VALIDATION SUMMARY

Procedure Name	**TOX-438-00 Cocaine-Metabolites Confirmation**
Validation Summary	Development and validation experiments were performed in April and May of 2023 by members of the toxicology discipline. Validation documentation was prepared **Redacted** **Redacted** Analytes evaluated: cocaine, benzoylecgonine, cocaethylene, ecgonine methyl ester, norcocaine All analytes were evaluated quantitatively from 10-1000 ng/mL and LODs evaluated at 1 ng/mL. Extraction of 0.2mL of blood samples was performed using SPE with analysis by LC/MS with full scan Orbitrap and targeted ion trap fragmentation of target analytes. **Results:** 1. Bias was evaluated using three replicates per concentration level. Three concentration levels were evaluated – low (30ng/mL), mid (500ng/mL), and high (800ng/mL) – over five calibration runs. Due to possible analyte instability, concentration pools of fortified samples were not used. Samples were fortified with each run.

Analyte	Desired Limit	Results			
cocaine	< ±20%	30	ng/mL	Low QC	2.96%
		500	ng/mL	Mid QC	-0.60%
		800	ng/mL	High QC	1.97%
BE	< ±20%	30	ng/mL	Low QC	13.77%
		500	ng/mL	Mid QC	0.59%
		800	ng/mL	High QC	4.03%
CE	< ±20%	30	ng/mL	Low QC	0.70%
		500	ng/mL	Mid QC	-0.26%
		800	ng/mL	High QC	2.84%
EME	< ±20%	30	ng/mL	Low QC	0.23%
		500	ng/mL	Mid QC	-1.43%
		800	ng/mL	High QC	2.55%
norcocaine	< ±20%	30	ng/mL	Low QC	-4.28%
		500	ng/mL	Mid QC	-5.15%
		800	ng/mL	High QC	-0.07%

2. Precision was assessed using triplicate analysis per concentration pool (low, mid, high) over five different runs. The maximum acceptable %CV is 20% at each concentration. Two types of precision studies were assessed: within-run and between-run.

Analyte	Desired Limit	Results		
cocaine	Within and Between Run < 20%	Low QC	Within:	2.04%
			Between:	4.77%
		Mid QC	Within:	2.03%
			Between:	7.14%
		High QC	Within:	1.16%
			Between:	4.99%
BE	Within and Between Run < 20%	Low QC	Within:	4.29%
			Between:	5.03%
		Mid QC	Within:	2.81%
			Between:	7.58%
		High QC	Within:	3.17%
			Between:	6.35%
CE	Within and Between Run < 20%	Low QC	Within:	1.76%
			Between:	4.85%
		Mid QC	Within:	2.43%
			Between:	7.42%
		High QC	Within:	1.51%
			Between:	4.69%
EME	Within and Between Run < 20%	Low QC	Within:	1.60%
			Between:	4.62%
		Mid QC	Within:	2.18%
			Between:	7.77%
		High QC	Within:	0.82%
			Between:	5.28%
norcocaine	Within and Between Run < 20%	Low QC	Within:	2.55%
			Between:	5.68%
		Mid QC	Within:	2.14%
			Between:	7.19%
		High QC	Within:	1.45%
			Between:	3.90%

3. Calibration model was determined by analyzing five sets of matrix-matched calibrator samples. Six different non-zero concentrations were used: 10, 150, 320, 525, 750, 1000 (all ng/mL). Two separate statistical software approaches were used to evaluate the weighting and model of the calibration curve. Using those results and evaluating the data led to the following calibration models and weighting:

Analyte	Linear or quad	Weighting results
Cocaine	Linear	$1/x^2$
BE	Quadratic	$1/x^2$
CE	Linear	$1/x^2$
EME	Linear	$1/x^2$
Norcocaine	Linear	$1/x^2$

The two statistical approaches used were:

