Memo To: James Hutchings, Ph.D., Toxicology Program Manager
From: Trista Wright, Ph.D., Toxicology Section Supervisor, Chad Harris, Senior Forensic Scientist
CC: Alka Lohmann, Technical Services Director
Date June 1, 2023
RE: Validation Plan
Validation of Phencyclidine, Ketamine, and Dextromethorphan using Miscellaneous Basic Drugs Quantitation and Confirmation by Liquid-Liquid Extraction Using LCMSMS

Validation Plan- Phencyclidine (PCP), Ketamine, and Dextromethorphan (Dxm) Quantitation and Confirmation by Solid Phase Extraction Using LCMSMS

It is proposed to validate a method for the quantitative analysis of PCP, Dxm, and ketamine using the Miscellaneous Basic Drugs Quantitation and Confirmation by LCMSMS method (Toxicology Procedures Manual, Section 31, Qualtrax Revision 26). Although PCP, Dxm, and ketamine are previously validated for quantitative analysis by GC or GCMS, a full validation will now be performed using the Miscellaneous Basic Drugs Quantitation and Confirmation by LCMSMS method. The target analytes, PCP, Dxm and ketamine will utilize PCP-d_5, Dxm-d_3, and ketamine-d_4 internal standard, respectively.

The sample preparation and instrumental parameters defined within the Miscellaneous Basic Drugs Quantitation and Confirmation by LCMSMS method will be utilized within this validation. The instrumental method is a positive ionization Dynamic MRM method. The optimized fragmentor voltage, collision energy, and cell accelerator voltage from the method development of PCP, Dxm, and ketamine will be employed. The parameters are listed in Table 1.

Table 1 Optimized voltages

Compound	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Fragmentor (V)	Collision Energy (V)	Cell Accelerator (V)
Dxm	272.2	171	164	44	4
Dxm	272.2	147	164	36	4
Dxm-d_3	275.2	215.1	140	28	4
Dxm-d_3	275.2	147	140	36	4
Ketamine	238.1	125	92	36	4
Ketamine	238.1	115	92	72	4
Ketamine-d_4	242.1	129	100	40	4
Ketamine-d_4	242.1	92.1	100	76	4
PCP	244.2	159	92	12	4
PCP	244.2	86.1	92	12	4
PCP-d_5	249.2	96.1	80	48	4
PCP-d_5	249.2	164.1	92	12	4

A validation plan is outlined herein pursuant to the 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).

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

1. Bias and Precision

This method is intended for the quantitative analysis of multiple matrices (e.g., postmortem blood, antemortem blood, and urine), bias and precision experiments shall be conducted for all matrix types.

a. Bias

Bias shall be measured using fortified matrix samples. To evaluate bias, a minimum of triplicate determinations per concentration (low, medium, and high) over a total of five batch analyses shall be evaluated per matrix type. The working range to be evaluated is 0.01 mg/L to 1.0 mg/L. The low concentration shall be no more than approximately three times the lowest end of the working range of the method and high concentration shall be within approximately 80% of the highest end of the working range. The low, medium, and high concentrations to be evaluated for bias will be 0.030 mg/L, 0.40 mg/L, and 0.80 mg/L.

The bias of the fortified pooled blood samples will be assessed using Equation 1.

Equation 1

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

Bias should be as low as possible, but shall not exceed ±20% at each concentration level when analyzing common biological fluids. The same data used in the evaluation of bias shall also be used for the determination of within-run and between-run precision.

b. Within-run Precision

Precision will be expressed as the percent coefficient of variation (%CV). During method validation, within-run precision is measured using pooled fortified matrix samples. A minimum of triplicate determinations per concentration (low, medium, and high) over a total of five batch analyses shall be evaluated. The low concentration shall be no more than approximately three times the lowest end of the working range of the method and high concentration shall be within approximately 80% of the highest end of the working range. The low, medium, and high concentrations to be evaluated for within-run precision will be 0.030 mg/L, 0.40 mg/L, and 0.80 mg/L, The within-run precision shall be calculated using Equation 2.

Equation 2

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

The within-run precision for each analytical run will be calculated. The analytical run with the largest within-run precision shall be utilized for the overall within-run precision of the process. Within-run precision shall not exceed 20% at each concentration level when analyzing common biological fluids.

c. Intermediate Precision

Intermediate precision will be measured using pooled fortified matrix samples. A minimum of triplicate determinations per concentration (as delineated above) over a total of five batch analyses shall be evaluated. The intermediate precision shall be calculated using Equation 3.

Equation 3

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

The intermediate precision will be calculated using the combined data from the multiple analyses over the minimum of five batches. The standard deviation and mean will be calculated to determine the intermediate precision. Intermediate precision shall not exceed 20% at each concentration level when analyzing common biological fluids.

