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6.1.3.3 Overview of Bioanalytical Methods

The assessment matrix for the biomarkers of exposure (BoExp) measured in PMI's clinical studies has been evaluated with regards to validity of the available methods, feasibility, robustness, and exposure time needed to assure reliable results.

Detailed procedures for sample collection and handling of samples were described in a separate sample handling manual and/or laboratory manual.

The measurements of BoExps were all conducted by (b) (4) A, to ensure consistency and interpretability of the results across all the studies, with the exception of carboxyhemoglobin (COHb). Because most of the methods are validated for a couple of days after time of sample collection, COHb in whole blood was sent to labs that were geographically closer to the clinical sites, i.e. to (b) (4). All the bioanalytical assays were carried out using validated methods and are documented in the Bioanalytical Reports and part of the Clinical Study Reports (Appendix 16.1.9 of each Clinical Study Report).

Table 1 and Table 2 present a summary of the validation parameters of analytical methods used for BoExp measurement in the PK studies (refer to section 6.2.1.2 for study summaries), while Table 3 and Table 4 present the summaries for the analytical methods used for BoExp measurement in Reduced Exposure studies (refer to section 6.1.3.4 for study summaries), using either LC-MS//MS methods, or spectrophotometry, with the accuracy, precision and validation range (i.e. lower limit to upper limit of quantification [LLOQ to ULOQ]) of each method. References to the methods numbers of the validation reports established by (b) (4) are also provided.

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Table 1 Summary of Analytical Methods Using LC-MS/MS Method for PMI's PK Studies(b) (4))

BoExp	Studies	Accuracy	Precision	Validation range	(b) (4) Reference
Nicotine (plasma)	All PK studies	Intra-batch[1]: -2.8 to 12% Inter-batch[2]: -0.9 to 5.5%	Intra-batch[1] : 0.9 to 7.1% Inter-batch[2]: 2.6 to 6.2%	0.2 to 10 ng/mL	AA33664-06 (b) (4) 2016a)
Cotinine (plasma)	ZRHR-PK-06-US	Intra-batch[1]:-7.2 to 10% Inter-batch[2]:-4.3 to 2.1%	Intra-batch[1]: 1.1 to 4.3% Inter-batch[2]: 2.1 to 5.2%	1 to 100 ng/mL	AA33664-06 (b) (4) 2016a)
Cotinine (plasma) [3]	All PK studies	Intra-batch[1]:-0.1 to 5% Inter-batch[2]:1.5 to 4.2%	Intra-batch[1]: 0.5 to 2.4% Inter-batch[2]: 1.1 to 2.2%	1 to 100 ng/mL	AA33664-01 (b) (4) 2016b)
trans-3'-hydroxycotinine (plasma) [3]	All PK studies	Intra-batch[1]:-6.6 to 4% Inter-batch[2]:-4.2 to 1.3%	Intra-batch[1]: 1.7 to 4.7% Inter-batch[2]: 2.3 to 3.7%	1 to 100 ng/mL	AA33664-01 (b) (4) 2016b)

[1] Repeatability

[2] Intermediate precision

[3] To assess CYP2A6 activity (molar ratio of *trans*-3'-hydroxycotinine/cotinine?

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Table 2 Summary of Analytical Methods using Spectrophotometry for PMI's PK Studies

BoExp	Studies	Accuracy	Precision	Validation range
COHb (b) (4)(b) (4)	ZRHM-PK-05-JP ZRHR-PK-02-JP	0.5 to 1.1%	Intra-batch[1]: 1.2 to 12.7% Inter-batch[2]: 1 to 19.7%	0 to 100 %
COHb (b) (4)	ZRHR-PK-01-EU ZRHR-PK-06-US	Intra-batch[1]: 1.3 to 16,2% Inter-batch[2]:0.1 to 10.0%	Intra-batch[1]: 1.2 to 3.5% Inter-batch[2]: 0.8 to 14.5%	0.5 to 100 %

