Appendix A6.1.3.35 Validation Summary ZZ33881-04 (Nicotine in Urine, Cotinine in Urine, trans-3′-Hydroxycotinine in Urine, Nicotine-N-glucuronide in Urine, Cotinine-N-glucuronide in Urine, and trans-3′-Hydroxycotinine-O-glucuronide in Urine)

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

Table 1. Validation Summary – Nicotine in Urine - ZZ33881-04

Information Requested	Data
Validation Summary	(b) (4) Validation Study ZZ33881-04
Analyte	Nicotine
Internal Standard (IS)	d_4-Nicotine
Method Description	Combined liquid-liquid and solid phase extraction with analysis/detection by LC-MS/MS
Limit of Quantitation (ng/mL)	50.0 ng/mL
Average Recovery of Drug (% Mean)	100% at 150 ng/mL 106% at 500 ng/mL 101% at 3750 ng/mL
Average Recovery of IS (% Mean)	113%
Standard Curve Concentrations (ng/mL)	50.0, 100, 200, 400, 800, 1500, 4000, and 5000 ng/mL
QC Concentrations (ng/mL)	LLOQ QC, 150, 500, and 3750 ng/mL
QC Intra-Batch Precision Range (% CV)	1.1 to 9.9%
QC Intra-Batch Accuracy Range (% Bias)	-13.3 to 13.0%
QC Inter-Batch Precision Range (% CV)	5.1 to 9.4%
QC Inter-Batch Accuracy Range (% Bias)	-2.2 to 1.2%
Bench-Top Stability (Hrs)	Short-Term Stability: 25 hours in polypropylene tubes at ambient temperature under white light Cumulative Short-Term Stability: 57 hours in polypropylene tubes at ambient temperature under white light (total of all thaw cycles)
Stock Stability (Days)	Long-Term Stability for Stock Solutions (Stock): N/AP
Processed Stability (Hrs)	Post-Preparative Stability: 229 hours in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 183 hours in a polypropylene 96 well plate at 5°C
Freeze-Thaw Stability (Cycles)	6 freeze (-20°C)-thaw (ambient temperature) cycles in polypropylene tubes under white light
Long-Term Storage Stability (Days)	Long-Term Stability: 476 days in polypropylene tubes at -20°C
Dilution Integrity	Up to 10,000 ng/mL, diluted 10-fold
Selectivity	No significant interference at the retention time and mass transition of nicotine was observed from

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	endogenous components in any of the 10 human urine lots screened or of d_4-nicotine (IS) in any of the 10 human urine lots screened

Additional Information – Nicotine in Urine - ZZ33881-04

Matrix		Human Urine	
Bioanalytical Method (BAM) SOP Number		BAM SOP ZZ33881-04	
Detector		AB SCIEX API 4000™	
Assay Volume Required		0.250 mL	
Regression Type		Weighted linear (1/concentration^2)	
Quantitation Method		Peak area ratio	
Multiple Analytes Measured by Assay		Nicotine at 150 ng/mL fortified with cotinine at 5000 ng/mL, *trans*-3′-hydroxycotinine at 5000 ng/mL, nicotine-*N*-glucuronide at 5000 ng/mL, cotinine-*N*-glucuronide at 20,000 ng/mL, and *trans*-3′-hydroxycotinine-*O*-glucuronide at 20,000 ng/mL	
Selectivity from Metabolites Not Quantified by the Method		Nicotine Oxide (500 ng/mL) Cotinine Oxide (500 ng/mL) Nornicotine (500 ng/mL) Norcotinine (500 ng/mL)	
Additional Selectivity Compound		Acetaminophen (300 µg/mL)	
Quality Control Samples		Precision (% CV)	Accuracy (% Bias)
Inter-Batch	**LLOQ**	9.4	-2.2
	Low	5.9	0.7
	Medium	5.1	1.2
	High	7.1	-0.8
Intra-Batch (Batch 11) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	6.1	13.0
	Low	4.4	5.3
	Medium	2.0	5.4
	High	1.5	3.5
Intra-Batch (Batch 15) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	2.9	-6.6
	Low	4.9	5.3
	Medium	2.0	3.8
	High	2.4	3.5
Intra-Batch (Batch 26) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	8.7	-0.8
	Low	5.4	2.0
	Medium	3.8	-1.8
	High	2.4	-13.3
Intra-Batch (Batch 28) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	9.9	-5.6
	Low	5.3	-1.3
	Medium	5.8	2.6
	High	5.2	4.3
Intra-Batch (Batch 33) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	5.2	-2.6
	Low	4.9	0.7
	Medium	2.7	2.6
	High	3.7	1.9

