath. Only 2.6% of filters were positive for cocaine from 1-9.5 h, BE and EME were detected in two specimens (0.72%) 6.5 and 12.5 h post-dosing, and no specimens were positive for norcocaine, following controlled administrations of 25 mg IV cocaine. This was the first report of EME detected in exhaled breath. When extracting the device itself, without the filters, 19.2% were positive for cocaine and 4.3% for BE up to 22 h after dosing, suggesting that the device reflects OF as well as lung microparticles, while the filter, based on the design of the device, reflects only drug-contaminated microparticles. Cocaine in breath after controlled IV administration identifies recent cocaine ingestion, but its absence does not preclude recent use.

Table 23. (Supplemental) Potential exogenous interferences analyzed at 500 µg/L in dried blood spots (DBS) by liquid chromatography-high resolution mass spectrometry and in whole blood by two dimensional-gas chromatography mass spectrometry.

	Interferences
Group 1	morphine, normorphine, morphine-3-glucuronide, morphine-6-glucuronide, codeine, norcodeine, 6-monoacetyl-morphine, 6-acetyl codeine, hydrocodone, hydromorphone, oxycodone, noroxycodone, oxymorphone, noroxymorphone
Group 2	diazepam, lorazepam, alprazolam, imipramine, clomipramine, fluoxetine, norfluoxetine
Group 3	Δ^9-tetrahydrocannibinol, 11-hydroxy-Δ^9-tetrahydrocannibinol, 11-nor-9-carboxy-Δ^9-tetrahydrocannibinol, cannabidiol, cannabinol
Group 4	methamphetamine, amphetamine, *p*-hydroxymethamphetamine, *p*–hydroxyamphetamine, hydroxymethoxymethamphetamine, hydroxymethoxyamphetamine, methylenedioxymethamphetamine, methylenedioxyamphetamine, methylenedioxyethylamphetamine, *p*-methoxyamphetamine, *p*-methoxymethamphetamine, ketamine, dextromethorphan, phentermine
Group 5	(±)-ephedrine, (±)-pseudoepehedrine, acetylsaliclic acid, ibuprofen, caffeine, acetaminophen
Group 6	cathinone, methcathinone, 4-fluoromethcathinone, methylone, ethylcathinone, α-pyrrolidinopentiophenone, buphedrone ephedrine, ethylone, 4-methoxymethcathinone, buphedrone, normephedrone, diethylcathinone, 3',4'-methylenedioxy-α-pyrrolidinopropiophenone, 4-methylephedrine, butylone, mephedrone, 4-methylethcathinone, 4-methyl-N-ethyl-norephedrine, 3',4'-methylenedioxy-α-pyrrolidinobutiophenone, Pentedrone, pentylone, 3',4'-dimethylmethcathinone, α-pyrrolidinopentiophenone, 4-methyl-α-pyrrolidinobutiophenone, 3',4'-methylenedioxypyrovalerone, pyrovalerone, benzedrone, naphyrone

Chapter 6 - Cocaine and metabolite concentrations in DBS and venous blood after controlled intravenous cocaine administration

(*As published in* Bioanalysis, *August 2015*)[6]

Abstract

Background: DBS are an increasingly common clinical matrix. *Methods & Results:* Sensitive and specific methods for DBS and venous blood cocaine and metabolite detection by LC-HRMS and 2D-GC-MS, respectively, were validated to examine correlation between concentrations following controlled intravenous cocaine administration. Linear ranges from 1-200 µg/L were achieved, with acceptable bias and imprecision. Authentic matched specimens' (392 DBS, 97 venous blood) cocaine and benzoylecgonine concentrations were qualitatively similar, but DBS had much greater variability (21.4-105.9% (%CV)) and were lower than in blood. *Conclusion:* DBS offer advantages for monitoring cocaine intake; however, differences between capillary and venous blood and DBS concentration variability must be addressed.

Introduction

Cocaine is a widely abused central nervous system stimulant worldwide [281,288,289], with approximately two million cocaine users in the US and Europe [260,281]. It is rapidly inactivated by plasma pseudocholinesterases and liver esterases *via* ester hydrolysis into EME and ecgonine (EG), and in the liver by carboxylesterases producing BE. Additionally, cocaine is metabolized by *N*-demethylation to norcocaine, an active although minor metabolite in humans [23]. Cocaethylene is another metabolite of cocaine that can be produced when used in combination with ethyl alcohol by the

[6] Ellefsen K.N. *et al.* Cocaine and metabolites concentrations in dried blood spots and venous blood after controlled intravenous administration. *Bioanalysis*. 2015; 7(16): 2041-56.

transesterfication of cocaine with ethyl alcohol *via* liver methylesterases. Cocaethylene also may be present in street and pharmaceutical cocaine [24]. Cocaine is unstable in biological matrices, hydrolyzing *in vitro* with conversion to BE, EME and EG spontaneously (without enzymes) at physiological temperature and pH [22].

DBS sampling refers to a microsampling technique of increasing interest in pharmaceutical, clinical and forensic settings [56]. DBS sampling is not a novel concept, as it was utilized over a century ago to monitor glucose concentrations in rabbits [56] and also proved useful in newborn infant screening programs [57,58]. Recently, the use of alternative matrices like DBS for detecting recent drug consumption is increasing due to easy, less invasive collection (via finger puncture) and increased stability compared to blood [59,60]. The increased analyte stability is especially important when rapid degradation of compounds in blood was documented [61,62]. Several studies demonstrated minimal analyte loss in DBS as compared to other biological matrices, like whole blood, for unstable compounds including benzodiazepines, zopiclone, 6-acetylmorphine (6-AM) and cocaine [60,64,290]. Additionally, DBS concentrations, unlike urine or hair, should reflect current concentrations in blood [65].

There are several published DBS applications for TDM [66-70]. DBS also was successfully applied to doping control [71] and drugs of abuse analysis including cannabinoids [72], opiates [62,73], MDMA [74], GHB [75], and cocaine [58-60,63,65,71,73,76-78].

Cocaine pharmacokinetics are well studied in blood, plasma and urine after various routes of administration, but less is known about cocaine disposition in DBS following controlled drug administration. Henderson *et al.* described the first qualitative

report of drugs of abuse in DBS to determine prenatal cocaine use in mothers utilizing a modified BE urine radioimmunoassay and found results correlated well with GC-MS determinations in venous blood [63]. Since then, multiple LC-based methods were published for the detection of cocaine and/or cocaine metabolites (including BE, EME, or cocaethylene) in DBS [56]; however, limited studies examined DBS results with corresponding venous blood concentrations from authentic specimens [58,59,65,73,77,78].

Mercolini *et al.* compared DBS and plasma cocaine, BE, and cocaethylene concentrations from one cocaine user utilizing LC coupled to spectrofluorimetric detection with a 20 µg/L LOQ [59]. DBS were collected via finger puncture; 10 µL blood was collected with a micropipette and dispersed onto the DBS card. The authors found that capillary DBS correlated well with plasma concentrations after an arbitrary hematocrit factor (1.62 for women and 1.92 for men) was applied. Others also reported good correlations between DBS and venous blood cocaine and metabolite concentrations from limited specimens obtained from polydrug users [78], DUID cases [73], and PM specimens [65]. A limitation of these studies is that the authors did not investigate capillary DBS, as Mercolini *et al.* performed [59]; rather, they dispersed whole blood onto DBS cards utilizing authentic venous blood. More studies are needed to demonstrate the equivalence between capillary DBS and venous blood before this alternative matrix can be utilized to determine recent drug consumption.

The present study compared cocaine and BE concentrations in DBS and venous blood after controlled IV cocaine administration. We developed and validated separate methods for quantifying cocaine and metabolites in DBS and in venous blood by LC-

HRMS) and 2D-GC-MS to determine if capillary DBS concentrations correlated well with blood concentrations.

Materials and Methods

Chemicals and Materials

Cocaine, norcocaine (1 g/L) and IS d_3-cocaine and d_3-norcocaine (100 mg/L) in acetonitrile, and BE (1 g/L) and d_8-BE (100 mg/L) in methanol were obtained from Cerilliant (Round Rock, TX, USA). SPE was performed with SOLA CX 10 mg 1 mL cartridges (Thermo Scientific, Fremont, CA) for DBS analyses and UCT Clean Screen DAU 200 mg 10 mL cartridges (United Chemical Technologies, Inc., PA) for blood analyses. *N*-methyl-*N*-(*t*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) + 1% *t*-butyl-dimethylchlorosilane (t-BDMCS) utilized for derivatization was from Regis® (Morton Grove, IL). Formic acid, methanol, acetonitrile, dichloromethane, isopropanol, ethyl acetate and water were acquired from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium hydroxide 28–30%, hydrochloric acid 37%, ammonium formate, potassium phosphate and sodium phosphate were from JT Baker (Phillipsburg, NJ, USA). All solvents employed in the extraction were HPLC grade, and LC-MS grade for the chromatographic system. Water for buffer preparation was purified-in-house by an ELGA Purelab Ultra Analytic purifier (Siemens Water Technologies, Lowell, MA, USA). Whatman 903 Protein Saver Cards™ and desiccant were obtained from Whatman GE Healthcare Bio-Sciences Corporation (Florham Park, NJ) and Tenderlett® lancets (incision depth 1.75 mm, length 0.94 mm) were acquired from ICT (Edison, NJ).

Preparation of Standard Solutions

DBS and blood calibrator working solutions at 10, 50, 100, 500, 1000 and 2000 µg/L were prepared by appropriate dilution in acetonitrile. QC working solutions were prepared in acetonitrile at low (30 µg/L), medium (750 µg/L) and high (1500 µg/L) concentrations from different lots than those employed for calibrators. IS containing d_3-cocaine, d_8-BE and d_3-norcocaine at 250 ng/L was prepared in acetonitrile for DBS. For blood analyses, an IS solution of 100 µg/L d_3-cocaine and 250 µg/L d_8-BE was prepared. A lower d_3-cocaine concentration was necessary due to presence of d_0-cocaine ions at higher concentrations (250 µg/L) interfering with linearity and potential increasing of LOQ.

Preparation of DBS Cards

Thirty µL calibrators or QC working solutions were added to 0.3 mL human blood aliquots to yield final concentration range (1–200 µg/L). Fifty microliters blank and prepared fortified blood were spotted onto 903 Whatman DBS paper cards (GE Healthcare Bio-Sciences, Pittsburgh, PA), and dried at room temperature a minimum of 3 h before extraction.

DBS Analysis

For sample extraction, a 3 mm diameter disc was punched manually and placed in a 10 mL polypropylene Sarstedt tube. A 1 mL aliquot 1% formic acid in water and 30 µL IS (250 ng/L d_3-cocaine, d_8-BE and d_3-norcocaine) was added. Tubes were capped, gently vortexed for 30s, sonicated for 15 min and centrifuged 4,000×g at 4°C for 5 min. SPE was performed onto SOLA CX cartridges preconditioned with methanol (0.5 mL) and ultrapure water (1 mL). Columns were washed with 0.5 mL 2% formic acid in

ultrapure water and 0.5 mL 2% formic acid in methanol. Cartridges were dried at 10 psi for 5 min and elution performed with 0.5 mL 5% ammonium hydroxide in methanol. After drying under compressed air at 40°C, samples were reconstituted in 150 µL mobile phase A (1 mmol/L ammonium formate+0.01% formic acid), vortexed briefly, and centrifuged at 4,000×g at 4 °C for 5 min. Twenty µL were injected onto the LC-HRMS system.

LC-HRMS was performed on a Thermo Scientific NCS-3500RS Ultimate 3000 Binary Rapid system coupled to a QExactive mass spectrometer (Thermo Scientific, Fremont, CA). Chromatographic separation was achieved with a Phenomenex Synergi Polar-RP 100A 100 x 2.0 mm 2.5 µm column and identically packed guard cartridges (10×2.1 mm 2.5 µm). Gradient elution was performed with mobile phase A and B (methanol) at 0.3 mL/min flow rate and 30°C. The initial composition (5% B) was maintained for 1.5 min, increased from 5 to 95% B over 5.5 min, held at 95% for 1.5 min, and returned to initial conditions over 0.5 min. A 1.5 min equilibration followed, yielding a total run time of 10.5 min.

The QExactive mass spectrometer was equipped with heated electrospray ionization source (HESI-II) and operated in positive ionization mode. The spray voltage was 3 kV, capillary temperature 350°C, heater temperature 425°C, S-lens RF level 50, sheath gas flow rate 50, auxiliary gas flow rate 13 and sweep gas 3 (manufacturer's units). The instrument was calibrated in the positive and negative mode every 25 h. The mass spectrometer acquired a targeted-MSMS scan at a resolution of 35,000 (full width at half maximum at *m/z* 200), AGC target 5 x 10^5 and maximum injection time 100 ms. Precursor ions are selected in the quadrupole with a 3 *m/z* window and subsequently

fragmented in the HCD cell. A full scan of all fragmented ions originating from the precursor ion was performed, and two specific product ions utilized for data analysis with a mass tolerance of 5 ppm (Table 24). Thermo TraceFinder Clinical Research 3.1 software was used for data collection and processing.

Table 24. Liquid chromatography-high-resolution mass spectrometry (LC-HRMS) parameters and retention times (RT) for cocaine, metabolites and internal standards in dried blood spots (DBS).

Analyte	Precursor ion (*m/z*)	Quantitative product ion (*m/z*)	Qualitative product ion (*m/z*)	NCE (%)	RT (min)
BE	290.1	168.1017	105.0338	50	6.42
d_8-BE	298.2	171.1205	110.0651	50	6.39
Cocaine	304.1	182.1174	82.0656	50	7.18
d_3-Cocaine	307.2	185.1363	85.0844	50	7.17
Norcocaine	290.1	136.0756	168.1017	45	7.33
d_3-Norcocaine	293.1	171.1205	136.0756	20	7.30

NCE: normalized collision energy, *RT:* retention time

Blood Analysis

Blank blood (0.25 mL) was fortified with 25 µL IS (100 µg/L d_3-cocaine, 250 µg/L d_8-BE) and their respective calibrator and QC concentrations yielding a final concentration range between 1-200 µg/L. Four mL phosphate buffer pH 6 (prepared with KH_2PO_4 and Na_2HPO_4) was added and Sarstedt polypropylene tubes were vortexed and centrifuged at 4,000 xg, 4ºC for 10 min. Supernatants were loaded onto UCT Clean Screen cartridges preconditioned with methanol (3 mL) and phosphate buffer pH 6 (3 mL). Columns were washed with water (6 mL), 0.1 mol/L HCl (3 mL) and methanol (3 mL) before drying under vacuum at 10 psi for 20 min. Analytes were eluted with 3 mL dichloromethane:isopropanol (80:20 v/v) with 2% ammonium hydroxide into conical glass centrifuge tubes. Eluates were evaporated to dryness under nitrogen at 40ºC and reconstituted with 20 µL ethyl acetate:MTBSTFA + 1% t-BDMS (50:50 v/v). Samples

were incubated for 40 min at 70ºC and centrifuged at 1,800 x g, 20ºC for 3 min before transferring to autosampler vials. The derivatized extracts (2 µL) were analyzed by an electron ionization 2D GC-MS method with modifications to the front inlet, back inlet and oven temperature programs [291].

Splitless injections (2 µL) were made onto a GC-MS system configured with a Deans switch, flame ionization detector (FID), 7683 autosampler, and 6890N GC interfaced to a 5973 mass selective detector (MSD) (Agilent Technologies, Wilmington, DE). The system also was equipped with a cryogenic focusing trap, mounted inside the GC oven (Joint Analytical Systems, Marlton, NJ) at the head of the second GC column and cooled with compressed air. The Deans switch connected two capillary chromatographic columns with a pneumatic valve directing output of the primary column (DBS-1MS, 15m x 0.25 mm, 0.25 µm; Agilent Technologies, Wilmington, DE) to either the FID or the inlet of the secondary column (ZB-50, 30m x 0.32mm, 0.25µm; Phenomenex, Torrance, CA). The inlet end of the secondary column was inserted through the cryogenic trap and the outlet directed to the MSD. Operating parameters are outlined in Table 29 (Supplemental).

Cocaine and BE were initially separated on the primary column, with analyte elution times on the primary column determined by injection of high concentration standards with the Deans switch regulator directing effluent to the FID at the beginning of the analytical sequence. For authentic specimens, the cryogenic trap was maintained at 100ºC to contain cocaine and BE based on elution times from the primary column. Immediately after the last analyte was cold-trapped, the oven temperature was lowered to 180ºC, the cryogenic trap ramped at 800ºC/min and analytes re-vaporized for separation

on the secondary column. The total run time was 18.63 min. The MSD was operated in EI selected ion monitoring (SIM) mode for cocaine and BE. A minimum of three ions for each analyte and IS were acquired. Target and qualifier ions are presented in Table 25.

Table 25. Gas chromatography mass selective detector parameters for cocaine, benzoylecgonine (BE) and their respective deuterated analogs in human venous blood.

Analyte	Target ion	Qualifier ions	Dwell time (msec)	Deans switch cuts (min)
Cocaine	82	94, 182, 303	30	3.70-4.10
d_3-Cocaine	85	185, 306	10	3.70-4.10
BE	282	346, 403	30	4.64-5.04
d_8-BE	285	354, 411	15	4.64-5.04

Participants

Eligibility criteria included healthy adults ages 18-50 years who smoked or used IV cocaine for at least six months and at least three times per month during the three months prior to screening and who were currently healthy, based on a comprehensive medical and psychological evaluation. Exclusion criteria included pregnant or nursing women; current physical dependence on any drug other than cocaine, caffeine, or nicotine; current clinically significant medical or psychiatric disorder; hemoglobin less than 12.5 g/dL and blood donation within eight weeks; current hypertension or blood pressure readings consistently above 140 mm Hg systolic or 90 mm Hg diastolic while at rest; heart rate consistently above 90 or below 50 bpm while at rest; abnormal 12-lead ECG; history of clinically significant adverse reaction to cocaine, acetazolamide, or quinine; or interest in drug abuse treatment within three months of study screening. Participants provided written informed consent to participate in this NIDA Institutional Review Board and Food and Drug Administration-approved study. Participants resided on a secure research unit for 13 days and 12 nights. The study's primary aims were to

evaluate potential pharmacodynamic and pharmacokinetic interactions between cocaine and acetazolamide and quinine. Acetazolamide and quinine are being considered as compliance markers for cocaine dependence treatment pharmacotherapies.

Authentic Specimens

Participants were administered a single 25 mg IV cocaine dose through a peripheral venous catheter on three separate days during the study (Days 1, 5, and 10). Cocaine was administered alone on Day 1, with oral acetazolamide on Day 5 and with oral quinine on Day 10. Venous blood and DBS were collected before and 30, 60 and 90 min after IV cocaine administration. Venous blood was collected in gray-top tubes. For DBS, capillary blood was collected by finger puncture onto a Whatman Protein Saver Card 903, with five spots per card. Collection cards were dried for a minimum of 3 h at room temperature and stored in a dry plastic bag with desiccants at -20ºC until analysis. Blood specimens also were stored at -20ºC until analysis. Only central punches from the DBS and specimens greater than 3mm (punch diameter disc) were included in the comparison between capillary DBS and venous blood.

Statistical Analyses

Visual inspection of data and evaluation by D'Agostino-Pearson normality test (omnibus K2) indicated non-normal data distribution. Therefore, statistical comparisons between blood and DBS concentrations were conducted with non-parametric tests. Correlations of cocaine and BE DBS to blood concentrations were performed with Spearman's rho correlation (r_s) and least-squares regression analysis in Prism Version 5.02 (GraphPad Software Inc, La Jolla, CA). In addition, Bland-Altman analysis plots were conducted to further analyze these data to examine difference between two

measurements as a function of the mean of the two measurements for each individual sample, resulting in a mean difference and standard deviation that is utilized to calculate 95% limits of agreement [292], which can be interpreted for clinical significance.

Results

Participants

Thirteen subjects (12 males, 8 black, 4 white, 1>one race) aged 35-50 (Table 30 (Supplemental)) participated in the study, with nine completing all dosing sessions. Two participants were medically discharged prior to the second cocaine dose on Day 5, one after the second cocaine dose on Day 5 and one prior to the third cocaine dose (Day 10). These participants were medically discharged for change in post-dosing ECG compared to pre-dosing ECG, abnormal pre-dosing ECG, or elevated heart rate and/or blood pressure prior to cocaine administration. Frequency of use among participants (smoked or intravenous) ranged from daily to once per week, with a median length of cocaine use of 16.6 years (range: 6-32 years) (Table 30 (Supplemental)).

DBS Method Validation Results

DBS method was validated following SWGTOX recommendations [264]. Linearity of peak area ratios versus theoretical concentrations was verified from 1 to 200 µg/L with $1/x^2$ weighted linear regression. Five calibration curves yielded determination coefficients (R^2) above 0.9930 ± 0.0027, with residuals within ± 15%. LODs were 0.5, 1 and 1 µg/L for benzoylecgonine, cocaine and norcocaine, respectively, with a 1 µg/L LOQ for all analytes. Figure 18a (Supplemental) illustrates a chromatogram of a DBS sample at LOQ. Bias and imprecision were evaluated in triplicate over five days (n = 15) at each QC concentration with a One-way ANOVA approach to calculate combined

within- and between-run imprecision. Within-run imprecision (<7.1%), between-run imprecision (<10.47%) and %bias (-4.6 – 7.4%) were satisfactory at all three QC concentrations (Table 26). Extraction efficiency for each analyte was measured at each QC concentration. Blank venous blood samples (*n*=5) were fortified with QC solution before spotting on DBS cards and extracted. These were compared to blank venous blood samples (*n*=5) spotted on DBS cards that were extracted and fortified with corresponding QC solution after sample extraction. Extraction efficiencies were 43.3-52.1% for cocaine, 48.6-64.5% for BE and 35.2-48.6% for norcocaine, with process efficiencies of 47.4-49.1%, 29.3-33.0%, and 28.0-29.4%, respectively. Ion suppression (*n*=10) was <43.9, 39.5 and 23.9% for cocaine, norcocaine and benzoylecgonine, respectively (%CV <24.2%). When corrected for IS, observed matrix effects were -13.8 to 16.9%, -15.1 to 10.0%, and -11.9 to 14.3% for cocaine, BE and norcocaine, respectively (%CV < 20.5%). Interferences from endogenous DBS compounds were not observed (*n*=10). Method selectivity was demonstrated by adding high concentrations (500 µg/L) of potentially interfering drugs and metabolites (Table 31 (Supplemental)) to negative samples. No analyte was detected >LOD, indicating no interferences with analytes of interest. No carryover was detected after the injection of a sample at 1,000 µg/L (5 x ULOQ). Analytes were stable in the autosampler for 48 h at low and high QC concentrations with percent differences between −7.4% and −0.1% (*n*=3). Short-term stability was evaluated at room temperature for 24 h, at 4°C and -20°C for 72 h and after three freeze-thaw cycles. Analytes were considered stable under evaluated conditions (% difference < 12.4%). Stability data are shown in Table 27.

Table 26. Within-run imprecision, between-run imprecision and %bias for cocaine, benzoylecgonine (BE) and norcocaine in dried blood spots (DBS) and cocaine and BE in blood at low (3 µg/L), medium (75 µg/L) and high (150 µg/L) concentrations. Extraction efficiency, process efficiency, and matrix effects for analytes at low (3 µg/L) and high (150 µg/L) concentrations.

Matrix	Analyte	Within-run imprecision (*n*= 15, %CV)			Between-run imprecision (*n*= 15, %CV)			% Bias (*n*=15)			Extraction efficiency (%CV) (*n*=5, %)		Process efficiency (*n*=5, %)		Matrix effect (%CV) (*n*=10, %)	
		Low	Med	High	Low	Med	High	Low	Med	High	Low	High	Low	High	Low	High
DBS	Cocaine	7.0	5.2	4.1	8.9	6.5	5.7	-2.5	7.4	4.3	43.3 (9.0)	52.1 (18.8)	47.4	49.1	-23.7 (10.0)	-43.9 (24.2)
	BE	7.1	4.9	3.1	7.5	5.5	5.7	-4.6	-1.6	-2.9	48.6 (1.7)	64.5 (10.0)	33.0	29.3	-2.4 (7.0)	-23.9 (20.9)
	Norcocaine	5.3	5.2	4.3	10.7	8.0	7.2	-2.6	4.4	2.3	35.2 (5.1)	48.6 (14.1)	28.0	29.4	-20.4 (9.6)	-39.5 (22.8)
Blood	Cocaine	3.3	4.6	1.6	3.4	4.2	2.5	6.7	-7.8	2.6	105.8 (12.6)	98.6 (6.8)	-	-	-	-
	BE	1.9	3.9	1.8	2.8	3.9	2.0	2.3	-3.2	9.1	87.6 (10.0)	87.3 (6.9)	-	-	-	-

Table 27.Short term stability data (%difference) of cocaine, benzoylecgonine (BE) and norcocaine (NC) in dried blood spots (DBS) and cocaine and BE in blood at low (L, 3 µg/L) and high (H, 150 µg/L) concentrations after storage at 4°C for 48 h in autosampler (DBS), room temperature for 72 h in autosampler (blood), at room temperature for 24 h, at 4°C and -20°C or 72h, and after three freeze-thaw cycles (over 72 h).

Matrix	Analyte	48 h 4°C / 72 h room temp autosampler (*n*=3)		Room temp 24h (*n*=3)		4°C 72 h (*n*=3)		-20°C 72 h (*n*=3)		3 freeze-thaw cycles (*n*=3)	
		L	H	L	H	L	H	L	H	L	H
DBS	Cocaine	-3.4	-4.3	-5.4	-9.3	-7.6	-8.9	-1.5	-6.7	-5.9	-6.0
	BE	-0.1	-7.1	-0.9	-6.5	-5.3	-8.0	-5.3	-4.0	-1.1	-3.2
	NC	-2.8	-0.2	-5.3	-12.4	-7.6	-6.7	-5.0	-6.2	-6.2	-4.5
Blood	Cocaine	-1.1	-8.4	-2.0	-3.8	-25.0	-23.4	-10.9	-13.2	-8.9	-13.2
	BE	-3.3	-11.0	1.3	-1.3	-6.4	-2.9	1.8	-1.4	3.2	-1.6

Blood Method Validation Results

Linearity from 1-200 µg/L with $1/x^2$ weighting was achieved for cocaine and BE in whole blood. Calibration curves (*n*=5) yielded R^2 above 0.9937 ±0.0029 with residuals <15%. LODs were 1 and 0.5 µg/L for cocaine and BE, respectively, with 1 µg/L LOQs <15%. LODs were 1 and 0.5 µg/L for cocaine and BE, respectively, with 1 µg/L LOQs (Figure 18b (Supplemental)). Within-run imprecision (<4.6%), between-run imprecision (<4.2%) and %bias (-7.8-9.1%) were acceptable for both analytes, at all three QC concentrations. Extraction efficiencies for cocaine were 98.6-106% and BE 87.3-87.6%. Table 26 highlights these data. Dilution integrity was acceptable (± 20%) at 1:2, 1:5 and 1:10 dilutions. Carryover was not detected for either analyte above their respective LODs after injection of blank specimens fortified 5x the ULOQ (1,000 µg/L). No exogenous interferences were observed from over 75 different compounds (500 µg/L) with the exception of norcocaine (Table 31 (Supplemental)); however, norcocaine concentrations are generally < 30 µg/L in clinical samples [39,293,294]. At the upper limit of linearity (200 µg/L) norcocaine did not produce an increase in cocaine concentrations at the low

QC concentration (3 µg/L). Compounds were stable (within ±25% of freshly prepared QCs) on the autosampler (room temperature) for 72 h, for 24 h at room temperature, 72 h at -20ºC and after three freeze-thaw cycles (Table 27).

Authentic Specimens

In total, 392 DBS (up to 5 spots per collection) and 97 venous blood specimens were analyzed after 25 mg/kg controlled IV cocaine administration on Days 1, 5 and 10. DBS and venous blood were collected 30, 60 and 90 min post cocaine dosing. Twenty-seven cocaine DBS and 19 BE DBS concentrations quantified above the ULOQ, and 1 cocaine DBS concentration was below the LOQ. All blood concentrations were within the method's limits of linearity. Norcocaine was not detected in any DBS specimens greater than the LOQ. Figure 15 illustrates chromatograms of cocaine and BE concentrations in DBS and matched blood samples from one participant 60 min after cocaine administration.

Table 28 summarizes cocaine and BE concentrations in DBS and venous blood on Days 1, 5 and 10 at 30, 60 and 90 min post-dosing. Cocaine DBS and venous blood concentrations ranged from 2.9-232.6 µg/L and 18.3-137.6 µg/L, respectively, with BE concentrations of 2.9-240.4 µg/L for DBS and 57.2-193.4 µg/L for blood. In general, median cocaine DBS concentrations were lower than their respective blood concentrations (Table 28); although, DBS concentration ranges were wider. The same trends also were observed for BE (Table 28). Figure 19 (Supplemental) illustrates the above-mentioned results graphically as box and whisker plots. In addition, large inter-subject-variability was observed with DBS collections at each time point (up to 5 spots per collection) as compared to blood specimens; %CV for cocaine ranged from 50.7-

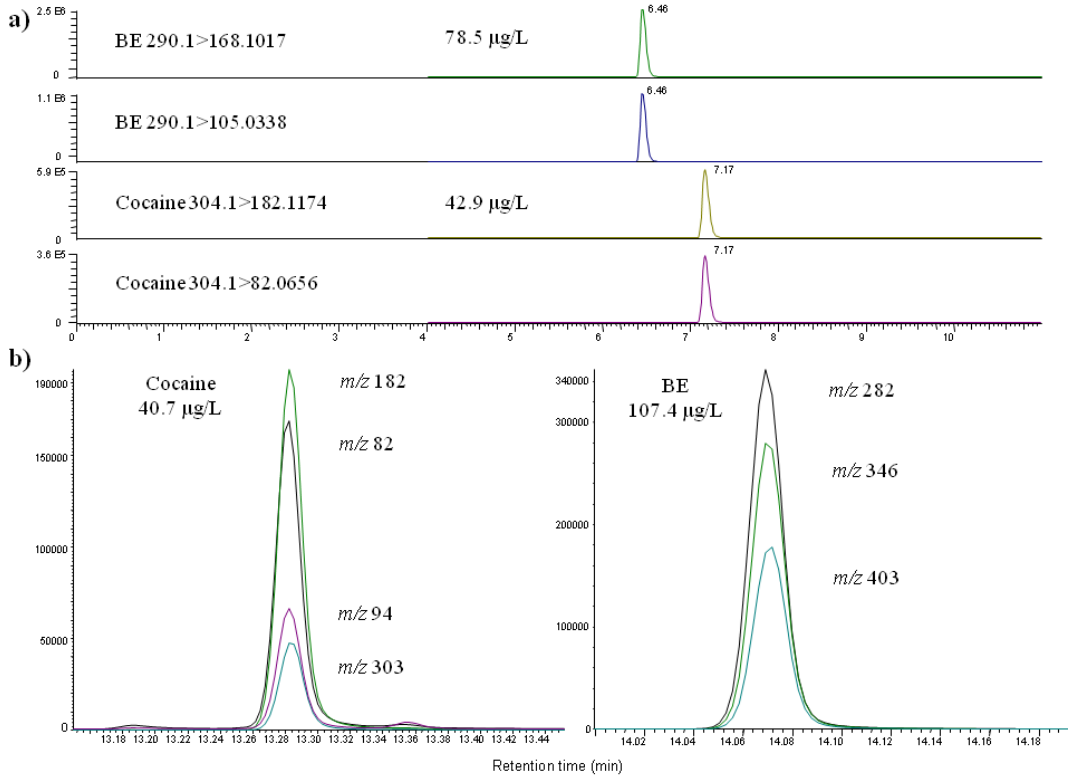


Figure 15. a) Liquid chromatography-tandem mass spectrometry chromatogram of a dried blood spot (DBS) specimen (ppt L) with 42.9 µg/L cocaine and 78.5 µg/L benzoylecgonine (BE). Norcocaine was not detected. b) Corresponding venous blood two dimensional (2D) gas chromatography-mass spectrometry chromatogram with 40.7 µg/L cocaine and 107.4 µg/L BE, 60 min after 25 mg IV cocaine.

Table 28. Cocaine and benzoylecgonine (BE) dried blood spot (DBS) and venous blood concentrations and standard deviations (SD) in simultaneously collected specimens (n=392 DBS, n=97 blood) from 13 participants, 30, 60 and 90 min following 25 mg/kg intravenous (IV) cocaine administration on three separate days (Days 1, 5 and 10). Table does not include 27 cocaine and 19 BE DBS that quantified above the upper limit of quantification (ULOQ) (>200µg/L) and 1 cocaine DBS detected below the LOQ.

	Day	Time (min)	Mean (µg/L)	Median (µg/L)	SD (µg/L)	Inter-Subject %CV	Median Intra-DBS %CV (range)	Minimum (µg/L)	Maximum (µg/L)	# of Samples
Cocaine DBS	1	30	81.4	62.3	50.0	61.5	20.0 (5.3-40.3)	19.0	232.6	47
		60	59.7	48.5	38.5	64.5	20.7 (7.5-80.4)	17.4	191.6	39
		90	72.1	47.9	53.8	74.6	29.7 (6.2-102)	17.6	219.2	51
	5	30	77.3	65.7	46.1	59.6	16.0 (4.9-54.3)	37.5	231.0	42
		60	50.1	46.4	25.4	50.7	24.0 (9.2-65.6)	19.5	139.4	43
		90	40.2	32.2	25.8	64.2	23.4 (7.2-122)	4.4	113.2	42
	10	30	68.2	59.4	52.7	77.2	15.4 (6.1-120)	2.9	232.4	34
		60	49.0	36.6	32.1	35.7	18.1 (7.3-62.3)	15.1	145.3	35
		90	56.7	31.7	60.1	105.9	25.2 (12.1-46.2)	3.8	215.7	31
Cocaine Blood	1	30	90.3	92.6	23.6	26.1	-	56.0	130.0	13
		60	53.9	53.0	23.1	43.0	-	30.2	115.3	13
		90	34.2	34.4	9.6	28.0	-	18.6	49.6	12
	5	30	91.6	93.2	23.6	25.8	-	57.4	121.9	10
		60	48.2	43.3	14.5	30.1	-	25.3	72.8	11
		90	32.2	30.8	7.2	22.5	-	18.3	42.0	11
	10	30	87.6	79.8	30.7	35.1	-	52.0	137.6	9
		60	50.8	46.0	18.6	36.6	-	30.0	84.9	9
		90	34.5	32.8	10.8	31.3	-	22.5	48.1	9
BE DBS	1	30	83.1	81.7	27.1	32.6	7.7 (2.8-26.8)	40.7	159.4	48
		60	89.4	83.4	33.0	36.9	10.0 (4.0-39.0)	50.1	240.4	42
		90	102.5	101.5	23.8	23.3	7.2 (1.6-20.5)	60.7	166.4	51
	5	30	101.7	96.1	35.3	34.7	13.6 (0.1-36.5)	51.2	199.7	45
		60	93.8	97.5	20.1	21.4	13.6 (5.3-32.9)	52.1	133.2	43
		90	98.7	104.5	36.3	36.8	10.8 (2.9-143)	1.1	197.6	44
	10	30	88.9	92.6	45.1	50.8	7.6 (4.7-119)	2.9	205.5	34
		60	103.1	101.2	28.6	27.7	13.1 (4.7-35.3)	51.1	175.5	35
		90	114.2	111.7	48.3	42.3	11.1 (7.1-27.9)	17.6	224.0	31

	Day	Time (min)	Mean (µg/L)	Median (µg/L)	SD (µg/L)	Inter-Subject %CV	Median Intra-DBS %CV (range)	Minimum (µg/L)	Maximum (µg/L)	# of Samples
BE Blood	1	30	86.6	82.3	22.6	26.1	-	57.2	132.6	13
		60	121.7	113.4	33.2	27.3	-	70.1	186.1	13
		90	133.5	127.5	30.1	22.6	-	77.9	180.5	12
	5	30	96.2	100.2	16.1	16.7	-	58.7	115.6	10
		60	132.9	139.7	29.7	22.4	-	76.4	178.2	11
		90	136.3	141.2	32.6	23.9	-	85.0	189.3	11
	10	30	94.4	94.0	23.1	24.5	-	60.3	126.9	9
		60	132.8	129.9	19.8	14.9	-	92.3	166.0	9
		90	141.2	142.0	24.0	17.0	-	111.9	193.4	9

105.9% (DBS) and 22.5-43.0% (blood), with BE %CV from 21.4-50.8% (DBS) and 14.9-27.3% (blood). Similarly, DBS %CVs indicate high intra-DBS-variability among the five spots per card, with medians (range) of 22.3% (4.9-122%) for cocaine and 9.8% (0.1-143%) for BE. Figure 16 illustrates median cocaine and BE concentrations in authentic DBS and venous blood samples following controlled IV cocaine administrations on Days 1, 5 and 10, 30, 60 and 90 min post-dosing, although capillary kinetics may be different from venous blood kinetics.

