Validation of Phencyclidine, Ketamine, and Dextromethorphan using Miscellaneous Basic Drugs Quantitation and Confirmation by Liquid-Liquid Extraction using Liquid Chromatography-Mass Spectrometry

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Abstract and Background

Phencyclidine (PCP), ketamine, and Dextromethorphan (Dxm) are hallucinogenic drugs commonly abused in the USA and are included in common drugs of abuse panels. Ketamine and PCP are more specifically classified as dissociative anesthetics, which can lead to confusion or a catatonic state in the individual (O'Malley and O'Malley 2022). Dextromethorphan, often found in cough suppressants, can cause both euphoria and hallucinations at elevated concentrations (Department of Justice/Drug Enforcement Administration Drug Fact Sheet 2020) (Journey, Agrawal and Stern 2023). Although PCP, ketamine and Dxm, have been previously validated for quantitative analysis by Gas Chromatography or Gas Chromatography Mass Spectrometry in a toxicological setting, a validated method has not been developed on Liquid Chromatography Mass Spectrometry (LCMSMS). Using the Miscellaneous Basic Drugs Quantitation and Confirmation by LCMSMS method and the target analytes; PCP-d5, Dxm-d3, and ketamine-d4 as internal standards, a full validation was performed. A standard validation protocol is outline in the Quality Manual and Toxicology Procedures Manual published by the Virginia Department of Forensic Science. The validation included bias and precision, sensitivity, linearity and calibration model, ionization enhancement, carryover, interferences, dilution integrity, stability, and robustness studies. As the research is still ongoing, the validation method is proving to be successful in the identification and quantification of the drugs with the method. The residual data was added to determine the calibration models and then analyzed using a single factor ANOVA and paired two sample t-tests. PCP, DXM, and ketamine were determined to be quadratic weighted. In addition to the linear/quadratic nature of the models the data analysis also determined them to all be best fit weighted 1/x. Residual plots were made to graph this data for each compound. Further investigation into the nature of these plot will additionally be conducted as the research continues. The successful quantitation and confirmation of PCP, Dxm, and ketamine using the Miscellaneous Basic Drugs Quantitation and Confirmation by LCMSMS method allows for the implication of a new method into the toxicology section standard practices. Future work connected to these findings can include the continuation of validation research concerning the Miscellaneous Basic Drugs method involving other toxicological drugs.

Introduction

Phencyclidine (PCP), Ketamine, and Dextromethorphan (Dxm) are hallucinogenic drugs commonly abused in the USA and are included in common drugs of abuse panels. Ketamine and PCP are more specifically classified as dissociative anesthetics, which can lead to confusion or a catatonic state in the individual (O'Malley and O'Malley 2022). Dextromethorphan, often found in cough suppressants, can cause both euphoria and hallucinations at elevated concentrations (Department of Justice/Drug Enforcement Administration Drug Fact Sheet 2020) (Journey, Agrawal and Stern 2023). Based on the Standard Practices for Method Validation in Forensic Toxicology from ANSI/ASB Standard 036 validation is defined as "the process of performing a set of experiments to establish objective evidence that a method is fit-for-purpose and to identify the method's limitations under normal operating conditions" (First Edition, 2019). The Virginia Department of Forensic Science (DFS) Western Laboratory aimed to perform such a study for the quantification and confirmation of these commonly abused drugs using the Miscellaneous Basic Drugs Quantitation and Confirmation method already in place. The successful quantitation and confirmation of PCP, Dxm, and ketamine using the method will allow toxicologists to confirm and quantify these common compounds within a currently established method, increasing efficiency without compromising accuracy.