[1] Repeatability

[2] Intermediate precision

Abbreviations: carboxyhemoglobin (COHb)

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Table 3 Summary of Analytical Methods Using LC-MS/MS Method for PMI's Reduced Exposure Studies (b) (4) (b) (4)

BoExp	Studies	Accuracy	Precision	Validation Range	(b) (4) Reference
Nicotine (plasma) SA arms	All reduced exposure studies	Intra-batch[1]: -2.8 to 12% Inter-batch[2]: -0.9 to 5.5%	Intra-batch[1] : 0.9 to 7.1% Inter-batch[2]: 2.6 to 6.2%	0.2 to 10 ng/mL	AA33664-06 (b) (4) 2016a)
Cotinine (plasma) SA arms	All reduced exposure studies	Intra-batch[1]: -0.1 to 5% Inter-batch[2]: 1.5 to 4.2%	Intra-batch[1] : 0.5 to 2.4% Inter-batch[2]: 1.1 to 2.2%	1 to 100 ng/mL	AA33664-06 (b) (4) 2016a)
Nicotine (plasma) THS and CC arms	ZRHR-REXC-03-EU ZRHR-REXC-04-JP ZRHM-REXA-08-US	Intra-batch[1]: 0.3 to 14.9% Inter-batch[2]: 3.7 to 5.7%	Intra-batch[1]: 0.8 to 4.5% Inter-batch[2]: 2.6 to 5.9%	1 to 50 ng/mL	AA33664-07 (b) (4) 2014a)
Cotinine (plasma) THS and CC arms	ZRHR-REXC-03-EU ZRHR-REXC-04-JP ZRHM-REXA-08-US	Intra-batch[1]:-7.2 to 10% Inter-batch[2]:-4.3 to 2.1%	Intra-batch[1]: 1.1 to 4.3% Inter-batch[2]: 2.1 to 5.2%	1 to 100 ng/mL	AA33664-07 (b) (4) 2014a)
Nicotine (plasma) THS and CC arms	ZRHM-REXA-07-JP	Intra-batch[1]: -3.2 to 14.0% Inter-batch[2]: -1.6 to 7.0%	Intra-batch[1]: 1.3 to 5.3% Inter-batch[2]: 1.7 to 6.6%	1 to 50 ng/mL	AA33664-08 (b) (4) 2014a)
Cotinine (plasma) THS and CC arms	ZRHM-REXA-07-JP	Intra-batch[1]:-3.2 to 7.8% Inter-batch[2]:-1.9 to 6.4%	Intra-batch[1]: 0.6 to 2.9% Inter-batch[2]: 1.5 to 3.1%	1 to 100 ng/mL	AA33664-08 (b) (4) 2014a)