2. Sensitivity

a. Estimated Limit of Detection (LOD)

The estimated limit of detection for this validation shall be defined as an administratively-defined decision point (threshold concentration). The administratively-defined decision point shall be established using two concentrations. The concentrations to be evaluated are 50% (0.005 mg/L) of the lowest calibrator concentration (threshold concentration) and 50% below the threshold concentration (0.0025 mg/L) within the method. These defined concentrations will be established as the decision point for reporting analytes within this method although a lower LOD may be analytically achievable.

The decision point shall be evaluated by fortifying, at minimum, three different blank matrix sources per matrix type (i.e., blank blood, postmortem blood, antemortem blood, urine, and liver). The three different blank matrix sources shall be analyzed over a minimum of three analyses to demonstrate that all predetermined detection and identification criteria are met.

Predetermined identification criteria:

Retention Time: ±3%
Qualifier Ratio: ±20%
Signal-to-Noise: ≥3.3

b. Lower Limit of Quantitation (LLOQ)

The lower limit of quantitation for this validation shall be established by evaluating the lowest non-zero calibrator for the method. For each matrix type (i.e., blank blood, postmortem blood, urine), a minimum of three different blank matrix sources shall be fortified at the lowest calibrator concentration and analyzed over a minimum of three analyses. A minimum of nine replicates per matrix source (27 replicates per matrix type) will be utilized to demonstrate that all detection, identification, bias, and precision criteria are met.

Predetermined acceptance criteria:

Retention Time: ±3%
Qualifier Ratio: ±20%
Signal-to-Noise: ≥10
Back Calculated Concentration: ±20%

3. Linearity and Calibration Model

The calibration model shall be established by determining the working range of analyte concentration over which the method shall be used. The working range to be evaluated shall be 0.01 mg/L to 1.0 mg/L. A total of seven non-zero calibrators (0.010 mg/L, 0.020 mg/L, 0.050 mg/L, 0.10 mg/L, 0.20 mg/L, 0.50 mg/L, 0.75 mg/L, 1.0 mg/L) will be evaluated. Within the working calibration range, there will be a correlation between peak area ratio of analyte and internal standard and the analyte concentration in the sample. The determined calibration model is the mathematical equation that describes this correlation.

To establish the calibration model, a minimum of five replicate determinations from different batches will be utilized. The calibration samples shall include the concentrations delineated in Table 2 for each target compound. A blank sample and a minimum of six different non-zero concentration levels shall be used to establish the calibration model. Although the least squares model for regression is preferred, the best and simplest model (e.g., weighted, unweighted, linear, quadratic) that best fits the data will be chosen. The origin shall be ignored in each calibration model, the correlation coefficient shall be ≥0.985, and the back calculated calibrator concentrations must be within ±20% of the target.

Table 2 Working range calibration sample concentrations

Volume of 1 µg/mL working solution (µg/mL)	Volume of 10 µg/mL working solution (µg/mL)	Final concentration (mg/L)
	100	1.0
	75	0.75
	50	0.50
	20	0.20
	10	0.10
50		0.050
20		0.020
10		0.010

The model will be established by residual analysis and statistical comparisons (ANOVA) between model fits. A plot of the residual values for each calibration type shall be generated to evaluate the effectiveness of the calibration model. The plot(s) will be visually evaluated to determine the model with homoscedasticity over the working range. Once established, the calibration model shall be utilized to obtain data regarding bias and precision, limit of quantitation, and dilution integrity within the validation.

4. Ionization Suppression/Enhancement

Ionization suppression and enhancement will be addressed with neat standards and post-extraction fortified samples. Two different sets of samples shall be prepared and their peak areas compared between sets. Neat standards, at low and high concentrations, will be prepared in neat extraction solvent and injected a minimum of six times each. The low and high concentrations evaluated during bias and precision will also be utilized in the determination of ionization suppression or enhancement. The responses will be averaged for the two different concentrations (0.030 mg/L and 0.80 mg/L). A minimum of ten duplicates of post-extraction fortified samples (matrix that is extracted and then fortified), per matrix type (i.e., blank blood, postmortem blood, antemortem blood, urine), will be prepared to compare to the neat standards. The responses will be averaged for the two concentrations. The ratio between the averages of the sets will then be used to assess ionization suppression or enhancement as shown in Equation 4.

Equation 4

$$Ion\ Suppression/Enhancement = \left(\frac{Average\ Post-Extraction\ Fortified\ Sample}{Average\ Neat\ Sample}\right) \times 100$$

The ionization suppression or enhancement will be evaluated for the qualifier and quantifier transitions for the analytes and internal standards within the method. If suppression or enhancement exceeds ±25% or the %CV exceeds 20%, an evaluation of the effect on limit of detection and bias shall be evaluated. The influence on the parameters shall be assessed by at least tripling the number of different sources of blank matrices used in the evaluation.