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Additional Information – Nicotine in Urine - ZZ33881-04			
Intra-Batch (Batch 35) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ** **Low** **Medium** **High**	6.2 3.1 4.7 4.1	-5.6 -0.7 3.0 2.1
Intra-Batch (Batch 37) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ** **Low** **Medium** **High**	7.5 1.1 2.5 2.4	-7.2 -8.0 -6.8 -7.5
Matrix Effect		No significant matrix effect was observed in 9 of the 10 human urine lots that were fortified with nicotine at the concentration of the LLOQ (50.0 ng/mL) or in any of the 10 human urine lots that were fortified with nicotine at the concentration of the high QC (3750 ng/mL) samples	
Sample Shipping Stability		18 days in polypropylene tubes at -80°C	
Long-Term Stability for Stock Solutions (Substock)		40 days at 100 µg/mL in water in polypropylene tubes at -20°C	
Long-Term Stability for Stock Solutions (Internal Standard Stock)		Refer to long-term stability for stock solutions data collected from unlabeled nicotine for labeled internal standard stability	
Long-Term Stability for Stock Solutions (Internal Standard Substock)		41 days at 10.0 µg/mL in water in polypropylene tubes at -20°C	
Short-Term Stability for Stock Solutions (Stock)		6 hours at approximately 1000 µg/mL in water in polypropylene tubes at ambient temperature under UV-shielded light	
Short-Term Stability for Stock Solutions (Substock)		6 hours at 100 µg/mL in water in polypropylene tubes 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 nicotine for labeled internal standard stability	
Short-Term Stability for Stock Solutions (Internal Standard Substock)		6 hours at 10.0 µg/mL in water in polypropylene tubes at ambient temperature under UV-shielded light 6 hours at 200 ng/mL in water in polypropylene tubes at ambient temperature under UV-shielded light	
Stability of Analyte During Sample Collection and Handling		45 minutes in non-colored high-density polyethylene containers at ambient temperature under white light, 4 hours in amber high-density polyethylene containers at ambient temperature under white light, 2 days in amber high-density polyethylene containers at 5°C in a refrigerator 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	
Sample Aliquot Frozen Storage Stability		Samples aliquoted manually at a volume of 0.250 mL, stored for 428 hours in a polypropylene 96 well plate at -20°C prior to extraction	

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Additional Information – Nicotine in Urine - ZZ33881-04	
Batch Size	96 injections

Table 2. Validation Summary – Cotinine in Urine - ZZ33881-04

Information Requested	Data
Validation Summary	(b) (4) Validation Study ZZ33881-04
Analyte	Cotinine
Internal Standard (IS)	d_3-Cotinine
Method Description	Combined liquid-liquid and solid phase extraction with analysis/detection by LC-MS/MS
Limit of Quantitation (ng/mL)	50.0 ng/mL
Average Recovery of Drug (% Mean)	120% at 150 ng/mL 114% at 500 ng/mL 109% at 3750 ng/mL
Average Recovery of IS (% Mean)	117%
Standard Curve Concentrations (ng/mL)	50.0, 100, 200, 400, 800, 1500, 4000, and 5000 ng/mL
QC Concentrations (ng/mL)	LLOQ QC, 150, 500, and 3750 ng/mL
QC Intra-Batch Precision Range (% CV)	1.8 to 5.8%
QC Intra-Batch Accuracy Range (% Bias)	-10.4 to 12.8%
QC Inter-Batch Precision Range (% CV)	4.3 to 8.4%
QC Inter-Batch Accuracy Range (% Bias)	-2.4 to 1.8%
Bench-Top Stability (Hrs)	Short-Term Stability: 25 hours in polypropylene tubes at ambient temperature under white light Cumulative Short-Term Stability: 57 hours in polypropylene tubes at ambient temperature under white light (total of all thaw cycles)
Stock Stability (Days)	Long-Term Stability for Stock Solutions (Stock): N/AP
Processed Stability (Hrs)	Post-Preparative Stability: 229 hours in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 79 hours in a polypropylene 96 well plate at 5°C
Freeze-Thaw Stability (Cycles)	6 freeze (-20°C)-thaw (ambient temperature) cycles in polypropylene tubes under white light
Long-Term Storage Stability (Days)	Long-Term Stability: 476 days in polypropylene tubes at -20°C
Dilution Integrity	Up to 10,500 ng/mL, diluted 10-fold
Selectivity	No significant interference at the retention time and mass transition of cotinine was observed from endogenous components in any of the 10 human urine lots screened or of d_3-cotinine (IS) in any of the 10 human urine lots screened

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Additional Information – Cotinine in Urine - ZZ33881-04

