Appendix A6.1.3.29 Validation Summary AA33664-07 (Nicotine and Cotinine in Plasma)

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TABLE OF TABLES

Table 1. Validation Summary – Nicotine in Plasma - AA33664-07 3
Table 2. Validation Summary – Cotinine in Plasma - AA33664-07 6

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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 – Nicotine in Plasma - AA33664-07

Information Requested	Data
Validation Summary	(b) (4) Validation Study AA33664-07
Analyte	Nicotine
Internal Standard (IS)	d_4-Nicotine
Method Description	Solid phase extraction with analysis/detection by LC-MS/MS
Limit of Quantitation (ng/mL)	1.00 ng/mL
Average Recovery of Drug (% Mean)	From Human Plasma (EDTA): 72% at 2.00 ng/mL 77% at 10.0 ng/mL 78% at 37.5 ng/mL From Water: 81% at 2.00 ng/mL 78% at 10.0 ng/mL 77% at 37.5 ng/mL
Average Recovery of IS (% Mean)	From Human Plasma (EDTA): 76% From Water: 77%
Standard Curve Concentrations (ng/mL)	1.00, 2.00, 4.00, 10.0, 17.5, 25.0, 37.6, 45.0, and 50.0 ng/mL
QC Concentrations (ng/mL)	LLOQ QC, 3.00, 7.00, and 37.5 ng/mL
QC Intra-Batch Precision Range (% CV)	0.8 to 4.5%
QC Intra-Batch Accuracy Range (% Bias)	-2.1 to 24.0%
QC Inter-Batch Precision Range (% CV)	5.0 to 7.4%
QC Inter-Batch Accuracy Range (% Bias)	2.9 to 7.0%
Bench-Top Stability (Hrs)	Short-Term Stability: 24 hours in polypropylene tubes at ambient temperature under white light Cumulative Short-Term Stability: 49 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: 142 hours in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 155 hours in a polypropylene 96 well plate at 5°C

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Information Requested	Data
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: 41 days in polypropylene tubes at -20°C (LLOQ); 220 days in polypropylene tubes at -20°C (QC A and D)
Dilution Integrity	Up to 100 ng/mL, diluted 10-fold
Selectivity	No significant interference at the retention time and mass transition of nicotine was observed from endogenous components in any of the 6 human plasma (EDTA) lots screened or of d_4-nicotine (IS) in any of the 6 human plasma (EDTA) lots screened

Additional Information – Nicotine in Plasma - AA33664-07	
Matrix	Human Plasma
Anticoagulant	K_2EDTA
Bioanalytical Method (BAM) SOP Number	BAM SOP AA33664-07
Detector	AB SCIEX API 4000™ or API 5000™
Assay Volume Required	0.300 mL
Regression Type	Weighted linear (1/concentration^2)
Quantitation Method	Peak area ratio
Multiple Analytes Measured by Assay	Nicotine at 3.00 ng/mL fortified with cotinine at 100 ng/mL
Over-the-Counter Cocktail Testing	Acetaminophen (25.0 µg/mL) Atorvastatin (100 ng/mL) Caffeine (20.0 µg/mL) Cetirizine (500 ng/mL) Dextromethorphan (25.0 ng/mL) Ethinyl Estradiol (0.500 ng/mL) Famotidine (200 ng/mL) Ibuprofen (20.0 µg/mL) Levonorgestrel (5.00 ng/mL) Metformin (2.00 µg/mL) Omeprazole (300 ng/mL) Ondansetron (200 ng/mL) Pseudoephedrine (500 ng/mL) Salicylic Acid (100 µg/mL)
Selectivity from Metabolites Not Quantified by the Method	*trans*-3′-Hydroxycotinine (4000 ng/mL) *trans*-3′-Hydroxycotinine Glucuronide (4000 ng/mL) Nicotine-Glucuronide (4000 ng/mL) Cotinine-Glucuronide (4000 ng/mL)

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Additional Information – Nicotine in Plasma - AA33664-07

