Appendix A6.1.3.32 Validation Summary ZZ34313-03 (Total NNAL and Total NNN in Urine)

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The following validation summary has been prepared by (b) (4) Full method validation reports and related SOPs are all available at (b) (4)
(b) (4)

Table 1. Validation Summary - Total NNAL – ZZ34313-03

Information Requested	Data
Validation Summary	(b) (4) Validation Study ZZ34313-03
Analyte	Total NNAL
Internal Standard (IS)	d_4-NNAL
Method Description	Solid phase extraction with analysis/detection by LC-MS/MS
Limit of Quantitation (pg/mL)	5.00 pg/mL
Average Recovery of Drug (% Mean)	88% at 15.0 pg/mL 82% at70.0 pg/mL 85% at 750 pg/mL
Average Recovery of IS (% Mean)	87%
Standard Curve Concentrations (pg/mL)	5.00, 10.0, 20.0, 50.0, 100, 200, 400, 800, and 1000 pg/mL
QC Concentrations (pg/mL)	LLOQ QC, 15.0, 70.0, and 750 pg/mL
QC Intra-Batch Precision Range (% CV)	1.6 to 43.0% (1.6 to 6.9 for acceptable PA batches only)
QC Intra-Batch Accuracy Range (% Bias)	-5.1 to 9.4% (-5.1 to 9.4 for acceptable PA batches only)
QC Inter-Batch Precision Range (% CV)	3.0 to 18.2% (3.3 to 6.5 for acceptable PA batches only)
QC Inter-Batch Accuracy Range (% Bias)	-2.3 to 3.0% (-2.5 to 4.0 for acceptable PA batches only)
Bench-Top Stability (Hrs)	Short-Term Stability: 26 hours in clear polypropylene tubes at ambient temperature under UV-shielded light Cumulative Short-Term Stability: 52 hours in clear polypropylene tubes at ambient temperature under UV-shielded light (total of all thaw cycles) Short-Term Stability: 25 hours (LLOQ QC) and 24 hours (dilution QC) in brown polypropylene tubes at ambient temperature under UV-shielded light Cumulative Short-Term Stability: 52 hours (LLOQ QC) and 53 hours (dilution QC) in brown polypropylene tubes at ambient temperature under UV-shielded light (total of all thaw cycles)
Stock Stability (Days)	Long-Term Stability for Stock Solutions (Stock): 98 days at approximately 100 µg/mL in methanol in a polypropylene container at -20°C

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Information Requested	Data
Processed Stability (Hrs)	Post-Preparative Stability: 126 hours in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 126 hours in a polypropylene 96 well plate at 5°C
Freeze-Thaw Stability (Cycles)	6 freeze (-20°C)-thaw (ambient temperature) cycles in clear polypropylene tubes under UV-shielded light 6 freeze (-20°C)-thaw (ambient temperature) cycles in brown polypropylene tubes under UV-shielded light
Long-Term Storage Stability (Days)	Long-Term Stability: 807 days in clear polypropylene tubes at -20°C; 318 days (LLOQ QC) and 296 days (dilution QC) in brown polypropylene tubes at -20°C
Dilution Integrity	Up to 2000 pg/mL, diluted 5-fold
Selectivity	No significant interference at the retention time and mass transition of total NNAL was observed from endogenous components in any of the 10 human urine lots screened or of d_4-NNAL (IS) in any of the 10 human urine lots screened

Additional Information - Total NNAL – ZZ34313-03

Matrix		Human Urine	
Bioanalytical Method (BAM) SOP Number		BAM SOP ZZ34313-03	
Detector		AB SCIEX API 4000™	
Assay Volume Required		2.00 mL	
Regression Type		Weighted linear (1/concentration^2)	
Quantitation Method		Peak area ratio	
Multiple Analytes Measured by Assay		Total NNAL at 15.0 pg/mL fortified with total NNN at 40.0 pg/mL	
Co-administered Compound Evaluation		Total NNAL concentration was evaluated in the presence of all analytes measured by the assay and other compounds users of tobacco products are exposed to	
Selectivity from Metabolites Not Quantified by the Method		Nornicotine (20.0, 40.0, and 100 ng/mL)	
Additional Selectivity Compounds		NAT (450 pg/mL) NAB (70.0 pg/mL)	
Quality Control Samples		Precision (% CV)	Accuracy (% Bias)
Inter-Batch	**LLOQ**	18.2	3.0
	Low	5.7	2.0
	Medium	3.0	-0.3
	High	5.6	-2.3
Inter-Batch (Acceptable PA Batches Only)	**LLOQ**	6.5	4.0
	Low	4.4	4.0
	Medium	3.3	-0.3
	High	4.1	-2.5