Matrix		Human Urine	
Bioanalytical Method (BAM) SOP Number		BAM SOP ZZ33881-04	
Detector		AB SCIEX API 4000™	
Assay Volume Required		0.250 mL	
Regression Type		Weighted linear (1/concentration^2)	
Quantitation Method		Peak area ratio	
Multiple Analytes Measured by Assay		Cotinine at 150 ng/mL fortified with nicotine at 5000 ng/mL, *trans*-3′-hydroxycotinine at 5000 ng/mL, nicotine-*N*-glucuronide at 5000 ng/mL, cotinine-*N*-glucuronide at 20,000 ng/mL, and *trans*-3′-hydroxycotinine-*O*-glucuronide at 20,000 ng/mL	
Selectivity from Metabolites Not Quantified by the Method		Nicotine Oxide (500 ng/mL) Cotinine Oxide (500 ng/mL) Nornicotine (500 ng/mL) Norcotinine (500 ng/mL)	
Additional Selectivity Compound		Acetaminophen (300 µg/mL)	
Quality Control Samples		Precision (% CV)	Accuracy (% Bias)
Inter-Batch	**LLOQ**	8.4	0.8
	Low	4.3	0.0
	Medium	4.6	1.8
	High	6.7	-2.4
Intra-Batch (Batch 26) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	5.0	12.8
	Low	5.1	1.3
	Medium	3.6	3.2
	High	3.2	-10.4
Intra-Batch (Batch 33) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	5.8	-2.4
	Low	4.2	2.7
	Medium	5.6	4.8
	High	4.1	4.8
Intra-Batch (Batch 35) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	4.9	-3.6
	Low	2.3	0.0
	Medium	1.9	1.6
	High	5.1	-1.3
Intra-Batch (Batch 37) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	3.0	-3.8
	Low	2.0	-4.0
	Medium	3.5	-2.8
	High	1.8	-2.9
Matrix Effect		No significant matrix effect was observed in any of the 10 human urine lots that were fortified with cotinine at the concentration of the LLOQ (50.0 ng/mL) or in any of the 10 human urine lots that were fortified with cotinine at the concentration of the high QC (3750 ng/mL) samples	
Sample Shipping Stability		18 days in polypropylene tubes at -80°C	
Long-Term Stability for Stock Solutions		40 days at 100 µg/mL in water in polypropylene tubes at	

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Additional Information – Cotinine in Urine - ZZ33881-04	
(Substock)	-20°C
Long-Term Stability for Stock Solutions (Internal Standard Stock)	refer to long-term stability for stock solutions data collected from unlabeled cotinine for labeled internal standard stability
Long-Term Stability for Stock Solutions (Internal Standard Substock)	41 days at 3.75 µg/mL in water in polypropylene tubes at -20°C
Short-Term Stability for Stock Solutions (Stock)	6 hours at approximately 1000 µg/mL in water in polypropylene tubes at ambient temperature under UV-shielded light
Short-Term Stability for Stock Solutions (Substock)	6 hours at 100 µg/mL in water in polypropylene tubes 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 cotinine for labeled internal standard stability
Short-Term Stability for Stock Solutions (Internal Standard Substock)	6 hours at 3.75 µg/mL in water in polypropylene tubes at ambient temperature under UV-shielded light 6 hours at 75.0 ng/mL in water in polypropylene tubes at ambient temperature under UV-shielded light
Stability of Analyte During Sample Collection and Handling	45 minutes in non-colored high-density polyethylene containers at ambient temperature under white light, 4 hours in amber high-density polyethylene containers at ambient temperature under white light, 2 days in amber high-density polyethylene containers at 5°C in a refrigerator 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
Sample Aliquot Frozen Storage Stability	Samples aliquoted manually at a volume of 0.250 mL, stored for 428 hours in a polypropylene 96 well plate at -20°C prior to extraction
Batch Size	96 injections

Table 3 Validation Summary – trans-3′-Hydroxycotinine in Urine - ZZ33881-04

Information Requested	Data
Validation Summary	(b) (4) Validation Study ZZ33881-04
Analyte	*trans*-3′-Hydroxycotinine
Internal Standard (IS)	d_3-*trans*-3′-Hydroxycotinine
Method Description	Combined liquid-liquid and solid phase extraction with analysis/detection by LC-MS/MS
Limit of Quantitation (ng/mL)	50.0 ng/mL
Average Recovery of Drug (% Mean)	49% at 150 ng/mL 53% at500 ng/mL 53% at 3750 ng/mL

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Average Recovery of IS (% Mean)	53%
Standard Curve Concentrations (ng/mL)	50.0, 100, 200, 400, 800, 1500, 4000, and 5000 ng/mL
QC Concentrations (ng/mL)	LLOQ QC, 150, 500, and 3750 ng/mL
QC Intra-Batch Precision Range (% CV)	1.3 to 7.5%
QC Intra-Batch Accuracy Range (% Bias)	-12.3 to 5.4%
QC Inter-Batch Precision Range (% CV)	3.2 to 6.9%
QC Inter-Batch Accuracy Range (% Bias)	-2.4 to 0.0%
Bench-Top Stability (Hrs)	Short-Term Stability: 25 hours in polypropylene tubes at ambient temperature under white light Cumulative Short-Term Stability: 57 hours in polypropylene tubes at ambient temperature under white light (total of all thaw cycles)
Stock Stability (Days)	Long-Term Stability for Stock Solutions (Stock): 41 days at approximately 1000 µg/mL in water in polypropylene tubes at -20°C
Processed Stability (Hrs)	Post-Preparative Stability: 229 hours in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 183 hours in a polypropylene 96 well plate at 5°C
Freeze-Thaw Stability (Cycles)	6 freeze (-20°C)-thaw (ambient temperature) cycles in polypropylene tubes under white light
Long-Term Storage Stability (Days)	Long-Term Stability: 476 days in polypropylene tubes at -20°C
Dilution Integrity	Up to 10,000 ng/mL, diluted 10-fold
Selectivity	No significant interference at the retention time and mass transition of *trans*-3′-hydroxycotinine was observed from endogenous components in any of the 10 human urine lots screened or of d_3-*trans*-3′-hydroxycotinine (IS) in any of the 10 human urine lots screened