1. The Pearring Regression Model Selection Spreadsheet (PRMSS)

2. An automated script run in RStudio. The script was obtained from Supplemental Data provided with an article from JAT (Desharnais, B., Camirand-Lemyre, F., Mireault, P., Skinner, C.D. (2017) Procedure for the Selection and Validation of a Calibration Model I – Description and Application. *Journal of Analytical Toxicology*, **41**, 261-268.)
Both approaches indicated that the weighting and model that best fit all analytes was linear $1/x^2$. However, when evaluating the individual QC results, the low control for BE failed 6 out of 15 times using this weighting and model. When the residuals plot were evaluated for BE, there seemed to be a more random scatter of results for quadratic 1/x2, indicating that perhaps that the quadratic model was best suited for BE. QC results for BE using a quadratic 1/x2 calibration model produced a failure of only 1 out of 15 replicates. The QC results combined with the evaluation of the residuals plots, led to changing the model for BE from linear to quadratic.

3. Carryover was evaluated by analyzing an extracted blank matrix immediately after the high calibrator in each of the five calibration runs. For all analytes, carryover was nonexistent or less than 4% of the low calibrator response.

4. Interferences

- Ten sources of blank postmortem whole blood were secured from previously analyzed cases and ante mortem whole blood from a medical supply company to evaluate matrix interferences. The blank matrix samples were extracted without the addition of internal standard and analyzed using the newly developed method. No interferences at the retention time for the target analytes were noted after analysis of the blank whole blood samples.
- One of the blank matrix samples was randomly selected and internal standard was added to the sample at 300ng/mL. The sample was then extracted and analyzed. This was to demonstrate that the internal standard would not interfere with the signal for the target analytes.
- One of the blank matrix samples was randomly selected and target analytes were added at 800 ng/mL, without addition of internal standard. The sample was extracted and analyzed. This was to evaluate whether the unlabeled analyte interferes with the signal for the deuterated analyte. The results demonstrated no interferences between the analytes and internal standards.
- To evaluate interferences from other commonly encountered analytes, neat solutions of 20-480 ng/mL of 44 DRUGS were injected. No interference was observed for the signal of the target analytes or internal standards.

5. Ionization suppression/enhancement was assessed for both target analytes and internal standards using the post-extraction addition technique. Two different sets of samples were prepared – a set of neat standards and a set of matrix samples fortified with neat standards after extraction – and the average analyte peak areas of each compared. This technique was performed at both a low and high concentration. Acceptable limits for suppression/enhancement were ±25% and ±20% for the CV of the suppression/enhancement.
   The results are below. Due to EME and BE having parameters outside of the acceptable limits, the number of matrix sources used to evaluate both LLOQ and LOD were tripled, from 3 lots to 9 lots.

Analyte	Desired Limit	Results	
cocaine	<25% suppression/ enhancement AND <20% for CV due to matrix	% Ionization Low QC:	-7.93
		% Ionization ISTD Low QC:	-7.96
		%CV LOW QC EXTRACTED:	8.01
		% Ionization High QC:	-15.82
		% Ionization ISTD High QC:	-15.72
		%CV HIGH QC EXTRACTED:	14.67
BE	<25% suppression/ enhancement AND <20% for CV due to matrix	% Ionization Low QC:	-0.76
		% Ionization ISTD Low QC:	-0.21
		%CV LOW QC EXTRACTED:	18.55
		% Ionization High QC:	-15.17
		% Ionization ISTD High QC:	-0.94
		%CV HIGH QC EXTRACTED:	28.84
CE	<25% suppression/ enhancement AND <20% for CV due to matrix	% Ionization Low QC:	-6.83
		% Ionization ISTD Low QC:	-6.9
		%CV LOW QC EXTRACTED:	8.6
		% Ionization High QC:	-6.25
		% Ionization ISTD High QC:	-6.36
		%CV HIGH QC EXTRACTED:	10.7
EME	<25% suppression/ enhancement AND <20% for CV due to matrix	% Ionization Low QC:	-12.45
		% Ionization ISTD Low QC:	-7.1
		%CV LOW QC EXTRACTED:	41.97
		% Ionization High QC:	-17.36
		% Ionization ISTD High QC:	-12.6
		%CV HIGH QC EXTRACTED:	36.03
norcocaine	<25% suppression/ enhancement AND <20% for CV due to matrix	% Ionization Low QC:	-9.74
		% Ionization ISTD Low QC:	-10.77
		%CV LOW QC EXTRACTED:	10.09
		% Ionization High QC:	-11.48
		% Ionization ISTD High QC:	-10.73
		%CV HIGH QC EXTRACTED:	12.23

