Document Title:	**Development and Validation of Two Innovative Quantitative Liquid Chromatography Tandem Mass Spectrometry Methods for Forensic Toxicology Laboratories: Novel Analysis of Designer Drugs and Simultaneous Method for Cocaine and Opioids in Biological Matrices**
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Project Title: Development and Validation of Two Innovative Quantitative Liquid Chromatography Tandem Mass Spectrometry Methods for Forensic Toxicology Laboratories: Novel Analysis of Designer Drugs and Simultaneous Method for Cocaine and Opioids in Biological Matrices

Final Summary Overview

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Purpose

The goal of this research project was to develop and validate two liquid chromatography tandem mass spectrometry methods (LC-MS/MS) for the analysis of whole blood and additional biological matrices in accordance with the guidelines promulgated by the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology.

The objectives for this project were:

1. Develop and validate a technique that addresses the recent proliferation of designer drugs (novel psychoactive substances) such as cannabimimetic agents and designer stimulants
2. Develop and validate a combined method for opioids, cocaine and its metabolites
3. Disseminate the methods to the toxicology community

The increased submission of novel psychoactive substances (NPS) to the Virginia Department of Forensic Science (VADFS) Controlled Substances Section indicated a need for a robust method within the Toxicology Section for detection of these compounds in biological matrices. Prior to this research project, VADFS did not have a method validated for the qualitative analysis of NPS. These structural analogs are manufactured to mimic the effects of scheduled compounds posing a twofold threat to the criminal justice system. These drugs pose legal concerns due to their ability to avoid provisions of drug laws and they have a high potential for abuse, which impacts toxicological analyses. The validation and implementation of new methods into forensic toxicology laboratories can be a tedious and time-consuming process. With limited resources and the ever-changing climate of NPSs, the development of validated methods within the project has the potential to facilitate a transition from independent laboratory developed methods to universally standardized methods, enabling a more globalized, consistent approach within the toxicology community.

Not only is it crucial for forensic laboratories to validate and implement new methods, it is also important to evaluate historical methods to increase laboratory productivity and efficiency. Many cases

submitted to the VADFS Toxicology Section involve a combination of drugs that, prior to this research project, required multiple sample extractions and quantitative analysis techniques. The combination of four quantitative analysis techniques, requiring a total of 8.0 mL of biological specimen, was previously required to quantitatively identify the compounds within the 1.0 mL combined quantitative opioid, cocaine, and cocaine metabolite LC-MS/MS method that has been developed within the scope of this project. These newly developed and validated methods have not only streamlined toxicology testing at VADFS, but are available to the toxicology community on the VADFS website for implementation across the United States.

Project Design and Methods

The methods were developed and validated to exceed the SWGTOX Standard Practices for Method Validation in Forensic Toxicology guidelines. The method development and validation process for the two methods was similar, including an evaluation of sample preparation techniques as well as optimal instrumental conditions that will not only demonstrate accuracy and precision, but also the efficiency for each method.

Qualitative Analysis of Novel Psychoactive Substances

To facilitate the identification of compounds to be evaluated within the NPS method, an evaluation of the compounds identified in the VADFS Controlled Substances Section since 2012 was completed. Compounds structurally similar to the identified compounds were also added to the method. The compounds within this method are delineated in Table 1. Once identified, instrumental method parameters were developed using an Agilent Technologies 1290 Infinity liquid chromatography system coupled to a 6430 quadruple mass spectrometer. The mass spectrometer instrumental conditions were optimized to include the identification of precursor and product ions, ionization source conditions, and instrument mode. Once the mass spectrometer instrumental conditions were optimized, the chromatographic conditions were evaluated to include flow rate, mobile phase composition, and gradient.

Table 1 Compounds included in the NPS qualitative analysis method

Target Compound	Target Compound
Methiopropamine	25I-NBF
3-Fluorophenmetrazine	25I-NBMD
4-APDB	BTCP
5-APDB	25I-NBOMe
3-Chloromethcathinone	TH-PVP
4-Chloromethcathinone	5F-AB-PINACA
6-APDB	AB-FUBINACA
Dibutylone (bk-DMBDB)	ADB-FUBICA
3-Chloroethcathinone	AB-PINACA
4/5/6-MAPB	3F-AMB
Mexedrone	4F/5F-AMB
Pentylone/N,N-Dimethylpentylone	4F-ADB
N-Ethylpentylone/N,N-Dietheylpentylone	5F-PB-22
5-DBFPV	AMB-FUBINACA (MMB-FUBMIACA and FUB-AMB)
Tenocyclidine (TCP)	SDB-006
4-Chloro-alpha-PVP	4-Fluoro-alpha-PVP
3-Methoxy-PCP	FUB-MDMB (MDMB-FUBINACA)
Mitragynine	5F-MN-18
Methoxyphenidine	MAB-CHMINACA (ADB-CHMINACA)
25H-NBOMe	PB-22
PV8	MMB-CHMICA
25C-NBOMe	XLR-11
25I-NBOH	MN-18
Clonazolam	SDB-005
25B-NBOMe	APINACA (AKB-48)