Additional Information – *trans*-3′-Hydroxycotinine in Urine - ZZ33881-04	
Matrix	Human Urine
Bioanalytical Method (BAM) SOP Number	BAM SOP ZZ33881-04
Detector	AB SCIEX API 4000™
Assay Volume Required	0.250 mL
Regression Type	Weighted linear (1/concentration^2)
Quantitation Method	Peak area ratio
Multiple Analytes Measured by Assay	*trans*-3′-Hydroxycotinine at 150 ng/mL fortified with nicotine at 5000 ng/mL, cotinine at 5000 ng/mL, nicotine-*N*-glucuronide at 5000 ng/mL, cotinine-*N*-glucuronide at 20,000 ng/mL, and *trans*-3′-hydroxycotinine-*O*-glucuronide at 20,000 ng/mL
Selectivity from Metabolites Not Quantified by the Method	Nicotine Oxide (500 ng/mL) Cotinine Oxide (500 ng/mL)

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Additional Information – *trans*-3′-Hydroxycotinine in Urine - ZZ33881-04			
		Nornicotine (500 ng/mL) Norcotinine (500 ng/mL)	
Additional Selectivity Compounds		Acetaminophen (300 µg/mL)	
Quality Control Samples		Precision (% CV)	Accuracy (% Bias)
Inter-Batch	**LLOQ**	6.9	-0.2
	Low	3.9	-1.3
	Medium	3.2	0.0
	High	5.1	-2.4
Intra-Batch (Batch 11) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	3.6	4.0
	Low	3.1	-0.7
	Medium	1.5	1.6
	High	1.3	-1.1
Intra-Batch (Batch 15) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	7.2	3.4
	Low	2.5	2.0
	Medium	3.0	1.8
	High	1.9	0.5
Intra-Batch (Batch 26) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	5.4	-0.8
	Low	5.1	-2.7
	Medium	4.4	-1.4
	High	4.3	-12.3
Intra-Batch (Batch 28) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	5.3	-5.4
	Low	2.8	-6.0
	Medium	4.0	-2.0
	High	2.1	-4.8
Intra-Batch (Batch 33) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	6.6	-2.0
	Low	2.5	-3.3
	Medium	1.6	-1.4
	High	2.0	-1.6
Intra-Batch (Batch 35) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	7.5	-5.6
	Low	2.0	0.7
	Medium	4.0	1.0
	High	2.0	1.9
Intra-Batch (Batch 37) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	4.9	5.4
	Low	1.9	1.3
	Medium	1.7	0.0
	High	1.8	0.0
Matrix Effect		No significant matrix effect was observed in any of the 10 human urine lots that were fortified with *trans*-3′-hydroxycotinine at the concentration of the LLOQ (50.0 ng/mL) or in any of the 10 human urine lots that were fortified with *trans*-3′-hydroxycotinine at the concentration of the high QC (3750 ng/mL) samples	
Sample Shipping Stability		18 days in polypropylene tubes at -80°C	
Long-Term Stability for Stock Solutions (Substock)		40 days at 100 µg/mL in water in polypropylene tubes at -20°C	
Long-Term Stability for Stock Solutions (Internal Standard Stock)		Refer to long-term stability for stock solutions data collected from unlabeled *trans*-3′-hydroxycotinine for labeled internal standard stability	

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Additional Information – *trans*-3′-Hydroxycotinine in Urine - ZZ33881-04	
Long-Term Stability for Stock Solutions (Internal Standard Substock)	41 days at 20.0 µg/mL in water in polypropylene tubes at -20°C
Short-Term Stability for Stock Solutions (Stock)	6 hours at approximately 1000 µg/mL in water in polypropylene tubes at ambient temperature under UV-shielded light
Short-Term Stability for Stock Solutions (Substock)	6 hours at 100 µg/mL in water in polypropylene tubes 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 *trans*-3′-hydroxycotinine for labeled internal standard stability
Short-Term Stability for Stock Solutions (Internal Standard Substock)	6 hours at 20.0 µg/mL in water in polypropylene tubes at ambient temperature under UV-shielded light 6 hours at 400 ng/mL in water in polypropylene tubes at ambient temperature under UV-shielded light
Stability of Analyte During Sample Collection and Handling	45 minutes in non-colored high-density polyethylene containers at ambient temperature under white light, 4 hours in amber high-density polyethylene containers at ambient temperature under white light, 2 days in amber high-density polyethylene containers at 5°C in a refrigerator 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
Sample Aliquot Frozen Storage Stability	Samples aliquoted manually at a volume of 0.250 mL, stored for 428 hours in a polypropylene 96 well plate at -20°C prior to extraction
Batch Size	96 injections