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BoExp	Studies	Accuracy	Precision	Validation Range	(b) (4) Reference
Cotinine (plasma) [3] THS, CC, and SA arms	All reduced exposure studies	Intra-batch[1]:0.1 to 5% Inter-batch[2]:1.5 to 4.2%	Intra-batch[1]: 0.5 to 2.4% Inter-batch[2]: 1.1 to 2.2%	1 to 100 ng/mL	AA33664-01 (b) (4) 2016b)
Trans-3'-hydroxycotinine (plasma) [3] THS, CC, and SA arms	All reduced exposure studies	Intra-batch[1]:-6.6 to 4% Inter-batch[2]:-4.2 to 1.3%	Intra-batch[1]: 1.7 to 4.7% Inter-batch[2]: 2.3 to 3.7%	1 to 100 ng/mL	AA33664-01 (b) (4) 2016b)
MHBMA* (urine) THS, CC, and SA arms	ZRHR-REXC-03-EU ZRHR-REXC-04-JP	Intra-batch[1]: -5.6 to 0.1% Inter-batch[2]: -2.1 to -10.6% %	Intra-batch[1]: 1.2 to 10% Inter-batch: 2.9 to 10.2%	0.10 to 20 ng/mL	VAA98876-02 (b) (4) 2016c)
MHBMA* (urine) THS, CC, and SA arms	ZRHM-REXA-07-JP ZRHM-REXA-08-US	Intra-batch[1]: -2.7 to 12.7% Inter-batch[2]: -3.3 to 9% %	Intra-batch[1]: 1.8 to 7.4% Inter-batch: 4.2 to 7%	0.10 to 20 ng/mL	VAA98876-02 (b) (4) 2016c)
Total 1-OHP (urine) THS, CC, and SA arms	All reduced exposure studies	Intra-batch[1]: 1.4 to 5.7% Inter-batch[2]: 3.4 to 7%	Intra-batch[1]: 1.4 to 5.7% Inter-batch[2]: 2.8 to 5.5%	10 to 2000 pg/mL	AA98876-03 (b) (4) 2016e)
S-PMA (urine) THS, CC, and SA arms	All reduced exposure studies	Intra-batch[1]: 7.2 to 18% Inter-batch[2]: 6.3 to 10%	Intra-batch[1]: 1.4 to 6.6% Inter-batch[2]: 2.2 to 12.9%	0.025 to 6.25 ng/mL	AA98876-01 (b) (4) 2013)
S-BMA (urine) THS, CC, and SA arms	All reduced exposure studies	Intra-batch[1]: -4.9% to 5.7% Inter-batch[2]: -9.9 to 6.3%	Intra-batch[1]: 0.8% to 2.7% Inter-batch[2]: 2.2 to 8.6%	0.1 to 25 ng/mL	AA98876-01 (b) (4) 2013)

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BoExp	Studies	Accuracy	Precision	Validation Range	(b) (4) Reference
4-ABP (urine) THS, CC, and SA arms	All reduced exposure studies	Intra-batch[1]: -4% to 9.3% Inter-batch[2]: 0.3 to 2.7%	Intra-batch[1]: 1 to 5.2% Inter-batch[2]: 2.9 to 6.4%	1.5 to 75 pg/mL	ZZ37540-01 ((b) (4) 2014c)
1-NA (urine) THS, CC, and SA arms	All reduced exposure studies	Intra-batch[1]: -5.3% to 8.9% Inter-batch[2]: -0.5% to 6%	Intra-batch[1]: 1.7% to 4.8% Inter-batch[2]: 2.8 to 4.5%	2.5 to 500 pg/mL	ZZ37540-01(b) (4) 2014c)
2-NA (urine) THS, CC, and SA arms	All reduced exposure studies	Intra-batch[1]: -6.1% to 5.6% Inter-batch[2]: 0.1% to 2.4%	Intra-batch[1]: 0.7% to 5.3% Inter-batch[2]: 2.8 to 5.8%	2.5 to 500 pg/mL	ZZ37540-01 ((b) (4) 2014c)
o-toluidine (urine) THS, CC, and SA arms	All reduced exposure studies	Intra-batch[1]: -6.9% to 6% Inter-batch[2]: -6.8% to 3.3%	Intra-batch[1]: 1.7% to 3.6% Inter-batch[2]: 2.3 to 4%	20 to 1000 pg/mL	ZZ37540-01(b) (4) 2014c)
CEMA (urine) THS, CC, and SA arms	All reduced exposure studies	Intra-batch[1]: -12.7% to 5.1% Inter-batch[2]: -4.4% to 0.6%	Intra-batch[1]: 1.4% to 7.5% Inter-batch[2]: 3.8 to 10%	0.275 to 207 ng/mL	ZZ36536-01 ((b) (4) 2015a)
3-HPMA (urine) THS, CC, and SA arms	All reduced exposure studies	Intra-batch[1]: -17 to 2.6% Inter-batch[2]: -6.3 to -0.7%	Intra-batch[1]: 1.2 to 10% Inter-batch[2]: -2.9 to 10.2%	20 to 5000 ng/mL	ZZ36536-01(b) (4) 2015a)
3-HMPMA (urine) THS, CC, and SA arms	All reduced exposure studies	Intra-batch[1]: -15.5% to 7.6% Inter-batch[2]: -13.5 to 1.6%.	Intra-batch[1]: 1.6% to 11.2% Inter-batch[2]: 2.8 to 7.6%.	20 to 5000 ng/mL	ZZ36536-01(b) (4) 2015a)

