Appendix A6.1.3.34 Validation Summary ZZ33881-03 (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 - ZZ33881-03

Information Requested	Data
Validation Summary	(b) (4) Validation Study ZZ33881-03
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)	10.0 ng/mL
Average Recovery of Drug (% Mean)	90% at 30.0 ng/mL 101% at 150 ng/mL 97% at 750 ng/mL
Average Recovery of IS (% Mean)	99%
Standard Curve Concentrations (ng/mL)	10.0, 20.0. 50.0, 100, 200, 400, 600, 800, and 1000 ng/mL
QC Concentrations (ng/mL)	LLOQ QC, 30.0, 150, and 750 ng/mL
QC Intra-Batch Precision Range (% CV)	1.8 to 9.3%
QC Intra-Batch Accuracy Range (% Bias)	-7.0 to 2.0%
QC Inter-Batch Precision Range (% CV)	2.5 to 5.9%
QC Inter-Batch Accuracy Range (% Bias)	-3.0 to 0.1%
Bench-Top Stability (Hrs)	Short-Term Stability: 24 hours in polypropylene tubes at ambient temperature under white light Cumulative Short-Term Stability: 54 hours in polypropylene tubes at ambient temperature under white light (total of all thaw cycles)
Stock Stability (Days)	N/AP
Processed Stability (Hrs)	Post-Preparative Stability: 180 hours in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 107 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: 440 days (LLOQ QC) and 476 days (dilution QC) in polypropylene tubes at -20°C
Dilution Integrity	Up to 8000 ng/mL, diluted 20-fold
Selectivity	No significant interference at the retention time and

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	mass transition of nicotine was observed from 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 - ZZ33881-03

Matrix		Human Urine	
Bioanalytical Method (BAM) SOP Number		BAM SOP ZZ33881-03	
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 30.0 ng/mL fortified with cotinine at 1000 ng/mL, *trans*-3′-hydroxycotinine at 1000 ng/mL, nicotine-*N*-glucuronide at 1000 ng/mL, cotinine-*N*-glucuronide at 2000 ng/mL, and *trans*-3′-hydroxycotinine-*O*-glucuronide at 5000 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**	5.3	-3.0
	Low	5.9	-2.3
	Medium	3.3	0.0
	High	2.5	0.1
Intra-Batch (Batch 118) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	5.1	-1.7
	Low	9.3	-2.0
	Medium	1.8	-1.3
	High	2.0	-1.3
Intra-Batch (Batch 125) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	3.7	-7.0
	Low	2.6	-4.3
	Medium	3.4	0.0
	High	3.1	0.5
Intra-Batch (Batch 130) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	4.7	-0.4
	Low	3.4	0.0
	Medium	3.8	2.0
	High	2.0	1.2
Matrix Effect		No significant matrix effect was observed in any of the 10 human urine lots that were fortified with nicotine at the concentration of the LLOQ (10.0 ng/mL) or in any of the 10 human urine lots that were fortified with nicotine at the concentration of the high QC (750 ng/mL) samples	
Sample Shipping Stability		21 days in polypropylene tubes at -80°C	

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Additional Information – Nicotine in Urine - ZZ33881-03	
Long-Term Stability for Stock Solutions (Substock)	40 days at 100 µg/mL in boxed 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 nicotine for labeled internal standard stability
Long-Term Stability for Stock Solutions (Internal Standard Substock)	41 days at 10.0 µg/mL in boxed water in polypropylene tubes at -20°C
Short-Term Stability for Stock Solutions (Stock)	6 hours at approximately 1000 µg/mL in boxed 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 boxed water in polypropylene tubes at ambient temperature under UV-shielded light
Short-Term Stability for Stock Solutions (Internal Standard)	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 boxed water in polypropylene tubes at ambient temperature under UV-shielded light 6 hours at 200 ng/mL in boxed 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
Sample Aliquot Frozen Storage Stability	Samples aliquoted manually at a volume of 0.250 mL, stored for 499 hours in a polypropylene 96 well plate at -20°C prior to extraction
Batch Size	96 injections