Table 4 Validation Summary – Nicotine-N-glucuronide in Urine - ZZ33881-04

Information Requested	Data
Validation Summary	(b) (4) Validation Study ZZ33881-04
Analyte	Nicotine-*N*-Glucuronide
Internal Standard (IS)	d_3-Nicotine-*N*-Glucuronide
Method Description	Combined liquid-liquid and solid phase extraction with analysis/detection by LC-MS/MS
Limit of Quantitation (ng/mL)	50.0 ng/mL
Average Recovery of Drug (% Mean)	48% at 150 ng/mL 51% at 500 ng/mL 51% at 3750 ng/mL
Average Recovery of IS (% Mean)	49%
Standard Curve Concentrations (ng/mL)	50.0, 100, 200, 400, 800, 1500, 4000, and 5000 ng/mL
QC Concentrations (ng/mL)	LLOQ QC, 150, 500, and 3750 ng/mL
QC Intra-Batch Precision Range (% CV)	1.0 to 7.6%
QC Intra-Batch Accuracy Range (% Bias)	-3.6 to 3.8%

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QC Inter-Batch Precision Range (% CV)	3.3 to 5.8%
QC Inter-Batch Accuracy Range (% Bias)	-1.9 to 1.4%
Bench-Top Stability (Hrs)	Short-Term Stability: 25 hours in polypropylene tubes at ambient temperature under white light Cumulative Short-Term Stability: 57 hours in polypropylene tubes at ambient temperature under white light (total of all thaw cycles)
Stock Stability (Days)	Long-Term Stability for Stock Solutions (Stock): 503 days at approximately 1000 µg/mL in water in polypropylene tubes at -20°C
Processed Stability (Hrs)	Post-Preparative Stability: 302 hours in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 185 hours in a polypropylene 96 well plate at 5°C
Freeze-Thaw Stability (Cycles)	6 freeze (-20°C)-thaw (ambient temperature) cycles in polypropylene tubes under white light
Long-Term Storage Stability (Days)	Long-Term Stability: 476 days in polypropylene tubes at -20°C
Dilution Integrity	Up to 10,000 ng/mL, diluted 10-fold
Selectivity	No significant interference at the retention time and mass transition of nicotine-*N*-glucuronide was observed from endogenous components in any of the 10 human urine lots screened or of d_3-nicotine-*N*-glucuronide (IS) in any of the 10 human urine lots screened

Additional Information – Nicotine-N-glucuronide in Urine - ZZ33881-04

Matrix		Human Urine	
Bioanalytical Method (BAM) SOP Number		BAM SOP ZZ33881-04	
Detector		AB SCIEX API 4000™	
Assay Volume Required		0.250 mL	
Regression Type		Weighted linear (1/concentration^2)	
Quantitation Method		Peak area ratio	
Multiple Analytes Measured by Assay		Nicotine-*N*-Glucuronide at 150 ng/mL fortified with nicotine at 5000 ng/mL, cotinine at 5000 ng/mL, *trans*-3′-hydroxycotinine at 5000 ng/mL, cotinine-*N*-glucuronide at 20,000 ng/mL, and *trans*-3′-hydroxycotinine-*O*-glucuronide at 20,000 ng/mL	
Selectivity from Metabolites Not Quantified by the Method		Nicotine Oxide (500 ng/mL) Cotinine Oxide (500 ng/mL) Nornicotine (500 ng/mL) Norcotinine (500 ng/mL)	
Additional Selectivity Compounds		Acetaminophen (300 µg/mL)	
Quality Control Samples		Precision (% CV)	Accuracy (% Bias)
Inter-Batch	**LLOQ**	5.8	1.4
	Low	4.0	0.7