Individual cocaine and BE venous blood concentrations were compared to median DBS concentrations, as up to five DBS were collected at each sampling time, versus a single blood sample. There were 87 and 90 whole blood-DBS pairs for cocaine and BE, respectively, to investigate agreement between the two matrices. Due to the high intra-DBS-variability, DBS median concentrations (for up to 5 spots) were employed for comparison of DBS and venous blood. Cocaine and BE blood and DBS concentrations significantly moderately correlated (Spearman's r_s 0.43 and 0.40, respectively; P <0.0001). Least-squares regression analyses also demonstrated a significant correlation between blood and DBS concentrations (P <0.05) with r^2 = 0.066 and 0.15 for cocaine and BE, respectively (Figure 17a); equations of the fitted lines were: y = 0.3882x + 40.04 for cocaine and y = 0.4314x + 47.24. Bland-Altman analysis plots yielded mean differences between blood and DBS cocaine concentrations of -5.10 ± 46.7 µg/L and 20.2 ± 39.4 µg/L for BE, with 95% limits of agreement from -96.6-86.4 µg/L and -56.9-97.3 µg/L, respectively (Figure 17b).

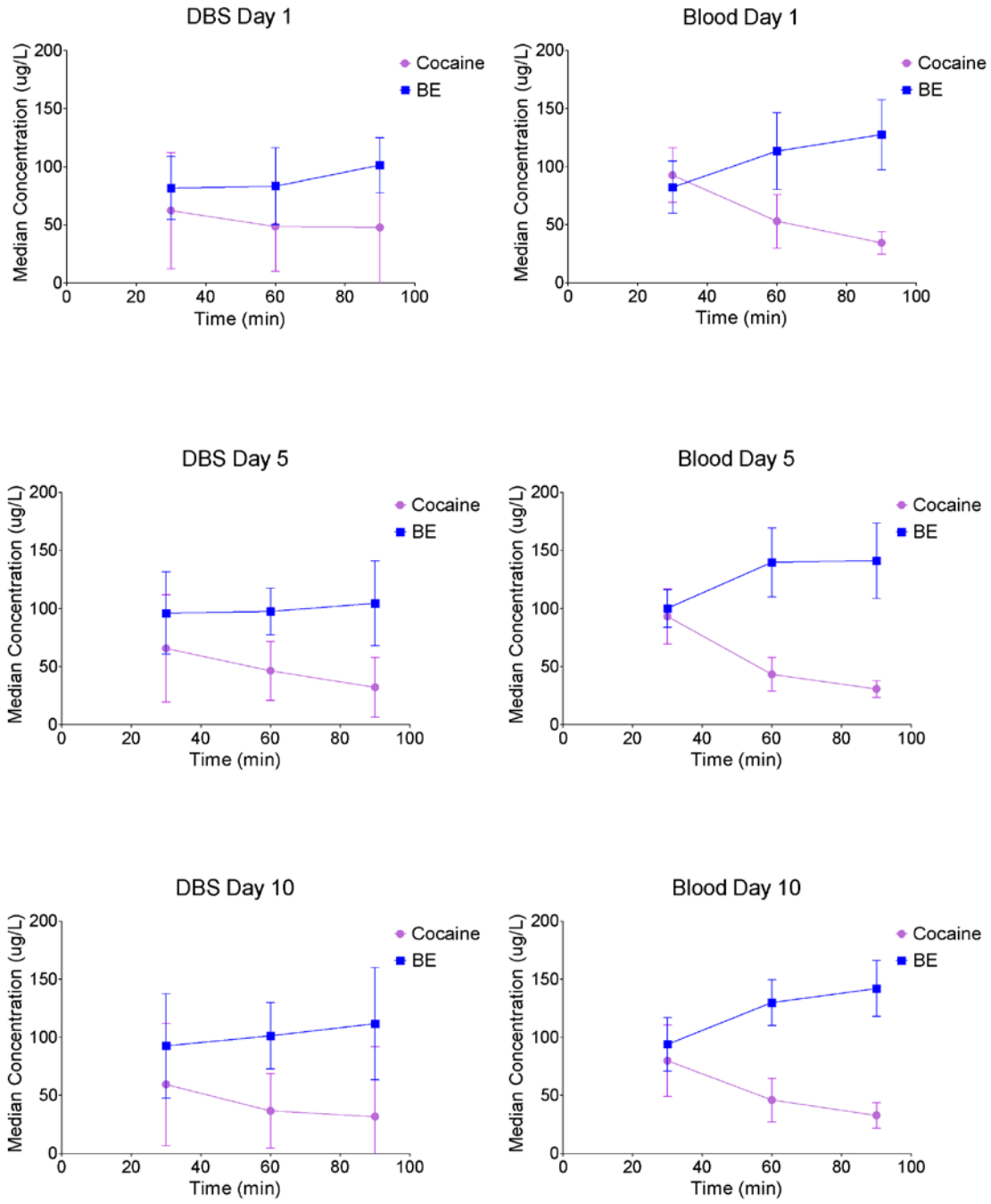


Figure 16. Median cocaine and benzoylecgonine (BE) concentrations in dried blood spot (DBS) and venous blood from 13 participants 30, 60 and 90 min following 25 mg/kg intravenous (IV) cocaine administrations on three separate days (Days 1, 5 and 10). Error bars represent one standard deviation constrained to 0.

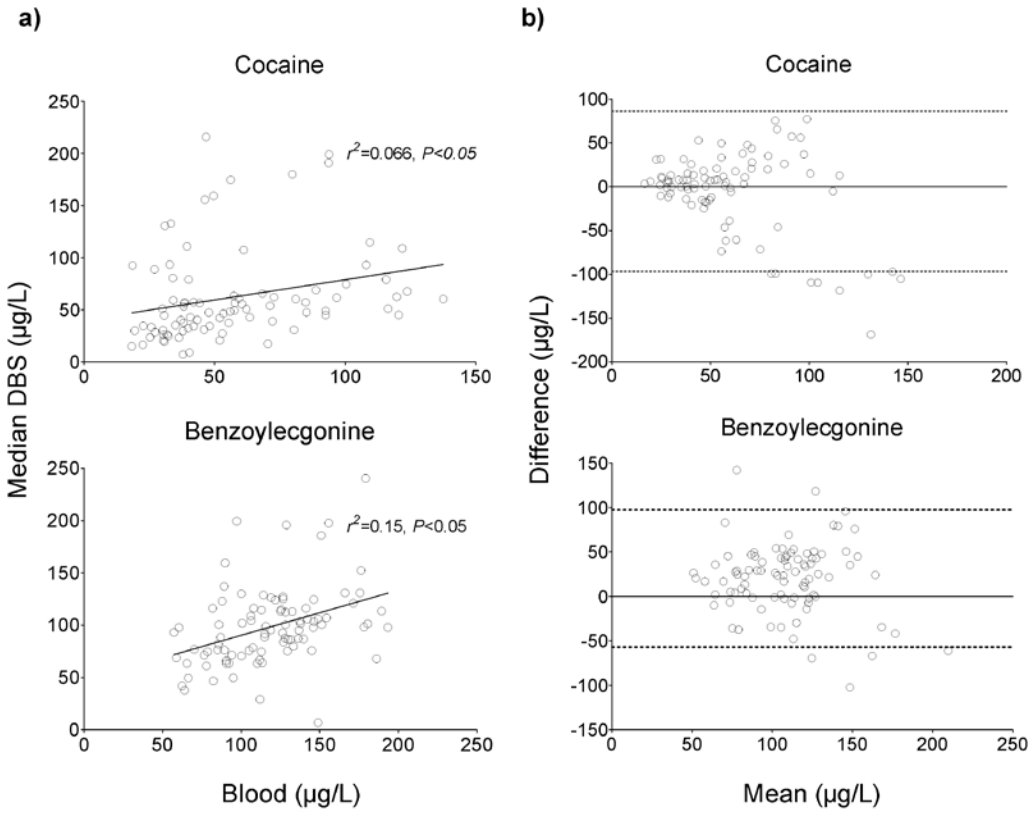


Figure 17. Comparison between venous blood and dried blood spot (DBS) cocaine and benzoylecgonine (BE) concentrations in simultaneously collected specimens from 13 participants 30, 60 and 90 min following 25 mg/kg intravenous (IV) cocaine administration on three separate days (Days 1, 5 and 10). Only paired venous blood-DBS specimens within the linear range were included in correlations and Bland-Altman analysis plots: cocaine (*n*=87) and BE (*n*=90). a) Correlation of venous blood with median DBS cocaine and BE concentrations. Regression was calculated by least-squares regression analysis. b) Bland-Altman analysis plots comparing blood and DBS collection methods for cocaine and BE detection. Dotted lines represent the 95% limits of agreement (mean difference ± 2SD).

significantly moderately correlated (Spearman's r_s 0.43 and 0.40, respectively; P <0.0001). Least-squares regression analyses also demonstrated a significant correlation between blood and DBS concentrations (P <0.05) with r^2 = 0.066 and 0.15 for cocaine and BE, respectively (Figure 17a); equations of the fitted lines were: y = 0.3882x + 40.04 for cocaine and y = 0.4314x + 47.24. Bland-Altman analysis plots yielded mean differences between blood and DBS cocaine concentrations of -5.10 ± 46.7 µg/L and 20.2 ± 39.4 µg/L for BE, with 95% limits of agreement from -96.6-86.4 µg/L and -56.9-97.3 µg/L, respectively (Figure 17b).

Discussion

The use of DBS as an alternative matrix in clinical and forensic toxicology is increasing as specimens can be collected easily in a less invasive manner (via finger puncture), with little potential for adulteration, increased stability over other matrices including blood, and have the potential to identify recent drug consumption. Here we describe cocaine and BE concentrations in DBS and simultaneously collected venous blood specimens following controlled IV cocaine administration; allowing evaluation of agreement between capillary DBS and blood concentrations. Previous studies did not investigate capillary DBS (obtained via finger puncture) compared to blood specimens collected simultaneously under controlled cocaine administration.

Fully validated analytical methods for the detection of cocaine, BE and norcocaine in DBS (LC-HRMS) and cocaine and BE in whole blood (2D-GC-MS) were developed to determine if capillary DBS correlate well with simultaneously collected blood specimens. A different analytical approach was utilized for the detection of DBS cocaine and metabolites than the technique implemented for venous blood to achieve the required low limits of quantification. Previous studies investigated cocaine and metabolites in DBS using LC-based methods [58-60,63,65,71,73,76-78], with reported LOQs ranging from 0.25-50 µg/L. In the present method, cocaine, BE and norcocaine quantification in DBS was achieved with low LOQs (1 µg/L) determined empirically, with lower analyte concentrations required to meet identification and quantification criteria. One previous study quantified cocaine with a lower LOQ (0.25 µg/L); however, this was determined empirically by calculating 5xLOD [71]. Norcocaine was not previously analyzed in other studies. Sensitive LOQs of 1 µg/L also were achieved for cocaine and BE in blood utilizing a 2D-GC-MS method, similarly to previous

publications [295-298]. Norcocaine was not quantified in venous blood in the current study as it chromatographed poorly and did not achieve clinically relevant sensitivity.

DBS sample preparation for this method required two steps: first, extraction of analytes from DBS paper (3 mm punched discs) with 1% formic acid in water, followed by SPE. Initially, a simple one-step solvent extraction procedure, as described in the literature [59,299,300], was attempted utilizing different solvents including methanol, methanol + 0.1% formic acid, acetonitrile, acetonitrile + 0.1% formic acid, water + 0.1% formic acid. Significant ion suppression was observed for all investigated solvents; therefore, a SPE procedure was employed to reduce matrix effects. Although we employed this additional step, ion suppression ranged from 2.4 to 43.9% (%CV <24.2). Matched deuterated IS were included to help compensate for observed DBS matrix effects (IS-corrected matrix effects ranged from -15.1 to 16.9%). Despite low extraction efficiencies (35.2-64.2%) and ion suppression (2.4-43.9%), a sensitive LOQ of 1 µg/L was achieved for all DBS analytes.

All analytes were stable in DBS under all tested conditions: room temperature 24 h, at 4°C and -20°C for 72 h, and after three freeze-thaw cycles. These results demonstrated that DBS for measurement of cocaine and metabolites could be collected, shipped and stored at ambient temperature, avoiding expensive refrigeration costs. Cocaine and BE also were stable in venous blood under all tested conditions (< -13.2%), except for cocaine at 4ºC for 72 h that demonstrated greater instability (25.0% loss). In authentic specimens, the loss observed in fortified stability samples may be different due to the presence of anticoagulants and preservatives in the gray-top blood collection tubes. It is possible that esterase activity would continue in authentic DBS specimens (which do

not contain sodium fluoride), reducing cocaine concentrations; however, when tested, we did not observe a difference between fortified DBS specimens allowed to dry the minimum 3 h (normal procedure) compared to specimens analyzed immediately following fortification with no drying time (n=5 at each QC concentration). Stability of DBS compared to blood was previously reported for cocaine [60] and other substances, such as phosphatidylethanol [61], zopiclone [64], and benzodiazepines. In this study, blood and DBS were stored at -20ºC until analysis to minimize analyte degradation.

One DBS method limitation was the limited amount of blood volume spotted onto cards and the small 3 mm punch from the DBS. The entire DBS eluent was evaporated to dryness to concentrate analytes and improve sensitivity. DBS were not reanalyzed if concentrations were greater than the ULOQ (200 µg/L). It is possible to dilute the extract with mobile phase so that concentrations initially >ULOQ fall within the linear range if the entire extract is not injected. Cocaine concentrations in 6.9% of DBS samples were >ULOQ, while 4.8% of BE concentrations were >ULOQ (n=392). High assay sensitivity was needed to quantify analytes in the small sample and obtain low LOQ. Increased sensitivity also was achieved by utilizing the QExactive mass spectrometer that offers high resolution and high mass accuracy, increasing specificity and sensitivity by improving the signal-to-noise ratio.

Another issue that was encountered was increased blood cocaine concentrations when norcocaine was present at high concentrations (500 µg/L); however, no interference was observed at the upper limit of linearity (200 µg/L). Norcocaine concentrations are generally much lower than 200 µg/L in blood, and certainly after 25 mg IV cocaine. A study examining cocaine and metabolite concentrations from patients admitted to the

emergency department reported mean norcocaine concentration after IV cocaine administration of 30 ± 100 µg/L (*n*=3) [294]; however, dosing information was unknown. Previously, after subcutaneous cocaine administration (121.3-228.2 mg) mean norcocaine concentrations were only 7.5 ± 2.0 µg/L (*n*=7) [293].

Previous studies applied analytical DBS methods for the detection of cocaine and metabolites in authentic specimens including newborn screening [58,63,77], cocaine and poly–drug users [59,78], DUID cases [73] and PM cases [65]; however, this study is the first to investigate cocaine and BE in capillary DBS compared to simultaneously collected blood specimens following controlled cocaine administration. Several studies reported good agreement between blood dispersed onto DBS cards utilizing authentic venous specimens [65,73,78], but these studies were primarily conducted to determine if DBS could be utilized for an alternative sample preparation procedure (reduce sample volume and sample preparation). Thus, capillary DBS obtained via finger puncture were not evaluated against venous blood concentrations. Mercolini *et al.* detected cocaine, BE and cocaethylene in capillary DBS and corresponding plasma specimens. They found DBS concentrations were in good agreement with plasma concentrations once hematocrit variability was considered [59]. They applied an arbitrary hematocrit correction factor (1.62 for women and 1.92 for men) to plasma concentrations; although, results were only documented for one case and one time point involving a cocaine user.

Both cocaine and BE were detected in all DBS and blood specimens collected 30, 60 and 90 min after controlled IV cocaine administration. Norcocaine was not detected in DBS and concentrations in venous blood were undetermined as this analyte was not included in the assay. In general, median cocaine and BE DBS concentrations were

slightly lower than their respective blood concentrations. This study also found that cocaine DBS concentration ranges were wider than blood concentrations ranging from 2.9-232.6 µg/L and 18.3-137.6 µg/L, and BE concentrations from 2.9-240.4 µg/L for DBS and 57.2-193.4 µg/L for blood, respectively. In addition, large intra-DBS- and inter-subject-variability was observed in cocaine and BE DBS concentrations as compared to their corresponding blood concentrations (Table 28). It is unlikely that observed discrepancies between the capillary DBS and venous blood cocaine and BE concentrations are a result of utilizing different analytical platforms, as both methodologies were successfully validated. Extraction efficiencies could contribute to observed differences. Additionally, it is not recommended to add >5% organic solvent to blood when preparing DBS, as it could affect the spreading of the spot and subsequent concentrations detected. Although 10% acetonitrile was added in this study during fortification, authentic DBS specimens quantified lower than calibrators, hence it does not appear to be a contributor to the concentration differences observed in this case. Variability in DBS concentrations also could be attributed to several factors, including volume of blood spotted onto the card, the hematocrit of the blood and the homogeneity of the spot [56,301]. Blood hematocrit levels can substantially affect DBS concentrations, as blood with higher hematocrit will contain less water per given volume of blood (or punch diameter), and therefore possibly increase analyte concentration, compared to blood with lower hematocrit [301,302]. Non-homogeneity of the blood spots also could contribute to the variability of DBS concentrations observed in this study, as several studies reported variation in analyte concentration across the spot, especially around the edges [303,304]. Extracting the entire blood spot and not just a fixed diameter can largely

avoid hematocrit bias and non-homogeneity of blood spots [56,301]; however, often the blood volumes spotted onto the cards in this study were inconsistent.

It is unlikely that hematocrit played a significant role in the observed discrepancy between DBS and venous blood concentrations. Typically, this is only a major issue in cases with strongly deviating hematocrit. Participants in this study were considered to have normal hematocrit values (38.5-50.0% men, 34.9-44.5% women), with hemoglobin levels >12.5 g/dL. Participants had hematocrit values between 38.2 and 46.0% at the time of screening for the study, within 9 weeks of cocaine administration. Additionally, blank blood obtained from the NIH blood bank for calibrators and QC samples had hematocrit values of at least 37.5%, similar to the population studied. To investigate if variable blood spot volumes or sample homogeneity contributed to intra-DBS variability, different volumes (50, 40, 30, 20 and 10 µL) of fortified venous blood were spotted onto DBS cards at low and high QC concentrations (n=3) and analyzed. In addition, peripheral punches also were obtained from the 50 µL specimens to assess sample homogeneity. No differences were observed between central and peripheral punches suggesting that sample homogeneity was unlikely a contributor to the observed DBS variability; however, small blood spot volumes (10 µL) demonstrated up to -20.9% decrease in concentration compared to 50 µL spots. This confirms our hypothesis that blood spot volumes contribute to the observed DBS variability.

In addition, to investigate if the DBS variability was due to the DBS approach itself, venous blood was fortified (n=5) at each of three QC concentrations, spotted onto the DBS cards and analyzed according to the previously described procedures. The venous blood also was analyzed. There was a significantly strong correlation between

venous DBS (vDBS) and venous blood for cocaine (r 0.93, R^2 0.9953) and BE (r 0.89, R^2 0.9885) concentrations. This suggests that the DBS approach *per se* was not the primary source of the observed variability; however, authentic vDBS may behave differently than fortified vDBS and should be investigated in future studies.

When DBS were statistically compared to corresponding blood concentrations, Spearman's correlation indicated that the two methods were significantly moderately correlated (r_s=0.43 cocaine, r_s=0.40 BE) to a degree unlikely due to random sampling error; however, linear regression analysis yielded r^2 of only 0.066 and 0.15 for cocaine and BE, indicating that one cannot accurately predict venous blood concentrations from those of DBS. Additionally, Bland-Altman analysis plots were conducted to further determine if the difference between the two collections methods were clinically acceptable, as Garcia Boy *et al.* did when evaluating venous DBS and blood for morphine [62]. We showed a mean difference between blood and DBS of -5.1 ± 46.7 µg/L for cocaine, with a 95% confidence interval spanning 183 µg/L in total. Although the mean difference between blood and DBS was close to zero, suggesting the two methods are similar, the wide confidence interval limits clinical usefulness for comparing analyte concentrations from two different collection methods. Additionally, the plot indicated inconsistent comparability between the two methods, i.e., scatter increased as the mean concentrations of blood and median DBS increased. The comparison for BE concentrations showed somewhat greater mean difference than for cocaine (20.2 ±39.4 µg/L) and a wide 95% confidence interval for BE (154.2 µg/L), especially in comparison to the linear range evaluated (1-200 µg/L). These statistical data suggest poor agreement between blood and DBS cocaine and BE concentrations.

The discrepancy between capillary DBS and venous blood cocaine and BE concentrations is primarily due to concentration differences between venous and capillary blood, based on results of vDBS and venous blood comparisons. Mohammed *et al.* reported significant differences in paracetamol capillary DBS and venous blood concentrations during absorption following oral administration [305]. The authors also stated that equivalent pharmacokinetics will only result after distribution equilibrium is attained. Drugs that are low in molecular weight, lipophilic and with low protein binding can traverse capillary walls more readily, thus contributing to concentration differences during the absorption phase [306]. It is possible that the observed discrepancies between cocaine and BE concentrations in capillary DBS and venous blood were due to inherent concentration differences between these two matrices, especially since sampling occurred rapidly after cocaine administration. We suggest that for cocaine and BE greater clinical acceptability for the capillary DBS method awaits resolution of inconsistent blood volume spots on the cards and further investigation of differences between capillary and venous blood following controlled drug administration.

Sensitive and specific methods for the detection of cocaine, BE and norcocaine in DBS (LC-HRMS) and cocaine and BE in blood (2D-GC-MS) were developed and validated to determine if capillary DBS concentrations correlate well with venous blood concentrations following controlled IV cocaine administration. These first data indicate that although cocaine and BE were detected in all DBS after controlled cocaine administration, there was significant variability (intra- and inter-) among DBS concentrations collected at the same time (up to 5 spots per card). There was not a good agreement in cocaine marker concentrations between DBS and blood collection methods.

DBS are an alternative matrix to blood for detecting recent cocaine consumption; however, DBS variability must be addressed and further investigation of differences between capillary and venous blood following controlled drug administration is warranted.

Future Perspective

Future research must address whether extracting the entire DBS, as opposed to only a 3mm disc as in this study, would help eliminate or reduce the variability observed in DBS. If DBS variability can be minimized, this collection method may prove advantageous for clinical and forensic toxicology applications. Sensitive LOQs are required to detect analyte concentrations in DBS, which was accomplished in this study. Another promising approach in utilizing DBS as an alternative matrix to blood is volumetric absorptive microsampling, believed to overcome hematocrit bias and homogeneity issues observed with conventional DBS sampling by sampling a fixed volume of blood [301]. Although cocaine and BE were detected in DBS, the large variability between DBS concentrations and lack of agreement between the two methods warrant further investigation.

Table 29. (Supplemental) Gas chromatography, mass spectrometry (MS), Deans switch, flame ionization (FID) and mass selective (MSD) detectors and back inlet (cold trap) method parameters for the detection and quantification of cocaine and benzoylecgonine (BE) in human venous blood.

Electron Ionization Parameters			
Deans switch		Back Inlet	
FID restrictor L.	212.7 cm	Initial T	250ºC
FID restrictor i.d.	0.180 mm	Initial time	2.5 min
Aux 3 pressure	12.9 psi	Ramp #1	800ºC/min
Front inlet		Final T; time	100ºC; 5.3 min
Flow mode	Constant pressure	Ramp #2	800ºC/min
Pressure	21.85 psi	Final T; time	250ºC; 0 min
Total flow	55.3 mL/min	Oven	
Inlet T	250ºC	Initial oven T	150ºC
Injection mode	Splitless	Initial oven hold	0.5 min
Injection volume	2 µL	Ramp #1	30ºC/min
Liner type	Single-taper glass wool	Final T; time	290ºC; 0.5 min
FID and MSD		Ramp #2	75ºC/min
FID T	250ºC	Final T; time	180ºC; 1.5 min
Hydrogen flow (OFF)	40 mL/min	Ramp #3	20ºC/min
Air flow (OFF)	450 mL/min	Final T; time	280ºC/min; 0 min
MSD reagent gas	Vacuum	Post Ramp	40ºC/min
MSD transfer line T	280ºC	Post T	320ºC; 4 min
MS source T	230ºC	Post time	4 min
MS quad T	150ºC	Column 1 pressure	21.85 psi
EM offset (total)	506 (2612)	Column 2 pressure	12.9 psi

Table 30. (Supplemental) Demographic and self-reported cocaine histories for 13 cocaine users.

PPT	Race	Sex	Age (years)	Weight (lbs)	Days used in past 14 (SM or IV)	Mean use (SM or IV)	Age first use (SM or IV) (years)
A	AA	M	44.0	176	4 SM	2-3/week	38
B	AA	M	38.1	202	11 SM	5/week	32
C	More than 1	M	42.4	162	5 IV 0 SM	3/week 3-4/month	32 21
D	W	M	35.2	165	3 SM	1/week	25
E	AA	M	45.3	206	0 IV 5 SM	N/A 4/week	34 18
F	W	M	44.0	197	5 SM	Daily	18
G	W	M	42.5	180	6 IV 7 SM	3/week 2/week	28 31
H	AA	M	43.7	150	14 SM	Daily	21
I	AA	M	49.9	175	10 SM	4-5/week	25
J	AA	M	46.5	176	1 SM	3/week	15
K	W	M	39.2	175	0 IV 0 SM	N/A Daily	35 11
L	AA	M	48.8	153	10 SM	5-6/week	18
M	AA	F	36.6	140	0 SM	2/week	20

PPT participant, *SM* smoked, *IV* intravenous, *AA* African-American, *W* White, *M* male

Table 31. (Supplemental) Potential exogenous interferences analyzed at 500 µg/L in dried blood spots (DBS) by liquid chromatography-high resolution mass spectrometry and in venous blood by two dimensional-gas chromatography mass spectrometry.

	Interferences
Group 1	morphine, normorphine, morphine-3-glucuronide, morphine-6-glucuronide, codeine, norcodeine, 6-monoacetyl-morphine, 6-acetyl codeine, hydrocodone, hydromorphone, oxycodone, noroxycodone, oxymorphone, noroxymorphone
Group 2	diazepam, lorazepam, alprazolam, imipramine, clomipramine, fluoxetine, norfluoxetine
Group 3	Δ^9-tetrahydrocannibinol, 11-hydroxy-Δ^9-tetrahydrocannibinol, 11-nor-9-carboxy-Δ^9-tetrahydrocannibinol, cannabidiol, cannabinol
Group 4	methamphetamine, amphetamine, *p*-hydroxymethamphetamine, *p*–hydroxyamphetamine, hydroxymethoxymethamphetamine, hydroxymethoxyamphetamine, methylenedioxymethamphetamine, methylenedioxyamphetamine, methylenedioxyethylamphetamine, *p*-methoxyamphetamine, *p*-methoxymethamphetamine, ketamine, dextromethorphan, phentermine, ecgonine, norcocaine, cocaethylene, norcocaethylene
Group 5	(±)-ephedrine, (±)-pseudoepehedrine, acetylsaliclic acid, ibuprofen, caffeine, acetaminophen
Group 6	cathinone, methcathinone, 4-fluoromethcathinone, methylone, ethylcathinone, α-pyrrolidinopentiophenone, buphedrone ephedrine, ethylone, 4-methoxymethcathinone, buphedrone, normephedrone, diethylcathinone, 3',4'-methylenedioxy-α-pyrrolidinopropiophenone, 4-methylephedrine, butylone, mephedrone, 4-methylethcathinone, 4-methyl-N-ethyl-norephedrine, 3',4'-methylenedioxy-α-pyrrolidinobutiophenone, Pentedrone, pentylone, 3',4'-dimethylmethcathinone, α-pyrrolidinopentiophenone, 4-methyl-α-pyrrolidinobutiophenone, 3',4'-methylenedioxypyrovalerone, pyrovalerone, benzedrone, naphyrone

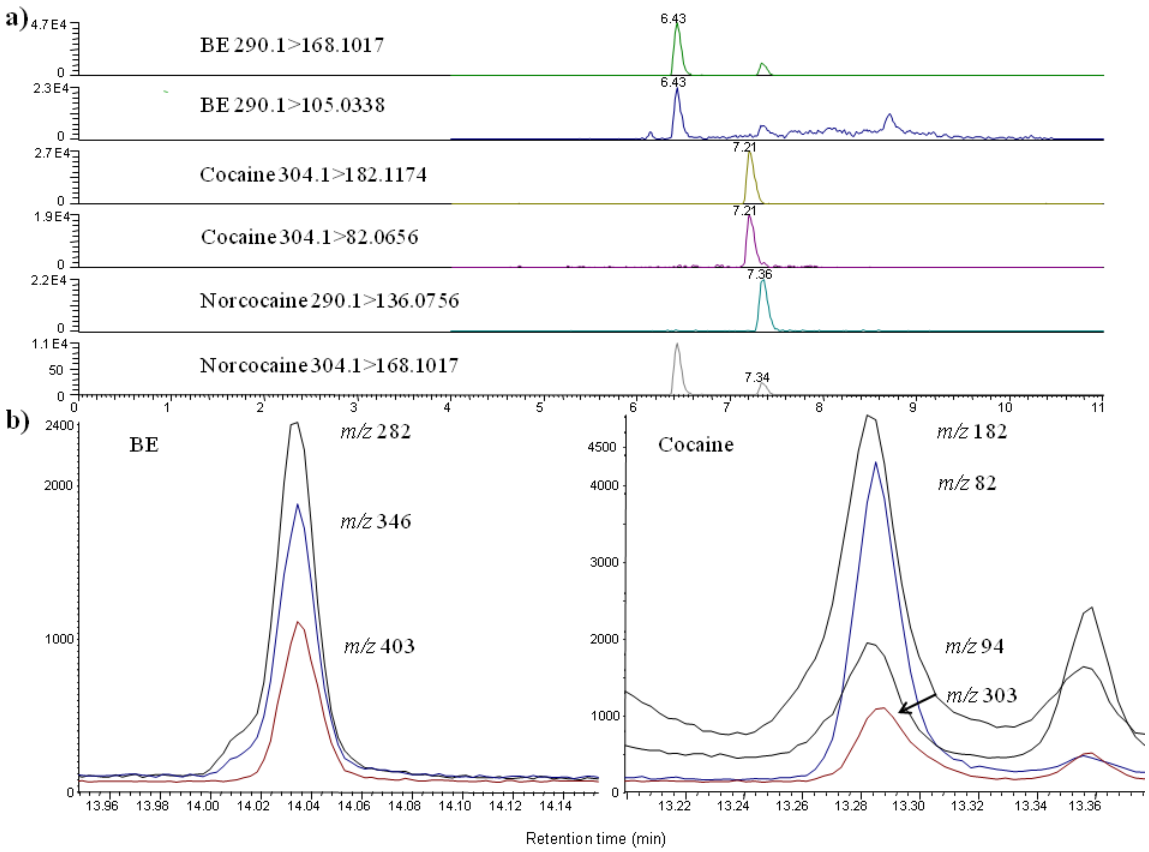


Figure 18. (Supplemental) a) Liquid chromatography-high resolution mass spectrometry chromatogram of a dried blood spot (DBS) sample fortified at the limits of quantification (1 µg/L) for cocaine, benzoylecgonine (BE) and norcocaine and b) two dimensional-gas chromatography mass spectrometry chromatogram of a venous blood sample fortified at the LOQ (1 µg/L) for cocaine and BE.

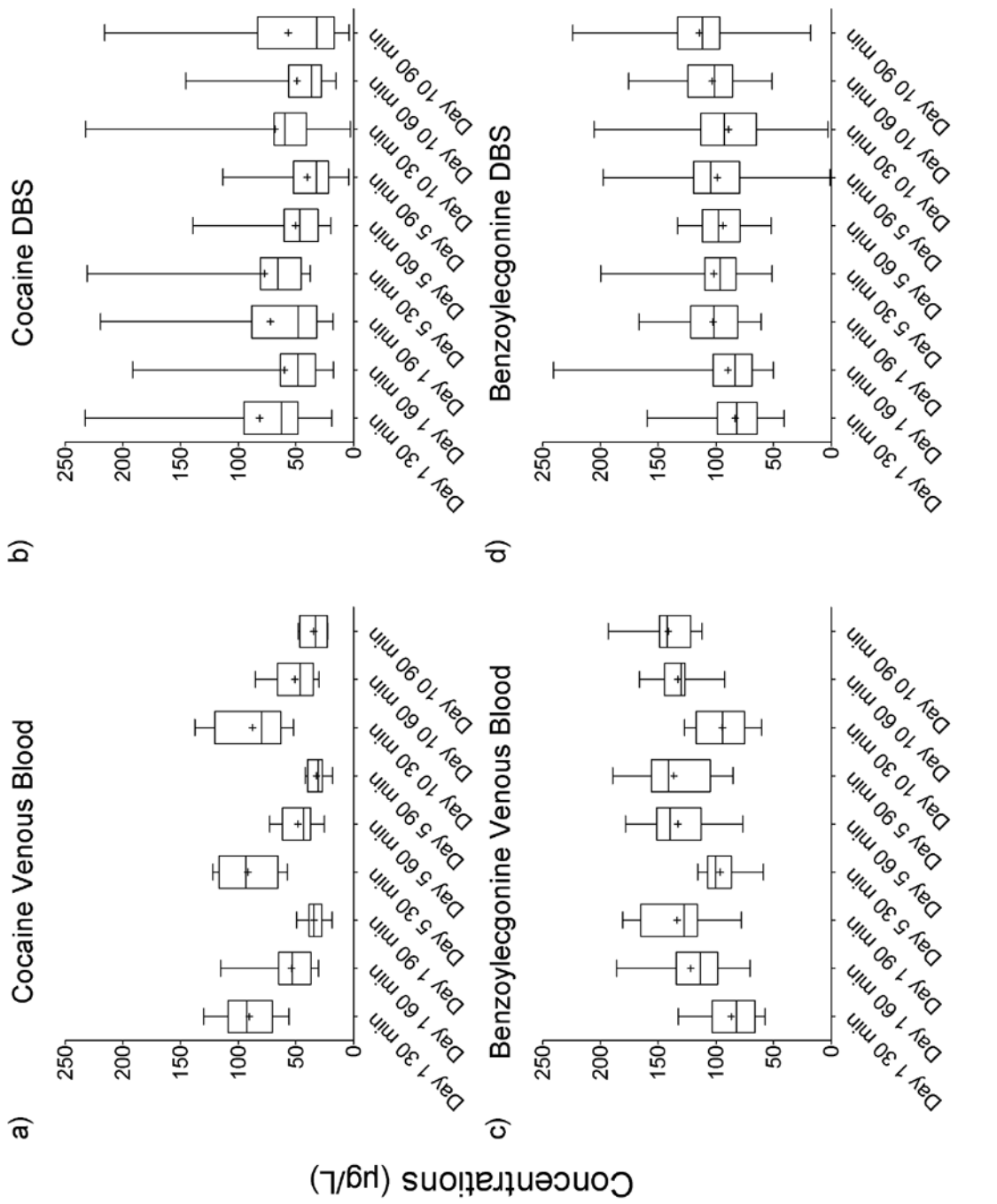


Figure 19. (Supplemental) Box and whisker graphs of a) cocaine venous blood (n = 97), b) cocaine dried blood spots (DBS) (n = 364), c) benzoylecgonine (BE) venous blood (n = 97), and d) BE DBS concentrations (n = 373) from 13 participants following 25 mg/kg intravenous (IV) cocaine administrations on three separate days (Days 1, 5 and 10). Medians represented by a line within the box and means represented by a + within the box. Boxes extend from the 25th to 75th percentiles with whiskers representing minimum and maximum concentrations. Figure does not include 27 cocaine and 19 BE DBS that quantified above the upper limit of quantification (ULOQ) and 1 cocaine DBS detected below the LOQ.