Methods

The method that was used within this research was the Miscellaneous Basic Drugs Quantitation and Confirmation by LCMSMS method can be found within the Toxicology Procedures Manual provided by DFS. Additionally, a standard validation protocol is outlined in the Quality Manual and Toxicology Procedures Manual. The validation included bias and precision, sensitivity, linearity and calibration model, ionization enhancement, carryover, interferences, dilution integrity, stability, and robustness studies. First, the extraction of PCP, Ketamine, and Dxm followed the standard operating procedure base drug screen. Calibrators, controls, and standards were made in accordance with the method with PCP-d5, Dxm-d3, and ketamine-d4 being the target analytes. Once samples were completely prepared, the samples were centrifuged at approximately 3500 RPM for 15 to achieve separation.The supernatant was transferred to a clean tube. Using a UTC Positive Pressure Manifold, the drugs were isolated from the samples by solid phase extraction (SPE). For the SPE, the column flow rate was approximately 1 mL/min. The cartridges were washed with nonpolar organic solvents in order to prepare the column. These solvents included hexane, methanol and deionized water. Once the column was prepared, the specimens were poured into the appropriate SPE column and eluted from the cartridges with approximately 1-2 mL/minute flow or around 5 psi. The target analyte is trapped within the sorbent bed of the SPE cartridge, while the matrix material within the solution is eluted away, further minimizing interference. Deionized water, 1.0 M acetic acid, and methanol is then eluted through the cartridges. The column was then dried for approximately 2 minutes at maximum pressure. Freshly prepared elution solvent, a mixture of methylene chloride, isopropanol and ammonium hydroxide (78:20:2 v/v/v) is used to break the bond that the analyte has to the absorbent bed, eluting the target solution into the centrifuge tube. Following the isolation of the drugs, the solution was dried down at approximately 50°C under nitrogen until no elution solvent was present. Finally, the drugs were reconstituted with 100μL of water with 0.1% formic acid (mobile phase A) and transferred to an autosampler vial for analysis on the LCMSMS.

The instrumental parameters are also defined within the Miscellaneous Basic Drugs Quantitation and Confirmation method. The instrumental method is a positive ionization dynamic MRM method. The optimized fragmentor voltage, collision energy, and cell accelerator voltage from the quantitative validation of PCP, Dxm, and ketamine will be employed seen in Table 1.

Figure 1. Picture of the LCMSMS that was used for this research. System ID "Moon." Model #5623016205. Agilent Technologies 6470 LC/TQ. Department of Forensic Science 2502

1. Bias and Precision
    a. Bias
    b. Within-run Precision
    c. Intermediate Precision
2. Sensitivity
    a. Limit of Detection (LOD)
    b. Lower Limit of Quantitation (LLOQ)
3. Linearity and Calibration Model
4. Ionization Suppression/Enhancement
5. Carryover
6. Interferences
    a. Endogenous Compounds
    b. Internal Standard
    c. Commonly Encountered Analytes
7. Dilution Integrity
8. Stability
9. Robustness
10. References

Figure 2. The validation plan followed within this research based of Quality Manual and Toxicology Procedures Manual. The validation plan is in accordance with ANSI/ASB Standard 036 Standard Practices for Method Validation in Forensic Toxicology (First Edition, 2019).

Compound	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Fragmentor (V)	Collision Energy (V)	Cell Accelerator (V)
Dmx	272.2	171	164	44	4
Dxm	272.2	147	164	36	4
Dxm-d_3	275.22	215.2	140	28	4
Dxm-d_3	275.22	147	140	36	4
Ketamine	238.1	125	92	36	4
Ketamine	238.1	115	92	72	4
Ketamine-d_4	242.13	129	100	40	4
Ketamine-d_4	242.13	92.1	100	76	4
PCP	244.21	159	92	12	4
PCP	244.21	86.1	92	12	4
PCP-d_5	249.24	96.1	80	48	4
PCP-d_5	249.24	164.1	92	12	4

Table 1. Optimized voltages used within the Positive Ionization Dynamic MRM method that were used within this research.

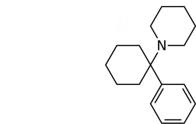

Figure 1. Structure of Phencyclidine

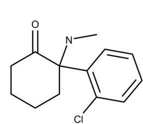

Figure 2. Structure of Ketamine

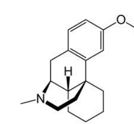

Figure 3. Structure of Dextromethorphan

Phencyclidine (Q 1/x)- Threshold				
RT Cal Mean	3.210	S/N > 3.3	Ratio Cal Mean	167.3
-3%	3.114		-20%	133.8
+3%	3.307		+20%	200.8
Phencyclidine (Q 1/x)- 50% Threshold				
RT Cal Mean	3.211	S/N > 3.3	Ratio Cal Mean	165.5
-3%	3.115		-20%	132.4
+3%	3.307		+20%	198.6