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Additional Information – Nicotine-N-glucuronide in Urine - ZZ33881-04			
	Medium	3.3	1.2
	High	3.3	-1.9
Intra-Batch (Batch 12)	**LLOQ**	3.7	1.6
Aliquot Method: Manual	**Low**	2.1	0.7
Extraction Method:	**Medium**	1.4	3.0
Automated	**High**	2.0	-2.7
Intra-Batch (Batch 16)	**LLOQ**	6.2	1.4
Aliquot Method: Manual	**Low**	3.5	0.0
Extraction Method:	**Medium**	2.5	3.0
Automated	**High**	6.1	-3.5
Intra-Batch (Batch 29)	**LLOQ**	5.4	-1.2
Aliquot Method: Manual	**Low**	4.1	1.3
Extraction Method:	**Medium**	1.9	2.2
Automated	**High**	1.0	0.0
Intra-Batch (Batch 38)	**LLOQ**	7.6	3.8
Aliquot Method: Manual	**Low**	6.0	2.0
Extraction Method:	**Medium**	2.1	-3.6
Automated	**High**	1.0	-1.9
Matrix Effect		No significant matrix effect was observed in any of the 10 human urine lots that were fortified with nicotine-*N*-glucuronide at the concentration of the LLOQ (50.0 ng/mL) or in any of the 10 human urine lots that were fortified with nicotine-*N*-glucuronide at the concentration of the high QC (3750 ng/mL) samples	
Sample Shipping Stability		18 days in polypropylene tubes at -80°C	
Long-Term Stability for Stock Solutions (Substock)		34 days at 50.0 µg/mL in water in polypropylene tubes at -20°C	
Long-Term Stability for Stock Solutions (Internal Standard Stock)		Refer to long-term stability for stock solutions data collected from unlabeled nicotine-*N*-glucuronide for labeled internal standard stability	
Long-Term Stability for Stock Solutions (Internal Standard Substock)		41 days at 125 µg/mL in water in polypropylene tubes at -20°C	
Short-Term Stability for Stock Solutions (Stock)		6 hours at approximately 1000 µg/mL in water in polypropylene tubes at ambient temperature under UV-shielded light	
Short-Term Stability for Stock Solutions (Substock)		6 hours at 50.0 µg/mL in water in polypropylene tubes 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 nicotine-*N*-glucuronide for labeled internal standard stability	
Short-Term Stability for Stock Solutions (Internal Standard Substock)		6 hours at 125 µg/mL in water in polypropylene tubes at ambient temperature under UV-shielded light 6 hours at 2500 ng/mL in water in polypropylene tubes at ambient temperature under UV-shielded light	
Stability of Analyte During Sample Collection and Handling		45 minutes in non-colored high-density polyethylene containers at ambient temperature under white light, 4 hours in amber high-density polyethylene containers at	

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Additional Information – Nicotine-N-glucuronide in Urine - ZZ33881-04	
	ambient temperature under white light, 2 days in amber high-density polyethylene containers at 5°C in a refrigerator 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
Sample Aliquot Frozen Storage Stability	Samples aliquoted manually at a volume of 0.250 mL, stored for 428 hours in a polypropylene 96 well plate at -20°C prior to extraction
Batch Size	96 injections

Table 5. Validation Summary – Cotinine-N-Glucuronide in Urine - ZZ33881-04

Information Requested	Data
Validation Summary	(b) (4) Validation Study ZZ33881-04
Analyte	Cotinine-*N*-Glucuronide
Internal Standard (IS)	d_3-Cotinine-*N*-Glucuronide
Method Description	Combined liquid-liquid and solid phase extraction with analysis/detection by LC-MS/MS
Limit of Quantitation (ng/mL)	200 ng/mL
Average Recovery of Drug (% Mean)	44% at 600 ng/mL 49% at 2000 ng/mL 52% at 15,000 ng/mL
Average Recovery of IS (% Mean)	53%
Standard Curve Concentrations (ng/mL)	200, 400, 800, 1600, 3200, 6000, 16,000, and 20,000 ng/mL
QC Concentrations (ng/mL)	LLOQ QC, 600, 2000, and 15,000 ng/mL
QC Intra-Batch Precision Range (% CV)	1.2 to 9.8%
QC Intra-Batch Accuracy Range (% Bias)	-11.5 to 3.7%
QC Inter-Batch Precision Range (% CV)	4.0 to 8.1%
QC Inter-Batch Accuracy Range (% Bias)	-4.0 to 1.2%
Bench-Top Stability (Hrs)	Short-Term Stability: 25 hours in polypropylene tubes at ambient temperature under white light Cumulative Short-Term Stability: 57 hours in polypropylene tubes at ambient temperature under white light (total of all thaw cycles)
Stock Stability (Days)	Long-Term Stability for Stock Solutions (Stock): 318 days at approximately 2000 µg/mL in water in a polypropylene container at -20°C
Processed Stability (Hrs)	Post-Preparative Stability: 302 hours in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 185 hours in a polypropylene 96 well plate at 5°C
Freeze-Thaw Stability (Cycles)	6 freeze (-20°C)-thaw (ambient temperature) cycles in

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	polypropylene tubes under white light
Long-Term Storage Stability (Days)	Long-Term Stability: 476 days in polypropylene tubes at -20°C
Dilution Integrity	Up to 40,000 ng/mL, diluted 10-fold
Selectivity	No significant interference at the retention time and mass transition of cotinine-*N*-glucuronide was observed from endogenous components in any of the 10 human urine lots screened or of d_3-cotinine-*N*-glucuronide (IS) in any of the 10 human urine lots screened