Quality Control Samples		Precision (% CV)	Accuracy (% Bias)
Inter-Batch	**LLOQ**	7.4	7.0
	Low	5.1	6.3
	Medium	5.0	4.1
	High	5.9	2.9
Intra-Batch (Batch 2)[1]	**LLOQ**	1.8	24.0
Aliquot Method: Manual	**Low**	1.8	10.3
Extraction Method:	**Medium**	2.6	4.4
Automated	**High**	1.8	-2.1
Intra-Batch (Batch 3)	**LLOQ**	2.2	4.0
Aliquot Method: Manual	**Low**	4.5	0.7
Extraction Method:	**Medium**	3.9	-0.1
Automated	**High**	1.5	0.0
Intra-Batch (Batch 4)	**LLOQ**	2.4	3.0
Aliquot Method: Manual	**Low**	3.9	3.3
Extraction Method:	**Medium**	2.5	1.7
Automated	**High**	2.1	-0.3
Intra-Batch (Batch 5)	**LLOQ**	1.6	5.0
Aliquot Method: Manual	**Low**	3.7	3.7
Extraction Method:	**Medium**	2.7	3.1
Automated	**High**	3.6	2.1
Intra-Batch (Batch 8)	**LLOQ**	1.7	7.0
Aliquot Method: Manual	**Low**	0.9	14.0
Extraction Method:	**Medium**	1.8	13.7
Automated	**High**	0.8	14.9
Intra-Batch (Batch 9)	**LLOQ**	1.5	1.0
Aliquot Method: Manual	**Low**	1.5	5.7
Extraction Method:	**Medium**	1.0	1.9
Automated	**High**	1.7	2.7
Matrix Effect		When an AB SCIEX API 4000™ detector was used, no significant matrix effect was observed in any of the 6 human plasma (EDTA) lots that were fortified with nicotine at the concentration of the LLOQ (1.00 ng/mL) or in any of the 6 human plasma (EDTA) lots that were fortified with nicotine at the concentration of the high QC (37.5 ng/mL) samples When an AB SCIEX API 5000™ detector was used, no significant matrix effect was observed in any of the 6 human plasma (EDTA) lots that were fortified with nicotine at the concentration of the LLOQ (1.00 ng/mL) or in any of the 6 human plasma (EDTA) lots that were fortified with nicotine at the concentration of the high QC (37.5 ng/mL) samples	

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

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Additional Information – Nicotine in Plasma - AA33664-07	
Hemolyzed Sample Integrity	No significant interference for nicotine was observed in any of the 3 hemolyzed human plasma (EDTA) lots (fortified with 5% whole blood) that were fortified at 0.600 ng/mL or in any of the 3 human plasma (EDTA) lots that were fortified at the concentration of the high QC (37.5 ng/mL) samples
Lipemic Sample Evaluation	No significant interference for nicotine was observed in any of the 3 lipemic human plasma (EDTA) lots that were fortified at 0.600 ng/mL or in any of the 3 lipemic human plasma (EDTA) lots that were fortified at the concentration of the high QC (37.5 ng/mL) samples
Sample Shipping Stability	15 days (LLOQ QC) and 11 days (dilution QC) in polypropylene tubes at -80°C
Long-Term Stability for Stock Solutions (Substock)	574 days at 100 µg/mL in water in a polypropylene container at -20°C 323 days at 1.00 ng/mL in water 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 nicotine for labeled internal standard stability
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)	7 hours at 5.00 ng/mL in water in a polypropylene container at ambient temperature under white light
Stability of Analyte During Sample Collection and Handling	Up to 120 minutes in human whole blood (EDTA) in polypropylene tubes at ambient temperature under white light
Sample Aliquot Frozen Storage Stability	Samples aliquoted manually at a volume of 0.300 mL, stored for 116 hours in a polypropylene 96 well plate at -20°C prior to extraction
Batch Size	192 injections

Table 2. Validation Summary – Cotinine in Plasma - AA33664-07

Information Requested	Data
Validation Summary	(b) (4) Validation Study AA33664-07
Analyte	Cotinine
Internal Standard (IS)	d_3-Cotinine
Method Description	Solid phase extraction with analysis/detection by LC-MS/MS
Limit of Quantitation (ng/mL)	1.00 ng/mL

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Information Requested	Data
Average Recovery of Drug (% Mean)	From Human Plasma (EDTA): 81% at 2.00 ng/mL 86% at 10.0 ng/mL 85% at 75.0 ng/mL From Water: 88% at 2.00 ng/mL 84% at 10.0 ng/mL 81% at 75.0 ng/mL
Average Recovery of IS (% Mean)	From Human Plasma (EDTA): 83% From Water: 83%
Standard Curve Concentrations (ng/mL)	1.00, 2.00, 4.00, 10.0, 25.0, 50.0, 75.0, 90.0, and 100 ng/mL
QC Concentrations (ng/mL)	LLOQ QC, 3.00, 10.0, 75.0 ng/mL
QC Intra-Batch Precision Range (% CV)	0.6 to 5.7%
QC Intra-Batch Accuracy Range (% Bias)	-6.7 to 32.0%
QC Inter-Batch Precision Range (% CV)	2.4 to 11.3%
QC Inter-Batch Accuracy Range (% Bias)	-4.3 to 10.0%
Bench-Top Stability (Hrs)	Short-Term Stability: 24 hours in polypropylene tubes at ambient temperature under white light Cumulative Short-Term Stability: 49 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: 142 hours in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 155 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: 41 days in polypropylene tubes at -20°C (LLOQ); 220 days in polypropylene tubes at -20°C (QC A and D)
Dilution Integrity	up to 200 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 6 human plasma (EDTA) lots screened or of d_3-cotinine (IS) in any of the 6 human plasma (EDTA) lots screened

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Additional Information – Cotinine in Plasma - AA33664-07