Chapter 7 - Validation of the Only Commercially Available Immunoassay for Synthetic Cathinones in Urine: Randox Drugs of Abuse V Biochip Array Technology

(*As published in* Drug Testing and Analysis, *July 2014*)[7]

Abstract

Deterrence of synthetic cathinone abuse is hampered by the lack of a high-throughput immunoassay screen. The Randox DOA-V biochip immunoassay contains two synthetic cathinones antibodies: BSI targets mephedrone/methcathinone and BSII targets MDPV/MDPBP. We evaluated DOA-V synthetic cathinones performance and conducted a full validation on the original assay with calibrators reconstituted in water, and the new assay with calibrators prepared in lyophilized urine; both utilized the same antibodies and were run on the fully automated Evidence® Analyzer. 20,017 authentic military urine specimens were screened and confirmed by LC-MS/MS for 28 synthetic cathinones. LODs for the original and new assays were 0.35 and 0.18 (BSI), and 8.5 and 9.2 µg/L (BSII), respectively. Linearity was acceptable (R^2>0.98); however, a large negative bias was observed with in-house prepared calibrators. Intra-assay imprecision was <20% BSI-II, while inter-assay imprecision was 18-42% BSI and <22% BSII. Precision was acceptable for Randox controls. Cross-reactivities of many additional synthetic cathinones were determined. Authentic drug-free negative urine pH <4 produced FP results for BSI (6.3 µg/L) and BSII (473 µg/L). Oxidizing agents reduced BSI and increased BSII results. Sensitivity, specificity, and efficiency of 100%, 52.1%, and 53.0%

[7] Ellefsen K.N. *et al.* Validation of the Only Commercially Available Immunoassay for Synthetic Cathinones in Urine: Randox Drugs of Abuse V Biochip Array Technology. *Drug Testing and Analysis*. 2015 Jul-Aug; 6(7-8): 728-738.

were obtained at manufacturer's proposed cutoffs (BSI 5 µg/L, BSII 30 µg/L). Performance improved if cutoff concentrations increased (BSI 7.5 µg/L, BSII 40 µg/L); however, there were limited confirmed positive specimens. Currently, this is the first and only fully validated immunoassay for preliminary detection of synthetic cathinones in urine.

Introduction

Designer drug intake grew rapidly around the world over the past several years [281,307,308]. These purported "legal highs" pose problems for law enforcement, military and public safety officials, and also toxicologists who must identify an unending variety of new drugs of abuse. Novel drugs are constantly being developed to circumvent legislative and regulatory efforts. These include new synthetic cathinones that are available online and in head shops or convenience stores.

Synthetic cathinones are novel stimulants intended to produce effects similar to those experienced with stimulants like methamphetamine, cocaine, MDMA [113-115]. Clinically reported symptoms of cathinone ingestion also include euphoria [97,114,115], agitation [115-119], combative and violent behavior [116,118], tachycardia [115,116,118], hallucinations [116,117], paranoia [116-118], and in some cases excited delirium [117] and death [119,127-130]. These drugs are sold online as "plant food," "bath salts," or "research chemicals" and are labeled "not for human consumption" in order to avoid potential regulation [95-97]. Abuse is documented across population groups from mid-to-late adolescents to older adults [107]. In 2010, the AAPC reported 304 phone calls regarding "bath salt" consumption [309]. This number increased dramatically in 2011 to 6,138 calls, decreased to 2,656 in 2012, with only 833 calls as of

October 31, 2013. Mephedrone, MDPV, and methylone are now Schedule I drugs in the U.S. based on the Controlled Substances Act [310,311]. As of November 28, 2012, at least 43 states banned cathinone derivatives [312].

Synthetic cathinones were identified in hair [197,219,220,251], whole blood (including PM) [108,116,119,127,128,130,156,157,165,204,223,225,226,229,251,313], plasma [253], serum [129,228,241], CSF [241], and urine [116,119,129,150,156-158,165,170,171,173,174,186,187,194,214-216,225,241,249]. Therefore, high-throughput screening methods and sensitive and specific GC- and LC-MS/MS confirmation methods for synthetic cathinones are needed for workplace (including military) and criminal justice programs. There are no government mandated screening and confirmation cutoffs for cathinone derivatives in any biological matrix.

Thus, there is a critical need for reliable screening assays that are rapid and cost efficient. Most amphetamine immunoassays do not readily detect a wide range of synthetic cathinones. Some cathinones, including MDPV, produced false-positive results in PCP immunoassays [117,232,233]. Additionally, Swortwood *et al.* reported positive Immunalysis and Neogen methamphetamine tests with synthetic cathinone concentrations starting at 1250 µg/L [230]. Greater cross-reactivity (2-25%) was observed with 40-450 µg/L mephedrone, methcathinone, methylone, 4-MEC, 4-FMC, butylone, and methedrone in the OraSure methamphetamine assay. However, with the continual development of novel cathinone derivatives, it is difficult to predict if immunoassays, not specifically designed for these drugs, have sufficient cross-reactivity for reliable screening for these compounds in urine. Recently, Randox developed a semi-quantitative screening system for synthetic cathinone detection in urine utilizing the Randox DOA-V

Biochip Array Technology. To date, this is the only commercially available immunoassay for urinary cathinone detection. The objective of this study was to fully validate the DOA-V as a sensitive and specific assay for screening synthetic cathinones in urine.

Material and Methods

Authentic Specimens

Authentic human urine specimens (*N*=20,017) collected between July 2011 and June 2012 were randomly selected from negative specimens analyzed from five Department of Defense forensic drug testing laboratories from service personnel serving around the world. Specimens were anonymized and shipped for analysis with the DOA-V synthetic cathinones assay between March and June 2012. These specimens, collected worldwide, had previously screened negative for amphetamines (d-amphetamine, d-methamphetamine, MDMA, methylenedioxyamphetamine), benzoylecgonine, opioids (codeine, morphine, hydrocodone, hydromorphone, oxycodone, oxymorphone), cannabinoids and PCP. Specimens were stored at room temperature once received, generally for two to four weeks prior to screening; however, some specimens were stored longer (up to 9 months) due to delays in receiving urine specimens from the military drug testing facilities after collection, time required to obtain waste water discharge approval, and autoanalyzer performance and maintenance issues. Based on the manufacturer's proposed cutoffs, a positive result for BSI (≥5 µg/L) or BSII (≥30 µg/L) was considered presumptive positive for synthetic cathinones. Presumptive positive specimens were stored at 4-7ºC prior to liquid chromatography high resolution tandem mass spectrometry (LC-HRMS) confirmation.

Reagents and Consumables

Randox (Crumlin, UK) released the original DOA-V assay, controls (QC, level 1 and 2), and calibrators (BSI 0-20.7 µg/L and BSII 0-1053 µg/L), with calibrator reconstitution in water. In July 2012, a new Randox DOA-V assay was released, with identical antibodies, requiring calibrator reconstitution in lyophilized urine (BSI 0-32.2 µg/L and BSII 0-1037 µg/L). Additional Evidence Analyzer universal reagents such as buffer wash, displacement fluid and signal reagent also were obtained from Randox. Mephedrone HCl, MDPV HCl, methylone HCl, R(+) methcathinone HCl, and S(-) methcathinone HCl standards were purchased from Cerilliant (Round Rock, TX), and buphedrone, butylone, *N*-ethylcathinone, ethylone, 3-FMC, 4-FMC, 4-MPBP, MDPBP, MDPPP, MDPV, 4-MEC, methedrone, 3-methoxymethcathinone, methylone, naphyrone, pentedrone, and pentylone were acquired from Cayman Chemicals (Ann Arbor, MI). All standards were stored at -20^{o}C. Intermediate (100 mg/L) and working concentrations (0.01–10 mg/L) were prepared with HPLC-grade methanol (Sigma-Aldrich, St. Louis, MO) and stored at 4–7^{o}C prior to analysis. Pooled negative urine samples from 10 individuals were utilized to prepare synthetic cathinone and interference standards, and for negative control samples after chromatographic evaluation to ensure negativity. Interference standards were purchased from Cerilliant and Sigma-Aldrich and prepared in in-house certified negative urine.

Immunoassay Screening on the Evidence® Analyzer

DOA-V is a competitive binding immunoassay on a chemically-modified biochip. Free antigens (e.g., synthetic cathinones) in the specimen compete with horseradish peroxidase-labeled analyte (conjugate) for binding sites on the immobilized polyclonal

antibodies. Signal reagent, containing a 1:1 mixture of luminol/enhancer and peroxide solution, is added to the biochip and produces a chemiluminescent signal when HRP-labeled analyte binds to the antibody sites. Chemiluminescent signals are detected with digital imaging technology (charged coupled device camera), with readings compared to calibrator signals, and signal input is inversely proportional to analyte concentration. The Randox Evidence® analyzer (EV 180-120) simultaneously runs multiple tests on one specimen (multiplexing) on one biochip, allowing laboratories to perform high-throughput analyses.

The DOA-V biochip has 11 different immobilized polyclonal antibodies, two of which bind to synthetic cathinones. BSI contains anti-methcathinone antibodies 100% cross-reactive to mephedrone, and BSII, anti-MDPV antibodies for the detection of MDPV/MDPBP. Other synthetic cathinones cross-react with BSI and BSII, including, but not limited to, methylone, flephedrone, naphyrone and pentedrone. DOA-V is specifically designed to run on the Evidence® paired with a computer and software for calibration, sample analysis, and historical data/result retrieval.

Instrument Calibration

DOA-V utilizes nine multi-analyte calibrator standards and an installation disk (CD-ROM). This disk contains product information, analyte concentration for each level, and four pre-defined performance parameters for each analyte. The software builds a derived calibration curve for competitive assays with pre-defined parameters (A, B, C, D), observed results and the following equation where *y* is the Relative Light Unit and *x* is the concentration, µg/L:

$$y = A + \frac{B+A}{1+(\frac{x}{C})^D}$$

The software constructs an optimal fit from the results with a 4-parameter curve fit method; a 4-parameter logistic (4PL) non-linear regression assuming a central turning point and two asymptotes at the terminal ends. A calibration curve for each analyte is displayed with a correlation coefficient (*r*). Target curve fit (*r*) is 0.949 or higher; FAIL is indicated if fit is lower, or if four or more calibrators are out of range. Calibrators were prepared each week, stored at 4–7^{o}C, and were stable for up to seven days. Calibration was performed daily and repeated within day only when a new immunoassay lot was assayed or if quality control sample results were unacceptable. A typical analytical batch included one negative, two different positive quality controls (analyzed in duplicate) and 86 samples. On average, six batches were analyzed daily.

Validation Procedures

Limits of Detection

Duplicates of 10 blank urine samples were assayed over three days (*n*=60). Mean observed concentrations and standard deviations (SD) for BSI & BSII were calculated. LOD was defined as mean observed concentration + 3SD.

Linearity

Calibrator concentrations were 0-20.7 µg/L (BSI) and 0-1053 µg/L (BSII) for the original, and 0-32.2 µg/L (BSI) and 0-1037 µg/L (BSII) for the new assays. Mephedrone HCl (100 mg/L) and MDPV HCl (100 mg/L) stock solutions were diluted with methanol to intermediate and working solutions; certified negative urine samples were fortified with mephedrone HCl, or MDPV HCl to final concentrations. Mephedrone in-house prepared linearity samples were 0.25, 0.50, 1, 2.5, 5.0, 7.5, 10, 20, 30, 40, and 50 µg/L (BSI), and for MDPV 10, 20, 30, 75, 135, 275, 555, 1035, 1050, 1100, and 1200 µg/L

(BSII). Each concentration was analyzed in triplicate per batch for five separately calibrated batches (*n*=15). Curve fit (*r*) for each antibody was derived from plotting mean RLUs (y) versus log mean concentration (x) for all 15 batches in GraphPad Prism, and linearity (R^2) for assay calibrators and in-house standards. In addition, % bias was determined for each concentration as 100 x (group mean observed concentration – known concentration)/ known concentration. Acceptable % bias was ±20% of target.

Imprecision

Low, medium, and high mephedrone (1.0, 5.0, 15 µg/L) and MDPV (30, 275, 555 µg/L) in-house standards were analyzed in quadruplicate in each of five batches (*n*=20). Mean observed concentrations, SD, %CV for intra- and inter-batch, and total imprecision were determined. Pooled intra-day, inter-day, and total imprecision were calculated for each concentration according to the procedure of Krouwer and Rabinowitz [314].

Cross-reactivity

Synthetic cathinones and structurally similar compounds were prepared at two concentrations based on the cross-reactivity profile provided by Randox in the current DOA-V product insert. Negative urine was fortified to final concentrations and analyzed in triplicate in one batch. Cross-reactivity (%) was determined as 100 x (BSI or II mean observed concentration)/target analyte concentration.

Interferences

Extensive exogenous and endogenous interferences were fortified in in-house certified negative urine (Table 36 (Supplemental)). Additionally, in-house certified negative urine samples pH were adjusted to pH 4.0 and pH 8.0 and evaluated for interferences. To evaluate peroxide and bleach as potential adulterants, mephedrone HCl

(10 µg/L) and MDPV HCl (275 µg/L) were mixed with negative urine containing either 10% bleach or 10% peroxide. Adulterant-free samples were included to establish baseline readings. A value <LOD was considered free from interference.

Carryover

Carryover was evaluated with in-house certified negative urine fortified at 250 µg/L mephedrone and 2000 µg/L MDPV. Fortified samples were analyzed followed by two negative urine samples. Negative samples with concentrations ≤LOD were considered without carryover.

Authentic Urine Specimen Analysis

Presumptive positive urine specimens based on the Randox recommended cutoffs for BSI (5 µg/L) and BSII (30 µg/L) were confirmed with a fully validated LC-HRMS confirmation method for 28 synthetic cathinones in urine [150]. LOQs were 0.5 µg/L for all synthetic cathinones, except buphedrone ephedrine (1 µg/L). In addition, presumptive negative specimens were selected at random from the original batches that provided presumptive positive specimens and analyzed by LC-HRMS. TP specimens screened and confirmed positive, and TN specimens were negative in both assays. FP specimens screened positive but no synthetic cathinones were identified by confirmation, and FN specimens screened negative but confirmed positive for one or more synthetic cathinones. Performance parameters were calculated as: sensitivity = 100 x (TP/TP+FN); specificity = 100 x (TN/TN+FP); and efficiency = 100 x (TP+TN/Total). Sensitivity, specificity, and efficiency of manufacturer's proposed cutoff concentrations, as well as 7.5 and 10 ug/L BSI cutoffs and 40 and 50 ug/L BSII cutoffs also were evaluated.

Additionally, performance was evaluated around cutoff concentrations of 2.5, 5.0, 7.5, and 10 µg/L for BSI and 20, 30, and 40 µg/L for BSII, and ±25% and ±50% of each of these cutoffs. Samples were analyzed in triplicate in one batch. Cutoffs were evaluated by examining the overlap between mean cutoff concentrations and ±25% and ±50% of each of these cutoffs.

Results

The original and new DOA-V were evaluated with 10% quality control samples (Negative, Control 1, and Control 2) included in each analytical batch. All calibrations had r>0.994 and quality control results within acceptable ranges.

Limits of Detection

LODs for the original and new assays were 0.35 (BSI), 8.5 (BSII), 0.18 (BSI) and 9.2 µg/L (BSII), respectively. LODs were 93-96% and 69-72% below the proposed cutoffs for BSI and BSII, respectively.

Linearity

Four PL curve fits for BSI-II were acceptable (R^2>0.99) in the original and new assays. BSI assay calibrators (original and new) were linear at concentrations below the manufacturer's proposed cutoff and upper limits of linearity (ULOL); R^2 ≥0.98 (1.3-20.73 µg/L) and R^2≥0.99 (1-32.2 µg/L), respectively. Mean % bias for BSI-II in the linear range was -19% to 19%. BSII assay calibrators (original and new) were linear (R^2≥0.99) above the proposed cutoff to the ULOL (original: 32.9-1053 µg/L, new: 43.1-1037 µg/L), exhibiting mean %bias across the linear range of -17% to 15%.

The in-house standard preparations for the original assays were linear (R^2≥0.99) from below the manufacturer's proposed cutoff (2.5 µg/L, with the proposed 5 µg/L

cutoff) to the ULOL (20 µg/L) for BSI. For BSII, linearity was documented between the manufacturer's proposed cutoff (30 µg/L) and ULOL (1050 µg/L); however, BSI %bias was -32 to -17% (median: -24%, mean: -24%, SD: 5.4%) between the proposed cutoff (5 µg/L) and 20 µg/L. BSII %bias was -49 to -34% (median: -40%, mean: -41%, SD: 5.9%) from the 30 µg/L proposed cutoff to 1050 µg/L (highest calibrator). Figure 20A and Figure 20B highlight the % bias for BSI and BSII across the linear ranges.

The new assays for BSI-II were linear ($R^2 \geq 0.98$) from below the manufacturer's proposed cutoff to the ULOL (BSI) and from the manufacturer's proposed cutoff to above the ULOL (BSII) for the in-house standard preparations (BSI: 2.5-30 µg/L, BSII: 30-1050 µg/L). BSI %bias ranged from -61 to -54% (median: -58%, mean: -58%, SD: 2.9%) between the proposed cutoff and 30 µg/L (Figure 20A). BSII % bias for 30-1050 µg/L was -54 to -11% (median: -30%, mean: -33%, SD: 16%) (Figure 20B).

Imprecision was assessed at 1, 5, and 15 µg/L for BSI and 30, 275, and 555 µg/L for BSII in the original assay. Intra-, inter-day and total imprecision are detailed in Table 32. BSI within-run imprecision (*n*=20) was <12% CV and BSII <17% CV. Inter-day imprecision (*n*=20) ranged from 18-41% and 17-40% CV for BSI and BSII, respectively. For BSI and BSII total imprecision (*n*=20) was 19-42% and 18-46% CV. Results for the new formulation also are in Table 32. BSI and BSII within-run imprecision (*n*=20) was respectively <7.2% and <8.3% CV for all concentrations. Inter-day imprecision (*n*=20) was 19-40% CV for BSI and 14-41% CV for BSII, and total imprecision (*n*=20) was 20-41% and 16-42% CV, respectively (Table 32). In-house precision was evaluated with fortified authentic urine samples prepared fresh each day, due to stability concerns with

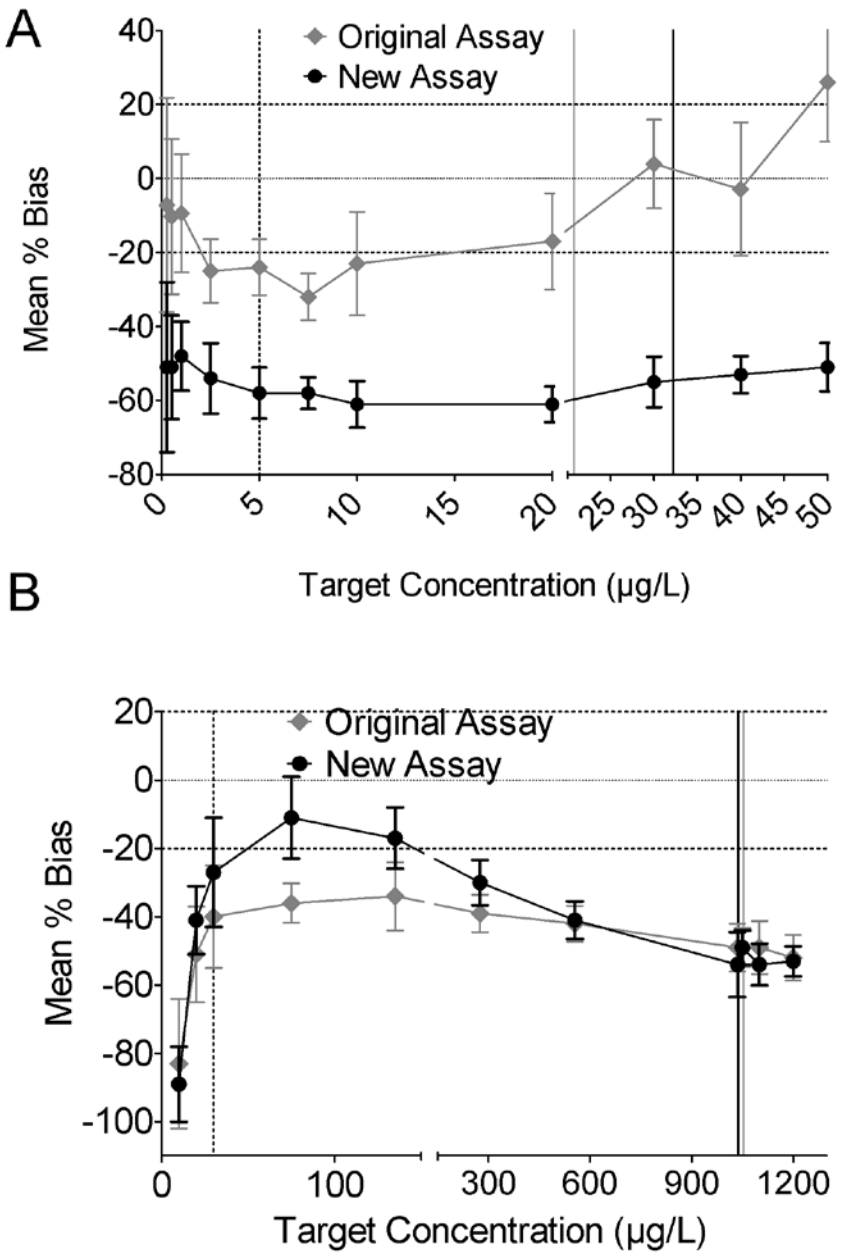


Figure 20. Mean % bias of the Randox DOA-V synthetic cathinone biochip A) BSI and B) BSII assays across their linear ranges using DOA-V original and new assays. Randox proposed cutoffs were 5 and 30 µg/L for BSI and BSII. The vertical line represent (••••) represents the manufacturer's proposed cutoffs for original and new assays and (—) highest calibrators (grey: original assay, black: new assay). Horizontal lines represent (⋯⋯) ± 20% acceptance criteria.

Table 32. Intra-, inter-, and total imprecision for Randox synthetic cathinone antibodies, BSI and BSII, for both original and new DOA-V assays at three concentrations (BSI 1, 5, 15 µg/L and BSII 30, 275, 555 µg/L).

Randox Biochip Array Technology		Intra-day imprecision (N=20) (CV%)			Inter-day imprecision (N=20) (CV%)			Total Imprecision (N=20) (CV%)		
		Low	Med	High	Low	Med	High	Low	Med	High
Original	BSI	12	8.2	9.9	31	18	41	33	19	42
	BSII	17	5.1	8.9	43	17	20	46	18	22
New	BSI	7.2	7.0	4.9	40	31	19	41	32	20
	BSII	7.6	5.3	8.3	41	15	14	42	16	16

the large negative % bias, and quality control sample instability noted after approximately 6 h.

Cross-reactivity

Cross-reactivity with the mephedrone antibody (BSI) varied (Figure 21A) and was lower than reported in the DOA-V product insert, especially for methylone. This study found that methedrone, 3-methoxymethcathinone and butylone cross-reacted with BSI, especially methedrone, with 57% (low) and 54% (high) cross-reactivity. 4-MPBP and MDPPP cross-reactivities were not evaluated by the manufacturer, but this study found less than 1% cross-reactivity with BSI. Pentylone, amphetamine, methamphetamine, ephedrine, pseudoephedrine, MDMA, MDEA, MDA, PMA, PMMA, MDPBP, MDPV, and naphyrone produced results <LOD.

MDPV cross-reactivities were much lower than the Randox reported cross-reactivity of 101% (Low: 37%, High: 42%). MDPBP cross-reactivities were 34% and 29% below reported 100% cross reactivity for low and high concentrations (Figure 21B). This study also found that naphyrone, 4-MPBP, MDPPP, pentedrone and butylone cross-reacted with BSII (Figure 21B). Butylone cross-reactivity with BSII was not tested by the manufacturer, but this study observed a low (<1.8%) cross-reactivity. No other tested

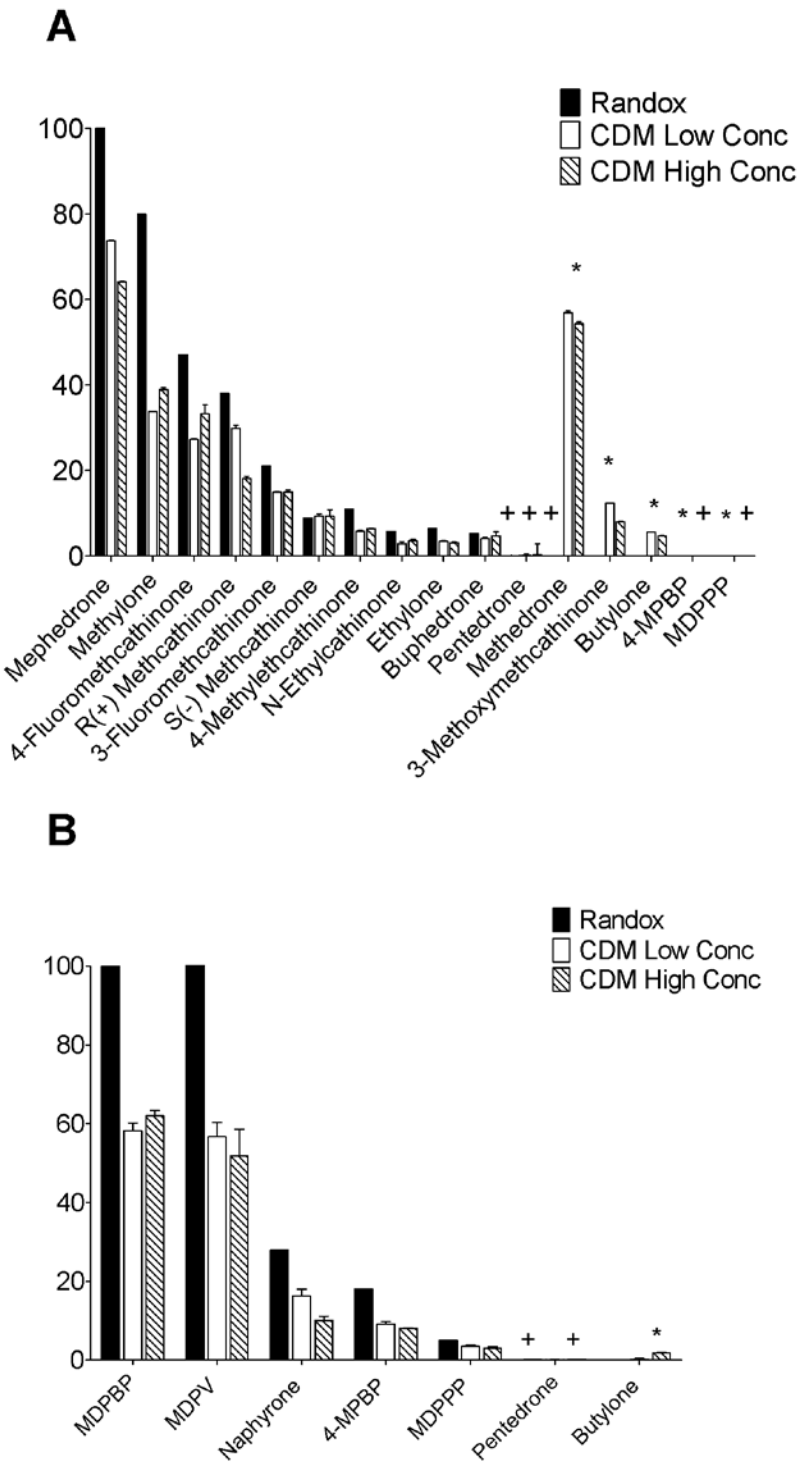


Figure 21.Randox and Chemistry and Drug Metabolism (CDM)-evaluated synthetic cathinone cross-reactivity with A) BSI (mephedrone) and B) BSII (MDPV). (*) represent no Randox cross-reactivity reported and (+) represent % cross-reactivity <1%.

substances demonstrated cross-reactivity with BSII >LOD including: mephedrone, methylone, 4-FMC, R(+) methcathinone, 3-FMC, 4-MEC, *N*-ethylcathinone, ethylone, buphedrone, methedrone, 3-methoxymethcathinone, pentylone, amphetamine, methamphetamine, ephedrine, pseudoephedrine, MDMA, MDEA, MDA, PMA, and PMMA.

Interferences

All interference standards quantified below the respective LODs, with several exceptions (Table 33). L-Ascorbic acid produced a BSI result three times the LOD and hemoglobin four times the LOD; however, no results exceeded the 5 µg/L Randox proposed cutoff. Drug-free certified negative urine adjusted to pH <4 produced false positive results greater than 20% for BSI (6.3 µg/L) and BSII (473 µg/L). Oxidizing agents (10% bleach or 10% peroxide) reduced BSI and increased BSII concentrations compared to non-adulterated specimens containing 10 µg/L mephedrone and 275 µg/L MDPV. The addition of bleach or peroxide to 10µg/L mephedrone reduced immunoassay results by 25 and 31%, respectively. The addition of bleach or peroxide to 275 µg/L MDPV produced results 64 and 5% above the concentration of MDPV alone, respectively.

Carryover

No carryover was detected in negative urine specimens following a 250µg/L mephedrone or 2000µg/L MDPV specimen.

Table 33. Interferences producing a measured concentration >LOD of BSI (0.35 µg/L) and/or BSII (8.46 µg/L) with the Randox DOA-V original assay.

Interference	Target Concentration	BSI Measured Concentration (µg/L)	BSII Measured Concentration (µg/L)
L-Ascorbic Acid	4.0 g/L	1.1	-
Hemoglobin	1.5 g/L	1.5	-
Urine, pH < 4	-	6.3	473
Mephedrone (10µg/L)	10 µg/L	8.6	-
Mephedrone (10µg/L, 10% Bleach)	10 µg/L	6.4	2.3
Mephedrone (10µg/L, 10% Peroxide)	10 µg/L	5.9	-
MDPV (275µg/L)	275 µg/L	-	157
MDPV (275µg/L, 10% Bleach)	275 µg/L	-	258
MDPV (275µg/L, 10% Peroxide)	275 µg/L	-	165

Authentic Urine Specimen Analysis

Based on BSI and BSII immunoassay screening, the overall presumptive positive rate was 0.53% (N=106) for one or more synthetic cathinones (Table 34). Among presumptive positives, 62.3% (N=66) were positive for BSI (mephedrone/methcathinone), 29.2% (N=31) for BSII (MDPV/MDPBP), and 8.5% (N=9) for both. Nine presumptive positive specimens were unavailable for confirmation due to inadequate specimen volume or laboratory error. Four specimens confirmed positive for methylone, α-PVP, pentedrone, pentylone, and/or pyrovalerone [150]. The presumptive positive confirmation rate was 4.1% (Table 34), yielding an overall positive rate for synthetic cathinones of 0.02% of 20,017 specimens.

Table 34. Randox DOA-V Biochip Immunoassay synthetic cathinone presumptive positive results for 20,017 screened urine specimens, and presumptive positive rates of synthetic cathinones (N=106). Presumptive positive urine specimens (N=97) confirmed by liquid chromatography-high resolution tandem mass spectrometry (LC-HRMS).

	Total # of Specimens (N=20,017)	Randox Presumptive Positive Rate	SC Presumptive Positive Rate	Total # of Confirmed Specimens (N=97)*	Positivity Rate (%)**
Total Presumptive Positive Specimens for Synthetic Cathinones	106	0.53%	N/A	4	4.1%
Presumptive Positive Specimens for BSI only	66	0.33%	62.3%	2	3.3%
Presumptive Positive Specimens for BSII only	31	0.15%	29.2%	1	3.6%
Total Presumptive Positive Specimens for BSI and BSII	9	0.04%	8.5%	1	11.1%

* 9 presumptive positive specimens were not available for confirmation
** Positivity Rate (%) = 100 x (number of positive by LC-HRMS/ number of positive by Randox)
Abbreviations: SC (synthetic cathinones)

Only 4 of 97 presumptive positive specimens confirmed positive for one or more synthetic cathinones at the proposed cutoffs of 5 µg/L (BSI) and 30 µg/L (BSII). All 101 presumptive negative urine specimens confirmed negative, yielding a total of 4 TP, 93 FP, 101 TN, and 0 FN. Sensitivity, specificity, and efficiency were 100%, 52.1%, and 53.0%, respectively. Additional cutoffs were evaluated to test if sensitivity, specificity, and efficiency could be improved (Table 35). Cutoff values of 7.5 µg/L for BSI only and 40 µg/L for BSII only yielded sensitivities, specificities, and efficiencies of 75, 76, and 76%, and 50, 89, and 88%, respectively. Furthermore, sensitivity of the DOA-V assay

Table 35. Evaluation of potential cutoffs for Randox DOA-V for the detection of synthetic cathinones in urine with confirmation results of authentic urine specimens (N=198).

Cutoff (µg/L)	BSI			BSII			BSI & BSII		
	5	7.5†	10	30	40†	50	5, 30*	7.5, 40†	10, 50
TP	3	3	2	2	2	1	4	4	2
FP	66	46	31	35	22	10	93	60	41
TN	128	148	163	159	172	184	101	134	153
FN	1	1	2	2	2	3	0	0	2
Sensitivity (%)	75.0	75.0	50.0	50.0	50.0	25.0	100	100	50
Specificity (%)	66.0	76.3	84.0	82.0	88.7	94.9	52.1	69.1	78.9
Efficiency (%)	66.2	76.3	83.3	81.3	87.9	93.4	53.0	69.7	78.3

improved when utilizing BSI and BSII cutoffs together (Figure 22). Optimal assay parameters were achieved when BSI 7.5 µg/L and/or 40 µg/L BSII cutoffs were applied, yielding sensitivity 100%, specificity 69% and efficiency 70%.

To evaluate the efficiency of the manufacturer's proposed cutoffs four cutoff concentrations for BSI (2.5, 5, 7.5, 10 µg/L) and three BSII cutoff concentrations (20, 30, 40 µg/L) and ±25% and ±50% around each cutoff were evaluated (Figure 23 and Figure 24). The consistent under recovery of expected concentrations at all proposed cutoffs and ±25% and ±50% of these concentrations limited evaluation of performance at these cutoffs. The manufacturer's proposed BSI 5 µg/L cutoff could be differentiated from samples fortified at ± 50% of this concentration in both assays despite % bias -36 to -28% in the original and -54 to -45% in the new formulation (Figure 23B). Good separation was achieved for both BSI assays at ±25% above the proposed cutoff of 7.5 µg/L (original % bias -43 to -28%, new % bias -56 to -52%) (Figure 23C). At the manufacturer's BSII proposed 30µg/L cutoff, good separation was achieved at ±25% of the cutoff concentration for the new assay (% bias -52 to -17%) but not for the original

assay (Figure 5B). At the proposed BSII 40 µg/L cutoff, samples fortified at ±50% of this cutoff for the original assay (% bias -36 to -21%) (Figure 24C) were distinguished.