Table 2. Validation Summary – Cotinine in Urine - ZZ33881-03

Information Requested	Data
Validation Summary	(b) (4) Validation Study ZZ33881-03
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)	10.0 ng/mL
Average Recovery of Drug (% Mean)	91% at 30.0 ng/mL 100% at 150 ng/mL 97% at 750 ng/mL
Average Recovery of IS (% Mean)	98%
Standard Curve Concentrations (ng/mL)	10.0, 20.0. 50.0, 100, 200, 400, 600, 800, and 1000 ng/mL

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QC Concentrations (ng/mL)	LLOQ QC, 30.0, 150, and 750 ng/mL
QC Intra-Batch Precision Range (% CV)	1.4 to 8.6%
QC Intra-Batch Accuracy Range (% Bias)	-10.6 to 5.3%
QC Inter-Batch Precision Range (% CV)	2.2 to 7.2%
QC Inter-Batch Accuracy Range (% Bias)	-4.3 to 2.0%
Bench-Top Stability (Hrs)	Short-Term Stability: 24 hours in polypropylene tubes at ambient temperature under white light Cumulative Short-Term Stability: 54 hours in polypropylene tubes at ambient temperature under white light (total of all thaw cycles)
Stock Stability (Days)	N/AP
Processed Stability (Hrs)	Post-Preparative Stability: 180 hours in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 107 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: 440 days (LLOQ QC) and 476 days (dilution QC) in polypropylene tubes at -20°C
Dilution Integrity	Up to 8000 ng/mL, diluted 20-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

Additional Information – Cotinine in Urine - ZZ33881-03

Matrix	Human Urine	
Bioanalytical Method (BAM) SOP Number	BAM SOP ZZ33881-03	
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 30.0 ng/mL fortified with nicotine at 1000 ng/mL, *trans*-3′-hydroxycotinine at 1000 ng/mL, nicotine-*N*-glucuronide at 1000 ng/mL, cotinine-*N*-glucuronide at 2000 ng/mL, and *trans*-3′-hydroxycotinine-*O*-glucuronide at 5000 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)

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Additional Information – Cotinine in Urine - ZZ33881-03			
Inter-Batch	**LLOQ**	7.2	-4.3
	Low	5.2	-1.7
	Medium	4.0	2.0
	High	2.2	1.3
Intra-Batch (Batch 118)	**LLOQ**	7.2	1.0
Aliquot Method: Manual	**Low**	8.6	0.0
Extraction Method:	**Medium**	4.4	1.3
Automated	**High**	2.6	1.3
Intra-Batch (Batch 125)	**LLOQ**	4.4	-10.6
Aliquot Method: Manual	**Low**	2.7	-3.3
Extraction Method:	**Medium**	4.0	0.0
Automated	**High**	2.4	0.5
Intra-Batch (Batch 130)	**LLOQ**	3.1	-3.8
Aliquot Method: Manual	**Low**	1.5	-2.0
Extraction Method:	**Medium**	1.4	5.3
Automated	**High**	1.7	2.0
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 (10.0 ng/mL) or in any of the 10 human urine lots that were fortified with cotinine at the concentration of the high QC (750 ng/mL) samples	
Sample Shipping Stability		21 days in polypropylene tubes at -80°C	
Long-Term Stability for Stock Solutions (Substock)		40 days at 100 µg/mL in boxed 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 for labeled internal standard stability	
Long-Term Stability for Stock Solutions (Internal Standard Substock)		41 days at 3.75 µg/mL in boxed water in polypropylene tubes at -20°C	
Short-Term Stability for Stock Solutions (Stock)		6 hours at approximately 1000 µg/mL in boxed 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 boxed water in polypropylene tubes at ambient temperature under UV-shielded light	
Short-Term Stability for Stock Solutions (Internal Standard)		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 boxed water in polypropylene tubes at ambient temperature under UV-shielded light 6 hours at 75.0 ng/mL in boxed 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	