Matrix		Human Plasma	
Anticoagulant		K_2EDTA	
Bioanalytical Method (BAM) SOP Number		BAM SOP AA33664-07	
Detector		AB SCIEX API 4000™ or API 5000™	
Assay Volume Required		0.300 mL	
Regression Type		Weighted linear (1/concentration^2)	
Quantitation Method		Peak area ratio	
Multiple Analytes Measured by Assay		Cotinine at 3.00 ng/mL fortified with nicotine at 50.0 ng/mL	
Over-the-Counter Cocktail Testing		Acetaminophen (25.0 µg/mL) Atorvastatin (100 ng/mL) Caffeine (20.0 µg/mL) Cetirizine (500 ng/mL) Dextromethorphan (25.0 ng/mL) Ethinyl Estradiol (0.500 ng/mL) Famotidine (200 ng/mL) Ibuprofen (20.0 µg/mL) Levonorgestrel (5.00 ng/mL) Metformin (2.00 µg/mL) Omeprazole (300 ng/mL) Ondansetron (200 ng/mL) Pseudoephedrine (500 ng/mL) Salicylic Acid (100 µg/mL)	
Selectivity from Metabolites Not Quantified by the Method		*trans*-3′-Hydroxycotinine (4000 ng/mL) *trans*-3′-Hydroxycotinine Glucuronide (4000 ng/mL) Nicotine-Glucuronide (4000 ng/mL) Cotinine-Glucuronide (4000 ng/mL)	
Quality Control Samples		Precision (% CV)	Accuracy (% Bias)
Inter-Batch	**LLOQ**	11.3	10.0
	Low	5.4	1.7
	Medium	2.8	-4.3
	High	2.4	-1.1
Intra-Batch (Batch 2)[2] **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	1.3	32.0
	Low	4.5	9.0
	Medium	1.1	-2.2
	High	2.3	-0.9
Intra-Batch (Batch 3) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	1.7	-0.7
	Low	2.2	-3.0
	Medium	1.9	-6.6
	High	1.0	-2.4

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

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Additional Information – Cotinine in Plasma - AA33664-07			
Intra-Batch (Batch 4) **Aliquot Method: Manual** **Extraction Method:** **Automated**	**LLOQ** **Low** **Medium** **High**	4.5 3.6 1.1 2.7	0.0 -2.3 -6.7 -2.0
Intra-Batch (Batch 5) **Aliquot Method: Manual** **Extraction Method:** **Automated**	**LLOQ** **Low** **Medium** **High**	5.7 3.1 2.6 2.1	7.0 0.0 -4.6 -1.9
Intra-Batch (Batch 8) **Aliquot Method: Manual** **Extraction Method:** **Automated**	**LLOQ** **Low** **Medium** **High**	1.8 0.9 1.1 0.6	10.0 5.0 -1.6 1.9
Matrix Effect		When an AB SCIEX API 4000™ detector was used, no significant matrix effect was observed in any of the 6 human plasma (EDTA) lots that were fortified with cotinine at the concentration of the LLOQ (1.00 ng/mL) or in any of the 6 human plasma (EDTA) lots that were fortified with cotinine at the concentration of the high QC (75.0 ng/mL) samples When an AB SCIEX API 5000™ detector was used, no significant matrix effect was observed in any of the 6 human plasma (EDTA) lots that were fortified with cotinine at the concentration of the LLOQ (1.00 ng/mL) or in any of the 6 human plasma (EDTA) lots that were fortified with cotinine at the concentration of the high QC (75.0 ng/mL) samples	
Hemolyzed Sample Integrity		No significant interference for cotinine was observed in any of the 3 hemolyzed human plasma (EDTA) lots (fortified with 5% whole blood) that were fortified at the concentration of the LLOQ (1.00 ng/mL) or in any of the 3 human plasma (EDTA) lots that were fortified at the concentration of the high QC (75.0 ng/mL) samples	
Lipemic Sample Evaluation		No significant interference for cotinine was observed in any of the 3 lipemic human plasma (EDTA) lots that were fortified at the concentration of the low QC (3.00 ng/mL) or in any of the 3 lipemic human plasma (EDTA) lots that were fortified at the concentration of the high QC (75.0 ng/mL) samples	
Sample Shipping Stability		15 days (LLOQ QC) and 11 days (dilution QC) in polypropylene tubes at -80°C	
Long-Term Stability for Stock Solutions (Substock)		2107 days at 100 µg/mL in water in a polypropylene container at -20°C 323 days at 5.00 ng/mL in water 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 cotinine for labeled internal standard stability	

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Additional Information – Cotinine in Plasma - AA33664-07	
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)	7 hours at 50.0 ng/mL in water in a polypropylene container at ambient temperature under white light
Stability of Analyte During Sample Collection and Handling	up to 120 minutes in human whole blood (EDTA) in polypropylene tubes at ambient temperature under white light
Sample Aliquot Frozen Storage Stability	samples aliquoted manually at a volume of 0.300 mL, stored for 116 hours in a polypropylene 96 well plate at -20°C prior to extraction
Batch Size	192 injections

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