Appendix A6.1.3.30 Validation Summary AA33664-08 (Nicotine and Cotinine in Plasma)

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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 - AA33664-08

Information Requested	Data
Validation Summary	(b) (4) Validation Study AA33664-08
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): 96% at 3.00 ng/mL 100% at 7.00 ng/mL 99% at 37.5 ng/mL From Water: 101% at 3.00 ng/mL 104% at 7.00 ng/mL 102% at 37.5 ng/mL
Average Recovery of IS (% Mean)	From Human Plasma (EDTA): 91% From Water: 96%
Standard Curve Concentrations (ng/mL)	1.00, 2.00, 4.00, 10.0, 17.5, 25.0, 37.5, 45.2, 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)	1.3 to 5.3%
QC Intra-Batch Accuracy Range (% Bias)	-3.2 to 14.0%
QC Inter-Batch Precision Range (% CV)	1.7 to 6.6%
QC Inter-Batch Accuracy Range (% Bias)	-1.6 to 7.0%
Bench-Top Stability (Hrs)	Short-Term Stability: 26 hours in polypropylene tubes at ambient temperature under white light Cumulative Short-Term Stability: 53 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

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Information Requested	Data
Processed Stability (Hrs)	Post-Preparative Stability: 124 hours (low QC) and 233 hours (high QC) in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 159 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: 211 days in polypropylene tubes at -20°C
Dilution Integrity	Up to 100 ng/mL, diluted 10-fold
Selectivity	When an AB SCIEX API 4000™ was used, 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 When an AB SCIEX API 5000™ was used, 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 - AA33664-08	
Matrix	Human Plasma
Anticoagulant	K_2EDTA
Bioanalytical Method (BAM) SOP Number	BAM SOP AA33664-08
Detector	AB SCIEX API 4000™ or API 5000™
Assay Volume Required	0.150 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 500 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)

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Additional Information – Nicotine in Plasma - AA33664-08

		Pseudoephedrine (500 ng/mL) Salicylic Acid (100 µg/mL)	
Selectivity from Metabolites Not Quantified by the Method		*trans*-3′-Hydroxycotinine (50.0 ng/mL) *trans*-3′-Hydroxycotinine Glucuronide (50.0 ng/mL) Nicotine-Glucuronide (50.0 ng/mL) Cotinine-Glucuronide (50.0 ng/mL)	
Quality Control Samples		Precision (% CV)	Accuracy (% Bias)
Inter-Batch	**LLOQ**	6.6	7.0
	Low	4.8	1.7
	Medium	2.7	-0.6
	High	1.7	-1.6
Intra-Batch (Batch 4) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	3.8	14.0
	Low	5.3	2.7
	Medium	2.5	-1.6
	High	1.3	-3.2
Intra-Batch (Batch 5) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	4.3	8.0
	Low	5.0	1.7
	Medium	3.1	-0.1
	High	1.5	-1.1
Intra-Batch (Batch 6) **Aliquot Method: Manual** **Extraction Method: Automated**	**LLOQ**	3.8	-1.2
	Low	4.5	0.3
	Medium	1.8	-0.3
	High	1.6	-0.8
Matrix Effect		When an AB SCIEX API 4000™ 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™ 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	
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 the concentration of the LLOQ (1.00 ng/mL) or in 2 of the 2 human plasma (EDTA) lots that were fortified at the concentration of the high QC (37.5 ng/mL) samples	

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Additional Information – Nicotine in Plasma - AA33664-08	
Lipemic Sample Evaluation	No significant interference for nicotine was observed in 2 of the 3 lipemic human plasma (EDTA) lots that were fortified at the concentration of the LLOQ (1.00 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	13 days 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)	20 hours at 12.5 ng/mL in water in a polypropylene container at ambient temperature under UV-shielded 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 0.150 mL, stored for 72 hours in a polypropylene 96 well plate at -20°C prior to extraction
Multiple Analyte Long-Term Stability (Frozen)	Against Cotinine: 211 days in polypropylene tubes at -20°C
Multiple Analyte Freeze-Thaw Stability	Against Cotinine: 6 freeze (-20°C)-thaw (ambient temperature) cycles in polypropylene tubes under white light
Multiple Analyte Short-Term Stability	Multiple Analyte Short-Term Stability Against Cotinine: 26 hours in polypropylene tubes at ambient temperature under white light Multiple Analyte Cumulative Short-Term Stability Against Cotinine: 53 hours in polypropylene tubes at ambient temperature under white light (total of all thaw cycles)
Batch Size	192 injections

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Table 2. Validation Summary – Cotinine in Plasma - AA33664-08