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Additional Information - Total NNAL – ZZ34313-03			
Intra-Batch (Batch 54)[1] **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ** **Low** **Medium** **High**	43.0 6.2 2.1 10.2	-1.0 -4.0 -0.3 -1.7
Intra-Batch (Batch 56) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ** **Low** **Medium** **High**	2.8 2.3 2.4 3.0	8.8 1.3 1.9 -0.4
Intra-Batch (Batch 60) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ** **Low** **Medium** **High**	4.4 6.9 3.8 4.0	9.4 3.3 0.7 -4.9
Intra-Batch (Batch 62) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ** **Low** **Medium** **High**	4.0 3.0 2.8 3.0	-3.4 6.0 -0.6 0.5
Intra-Batch (Batch 67) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ** **Low** **Medium** **High**	4.6 3.1 1.6 3.4	1.0 5.3 -3.1 -5.1
Matrix Effect		No significant matrix effect was observed in any of the 10 human urine lots that were fortified with total NNAL at the concentration of the LLOQ (5.00 pg/mL) or in any of the 10 human urine lots that were fortified with total NNAL at the concentration of the high QC (750 pg/mL) samples	
Sample Shipping Stability		9 days in clear polypropylene tubes at -80°C 4 days in brown polypropylene tubes at -80°C	
Long-Term Stability for Stock Solutions (Substock)		98 days at 3600 ng/mL in methanol in a polypropylene container at -20°C	
Long-Term Stability for Stock Solutions (Substock)		98 days at 200 ng/mL in methanol in a polypropylene container at -20°C	
Long-Term Stability for Stock Solutions (Internal Standard)		Refer to long-term stability for stock solutions data collected from unlabeled NNAL for labeled internal standard stability	
Short-Term Stability for Stock Solutions (Stock)		18 hours at approximately 100 µg/mL in methanol in a polypropylene container at ambient temperature under UV-shielded light	
Short-Term Stability for Stock Solutions (Substock)		18 hours at 3600 ng/mL in methanol in a polypropylene container at ambient temperature under UV-shielded light 18 hours at 200 pg/mL in methanol in a polypropylene container at ambient temperature under UV-shielded light	

[1] Batch fails intra-day PA, but passes batch acceptance criteria.

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Additional Information - Total NNAL – ZZ34313-03	
Short-Term Stability for Stock Solutions (Internal Standard Stock)	Refer to short-term stability for stock solutions data collected from unlabeled NNAL for labeled internal standard stability
Short-Term Stability for Stock Solutions (Internal Standard Substock)	7 hours at 18.0 pg/mL in methanol in a polypropylene container at ambient temperature under UV-shielded light
Stability of Analyte During Sample Collection and Handling	45 minutes in non-colored high-density polyethylene void containers at ambient temperature under white light, 4 hours in amber high-density polyethylene refrigeration containers at ambient temperature under white light, and approximately 2 days in amber high-density polyethylene refrigeration containers at 5°C Up to 96 hours in smoker human urine in high-density polyethylene containers at 5°C, 20°C, and 40°C under UV-shielded light
Co-administered Compound Long-Term Stability (Frozen)	Against All Analytes Measured by the Assay and Other Compounds Users of Tobacco Products are Exposed To: 349 days (low QC) and 179 days (high QC) in clear polypropylene tubes at -20°C
Co-administered Compound Freeze-Thaw Stability	Against All Analytes Measured by the Assay and Other Compounds Users of Tobacco Products are Exposed To: 6 freeze (-20°C)-thaw (ambient temperature) cycles in clear polypropylene tubes under UV-shielded light
Co-administered Compound Short-Term Stability	Co-administered Compound Short-Term Stability Against Total All Analytes Measured by the Assay and Other Compounds Users of Tobacco Products are Exposed To: 26 hours in clear polypropylene tubes at ambient temperature under UV-shielded light Co-administered Compound Cumulative Short-Term Stability Against All Analytes Measured by the Assay and Other Compounds Users of Tobacco Products are Exposed To: 53 hours in clear polypropylene tubes at ambient temperature under UV-shielded light (total of all thaw cycles)
Batch Size	120 injections