Figure 4. LOD results for Phencyclidine looking at both the original threshold and 50% of the threshold

Ketamine (Q 1/x)- Threshold				
RT Cal Mean	1.691	S/N > 3.3	Ratio Cal Mean	22.6
-3%	1.641		-20%	18.1
+3%	1.742		+20%	27.2
Ketamine (Q 1/x)- 50% Threshold				
RT Cal Mean	1.692	S/N > 3.3	Ratio Cal Mean	23.4
-3%	1.641		-20%	18.7
+3%	1.743		+20%	28.1

Figure 5. LOD results for Ketamine looking at both the original threshold and 50% of the threshold

Dextromethorphan (Q 1/x)- Threshold				
RT Cal Mean	3.471	S/N > 3.3	Ratio Cal Mean	75.8
-3%	3.366		-20%	60.6
+3%	3.575		+20%	91.0
Dextromethorphan (Q 1/x)- 50% Threshold				
RT Cal Mean	3.473	S/N > 3.3	Ratio Cal Mean	76.7
-3%	3.369		-20%	61.3
+3%	3.578		+20%	92.0

Figure 6. LOD results for Dextromethorphan looking at both the original threshold and 50% of the threshold

Mean	0.0359	0.334	0.727	0.0316	0.357	0.704	0.0336	0.376	0.744
SD	0.0015	0.033	0.037	0.0016	0.019	0.038	0.0012	0.020	0.026
Precision (%CV)	**4.19**	**9.78**	**5.02**	**4.96**	**5.29**	**5.35**	**3.47**	**5.28**	**3.47**
Accuracy (Bias)%	**19.6**	**-16.4**	**-9.1**	**5.2**	**-10.9**	**-12.0**	**12.2**	**-6.1**	**-6.9**
Accuracy + 100	**120**	**84**	**91**	**105**	**89**	**88**	**112**	**94**	**93**

Figure 7. Between Run Precision/Intermediate Precision (%CV) and Accuracy of Postmortem Blood.

Mean	0.0297	0.370	0.841	0.0305	0.384	0.788	0.0308	0.387	0.835
SD	0.0038	0.065	0.024	0.0024	0.059	0.018	0.0035	0.066	0.019
Precision (%CV)	**12.76**	**17.70**	**2.80**	**7.96**	**15.37**	**2.29**	**11.44**	**17.09**	**2.22**
Accuracy (Bias)%	**-1.1**	**-7.5**	**5.1**	**1.6**	**-4.0**	**-1.5**	**2.6**	**-3.4**	**4.4**
Accuracy + 100	**99**	**92**	**105**	**102**	**96**	**99**	**103**	**97**	**104**

Figure 8. Between Run Precision/Intermediate Precision (%CV) and Accuracy of Negative Blood.

Mean	0.0290	0.508	0.882	0.0312	0.421	0.812	0.0311	0.473	0.851
SD	0.0015	0.012	0.036	0.0017	0.010	0.030	0.0011	0.010	0.032
Precision (%CV)	**5.20**	**2.41**	**4.08**	**5.38**	**2.28**	**3.73**	**3.66**	**2.11**	**3.78**
Accuracy (Bias)%	**-3.3**	**27.0**	**10.3**	**3.9**	**5.3**	**1.5**	**3.6**	**18.4**	**6.3**
Accuracy + 100	**97**	**127**	**110**	**104**	**105**	**101**	**104**	**118**	**106**

Figure 9. Between Run Precision/Intermediate Precision (%CV) and Accuracy of Urine.