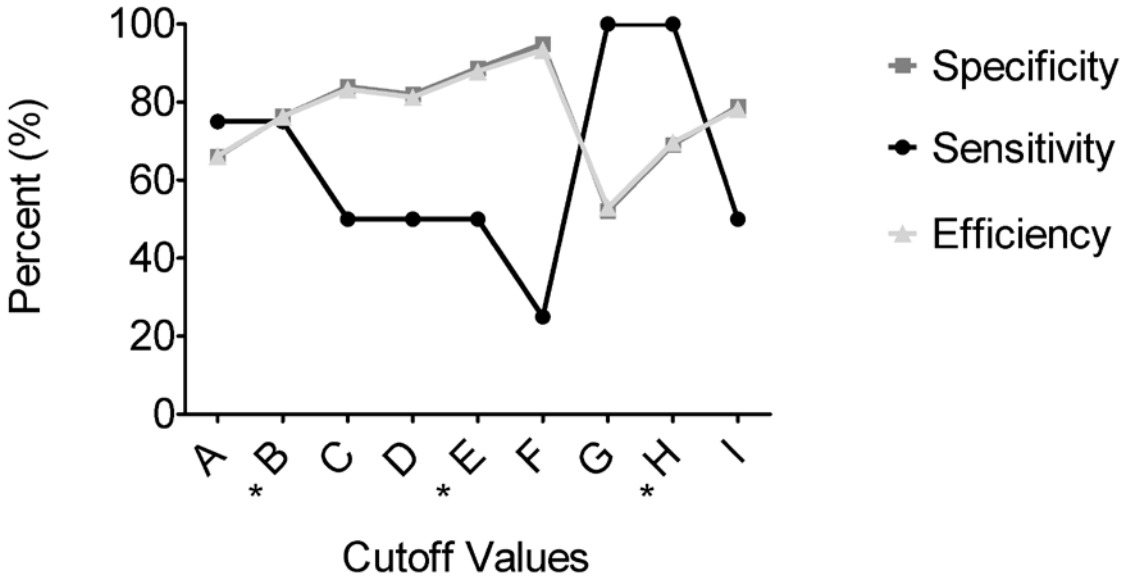


Figure 22. Sensitivity, specificity, and efficiency of Randox DOA-V for the detection of synthetic cathinones in authentic confirmed urine specimens (N=198) evaluated with different cutoffs: A) BSI (5 µg/L), B*) BSI (7.5 µg/L), C) BSI (10 µg/L), D) BSII (30 µg/L), E*) BSII (40 µg/L), F) BSII (50 µg/L), G) BSI (5 µg/L) & BSII (30 µg/L) (Randox proposed cutoffs), H*) BSI (7.5 µg/L) & BSII (40 µg/L), I) BSI (10 µg/L) & BSII (50 µg/L). Only four specimens were confirmed positive via LC-HRMS. * Represents optimal cutoff concentrations for BSI only (B), BSII only (E), and BSI & BSII (H).

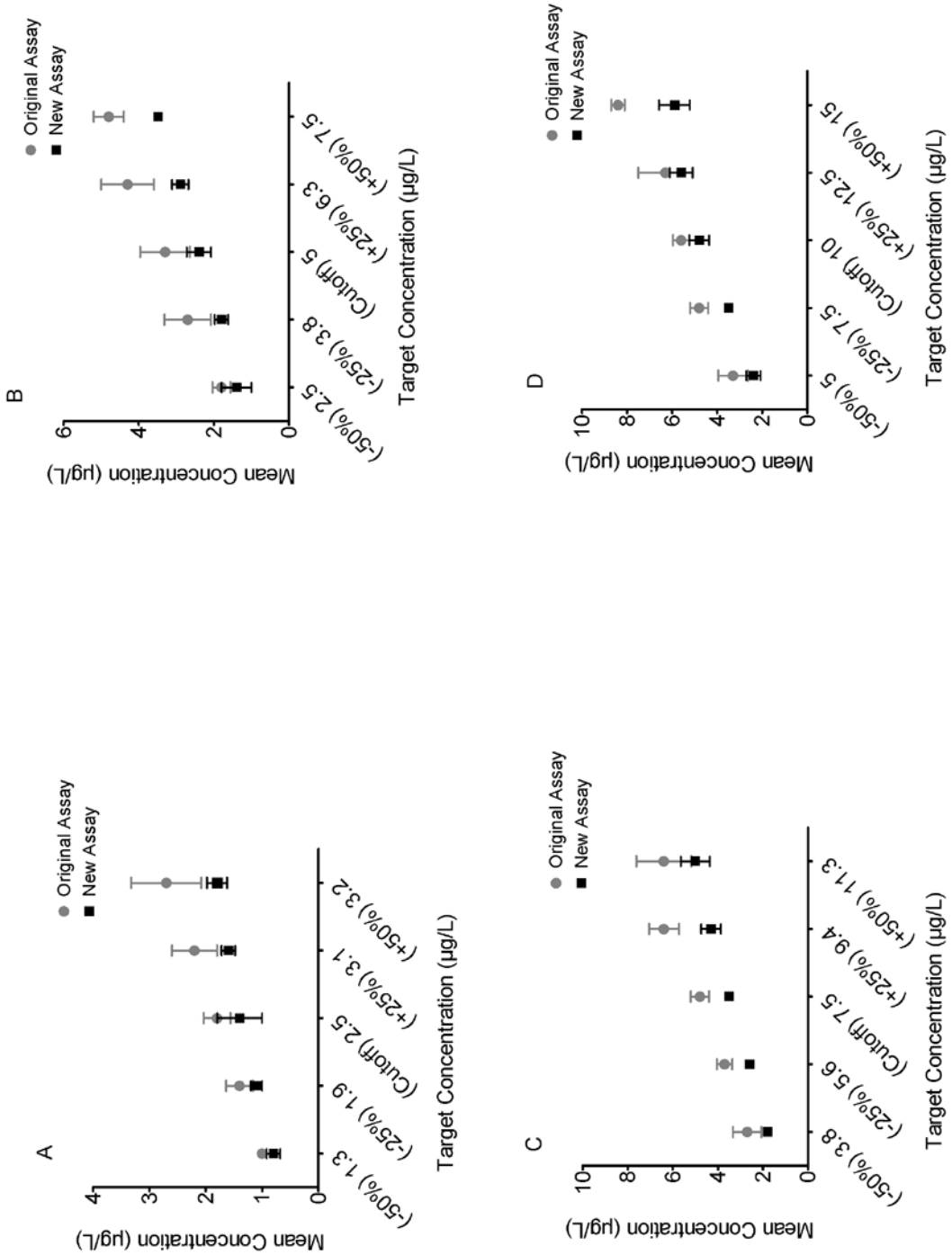


Figure 23. Performance around cutoff concentrations of Randox synthetic cathinone BSI biochip for original and new DOA-V kits. Mephedrone fortified negative urine samples at cutoff A) 2.5, B) 5, C) 7.5, and D) 10 µg/L, and ± 25% and ± 50% of cutoffs. Error bars represent ± 2SD from mean observed concentration.

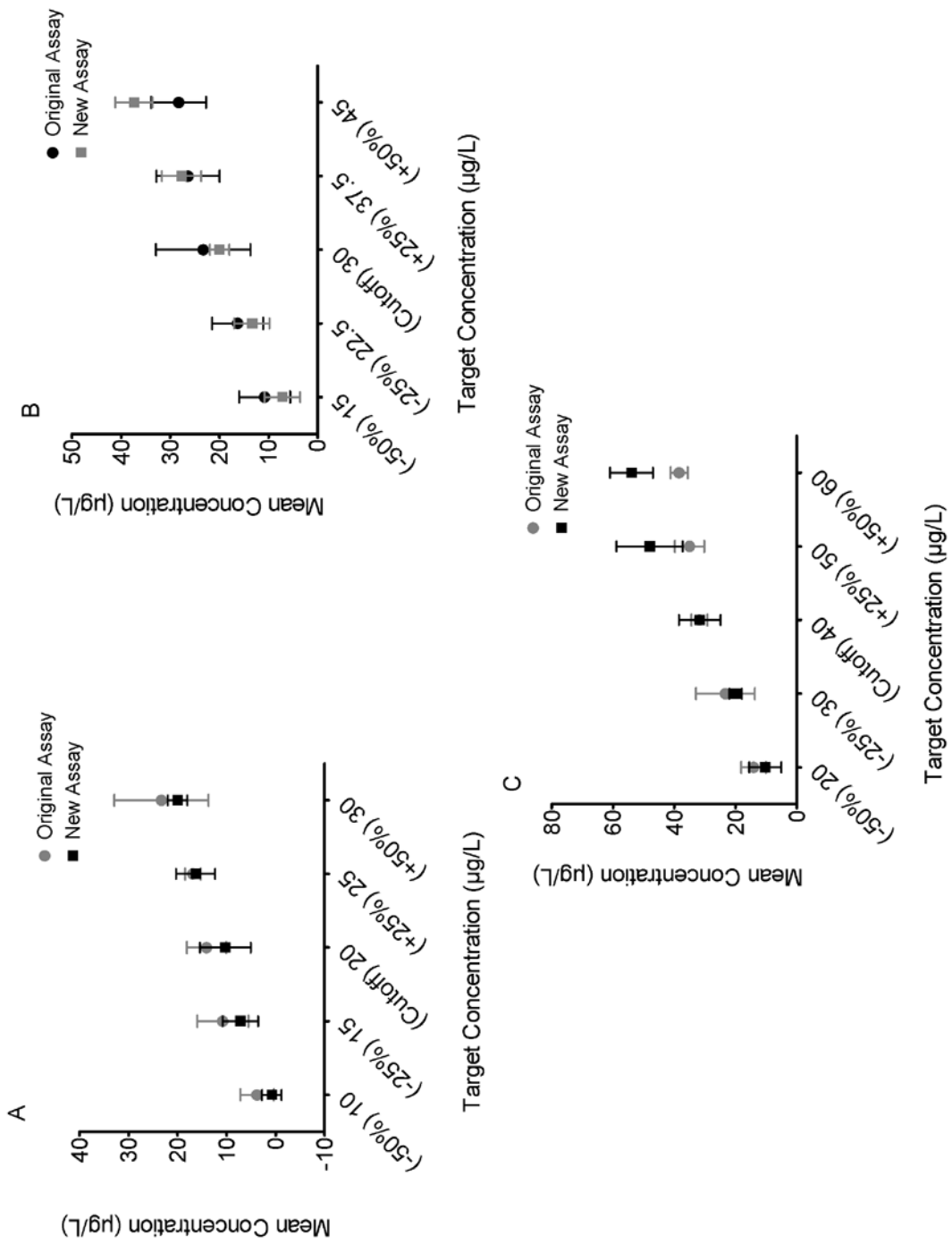


Figure 24. Performance around cutoff concentrations of Randox synthetic cathinone BSII biochip for original and new DOA-V assays. MDPV fortified negative urine samples at A) 20, B) 30, and C) 40 µg/L cutoffs, and ± 25% and ± 50% of these cutoffs. Error bars represent ± 2SD from the mean observed concentration.

Discussion

The objectives of this study were to determine if the DOA-V provides a reliable screen for the detection of synthetic cathinones in urine and to evaluate the feasibility of this assay in a high-throughput laboratory setting. Based on observations, the DOA-V's main limitation for the original and new assays was the high negative %bias. Concentrations for both mephedrone and MDPV were consistently lower and below acceptable criteria (±20%), despite daily preparation of fortified samples.

Due to low recovery in BSI (mephedrone) and BSII (MDPV) in the original assay and the high negative % bias for both BSI and BSII with the DOA-V new formulation, linearity, imprecision, and cutoff evaluation experiments were repeated with freshly fortified samples to ensure that the low recovery was not due to stability issues. Results did not improve and analyte recovery remained low.

The DOA-V demonstrated acceptable intra-assay imprecision for both formulations with three Randox control concentrations, but was unacceptable for some inter-assay and total imprecision measures. It is possible that the variability observed among the inter-day and total imprecision is due to different analysts preparing samples each day. Due to stability concerns, quality control samples were fortified each day to ensure that samples contained intact synthetic cathinones at the specified concentrations. This also could have increased imprecision to a small degree. It is more likely that the variability was related to the use of calibrators over several days that may have been less stable than indicated by the manufacturer's instructions (stable for up to seven days when refrigerated). In addition, the high negative percent bias could have contributed to poor imprecision at lower quality control concentrations.

Method validation results demonstrated optimal performance of mephedrone in both DOA-V kits above the proposed Randox cutoff: 7.5 µg/L, as it was determined that 95% of samples did not overlap with concentrations ±25% of the cutoff concentration. Optimal BSII (MDPV) performance was observed above the proposed Randox cutoff with the original assay (40 µg/L). These results differed from what was observed for BSII with the new formulation. Noticeable separation of MDPV concentrations was demonstrated at both 30 and 20 µg/L cutoffs.

Based on the evaluation of alternative cutoffs, poor performance around the manufacturer's proposed cutoffs and good sensitivity, specificity, and efficiency observed at cutoff concentrations of 7.5 and 40 µg/L suggest implementation of higher proposed cutoffs for synthetic cathinone detection in urine with the DOA-V assay. However, based on this method validation alone, it is hard to establish proposed cutoff values for the detection of mephedrone and MDPV for both the original and new DOA-V assays because of the high negative % bias. Additionally, limited specimens (N=4) were confirmed positive for synthetic cathinones in urine. More positive specimens are needed for accurate assessments of DOA-V assay parameters (sensitivity, specificity, and efficiency) utilizing alternative cutoff concentrations.

BSI and BSII cross-reactivities were consistently lower than Randox reported results, especially for methylone. This difference in cross-reactivity could be attributed to low analyte recovery observed in this validation. In addition, Randox cross-reactivities were assessed with nine concentrations in calibrator base (containing phosphate buffer) and not in authentic urine; therefore, matrix interferences may have contributed to the low cross-reactivities observed during our method validation. This validation also

examined additional synthetic cathinone cross-reactivities that had not been previously reported. Five compounds exhibited cross-reactivity to mephedrone antibody, most notably methedrone (54-57%). Methedrone only differs from mephedrone by a methoxy group on the 4′ position. The 3′ methedrone regioisomer also demonstrated cross-reactivity with BSI, although to a lesser degree than methedrone. Butylone, a positional isomer of ethylone, had comparable cross-reactivity to the reported Randox cross-reactivity of ethylone. Both 4-MPBP and MDPPP exhibited cross-reactivity to mephedrone when present at high concentrations, albeit with minimal cross-reactivity (0.02%). Only one additional synthetic cathinone (butylone) demonstrated 1.8% cross-reactivity with BSII.

Based on our cross-reactivity data, it appears that 4-methylphenyl-based compounds, such as methylone and methedrone, are cross-reactive primarily to BSI, whereas MDPV-like compounds, such as MDPPP and naphyrone, are primarily cross-reactive with BSII. Those compounds containing mixed variations, like 4-MPBP, appear to have cross-reactivity to both BSI and BSII. It is hypothesized that newer synthetic cathinones and cathinones not analyzed during cross-reactivity experiments with similar structural features would behave in a similar manner. Increased BSI readings occurred when samples contained L-ascorbic acid (4 g/L) or hemoglobin (1.5 g/L), but concentrations were less than the 5 µg/L proposed cutoff. Negative urine adjusted to pH 4 also interfered with BSI and BSII assays; concentrations were above the proposed cutoffs and yielded false positive screening results. The addition of oxidizing agents, bleach or peroxide, to mephedrone-fortified samples reduced concentrations as compared to

mephedrone alone. In contrast, when these adulterants were added to MDPV-fortified urine, concentrations increased.

Another study limitation is the unknown stability of synthetic cathinones in authentic urine specimens. Specimens were stored at room temperature generally for two to four weeks prior to screening. Because urine specimens are rarely assayed immediately after collection, the stability of synthetic cathinones in urine is an important issue, but minimal data are available. Mephedrone urine concentrations decreased by 60% when samples were stored at room temperature for up to 14 days, but were stable at 4ºC over the same time period [147]. MDPV was stable in urine for 14 days at room temperature and 4ºC. Soh and Elliot reported that 4-MEC was not detectable in blood after 14 days at room temperature, with a corresponding 54% loss in plasma [148]. Two additional studies reported that substitutions on the benzene ring, nitrogen, and phenethylamine backbone affected synthetic cathinone stability [204,235].

Cathinones including the pyrrolidinyl moiety, such as MDPV, MDPPP, MDPBP, α-PVP and 4-MPBP, are more stable. This enhanced stability may be due to steric hindrance by the bulky pyrrolidinyl group adjacent to the ketone. This may block the electrophilic carbon reducing its reactivity. Therefore, reduction of the ketone group to its corresponding alcohol may not occur as readily as for cathinones with the pyrrolidinyl substituent. Tsujikawa *et al.* also reported that tertiary amines, such as dimethylcathinone, are more stable [235]. However, these authors hypothesized the enhanced stability was attributed to sensitivity to oxidative deamination, as tertiary amines do not readily undergo oxidative deamination like secondary aliphatic amines. In addition, Zaitsu *et al.* found that methylone does not readily reduce to its corresponding

alcohol like butylone and ethylone and suggested this may be due to the compound's structural affinity differences to reductive enzymes [236]. Concheiro *et al.* previously documented short-term stability findings during confirmation method validation and showed that most synthetic cathinones were stable for 72 h at 4ºC, with the exception of benzedrone (27% loss) [150]. However, many synthetic cathinones were not stable for 24h at room temperature (20% to 67.6% loss). Only the metabolites buphedrone ephedrine, 4-methylephedrine, and 4-MEC metabolite, as well as the synthetic cathinones diethylcathinone, MDPPP, MDPBP, α-PVP, 4-MPBP and MDPV, were stable at room temperature for 24 h. There are no published long-term synthetic cathinone stability studies.

The majority of presumptive positive urine specimens (62.3%, *N*=66) screened positive on BSI, which targets mephedrone and methcathinone, with cross-reactivity to other cathinones; these were reported unstable at room temperature based on stability data [150]. The confirmation rate of presumptive positive specimens was only 4.1% by LC-HRMS, indicating a high FP screening rate (95.9%). FP results could be due to cross-reactivity with other cathinones not present in the confirmatory method (although this confirmation assay is the most comprehensive method to date and parent synthetic cathinones are well represented in urine), instability and degradation of the specimens over the interval between screening and confirmatory testing, or inappropriately low cutoff concentrations recommended by the manufacturer. Multiple cathinones exhibited cross-reactivity to mephedrone and MDPV in the screening assay; however, these cathinones were included in the confirmation method, and so should have been identified. The urine specimens were stored refrigerated (4ºC) up to a year before confirmation

analysis. The synthetic cathinone stability findings in urine at 4ºC suggest that the high FP screening rate and low confirmation rate could be due to analyte degradation, although it is not possible to definitively determine the reason unless new fresh urine specimens are analyzed.

Inclusion of only four metabolites in the confirmatory assay (due to lack of commercially available metabolite standards) could also contribute to the FP screening rate. It is possible that metabolites present in urine but not included in the confirmation contributed to the low synthetic cathinones confirmation rate. However, this is unlikely, because for most synthetic cathinones, the parent drug is often detected in urine specimens in high concentrations urine [116,119,128,129,156,157,215,225,241], along with metabolites.

Little is known about the prevalence of synthetic cathinones despite the growing media attention and regulation of many of these cathinone derivatives. Kriikku *et al.* reported the prevalence of MDPV among drivers apprehended for DUID in Finland from August 2009 to August 2010. The study found that 5.7% (*n*=259) of all confirmed DUID cases, excluding alcohol-only cases (*n*=4570), tested positive for MDPV [108]. A recent study administered a self-report survey to 2349 students at a large university in the Southeastern United States and found only 25 (1.0%) students reporting at least one synthetic cathinone intake in their lifetime [109]. These authors hypothesized that the low prevalence of cathinone use suggests there may not be an epidemic of illicit use of these compounds. Similarly, the overall presumptive positive rate (0.53%) and presumptive positive confirmation rate (4.1%) were low in this study. However, due to storage

conditions and stability issues with synthetic cathinones it is difficult to draw definitive conclusions.

Although the original DOA-V assay had a sensitivity of 100%, its specificity and efficiency were low (52.1% and 53.0%), potentially due to stability issues. As discussed previously, assay performance may be improved with alternative cutoffs. The Randox DOA-V Biochip Array Technology paired with the Randox Evidence® Analyzer is capable of testing 90 samples for 11 different analytes in approximately one to two hours. In particular, it is a sensitive assay capable of identifying urinary synthetic cathinones. To date, this is the only commercially available immunoassay capable of detecting synthetic cathinones in urine.

Several confirmation and screening methods utilizing LC-MS/MS and GC-MS were previously published for the determination of synthetic cathinones in urine [158,170,171,173,174,186,187,194,213-216,225,228]. Previous analytical methods included no more than 12 synthetic cathinones, while this recently developed LC-HRMS confirmation method includes 24 synthetic cathinones and four metabolites [150]. It is difficult for immunoassay screening methods to detect new emerging synthetic cathinones, as they are limited to the antibody's affinity and cross-reactivity to the target compound. LC-MS/MS methods incorporating a wide range of cathinones in a rapid screening method is better suited to detect novel designer drugs compared to immunoassays for high-throughput laboratories, due to the long lead time to raise antibodies to new synthetic cathinones and to optimize new commercial immunoassays for newly introduced designer drugs. However, it is difficult for laboratories to conduct high throughput chromatographic assays.

Conclusion

This is the first method validation of the Randox Biochip Array Technology for the detection of synthetic cathinones in urine. Results demonstrated a large negative percent bias outside of acceptable limits for the detection of BSI (mephedrone) and BSII (MDPV). Cross-reactivity data in authentic urine were provided for many synthetic cathinones to suggest compounds that might or might not be detected by the assay. A smaller number of synthetic cathinone cross-reactivity data were provided in the package insert, but data were acquired with synthetic cathinones in calibrator base (phosphate buffer), rather than urine. The Randox DOA-V biochip array is currently the only immunoassay available for preliminary detection of synthetic cathinones in urine, and also is capable of high-throughput and requires only a small urine sample volume on the Evidence® Analyzer. This study provides critical data for scientists faced with the need to identify potential synthetic cathinone intake in clinical and forensic cases.

Table 36. Interferences analyzed for BSI and BSII with the Randox DOA-V assay.

Sample Code	Compounds Analyzed	Target Concentration
INTFMIX001	buprenorphine, norbuprenorphine	1 mg/L
INTFMIX002	cocaine, benzoylecgonine, norcocaine, nor-benzoylecgonine	1 mg/L
INTFMIX003	ecgonine ethyl ester, ecgonine methyl ester, anhydroecgonine methyl ester, ecgonine	1 mg/L
INTFMIX004	cocaethylene, norcocaethylene	1 mg/L
INTFMIX005	m-hydroxy-cocaine, p-hydroxy-cocaine, m-hydroxy-benzoylecgonine, p-hydroxy-benzoylecgonine	1 mg/L
INTFMIX007	Δ^9-tetrahydrocannibinol (THC), 11-hydroxy-Δ^9-tetrahydrocannibinol (11-OH-THC), 11-nor-9-carboxy-Δ^9-tetrahydrocannibinol (THC-COOH)	1 mg/L
INTFMIX008	morphine, normorphine, morphine-3-glucuronide, morphine-6-glucuronide	1 mg/L
INTFMIX009	codeine, norcodeine, 6-acetylmorphine, 6-acetylcodeine	1 mg/L
INTFMIX010	hydrocodone, hydromorphone, oxycodone	1 mg/L
INTFMIX011	noroxycodeine, oxymorphone, nor-oxymorphone	1 mg/L
INTFMIX012	propoxyphene, 3,4-methylenedioxyphenyl-2-butanamine (BDB), N-methyl-BDB	1 mg/L
INTFMIX013	R-cathinone, ethylamphetamine, 4-bromo-2,5-dimethoxyphenethylamine (2C-B)	1 mg/L
INTFMIX014	diazepam, lorazepam, oxazepam, alprazolam	1 mg/L
INTFMIX015	imipramine, clomipramine, fluoxetine, norfluoxetine	1 mg/L
INTFMIX016	parexetine, 7-aminoclonazepam, 7-aminoflunitrazepam, 7-aminonitrazepam	1 mg/L
INTFMIX017	clonidine, ibuprofen, pentazocine, caffeine	1 mg/L
INTFMIX018	diphenydramine, chlorpheniramine, brompheniramine	1 mg/L
INTFMIX019	acetylsalicylic acid, acetaminophen, phencyclidine	1 mg/L
INTFMIX020	bromazepam, clonazepam, flurazepam	1 mg/L
INTFMIX021	nitrazepam, flunitrazepam, temazepam, nordiazepam	1 mg/L
INTFMIX022	nicotine, cotinine, hydroxy-cotinine, Nnrcotinine	1 mg/L
INTFMIX023	methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP)	1 mg/L
INTFMIX024	paramethoxyamphetamine, paramethoxymetamphetamine	1 mg/L
INTFMIX025	methamphetamine, amphetamine, hydroxyamphetamine, hydroxymethoxyamphetamine, methylenedioxymethamphetamine, methylenedioxyamphetamine, methylenedioxyethylamphetamine	1 mg/L

Sample Code	Compounds Analyzed	Target Concentration
INTFMIX026	hydroxymethoxymethamphetamine	1 mg/L
INTFMIX027	Ephedrine, pseudoephedrine	1 mg/L
INTFMIX028	ketamine, dextromethorphan, phentermine	1 mg/L
INTFMIX029	cannabigerol	1 mg/L
INTFMIX030	L-ascorbic acid	4 g/L
INTFMIX031	sodium chloride	40 µg/L
INTFMIX032	creatinine	7.5 g/L
INTFMIX033	glucose	30 g/L
INTFMIX034	riboflavin	75 mg/L
INTFMIX035	human serum albumin (HSA)	5 g/L
INTFMIX036	hemoglobin	1.5 g/L
INTFMIX037	urea	35 g/L
INTFMIX038	oxalic acid	1 µg/L
INTFMIX039	ranitidine	100 mg/L
INTFMIX040	ethanol	5 g/L
INTFMIX041	acetone	10 g/L
INTFMIX042	THC-glucuronide, THC-COOH-glucuronide	1 mg/L
INTFMIX043	cannabidiol, cannabinol	1 mg/L
INTFMIX044	urine (pH 6.5) adjusted to pH 4 with acetic acid	-
INTFMIX045	urine (pH 6.5) adjusted to pH 8 with potassium phosphate dibasic	-
INTFMIX046	JWH018	100 µg/L
INTFMIX047	JWH018, 10% Bleach	100 µg/L
INTFMIX048	JWH018, 10% Peroxide	100 µg/L
INTFMIX049	JWH250	100 µg/L
INTFMIX050	JWH250, 10% Bleach	100 µg/L
INTFMIX051	JWH250, 10% Peroxide	100 µg/L
INTFMIX052	mephedrone	10 µg/L
INTFMIX053	mephedrone, 10% Bleach	10 µg/L
INTFMIX054	mephedrone, 10% Peroxide	10 µg/L
INTFMIX055	MDPV	275 µg/L
INTFMIX056	MDPV, 10% Bleach	275 µg/L
INTFMIX057	MDPV, 10% Peroxide	275 µg/L

Chapter 8 - Quantification of methylone and metabolites in rat and human plasma by liquid chromatography-tandem mass spectrometry

(*As published in* Forensic Toxicology, *2015*)[8]

Abstract

Methylone is a commonly abused synthetic cathinone marketed as a "legal" alternative to "ecstasy" or cocaine. Previous studies examined the metabolism of methylone in vitro and in vivo; HMMC was identified as the primary metabolite, with other reported minor metabolites, 3,4-methylenedioxycathinone (MDC) and 3,4-dihydroxymethcathinone (HHMC). However, limited information is known about methylone and its metabolites' pharmacokinetics. We developed and fully validated a method for the simultaneous quantification of methylone, HMMC, MDC and HHMC by liquid chromatography-tandem mass spectrometry in 100 µL rat and human plasma. β-Glucuronidase was utilized for plasma hydrolysis, followed by perchloric acid protein precipitation and solid-phase extraction utilizing cation exchange columns. Chromatographic separation was performed with a Synergi Polar column in gradient mode, and analytes were determined by two MRM transitions. Linear ranges of 0.5-1,000 µg/L (methylone, HMMC and MDC) and 10-1,000 µg/L (HHMC) were achieved. Bias and imprecision were acceptable, although quantitation of HHMC exhibited variability (16.2-37%). Extraction efficiencies and ion suppression were 89.9-104% (HHMC, 15.9-16.2%) and <11.4%, respectively. Methylone and metabolites were stable in plasma for 24 h at room temperature, 72 h at 4ºC, and after three freeze-thaw cycles (except a 60% HHMC increase). Human and rat plasma were cross-validated, documenting that rat plasma

[8] Ellefsen K.N. *et al.* Quantification of methylone and metabolites in rat and human plasma by liquid chromatography-tandem mass spectrometry. *Forensic Toxicology*. 2015; 7(16): 2041-56.

quality control samples were accurately quantified against a human plasma calibration curve (-23.8 to 12% bias). As proof of method, rat plasma specimens were analyzed pre-injection and after subcutaneous administration of methylone at 6 mg/kg from 15 to 480 min post-dosing. Methylone, HMMC, MDC and HHMC concentrations ranged from 1.1-1,310 µg/L, 11.2-194 µg/L, 1.9-152 µg/L and 24.7-188 µg/L, respectively.

Introduction

Abuse of novel psychoactive substances is an ongoing problem around the world. Synthetic cathinones appeared in the US market in 2010 as legal alternatives to MDMA, amphetamine, or cocaine [98]. They are stimulant-like drugs derived from cathinone, the active ingredient of the khat plant *Catha edulis*. In 2011, the AAPC received 6,138 synthetic cathinones calls [100]. As a result of this increased presence, the synthetic cathinones MDPV, mephedrone, and methylone were regulated as Schedule I controlled substances in the US. Despite scheduling, methylone remains one of the 25 most frequently identified drugs in the US [289].

Reports of synthetic cathinones from state and local forensic laboratories increased from 142 reports in the first half of 2010 to 7,997 during the first half of 2013 [98], with methylone detected in 65% of reports in 2013. The DEA and CBP also detected methylone in just over half (51%) of the reported 518 synthetic cathinone cases from January to June 2013 [98]. A recent study administered a self-reported survey to students at a large university in the Southeastern US and found that 1.1% of the population surveyed (n=2,394) used synthetic cathinones at least once in their lifetime [315]. Another study analyzing 34,561 urine samples submitted for designer stimulant

testing, between February 2011 and January 2013, reported methylone as the 4th most detected synthetic cathinone (*n*=186) only behind α-PVP, MDPV, and pentedrone [112].

From a chemical structure perspective, methylone only differs from MDMA by the presence of a keto moiety on the beta carbon (Figure 25a). It was initially patented as an anti-depressant and anti-Parkinsonian drug in 1996; however, it was never developed into a pharmaceutical product [316,317]. Drug users take methylone for its euphoric and psychostimulant properties, but its reported adverse effects include increased body temperatures, agitation, confusion, psychosis, seizures, and tachycardia [95,96,116]. Additionally, several fatalities were linked to methylone consumption [127,128,156,158,257,258].

Limited data are available on methylone's pharmacology. Recent preclinical studies demonstrated that like other stimulants, methylone interacts with the monoamine transporters [134-137]. Baumann et al. [135] reported that methylone acts as a fully efficacious transporter releaser at DA, NE, and 5-HT transporters, to produce *in vivo* neurochemical effects resembling MDMA. The *in vitro* metabolism of methylone was studied in HLM [161,172], with the identification of various metabolites including dihydromethylone, normethylone or MDC, *N*-hydroxymethylone, 3-hydroxy-methylenedioxymethcathinone, and most notably HHMC. *In vivo* studies analyzing rat urine after methylone administration, and human urine from acutely intoxicated individuals, revealed HMMC as the primary metabolite in both species [170,174]. Other minor metabolites included the *N*-demethylated metabolite (MDC), and 3-hydroxy-4-methoxymethcathinone. These studies also reported that hydroxylated methylone metabolites were primarily conjugated. A recent study

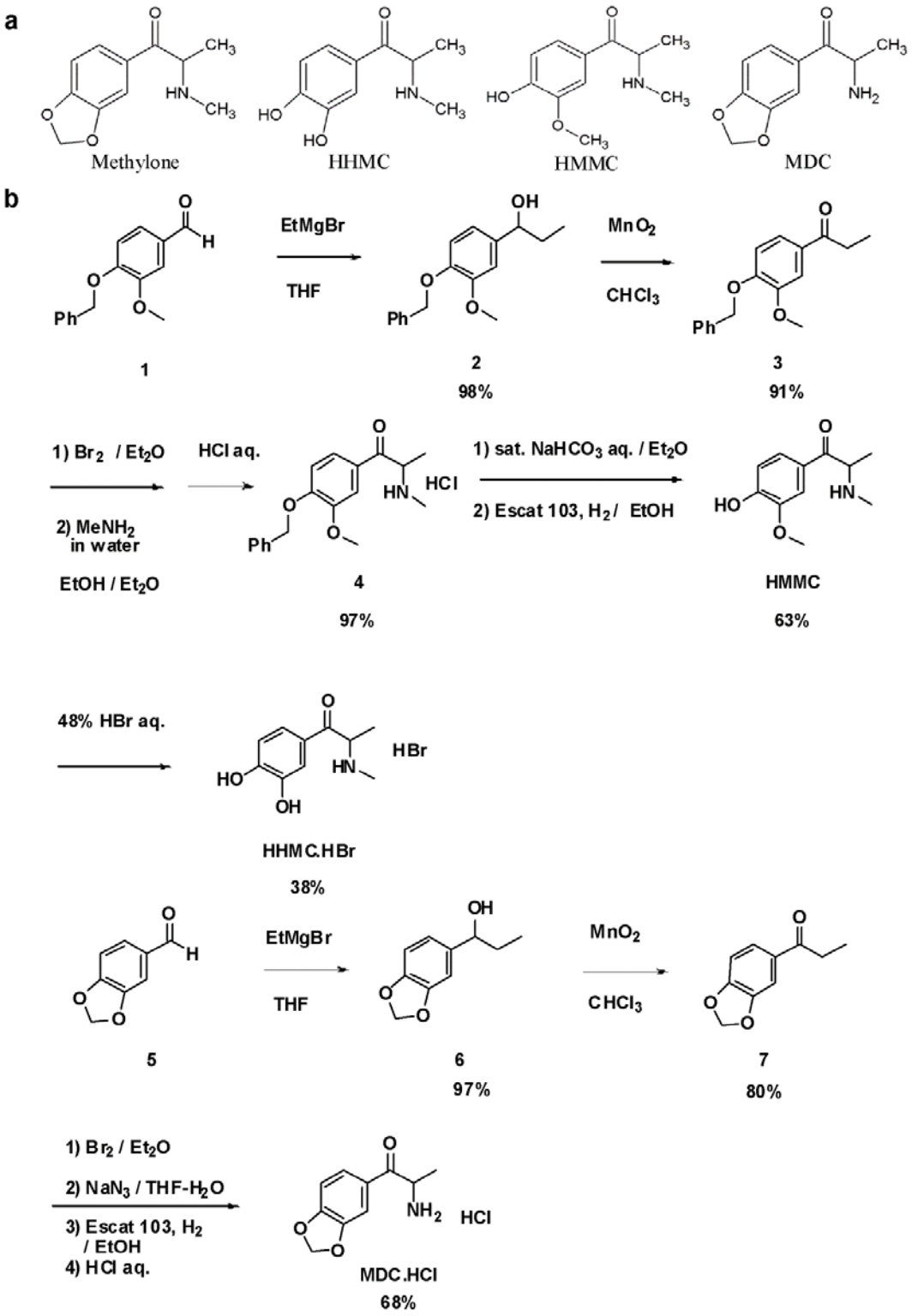


Figure 25. a) Structures of methylone, 3,4-dihydroxymethcathinone (HHMC), 4-hydroxy-3-methoxymethcathinone (HMMC), and 3,4-methylenedioxycathinone (MDC), b) Chemical synthesis of HMMC, HHMC-HBr, and MDC-HCl.

investigating oral and intravenous methylone administration in rats identified four metabolites in rat blood: HMMC, 3-hydroxy-4-methoxymethcathinone, MDC, and 3-hydroxy-methylenedioxymethcathinone. The intermediate metabolite HHMC, a precursor of the two hydroxylated metabolites (HMMC and 3-hydroxy-4-methoxymethcathinone), was not detected potentially due to a relatively short half-life [152].

