# Appendix A6.1.3.28 Validation Summary AA33664-06 (Nicotine and Cotinine in Plasma)

**Confidentiality Statement**

*Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.*

## TABLE OF TABLES

Table 1. Validation Summary – Nicotine in Plasma - AA33664-06 ..... 3
Table 2. Validation Summary – Cotinine in Plasma - AA33664-06 ..... 7

**Confidentiality Statement**

*Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.*

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 Plasma - AA33664-06**

| Information Requested | Data |
|---|---|
| **Validation Summary** | (b) (4) Validation Study AA33664-06 |
| **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)** | 0.200 ng/mL |
| **Average Recovery of Drug (% Mean)** | From Human Plasma (EDTA):<br>81% at 0.600 ng/mL<br>79% at 1.20 ng/mL<br>84% at 7.50 ng/mL<br>From Water:<br>80% at 0.600 ng/mL<br>76% at 1.20 ng/mL<br>78% at 7.50 ng/mL |
| **Average Recovery of IS (% Mean)** | From Human Plasma (EDTA):<br>78%<br>From Water:<br>76% |
| **Standard Curve Concentrations (ng/mL)** | 0.200, 0.400, 0.800, 1.00, 2.50, 5.00, 7.50, 9.00, and 10.0 ng/mL |
| **QC Concentrations (ng/mL)** | LLOQ QC, 0.600, 1.20, and 7.50 ng/mL |
| **QC Intra-Batch Precision Range (% CV)** | 0.9 to 7.1% |
| **QC Intra-Batch Accuracy Range (% Bias)** | -2.8 to 12.0% |
| **QC Inter-Batch Precision Range (% CV)** | 2.6 to 6.2% |
| **QC Inter-Batch Accuracy Range (% Bias)** | -0.9 to 5.5% |
| **Bench-Top Stability (Hrs)** | Short-Term Stability: 27 hours in polypropylene tubes at ambient temperature under white light |
| **Stock Stability (Days)** | Long-Term Stability for Stock Solutions (Stock): N/AP |
| **Processed Stability (Hrs)** | Post-Preparative Stability: 321 hours in a polypropylene 96 well plate at 5°C<br>Processed Sample Integrity: 272 hours in a polypropylene 96 well plate at 5°C |

**Confidentiality Statement**

*Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.*

| 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: 1041 days in polypropylene tubes at -20°C |
| **Dilution Integrity** | Up to 20.0 ng/mL, diluted 10-fold<br>Up to 40.0 ng/mL, diluted 5-fold<br>Up to 60.0 ng/mL, diluted 10-fold<br>Up to 150 ng/mL, diluted 25-fold |
| **Selectivity** | No significant interference at the retention time and mass transition of nicotine was observed from endogenous components in 5 of the 6 human plasma (EDTA) lots screened or of $d_4$-nicotine (IS) in any of the 6 human plasma (EDTA) lots screened<br>Precolumn Method: 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 | |
|---|---|
| **Matrix** | Human Plasma |
| **Anticoagulant** | $K_2$EDTA |
| **Bioanalytical Method (BAM) SOP Number** | BAM SOP AA33664-06 |
| **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 0.600 ng/mL fortified with cotinine at 100 ng/mL |
| **Over-the-Counter Cocktail Testing** | Acetaminophen (25.0 µg/mL)<br>Atorvastatin (100 ng/mL)<br>Caffeine (20.0 µg/mL)<br>Cetirizine (500 ng/mL)<br>Dextromethorphan (25.0 ng/mL)<br>Ethinyl Estradiol (0.500 ng/mL)<br>Famotidine (200 ng/mL)<br>Ibuprofen (20.0 µg/mL)<br>Levonorgestrel (5.00 ng/mL)<br>Metformin (2.00 µg/mL)<br>Omeprazole (300 ng/mL)<br>Ondansetron (200 ng/mL)<br>Pseudoephedrine (500 ng/mL)<br>Salicylic Acid (100 µg/mL) |