7. Limit of detection (LOD) was evaluated in nine different matrices over three separate runs. An LOD value of 1 ng/mL was administratively chosen to be evaluated. All detection/identification criteria were met for all replicates, except for EME. The LOD for EME will be 10ng/mL based on the quality of the ion trap MS^2 data.

8. Lower limit of quantitation (LLOQ) was evaluated using nine different matrixes over three separate runs. The lowest non-zero calibration (10ng/mL) was used as the LLOQ. All detection, identification, bias, and precision criteria were met.

Analyte	Parameter	Desired Limit	Results		
Cocaine	Bias	< ±20%	10ng/mL	LLOQ	4.13%
	Precision	Within and Between Run <20%	LLOQ	Within:	1.81%
				Between:	4.84%
BE	Bias	< ±20%	10ng/mL	LLOQ	-1.58%
	Precision	Within and Between Run <20%	LLOQ	Within:	11.63%
				Between:	13.52%
CE	Bias	< ±20%	10ng/mL	LLOQ	3.16%
	Precision	Within and Between Run <20%	LLOQ	Within:	3.04%
				Between:	5.53%
EME	Bias	< ±20%	10ng/mL	LLOQ	3.12%
	Precision	Within and Between Run <20%	LLOQ	Within:	7.77%
				Between:	9.77%
Norcocaine	Bias	< ±20%	10ng/mL	LLOQ	3.50%
	Precision	Within and Between Run <20%	LLOQ	Within:	1.78%
				Between:	4.51%

9. Dilution integrity was assessed by evaluating the effect of a 10x dilution on the method's bias and precision. 8000ng/mL samples were prepared and then diluted 10x with negative matrix to bring the concentration into the calibration range. Triplicate analysis of this control level was evaluated over five different runs.

Analyte	Parameter	Desired Limit	Results		
Cocaine	Bias	< ±20%	8000ng/mL	10x dilution	-4.07%
	Precision	Within and Between Run <20%	10x dilution	Within:	1.74%
				Between:	3.20%
BE	Bias	< ±20%	8000ng/mL	10x dilution	-11.56%
	Precision	Within and Between Run <20%	10x dilution	Within:	3.98%
				Between:	6.12%
CE	Bias	< ±20%	8000ng/mL	10x dilution	-3.35%
	Precision	Within and Between Run <20%	10x dilution	Within:	2.00%
				Between:	2.99%
EME	Bias	< ±20%	8000ng/mL	10x dilution	-6.36%
	Precision	Within and Between Run <20%	10x dilution	Within:	2.06%
				Between:	2.50%
Norcocaine	Bias	< ±20%	8000ng/mL	10x dilution	-6.82%
	Precision	Within and Between Run <20%	10x dilution	Within:	1.74%
				Between:	2.55%

10. Processed sample stability was evaluated at two different concentrations – low (30ng/mL) and high (800ng/mL). Several samples at each level were extracted. The reconstituted samples of each level were pooled and then divided out into four separate autosampler vials. The first of these vials was injected three times on the same day the extraction was performed. The average ratio of analyte/internal standard of those three injections represents the day zero sample. The remaining vials were kept in the cooled autosampler until analyzed. The other three analyses occurred on days 1, 4, and 7. Analyte/internal standard ratios from the triplicate analyses were averaged and compared to the day zero average. As the required bias is ±20%, analytes would be considered stable until a change of more than 20% from the day zero ratio. The data suggests that all analytes remained stable within 10% from the day zero value for the entire 7 day period of the study.

APPROVALS			
Technical Approval	Redacted	Date	7/14/2023
Unit Chief Approval		Date	7/18/2023