Additional Information – Cotinine-N-Glucuronide in Urine - ZZ33881-04

Matrix		Human Urine	
Bioanalytical Method (BAM) SOP Number		BAM SOP ZZ33881-04	
Detector		AB SCIEX API 4000™	
Assay Volume Required		0.250 mL	
Regression Type		Weighted linear (1/concentration^2)	
Quantitation Method		Peak area ratio	
Multiple Analytes Measured by Assay		Cotinine-*N*-Glucuronide at 600 ng/mL fortified with nicotine at 5000 ng/mL, cotinine at 5000 ng/mL, *trans*-3′-hydroxycotinine at 5000 ng/mL, nicotine-*N*-glucuronide at 5000 ng/mL, and *trans*-3′-hydroxycotinine-*O*-glucuronide at 20,000 ng/mL	
Selectivity from Metabolites Not Quantified by the Method		Nicotine Oxide (500 ng/mL) Cotinine Oxide (500 ng/mL) Nornicotine (500 ng/mL) Norcotinine (500 ng/mL)	
Additional Selectivity Compounds		Acetaminophen (300 µg/mL)	
Quality Control Samples		Precision (% CV)	Accuracy (% Bias)
Inter-Batch	**LLOQ**	8.1	-4.0
	Low	7.1	1.2
	Medium	4.0	-2.5
	High	4.3	-3.3
Intra-Batch (Batch 12) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	9.8	-2.5
	Low	7.5	3.5
	Medium	4.2	-5.5
	High	4.5	-4.0
Intra-Batch (Batch 16) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	3.4	-5.5
	Low	8.9	3.7
	Medium	4.9	-2.5
	High	5.8	-5.3
Intra-Batch (Batch 29) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	3.1	-11.5
	Low	4.4	-3.5
	Medium	2.2	0.0
	High	1.2	0.7
Intra-Batch (Batch 38) **Aliquot Method: Manual**	**LLOQ**	5.7	3.0
	Low	5.8	0.8

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Additional Information – Cotinine-N-Glucuronide in Urine - ZZ33881-04			
Extraction Method: Automated	**Medium**	2.4	-0.5
	High	2.2	-3.3
Matrix Effect		No significant matrix effect was observed in any of the 10 human urine lots that were fortified with cotinine-*N*-glucuronide at the concentration of the LLOQ (200 ng/mL) or in any of the 10 human urine lots that were fortified with cotinine-*N*-glucuronide at the concentration of the high QC (15,000 ng/mL) samples	
Sample Shipping Stability		18 days in polypropylene tubes at -80°C	
Long-Term Stability for Stock Solutions (Substock)		41 days at 50.0 µg/mL in water in polypropylene tubes at -20°C	
Long-Term Stability for Stock Solutions (Internal Standard)		Refer to long-term stability for stock solutions data collected from unlabeled cotinine-*N*-glucuronide for labeled internal standard stability	
Short-Term Stability for Stock Solutions (Stock)		6 hours at approximately 2000 µg/mL in water in a polypropylene container at ambient temperature under white light	
Short-Term Stability for Stock Solutions (Substock)		6 hours at 100 µg/mL in water in a polypropylene container at ambient temperature under white light	
Short-Term Stability for Stock Solutions (Internal Standard Stock)		Refer to short-term stability for stock solutions data collected from unlabeled cotinine-*N*-glucuronide for labeled internal standard stability	
Short-Term Stability for Stock Solutions (Internal Standard Substock)		6 hours at 50.0 µg/mL in water in polypropylene tubes at ambient temperature under UV-shielded light 6 hours at 1000 ng/mL in water in polypropylene tubes at ambient temperature under UV-shielded light	
Stability of Analyte During Sample Collection and Handling		45 minutes in non-colored high-density polyethylene containers at ambient temperature under white light, 4 hours in amber high-density polyethylene containers at ambient temperature under white light, 2 days in amber high-density polyethylene containers at 5°C in a refrigerator 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	
Sample Aliquot Frozen Storage Stability		Samples aliquoted manually at a volume of 0.250 mL, stored for 428 hours in a polypropylene 96 well plate at -20°C prior to extraction	
Batch Size		96 injections	

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Table 6. Validation Summary – trans-3′-Hydroxycotinine-O-Glucuronide in Urine - ZZ33881-04

Information Requested	Data
Validation Summary	(b) (4) Validation Study ZZ33881-04
Analyte	*trans*-3′-Hydroxycotinine-*O*-Glucuronide
Internal Standard (IS)	d_3-*trans*-3′-Hydroxycotinine-*O*-Glucuronide
Method Description	Combined liquid-liquid and solid phase extraction with analysis/detection by LC-MS/MS
Limit of Quantitation (ng/mL)	200 ng/mL
Average Recovery of Drug (% Mean)	39% at 600 ng/mL 45% at 2000 ng/mL 46% at 15,000 ng/mL
Average Recovery of IS (% Mean)	40%
Standard Curve Concentrations (ng/mL)	200, 400, 800, 1600, 3200, 6000, 16,000, and 20,000 ng/mL
QC Concentrations (ng/mL)	LLOQ QC, 600, 2000, and 15,000 ng/mL
QC Intra-Batch Precision Range (% CV)	1.3 to 8.3%
QC Intra-Batch Accuracy Range (% Bias)	-2.5 to 3.0%
QC Inter-Batch Precision Range (% CV)	2.4 to 5.3%
QC Inter-Batch Accuracy Range (% Bias)	0.0 to 1.0%
Bench-Top Stability (Hrs)	Short-Term Stability: 25 hours in polypropylene tubes at ambient temperature under white light Cumulative Short-Term Stability: 57 hours in polypropylene tubes at ambient temperature under white light (total of all thaw cycles)
Stock Stability (Days)	Long-Term Stability for Stock Solutions (Stock): N/AV
Processed Stability (Hrs)	Post-Preparative Stability: 302 hours in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 185 hours in a polypropylene 96 well plate at 5°C
Freeze-Thaw Stability (Cycles)	6 freeze (-20°C)-thaw (ambient temperature) cycles in polypropylene tubes under white light
Long-Term Storage Stability (Days)	Long-Term Stability: 476 days in polypropylene tubes at -20°C
Dilution Integrity	Up to 40,000 ng/mL, diluted 10-fold
Selectivity	No significant interference at the retention time and mass transition of *trans*-3′-hydroxycotinine-*O*-glucuronide was observed from endogenous components in any of the 10 human urine lots screened or of d_3-*trans*-3′-hydroxycotinine-*O*-glucuronide (IS) in any of the 10 human urine lots screened