Upon instrumental optimization, the sample preparation procedure was developed. Various sample preparation procedures including protein precipitation and solid phase extraction were evaluated. The optimal sample preparation procedure was determined to be the procedure that utilized the least amount of biological matrix while still maintaining accurate and precise results. Also during development, parameters such as recovery, ionization suppression and enhancement, and limit of detection were preliminarily assessed. The optimized method was then validated in accordance with SWGTOX Standard Practices for Method Validation in Forensic Toxicology guidelines.

The optimized method utilized 0.5 mL of biological matrix that was diluted with 1.0 mL of 0.1 M phosphate buffer prior to solid phase extraction. The solid phase extraction was a traditional solid phase extraction (United Chemical Technologies CSDAU200 CleanScreen Columns) that included column conditioning prior to addition of the sample. The columns were washed with 1.0 mL water and 1.0 mL acetate buffer (pH 4) prior to two elution steps. The first elution was 1.0 mL of methanol while the second was 3.0 mL of 78:20:2 dicholormethane:isopropanol:ammonium hydroxide. Prior to evaporation, 40 µL of

HCl in isopropanol was added to each sample. Samples were evaporated to dryness at approximately 50°C. Finally, samples were reconstituted in 50 µL of starting mobile phase.

Quantitative Analysis of Opioids, Cocaine, and Cocaine Metabolites

The compounds evaluated during method development included opioids, cocaine, and cocaine metabolites. A complete list of compounds is listed in Table 2. Instrumental method parameters were developed using an Agilent Technologies 1290 Infinity liquid chromatography system coupled to a 6430 quadruple mass spectrometer. The mass spectrometer instrumental conditions were optimized to include the identification of precursor and product ions, ionization source conditions, and instrument mode. Once the mass spectrometer instrumental conditions were optimized, the chromatographic conditions were evaluated to include flow rate, mobile phase composition, and gradient.

Table 2 Compounds evaluated within the opioids, cocaine, and cocaine metabolite method

Target Compound	Target Compound
Morphine	Tramadol
Oxymorphone	Cocaine
Hydromorphone	Meperidine
Codeine	Acetyl Fentanyl
Oxycodone	Cocaethylene
6-Monoacetylmorphine	Fentanyl
Hydrocodone	Methadone
Benzoylecgonine	

Upon completion of the instrumental method development, two sample preparation methods were developed. The sample preparation methods include a solid phase extraction and protein precipitation. The methods were developed using the same volume of biological matrix (1.0 mL). During development, the methods were compared to each other by evaluating ionization suppression and enhancement, recovery, accuracy and precision, and limit of detection. Both methods met development guidelines. Therefore, the solid phase extraction and protein precipitation methods were both validated in accordance with SWGTOX Standard Practices for Method Validation in Forensic Toxicology guidelines.

The optimized protein precipitation method utilized 1.0 mL of biological matrix. To each sample, 2.0 mL of acetonitrile was added and the samples were vortexed for 15-30 seconds. The samples were

centrifuged at approximately 2800 rpm for 15 minutes to achieve separation. The samples were then frozen at approximately -20°C for 30 minutes. The uppermost layer was then transferred and evaporated under nitrogen at approximately 60°C. Samples were then reconstituted in 200 µL of 0.01% formic acid and 5 mM ammonium formate in water.

The optimized solid phase extraction procedure required 1.0 mL of biological matrix for extraction using United Chemical Technologies CSDAU200 CleanScreen Columns. To prepare the biological specimens, 2.0 mL of water was added to each sample and vortexed. The samples were incubated for five minutes prior to centrifugation at approximately 2500 rpm for 15 minutes. After centrifugation, 1.0 mL of 0.1 M phosphate buffer was added to the remaining sample. The solid phase extraction columns were conditioned prior the addition of sample. After sample addition, the columns were washed with water, 0.5 M acetic acid and methanol. The compounds were then eluted with 3.0 mL of 78:20:2 dicholormethane:isopropanol:ammonium hydroxide. Samples were evaporated to dryness at 50-60°C and finally reconstituted in 200 µL of 0.01% formic acid and 5 mM ammonium formate in water.