Currently, there are no published quantitative methods for the analysis of methylone and its main metabolites in plasma. It is critical to develop assays to detect methylone and metabolites to determine their presence and concentrations in forensic and clinical settings. Also, it is important to quantitatively characterize methylone pharmacokinetics and its metabolites in rodent models, since preclinical safety data are needed before controlled administration studies in humans can be conducted. Here, we report the development and full validation of a method for the simultaneous quantification of methylone, HMMC and MDC, and the dihydroxy intermediate HHMC by LC-MS/MS. This method will be utilized to characterize the pharmacokinetics of methylone and its metabolites after controlled administration in rats.

Materials and methods

Chemicals and Materials

Methylone (1 g/L) and methylone-d_3 (100 mg/L) were purchased from Cerilliant (Round Rock, TX, USA). HMMC, MDC and HHMC were synthesized and purified by the Drug Design and Synthesis Section of the NIDA, Intramural Research Program, Baltimore, MD, USA, in powder form. SPE was performed with SOLA SCX 10 mg 1 mL cartridges (Thermo Scientific, Fremont, CA, USA). Glacial acetic acid, formic acid (FA), hydrochloric acid HCl (36.5-38%), methanol, acetonitrile, water, methylene

chloride, ethylenediaminetetraacetic acid (EDTA) and sodium metabisulfite (SMBS) were purchased from Fisher Scientific (Fair Lawn, NJ, USA); isopropranol and 4-methylcatechol from Sigma (Milwaukee, WI, USA) and ammonium hydroxide (28-30%) and perchloric acid (69-72%) from JT Baker (Phillipsburg, NJ, USA). All solvents employed in the extraction were of the HPLC grade and LC-MS grade in the chromatographic system. β-Glucuronidase from Red Abalone (>100,000 U/mL) was obtained from Kura Biotec (Inglewood, CA, USA). Water for EDTA, 4-methylcatechol and SMBS solution preparation was purified in-house by an ELGA Purelab Ultra Analytic purifier (Siemens Water Technologies, Lowell, MA, USA).

Chemical Synthesis of 3,4-dihydroxymethcathinone, 4-hydroxy-3-methoxymethcathinone, and 3,4-methylenedioxycathinone

The synthetic route to HHMC, HMMC, and MDC is shown in Figure 25b. These compounds were synthesized according to the procedures of Kamata et al. [174] with modifications. Briefly, commercially available benzaldehyde **1** was treated with EtMgBr to produce the desired alcohol **2** in 98% yield. Oxidation of **2** was easily accomplished with manganase dioxide in refluxing chloroform to create a ketone **3** in 91% yield. The ketone **3** was brominated to give the crude bromide. The crude bromide was treated with methyl amine to give crude **4** that was converted to the pure HCl salt **4**. Hydrogenolysis of the free base of **4** gave the desired HMMC in 63% yield. HMMC was treated with 48% HBr aq. to produce the HBr salt (HHMC-HBr) in 38% yield. Reaction of a commercially available aldehyde **5** with EtMgBr created the desired alcohol **6** in 97% yield. Oxidation of **6** was carried out with manganase dioxide in refluxing chloroform to give a ketone **7** in 80% yield. Bromination of **7** provided a crude product that was treated

with NaN_3 to give the desired azide derivative. Hydrogenolysis of azide resulted in the crude MDC that was converted to the pure HCl salt (MDC-HCl) in 68% yield.

Instrumentation

LC-MS/MS was performed on a Shimadzu LC system (Columbia, MD, USA) coupled with an ABSciex 3200 QTrap® mass spectrometer with a TurboIonSpray source (Foster City, CA, USA). The LC system consisted of a DGU-20A3 degasser, LC-20ADXR pumps, and a CTO-10AC column oven. SPE was performed with a negative pressure manifold. Evaporation under nitrogen was completed using a TurboVap LV® evaporator from Zymark (Hopkinton, MA, USA).

Preparation of Standard Solutions

Stock solutions (1 g/L) of all analytes were prepared in methanol. Calibrator working solutions were prepared in 3% 250 mM SMBS, 3% 250 mM EDTA in 0.01 M FA (SMBS+EDTA+FA mixture) at 0.5, 1, 5, 10, 25, 50, 100, 500 and 1000 µg/L. Quality control (QC) working solutions also were prepared in the same manner, on different days, at 1.5 (low 1), 30 (low 2), 300 (medium), and 750 µg/L (high). An IS solution (100 µg/L) of methylone-d_3 also was prepared in the SMBS+EDTA+FA mixture.

Animals and Surgery

Methylone and its metabolites were quantified in rat plasma specimens obtained after SC administration of methylone at 6.0 mg/kg. To obtain pooled plasma for hydrolysis optimization, twelve male Sprague-Dawley rats were anesthetized with 60 mg/kg pentobarbital and indwelling jugular catheters were surgically implanted. One week after surgery, rats received a methylone injection in the home cage, and 4 h later

single blood specimens (1200 µL) were withdrawn via the catheter and transferred to collection tubes. Blood was centrifuged at 3,000 rpm for 10 min at 4°C. Plasma from all rats was combined into a single plasma pool and stored at -80°C until analysis. For the time-course study, six male Sprague-Dawley rats received surgically-implanted jugular catheters under pentobarbital anesthesia as noted above. One week after surgery, rats were brought from the vivarium to the laboratory in their home cages, and polyethylene extension tubes were connected to the catheters. Rats received 0.5 mL IV 48 IU/mL heparin saline, and control blood specimens (300 µL) were collected via the catheter. Immediately thereafter, rats received SC injection of methylone at 6 mg/kg, with blood specimens collected at 15 min, 30 min, 1, 2, 4, and 8 h post-injection. Plasma was collected and specimens from each individual rat were stored at -80°C prior to analysis as described above. Animal use procedures followed the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the National Institute on Drug Abuse Animal Care and Use Committee.

Sample Preparation

Sample preparation was modeled after previously described methods [206,318], with modifications. Twenty microliters of 250 mM SMBS, 10 µL of 250 mM EDTA, 50 µL of internal standard, and 100 µL of either standard or SMBS+EDTA+FA mixture for authentic specimens were added to 100 µL rat plasma in 1.5 mL microcentrifuge tubes and gently vortexed. Cleavage of metabolite conjugates was performed by incubating the specimens with 10 µL β-Glucuronidase with the specimens at 50°C for 60 min. Specimens were then cooled to room temperature before addition of 20 µL 4-

methylcatechol. Protein precipitation was performed with 10 µL concentrated perchloric acid followed by centrifugation (15,000 x *g*, 10 min).

To further improve analyte recovery and reduce matrix effects, supernatants were loaded onto SOLA SCX cartridges preconditioned with methanol (500 µL) and water (500 µL) utilizing a negative pressure manifold. Columns were washed with 1 M acetic acid (500 µL) and methanol (500 µL) before drying columns under nitrogen using negative pressure at 10 psi for 5 min. Elution was performed with 5% ammonium hydroxide in methylene chloride/isopropanol (60:40 v/v). Acidic methanol (1% HCl) (50 µL) was added to eluates prior to evaporating to dryness under nitrogen for 20 min at 40ºC. Specimens were reconstituted in 200 µL mobile phase A (0.1% formic acid in water), vortexed briefly, and centrifuged (4,000 x *g*, 5 min).

Hydrolysis Optimization and Performance

Pooled rat plasma specimens, were utilized to optimize hydrolysis parameters, as conjugated methylone metabolite reference materials were not commercially available. Temperature (37, 50, and 70ºC), incubation time (60, 90, and 120 min) and amount of enzyme (10, 20, and 40 µL) were evaluated against standard conditions, previously described in Sample Preparation (50ºC, 60 min, 10 µL), in triplicate to maximize the recoveries of methylone and its metabolites. One-way ANOVA followed by Tukeys *post-hoc* test evaluated hydrolysis conditions. Data were considered significant if $P < 0.05$. In addition, after hydrolysis conditions were optimized, authentic specimens were analyzed with and without hydrolysis, and evaluated with a paired *t*-test for significance.

Liquid Chromatography-Tandem Mass Spectrometry

Chromatographic separation was achieved at 40ºC with a Phenomenex Synergi Polar-RP column (100 x 2 mm, particle size 2.5 µm) and identically packed defender guard cartridges (10 x 20 mm, particle size 2.5 µm). Gradient elution was performed with 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) at 0.3 mL/min of flow rate. Initial composition (5% B) was increased to 50% over 5 min, from 50% to 95% over 0.5 min, held at 95% for 1 min, and returned to initial conditions over 0.5 min. A 2 min equilibration followed, yielding a total run time of 9 min.

MS conditions were: interface, electrospray ionization (ESI) in positive mode; IonSpray voltage, 3.5 kV; capillary temperature, 600ºC; curtain gas, 50; ion source gas 1 and 2,50 and 30, respectively; nitrogen collision gas, medium for all experiments; dwell time, 50 ms; collision cell exit potential (CXP), 4. Analyst software version 1.5.1 was used for data collection and processing. Multiple reaction monitoring transitions and MS parameters for methylone, methylone-d_3, HMMC, MDC, and HHMC are outlined in Table **37**.

The following criteria identified compounds: presence of two characteristic transitions, ion ratio of the quantifying ion/qualifying ion within ±20% of the average of all calibrators, and RT within ±0.2 min.

Table 37. Parameters for analysis of methylone, its metabolites and internal standard by liquid chromatography- tandem mass spectrometry.

Analyte	Precursor ion (*m/z*)	Product ions (*m/z*)	DP	EP	CEP	CE	RT (min)
HHMC	196.1	**160.1**, 132.2	31	3.5	12	21, 31	1.93
HMMC	210.1	**160.2**, 132.3	26	5.5	18	25, 39	2.87
MDC	194.1	**146.0**, 118.1	31	4	14	21, 35	3.39
Methylone	208.2	**160.2**, 132.2	36	5	14	23, 35	3.67
Methylone-d_3	211.1	**163.1**, 135.1	26	11	18	25, 35	3.67

Quantification ions are in bold

DP declustering potential, *EP* entrance potential, *CEP* cell exit potential, *CE* collision energy, *HHMC* 3,4-dihydroxymethcathinone, *HMMC* 4-hydroxy-3-methoxymethcathinone, *MDC* 3,4-methylendixoycathinone, *RT* retention time

Method Validation

Method validation was performed based on the standard practices for method validation of the SWGTOX [264]. Validation parameters included linearity, LOD, LOQ, bias and imprecision, ionization suppression/enhancement, extraction efficiency, process efficiency, interference studies, carryover, dilution integrity and autosampler and short-term stability studies. Linearity (r^2) was determined by least squares regression with ≥6 nonzero calibrators on 5 days. Acceptable linearity was achieved when r^2 ≥0.99 and calibrators quantified within ±15%. LODs and LOQs were evaluated with decreasing analyte concentrations; LOD was the lowest concentration with acceptable chromatography, signal/noise ratio ≥3, with the ratio of ions within ±20% of the average ratio of the calibrators. LOQs were the lowest concentrations that met LOD criteria, a signal/noise ratio of at least 10, bias and imprecision within ±20% of target concentrations. LOD and LOQ were evaluated in triplicate on three different days (n=9).

Assay bias and imprecision were determined at four concentrations (1.5 µg/L (low 1), 30 µg/L (low 2), 300 µg/L (medium) and 750 µg/L (high)) in triplicate over five days (overall n=15). Bias was evaluated for each concentration as the percent error.

Acceptable bias was ±20% of target. Imprecision was expressed as percent coefficient of variation (%CV) and determined by the one-way ANOVA approach to calculate combined within- and between-run imprecision. Acceptable imprecision was ≤15 %CV.

Ion suppression/enhancement, extraction efficiency, and process efficiency for each analyte were measured at low (30 µg/L) and high (750 µg/L) QC concentrations as described by Matuszewski et al. [285]. Ion suppression/enhancement was assessed by comparing analyte peak areas of neat samples (*n*=6) (Set 1) to peak areas of ten different blank samples fortified with analyte and internal standard after extraction (Set 2). Extraction efficiency was examined by comparing analyte peak areas of five different samples fortified at low and high concentrations with internal standard before extraction (Set 3), to peak areas of Set 2. Process efficiency examined the overall effect of extraction efficiency and ion suppression/enhancement on quantification of analytes and was calculated by comparing mean peak areas of Set 3 with mean peak areas of Set 1 at low and high concentrations.

Endogenous and exogenous interferences were evaluated. Interferences from endogenous matrix components were investigated by analyzing plasma samples from nine individuals and one rat without the addition of internal standard. Exogenous interferences including an amphetamine mixture (amphetamine, methamphetamine, MDMA, MDEA, MDA, and phentermine), a stimulant mixture (BE, cocaethylene, cocaine, and EME), and a benzodiazepine mixture (alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam, and temazepam) were analyzed in neat samples with interferences equivalent to 1,000 µg/L. Interferences were considered insignificant if analytes of interest were <LOD. Lack of carryover was demonstrated by

injecting triplicate internal standard-fortified blank samples after a sample fortified with analytes at 2,500 µg/L. Carryover was considered negligible if the measured concentration was <LOD.

Dilution integrity was evaluated by diluting 2,500 and 1,500 µg/L samples in SMBS+EDTA+FA mixture to achieve 10-fold and 2-fold dilutions, respectively. After internal standard addition, samples were extracted as previously described. Dilution integrity was maintained if samples quantified within ±20% of target. Autosampler stability was investigated by reinjecting low (30 µg/L) and high (750 µg/L) QC samples stored 48 h at 4ºC on the autosampler (*n*=3) and calculating results against the original calibration curve. In addition, short-term stability was evaluated with plasma fortified at low and high QC concentrations and stored for 24 h at room temperature (*n*=3), 72 h at 4ºC (*n*=3) and -20ºC (*n*=3), and after three freeze-thaw cycles (*n*=3). IS was added to each sample just prior to extraction and processed as described. Stability was considered acceptable if QC samples quantified within ± 20% of freshly prepared QC samples (*n=3*).

Results

Hydrolysis Optimization and Performance

Hydrolysis conditions, including temperature, time, and enzyme amount, were evaluated for optimal recovery of methylone and metabolites. Overall significant differences between hydrolysis conditions were observed for methylone, HMMC, and MDC, with significant decreases in peak areas when specimens were incubated at 70ºC versus 50ºC (P <0.05). No significant differences were observed between amounts of enzyme utilized and times of incubation. Therefore, to save on cost and time, 10 µL enzyme and 60 min incubation were utilized for hydrolysis. No significant differences

were observed between hydrolysis conditions for HHMC. Therefore, optimal recovery of methylone and metabolites was achieved when specimens were incubated at 50ºC for 60 min with 10 µL enzyme.

Methylone and metabolite concentrations in rat plasma, with and without hydrolysis (n=3 each), were examined 4 h after rats were administered 6 mg/kg methylone subcutaneously. Methylone (with hydrolysis 26.5 µg/L; without hydrolysis 26.4 µg/L) and MDC (with hydrolysis 42.7; without hydrolysis 41.6 µg/L) mean concentrations did not change significantly with or without hydrolysis; however, HMMC (with hydrolysis 183.0 µg/L; without hydrolysis 2.8 µg/L) and HHMC (with hydrolysis 38.4 µg/L; without hydrolysis <LOQ) mean concentrations significantly decreased (P <0.05) without the deconjugation step to concentrations near or below their respective LOQs.

Method Validation

Linearity of analyte-to-internal standard peak area ratio versus theoretical concentration was verified in plasma samples from 0.5 (methylone, HMMC and MDC) or 10 (HHMC) to 1000 µg/L with $1/x^2$ weighted linear regression. The $1/x^2$ weighting was selected for this method as correlation coefficients were acceptable and data demonstrated homoscedasticity, or random distribution of the individual residuals around the zero line, suggesting that this linear model was optimal in comparison to other models. Calibration curves from five separate days yielded determination coefficients (r^2) above 0.99 ± 0.002 with residuals within ±15%, except HHMC (0.986 ± 0.014). LODs were 0.25 µg/L for all analytes except HHMC (10 µg/L), and LOQs were 0.5 µg/L for all

analytes except HHMC (also 10 µg/L). Figure 26a shows a chromatogram of a human plasma sample with all analytes at LOQs.

Bias and imprecision results are presented in Table 38. For all analytes, bias was between -16.8 to 9.5%, and within- and between-run imprecision was < 8.8%, except for HHMC, which displayed greater imprecision (16.2-37.0%). Extraction efficiencies and process efficiencies were 89.9-104% and 83.3-96.9%, respectively, for methylone, HMMC, and MDC (Table 2). HHMC exhibited extraction efficiencies between 15.9-16.2% and process efficiencies between 15.2-15.6%. Ion suppression was less than 11.4% for all analytes with %RSD < 12.7 (Table 38).

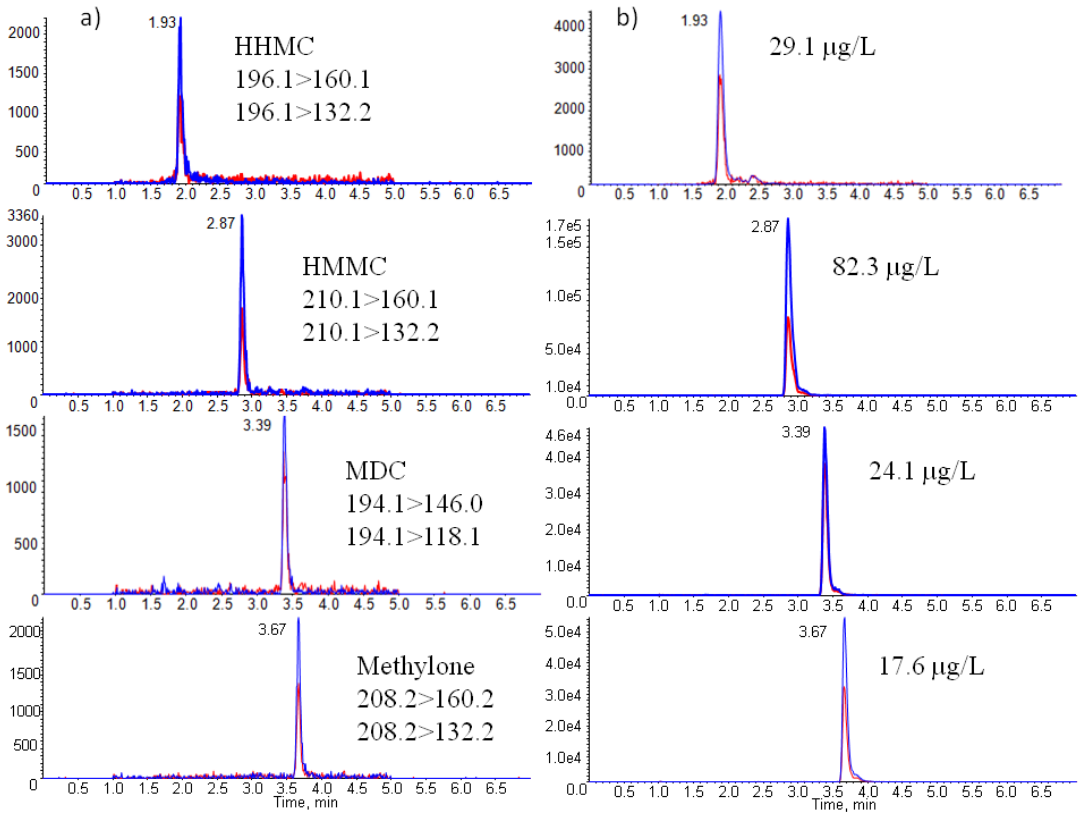


Figure 26. Multiple reaction monitoring chromatograms for a) LOQs for HHMC (10 µg/L), HMMC, MDC, and methylone (0.5 µg/L) fortified in human plasma b) authentic rat plasma specimen for HHMC, HMMC, MDC and methylone after subcutaneous administration of methylone at 6 mg/kg collected 4 h post-dosing.

Table 38. Within-run, between-run imprecision and % bias for methylone, HMMC, MDC and HHMC in plasma at low1 (1.5 µg/L), low2 (30 µg/L), medium (Med) (300 µg/L), and high (750 µg/L) concentrations and analyte extraction efficiency, process efficiency, and ion suppression/enhancement in human plasma at low (30 µg/L) and high (750 µg/L) concentrations.

	Methylone				HMMC				MDC				HHMC			
	Low_1	Low_2	Med	High	Low_1	Low_2	Med	High	Low_1	Low_2	Med	High	Low_1	Low_2	Med	High
Within-run imprecision (*n*=15, %CV)	5.8	4.2	2.7	3.2	5.9	5.8	1.7	2.9	5.3	5.0	3.4	4.6	-	28.5	20.0	26.2
Between-run imprecision (*n*=15, %CV)	5.4	4.3	3.2	5.7	8.8	5.5	2.4	3.0	5.0	6.5	4.3	5.8	-	37.0	21.2	26.8
% Bias (*n*=15)	0.2	1.8	-5.2	-4.0	-6.7	9.5	-13	-16.8	-4.1	-3.0	-1.5	4.1	-	-12.8	-5.2	6.7
Extraction efficiency % (*n*=5)	-	104	-	97.2	-	104	-	89.9	-	102	-	90.7	-	15.9	-	16.2
Process efficiency % (*n*=5)	-	91.7	-	88.7	-	96.9	-	87.8	-	90.1	-	83.3	-	15.2	-	15.6
Ion suppression/ enhancement (%RSD) *n*=10	-	-11.4 (12.7)	-	-8.7 (5.5)	-	-6.8 (8.7)	-	-2.4 (1.1)	-	-11.3 (10.5)	-	-8.2 (4.4)	-	-4.6 (11.1)	-	-3.4 (3.8)

Under described conditions, no interference from any extractable endogenous plasma compound was observed in 9 human plasma samples, and 1 rat plasma sample. Addition of potentially interfering drugs and metabolites at 1,000 µg/L to neat samples did not produce any interfering peaks. No carryover was observed for all four analytes after a sample fortified at 2,500 µg/L.

Dilution integrity was evaluated for 1:2, and 1:10 dilutions. Diluted samples (750 µg/L targeted) were quantified within -16.9 to 4.1% of target (*n=2*) for a 1:2 dilution, and within -7.4 to 0.6% of target (250 µg/L) (*n=2*) for a 1:10 dilution for methylone, HMMC, and MDC. Extracted analytes were stable in the autosampler for 48 h with % differences between -11.5 and 3.9% (Table **39**). When stored at room temperature for 24 h, at 4ºC for 72 h, and after three free-thaw cycles methylone, HMMC, and MDC were stable (-15.8 to -0.2%); however, HHMC was not stable (-14.1 to 60.6%).

Table 39. Stability data (% difference) for methylone, HMMC, MDC, and HHMC at low (30 µg/L) and high (750 µg/L) concentrations after storage in autosampler at 4ºC for 48 h, at room temperature for 24 h, at 4ºC for 72 h, and after 3 freeze-thaw cycles (over 72 h).

Stability conditions (*n*=3)	Methylone		HMMC		MDC		HHMC	
	Low	High	Low	High	Low	High	Low	High
48 h at 4ºC in autosampler	0.9	-11.5	3.5	-7.2	3.9	-8.5	3.5	3.2
24 h at room temperature	-6.2	-14.2	-3.1	-11.2	-8.1	-14.0	33.6	-14.1
72 h at 4ºC	-6.5	-15.8	-4.4	-10.9	-5.2	-13.2	-3.1	-10.6
3 freeze-thaw cycles	-1.3	-14.6	-0.2	-10.6	-5.5	-11.3	60.6	37.3

Human-rat plasma cross validation was investigated by assaying rat plasma from drug-naïve rats fortified at all QC concentrations (*n=5*) against a calibration curve fortified into human plasma. Imprecision (%CV) was < 11.5% and % bias between -16.9 to 12.1% for methylone, HMMC, and MDC (Table **40**). For HHMC bias ranged from -23.8 to -17.1% with %CV < 25.7%. Due to the poor validation performance of HHMC, concentrations were considered semi-quantitative.

Table 40. Human-rat plasma cross validation data for methylone, HMMC, MDC, and HHMC including % bias and % CV of fortified rat plasma at low 1 (1.5 µg/L), low 2 (30 µg/L), medium (300 µg/L), and high (750 µg/L) concentrations against a human plasma calibration curve.

Analyte	% Bias (%CV) (*n*=5)			
	Low1	Low2	Med	High
Methylone	12.1 (11.5)	-2.9 (3.7)	-0.3 (4.1)	-5.3 (3.9)
HMMC	-9.7 (10.7)	4.0 (2.3)	-12.5 (4.9)	-16.9 (2.0)
MDC	5.3 (9.0)	-11.8 (5.2)	1.1 (5.3)	-0.2 (1.7)
HHMC	-	-17.1 (15.1)	-23.8 (12.4)	-18.3 (25.7)

Authentic Specimens

As proof of method, plasma specimens from individual rats were analyzed for methylone and metabolites following SC administration of methylone at 6 mg/kg from 15 to 480 min post-dosing. In the specimen collected before dosing, all analytes were absent. After SC dosing, all specimens were positive for methylone, HMMC, MDC and HHMC with concentrations ranging from 1.1 to 1,310 µg/L, 11.2 to 194 µg/L, 1.9 to 152 µg/L and 24.7 to 187 µg/L, respectively.

Discussion

A sensitive and specific LC-MS/MS method for the detection of methylone and its metabolites, HMMC, MDC and HHMC in plasma was developed and validated. Currently, there are no published quantitative methods for the analysis of methylone and

its main metabolites in plasma. A previous study quantified methylone only in rat plasma specimens obtained via repeated venipuncture under isoflurane anesthesia, with a 10 µg/L LOQ in 100 µL plasma [152]. In the present study, we obtained repeated plasma specimens from unanesthetized freely-behaving rats bearing indwelling jugular catheters. Our LOQs were 0.5 µg/L for methylone, HMMC and MDC, and 10 µg/L for HHMC, in 100 µL plasma. Lower LOQs may be achieved with a larger specimen volume; however, a limitation with pharmacokinetic animal studies is the low amount of specimen available for analyses. Methylone blood concentrations from reported intoxication and fatality cases ranged from 7 to 3,400 µg/L [127,128,156,158,257,258]; therefore, LOQs in this new method are more than adequate to detect methylone and metabolites in authentic cases, even with low specimen volume.

Sample preparation for this method required three steps: deconjugation of possible phase II metabolites, protein precipitation, and SPE. Deconjugation was performed because prior metabolism studies demonstrated that methylone metabolites are primarily conjugated [170,174], and commercial conjugated reference standards were unavailable. HHMC and HMMC had significant decreases in concentrations when there was no hydrolysis procedure, similar to MDMA metabolites, 3,4-dihydroxymethamphetamine (HHMA) and 4-hydroxy-3-methoxymethamphetamine (HMMA) [319,320]. These results indicate that HHMC and HMMC are present in rat plasma primarily as conjugated metabolites, which coincides with previous reports [170,174,319,320]. Protein precipitation was achieved with perchloric acid after the addition of 4-methylcatechol, which was added to reduce possible adsorption of the dihydroxy-metabolite to precipitated proteins. Cold acetonitrile also was evaluated for

protein precipitation; however, analyte recoveries were lower and interference/background noise larger than observed with perchloric acid.

Despite adequate analyte recoveries with perchloric acid protein precipitation, large matrix effects and endogenous interferences were observed during method development; therefore, SPE was employed to reduce these effects. The SPE method employed in this study minimized ion suppression, as less than 11.4% suppression was observed for all four analytes; however, recovery of the intermediate dihydroxy metabolite, HHMC, was difficult, due to its polarity. Several different elution solvents were evaluated during method development including 2% ammonium hydroxide in methylene chloride/isopropanol (80:20 v/v), 5% ammonium hydroxide in methylene chloride/isopropanol (60:40 v/v), and methanol/ammonium hydroxide (95:5 v/v). Optimal HHMC recovery was accomplished with methanol/ammonium hydroxide (95:5 v/v) (49.2%); although, recovery of the three remaining analytes were poor (64.6-81.7%). Taking into account that HMMC and MDC are the main metabolites of methylone, 5% ammonium hydroxide in methylene chloride/isopropanol (60:40 v/v) was utilized to recover all four compounds from the SPE cartridges. It also is important to note that SMBS and EDTA were utilized to prevent analyte oxidation similar to MDMA and MDPV metabolite quantification in plasma [206,318].

Validation results of methylone and its main metabolites, HMMC and MDC, were acceptable. Extraction efficiencies and process efficiencies were between 89.9-104% and 83.3-96.9%, respectively. The performance of the intermediate dihydroxy metabolite (HHMC), however, was not optimal. This is most likely due to its low extraction (15.9-16.2%), process efficiencies (15.2-15.6), and analyte instability. Percent bias was

acceptable for HHMC (-12.8 to 6.7%), although imprecision was greater than 20%. Similar process and extraction efficiencies were recently reported with the dihydroxy MDPV metabolite, 3,4-catechol-PV (39-44.9% and 35.6-45.3%, respectively) [206]. Authors attributed this loss to partitioning of the polar analyte into the aqueous layer after centrifugation and protein precipitation. Due to poor HHMC performance in the method validation, concentrations are considered semi-quantitative. No previous studies reported HHMC identification, potentially due to the analyte's short half-life in blood [152].

Another important issue to consider with synthetic cathinones when quantifying them in various matrices is their stability. Methylone, HMMC, and MDC were stable under each short-term stability condition tested: 24 h at room temperature, 72 h at 4ºC, and after 3 freeze-thaw cycles. Other studies examining synthetic cathinone stability in plasma demonstrated varying results. Johnson and Botch-Jones [147] reported MDPV to be stable at room temperature, 4ºC, and -20ºC for up to 14 days, whereas mephedrone exhibited a 30% loss after 14 days at 4ºC, and was not detected after 7 days at room temperature. Soh and Elliot detected a 54% loss of 4-MEC in plasma after 14 days at room temperature [148]. The stability of methylone, HMMC, and MDC observed in the present study may be attributed to SMBS and EDTA use during sample storage and preparation, as it prevents oxidation, combined with the fact that methylone does not readily reduce to its corresponding alcohol [112,236]. HHMC demonstrated varying results in terms of stability (-10.6 to 60.6% difference). It is difficult to determine if this was due to analyte instability, or poor extraction and process efficiencies.

The human-rat plasma cross-validation results demonstrated that it is possible to quantify methylone and metabolites in rat plasma utilizing human plasma for calibration,

as volumes of drug-free rat plasma are often limited. The results of this study demonstrate that this assay can be utilized for preclinical, clinical or forensic applications. Proof of concept was demonstrated with plasma from rats dosed with 6 mg/kg methylone subcutaneously. The highest concentrations observed for methylone, HMMC, MDC and HHMC were 1,310 µg/L (15 min post-dosing), 194 µg/L (60 min post-dosing), 152 µg/L (60 min post-dosing) and 185 µg/L (60 min post-dosing), respectively. Plasma concentrations of methylone, HMMC, MDC and HHMC were 1.1, 11.2, 1.9 and 24.7 µg/L at 480 min post-dose, respectively. These preliminary data indicate that methylone plasma C_{max} is higher than metabolites, although metabolites appear to have longer windows of detection. This suggests that the metabolites could be good markers to detect methylone abuse, in addition to the parent compound; however, further investigation is warranted.

Conclusions

A sensitive and specific LC-MS/MS method for simultaneous quantification of methylone and metabolites, HMMC, MDC and HHMC, in plasma was developed and validated. Only 100 µL specimen was required for LOQs of 0.5 µg/L for all analytes except HHMC (10 µg/L). Quantification methods are needed to characterize pharmacokinetics of methylone and its metabolites for preclinical, clinical and forensic studies. Methylone remains one of the most commonly used synthetic cathinones in the United States despite scheduling efforts, and as such, quantification data for this analyte and its metabolites will aid in the interpretation of results in forensic and clinical settings, and may suggest mechanisms of methylone toxicity.

Chapter 9 - 4-methoxy-α-PVP: *In silico* prediction, metabolic stability, and metabolite identification by human hepatocyte incubation and high resolution-mass spectrometry

(*As published* in Forensic Toxicology, *2016*)[9]

Abstract

NPS are continuously developed to circumvent legislative and regulatory efforts. A new synthetic cathinone, 4-methoxy-α-PVP, was identified for the first time in illegal products; however, the metabolism of this compound is not known. Complete metabolic profiles are needed for these novel psychoactive substances to enable identification of their intake and to link adverse effects to the causative agent. This study assessed 4-methoxy-α-PVP metabolic stability with HLM and identified its metabolites after HLM and hepatocyte incubations followed by HRMS. The high-resolution full scan data dependent mass spectrometry method, with (1) and without (2) inclusion list of predicted metabolites, and with full scan and all-ion-fragmentation (AIF) (3) to identify potential unexpected metabolites were employed on a Thermo Scientific QExactive HRMS. *In silico* predictions were performed and compared to *in vitro* results. Scans were thoroughly mined with different data processing algorithms using WebMetabase (Molecular Discovery). 4-methoxy-α-PVP exhibited a long half-life of 79.7 min in HLM, with an intrinsic clearance of 8.2 $ml \cdot min^{-1} \cdot kg^{-1}$. In addition, this compound is predicted to be a low-clearance drug with an estimated human hepatic clearance of 5.8 $ml \cdot min^{-1} \cdot kg^{-1}$. Eleven 4-methoxy-α-PVP metabolites were identified, generated by *O*-demethylation, hydroxylation, oxidation, ketone reduction, *N*-dealkylation, and glucuronidation. The

[9] Ellefsen K.N *et al.* 4-methoxy-α-PVP: *In silico* prediction, metabolic stability, and metabolite identification by human hepatocyte incubation and high resolution-mass spectrometry. *Forensic Toxicology*. 2016; 34(1): 61-75.

most dominant metabolite in HLM and human hepatocyte samples was 4-hydroxy-α-PVP, also predicted as the #1 *in silico* metabolite, and is suggested to be a suitable analytical target in addition to the parent compound.

Introduction

In recent years, NPS appeared rapidly on the drug market in an effort to bypass controlled substances' legislation. The EMCDDA reported 41 NPS identified for the first time across Europe in 2010, 81 in 2013 and 101 in 2014 [92]. These NPS are continuously developed to circumvent legislative and regulatory efforts, with limited available pharmacological and toxicological data. NPS encompass a wide range of compounds including synthetic cannabinoids, phenethylamines, tryptamines, piperazines, ketamine, cathinones, and other plant-based psychoactive substances [94].

Synthetic cathinones emerged on the designer drug market as popular "legal" alternatives to illicit drugs in the late 2000's, and are marketed as "legal highs" and "not for human consumption". They are stimulant-like drugs derived from cathinone, the active ingredient of the khat plant *Catha Edulis,* with adverse effects including hyperthermia, agitation, confusion, psychosis, seizures, and tachycardia [95,116,124,128,321]. A variety of synthetic cathinones, alone and in combination with other illicit drugs, were detected in acute intoxications [116,228,241], impaired driving cases [108,158,165,244], and fatalities [116,127,129,158,159,202,222,223,225,226,251,257].

4-methoxy-α-PVP (4-methoxy-α-pyrrolidinovalerophenone) was identified for the first time in illegal products purchased in Japan in 2013 [322] and also recently identified by Customs in Berlin [323]. 4-methoxy-α-PVP is a substituted cathinone containing a

pyrrolidinyl moiety and a methoxy group on the 4′ position of the aromatic ring (Figure 27). Its structure is similar to other α-pyrrolidinophenone derivatives like MDPV and α-α-PVP, both schedule I compounds [101,103]. The mechanism of action of 4-methoxy-α-PVP is unknown; however, based on structural similarities to MDPV and α-PVP, it is hypothesized that 4-methoxy-α-PVP would act similarly as a monoamine transporter blocker increasing the amount of extracellular DA [137,142-144]. Extracellular DA increases pose a higher risk for addiction [142,144]. In addition, the high lipophilicity of the pyrrolidine ring increases permeability through the blood-brain-barrier, and hence, increased potency and abuse potential [137,145,146].