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Table 2. Validation Summary – Total NNN in Urine - ZZ34313-03

Information Requested	Data
Validation Summary	(b) (4) Validation Study ZZ34313-03
Analyte	Total NNN
Internal Standard (IS)	d_4-NNN
Method Description	Solid phase extraction with analysis/detection by LC-MS/MS
Limit of Quantitation (pg/mL)	0.200 pg/mL
Average Recovery of Drug (% Mean)	64% at 0.600 pg/mL 62% at 2.80 pg/mL 66% at 30.0 pg/mL
Average Recovery of IS (% Mean)	66%
Standard Curve Concentrations (pg/mL)	0.200, 0.400, 0.800, 2.00, 4.00, 8.00, 16.0, 32.0, and 40.0 pg/mL
QC Concentrations (pg/mL)	LLOQ QC, 0.600, 2.80, and 30.0 pg/mL
QC Intra-Batch Precision Range (% CV)	1.5 to 56.1% (1.5 to 11.5 for acceptable PA batches only)
QC Intra-Batch Accuracy Range (% Bias)	-5.0 to 12.5% (-5.0 to 12.5 for acceptable PA batches only)
QC Inter-Batch Precision Range (% CV)	4.4 to 24.6% (2.9 to 9.8 for acceptable PA batches only)
QC Inter-Batch Accuracy Range (% Bias)	-0.3 to 3.5% (-1.3 to 3.5 for acceptable PA batches only)
Bench-Top Stability (Hrs)	Short-Term Stability: 26 hours in clear polypropylene tubes at ambient temperature under UV-shielded light Cumulative Short-Term Stability: 52 hours in clear polypropylene tubes at ambient temperature under UV-shielded light (total of all thaw cycles) Short-Term Stability: 24 hours in brown polypropylene tubes at ambient temperature under UV-shielded light Cumulative Short-Term Stability: 53 hours in brown polypropylene tubes at ambient temperature under UV-shielded light (total of all thaw cycles)
Stock Stability (Days)	Long-Term Stability for Stock Solutions (Stock): 98 days at approximately 100 µg/mL in methanol in a polypropylene container at -20°C
Processed Stability (Hrs)	Post-Preparative Stability: 179 hours in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 160 hours in a polypropylene 96 well plate at 5°C
Freeze-Thaw Stability (Cycles)	6 freeze (-20°C)-thaw (ambient temperature) cycles in clear polypropylene tubes under UV-shielded light 6 freeze (-20°C)-thaw (ambient temperature) cycles in brown polypropylene tubes under UV-shielded light
Long-Term Storage Stability (Days)	Long-Term Stability: 807 days in clear polypropylene tubes at -20°C, 318 days (LLOQ QC) and 296 days (dilution QC) in brown polypropylene tubes at -20°C
Dilution Integrity	up to 80.0 pg/mL, diluted 5-fold

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Information Requested	Data
Selectivity	No significant interference at the retention time and mass transition of total NNN was observed from endogenous components in 9 of the 10 human urine lots screened or of d_4-NNN (IS) in any of the 10 human urine lots screened

Additional Information – Total NNN in Urine - ZZ34313-03			
Matrix		Human Urine	
Bioanalytical Method (BAM) SOP Number		BAM SOP ZZ34313-03	
Detector		AB SCIEX Triple Quad™ 6500	
Assay Volume Required		2.00 mL	
Regression Type		Weighted linear (1/concentration^2)	
Quantitation Method		Peak area ratio	
Multiple Analytes Measured by Assay		Total NNN at 0.200 pg/mL fortified with total NNAL at 1000 pg/mL	
Co-administered Compound Evaluation		Total NNN concentration was evaluated in the presence of all analytes measured by the assay and other compounds users of tobacco products are exposed to	
Selectivity from Metabolites Not Quantified by the Method		Nornicotine (20.0, 40.0, and 100 ng/mL)	
Additional Selectivity Compounds		NAT (450 pg/mL) NAB (70.0 pg/mL)	
Quality Control Samples		Precision (% CV)	Accuracy (% Bias)
Inter-Batch	**LLOQ**	24.6	3.5
	Low	4.4	1.3
	Medium	5.9	1.4
	High	4.8	-0.3
Inter-Batch (Acceptable PA Batches Only)	**LLOQ**	9.8	3.5
	Low	4.3	0.5
	Medium	2.9	0.0
	High	3.2	-1.3
Intra-Batch (Batch 57) Aliquot Method: Manual Extraction Method: Automated	**LLOQ**	6.4	12.5
	Low	4.4	3.0
	Medium	2.5	2.1
	High	2.3	1.3
Intra-Batch (Batch 61) Aliquot Method: Manual Extraction Method: Automated	**LLOQ**	11.5	-3.5
	Low	4.5	-0.3
	Medium	2.6	1.1
	High	3.2	-0.7
Intra-Batch (Batch 59)[2] Aliquot Method: Manual Extraction Method: Automated	**LLOQ**	56.1	2.5
	Low	2.2	5.2
	Medium	10.7	6.4
	High	7.7	3.7