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Additional Information – Cotinine in Urine - ZZ33881-03	
	refrigerator
Sample Aliquot Frozen Storage Stability	Samples aliquoted manually at a volume of 0.250 mL, stored for 499 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 - ZZ33881-03

Information Requested	Data
Validation Summary	(b) (4) Validation Study ZZ33881-03
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)	10.0 ng/mL
Average Recovery of Drug (% Mean)	49% at 30.0 ng/mL 52% at 150 ng/mL 51% at 750 ng/mL
Average Recovery of IS (% Mean)	50%
Standard Curve Concentrations (ng/mL)	10.0, 20.0. 50.0, 100, 200, 400, 600, 800, and 1000 ng/mL
QC Concentrations (ng/mL)	LLOQ QC, 30.0, 150, and 750 ng/mL
QC Intra-Batch Precision Range (% CV)	1.1 to 11.5%
QC Intra-Batch Accuracy Range (% Bias)	-9.5 to 0.3%
QC Inter-Batch Precision Range (% CV)	1.9 to 8.1%
QC Inter-Batch Accuracy Range (% Bias)	-3.9 to -0.7%
Bench-Top Stability (Hrs)	Short-Term Stability: 24 hours in polypropylene tubes at ambient temperature under white light Cumulative Short-Term Stability: 54 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 boxed water in polypropylene tubes at -20°C
Processed Stability (Hrs)	Post-Preparative Stability: 180 hours in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 107 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: 440 days (LLOQ QC) and 476 days (dilution QC) in polypropylene tubes at -20°C
Dilution Integrity	Up to 8000 ng/mL, diluted 20-fold

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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

Matrix		Human Urine	
Bioanalytical Method (BAM) SOP Number		BAM SOP ZZ33881-03	
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 30.0 ng/mL fortified with nicotine at 1000 ng/mL, cotinine at 1000 ng/mL, nicotine-*N*-glucuronide at 1000 ng/mL, cotinine-*N*-glucuronide at 2000 ng/mL, and *trans*-3′-hydroxycotinine-*O*-glucuronide at 5000 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**	8.1	-3.9
	Low	6.2	-2.0
	Medium	1.9	-0.7
	High	2.4	-0.9
Intra-Batch (Batch 118) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	11.5	-2.4
	Low	9.1	-1.0
	Medium	2.8	-0.7
	High	2.5	-1.1
Intra-Batch (Batch 125) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	2.9	0.0
	Low	5.4	-3.3
	Medium	1.1	-0.7
	High	3.0	-2.1
Intra-Batch (Batch 130) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	3.3	-9.5
	Low	3.8	-1.3
	Medium	1.3	0.0
	High	1.2	0.3
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 (10.0 ng/mL) or in any of the 10 human urine lots that were fortified with *trans*-3′-hydroxycotinine at the	

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Additional Information – *trans*-3′-Hydroxycotinine	
	concentration of the high QC (750 ng/mL) samples
Sample Shipping Stability	21 days in polypropylene tubes at -80°C
Long-Term Stability for Stock Solutions (Substock)	40 days at 100 µg/mL in boxed 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 *trans*-3′-hydroxycotinine for labeled internal standard stability
Long-Term Stability for Stock Solutions (Internal Standard Substock)	41 days at 20.0 µg/mL in boxed water in polypropylene tubes at -20°C
Short-Term Stability for Stock Solutions (Stock)	6 hours at approximately 1000 µg/mL in boxed 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 boxed water in polypropylene tubes at ambient temperature under UV-shielded light
Short-Term Stability for Stock Solutions (Internal Standard)	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 boxed water in polypropylene tubes at ambient temperature under UV-shielded light 6 hours at 400 ng/mL in boxed 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
Sample Aliquot Frozen Storage Stability	Samples aliquoted manually at a volume of 0.250 mL, stored for 499 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 - ZZ33881-03