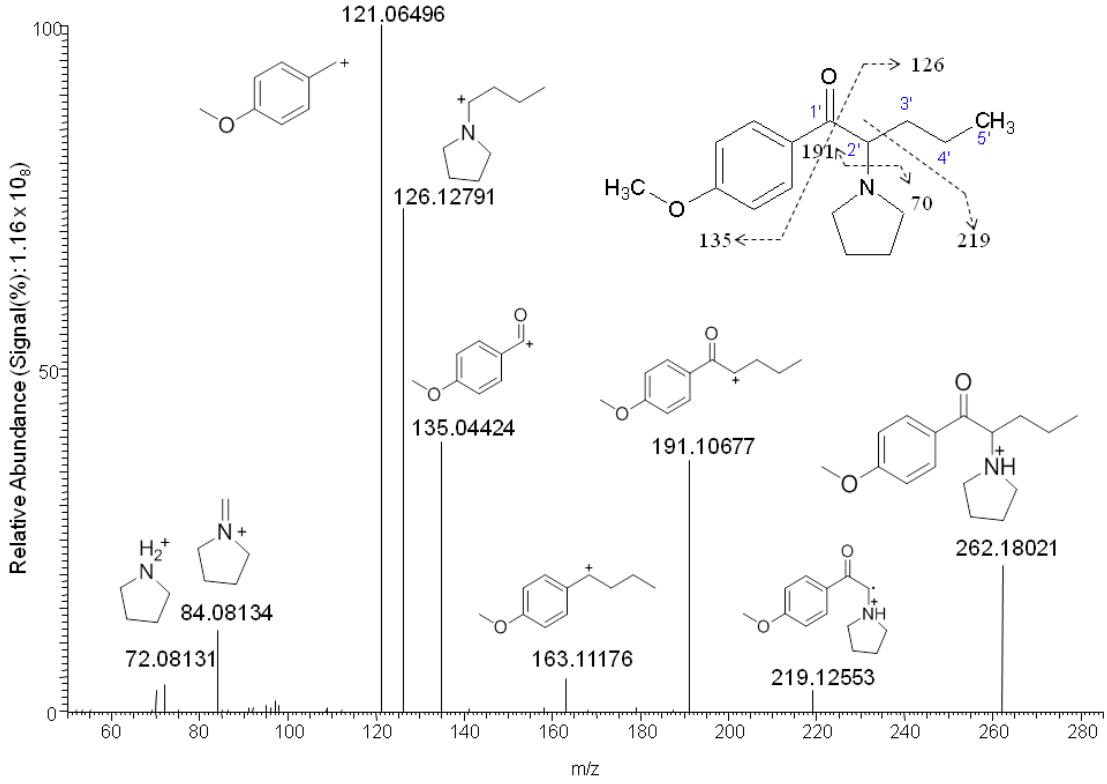


Figure 27. Product ion spectrum of 4-methoxy-α-PVP and its fragmentation pattern.

The metabolic pathways of other α-pyrrolidinophenones, including MDPV, α-PVP, α-PBP, α-pyrrolidinopropiophenone (α-PPP), 4-MPPP and 4-methoxy-α-PPP, were previously investigated [112,155,162,170,175,179,180-182].There are no metabolism studies for 4-methoxy-α-PVP. A recent review of the pharmacology of α-pyrrolidinophenones outlined the major metabolic pathways of other structurally similar compounds including: reduction of the keto moiety to the corresponding alcohol, hydroxylation followed by oxidation of the pyrrolidine ring to the lactam (2″-oxo), hydroxylation and carboxylation of the 4′-methyl group, *O*-demethylation of the 4′-methoxy group and demethylenation followed by *O*-methylation of the 3′, 4′-methylenedioxy moiety [324].

A promising approach to elucidate metabolites of novel psychoactive substances includes *in silico* metabolite predictions, HLM and human hepatocyte incubations, followed by analysis with HRMS and software-assisted data mining [325,326]. Complete metabolic profiles are needed for 4-methoxy-α-PVP to enable identification of intake and link adverse effects to the causative agent. We evaluated 4-methoxy-α-PVP *in silico* metabolism predictions, assessed metabolic stability with HLM, and identified metabolites after HLM and human hepatocyte incubations followed by HRMS.

Materials and Methods

Chemicals and Reagents

4-methoxy-α-PVP HCl was purchased from Cayman Chemicals (Ann Arbor, Michigan, USA) and diclofenac was obtained from Toronto Research Chemicals (Toronto, Canada). 50-donor pooled HLM, 10-donor pooled cryopreserved human hepatocytes, InVitro Gro HT medium for thawing, InVitro Gro KHB (Krebs-Henseleit

buffer) for washing and incubation were acquired from BioreclamationIVT (Baltimore, Maryland, USA) and NADPH-regenerating system (Solution A), and glucose-6-phosphate dehydrogenase (Solution B) were purchased from Corning Gentest (Woburn, Massachusetts, USA). LC-MS grade water and formic acid were from Fisher Scientific (Fair Lawn, New Jersey, USA), and LC-MS grade acetonitrile from Sigma Aldrich (St. Louis, Montana, USA).

In silico Metabolite Prediction

MetaSite software (v.5.0.3; Molecular Discovery, Pinner, UK) was utilized to predict *in silico* 4-methoxy-α-PVP metabolites. The structure of 4-methoxy-α-PVP was imported into the software and predictions generated using the CYP450 liver model, reactivity correction, 39 common biotransformations, and a minimum mass threshold of 50 Da for predicted metabolites. The Predicted Sites of Metabolism were are a consensus from liver CYP isoforms and FMO3. The computational procedure considers both thermodynamic and kinetic factors. Potential metabolites were assigned a probability score representing the likelihood of being generated, with 100% being the maximum score. In addition, the top predicted *in silico* metabolite (100% probability score) was imported into the software to predict second-generation metabolites.

Metabolic Stability of 4-methoxy-α-PVP in HLM

To investigate the metabolic stability of 4-methoxy-α-PVP, 1 µmol/L drug was incubated with 50-donor-pooled HLM. Water bath incubations were performed on a Precision reciprocal shaking bath (Winchester, VA, USA). The reaction mixture contained 780 µL purified water, 100 µL 0.5 mol/L potassium phosphate buffer pH 7.4, 10 µL Solution B, and 10 µL 4-methoxy-α-PVP (100 µmol/L). Samples were vortexed to

homogenize the solutions and 50 µL HLM (20 mg protein/mL suspension) were added to the mixture and pre-incubated at 37ºC for approximately 3 min. Organic solvent percentage (methanol) was 1%. The reaction was initiated with the addition of 50 µl Solution A and 100 µL samples were collected at 0, 3, 8, 13, 20, 30, 45 and 60 min. The reaction was stopped with an equal volume (100 µL) of cold acetonitrile. Samples were then centrifuged at 15,000 x *g* for 5 min at 4ºC and subsequently stored at -80ºC prior to analysis.

Incubation of 4-methoxy-α-PVP with Cryopreserved Primary Human Hepatocytes

For the human hepatocyte experiments, 10 µmol/L 4-methoxy-α-PVP was incubated at 37ºC with pooled cryopreserved human hepatocytes. Hepatocytes were incubated in a Forma^{TM} Steri-Cycle^{TM} CO_2 incubator from Thermo Scientific. Cells were thawed in and washed with InVitro Gro HT medium and centrifuged at 50 x *g* for 5 min at room temperature. Supernatant was aspirated and InVitro Gro KHB was added to wash the hepatocytes again. After centrifugation and removal of the supernatant, the cell pellet was re-suspended in 2 mL buffer. Cell viability was assessed with the Trypan blue exclusion method assuring >80% viability. The reaction mixture contained 250 µL 4-methoxy-α-PVP in buffer (20 µmol/L) and 250 µL cell suspension yielding a final drug concentration of 10 µmol/L and final cell concentration of $1x10^6$ cells/ml. Samples (500 µL) were collected at 0, 1 and 3 h based on HLM half-life calculations and the reaction stopped with 500 µL cold acetonitrile. Specimens were stored at -80ºC prior to analysis. Diclofenac also was incubated as a positive control for human hepatocyte functional viability.

Instrumentation

LC-HRMS was performed on a Thermo Scientific NCS-3500RS Ultimate 3000 Binary Rapid system coupled to a Thermo Scientific QExactive mass spectrometer (Thermo Scientific, Fremont, CA). The Ultimate 3000 RSLCnano system consisted of a degasser, a tertiary loading pump, a binary eluting pump, a column oven and an RS Autosampler. The QExactive contained a heated electrospray ionization source (HESI-II) and was operated in positive ionization mode. The spray voltage was 3 kV, capillary temperature 350^0C, heater temperature 425^0C, S-lens RF level 50, sheath gas flow rate 50, auxiliary gas flow rate 13 and sweep gas 3 (manufacturer's units). Nitrogen was used for spray stabilization, for collision-induced dissociation experiments in the HCD cell, and as the damping gas in the C-trap. The instrument was calibrated in the positive and negative mode every 25 h.

LC-HRMS for HLM Samples

Chromatographic separation of HLM samples, diluted 100-fold with mobile phase A (0.1% formic acid in water), was achieved with an Accucore C18 column (2.6 µm, 100 x 2.1 mm) and C18 guard cartridge (4 x 2.0 mm) with a 0.4 mL/min flow rate at 35ºC. Gradient elution was performed with 2% B (0.1% formic acid in acetonitrile) for 2 min, increased to 10% B at 10 min, then ramped to 30% B at 14 min, from 30 to 95% in 2 min, held at 95% for 1 min, and returned to initial conditions over 1 min. A 2 min equilibration followed, yielding a total run time of 20 min.

Full scan $ddMS^2$ data were collected from 100-600 *m/z* at 70,000 resolution with AGC target 1.0×10^6, and maximum injection time 250 ms. Data-dependent MS^2 scans were triggered at the apex of the peak (3-8 s) when an underfill ratio of 5% was reached

(intensity 8.3 x 10^4) for the 5 most intense peaks per cycle (TopN of 5). MS scans were acquired at a resolution of 17,500 with ACG of 2 x 10^5, maximum injection time of 120 ms, and stepped normalized collision energy (NCE) 50 ±30%.

LC-HRMS for Human Hepatocytes

Hepatocyte samples were diluted 5-fold with mobile phase A, and chromatographically separated utilizing a Synergi 4 Hydro-RP column (80Å, 150 x 2 mm) and C18 guard cartridge (4 x 2.0 mm) within 30 min at 0.4 mL/min flow rate. Initial conditions (2% B) were held for 2 min, increased to 95% B for 18 min, held at 95% B for 5 min, and returned to initial conditions over 1 min. A 4 min equilibration followed, yielding a total run time of 30 min.

Hepatocytes incubations were analyzed with three different MS methods: full scan and $ddMS^2$, with (1) and without (2) an inclusion list of predicted metabolites, and with a full scan and AIF method (3). Full scan and $ddMS^2$ data (without an inclusion list) were acquired as previously described for HLM samples. In addition, an inclusion list was generated by MetaSite software based on its *in silico* metabolism predictions and imported into the full scan $ddMS^2$ acquisition method. Dd-MS^2 scans were triggered if the precursor ions from the inclusion list were detected above 8.3x10^4 intensity threshold. To identify potential unexpected metabolites, full scan and AIF data were acquired. AIF experiments were performed at 35,000 resolution over a scan range of 100-600 *m/z*, with ACG target 5 x 10^5, maximum injection time of 120 ms, and a stepped NCE 50 ± 30%.

Data Analysis

In vitro microsomal $t_{1/2}$ and microsomal intrinsic clearance ($CL_{int, micr}$) of 4-methoxy-α-PVP were calculated on the model described by Baranczewski *et al* [327].

This microsomal intrinsic clearance was then scaled to whole liver dimensions to calculate the intrinsic clearance (CL_{int}) by multiplying two factors: the content of microsomal protein/g liver tissue (~45mg/g) and liver weight/kg body weight (~20 g/kg), according to McNaney *et al* [328]. Human hepatic clearance (CL_H) and extraction ratio (ER) were calculated [327] without consideration of plasma protein binding.

Mass spectra acquired by the QExactive were analyzed with WebMetaBase software (v.2.0.2, Molecular Discovery, Pinner, UK) for metabolite candidates in the microsomes and hepatocyte incubations. Raw data were submitted as a batch, which included a substrate (parent compound, 4-methoxy-α-PVP) structure mol file, a blank file (mobile phase), a substrate file (neat standard) to analyze the substrate fragmentation pattern, and raw data files for each incubation time point for hepatocyte incubations (t=0, 1, 3 h) and for microsomes (t=0, 3, 8, 13, 20, 30, 45, 60 min). Processing parameters for hepatocyte incubations included: “hepatocyte metabolic system”; retention time window 2-25 min; chromatogram, MS, and MS/MS auto filter thresholds of 0.98; same peak tolerance of 0.010; 3 metabolite generations, minimum metabolite generation mass of 50 Da, and expected metabolites were rescue enabled (selects peaks with an equivalent *m/z* to a known metabolite regardless of peak area, provided there is MS^2 data associated with the peaks). For the HLM incubations similar processing parameters were chosen, except the metabolic system selected was microsomes and the RT window was 2-18 min due to the different chromatography. The software auto-detects chromatographic peaks related to the parent compound and its metabolites, proposes potential structures based on fragmentation patterns for each detected peak and Markush structures (chemical structures with functional groups highlighted based on site reactivity), and ranks potential

structures compatible with extracted fragment information. Structures of potential metabolite candidates were assigned based on retention times, mass shift between theoretical mass and observed mass (<5 ppm), peak abundance and fragmentation pattern. Metabolites were thoroughly examined against literature reports of structurally similar compounds and were eventually compared to the *in silico* predictions.

Results

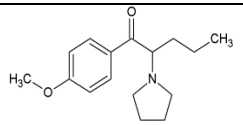
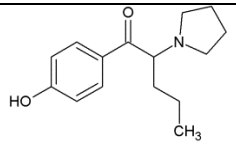
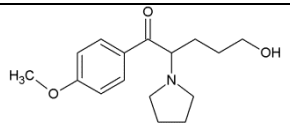
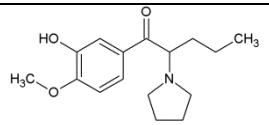
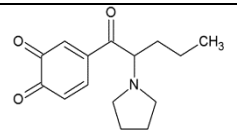
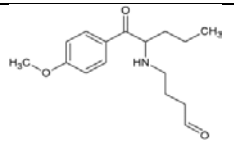
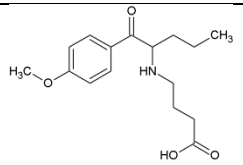
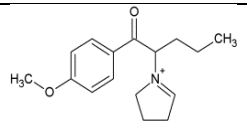
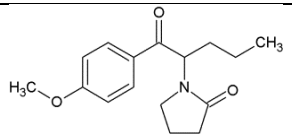
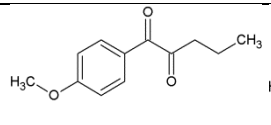
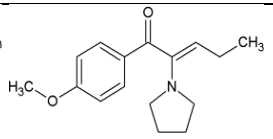
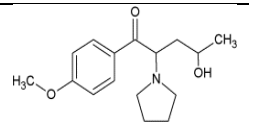
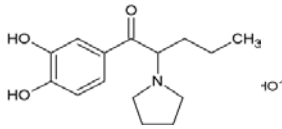
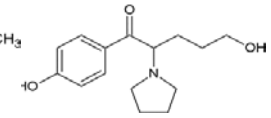
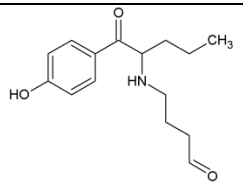
In silico Metabolite Prediction

Eleven predicted *in silico* metabolites were generated with MetaSite software and are summarized in Table 41 (scores >20%), with three second generation metabolites predicted from the highest ranked *in silico* metabolite. The top three metabolites predicted *in silico* were the *O*-demethylated metabolite, the metabolite hydroxylated at C5′ position and the metabolite hydroxylated at the phenyl ring. Utilizing the top predicted first generation metabolite (4-hydroxy-α-PVP), three second generation metabolites were predicted including the aromatic hydroxylated metabolite, the metabolite hydroxylated at C5′ position and the metabolite resulting from ring opening and oxidation (Table 41).

Metabolic Stability Assessment with HLM

4-methoxy-α-PVP exhibited an *in vitro* $T_{1/2}$ of 79.7 ± 1.3 min in HLM, with a microsomal intrinsic clearance $CL_{int,\ micr}$ of 8.7 $\mu L \cdot min^{-1} \cdot mg^{-1}$ and intrinsic clearance CL_{int} of 8.2 $mL \cdot min^{-1} \cdot kg^{-1}$. Hepatic clearance was calculated to 5.8 $mL \cdot min^{-1} \cdot kg^{-1}$ and the extraction ratio ER was 0.29.

Table 41. In silico predicted metabolites for 4-methoxy-α-PVP in decreasing order based on score. Second generation metabolites were generated by importing first generation metabolites with scores of 100% into the in silico software (only P1 had a score of 100%)

First Generation	**4-methoxy-α-PVP**	**P1***	**P2**	**P3**	**P4**	**P5**
						
Reaction	Parent	*O*-demethylation	Hydroxylation	Hydroxylation	Ortho *O*-dearylation	Ring opening
Mass (Da)	261.1728	247.1572	277.1678	277.1678	261.1365	277.1678
Log D (pH 4)	0.18	-0.31	-0.88	-0.14	-0.75	0.02
Score (%)	N/A	100	36	32	32	26
	P6	**P7***	**P8***	**P9**	**P10**	**P11**
						
Reaction	Ring opening	Iminium formation	Carbonylation	*N*-dealkylation	Dehydrogenation	Hydroxylation (C4)
Mass (Da)	293.1627	260.1650	275.1521	206.0943	259.1573	277.1678
Log D (pH 4)	0.05	2.07	2.80	2.05	0.73	-0.75
Score (%)	26	26	26	25	25	19.5
Second Generation	**P1a**	**P1b**	**P1c**			
						
Reaction	*O*-Demethylation & hydroxylation	*O*-Demethylation & hydroxylation	*O*-Demethylation & *N*-dealkylation (aldehyde)			
Mass (Da)	263.1521	263.1521	263.1521			
Log D (pH 4)	-0.26	-1.37	-0.47			
Score (%)	100	62.2	61.1			

* Identified in 3 h human hepatocyte incubation

Identification of Human Liver Microsome Metabolites

In the HLM samples, the *O*-demethylated metabolite (M3 in the hepatocyte samples) was the only metabolite detected. Peak areas increased from the 20 to 60 min incubation times.

Metabolite Profiling with Human Hepatocytes

Diclofenac (positive control) and its metabolites, 4-hydroxydiclofenac and diclofenac β-D acyl glucuronide, were observed in the hepatocyte samples confirming hepatocyte metabolic activity. Accurate full scan and product ion spectra were obtained and thoroughly data-mined, identifying eleven 4-methoxy-α-PVP metabolites (Table 42) with mass measurement errors <1.5 ppm. Metabolites eluted between 4.9 and 13.8 min with 4-methoxy-α-PVP parent at 9.1 min (Table 42). Ten phase I metabolites (M1-M7, M9-M11) and one phase II metabolite (M8) were identified in the hepatocyte samples. In the 1 h sample, M1, M3-M7, and M9 were the only metabolites detected with 91.0% of 4-methoxy-α-PVP unchanged. All metabolites were found in the 3 h sample with 78.7% parent compound remaining. Consistent with ongoing metabolism, 4-methoxy-α-PVP peak areas decreased during incubation while in general metabolite peak areas increased from 1 to 3 h incubation. The metabolic reactions observed included: *O*-demethylation (M3), *O*-demethylation + *N*-dealkylation (M1), *O*-demethylation + ketone reduction (M2), hydroxylation (M4, M6), pyrrolidine ring opening + hydroxylation (M5), ketone reduction (M7), carbonylation (M11), aliphatic *N*-oxidation (M9), and iminium ion formation (M10). One glucuronide also was noted (M8); however, a product ion spectrum for M8 was not obtained, as the intensity threshold criterion was not met. Based

Table 42. 4-methoxy-α-PVP phase I metabolites identified after incubation with human hepatocytes, sorted by retention time (RT). Rank was based on MS peak areas. Fragments are expressed in nominal mass.

ID	Metabolic Reaction	RT (min)	Elemental Composition	*m/z*	Mass Error (ppm)	Characteristic Fragments	MS % Peak Areas		Ranking	
							1 h	3 h	1 h	3 h
M1	*O*-demethylation + *N*-dealkylation	4.87	$C_{11}H_{15}NO_2$	194.1177	0.52	135, 107, 58	$2.2e^7$	$2.9e^7$	4	6
M2	Ketone reduction + *O*-demethylation	7.26	$C_{15}H_{23}NO_2$	250.1800	0.80	189, 232, 107, 70	-	$1.3e^7$	-	9
M3	*O*-demethylation	7.48	$C_{15}H_{21}NO_2$	248.1643	0.81	107, 126, 121, 72, 177	$4.7e^8$	$7.5e^8$	1	1
M4	Hydroxylation	8.51	$C_{16}H_{23}NO_3$	278.1748	1.08	121, 142, 135, 191	$1.2e^7$	$2.7e^7$	6	7
M5	Pyrrolidine ring opening + hydroxylation	8.65	$C_{16}H_{25}NO_3$	280.1906	0.36	121, 190, 262, 148, 135, 144	$1.4e^7$	$1.2e^7$	5	10
M6	Di-hydroxylation	8.78	$C_{16}H_{23}NO_4$	294.1699	0.31	121, 87, 140, 276, 135, 191	$8.0e^7$	$1.9e^8$	2	2
M7	Ketone reduction	8.96	$C_{16}H_{25}NO_2$	264.1957	0.38	246, 203, 188, 121, 175, 72	$4.8e^7$	$1.1e^8$	3	3
M9	Aliphatic *N*-oxidation	9.81	$C_{16}H_{23}NO_3$	278.1749	0.72	126, 86, 135, 163, 121	$9.3e^6$	$1.8e^7$	7	8
M10	Iminium ion formation	11.48	$C_{16}H_{21}NO_2$	260.1644	1.54	175, 86, 121, 70	-	$3.6e^7$	-	5
M11	Carbonylation	13.83	$C_{16}H_{21}NO_3$	276.1593	0.36	140, 98, 121, 135, 69, 191	-	$3.9e^7$	-	4
	Parent	9.10	$C_{16}H_{23}NO_2$	262.1798	1.53	121, 126, 135. 191, 84, 163, 219, 72, 70	$6.6e^9$	$4.4e^9$	-	-

on observed peak areas, M3 was the most intense metabolite in hepatocyte incubations, followed by M6 and M7.

All metabolites were identified with the full scan and dd-MS^2 method, with the exception of M8. This glucuronidated metabolite was identified with our full scan and dd-MS^2 method with the *in silico* generated inclusion list. Besides M8, this acquisition method did not detect any additional metabolites and only found 5/10 metabolites (M3, M4, M6, M7 and M11) identified with the full scan and dd-MS^2 method. The full scan and AIF method did not detect any additional unexpected metabolites. Compared to the full scan and dd-MS^2 method, the full scan and AIF did not identify M5 (pyrrolidine ring opening + hydroxylation), the lowest prevalence metabolite other than M8.

Discussion

A variety of different product ions were observed for 4-methoxy-α-PVP (Figure 27), with *m/z* 121 the most intense fragment ion at the given collision energy conditions. The base peak is generated by cleavage between the β-keto (C1′) and aromatic ring. Cleavage of the bond between the β-keto (C1′) and alpha (C2′) carbon generated the next highest intensity ions at *m/z* 126 and 135, similar to cleavage that was previously reported for α-PVP [182]. Fragmentation of the bond between the pyrrolidine ring and alpha carbon (C2′) produced *m/z* 191 and 72 ions. Other distinct product ions included *m/z* 219 (loss of C_3H_7 radical) and 84. The unique fragmentation pattern of 4-methoxy-α-PVP was utilized for metabolite structure elucidation.

Identification of O-demethylated Metabolites

4-hydroxy-α-PVP (M3), the product of *O*-demethylation of the 4-methoxy moiety, was the most intense metabolite in the HLM and 1 and 3 h hepatocyte samples

(Table 42). Figure 28 illustrates the product ion spectra for all *O*-demethylated metabolites. M3 product ion spectrum showed three distinct fragments of the precursor 4-methoxy-α-PVP molecule (*m/z* 126, 72, and 84) suggesting no modifications to the pyrrolidine ring and aliphatic chain of the molecule. The characteristic fragments of M3 included *m/z* 107 and 121 indicating *O*-demethylation at the methoxy group and subsequent formation of the tropylium ion and bond cleavage between the β-keto (C1′) and alpha (C2′) carbons. Further reduction of M3 at the keto moiety yielded M2 at *m/z* 250. M2 product ion spectrum showed a characteristic *m/z* 189 fragment produced by cleavage of the carbonyl and terminal C_3H_7 radical, and a *m/z* 232 fragment from loss of water. In addition, *N*-dealkylation of the pyrrolidine ring of M3 yielded M1. This metabolite product ion spectrum showed characteristic fragments *m/z* 135, 107, and 58 suggesting modifications to the pyrrolidine ring and 4-methoxy moiety.

Identification of Hydroxylated Metabolites

Three hydroxylated metabolites, one monohydroxylated (M4), one di-hydroxylated (M6) and one pyrrolidine ring opening and hydroxylated metabolite (M5) were identified. Figure 29 illustrates the product ion spectra for all hydroxylated metabolites. Hydroxylations occurred at the pyrrolidine ring, as three characteristic fragments from 4-methoxy-α-PVP were identified in each metabolite (*m/z* 121, 135, and191). M6 was the second most intense peak after 1 and 3 h incubations showing fragments at *m/z* 158, 140, 276, 98, and 87, suggesting di-hydroxylation at the pyrrolidine ring. M4 showed distinct fragments including *m/z* 142, 100, and 70 supporting a monohydroxylation on the pyrrolidine ring. The exact position of the hydroxyl group is

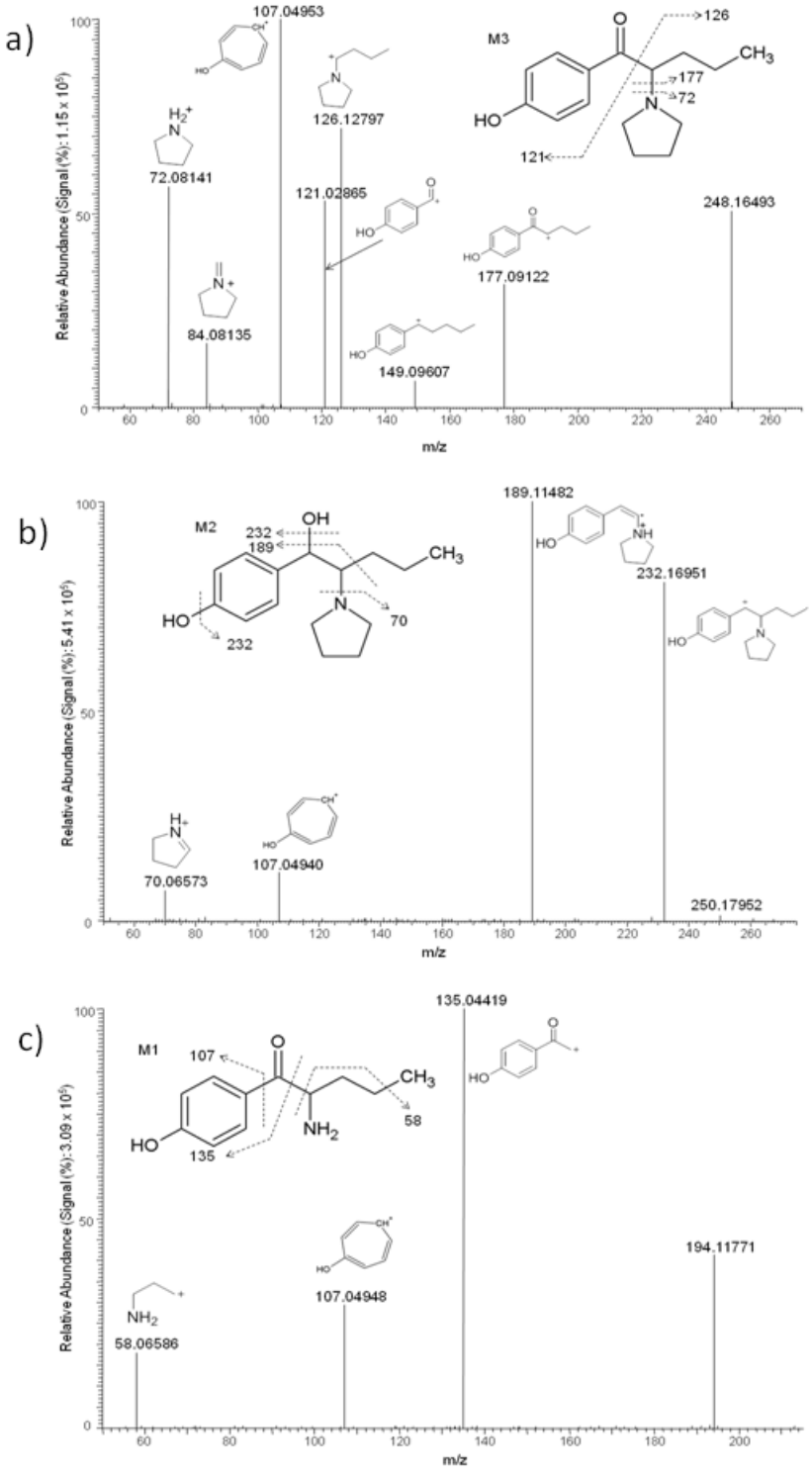


Figure 28. 4-methoxy-α-PVP metabolites product ion spectra and assigned fragmentation patterns for metabolites generated by *O*-demethylation: a) 4-hydroxy-α-PVP M3, b) *O*-demethylated and ketone reduced metabolite M2, and c) the *O*-demethylated and *N*-dealkylated metabolite M1.

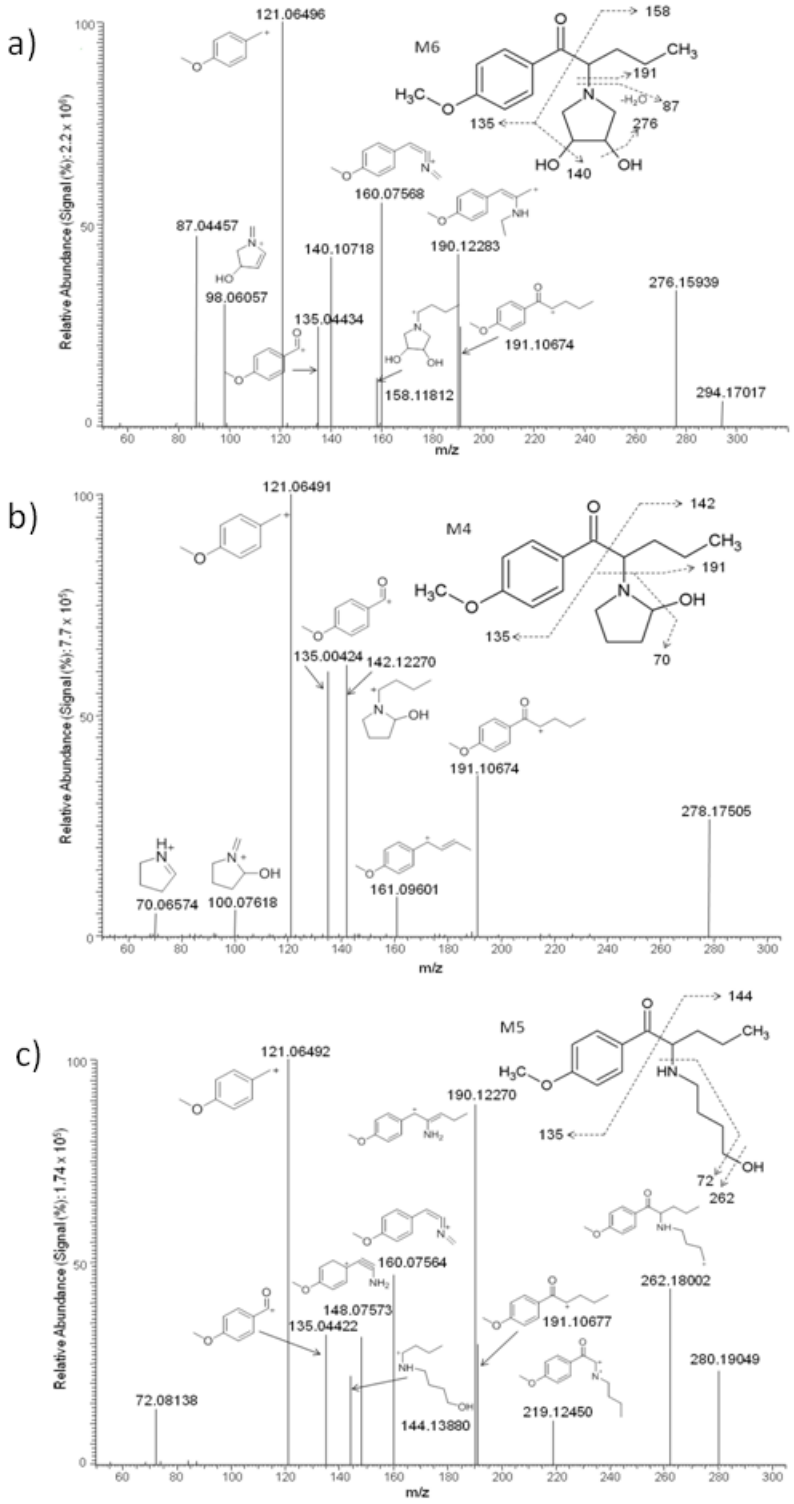


Figure 29. Product ion spectra and assigned fragmentation pattern for hydroxylated 4-methoxy-α-PVP for metabolites: a) the di-hydroxylated metabolite on the pyrrolidine ring M6, b) the monohydroxylated metabolite on the pyrrolidine ring M4, and c) the ring opening and hydroxylated metabolite M5. The exact location of the di-hydroxylated metabolite is unknown; however, the position of the hydroxyl group at the 2″ in M4 is suggested based on previously identified α-pyrrolidine metabolites.

unclear, although, previous metabolism studies identified 2″ OH-α-PVP in the structurally similar compound α-PVP [155,181,182]. It is expected that similar metabolism could be observed with M4. The last identified hydroxyl metabolite (M5) was the product of the pyrrolidine ring opening and hydroxylation on the terminal end, similar to what was reported for α-PVP [329]. Characteristic fragments were *m/z* 262, 144, and 219.

Metabolite Generated by Ketone Reduction

The third most prevalent metabolite in the 1 and 3 h hepatocyte samples was the ketone reduced metabolite, M7 (Figure 30). The base peak *m/z* 246 resulted from the loss of water. Other characteristic fragments included *m/z* 203, 188, 175, and 121 and 72 as observed with the precursor molecule.