**Confidentiality Statement**

*Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.*

## Additional Information – Nicotine in Plasma

| | | | |
|---|---|---|---|
| **Selectivity from Metabolites Not Quantified by the Method** | | *trans*-3′-Hydroxycotinine (50.0 ng/mL)<br>*trans*-3′-Hydroxycotinine-Glucuronide (50.0 ng/mL)<br>Nicotine-Glucuronide (50.0 ng/mL)<br>Cotinine-Glucuronide (50.0 ng/mL) | |
| **Quality Control Samples** | | Precision (% CV) | Accuracy (% Bias) |
| **Inter-Batch** | **LLOQ** | 6.2 | 5.5 |
| | **Low** | 3.7 | -0.8 |
| | **Medium** | 2.6 | 0.8 |
| | **High** | 2.9 | -0.9 |
| **Intra-Batch (Batch 9)**<br>**Aliquot Method: Manual**<br>**Extraction Method: Automated** | **LLOQ** | 3.9 | 1.5 |
| | **Low** | 3.3 | -2.0 |
| | **Medium** | 2.9 | 0.0 |
| | **High** | 2.0 | -1.7 |
| **Intra-Batch (Batch 10)**<br>**Aliquot Method: Manual**<br>**Extraction Method: Automated** | **LLOQ** | 4.3 | 4.5 |
| | **Low** | 1.8 | -2.2 |
| | **Medium** | 1.6 | -0.8 |
| | **High** | 2.1 | -1.5 |
| **Intra-Batch (Batch 11)**<br>**Aliquot Method: Manual**<br>**Extraction Method: Automated** | **LLOQ** | 4.8 | 12.0 |
| | **Low** | 2.7 | 0.7 |
| | **Medium** | 2.1 | 1.7 |
| | **High** | 0.9 | -2.8 |
| **Intra-Batch (Batch 14)**<br>**Aliquot Method: Manual**<br>**Extraction Method: Automated** | **LLOQ** | 7.1 | 4.0 |
| | **Low** | 5.7 | 1.3 |
| | **Medium** | 2.8 | 2.5 |
| | **High** | 3.0 | 3.2 |
| **Matrix Effect** | | 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 low QC (0.600 ng/mL) or in any of the 6 human plasma (EDTA) lots that were fortified with nicotine at the concentration of the high QC (7.50 ng/mL) samples<br>Precolumn Method: 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 low QC (0.600 ng/mL) or in any of the 6 human plasma (EDTA) lots that were fortified with nicotine at the concentration of the high QC (7.50 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 low QC (0.600 ng/mL) or in any of the 3 human plasma (EDTA) lots that were fortified at the concentration of the high QC (7.50 ng/mL) samples | |

**Confidentiality Statement**

*Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.*

| Additional Information – Nicotine in Plasma | |
|---|---|
| **Lipemic Sample Evaluation** | No significant interference for nicotine was observed in any of the 3 lipemic human plasma (EDTA) lots that were fortified at the concentration of the low QC (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 (7.50 ng/mL) samples |
| **Sample Shipping Stability** | 19 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<br>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 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)** | 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 UV-shielded light |
| **Sample Aliquot Frozen Storage Stability** | Samples aliquoted manually at a volume of 0.300 mL, stored for 164 hours in a polypropylene 96 well plate at -20°C prior to extraction |
| **Batch Size** | 192 injections |

**Confidentiality Statement**

*Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.*

## Table 2. Validation Summary – Cotinine in Plasma - AA33664-06

| Information Requested | Data |
|---|---|
| **Validation Summary** | (b) (4) Validation Study AA33664-06 |
| **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 |
| **Average Recovery of Drug (% Mean)** | From Human Plasma (EDTA) (50.0 ng/mL $d_3$-Cotinine Working Intrernal Standard Concentration):<br>75% at 3.00 ng/mL<br>78% at 12.0 ng/mL<br>83% at 75.0 ng/mL<br>From Water (50.0 ng/mL $d_3$-Cotinine Working Intrernal Standard Concentration):<br>78% at 3.00 ng/mL<br>78% at 12.0 ng/mL<br>82% at 75.0 ng/mL<br>From Human Plasma (EDTA) (5.00 ng/mL $d_3$-Cotinine Working Intrernal Standard Concentration):<br>78% at 2.00 ng/mL<br>83% at 25.0.0 ng/mL<br>87% at 90.0 ng/mL<br>From Water (5.00 ng/mL $d_3$-Cotinine Working Intrernal Standard Concentration):<br>76% at 2.00 ng/mL<br>73% at 25.0 ng/mL<br>79% at 90.0 ng/mL |
| **Average Recovery of IS (% Mean)** | From Human Plasma (EDTA) (50.0 ng/mL $d_3$-Cotinine Working Intrernal Standard Concentration):<br>77%<br>From Water (50.0 ng/mL $d_3$-Cotinine Working Intrernal Standard Concentration:<br>78%<br>From Human Plasma (EDTA) (5.00 ng/mL $d_3$-Cotinine Working Intrernal Standard Concentration):<br>83%<br>From Water (5.00 ng/mL $d_3$-Cotinine Working Intrernal Standard Concentration):<br>76% |
| **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, 12.0, and 75.0 ng/mL |
| **QC Intra-Batch Precision Range (% CV)** | 0.5 to 2.4% |
| **QC Intra-Batch Accuracy Range (% Bias)** | -0.1 to 5.0% |
| **QC Inter-Batch Precision Range (% CV)** | 1.1 to 2.2% |
| **QC Inter-Batch Accuracy Range (% Bias)** | 1.5 to 4.2% |