[2] Batch fails intra-day PA, but passes batch acceptance criteria.

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Additional Information – Total NNN in Urine - ZZ34313-03			
Intra-Batch (Batch 63) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ** **Low** **Medium** **High**	10.6 4.5 1.5 1.7	2.5 1.7 0.4 -1.0
Intra-Batch (Batch 68) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ** **Low** **Medium** **High**	3.7 2.3 1.7 1.6	2.5 -2.5 -3.2 -5.0
Matrix Effect		No significant matrix effect was observed in 7 of the 10 human urine lots that were fortified with total NNN at the concentration of the LLOQ (0.200 pg/mL) or in any of the 10 human urine lots that were fortified with total NNN at the concentration of the high QC (30.0 pg/mL) samples	
Sample Shipping Stability		9 days in clear polypropylene tubes at -80°C 4 days in brown polypropylene tubes at -80°C	
Long-Term Stability for Stock Solutions (Substock)		98 days at 400 ng/mL in methanol in a polypropylene container at -20°C 102 days at 8.00 pg/mL in methanol in a polypropylene container at -20°C	
Long-Term Stability for Stock Solutions (Internal Standard)		Refer to long-term stability for stock solutions data collected from unlabeled NNN for labeled internal standard stability	
Short-Term Stability for Stock Solutions (Stock)		18 hours at approximately 100 µg/mL in methanol in a polypropylene container at ambient temperature under UV-shielded light	
Short-Term Stability for Stock Solutions (Substock)		18 hours at 400 ng/mL in methanol in a polypropylene container at ambient temperature under UV-shielded light 17 hours at 8.00 pg/mL in methanol in a polypropylene container at ambient temperature under UV-shielded light	
Short-Term Stability for Stock Solutions (Internal Standard Stock)		Refer to short-term stability for stock solutions data collected from unlabeled NNN for labeled internal standard stability	
Short-Term Stability for Stock Solutions (Internal Standard Substock)		7 hours at 2.00 pg/mL in methanol in a polypropylene container at ambient temperature under UV-shielded light	
Stability of Analyte During Sample Collection and Handling		45 minutes in non-colored high-density polyethylene void containers at ambient temperature under white light, 4 hours in amber high-density polyethylene refrigeration containers at ambient temperature under white light, and approximately 2 days in amber high-density polyethylene refrigeration containers at 5°C Up to 96 hours in smoker human urine in high-density polyethylene containers at 5°C, 20°C, and 40°C under UV-shielded light	

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Additional Information – Total NNN in Urine - ZZ34313-03	
Co-administered Compound Long-Term Stability (Frozen)	Against All Analytes Measured by the Assay and Other Compounds Users of Tobacco Products are Exposed To: 358 days (low QC) and 179 days (high QC) in clear polypropylene tubes at -20°C
Co-administered Compound Freeze-Thaw Stability	Against All Analytes Measured by the Assay and Other Compounds Users of Tobacco Products are Exposed To: 6 freeze (-20°C)-thaw (ambient temperature) cycles in clear polypropylene tubes under UV-shielded light
Co-administered Compound Short-Term Stability	Co-administered Compound Short-Term Stability Against All Analytes Measured by the Assay and Other Compounds Users of Tobacco Products are Exposed To: 26 hours in clear polypropylene tubes at ambient temperature under UV-shielded light Co-administered Compound Cumulative Short-Term Stability Against All Analytes Measured by the Assay and Other Compounds Users of Tobacco Products are Exposed To: 52 hours (low concentration) and 53 hours (high concentration) in clear polypropylene tubes at ambient temperature under UV-shielded light (total of all thaw cycles)
Batch Size	120 injections

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