Information Requested	Data
Validation Summary	(b) (4) Validation Study ZZ33881-03
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)	10.0 ng/mL
Average Recovery of Drug (% Mean)	47% at 30.0 ng/mL 52% at 150 ng/mL 54% at 750 ng/mL

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Average Recovery of IS (% Mean)	51%
Standard Curve Concentrations (ng/mL)	10.0, 20.0. 50.0, 100, 200, 400, 600, 800, and 1000 ng/mL
QC Concentrations (ng/mL)	LLOQ QC, 30.0, 150, and 750 ng/mL
QC Intra-Batch Precision Range (% CV)	1.5 to 9.2%
QC Intra-Batch Accuracy Range (% Bias)	-11.3 to 2.7%
QC Inter-Batch Precision Range (% CV)	3.7 to 8.3%
QC Inter-Batch Accuracy Range (% Bias)	-5.0 to 0.0%
Bench-Top Stability (Hrs)	Short-Term Stability: 24 hours in polypropylene tubes at ambient temperature under white light Cumulative Short-Term Stability: 54 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): 758 days at approximately 1000 µg/mL in boxed water in polypropylene tubes at -20°C
Processed Stability (Hrs)	Post-Preparative Stability: 181 hours in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 108 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: 440 days (LLOQ QC) and 476 days (dilution QC) in polypropylene tubes at -20°C
Dilution Integrity	Up to 8000 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	
Matrix	Human Urine
Bioanalytical Method (BAM) SOP Number	BAM SOP ZZ33881-03
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 30.0 ng/mL fortified with nicotine at 1000 ng/mL, cotinine at 1000 ng/mL, *trans*-3′-hydroxycotinine at 1000 ng/mL, cotinine-*N*-glucuronide at 2000 ng/mL, and *trans*-3′-hydroxycotinine-*O*-glucuronide at 5000 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 – Nicotine-*N*-Glucuronide			
		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**	8.3	-4.2
	Low	5.5	-5.0
	Medium	5.2	0.0
	High	3.7	-3.9
Intra-Batch (Batch 120) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	5.3	-0.4
	Low	6.4	-4.0
	Medium	5.2	-0.7
	High	5.5	-5.5
Intra-Batch (Batch 126) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	3.9	-11.3
	Low	5.9	-5.0
	Medium	5.0	2.7
	High	2.9	-2.8
Intra-Batch (Batch 131) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	9.2	-0.8
	Low	5.1	-6.0
	Medium	4.5	-3.3
	High	1.5	-3.2
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 (10.0 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 (750 ng/mL) samples	
Sample Shipping Stability		21 days in polypropylene tubes at -80°C	
Long-Term Stability for Stock Solutions (Substock)		34 days at 50.0 µg/mL in boxed 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 nicotine-*N*-glucuronide for labeled internal standard stability	
Long-Term Stability for Stock Solutions (Internal Standard Substock)		41 days at 125 µg/mL in boxed water in polypropylene tubes at -20°C	
Short-Term Stability for Stock Solutions (Stock)		6 hours at approximately 1000 µg/mL in boxed 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 boxed water in polypropylene tubes at ambient temperature under UV-shielded light	
Short-Term Stability for Stock Solutions (Internal Standard)		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 boxed water in polypropylene tubes at ambient temperature under UV-shielded light 6 hours at 2500 ng/mL in boxed water in polypropylene tubes at ambient temperature under UV-shielded light	

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Additional Information – Nicotine-*N*-Glucuronide	
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
Sample Aliquot Frozen Storage Stability	Samples aliquoted manually at a volume of 0.250 mL, stored for 499 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 - ZZ33881-03