**Confidentiality Statement**

*Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.*

| Information Requested | Data |
|---|---|
| **Bench-Top Stability (Hrs)** | Short-Term Stability: 27 hours in polypropylene tubes at ambient temperature under white light |
| **Stock Stability (Days)** | Long-Term Stability for Stock Solutions (Stock): N/AP |
| **Processed Stability (Hrs)** | Post-Preparative Stability: 321 hours in a polypropylene 96 well plate at 5°C<br>Processed Sample Integrity: 272 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: 739 days in polypropylene tubes at -20°C |
| **Dilution Integrity** | up to 200 ng/mL, diluted 10-fold<br>up to 600 ng/mL, diluted 10-fold<br>up to 1000 ng/mL, diluted 25-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<br>Precolumn Method: 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 | |
|---|---|
| **Matrix** | Human Plasma |
| **Anticoagulant** | $K_2$EDTA |
| **Bioanalytical Method (BAM) SOP Number** | BAM SOP AA33664-06 |
| **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 10.0 ng/mL |

**Confidentiality Statement**

*Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.*

## Additional Information– Cotinine in Plasma

| | | | |
|---|---|---|---|
| **Over-the-Counter Cocktail Testing** | | Acetaminophen (25.0 µg/mL)<br>Atorvastatin (100 ng/mL)<br>Caffeine (20.0 µg/mL)<br>Cetirizine (500 ng/mL)<br>Dextromethorphan (25.0 ng/mL)<br>Ethinyl Estradiol (0.500 ng/mL)<br>Famotidine (200 ng/mL)<br>Ibuprofen (20.0 µg/mL)<br>Levonorgestrel (5.00 ng/mL)<br>Metformin (2.00 µg/mL)<br>Omeprazole (300 ng/mL)<br>Ondansetron (200 ng/mL)<br>Pseudoephedrine (500 ng/mL)<br>Salicylic Acid (100 µg/mL) | |
| **Selectivity from Metabolites Not Quantified by the Method** | | *trans*-3′-Hydroxycotinine (50.0 ng/mL)<br>*trans*-3′-Hydroxycotinine-Glucuronide (50.0 ng/mL)<br>Nicotine-Glucuronide (50.0 ng/mL)<br>Cotinine-Glucuronide (50.0 ng/mL) | |
| **Quality Control Samples** | | Precision (% CV) | Accuracy (% Bias) |
| **Inter-Batch** | **LLOQ** | 2.2 | 4.0 |
| | **Low** | 1.9 | 1.7 |
| | **Medium** | 1.1 | 4.2 |
| | **High** | 1.3 | 1.5 |
| **Intra-Batch (Batch 8)**<br>**Aliquot Method: Manual**<br>**Extraction Method: Automated** | **LLOQ** | 2.0 | 5.0 |
| | **Low** | 1.5 | 0.3 |
| | **Medium** | 1.2 | 3.3 |
| | **High** | 1.0 | 1.6 |
| **Intra-Batch (Batch 9)**<br>**Aliquot Method: Manual**<br>**Extraction Method: Automated** | **LLOQ** | 1.9 | 2.0 |
| | **Low** | 2.1 | 1.7 |
| | **Medium** | 0.5 | 4.2 |
| | **High** | 0.7 | 1.9 |
| **Intra-Batch (Batch 10)**<br>**Aliquot Method: Manual**<br>**Extraction Method: Automated** | **LLOQ** | 2.4 | 4.0 |
| | **Low** | 1.9 | 2.0 |
| | **Medium** | 0.9 | 4.2 |
| | **High** | 1.4 | 1.6 |
| **Intra-Batch (Batch 11)**<br>**Aliquot Method: Manual**<br>**Extraction Method: Automated** | **LLOQ** | 1.5 | 3.0 |
| | **Low** | 1.3 | 1.7 |
| | **Medium** | 1.4 | 3.3 |
| | **High** | 1.1 | -0.1 |
| **Intra-Batch (Batch 14)**<br>**Aliquot Method: Manual**<br>**Extraction Method: Automated** | **LLOQ** | 2.4 | 5.0 |
| | **Low** | 1.8 | 3.7 |
| | **Medium** | 0.9 | 5.0 |
| | **High** | 0.9 | 2.4 |

**Confidentiality Statement**

*Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.*

| Additional Information– Cotinine in Plasma | |
|---|---|
| **Matrix Effect** | No significant matrix effect was observed in 5 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<br>Precolumn Method: No significant matrix effect was observed in 5 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** | 19 days in polypropylene tubes at -80°C |
| **Long-Term Stability for Stock Solutions (Substock)** | 160 days at 100 µg/mL in water in a polypropylene container at -20°C<br>323 days at 5.00 ng/mL in water in a polypropylene container at -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 |
| **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 UV-shielded light |
| **Sample Aliquot Frozen Storage Stability** | samples aliquoted manually at a volume of 0.300 mL, stored for 164 hours in a polypropylene 96 well plate at -20°C prior to extraction |
| **Batch Size** | 192 injections |

**Confidentiality Statement**

*Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.*