Other Identified Metabolites

Two metabolites were products of oxidation reactions (M11 and M9). M11 was the result of carbonylation of the pyrrolidine ring, a common biotransformation for α-pyrrolidinophenones [324]. Characteristic fragments of this late eluting compound included *m/z* 140, 98, 86, 121, and 191. The amide is less basic than the tertiary amine in the parent compound and therefore, is less likely to be protonated and as water soluble as the parent. The *N*-oxide metabolite (M9) also eluted after the parent compound, with the simultaneous appearance of *m/z* 126 and 135 and an *m/z* 86 fragment that was not observed in the monohydroxylated metabolite (M4), thus suggesting this oxidation reaction. Other distinct fragments included *m/z* 163, 121, and 191. In addition to these oxidative metabolites, the formation of the iminium ion was proposed (M10). This compound also was predicted *in silico* (P7). Fragments included *m/z* 175, 121, 86,

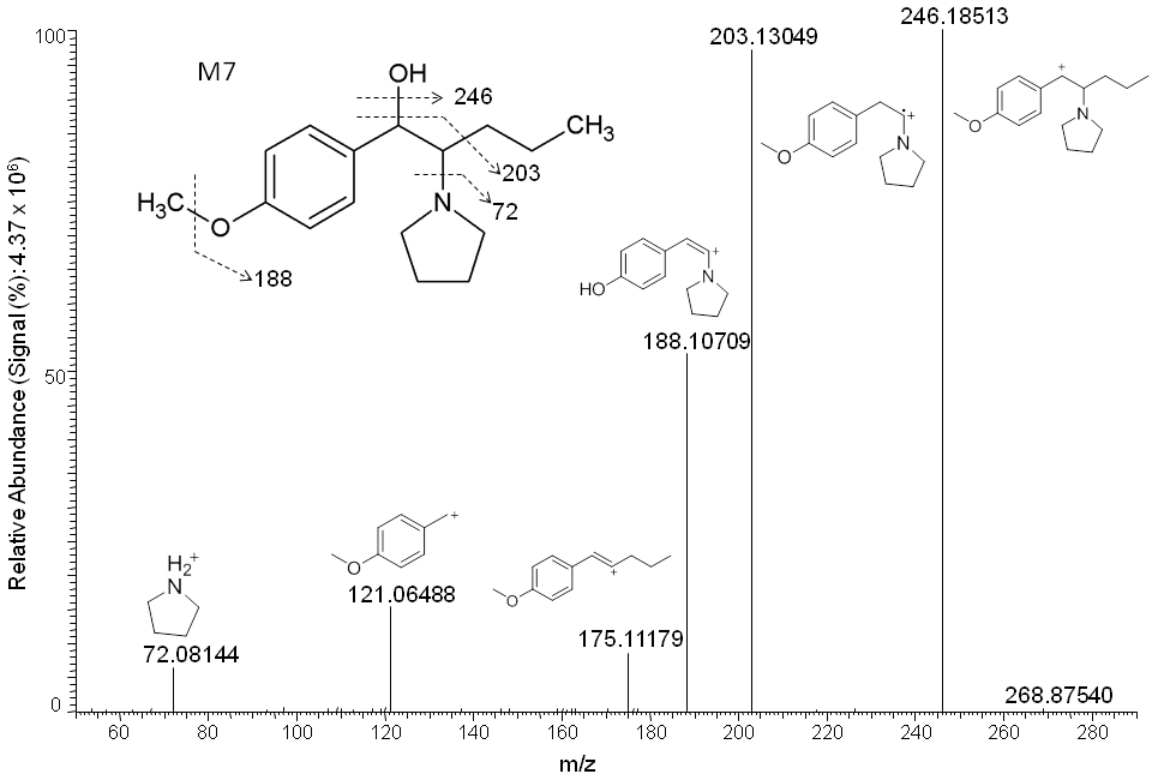


Figure 30. Product ion spectrum and fragmentation pattern for the metabolite generated by ketone reduction M7.

98,160, and 70. The *m/z* 70 fragment suggests the formation of an iminium ion at the pyrrolidine ring, otherwise fragment *m/z* 72 should be observed as in the parent compound (Figure 27) after cleavage of the pyrrolidine ring. Figure 31 highlights the product ion spectra for these metabolites.

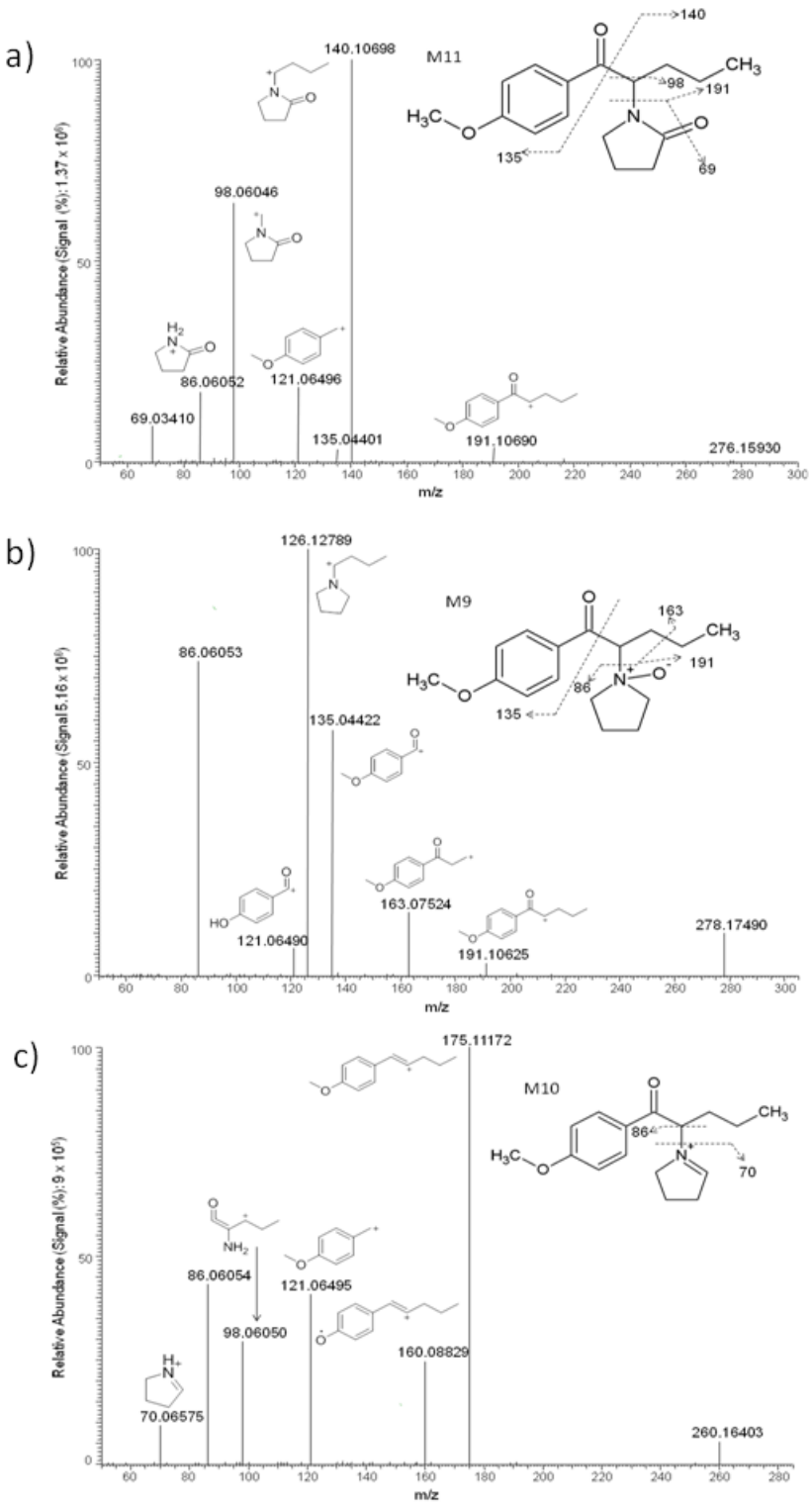


Figure 31. 4-methoxy-α-PVP metabolites and assigned fragmentation patterns for metabolites generated by a) carbonylation of the pyrrolidine ring M11, b) aliphatic N-oxidation M9, c) and iminium ion formation M10. The location of the carbonyl moiety at the 2″ position is based on previously identified α-pyrrolidine lactams.

Prevalence of 4-methoxy-α-PVP Metabolites

Most likely, the most intense MS peak areas observed *in vitro* will be the major 4-methoxy-α-PVP metabolites *in vivo*, or the precursors of second-generation *in vivo* metabolites. The most abundant metabolite in the hepatocyte samples at both 1 and 3 h was 4-hydroxy-α-PVP (M3, RT 7.48 min), resulting from *O*-demethylation. This also was the only metabolite observed in the HLM samples and the highest predicted *in silico* metabolite. The next most abundant hepatocyte metabolites were di-hydroxylations of the pyrrolidine ring (M6, RT 8.78 min) and ketone reduction to the corresponding alcohol (M7, RT 8.96 min). Other major metabolites of 4-methoxy-α-PVP may exist and may have been missed in human hepatocyte and microsomal incubations due to short incubation times, and the long half-life of parent 4-methoxy-α-PVP. Based on our results, 4-hydroxy-α-PVP is the major metabolite of 4-methoxy-α-PVP and could be utilized in combination with the parent compound as potential markers. A metabolic pathway for 4-methoxy-α-PVP in hepatocytes is illustrated in Figure 32.

Metabolic Stability Assessment in Human Liver Microsomes

4-methoxy-α-PVP exhibited a long half-life (79.7 ± 1.3 min) *in vitro*, identified by the slow 9% and 21.3% decrease of parent compound at 1 and 3 h in the hepatocyte samples. Based on the intrinsic clearance, extraction ratio and half-life observed in this study, 4-methoxy-α-PVP is predicted to be slowly metabolized. Low clearance compounds typically have CL_{int} <15 $ml \cdot min^{-1} \cdot kg^{-1}$ [328] and extraction ratios <0.3 [330]. It is important to note that predicted hepatic clearance can vary among individuals based on enzymatic polymorphisms.

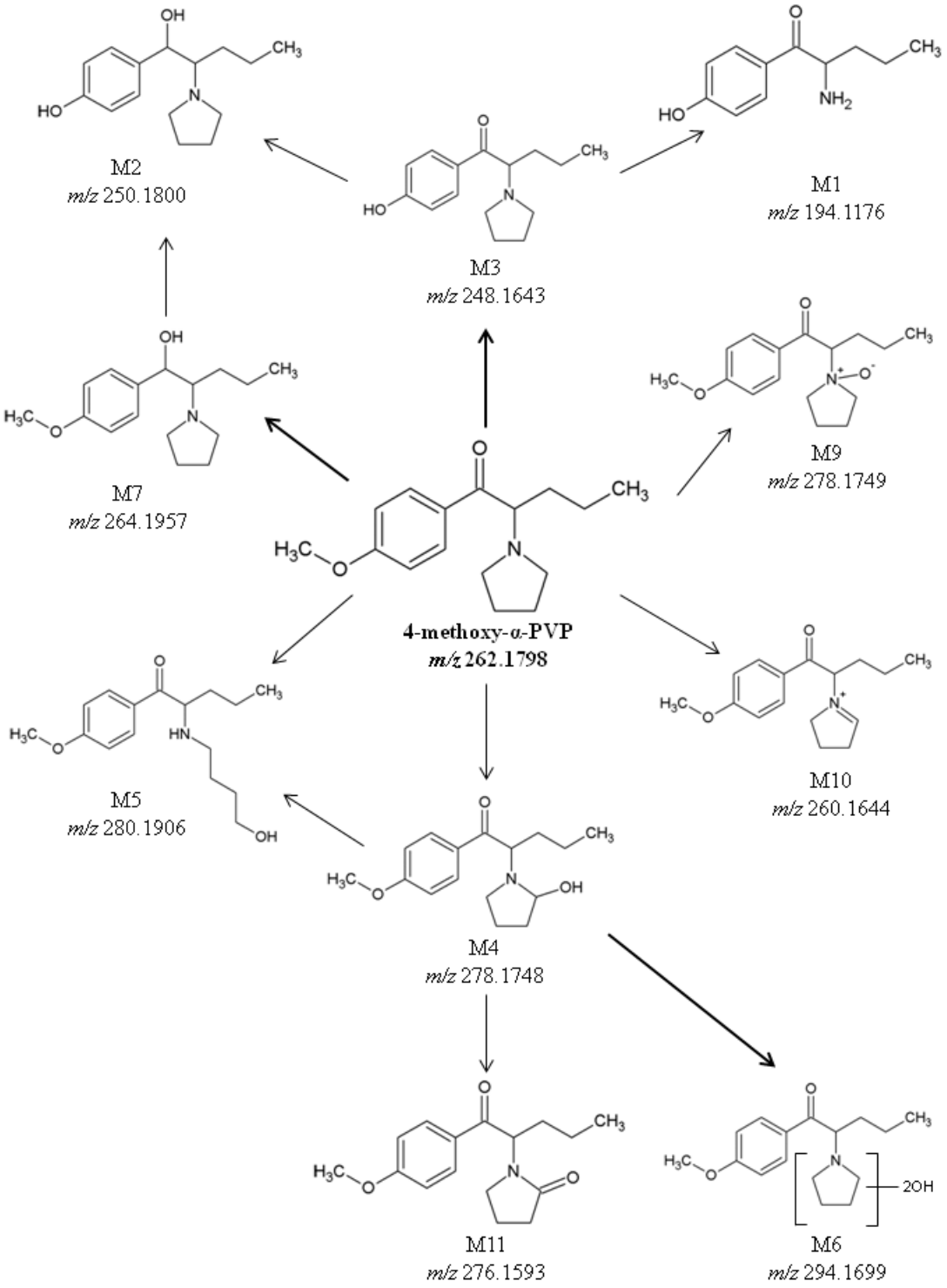


Figure 32. Proposed metabolic pathways for 4-methoxy-α-PVP in human hepatocytes. Position of di-hydroxylation on the pyrrolidine ring in M6 is unknown and represented with Markush structures.

Comparison with Other Synthetic Cathinones

In general, 4-methoxy-α-PVP followed the metabolic patterns identified in other structurally similar synthetic cathinones, i.e. multiple hydroxylation products, carbonylation, ring opening and oxidation reactions, ketone reduction, and *O*-demethylation [324]. The major metabolic pathway identified in our study was *O*-demethylation of the 4-methoxy group, similar to 4-methoxy-α-PPP (a 4-methoxy-α-pyrrolidinophenone with a three carbon alkyl chain) in human liver microsomes by Springer *et al* [175].

Several studies investigated the metabolism of α-PVP, the closest analog to 4-methoxy-α-PVP. An *in vivo* study examining rat urine following a 20 mg/kg α-PVP dose reported multiple hydroxylation products of α-PVP, carbonylation of the 2′ position of the pyrrolidine ring, ring opening and oxidation reactions, as well as degradation of the pyrrolidine ring to the corresponding primary amine [182]. For α-PVP and α-PBP in authentic human urine specimens, Uralets *et al* reported *N*-dealkylation to the primary amine metabolite that subsequently underwent ketone reduction [112]. The authors suggested that the pyrrolidine ring hinders direct keto reduction. No information was provided about the time of urine collection and how long after dosing the specimens were collected. However, ketone reduction was identified by Tyrkko *et al.* while investigating the phase I metabolites of α-PVP *in vitro* utilizing HLM, and in authentic human urine [162]. Similarly, Shima *et al.* found that the two major metabolic pathways of α-PVP identified in human urine were the reduction of the ketone to the corresponding alcohol and the carbonylation of the 2′ position of the pyrrolidinophenone ring to produce the 2″-oxo metabolite [181], which was also confirmed by Namera et al. [155]. Alpha-PVP and

α-PBP do not contain the 4-methoxy moiety, and therefore, cannot produce *O*-demethylated metabolites.

We identified all α-PVP metabolic biotransformations, as discussed above, in our study of the novel 4-methoxy-α-PVP, with the exception of *N*-dealkylation of the pyrrolidine ring to the corresponding primary amine. Since formation of the primary amine metabolite was one of the major pathways for α-PVP, we carefully checked all our raw data, and although we did not identify this metabolite, we did find the metabolite resulting from degradation of the pyrrolidine ring in combination with *O*-demethylation (M1). In agreement with Tyrkko and Shima, we also identified a metabolite resulting from direct reduction of the keto moiety for 4-methoxy-α-PVP (M7) as also observed for α-PVP. Ketone reduction and carbonylation of the pyrrolidine ring were ranked 3rd and 4th among the most prevalent metabolites, respectively.

It is important to note that although incubation with hepatocytes reflects liver metabolism, it cannot reflect additional processes involved in drug elimination including extrahepatic metabolism, enterohepatic circulation, and renal filtration, re-absorption and secretion. In addition, distribution processes can alter parent and metabolite patterns and concentrations that hepatocytes cannot simulate. Metabolites identified in these experiments may be present in human urine and blood after 4-methoxy-α-PVP intake; however, it is important to confirm these metabolites in authentic specimens as hepatocytes only reflect one portion of drug elimination.

In silico Prediction of 4-methoxy-α-PVP Metabolites

In silico metabolite prediction software was utilized to predict 4-methoxy-α-PVP metabolic candidates and evaluate them against identified *in vitro* metabolites. Eleven phase I metabolites with a probability score >20% were predicted *in silico,* while ten phase I metabolites were identified in the 3 h hepatocyte samples. Three *in silico* metabolites (>20%) matched metabolites identified in the 3 h hepatocyte samples, namely M3, which corresponds to P1 scoring 100% *in silico* and also the most predominant HLM and hepatocyte metabolite, M10 (→ P7), M11 (→ P8). Metabolites resulting from hydroxylation of the pyrrolidine ring (M4) and ketone reduction (M7) also were predicted *in silico,* although scores were less than 20% (19.1% and 8.2%, respectively). Five hepatocyte biotransformations were not predicted *in silico;* however, these metabolites involved combinations of phase I biotransformations, which the software does not readily predict. Manufacturers suggest analyzing the top predicted metabolites (100% scores) in the software in order to identify potential second generation metabolites. In our case, none of the predicted second generation metabolites were identified *in vitro* in this study; a possible reason might be the low clearance of the parent compound. Although *in silico* prediction software has limitations, it is a valuable investigative tool for metabolite identification, especially for novel psychoactive substances. Limited information for these compounds exists, so *in silico* prediction software can aid in developing a chromatographic method as it suggests metabolites that may be less polar than the parent. In addition, the software also can be utilized to import an inclusion list for MS acquisition methods.

Conclusions

4-methoxy- α-PVP followed the metabolic patterns comparable to other pyrrolidinophenones, including *O*-demethylation, ketone reduction, multiple hydroxylation products, ring opening and oxidation reactions, *N*-dealkylation, and carbonylation to the corresponding lactam. The combination of different HRMS acquisition methods proved useful for identifying potential metabolites, as these are the first data identifying 4-methoxy-α-PVP metabolites that are good targets for documenting drug intake in forensic and clinical investigations. Based on our results, 4-hydroxy-α-PVP, the predominant metabolite, and the parent compound are good markers to identify consumption. This study demonstrated the applicability of utilizing *in vitro* techniques coupled with HRMS methods to elucidate the metabolic profiles of emerging novel psychoactive substances and comparison with *in silico* metabolite predictions. It is necessary to elucidate the metabolic pathways of these new psychoactive substances to enable linkage to adverse effects and recent intake.

Chapter 10 – Conclusions

The primary objective of this research was to advance analysis and interpretation of classic and novel stimulant test results. First, we determined whether alternative matrices (OF, breath and DBS) are suitable for detection of classical stimulants, e.g. cocaine, in forensic, clinical and workplace drug testing programs. Second, we evaluated new OF on-site roadside screening tests for detection of cocaine, comparing performance of screening immunoassays to an evidentiary OF collection device, and characterized cocaine pharmacodynamics and plasma and OF pharmacokinetics following controlled cocaine administration. Next, we developed and evaluated analytical screening and confirmation methods for novel stimulants, e.g. synthetic cathinones, and human hepatocytes and HRMS techniques to elucidate optimal metabolites for identification of a new synthetic cathinone, 4-methoxy-α-PVP.

The primary aims of this research were to 1) evaluate cocaine's pharmacodynamics and plasma and OF pharmacokinetics after controlled IV administration; 2) evaluate an on-site roadside screening device for cocaine and BE in OF for DUID, workplace, pain management, and clinical drug testing programs; 3) characterize cocaine and metabolites in breath and DBS alternative matrices following controlled IV cocaine administration; 4) conduct a comprehensive literature review of synthetic cathinones including their epidemiology, legal status, pharmacodynamics, pharmacokinetics, clinical implications, and analytical methods for detection in seized products and biological matrices; 5) evaluate performance of an immunoassay for synthetic cathinones in urine and determine the prevalence of synthetic cathinone intake in the US military; 6) develop and validate a methylone and metabolites' quantification

method in rat and human plasma; and 7) characterize *in vitro* Phase I and Phase II metabolism of 4-methoxy-α-PVP, a new synthetic cathinone.

Pharmacodynamic Effects and Relationships to Plasma and Oral Fluid Pharmacokinetics after Intravenous Cocaine Administration

Cocaine pharmacokinetics are well studied in blood, plasma, and urine after various administration routes, but less is known about cocaine and metabolite disposition in OF, OF-plasma ratios, and correlations of cocaine pharmacodynamic effects with OF and plasma concentrations following controlled cocaine administration. Pharmacodynamic effects may correlate with cocaine OF concentrations providing further support for OF as an alternative to blood for DUID interpretation and impairment [10,15]. We characterized cocaine's pharmacodynamics and plasma and OF pharmacokinetics and evaluated OF detection windows in commercially available OF collection devices (Quantisal, Oral-Eze and StatSure) not previously studied following controlled IV cocaine administration.

IV cocaine administration produced the expected increases in heart rate, blood pressure, and subjective ratings within the first 15 minutes, persisting up to 1 h ("Rush"). Subjective measures were strongly correlated with plasma cocaine concentrations and weakly-moderately correlated with OF concentrations. Prior to this research, no studies examined concentration-effect curves for subjective measures and OF cocaine concentrations. Peak subjective effects (for some measures) occurred prior to peak OF cocaine concentrations, whereas they occurred simultaneously with observed peak plasma concentrations and subjective measures, most likely due to significantly earlier plasma T_{max} compared to OF $T_{max.}$ The clockwise hysteresis observed for the relationship between OF concentrations and subjective measures suggests the effect of cocaine

concentration on acute subjective effects is less on the descending limb of the hysteresis curve, potentially due to acute tolerance development.

In support of our hypothesis, we observed significant correlations between cocaine and, for the first time, BE, OF and plasma concentrations following IV cocaine. However, high inter-subject variability in OF concentrations and OF/P ratios were observed, making prediction of individual plasma cocaine concentrations from individual OF concentrations inaccurate. Similar findings were noted in other studies [31,268]. Consistent with previous reports, cocaine OF/P ratios were typically ≥1 from 0.17-12.5 h, and cocaine OF $AUC_{0\text{-}69h}$ (582 h x µg/L) tended to be greater than cocaine plasma $AUC_{0\text{-}21h}$ (345 h x µg/L). Significant increases in BE plasma $AUC_{0\text{-}21h}$ were observed compared to OF $AUC_{0\text{-}21h}$, consistent with previously reported BE OF/P ratios (<1). BE OF/P ratios were generally ≥1 (like cocaine) up to 1 h post-cocaine, despite increased plasma $AUC_{0\text{-}21h}$ compared to OF $AUC_{0\text{-}21h}$, before OF/P ratios decreased to <1 from 1-21 h post-dosing.

Cocaine T_{max} was significantly longer in Quantisal OF (0.5 h) compared to plasma (0.017 h), whereas BE's T_{max} was significantly shorter in Quantisal OF (0.5 h) compared to plasma (2.0 h). We found no significant differences between cocaine and BE half-lives in Quantisal OF compared to plasma, similar to a study with 25 mg IV cocaine [37]. Although there were no significant differences in cocaine and BE Quantisal OF and plasma $T_{1/2}$, we did observe a trend that cocaine Quantisal OF T_{last} was longer than in plasma; plasma and Quantisal OF BE T_{last} could not be statistically compared, as BE plasma concentrations were still positive at the last measurement (21 h).

OE and SS OF collection devices also were effective in monitoring cocaine and BE disposition in OF. However, SS OF concentrations trended lower and detection times were slightly shorter than those for OE. Significant differences in pharmacokinetics parameters were only observed for cocaine $t_{1/2}$; SS exhibited significantly shorter cocaine $t_{1/2}$, with cocaine eliminated more quickly than in OE OF specimens. These observed differences could be due to differences in analyte recovery from the absorbent pad, and/or buffer pH and composition in the OF collection devices. Although median OF cocaine T_{max} in this study was consistent with previous findings after SC administration [31], median BE T_{max} occurred much earlier: 0.5 h versus 2-2.1 h, respectively. However, the previous SC cocaine study collected OF via citric-acid stimulated expectoration. This could contribute to the observed T_{max} difference, as stimulation increases bicarbonate excretion, increasing OF pH and thus affecting cocaine and BE ion trapping into OF, thereby potentially increasing the time required to reach C_{max}.

SAMHSA (8 µg/L) and DRUID (10 µg/L) OF cocaine cutoffs yielded OF detection windows similar to the time course of cocaine's impairing effects, making them useful for roadside DUID and other home and workplace accident investigations. Cocaine OF detection times (6.5 h at SAHMSA and DRUID cutoffs) from the Quantisal collection device after IV cocaine were similar to those reported with OE and SS devices (4-6.5 h) containing stabilizing/elution buffers at 8 and 10 µg/L confirmation cutoffs following IV cocaine. Longer detection windows were achieved for BE (21-28 h) in this study compared to IV, IN, and SM cocaine administration (5.0-8.7 h) with 8 µg/L cutoffs [39]; however, those were collected via citric acid stimulated expectoration with no stabilizers or elution buffer. Additionally, including cocaine in the confirmation did not widen

detection windows compared to BE only testing, as reported previously [31], due to BE being present in the OF immediately after controlled IV cocaine administration and perhaps to the presence of stabilizing and elution buffers in the collection devices. These data advance our understanding of OF as an alternative matrix to blood and plasma for identifying cocaine intake, defining pharmacokinetic parameters at different confirmation cutoffs, and aiding different drug testing programs in the selection of confirmation analytes and cutoffs to best achieve their monitoring goals.

On-site Roadside Screening for Cocaine and BE in Oral Fluid

DUID is a major worldwide problem. Law enforcement requires sensitive, specific, reliable and easy to use devices to detect drug intake. The Draeger DrugTest 5000 is an OF on-site screening device that detects cocaine along with five other drug classes. Recently, a newly developed BE test-strip for the DrugTest 5000 device (20µg/L cutoff) with equivalent cross reactivity for cocaine and BE was manufactured. Prior to our research, performance of this new BE test-strip was not evaluated, and detection rates at different confirmation cutoffs for cocaine and BE were not determined.

The DrugTest 5000 is a sensitive, specific, and efficient on-site OF screening device for both cocaine and BE following controlled administration of IV cocaine at proposed SAMHSA (8 µg/L) and DRUID (10 µg/L) confirmation cutoffs. Cocaine test-strip sensitivity, specificity and efficiency ranged from 85.5-100% and 83.3-100% for cocaine only confirmation at 8 and 10 µg/L. For the BE test-strip, sensitivity, specificity and efficiency ranged from 85.5-97.5% and 78.4-97.4% for cocaine and/or BE confirmation at 8 and 10µg/L cutoffs, respectively. Performance characteristics of the DrugTest 5000 cocaine test-strip with cocaine only confirmation at 10 µg/L were

consistent with previous reports (performance characteristics 82-100%) [89,91]. We reported greater sensitivities (94.1-100%) than what were previously reported with a positive cocaine test-strip and cocaine only confirmation at ≥10 μg/L; however, specificity (83.3-85.9%) and efficiency (89.9-90.8%) were lower potentially due to our longer monitoring windows (69 h).

Additionally, in support of our hypotheses, we demonstrated that the BE test-strip had longer detection windows compared to the cocaine test-strip and that different cutoffs and confirmation analytes influence performance characteristics, detection rates and windows of detection. Cocaine test-strip median T_{last} for screening only results was 6.5 h, and 6.5 h with OE and 4 h for SS OF confirmation for cocaine and/or BE at 1, 8 and 10 μg/L. For the BE test-strip, median T_{last} was 12.5 h for screening only and confirmation for cocaine and/or BE at all three cutoffs. Based on overall results of this study, the cocaine DrugTest 5000 with cocaine only OF confirmation is a suitable option for monitoring the acute cocaine intoxication phase of DUID, whereas the BE DrugTest 5000 with cocaine and/or BE confirmation is better suited for workplace drug testing, drug court, parole, pain management, drug treatment monitoring, and both the acute cocaine intoxication and cocaine crash/fatigue phase of DUID.

Characterization of Cocaine and Metabolites in Breath and DBS Alternative Matrices

OF offers many advantages as described previously, but it also has disadvantages including small sample volume, especially after intake of sympathomimetic drugs, and interpretation of results due to oral mucosal contamination. These factors may produce a poor correlation for OF/plasma concentrations early in the time course. Alternative matrices like breath and DBS also may be suitable for detecting drug use.

Breath was previously investigated as an alternative matrix for detecting recent cocaine use; however, there were no controlled cocaine administration studies investigating the drug's disposition in breath prior to this research. We developed and validated a sensitive and specific LC-HRMS method for the determination of cocaine, BE, EME and norcocaine with linear ranges from 25-1,000 pg/filter and acceptable validation parameters. Only 2.6% of breath specimens collected were positive for cocaine and 0.72% positive for BE and EME, with concentrations lower than previously reported. Following a methanolic extraction of the whole breath device, we observed increased % positives (for cocaine and BE) and higher concentrations more similar to previous reports, suggesting that the device reflects drug in OF as well as drug-laden microparticles, also identified in the breath filters. Based on these results, cocaine in breath can identify recent cocaine ingestion, but its absence does not preclude recent use.

Additionally, little information is known about cocaine disposition in DBS following controlled cocaine administration. A few studies examined DBS results with corresponding venous blood cocaine concentrations from authentic specimens [58,59,65,73], but, no studies investigated the equivalence between true capillary DBS, as opposed to venous DBS, and venous blood after controlled cocaine administration. We developed sensitive and specific methods for the detection of cocaine, BE and norcocaine in DBS (LC-HRMS) and cocaine and BE in blood (2D-GC-MS) with a 1 µg/L LOQ to determine if capillary DBS concentrations correlated with venous blood concentrations. As hypothesized, the two methods were significantly correlated, although only moderately, with R^2 values indicating we could not accurately predict venous blood concentrations from those of DBS. Although cocaine and BE were detected in all DBS,

DBS concentrations were lower and ranges wider than their respective venous blood concentrations, with significant variability (intra- and inter-) among DBS concentrations collected at the same time (up to five spots per card) also observed. These DBS discrepancies (and highly variable DBS concentrations) could be attributed to blood volumes spotted onto the cards, differences in extraction efficiency and/or possible venous-capillary blood differences. DBS are an alternative matrix to blood for detecting recent consumption; however, further investigation into its limitations following controlled drug administration is warranted.

Synthetic Cathinone Pharmacokinetics, Analytical Methods and Toxicological Findings in Human Performance and Postmortem Cases

The continual emergence of novel synthetic cathinones poses constant challenges for both clinical and forensic toxicologists. Few data are available on the pharmacology of these substances and modifications to existing analytical methods are needed to detect emerging NPS. To support our hypothesis that synthetic cathinones are NPS that appeal to the general public due to their purported psychoactive effects similar to other illicit drugs like cocaine and MDMA, and their lack of detection in routine drug tests, we conducted a comprehensive literature review addressing *in vitro* and *in vivo* synthetic cathinone pharmacokinetics, analytical methods for detection and quantification in biological matrices, and toxicological findings from human performance and PM cases.

To help identify NPS, simultaneous targeted quantitative and non-targeted screening methods by HRMS offer advantages, as they allow for retrospective data analysis and easier addition of new synthetic cathinones to existing methods. However, a challenging issue with synthetic cathinones is their apparent instability in biological matrices and thermal degradation. In addition, identification of suitable biomarkers, via *in*

vitro studies and the development of commercial reference standards must be addressed in order to document intake of these compounds and link toxicity to causative agents. Lack of controlled administration studies in humans, complicate synthetic cathinone interpretation in biological matrices as dosing information is typically unknown. Furthermore, antemortem and PM concentrations often overlap and other substances are typically found in combination with cathinone derivatives, further confounding results. Laboratories must be capable of responding quickly, adapting to changing NPS trends, and improving analytical methodologies for the detection of these highly abused compounds.

Immunoassay Performance for Synthetic Cathinone Detection in Urine

Deterrence of synthetic cathinone abuse is hampered by the lack of a high-throughput immunoassay screen, especially since most immunoassays do not readily detect synthetic cathinones [217,231,234]. Randox developed a semi-quantitative screening system for synthetic cathinone detection in urine utilizing the Randox DOA-V, the only commercially available immunoassay for urinary cathinone detection. Prior to this research, performance of this assay was not evaluated and it was unknown if this was a reliable assay for screening synthetic cathinones in urine.

Based on our validation, the Randox DOA-V for synthetic cathinone screening consistently exhibited a high negative % bias; concentrations for mephedrone (BSI) and MDPV (BSII) were consistently low and below acceptable criteria (±20%). We hypothesized that the Randox DOA-V biochip array immunoassay was a sensitive, specific and efficient assay for urine cathinone screening; however, specificity, sensitivity and efficiency of 100%, 52.1% and 53.0% were obtained at the manufacturer's proposed

cutoffs (BSI 5 µg/L, BSII 30 µg/L). Performance was improved at higher cutoff concentrations (BSI 7.5 µg/L, BSII 40 µg/L), confirming our hypothesis that alternative screening cutoffs compared to manufacturer's cutoffs may improve performance. Based on this method validation alone, it was difficult to establish proposed cutoffs due to the high negative % bias and the limited confirmed urine specimens (*n*=4) by LC-HRMS.

After screening urine specimens from 20,017 military personnel worldwide with the Randox DOA-V, overall presumptive positive rate was 0.53% (*n*=106) for one or more synthetic cathinones; only 4.1% presumptive positive specimens confirmed positive for one or more synthetic cathinones at the manufacturer's proposed cutoffs, suggesting a high false positive rate. One study limitation was the unknown stability of synthetic cathinones in urine; specimens were stored at room temperature generally two to four weeks prior to screening, and urine specimens were stored up to a year (4°C) prior to confirmation. However, it is not possible to definitively determine if the high false positive screening rate and low confirmation rate could be from analyte instability and degradation, inappropriately low cutoff concentrations, or cross-reactivity to additional synthetic cathinones not present in the confirmation method (although this confirmation method was the most comprehensive to date at the time of research) without fresh specimens. LC-MS/MS screening methods incorporating a wide range of compounds may be better suited for detection of these NPS compared to immunoassays designed to detect synthetic cathinones in high prevalence at the time of assay development.

Quantification Method for Methylone and Metabolites in Rat and Human Plasma

Prior to our research, there were no published quantitative methods for the analysis of methylone and its main metabolites in plasma. It is critical to develop assays

to detect methylone and metabolites to determine their presence and concentrations in forensic and clinical settings, as it remains one of the 25 most frequently identified drugs in the US despite scheduling [106]. In addition, limited data on methylone and metabolite pharmacokinetics exists.

A sensitive and specific LC-MS/MS method was developed for the simultaneous quantification of methylone and its metabolites, HMMC, MDC and HHMC, in human and rat plasma with low specimen volume (100 µL). Low specimen volumes are important for animal pharmacokinetic studies as low amounts of specimen are available for analyses. Sensitive LOQs were achieved with only 100 µL specimen; 0.5 µg/L for methylone, HMMC and MDC, and 10 µg/L for HHMC. Only one prior study quantified methylone in rat plasma, however, the LOQ was 10 µg/L in 100 µL specimen. Our new method will allow for further investigation of methylone pharmacokinetics through application of this method to rat plasma specimens obtained from a preclinical study examining the pharmacodynamics and pharmacokinetics in rats following SC methylone administration (data not yet published).

Preliminary data were obtained from a rat 15-480 min post-dosing following 6 mg/kg SC methylone. The highest concentrations observed for methylone, HMMC, MDC and HHMC were 1,310 µg/L (15 min post-dosing), 194 µg/L (60 min post-dosing), 152 µg/L (60 min post-dosing) and 185 µg/L (60 min post-dosing), respectively. Plasma concentrations of methylone, HMMC, MDC and HHMC were 1.1, 11.2, 1.9 and 24.7 µg/L at 480 min post-dose, respectively. These preliminary data support our hypothesis that methylone plasma C_{max} would be higher than its metabolites, although metabolites will exhibit longer detection windows.