Information Requested	Data
Validation Summary	(b) (4) Validation Study ZZ33881-03
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)	20.0 ng/mL
Average Recovery of Drug (% Mean)	46% at 60.0 ng/mL 48% at 300 ng/mL 51% at 1500 ng/mL
Average Recovery of IS (% Mean)	47%
Standard Curve Concentrations (ng/mL)	20.0, 40.0, 100, 200, 400, 800, 1200, 1600, and 2000 ng/mL
QC Concentrations (ng/mL)	LLOQ QC, 60.0, 300, and 1500 ng/mL
QC Intra-Batch Precision Range (% CV)	3.9 to 11.3%
QC Intra-Batch Accuracy Range (% Bias)	-9.5 to 10.2%
QC Inter-Batch Precision Range (% CV)	6.3 to 11.5%
QC Inter-Batch Accuracy Range (% Bias)	-3.5 to 4.2%
Bench-Top Stability (Hrs)	Short-Term Stability: 24 hours in polypropylene tubes at ambient temperature under white light Cumulative Short-Term Stability: 54 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 120 days at approximately 1000 µg/mL in boxed water in polypropylene tubes at -20°C
Processed Stability (Hrs)	Post-Preparative Stability: 181 hours in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 108 hours in a polypropylene 96 well plate at 5°C

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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: 440 days (LLOQ QC) and 476 days (dilution QC) in polypropylene tubes at -20°C
Dilution Integrity	Up to 16,000 ng/mL, diluted 20-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

Matrix		Human Urine	
Bioanalytical Method (BAM) SOP Number		BAM SOP ZZ33881-03	
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 60.0 ng/mL fortified with nicotine at 1000 ng/mL, cotinine at 1000 ng/mL, *trans*-3′-hydroxycotinine at 1000 ng/mL, nicotine-*N*-glucuronide at 1000 ng/mL, and *trans*-3′-hydroxycotinine-*O*-glucuronide at 5000 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**	11.5	-3.5
	Low	9.1	4.2
	Medium	8.0	1.0
	High	6.3	-1.3
Intra-Batch (Batch 120) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	10.4	-5.5
	Low	9.7	-3.0
	Medium	4.7	-3.7
	High	6.0	-2.7
Intra-Batch (Batch 126) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	6.2	-9.5
	Low	8.2	10.2
	Medium	8.1	1.7
	High	3.9	-0.7
Intra-Batch (Batch 137) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	11.3	5.5
	Low	4.7	5.5
	Medium	8.4	5.3
	High	9.0	-1.3
Matrix Effect		No significant matrix effect was observed in 9 of the	

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Additional Information – Cotinine-*N*-Glucuronide	
	10 human urine lots that were fortified with cotinine-*N*-glucuronide at the concentration of the LLOQ (20.0 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 (1500 ng/mL) samples
Sample Shipping Stability	21 days in polypropylene tubes at -80°C
Long-Term Stability for Stock Solutions (Substock)	34 days at 100 µg/mL in boxed 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
Long-Term Stability for Stock Solutions (Internal Standard Substock)	41 days at 50.0 µg/mL in boxed water in polypropylene tubes at -20°C
Short-Term Stability for Stock Solutions (Stock)	6 hours at approximately 1000 µg/mL in boxed 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 boxed water in polypropylene tubes at ambient temperature under UV-shielded light
Short-Term Stability for Stock Solutions (Internal Standard)	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 boxed water in polypropylene tubes at ambient temperature under UV-shielded light 6 hours at 1000 ng/mL in boxed 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
Sample Aliquot Frozen Storage Stability	Samples aliquoted manually at a volume of 0.250 mL, stored for 499 hours in a polypropylene 96 well plate at -20°C prior to extraction
Batch Size	96 injections

Table 6. Validation Summary – trans-3′-Hydroxycotinine-O-glucuronide in Urine - ZZ33881-03

Information Requested	Data
Validation Summary	(b) (4) Validation Study ZZ33881-03
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