Volume of 1 μg/mL working solution (μg/mL)	Volume of 10 μg/mL working solution (μg/mL)	Final concentration (mg/L)
	10	1.0
	75	0.75
	50	0.50
	20	0.20
	10	0.10
50		0.050
20		0.020
10		0.010

Figure 10. Working range calibration sample concentrations. For the calibration model, calibration samples included the concentrations delineated in this figure for each target compound.

$$Bias\ (\%)\ Concentration_x = \left(\frac{Mean\ of\ Calculated\ Concentration_x - Expected\ Concentration_x}{Expected\ Concentration_x}\right) \times 100$$

$$Within-run\ Precision\ (\%CV) = \left(\frac{Standard\ Deviation\ of\ Batch\ Mean}{Calculated\ Mean\ of\ Batch}\right) \times 100$$

$$Intermediate\ Precision\ (\%CV) = \left(\frac{Standard\ deviation\ of\ combined\ means}{Calculated\ grand\ mean}\right) \times 100$$

Figure 11. Important equations that were used throughout the validation procedure.

Results and Conclusions

The procedure was carried out successfully according to the Department of Forensic Science's toxicology standard operating procedures. Additionally, the validation plan outlined and followed was based on the Quality Manual and Toxicology Procedures Manual in accordance with ANSI/ASB Standard 036 Standard Practices for Method Validation in Forensic Toxicology (First Edition, 2019). As the research is still ongoing, the validation method is proving to be successful in the identification and quantification of PCP, Ketamine and Dextromethorphan. Following the validation outline, accuracy (bias) and precision studies of negative blood, medical examiners, and urine samples were completed. Bias and precision was assessed by analyzing samples with target compounds at three different concentrations (0.03 mg/L, 0.4 mg/L, 0.8 mg/L) over a total of five batch analyses.The acceptance criterion for the pooled bias and the within-run precision was ≤20% for the %CV at each concentration level. The intermediate precision was calculated. No significant impact on bias was noted for the samples tested. All matrices were consistent when compared to the blank blood calibration curve. The within-run and intermediate precision was within the predetermined acceptance criterion. The sensitivity of the research is being validated by limit of detection (LOD) and Lower Limit of Quantitation (LLOQ). The Limit of Detection was defined as an administratively-defined decision point that was determined using two concentrations. The concentrations that were evaluated were 0.005 mg/L and 0.0025 mg/L. The results of the LOD portion were successful for each compound and the estimated limit of detection for all target compounds was determined to be 0.005 mg/L. The LLOQ portion of the validation is currently underway. The best fit calibration model was determined using multiple statistical analysis techniques as well as the analysis of residual plots.The residual data was added to determine the calibration models and then analyzed using a single factor ANOVA and paired two sample t-tests. The linearity and calibration model was determined to be quadratic weighted. Once established, the calibration model was utilized to obtain data regarding accuracy and precision, limit of quantitation, and dilution integrity within the validation. The carryover was evaluated by analyzing blank matrix samples immediately following progressively higher concentrations of fortified matrix within the injection sequence. The highest analyte concentration at which no analyte carryover is observed, in the blank matrix, is determined to be the concentration at which the method is free from carryover. Within the research, no blank matrix samples immediately following any fortified matrix sample had indications of carryover. Interferences were assessed by monitoring the qualifier and quantifier ions for the target compounds. For the endogenous interferences, a total of ten matrix sources per matrix type were evaluated without the addition of the internal standard. The samples were evaluated for the presence of instrumental response for the analyte and internal standard. No endogenous interferences were identified.The effect of sample dilution on the bias and precision of samples was evaluated using a large volume dilution. When assessing large volume dilution, a pooled blood sample fortified at the highest calibrator concentration was prepared. All dilutions were within the predetermined acceptance criterion for bias.

The validation research performed at the Department of Forensic Science is ongoing, but has currently been successful in the quantification and confirmation of phencyclidine, ketamine, and dextromethorphan. The validation studied already performed has produced successful results. The ultimate goal of these findings is the implementation of the method into toxicology standard practices.

Future Work

1. Transition of research to further validate other important toxicological drugs concerning the Miscellaneous Basic Drugs method.
2. Complete the validation research by completing the interference study, ion suppression, and Lower Limit of Quantitation (LLOQ). The LLOQ will be establish by evaluating the lowest non-zero calibrator for the method. The ionization suppression will be evaluated by assessing the instrumental response of post-extraction fortified samples and neat standards. Interference will be assessed by evaluating the potential interferences of the internal standard and commonly encountered analytes.

References and Acknowledgments

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