Data Analysis

Qualitative Analysis of Novel Psychoactive Substances

The qualitative validation of NPS using LC-MS/MS was completed by evaluating the method sensitivity, ionization suppression and enhancement, recovery, carryover, interferences, and stability. Method sensitivity was assessed by determining the limit of detection for each compound. The limit of detection was assessed for a total of ten batch analyses with triplicate determinations per batch using blank blood. Decreasing concentrations were evaluated within each batch to determine the limit of detection for each compound. The limit of detection was defined as the lowest concentration that was reliably able to meet the predetermined acceptance criterion. The acceptance criteria included a signal-to-noise ratio of greater than three, qualifier ratios within ±20%, and acceptable peak shape.

Ionization suppression and enhancement was evaluated using pre-extracted and post-extracted fortified samples. Two concentrations were evaluated in triplicate for each biological matrix source to determine the amount of ionization suppression and enhancement for each matrix type. Four sources of blank blood, four sources of postmortem blood, four sources of antemortem blood, four sources of liver, and two sources of urine were evaluated. Recovery was also assessed during the validation. Pre-extraction fortified samples were compared to post-extraction fortified samples to establish the recovery for each compound. The same matrices utilized to evaluate ionization suppression and enhancement were utilized in the evaluation of recovery. Carryover was evaluated by analyzing blank matrix samples directly following increasing concentration of extracted sample. Carryover was evaluated at 0.75 mg/L and 1.0 mg/L in triplicate for a total of ten batch analyses.

To evaluate interferences, commonly encountered analytes, endogenous compounds, and contribution from compound to internal standard and internal standard to compound were evaluated. The evaluation of interferences was also evaluated by assessing available isomers. Isomers were evaluated individually to identify retention time and qualifier ratios. Stability was evaluated by extracting two concentrations and analyzing each sample every twenty-four hours for a seven-day period with triplicate injections at each time point. The instrumental response from subsequent injections was compared to the day one instrumental response.

Quantitative Analysis of Opioids, Cocaine, and Cocaine Metabolites

The quantitative validation of opioids, cocaine, and cocaine metabolites was completed in the same manner for both the solid phase extraction and protein precipitation procedures. The methods were evaluated by assessing accuracy and precision, sensitivity (limit of detection and limit of quantitation), linearity and calibration model, ionization suppression and enhancement, recovery, carryover, interferences, dilution integrity, and stability.

Accuracy and precision were evaluated using pooled blood samples fortified with the target compounds at three different concentrations in triplicate within each batch for a total of five batch analyses. The acceptance criterion for pooled accuracy and precision was ±20%. The precision was assessed using both within-run precision and intermediate precision. Within-run precision was the batch with the highest imprecision, while intermediate precision was the calculated precision for all batch analyses combined.

The limit of detection was assessed by fortifying three blank blood sources with target compounds. Decreasing concentrations were evaluated for each target compound using the three fortified blank blood sources. The lowest concentration that was capable of achieving acceptable predetermined identification criteria was considered the compound's limit of detection. The limit of quantitation was evaluated by analyzing seven blank blood samples fortified with target compound at the lowest calibrator concentration. Extrapolation lower than the concentration of the lowest calibrator would result in a qualitative measurement and would not be used for quantitative purposes. The seven replicates were evaluated to ensure that the lowest calibrator concentration was capable of reproducibly achieving the predetermined acceptance criterion of retention time, qualifier ratio, signal-to-noise, and back-calculated concentration.

The best-fit calibration model was determined using multiple statistical analysis techniques as well as the analysis of residual plots. A total of three fortified blank blood sources over twelve batch analyses were analyzed to determine the calibration model for each target for the protein precipitation validation. A total of three fortified blank blood sources over sixteen batch analyses were analyzed to determine the calibration model for each target for the solid phase extraction validation.

The accuracy and precision of dilutions were also evaluated for the protein precipitation and solid phase extraction procedures. Both large-volume and small-volume dilutions were evaluated to simulate a concentration above the calibration range and small sample volume, respectively. Ionization

suppression and enhancement, recovery, carryover, and stability were evaluated in a similar manner to the NPS qualitative validation. A total of five sources of blank blood, three postmortem blood, four liver, and two urine sources were evaluated for ionization suppression and enhancement as well as recovery.