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BoExp	Studies	Accuracy	Precision	Validation Range	(b) (4) Reference
HEMA (urine) THS, CC, and SA arms	All reduced exposure studies	Intra-batch[1]: -5.4% to 9.8% Inter-batch[2]: -2% to 4.9%	Intra-batch[1]: 2.2% to 5.8% Inter-batch[2]: 3.2 to 6.1%	0.1 to 20 ng/mL	ZZ38073-01 ((b) (4) 2014e)
3-OH-B[a]P (urine) THS, CC, and SA arms	All reduced exposure studies	Intra-batch[1]:-10.8% to 7.4% Inter-batch[2]:-3-6% to 0.2%	Intra-batch[1]: 2.3% to 13.6% Inter-batch[2]: 3.6 to 10.9%	50 to 2000 fg/mL	ZZ42666-(b) (4) 2016f)
Total NNAL (urine) THS, CC, and SA arms	All reduced exposure studies	Intra-batch[1]: -4.4% to 5% Inter-batch[2]: -0.8 to 0.4%	Intra-batch[1]: 1.7% to 3.9% Inter-batch[2]: 1.9 to 5.3%	5 to 1000 pg/mL	ZZ34313-(b) (4) 2015b)
Total NNN (urine) THS, CC, and SA arms	All reduced exposure studies	Intra-batch[1]: -4% to 6.7% Inter-batch[2]: -2.3 to 1.0%	Intra-batch[1]: 0.8 to 12% Inter-batch[2]: 1.1 to 8.4%	0.2 to 40 pg/mL	ZZ34313-03(b) (4) 2015b)
Paraxanthine (plasma) THS, and CC arms	All reduced exposure studies	Intra-batch[1]: -9.5% to 4.0% Inter-batch[2]: -3.3 to -0.6%	Intra-batch[1]: 0.7% to 4.7% Inter-batch[2]: 1.9 to 6.5%	20 to 5000 ng/mL	ZZ25187-02 ((b) (4) 2014d)
Caffeine (plasma) THS, and CC arms	All reduced exposure studies	Intra-batch[1]: -10.5% to 8.0% Inter-batch[2]: -6 to -3.6%	Intra-batch[1]: 1.1% to 8% Inter-batch[2]: 4.3 to 7.9%	20 to 5000 ng/mL	ZZ25187-02 (b) (4) 2014d)
Nicotine # (urine) THS, and CC arms	All reduced exposure studies	Intra-batch[1]: -13.3 to 13.0% Inter-batch[2]: -2.2 to 1.2%	Intra-batch[1]: 1.1 to 9.9% Inter-batch[2]: 5.1 to 9.4%	50 to 5000 ng/mL	ZZ33881-04 ((b) (4) 2014f)