Information Requested	Data
Validation Summary	(b) (4) Validation Study AA33664-08
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)	5.00 ng/mL
Average Recovery of Drug (% Mean)	From Human Plasma (EDTA): 97% at 15.0 ng/mL 97% at 50.0 ng/mL 98% at 375 ng/mL From Water: 101% at 15.0 ng/mL 104% at 50.0 ng/mL 102% at 375 ng/mL
Average Recovery of IS (% Mean)	From Human Plasma (EDTA): 97% From Water: 103%
Standard Curve Concentrations (ng/mL)	5.00, 10.0, 20.0, 50.0, 125, 250, 375, 450, and 500 ng/mL
QC Concentrations (ng/mL)	LLOQ QC, 15.0, 50.0, and 375 ng/mL
QC Intra-Batch Precision Range (% CV)	0.6 to 2.9%
QC Intra-Batch Accuracy Range (% Bias)	-3.2 to 7.8%
QC Inter-Batch Precision Range (% CV)	1.5 to 3.1%
QC Inter-Batch Accuracy Range (% Bias)	-1.9 to 6.4%
Bench-Top Stability (Hrs)	Short-Term Stability: 26 hours in polypropylene tubes at ambient temperature under white light Cumulative Short-Term Stability: 53 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: 124 hours (low QC) and 233 hours (high QC) in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 159 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: 211 days in polypropylene tubes at -20°C
Dilution Integrity	up to 800 ng/mL, diluted 10-fold

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Information Requested	Data
Selectivity	When an AB SCIEX API 4000™ was used, 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 When an AB SCIEX API 5000™ was used, 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

Additional Information – Cotinine in Plasma - AA33664-08		
Matrix	Human Plasma	
Anticoagulant	K_2EDTA	
Bioanalytical Method (BAM) SOP Number	BAM SOP AA33664-08	
Detector	AB SCIEX API 4000™ or API 5000™	
Assay Volume Required	0.150 mL	
Regression Type	Weighted linear (1/concentration^2)	
Quantitation Method	Peak area ratio	
Multiple Analytes Measured by Assay	Cotinine at 15.0 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 (50.0 ng/mL) *trans*-3′-Hydroxycotinine Glucuronide (50.0 ng/mL) Nicotine-Glucuronide (50.0 ng/mL) Cotinine-Glucuronide (50.0 ng/mL)	
Quality Control Samples	Precision (% CV)	Accuracy (% Bias)

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Additional Information – Cotinine in Plasma - AA33664-08			
Inter-Batch	**LLOQ**	3.1	6.4
	Low	1.8	0.0
	Medium	1.5	-1.0
	High	1.7	-1.9
Intra-Batch (Batch 4)	**LLOQ**	2.2	6.8
Aliquot Method: Manual	**Low**	0.6	-1.3
Extraction Method:	**Medium**	2.2	-0.4
Automated	**High**	1.3	-1.3
Intra-Batch (Batch 5)	**LLOQ**	2.9	7.8
Aliquot Method: Manual	**Low**	2.3	0.0
Extraction Method:	**Medium**	1.0	-1.6
Automated	**High**	1.0	-3.2
Intra-Batch (Batch 6)	**LLOQ**	1.9	2.8
Aliquot Method: Manual	**Low**	1.2	0.0
Extraction Method:	**Medium**	1.1	-0.2
Automated	**High**	1.3	0.0
Matrix Effect		When an AB SCIEX API 4000™ 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 (5.00 ng/mL) or in any of the 6 human plasma (EDTA) lots that were fortified with cotinine at the concentration of the high QC (375 ng/mL) samples When an AB SCIEX API 5000™ 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 (5.00 ng/mL) or in any of the 6 human plasma (EDTA) lots that were fortified with cotinine at the concentration of the high QC (375 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 (5.00 ng/mL) or in any of the 2 human plasma (EDTA) lots that were fortified at the concentration of the high QC (375 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 LLOQ (5.00 ng/mL) or in any of the 3 lipemic human plasma (EDTA) lots that were fortified at the concentration of the high QC (375 ng/mL) samples	
Sample Shipping Stability		13 days in polypropylene tubes at -80°C	

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Additional Information – Cotinine in Plasma - AA33664-08	
Long-Term Stability for Stock Solutions (Substock)	69 days at 250 µg/mL in water in a polypropylene container at -20°C 39 days at 200 µg/mL in water in a polypropylene container at -20°C 160 days at 100 µg/mL in water in a polypropylene container at -20°C 299 days at 10.0 ng/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
Short-Term Stability for Stock Solutions (Substock)	16 hours at 250 ng/mL in water in a polypropylene container 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)	20 hours at 125 ng/mL in water in a polypropylene container at ambient temperature under UV-shielded 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 0.150 mL, stored for 72 hours in a polypropylene 96 well plate at -20°C prior to extraction
Multiple Analyte Long-Term Stability (Frozen)	Against Nicotine: 211 days in polypropylene tubes at -20°C
Multiple Analyte Freeze-Thaw Stability	Against Nicotine: 6 freeze (-20°C)-thaw (ambient temperature) cycles in polypropylene tubes under white light
Multiple Analyte Short-Term Stability	Multiple Analyte Short-Term Stability Against Nicotine: 26 hours in polypropylene tubes at ambient temperature under white light Multiple Analyte Cumulative Short-Term Stability Against Nicotine: 53 hours in polypropylene tubes at ambient temperature under white light (total of all thaw cycles)
Batch Size	192 injections

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