Findings

Qualitative Analysis of Novel Psychoactive Substances

The majority of the compounds within the method passed the comprehensive qualitative validation, with the exception of 4-fluoro-alpha-PVP and APINACA. Several compounds had a high limit of detection of 0.1 mg/L or greater within the method and may not be applicable to all laboratories. These compounds include 3-chloromethcathinone, 3-chloroethcathinone, MN-18, SDB-005, and APINACA. All other compounds had a limit of detection of 0.05 mg/L down to 0.001 mg/L.

Significant ionization suppression was identified in the majority of compounds, with some compounds (MN-18, SDB-005, and APINACA) indicating significant ionization enhancement. To determine the effect of ionization suppression and enhancement for each matrix type, a limit of detection determination was completed in antemortem blood, postmortem blood, and liver. It was determined that, although ionization suppression exists in antemortem and postmortem blood, there was no effect on the limit of detection. On the other hand, ionization suppression in liver samples indicated a significant effect on the limit of detection. Therefore, the method was determined to be inadequate for liver analysis.

No carryover was noted during the validation and no effect was observed for interference studies including endogenous compounds, internal standard and analyte contribution, and commonly encountered analytes. Several compounds were determined to be unstable 24 hours after initial analysis, including cathinone compounds, mitragynine, and NACA compounds. Dilution integrity experiments were not conducted as part of the validation given the qualitative nature of the method. Overall, the method provides reliable screening results for several NPS compounds that have not been previously detected in many laboratories across the United States.

Quantitative Analysis of Opioids, Cocaine, and Cocaine Metabolites

The protein precipitation and solid phase extraction procedures both passed the comprehensive validation for the quantitative analysis of opioids, cocaine, and cocaine metabolites. No significant differences were noted between the two methods when performing a comparative analysis of the results. One noted difference was during the evaluation of urine samples. The protein precipitation method produced retention time shifting for early eluting compounds including morphine, oxymorphone, and hydromorphone. The amount of retention time shifting varied per urine sample. The shifting was due to the concentration of creatinine in the urine sample. The utilization of matrix-matched calibrators (blank urine) did not correct for the retention time shifting. This phenomenon was not present with the solid phase extraction procedure.

No carryover or interferences from endogenous compounds, internal standard, analyte, or commonly encountered analytes was noted during the validation of both methods. The evaluation of small-volume dilution for both procedures produced accurate and precise results for a 1:2, 1:5, 1:10 and 1:20 dilution using mechanical pipettes. All compounds were stable for seven days following extraction for both sample preparation procedures. During stability analysis, samples were maintained on the autosampler and subject to laboratory conditions to simulate an abrupt termination in sample analysis.

The cost of the protein precipitation procedure was significantly less than the solid phase extraction procedure. The solid phase extraction columns alone cost $2.50-$3.00 per sample, which was not required for the protein precipitation procedure. In addition, the solid phase extraction procedure was more time consuming when utilizing manual solid phase extraction techniques.

Implication for Criminal Justice Policy and Practice in the United States

The methods developed and validated within this project have several implications for criminal justice practices in the United States. The novel psychoactive substances method contains over forty-five compounds that are not typically evaluated for in forensic laboratories. Given the broad sample

preparation procedure and the instrumentation utilized, the method also possesses the ability to have additional compounds added to continue to combat the ever changing climate of novel psychoactive substances.

The opioid, cocaine, and cocaine metabolite method possesses the ability to streamline analyses within a forensic laboratory. Traditional analytical workflows in forensic toxicology are performed by drug classification. This newly developed method combines compounds that are commonly encountered in a single biological sample. At VADFS, the method combined four qualitative analysis methods that required a total of 8.0 mL of biological sample to perform the analysis. The same compounds can now be evaluated in a single analytical technique utilizing only 1.0 mL of biological specimen.

The information obtained within this research project has been disseminated using several different forums. Two standard operating procedures for quantitation and confirmation of opioids and cocaine using LC-MS/MS and qualitative analysis of novel psychoactive compounds has been published in the VADFS Toxicology Procedure Manual and is available for public access on the VADFS website at https://www.dfs.virginia.gov/documentation-publications/manuals/. In addition, a platform presentation was given at the SOFT-TIAFT joint conference in Boca Raton, Florida in January 2018. The platform presentation was a comparison of the two sample preparation methods. Furthermore, data was also shared during the "Where the Wild Things Are-Method Development" workshop. An invitation was also accepted to present at the 2019 NIJ Forensic Science R&D Symposium at the American Academy of Forensic Sciences meeting in February 2019.