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BoExp	Studies	Accuracy	Precision	Validation Range	(b) (4) Reference
Nicotine # (urine) SA arms	All reduced exposure studies	Intra-batch[1]: -7.0 to 2.0% Inter-batch[2]: -3.0 to 0.1%	Intra-batch[1]: 1.8 to 9.3% Inter-batch[2]: 2.5 to 5.9%	10 to 1000 ng/mL	ZZ33881-03 ((b) (4) 2016d)
Nicotine-*N*-glucuronide # (urine) THS, and CC arms	All reduced exposure studies	Intra-batch[1]: -3.6 to 3.8% Inter-batch[2]:-1.9 to 1.4%	Intra-batch[1]:1 to 7.6% Inter-batch[2]: 3.3 to 5.8%	50 to 5000 ng/mL	ZZ33881-04 (b) (4) 2014f)
Nicotine-*N*-glucuronide # (urine) SA arms	All reduced exposure studies	Intra-batch[1]: -11.3 to 2.7% Inter-batch[2]: -5.0 to 0.0%	Intra-batch[1]:1.5 to 9.2% Inter-batch[2]: 3.7 to 8.3%	10 to 1000 ng/mL	ZZ33881-03 ((b) (4) 2016d)
Cotinine # (urine) THS, and CC arms	All reduced exposure studies	Intra-batch[1]: -10.4 to 12.8% Inter-batch[2]: -2.4 to 1.8%	Intra-batch[1] : 1.8 to 5.8% Inter-batch[2]: 4.3 to 8.4%	50 to 5000 ng/mL	ZZ33881-04 (b) (4) 2014f)
Cotinine # (urine) SA arms	All reduced exposure studies	Intra-batch[1]: -10.6 to 5.3% Inter-batch[2]: -4.3 to 2.0%	Intra-batch[1] : 1.4 to 8.6% Inter-batch[2]: 2.2 to 7.2%	10 to 1000 ng/mL	ZZ33881-03 ((b) (4) 2016d)
Cotinine *N*-Glucuronide # (urine) THS, and CC arms	All reduced exposure studies	Intra-batch[1]: -11.5 to 3.7% Inter-batch[2]: -4 to 1.2%	Intra-batch[1]: 1.2 to 9.8% Inter-batch[2]: 4 to 8.1%	200 to 20000 ng/mL	ZZ33881-04 ((b) (4) 2014f)
Cotinine *N*-Glucuronide # (urine) SA arms	All reduced exposure studies	Intra-batch[1]: -9.5 to 10.2% Inter-batch[2]: -3.5 to 4.2%	Intra-batch[1]: 3.9 to 11.3% Inter-batch[2]: 6.3 to 11.5%	20 to 2000 ng/mL	ZZ33881-03 ((b) (4) 2016d)

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BoExp	Studies	Accuracy	Precision	Validation Range	(b) (4) Reference
trans.3' hydroxy-cotinine # (urine) THS, and CC arms	All reduced exposure studies	Intra-batch[1]: -12.3 to 5.4% Inter-batch[2]: -2.4 to 0.0%	Intra-batch[1]: -1.3 to 7.5% Inter-batch[2]: 3.2 to 6.9%	50 to 5000 ng/mL	ZZ33881-04 (b) (4) 2014f)
trans 3'-hydroxy-cotinine # (urine) SA arms	All reduced exposure studies	Intra-batch[1]: -9.5 to 0.3% Inter-batch[2]: -3.9 to 0.7%	Intra-batch[1]: 1.1 to 11.5% Inter-batch[2]: 1.9 to 8.1%	10 to 1000 ng/mL	ZZ33881-03 (b) (4) 2016d)
trans-3'-hydroxy-cotinine-*O*-glucuronide # (urine) THS and CC arms	All reduced exposure studies	Intra-batch[1]: -2.5 to 3% Inter-batch[2]: 0.0 to 1.0%	Intra-batch[1]: 1.3 to 8.3% Inter-batch[2]: 2.4 to 5.3%	200 to 20000 ng/mL	ZZ33881-04 (b) (4) 2014f)
trans-3' hydroxy-cotinine-*O*-glucuronide # (urine) SA arms	All reduced exposure studies	Intra-batch[1]: -7.8 to 0.0% Inter-batch[2]: -6.0% to -3.3%	Intra-batch[1]: 1.6 to 8.9% Inter-batch[2]: 3.5 to 7.4%	50 to 5000 ng/mL	ZZ33881-03 (b) (4) 2016d)

[1] Repeatability

[2] Intermediate precision

[3] To assess CYP2A6 activity (molar ratio of trans-3'-hydroxycotinine/cotinine?