5. Carryover

Carryover will be 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. Analyte carryover is indicated by a response greater than 10% of the LLOQ. This concentration shall be confirmed using triplicate analysis with a minimum of three sources per matrix type.

6. Interferences

To assess for interference, the qualifier and quantifier ions for each analyte and internal standard within the method shall be monitored. Interferences below the limit of detection for the method may be deemed insignificant. If present, the impact on identification and quantitation shall be evaluated. If the instrumental response is less than 10% of the LLOQ response for the qualifier or quantifier ions, the impact is deemed insignificant.

a. Endogenous Compounds

Where possible, a minimum of ten negative matrix samples from different sources without the addition of an internal standard shall be analyzed for possible endogenous interferences. A minimum of ten matrix samples for each matrix type (i.e., blank blood, postmortem blood, antemortem blood, urine, and liver) within the validation should be evaluated, whenever possible.

b. Internal Standard

To evaluate potential interferences of the internal standard by a high concentration of analyte, samples shall be fortified with the highest calibrator concentration (1.0 mg/L) without internal standard and analyzed for the absence of response for the internal standard. A single blank matrix (i.e., blank blood, postmortem blood, antemortem blood, urine) sample, per matrix type shall be evaluated.

To evaluate potential interferences from the method's internal standard concentration to a low concentration of analyte, matrix shall be fortified with an appropriate concentration of internal standard (0.50 mg/L) without the analyte of interest and analyzed for the absence of response for the analyte. A single blank matrix (i.e., blank blood, postmortem blood, antemortem blood, urine) sample, per matrix type shall be evaluated.

c. Commonly Encountered Analytes

Analytes which may be expected to be present in case samples shall be evaluated for their potential to interfere with the method's analytes. Matrix samples shall be fortified with

commonly encountered drugs, metabolites, and other structurally similar compounds at high concentrations (i.e., highest calibrator concentration from current methods).

Potential interferents to be evaluated:

Barbiturates (30 mg/L)
Amphetamines (2.0 mg/L)
Benzodiazepines (2.0 mg/L)
Carisoprodol and meprobamate (100 mg/L)
Anti-epileptic drugs (40 mg/L)
Basic drugs from previously made mixes (6.0 mg/L)
Acid/neutral drugs from previously made mixes (6.0 mg/L)
Opioids and cocaine (0.2/2.0/1.0 mg/L)
Fentanyl derivatives (0.05/0.1 mg/L)
Novel psychoactive substances (1.0 mg/L)
Cannabinoids (0.1/0.5 mg/L THC/Carboxy-THC)

7. Dilution Integrity

The dilution integrity will be assessed for scenarios including concentrations above the ULOQ with sufficient sample volume (large volume). The large volume dilution will be evaluated using 1.0 mL of matrix and diluting with blank matrix. Common dilution ratios (1:2 and 1:10) will be evaluated for bias and precision per matrix type utilizing the experiments delineated in Section 1. The concentration will be adjusted depending upon the dilution factor and the adjusted concentration must be within the predetermined acceptance criteria (±20% of the undiluted target concentration) for both bias and precision.

8. Stability

During the validation period, the stability of extracted samples that are not analyzed immediately shall be addressed. Extracted samples shall be stored in autosampler vials on the instrument throughout the stability evaluation process. This enables the simulation of an abrupt abortion, delay, or interruption during instrumental analysis.

At minimum, a single blank matrix source, per matrix type (i.e., blank blood, postmortem blood, antemortem blood, urine), will be extracted at two concentrations (0.80 and 0.03 mg/L) and analyzed at minimum every twenty-four hours for a seven day period with triplicate injections at each time point. For day one instrumental response, samples will be extracted and immediately analyzed. The responses will be averaged and all other responses from subsequent time points will be evaluated against the average response. The average instrumental responses for each time point will be compared to the day one instrumental response and plotted. Compounds are considered stable if the average signal response of the triplicate injections for a time point falls within the method's predefined acceptable bias (i.e., ±20%). For example, if the peak area

increases above 120% or decreases below 80% of the original response the compound is no longer deemed stable. Alternatively, the ratio of peak area of analyte to internal standard may be utilized in the stability evaluation as opposed to peak area.

The stability should be carried out by injecting samples from the same autosampler vial throughout the stability experiments. For methods with larger sample volumes, it may be necessary to extract multiple samples and pool the extracts together for analysis.

9. Robustness

Robustness will be determined by performing the validation on multiple instruments. Validation experiments should encompass all models of instruments within the laboratory.

10. References

Virginia Department of Forensic Science Quality Manual, Qualtrax Revision 26, **2023**.

Virginia Department of Forensic Science Toxicology Procedures Manual, Qualtrax Revision 26, **2023**.

ANSI/ASB Standard 036, Standard Practices for Method Validation in Forensic Toxicology. 1st Edition. **2019**.