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Limit of Quantitation (ng/mL)	50.0 ng/mL
Average Recovery of Drug (% Mean)	42% at 150 ng/mL 49% at 750 ng/mL 49% at 3750 ng/mL
Average Recovery of IS (% Mean)	44% (221 ng/mL) 36% (1000 ng/mL)
Standard Curve Concentrations (ng/mL)	50.0, 100. 250, 500, 1000, 2000, 3000, 4000, and 5000 ng/mL
QC Concentrations (ng/mL)	LLOQ QC, 150, 750, and 3750 ng/mL
QC Intra-Batch Precision Range (% CV)	1.6 to 8.9%
QC Intra-Batch Accuracy Range (% Bias)	-7.8 to 0.0%
QC Inter-Batch Precision Range (% CV)	3.5 to 7.4%
QC Inter-Batch Accuracy Range (% Bias)	-6.0 to -3.3%
Bench-Top Stability (Hrs)	Short-Term Stability: 24 hours in polypropylene tubes at ambient temperature under white light Cumulative Short-Term Stability: 54 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): 120 days at approximately 1000 µg/mL in boxed water in polypropylene tubes at -20°C
Processed Stability (Hrs)	Post-Preparative Stability: 181 hours in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 108 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: 440 days (LLOQ QC) and 476 days (dilution QC) in polypropylene tubes at -20°C
Dilution Integrity	Up to 40,000 ng/mL, diluted 20-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 No significant interference at the retention time and mass transition of d_3-*trans*-3′-hydroxycotinine-*O*-glucuronide (IS) (62.5 ng/mL) was observed from endogenous components in any of the 6 human urine lots screened

Additional Information – *trans*-3′-Hydroxycotinine-*O*-Glucuronide	
Matrix	Human Urine
Bioanalytical Method (BAM) SOP Number	BAM SOP ZZ33881-03
Detector	AB SCIEX API 4000™

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Additional Information – *trans*-3′-Hydroxycotinine-*O*-Glucuronide			
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 150 ng/mL fortified with nicotine at 1000 ng/mL, cotinine at 1000 ng/mL, *trans*-3′-hydroxycotinine at 1000 ng/mL, nicotine-*N*-glucuronide at 1000 ng/mL, and cotinine-*N*-glucuronide at 2000 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**	6.1	-6.0
	Low	7.4	-3.3
	Medium	6.4	-3.6
	High	3.5	-5.9
Intra-Batch (Batch 120) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	6.1	-7.8
	Low	6.6	-6.0
	Medium	6.5	-2.7
	High	4.2	-3.7
Intra-Batch (Batch 126) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	6.9	-3.8
	Low	8.9	0.0
	Medium	5.3	-5.1
	High	1.6	-6.1
Intra-Batch (Batch 131) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	5.6	-6.4
	Low	5.9	-5.3
	Medium	8.1	-3.2
	High	3.1	-7.7
Matrix Effect		No significant matrix effect was observed in 9 of the 10 human urine lots that were fortified with *trans*-3′-hydroxycotinine-*O*-glucuronide 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-*O*-glucuronide at the concentration of the high QC (3750 ng/mL) samples	
Sample Shipping Stability		21 days in polypropylene tubes at -80°C	
Long-Term Stability for Stock Solutions (Substock)		34 days at 250 µg/mL in boxed 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 *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 boxed water in polypropylene tubes at -20°C	
Short-Term Stability for Stock Solutions		6 hours at approximately 1000 µg/mL in boxed water in	

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Additional Information – *trans*-3′-Hydroxycotinine-*O*-Glucuronide	
(Stock)	polypropylene tubes at ambient temperature under UV-shielded light
Short-Term Stability for Stock Solutions (Substock)	6 hours at 250 µg/mL in boxed water in polypropylene tubes at ambient temperature under UV-shielded light
Short-Term Stability for Stock Solutions (Internal Standard)	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 boxed water in polypropylene tubes at ambient temperature under UV-shielded light 6 hours at 552 ng/mL in boxed 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
Sample Aliquot Frozen Storage Stability	Samples aliquoted manually at a volume of 0.250 mL, stored for 499 hours in a polypropylene 96 well plate at -20°C prior to extraction
Batch Size	96 injections

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