Metabolic Profiling of Synthetic Cathinone 4-methoxy-α-PVP

It is necessary to determine the most important metabolites of new NPS so they can be included in analytical methods to document intake and link adverse effects to causative agents. A new synthetic cathinone, 4-methoxy-α-PVP (4-methoxy-α-pyrrolidinovalerophenone) was identified for the first time in illegal products purchased in Japan in 2013 [322] and also recently identified by Customs in Berlin [323]. Prior to our research, there were no metabolism studies for 4-methoxy-α-PVP. A successful approach utilized in our laboratory to elucidate NPS metabolite structures and biotransformation pathways involves determining metabolic stability with HLM, metabolite profiling with human hepatocyte incubations and HRMS and comparing *in vitro* metabolites with *in silico* software predictions.

In support of our final aim, we hypothesized that 4-methoxy-α-PVP would have a similar metabolic profile to other α-pyrrolidinophenones' metabolism, including *O*-demethylation, ketone reduction, multiple hydroxylations, ring opening and oxidation reactions, *N*-dealkylation, and carbonylation to the corresponding lactam. We identified 10 Phase I and 1 Phase II metabolite generated by *O*-demethylation, hydroxylation, oxidation, ketone reduction and glucuronidation, supporting our hypothesis. In addition to the parent compound, 4-OH-α-PVP was identified as a suitable biomarker to identify drug consumption; however, a limitation was our inability to confirm these predicted *in vitro* metabolites in authentic specimens. Our study findings also demonstrated a relatively long half-life of 79.7 min and predicted 4-methoxy-α-PVP to be a low clearance compound based on hepatic clearance and extraction ratios. *In silico* prediction software was unable to identify the majority of identified metabolites, suggesting that it should not be implemented as a standalone method to identify NPS metabolites, rather to

compliment distinguishing chromatographic elution order and likely reaction sites. Metabolic profiling with human hepatocytes and HRMS are valuable alternatives to controlled drug administration studies, especially with the continued emergence of NPS and limited preclinical safety data.

Future Directions

Additional research is needed to investigate identification of cocaine markers in alternative matrices for use in DUID, workplace, clinical and anti-doping programs. The present research primarily focused on detection of cocaine markers in OF, breath and DBS following controlled IV cocaine administration. Other investigations are needed to establish the disposition of cocaine and its metabolites in alternative matrices following other routes of administration, as it is unclear how route will alter cocaine and its metabolite pharmacokinetic parameters and detection windows. Furthermore, the cocaine dose administered in this research was lower than what is typically self-administered in the community in order to minimize adverse effects. Results may differ with higher doses and repeated dosing. Additionally, as new techniques appear for on-site screening of cocaine and metabolites in alternative matrices, they too will need to be evaluated to determine their usefulness for workplace drug testing, parole, pain management, drug treatment and DUID programs.

The increased prevalence and continuous emergence of new NPS in recent years poses many challenges for law enforcement, military, public safety officials, and also forensic and clinical toxicologists. Despite scheduling of many NPS, clandestine laboratories are constantly developing modified derivatives. Although both qualitative and quantitative methods were developed to detect numerous NPS substances,

laboratories cannot keep pace and reference standards are not always available. Simultaneous targeted-non-targeted HRMS methods should be considered to detect and quantify these compounds. Future research should also address the detection and quantification of NPS in alternative matrices as they offer many advantages over traditional matrices like blood and urine. Elucidation of NPS metabolites via *in vitro* and *in vivo* metabolism studies also continue to identify analytical targets, link causative agents to toxicities and provide necessary preclinical safety data to support controlled drug administration studies. Additionally, more preclinical *in vivo* drug administration studies are needed to characterize NPS pharmacodynamics and pharmacokinetics to aid in interpretation of results.

Finis

Despite decreasing cocaine prevalence in the US, it remains one of the most widely used illicit stimulants worldwide. Pharmacodynamics and pharmacokinetics are well established in biological matrices like blood and urine; however, less is known about cocaine and metabolites' disposition in alternative matrices. This research developed and validated methods to identify cocaine and metabolites in OF, breath and DBS. We provided evidence that OF is a suitable matrix to identify cocaine use following IV cocaine. In contrast, although cocaine and metabolites were identified in breath and DBS, further research is necessary to address their limitations, such as low prevalence and variability, before these alternative matrices can be routinely implemented in a variety of drug testing programs. Recently, NPS including synthetic cathinones, emerged on the drug market with no shortage of challenges for clinical and forensic toxicologists. Analytical methods in biological matrices and limited pharmacokinetic data hinder

interpretation of results in human performance and postmortem cases. Furthermore, limited commercially available reference standards, instability and thermal degradation pose additional problems for detecting and quantifying these compounds in biological matrices. Immunoassays do not readily detect synthetic cathinones, and those specifically developed for cathinone detection exhibited large negative % bias and poor specificity and efficiency. Inclusion of metabolites in confirmatory methods is imperative for quantification and characterization after preclinical *in vivo* drug administration to expand our knowledge of these psychoactive stimulants. Metabolite profiling with HLM and human hepatocytes and HRMS also is critical to determine analytical targets, in addition to parent compounds, and to link toxicity to causative agents.

Additional Projects

As a predoctoral visiting fellow at the NIDA Intramural Research Program, I was the lead associate investigator for a NIDA Institutional Review Board and Food and Drug Administration (FDA)-approved study entitled "Pharmacodynamic and pharmacokinetic interactions between intravenous cocaine and acetazolamide or quinine." Specifics are outlined in Appendix A. The study's primary aims were to evaluate potential pharmacodynamic and pharmacokinetic interactions between cocaine and acetazolamide and quinine, as they are considered compliance markers for cocaine dependence treatment. These data are being evaluated by other co-investigators. Many people participated in the development and approval of the clinical protocol, as well as specimen and data analysis. As lead associate investigator, I was involved in designing and writing the clinical protocol, the FDA Investigational New Drug Application, standard operating procedures, protocol amendments and problem reports. My role in specimen and data

analysis included developing and validating methods for the detection of cocaine and metabolites in breath, OF, venous blood and DBS, and plasma and OF pharmacokinetics, DrugTest 5000 OF analysis, breath and DBS performance, and subjective and physiological effects.

Additionally, I also was involved in multiple projects aside from my dissertation research, including a Department of Defense, Drug Testing and Program Policy, Operational Readiness and Safety contract with NIDA, under which we conducted a military-prevalence study of NPS, involving immunoassay screening of 20,017 urine specimens and confirmation of presumptive positive tests. Furthermore, I participated in the NIDA Designer Drug Research Unit's research investigating preclinical pharmacology and toxicology of synthetic cathinones and other NPS. Below is a brief summary of research objectives, methods, and findings for each project.

Simultaneous quantification of 28 synthetic cathinones and metabolites in urine by liquid chromatography-high resolution mass spectrometry[10]

Synthetic cathinones are novel stimulants derived from cathinone, with amphetamines or cocaine-like effects, often labeled "not for human consumption" and considered "legal highs". Emergence of these new designer drugs complicate interpretation of forensic and clinical cases, with introduction of many new analogs designed to circumvent legislation and vary effects and potencies. We developed a method for the simultaneous quantification of 28 synthetic cathinones, including four metabolites, in urine by liquid chromatography coupled to high resolution mass

[10] Abstract from Concheiro et al. *Anal. Bioanal. Chem.* 2013: 405(29): 9437-48.

spectrometry (LC-HRMS). These cathinones include cathinone, methcathinone, and synthetic cathinones position-3'-substituted, N-alkyl-substituted, ring-substituted, methylenedioxy-substituted, and pyrrolidinyl-substituted. One mL phosphate buffer pH 6 and 25 μL IStd solution were combined with 0.25 mL urine, and subjected to solid phase cation exchange extraction (SOLA SCX). The chromatographic reverse-phase separation was achieved with a gradient mobile phase of 0.1% formic acid in water and in acetonitrile in 20 min. We employed a Q Exactive high resolution mass spectrometer, with compounds identified and quantified by target-MSMS experiments. The assay was linear from 0.5–1 to 100 μg/L, with limits of detection of 0.25–1 μg/L. Imprecision (*n*=20) was <15.9% and accuracy (*n*=20) 85.2–118.1%. Extraction efficiency was 78.9–116.7% (CV 1.4–16.7%, *n*=5), process efficiency 57.7–104.9%, and matrix effects from −29.5% to 1.5% (CV 1.9–13.1%, *n*=10). Most synthetic cathinones were stable at 4°C for 72 h (*n*=27) and after 3 freeze-thaw cycles (*n*=26), but many (*n*=19) were not stable at room temperature for 24 h (losses up to −67.6%). The method was applied to authentic urine specimens from synthetic cathinone users. This method provides a comprehensive confirmation method for 28 synthetic cathinones in urine, with good selectivity and specificity.

Method validation ofthe biochip array technology for synthetic cannabinoids detection in urine[11]

Synthetic cannabinoids (SC) are widely-abused cannabimimetic drugs that do not screen positive in traditional cannabinoids immunoassays, making detection difficult.

[11] Abstract from Castaneto et al. *Bioanalysis*. 2014; 6(21): 2919-2930.

The first commercially-available immunoassay for urinary SC was validated. Limits of detection (5-20 µg/L), imprecision (<13.1% intra-, <37.7% inter-assay), and cross-reactivity profiles of 22 SC and 37 metabolites were obtained. A large negative bias (-80.8 to -28.0%) was observed. Sensitivity (98.3%), specificity (48.1%) and efficiency (53.9%) were determined from screening 20,017 urine specimens and confirming 1432 presumptive positive and 1069 selected negative specimens by LC-MS/MS. Cutoff optimization improved performance to 87.6% sensitivity, 85.2% specificity, and 85.4% efficiency. This high-throughput urine SC assay has good sensitivity and improved specificity and efficiency at modified cutoff concentrations.

3,4-Methylenedioxypyrovalerone (MDPV) and metabolites quantification in human and rat plasma by liquid chromatography-high resolution mass spectrometry[12]

Synthetic cathinones are recreational drugs that mimic the effects of illicit stimulants like cocaine, amphetamine or Ecstasy. Among the available synthetic cathinones in the United States, 3,4-methylenedioxypyrovalerone (MDPV) is commonly abused and associated with dangerous side effects. MDPV is a dopamine transporter blocker 10-fold more potent than cocaine as a locomotor stimulant in rats. Previous *in vitro* and *in vivo* studies examining MDPV metabolism reported 3,4-dihydroxypyrovalerone (3,4-catechol-PV) and 4-hydroxy-3-methoxypyrovalerone (4-OH-3-MeO-PV) as the two primary metabolites. We developed and validated a liquid chromatography-high resolution mass spectrometry method to quantify MDPV and its primary metabolites in 100 µL human and rat plasma. Plasma hydrolysis was followed by protein precipitation before analysis. Limits of detection were 0.1 µg L^{-1}, with linear

[12] Abstract from Anizan et al. *Anal. Chim. Acta.* 2014; 827: 54-63.

ranges from 0.25-1000 µg L^{-1}. Process efficiency, matrix effect, total imprecision (%CV) and accuracy (%target) were 36 to 93%, -8 to 12%, 2.1 to 7.3% and 86 to 109%, respectively. MDPV and metabolites were stable at room temperature for 24h, 4°C for 72h and after 3 freeze-thaw cycles with less than 10% variability. Human-rat plasma cross validation demonstrated that rat plasma could be accurately quantified against a human plasma calibration curve. As proof of this method, rat plasma specimens were analyzed after intraperitoneal and subcutaneous dosing with MDPV (0.5 mg kg^{-1}). MDPV, 3,4-catechol-PV and 4-OH-3-MeO-PV concentrations ranged from not detected to 107.5 µg L^{-1}.

In vitro, In vivo and In silico Metabolic Profiling of α-Pyrrolidinopentiothiophenone, a Novel Thiophene Stimulant[13]

Little or no pharmacological or toxicological data are available for novel psychoactive substances when they first emerge, making their identification and interpretation in biological matrices challenging.

A new synthetic cathinone, α-pyrrolidinopentiothiophenone (α-PVT), was incubated with hepatocytes and samples were analyzed using liquid chromatography coupled to a Q Exactive™ Orbitrap mass spectrometer. Authentic urine specimens from suspected α-PVT cases also were analyzed. Scans were data mined with Compound Discoverer™ for identification and structural elucidation of metabolites. Seven α-PVT metabolites were identified in hepatocyte incubations, and in the authentic urine samples, also with an additional monohydroxylated product and a glucuronide of low intensity. α-

[13] Abstract from Swortwood et al. *Bioanalysis.* 2016; 8(1): 65-82.

PVT dihydroxypyrrolidinyl, α-PVT 2-ketopyrrolidinyl, α-PVT hydroxythiophenyl, and α-PVT thiophenol had the most intense *in vivo* signals.

First Metabolic Profile of PV8, a Novel Synthetic Cathinone, in Human Hepatocytes and Urine by High-Resolution Mass Spectrometry[14]

Novel psychoactive substances (NPS) are constantly emerging onto the illicit drug market making identification of new cathinones and their metabolites difficult. Recently, PV8, a synthetic pyrrolidinophenone, was identified in illegal products purchased in Japan in 2013 and in seized products in the Netherlands and Germany. There are no controlled PV8 administration studies, and no pharmacodynamic and pharmacokinetic data. The objective was to determine PV8's metabolic stability in human liver microsome (HLM) incubation and its metabolism following human hepatocyte incubation and high-resolution mass spectrometry (HRMS) with a Thermo Scientific Q-Exactive. Data were acquired with a full-scan data-dependent mass spectrometry method. Scans were thoroughly data mined with different data processing algorithms and analyzed in WebMetaBase. PV8 exhibited a relatively short 28.8 min half-life, with an intrinsic 24.2 μL/min/mg microsomal clearance. This compound is predicted to be an intermediate clearance drug with an estimated human 22.7 mL/min/kg hepatic clearance. Metabolic pathways identified *in vitro* included: hydroxylation, ketone reduction, carboxylation, *N*-dealkylation, iminium formation, dehydrogenation, *N*-oxidation, and carbonylation. The top three *in vitro* metabolic pathways were di-hydroxylation > ketone reduction > γ-lactam formation. Authentic urine specimen analyses revealed the top three metabolic pathways were aliphatic hydroxylation > ketone reduction + aliphatic hydroxylation >

[14] Abstract from Swortwood et al. *Anal. Bioanal. Chem.* 2016, *In press*.

aliphatic carboxylation, although the most prominent peak was parent PV8. These data provide useful urinary metabolite targets (aliphatic hydroxylation, aliphatic hydroxylation + ketone reduction, aliphatic carboxylation, and di-hydroxylation) for forensic and clinical testing and also focus reference standard companies' synthetic efforts to provide commercially available standards needed for PV8 biological specimen testing.

APPENDIX A – Clinical Protocol Description (Pharmacodynamic and pharmacokinetic interactions between intravenous cocaine and acetazolamide or quinine)

Study Objectives

The primary goals of this study are to evaluate pharmacodynamic and pharmacokinetic interactions between IV cocaine and oral acetazolamide and oral quinine in plasma and urine, in order to determine the suitability of acetazolamide and quinine as markers for medication adherence in studies of cocaine addiction treatment. Pharmacodynamic (including cocaine subjective effects) interactions are evaluated, along with pharmacokinetic interactions, in accordance with FDA recommendations and the NIDA Division of Pharmacotherapies and Medical Consequences of Drug Abuse format.

Primary objectives

- Determine whether multiple oral doses of acetazolamide significantly alter the pharmacokinetic parameters of IV cocaine in plasma and urine in terms of maximum concentration (C_{max}), time to C_{max} (T_{max}), or area under the curve (AUC).
- Determine whether multiple oral doses of quinine significantly alter the pharmacokinetic parameters of IV cocaine in plasma and urine in terms of C_{max}, T_{max}, or AUC.
- Determine whether a single IV dose of cocaine significantly alters the pharmacokinetic parameters of multiple oral doses of acetazolamide or quinine in plasma and urine in terms of C_{max}, T_{max}, or AUC.
- Determine whether multiple oral doses of acetazolamide significantly alter the subjective and physiological effects of IV cocaine.
- Determine whether multiple oral doses of quinine significantly alter the subjective and physiological effects of IV cocaine.

Secondary objectives

- Evaluate the performance characteristics of the Draeger on-site oral fluid screening device for detection of controlled IV administration of cocaine.
- Evaluate the performance characteristics of the Quantisal, Oral-Eze and Stat-Sure, devices for the detection of IV cocaine.
- Evaluate the performance characteristics of breath for the detection of controlled IV administration of cocaine.
- Determine whether butyrylcholinesterase or carboxylesterase genotypes influence plasma enzyme activity.
- Determine whether plasma butyrylcholinesterase activity phenotype influences the pharmacokinetic parameters of IV cocaine.
- Determine the contribution of plasma carboxylesterase activity to the metabolism of IV cocaine.

- Evaluate the performance characteristics of dried blood spots (DBS) for the detection of cocaine and metabolites
- Evaluate the performance characteristics of AutoSense for monitoring physiological measures during cocaine use
- Evaluate the acute and chronic effects of cocaine on concentrations of leptin and other appetitive peptides

Description of study population

Subjects are healthy adults (male or female) with a history of smoked or IV cocaine use. Up to 30 subjects will be enrolled to achieve 10 completers for each of the acetazolamide and quinine phases of the study. Non-completers will be replaced.

Inclusion criteria

- Age 18-50 years old
- Smoked or used IV cocaine for at least six months and at least three times per month during the three months prior to screening. Urine test positive for cocaine within the prior 6 months
- Adequate venous access for catheter placement
- Serum sodium and potassium concentrations within normal limits (based on Johns Hopkins Bayview Medical Center clinical laboratory)
- Women with reproductive potential must use a medically acceptable form of contraception for the duration of the study. Medically acceptable forms of contraception include: oral contraceptive, intrauterine device (IUD), depot hormonal preparation (ring, injection implant), or a barrier method of contraception such as a diaphragm, sponge with spermicide, or a condom.

Exclusion criteria

- Current physical dependence on any drug other than cocaine, caffeine, or nicotine
- Current clinically significant medical or psychiatric disorder, such as heart disease, kidney disease, liver disease, adrenal insufficiency, myasthenia gravis, glucose-6-phosphate dehydrogenase deficiency, epilepsy, stroke, optic neuritis, hyperthyroidism, glaucoma; or psychosis, panic attacks, depression, or mania
- Current sulfa allergy
- Currently seeking treatment for a cocaine use disorder or in such treatment within the prior 3 months
- Current hypertension or blood pressure readings consistently above 140 mm Hg systolic or 90 mmHg diastolic while at rest
- Heart rate consistently above 90 bpm or below 50 bpm while at rest
- History of premature coronary artery disease or heart attack before age 50 in a first degree biological relative

- QTc greater than 450 ms or evidence of heart block, ischemia, or other clinically significant cardiovascular disease on a 12-lead resting ECG with three-minute rhythm strip
- Hemoglobin less than 12.5 g/dL
- Blood donation within 8 weeks of study entry
- History of clinically significant adverse reaction to ingestion of cocaine, acetazolamide, or quinine
- Hypersensitivity to acetazolamide, sulfonamides, sulfonamide derivatives, quinine, mefloquine or quinidine
- Women who are pregnant or nursing
- Currently on anti-hypertensive medication

Study Design and Methods

Study overview

This is a single site, open-label, non-treatment study involving a single visit after enrollment. Each participant spends a 13-day, 12-night stay on a secure research unit on the Johns Hopkins Bayview campus in Baltimore, MD. Subjects spend their days at the NIDA IRP clinical research area in the NIH Biomedical Research Center (BRC) undergoing research procedures.

After admission, subjects spend Day -1 and 0 undergoing baseline assessments, training, and medication/drug washout. Single doses of 25 mg IV cocaine are given by the physician the morning of Days 1, 5 and 10. Single oral doses of 15 mg acetazolamide are given on Days 2-5 and of 80 mg quinine on days 7-10. Day 6 is a washout day, with no medication given. Subjects are discharged on Day 11.

Potential participants in this study are recruited and screened under a separate NIDA screening protocol (06-DA-N415) with separate investigators and study staff.

Recruitment/Screening

Cocaine users are recruited from the community with print, radio, television and web-based advertisements, previously approved by the NIDA IRB. Participants will be enrolled in Protocol 06-DA-N415 (Evaluation of potential research subjects – screening protocol for clinical studies) and screened for study entry.

Study and Procedures

To ensure validity of the pharmacokinetic data, participants will be questioned prior to admission to verify that they have not taken any prescription or over-the-counter medication, grapefruit juice, or herbal supplement within the prior 14 days. Oral contraceptives are excluded from this requirement.

Subjects are admitted to a secure research unit after noon on Day -1, undergo a 12-lead resting ECG with 3-min rhythm strip and baseline physiological assessments (vital signs). The next day (Day 0), subjects undergo training.

On Days 1, 5, and 10 at around 11 a.m., subjects receive 25 mg IV cocaine hydrochloride dissolved in sterile saline over 10 sec. Subjective and physiological measures are assessed beforehand and periodically thereafter for the next 3 h. Venous blood, oral fluid, and breath specimens for assay of cocaine and metabolites are collected beforehand and periodically thereafter for 70 h. Dried blood spot specimens are collected on Days 1, 5, and 10 up to 3 times daily over 1.5 h. On Days 2-5, subjects receive an oral dose of 15 mg acetazolamide around 10 a.m. On Days 4-8, venous blood specimens for assay of acetazolamide are collected before acetazolamide dosing and periodically thereafter for 71 h. A 12-lead resting ECG with 3-min rhythm strip is done prior to cocaine dosing and 3-4 h afterwards.

Day 6 is a washout day, with no medication given; blood and oral fluid will be collected once and all voided urine is collected by the participant.

On Days 7-10, subjects receive an 80 mg oral quinine dose around 9 a.m. On Days 9 and 10, venous blood specimens for assay of quinine are collected beforehand and periodically thereafter for 23 h.

On Day 11, venous blood, oral fluid, and breath specimens are collected and subjects are discharged from the study in the late afternoon.

The AutoSense device will be worn by participants to collect physiological measures on Days 1, 5, and 10, as well as 4 additional days in which cocaine is not administered (Days 3, 4, 8, and 9) for up to12 hours each day. All voided urine is collected throughout the study by the participant themselves.

Specimen collection and analysis

Plasma and urine specimens will be analyzed for cocaine, benzoylecgonine, norcocaine and ecgonine methyl ester, and acetazolamide and quinine by GCMS, GCMSMS, or LCMSMS. Oral fluid specimens will be analyzed by the Draeger 5000 on-site test for cocaine, and by GCMS, GCMSMS, or LCMSMS for cocaine, benzoylecgonine, norcocaine and ecgonine methyl ester in specimens collected with the Quantisal, Oral-Eze and Stat-Sure devices. Breath specimens will be analyzed by GCMS, GCMSMS, or LCMSMS for cocaine, benzoylecgonine and ecgonine methyl ester.

Plasma and whole blood specimens

Peripheral venous blood collection varies with the day of the study, up to 19 times in 24 h according to the schedule in Appendix 2. Blood is collected prior to dosing and for up to 71 h after dosing. Specimens are collected in 5 (cocaine analysis only) or 10 mL (cocaine and marker analysis) gray-top (sodium fluoride, potassium oxalate) Vacutainer® tubes and stored on ice prior to centrifugation. For specimens where whole blood is collected in conjunction with dried blood spots, 1 mL of whole blood is transferred to one cryotube prior to centrifugation and storage of plasma specimens in cryotubes at -20ºC until analysis. No additional blood is needed for the whole blood specimen. This will come from blood already collected at these time points. For specimens collected for the analysis of acetazolamide, 1 mL of whole blood is transferred to one cryotube prior to centrifugation and storage of plasma specimens in cryotubes at -20°C until analysis. For specimens collected for the analysis of quinine or cocaine, plasma is separated by

centrifugation and stored in cryotubes at -20°C until analysis. 481.5 mL total blood is collected during the study (444 for pharmacokinetics, 10 mL for genotyping, 20 mL for BChE/carboxylesterase activity, and 7.5 mL for serum concentrations of leptin and other appetitive peptides; no more than 22.5 mL blood is collected at any single time point). Cocaine, benzoylecgonine, norcocaine, ecgonine methylester, acetazolamide, and quinine will be quantified in plasma by the University of California, San Francisco (UCSF) under the NIDA analysis contract. Acetazolamide will be also quantified in whole blood by UCSF under the NIDA analysis contract. Whole blood collected in conjunction with dried blood spot specimens will be analyzed for cocaine and metabolites by the Chemistry and Drug Metabolism (CDM) Section of the Intramural Research Program (IRP) of NIDA. No additional blood is needed for the whole blood specimen. This will come from blood already collected at these time points.

Dried blood spot specimens

Dried blood spot specimens (DBS) are collected from each participant up to 3 times in 1.5 h according to the schedule in appendix 2. DBS collections are performed on Days 1, 5, and 10 (Pharmacokinetic Session 1, 3, and 5). For DBS specimen collection, whole blood is collected via finger prick onto a collection card. The collection card is dried for at least 4 hours at room temperature, and stored frozen in a dry plastic bag with desiccant at -20ºC until analysis. Cocaine, ecgonine methyl ester, norcocaine and benzoylecgonine will be quantified in DBS by CDM, IRP, NIDA. Participants may be enrolled in the study if they refuse dried blood spot collection.

Urine specimens

Participants collect all voided urine *ad libitum* in polypropylene bottles during their entire stay on the research unit. Participants are responsible for labeling urine bottles with their name, date and time collected and completing an inventory of specimens that is checked by unit staff. Specimens are temporarily stored in a refrigerator until retrieved by research staff. The total volume of each urine void is measured and recorded. Urine specimens are pooled for 3-hour intervals by research staff, except for each morning's first urination, which is kept separate. Aliquots are stored frozen at –20°C until analysis. Cocaine, benzoylecgonine, ecgonine methyl ester, norcocaine, acetazolamide, and quinine will be quantified in urine by the University of California San Francisco (UCSF) under the NIDA contract. Additional urine aliquots will be stored frozen at –20°C at NIDA for future screening and confirmatory testing for cocaine and metabolites.

Oral fluid specimens

OF specimens are collected with the Quantisal,™ Oral-Eze, and Stat-Sure oral fluid collection devices and the Draeger on-site collection system. OF specimens may be collected again if an invalid result is obtained. Specimens are stored at -20ºC until analysis. Cocaine, ecgonine methylester, norcocaine and benzoylecgonine will be

quantified in oral fluid by the Chemistry and Drug Metabolism (CDM) Section of the Intramural Research Program (IRP) of NIDA.

Breath specimens

Breath specimens are collected in a sampling device consisting of a mouthpiece, collection chamber and filter. Participants are asked to breathe more deeply than normal into the mouthpiece and the exhaled breath passes through a filter that adsorbs cocaine and its metabolites. After collection, the filters are stored at -20ºC until analysis. Cocaine, ecgonine methyl ester, benzoylecgonine, and norcocaine will be quantified in breath filters by the Chemistry and Drug Metabolism (CDM) Section of the Intramural Research Program (IRP) of NIDA.

Genotyping

Up to 10 mL of peripheral venous whole blood will be collected after enrollment and stored frozen until later extraction of DNA for genotyping. Participants may be enrolled in the study even if they refuse genotyping.

BChE and carboxylesterase enzyme activity

10 mL of peripheral venous whole blood for BChE and carboxylesterase enzyme activity is collected twice: once after enrollment and before the first cocaine administration, and again on Day 11. Blood is collected into chilled heparinized plastic tubes kept on ice, centrifuged (4000 x g, 10 min) within 2 h of collection to obtain plasma, and the plasma frozen until shipment on dry ice to the collaborating laboratory (Neuropharmacology Laboratory, Mayo Clinic, Rochester, MN). No personally identifiable information will be provided to the collaborating laboratory.
The assay employs selective solvent-extraction of radiolabeled benzene released by hydrolytic cleavage of benzene ring-labeled ^{3}H-cocaine (50 Ci/mmol, New England Nuclear, Boston MA) mixed with varying amounts of unlabeled (-)-cocaine (obtained from Sigma Chemical, St Louis MO). To initiate reactions, 50 nCi of substrate is mixed with enzyme in 0.1 M sodium phosphate, pH 7.4, in a final volume of 100 µl. Reactions proceed for up to 60 min (typically at 23°C) and are stopped by adding 300 µl of 0.02 M HCl, which neutralizes the liberated benzoic acid for selective extraction into 4 ml of toluene for scintillation counting in standard toluene-based fluor. Each specimen will be assayed with a wide range of cocaine concentrations (0.1 to 100 µM) likely to encompass the range of plasma cocaine concentrations found in human cocaine users. Kinetic data will be analyzed with a non-linear least squares regression fit to the Michaelis-Menten model to obtain best estimates of Km and Vmax with associated standard errors.

Carboxylesterase activity will be evaluated with a spectrophotometric assay employing p-nitrophenyl acetate as a substrate. In addition, the contribution of carboxylesterase to cocaine hydrolysis will be evaluated by the addition of selective inhibitors of that enzyme or of BChE to the reaction.

Serum concentrations of leptin and other appetitive peptides

2.5 mL of peripheral venous whole blood will be collected three times, for a total of 7.5 mL of blood, to evaluate basal and post-cocaine administration serum concentrations of leptin and additional appetitive hormones including ghrelin, GLP-1, amylin, and insulin. Blood collections will occur on Day 0 at 10:00 am, Day 1 at 10:00 am (1 h prior to cocaine dosing), and Day 1 at 12:00 pm (1 hour post-dose). Blood is collected into red-white SST tubes containing inhibitors to prevent the metabolism of hormones like ghrelin, GLP-1, and amylin, then centrifuged (1000 x g, 10 min at 25ºC) within 2 h of collection to obtain serum. The serum is aliquoted into small storage tubes for storage at -80ºC until analysis. Specimens will be analyzed by the Clinical Psychoneuroendocrinology and Neuropsychopharmacology (CPN) section, Laboratory of Clinical and Translational Studies (LCTS), NIAAA-IRP.

Physiological measures

Heart rate and rhythm, blood pressure, respiratory rate, and peripheral pulse oximetry are measured by standard, non-invasive clinical methods. Heart rate, blood pressure, and respiratory rate are measured twice before and periodically for 3 h after cocaine administration on Days 1, 5, and 10 (Appendix 3). In addition, heart rate and rhythm and peripheral pulse oximetry is monitored continuously for 1 h after each cocaine administration.

Physiological monitoring with AutoSense

Physiological measures of the participant are monitored with the AutoSense device on days of cocaine administration (Days 1, 5, and 10), and additionally 4 days where cocaine is not administered (Days 3, 4, 8, and 9) for up to 12 hours each day. The AutoSense will be removed at the end of each day. Parameters measured by the AutoSense include respiration rate, heart rate, heart-rate variability, skin temperature, skin conductance, and activity level. Sensors are attached to an elastic belt that is worn around the participants' chest. Two electrodes, similar to those used in ECGs, are placed on the chest and connected to the sensors in the belt. During each of the specified days, these electrodes will be placed on the participants' chest by a nurse. The sensors on the body communicate wirelessly to a smartphone, which processes the sensor measurements in real time to infer behavioral events of interest (stress, speaking, smoking, etc.). The data generated from this study will be analyzed to develop an algorithm for detection of cocaine use in the field without the need for self-report by participants. Additionally, participants will be asked to push a button on the AutoSense smart phone each time they start and finish smoking a cigarette. A picture and description of the device are shown in Appendix 8. Participants may be enrolled in the study even if they refuse to participate in the AutoSense portion of the study.

Subjective effect measures

Subjective response to cocaine is measured with thirteen 100-mm visual-analogue scales (VAS): "crave cocaine," "want cocaine," "like cocaine," "strength of drug effect," "good drug effect," "bad drug effect," "high," "rush," "elated," "stimulated," "suspicious," "anxious," and "tired." These measures are taken once before and periodically for 3 h after cocaine administration on Days 1, 5, and 10 (Appendix 3). Each VAS is anchored by "not at all" at left end and "most ever" at right end.

End of Participation

At the end of the study schedule on Day 11, participants will have vital signs and a standardized Neuromotor Examination performed by a nurse to establish that it is safe to discharge them to home. This examination also will be performed to establish the safety of discharge in participants withdrawn from the study prior to completion.

This is not a treatment study, and participants have no active medical condition requiring treatment, so there is no need to transfer care to a non-NIDA provider.

Outcome measures

Primary outcome measures

The primary outcome measures are heart rate, blood pressure, and subjective responses to IV cocaine and C_{max}, T_{max}, and AUC in plasma and urine for IV cocaine, oral acetazolamide, and oral quinine. These measures address the primary scientific objective of the study, viz., are there significant pharmacodynamic and pharmacokinetic interactions among cocaine, acetazolamide, and quinine.

Secondary outcome measures

Secondary outcome measures include the following:

- Enzymatic activity of BChE and carboxylesterase in plasma
- Cocaine pharmacokinetics and windows of detection in OF collected with Quantisal, StatSure and Oral-Eze collection devices.
- Performance of and windows of cocaine detection with the Draeger on-site oral fluid cocaine test
- Cocaine pharmacokinetics and windows of detection in breath.
- Genotype of BChE and hCES-2
- Performance of dried blood spots in the detection of IV cocaine
- Performance of AutoSense for monitoring physiological measures during cocaine use
- Effects of acute and chronic cocaine on serum concentrations of leptin and other appetitive peptides

Subject safety monitoring

Participants' responses to study medications will be closely monitored by clinical staff and study personnel.

Subjects will not receive any dose of study medication unless all of the following criteria are satisfied prior to dosing:

- Systolic BP within 95-140 mm Hg and diastolic BP within 45-90 mm Hg, and heart rate within 50-90 bpm while at rest. BP and HR will be measured within 15 minutes before administration of cocaine. In the event vital signs are not within dosing limits, study staff may repeat measures every 5 minutes for up to 3 times, and again 15 minutes after the last 5 minute check. If BP and/or HR are still outside of the dosing limit, the dose will not be given. If BP and HR are within dosing limits at any repeat time point, the dose may be given. The timing of the dose will be adjusted as needed and post-dosing collection schedule on dosing day will be adjusted accordingly.
- No ingestion within the prior 7 days of high-dose aspirin, phenytoin, primidone, carbonic anhydrase inhibitors, folic acid antagonists, amphetamines, methenamine, lithium, sodium bicarbonate, cyclosporine, class IA antiarrhythmic agents (quinidine, procainamide, disopyramide), class III antiarrhythmic agents (amiodarone, sotalol, dofetilide), astemizol, cisapride, terfenadine, pimozide, halofantrine, macrolide antibiotics like erythromycin, or rifampin.

After cocaine dosing, subjects will have:

- Continuous monitoring of heart rate and rhythm (one-lead chest ECG) and peripheral pulse oximetry for one hour
- BP checked every 5 min for 15 min, then every 15 min for the next 45 min
- Nurse present in or adjacent to the study room for one hour
- Physician certified in ACLS present during dosing and for at least 30 minutes after administration or until vital signs return to within 10% of baseline, whichever occurs later.

A subject will receive no further study medication and be withdrawn from the study when it is clinically appropriate to do so should any of the following occur during a study session:

- Any serious adverse event
- Premature ventricular contractions greater than 1/min or premature supraventricular contractions greater than 3/min over a period of 5 min
- ECG suggestive of acute cardiac ischemia
- Heart rate above 0.85 times (220 minus age) for more than 1 min
- Systolic BP above 160 mm Hg or diastolic BP above 100 mm Hg for more than 2 consecutive readings
- O_2 saturation less than 95% for more than 30 s while breathing room air
- Acute non-traumatic chest pain
- Acute disorientation or loss of consciousness
- Refusal to cooperate with required monitoring and safety procedures

- Behavior which is disruptive or threatening to staff or others on the clinical research unit.

In the event of a severe reaction or medical emergency, the following resources are available:

- ACLS-certified nurse available adjacent to the study room, ACLS-certified physician available in the building, and crash cart with defibrillator and suction and emergency medicine box (containing appropriate anti-convulsant and anti-hypertensive medications) available adjacent to the study room. In case of a medical emergency requiring urgent evaluation or treatment, 911 will be called to transport the participant via ambulance to the nearest available emergency department.

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