* Method for MHBMA had to be revalidated due to laboratory transfer of the method (from Lincoln to Zurich)

Nicotine, nicotine-*N*-glucuronide, cotinine, cotinine-*N*-glucuronide, *trans*-3'-hydroxy-cotinine, *trans*-3'-hydroxy-cotinine-*O*-glucuronide are all parts of the nicotine equivalents (NEQ) measurement. A lower range of detection was applied for smoking abstinent arms (SA).

Abbreviations: monohydroxybutenyl mercapturic acid (MHBMA), 3-hydroxypropylmercapturic acid (3-HPMA), *S*-phenylmercapturic acid (S-PMA), Total 1-hydroxypyrene (1-OHP), Total N-nitrosonornicotine (NNN), 4-aminobiphenyl (4-ABP), 1-aminonaphthalene (1-NA), 2-aminonaphthalene (2-NA), 2-cyanoethylmercapturic acid (CEMA), 2-hydroxyethyl mercapturic acid (HEMA), 3-hydroxy(a)benzopyrene (3-OH-B[a]P), 3-hydroxy-1-methylpropylmercapturic acid (3-HMPMA), *S*-benzylmercapturic acid (S-BMA), Total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), Nicotine equivalents (NEQ) is derived from free nicotine, nicotine *N*-glucuronide, free cotinine, cotinine-*N*-glucuronide, free *trans*-3'-hydroxycotinine, *trans*-3'-hydroxycotinine-*O*-glucuronide, smoking abstinence (SA), conventional cigarette (CC), Tobacco Heating System (THS)

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Table 4 Summary of Analytical Methods using Spectrophotometry for PMI's Reduced Exposure Studies

BoExp	Studies	Accuracy	Precision	Validation Range
COHb	ZRHR-REXC-03-EU	<0.25%	Intra-batch[1]: 3.8%	0 to 100 %
(b) (4)			Inter-batch[2]: 1.82 to 4.86%	
COHb	ZRHR-REXC-04-JP	0.5 to 1.1%	Intra-batch[1]: 1.2 to 12.7%	0 to 100 %
(b) (4)	ZRHM-REXA-07-JP		Inter-batch[2]: 1 to 19.7%	
COHb	ZRHM-REXA-08-US	Intra-batch[1]: 1.3 to 16,2%	Intra-batch[1]: 1.2 to 3.5%	0.5 to 100 %
		Inter-batch[2]:0.1 to 10%	Inter-batch[2]: 0.8 to 14.5%	
Creatinine	All reduced exposure studies	Intra-batch[1]: -1.3 to 0.3%	Intra-batch[1]: 1.2% to 3.5%	12.31 to 282.6 mg/dL
		Inter-batch[2]:0 %.	Inter-batch[2]: 1.1 to 1.9%	

[1] Repeatability

[2] Intermediate precision

Abbreviations: carboxyhemoglobin (COHb)

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In the following sections, an overview of all methods, except for COHb measurement, including a brief description of the method validation and of the biological matrix, are provided.

6.1.3.3.1 *Validation of the Nicotine/Cotinine/trans-3′-Hydroxycotinine in Plasma Assay (AA33664-01)*

The full validation report is archived at (b) (4) Study AA33664-01) and is available on request (b) (4).

A summary of the validation parameters tested and the results obtained during the method validation is provided in Appendix A6.1.3.27.

In PMI's studies, the molar ratio of *trans*-3′-hydroxycotinine/cotinine, as measured with this assay, was calculated to assess CYP2A6 activity (in %).

