Memo To: James Hutchings, Ph.D., Toxicology Program Manager
From: Rebecca Wagner, Ph.D., Chemistry Research Section Supervisor
CC: Alka Lohmann, Technical Services Director
Date September 15, 2023
RE: Validation Plan
Addition of Xylazine and Dexmedetomidine to the Opioid, Cocaine, Benzoylecgonine and Cocaethylene Quantitation and Confirmation by LCMSMS Method

Validation Plan – Addition of Xylazine and Dexmedetomidine to the Opioid, Cocaine, Benzoylecgonine and Cocaethylene Quantitation and Confirmation by LCMSMS Method

It is proposed to add xylazine and dexmedetomidine to the existing Toxicology Procedures Manual Section 28, Opioid, Cocaine, Benzoylecgonine and Cocaethylene Quantitation and Confirmation by LCMSMS method (Qualtrax Revision 28). The validation will be performed using the liquid-liquid extraction option (Section 28.6.7.1) within the method. The target analytes and associated internal standards are listed in Table 1.

Table 1 Target compounds and internal standards

Quantitative Targets	Internal Standard
Xylazine	Xylazine-D_6
Dexmedetomidine	Medetomidine-$^{13}C,D_3$

The target compounds and internal standards were optimized using the Agilent Technologies Optimizer software. The optimized transition ions and instrumental settings are listed in Table 2.

Table 2 Optimized instrumental settings

Compound	Precursor	Product	RT	Fragmentor (V)	Collision Energy (V)	Cell Accelerator (V)
Xylazine	221.1	164	4.40	145	24	7
		90		145	20	7
Xylazine-D_6	227.1	170	4.40	140	24	7
		90		140	20	7
Dexmedetomidine	201.1	95	5.00	110	24	7
		68.1		110	40	7
Medetomidine-$^{13}C,D_3$	205.16	99	4.90	110	16	7
		72.1		110	36	7

The working range for xylazine and dexmedetomidine will be 0.001 mg/L to 0.2 mg/L. A validation plan is outlined herein pursuant to the Quality Manual (Qualtrax Revision 27) and Toxicology Procedures Manual (Qualtrax Revision 28). The validation plan is in accordance with the 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. Estimated 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

The method is intended for the quantitative analysis of multiple matrices (i.e., blank blood, 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. The working range to be evaluated is 0.001 mg/L to 0.2 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 0.003 mg/L, 0.08 mg/L, and 0.16 mg/L.

The bias of the fortified pooled 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 0.003 mg/L, 0.08 mg/L, and 0.16 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

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

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 estimated using two concentrations. The concentrations to be evaluated are 50% and 75% below the lowest calibrator concentration (0.001 mg/L) within the method. These defined concentrations will be established as the decision point for reporting analytes within this method although a lower estimated 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, and urine). 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 (0.001 mg/L) for the method. For each matrix type (e.g., blank blood, postmortem blood, antemortem blood, and 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 0.001 mg/L to 0.2 mg/L. A total of eight non-zero calibrators (0.001 mg/L, 0.002 mg/L, 0.005 mg/L, 0.01 mg/L, 0.025 mg/L, 0.075 mg/L, 0.01 mg/L, 0.2 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 3 for each target compound. A blank sample and the eight 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 3 Working range calibration sample concentrations

Amount of 1.0 mg/L stock solution (µL)	Amount of 0.1 mg/L stock solution (µL)	Final concentration (mg/L)
	10	0.001
	20	0.002
	50	0.005
	100	0.01
25		0.025
75		0.075
100		0.1
200		0.2

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. Low and high concentrations will be utilized in the determination of ionization suppression or enhancement. The responses will be averaged for the two different concentrations (0.003 mg/L and 0.16 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, and 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, and urine) 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 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, and 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 (concentration delivered within method) 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, and 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 method).

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 substance (1.0 mg/L)
Cannabinoids (0.1/0.5 mg/L)

In addition to commonly encountered analytes, each drug within the method will be evaluated individually.

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.

In addition to a large volume dilution evaluation, a small volume evaluation will be performed for samples with minimal specimen volume. The small volume dilution will be evaluated using volumes less than 1.0 mL. Common dilution ratios (1:2 and 1:10) will be evaluated for bias and precision per matrix type utilizing 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, and urine), will be extracted at two concentrations (high [0.16 mg/L] and low [0.003 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.

9. Robustness

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

10. References

Virginia Department of Forensic Science Quality Manual, Qualtrax Revision 27, **2023.**

Virginia Department of Forensic Science Toxicology Procedures Manual, Qualtrax Revision 28, **2023**.