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Additional Information – *trans*-3′-Hydroxycotinine-O-Glucuronide in Urine - ZZ33881-04

Matrix		Human Urine	
Bioanalytical Method (BAM) SOP Number		BAM SOP ZZ33881-04	
Detector		AB SCIEX API 4000™	
Assay Volume Required		0.250 mL	
Regression Type		Weighted linear (1/concentration^2)	
Quantitation Method		Peak area ratio	
Multiple Analytes Measured by Assay		*trans*-3′-Hydroxycotinine-*O*-Glucuronide at 600 ng/mL fortified with nicotine at 5000 ng/mL, cotinine at 5000 ng/mL, *trans*-3′-hydroxycotinine at 5000 ng/mL, nicotine-*N*-glucuronide at 5000 ng/mL, and cotinine-*N*-glucuronide at 20,000 ng/mL	
Selectivity from Metabolites Not Quantified by the Method		Nicotine Oxide (500 ng/mL) Cotinine Oxide (500 ng/mL) Nornicotine (500 ng/mL) Norcotinine (500 ng/mL)	
Additional Selectivity Compounds		Acetaminophen (300 µg/mL)	
Quality Control Samples		Precision (% CV)	Accuracy (% Bias)
Inter-Batch	**LLOQ**	5.3	0.0
	Low	3.0	0.8
	Medium	3.4	1.0
	High	2.4	0.7
Intra-Batch (Batch 12) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	4.5	1.5
	Low	2.6	0.2
	Medium	4.0	4.0
	High	1.4	0.7
Intra-Batch (Batch 16) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	4.6	-2.0
	Low	2.9	0.0
	Medium	1.3	3.0
	High	2.5	2.0
Intra-Batch (Batch 29) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	2.9	0.5
	Low	1.6	0.5
	Medium	1.7	0.5
	High	1.6	1.3
Intra-Batch (Batch 38) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	8.3	-0.5
	Low	4.2	2.8
	Medium	1.9	-2.5
	High	3.2	-0.7
Matrix Effect		No significant matrix effect was observed in any of the 10 human urine lots that were fortified with *trans*-3′-hydroxycotinine-*O*-glucuronide at the concentration of the LLOQ (200 ng/mL) or in any of the 10 human urine lots that were fortified with *trans*-3′-hydroxycotinine-*O*-glucuronide at the concentration of the high QC (15,000 ng/mL) samples	

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Additional Information – *trans*-3′-Hydroxycotinine-O-Glucuronide in Urine - ZZ33881-04	
Sample Shipping Stability	18 days in polypropylene tubes at -80°C
Long-Term Stability for Stock Solutions (Substock)	34 days at 250 µg/mL in water in polypropylene tubes at -20°C
Long-Term Stability for Stock Solutions (Internal Standard Stock)	Refer to long-term stability for stock solutions data collected from unlabeled *trans*-3′-hydroxycotinine-*O*-glucuronide for labeled internal standard stability
Long-Term Stability for Stock Solutions (Internal Standard Substock)	41 days at 27.6 µg/mL in water in polypropylene tubes at -20°C
Short-Term Stability for Stock Solutions (Stock)	N/AV
Short-Term Stability for Stock Solutions (Substock)	6 hours at 250 µg/mL in water in polypropylene tubes 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 *trans*-3′-hydroxycotinine-*O*-glucuronide for labeled internal standard stability
Short-Term Stability for Stock Solutions (Internal Standard Substock)	6 hours at 27.6 µg/mL in water in polypropylene tubes at ambient temperature under UV-shielded light 6 hours at 552 ng/mL in water in polypropylene tubes at ambient temperature under UV-shielded light
Stability of Analyte During Sample Collection and Handling	45 minutes in non-colored high-density polyethylene containers at ambient temperature under white light, 4 hours in amber high-density polyethylene containers at ambient temperature under white light, 2 days in amber high-density polyethylene containers at 5°C in a refrigerator 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
Sample Aliquot Frozen Storage Stability	Samples aliquoted manually at a volume of 0.250 mL, stored for 428 hours in a polypropylene 96 well plate at -20°C prior to extraction
Batch Size	96 injections

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