6.1.3.3.1.1 *Description of the Analytical Method*

This analytical method was developed at (b) (4), according to the current validation SOP GL-BIO-10601. This SOP was written based on the GLP principles described in 21 CFR part 58 and the Guidance for Industry – Bioanalytical Method Validation (FDA (Food and Drug Administration) 2001). The method is documented in BAM AA33664-01. (b) (4) (b) (b) (4) (b) (4)).

An aliquot of human plasma (EDTA) containing each analyte and internal standard was extracted using a solid phase extraction procedure. The extracted samples were analyzed by an HPLC equipped with an AB SCIEX API 5000™ or QTRAP® 5500 mass spectrometer. Positive ions were monitored in the multiple reaction monitoring (MRM) mode. Quantitation was determined using a weighted linear regression analysis ($1/x^2$) of peak area ratios of each analyte and internal standard.

6.1.3.3.1.2 *Biological Matrix*

Human plasma, with K_2EDTA as anticoagulant, was purchased from (b) (4). and collected in-house at (b) (4). Human plasma (EDTA), free of significant interference at the retention time and mass transitions of nicotine, cotinine, *trans*-3′-hydroxycotinine, d_3-nicotine (IS), and d_3-cotinine (IS), d_3-*trans*-3′-hydroxycotinine (IS) was used to prepare quality control (QC) samples. Water was used to prepare calibration standards and used as control matrix.

6.1.3.3.1.3 *Validation Components*

The minimum requirements for validation included an assessment of accuracy, precision, response function, carryover, sensitivity, selectivity, matrix effect, multiple analytes measured by assay, known metabolites/isomers/endogenous compound evaluation, hemolyzed sample integrity, lipemic sample evaluation, recovery, stability (sample shipping, long-term, freeze-thaw, short-term, post-preparative, long-term stability for stock solutions, short-term stability for stock solutions, stability of analyte during sample

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collection and handling, and sample aliquot frozen storage stability), integrity (dilution integrity and processed sample integrity), and stress test.

6.1.3.3.1.3.1 Reference Standards

The reference standards used for the definitely quantitative assays used to measure nicotine, cotinine and *trans*-3′-hydroxycotinine were purchased from a reputable synthetic laboratory and provided as a solution of known concentration in ampoules. Certification for all reference materials was complete.

6.1.3.3.1.3.2 Internal Standards

The deuterated stable label internal standards utilized for the assays were also purchased from a reputable synthetic laboratory. The solutions of known concentration were provided in ampoules. Each internal standard reference solution was also assessed for the presence of unlabeled analyte.

6.1.3.3.1.3.3 Standard Curve Performance

The establishment of an assay to measure nicotine concentrations below 10 ng/mL requires great diligence in the elimination of sources of contamination. Nicotine is nearly ubiquitous even in the laboratory environment. Nanogram levels of nicotine have been observed on new plastic tubes, bottles, pipette tips, 96-well plates. Double deionized NANOpureTM water was also found to contain small amounts of nicotine which could concentrate during sample extraction.

To eliminate the different sources of contamination the following steps were taken. Only non-smokers processed the clinical study samples, transferring the plasma from centrifuged vacutainers to pre-rinsed storage tubes using pre-rinsed plastic pipettes. The pre-rinsed supplies for the clinical study sample collection were supplied by (b) (4) to the central laboratories responsible for assembling the collection kits. The storage tubes were capped and the pipettes were bagged to avoid the possibility of contamination occurrence after the pre-rinsing.

All surfaces used to perform the sample extraction including beakers, pipette tips, and 96-well collection plates were pre-rinsed prior to use. All solutions used in the sample extraction process were sub-aliquoted into pre-rinsed bottles and only used on a single occasion. Additional volume, if it remained unused at the end of the day, was discarded to avoid contamination from exposure to nicotine in the air. Sub-aliquots of the solvents were pre-screened prior to use to ensure the absence of nicotine contamination.

Standard calibrator performance was acceptable, though on occasion, small contaminations could result in the loss of the lower limit of quantitation for nicotine. This issue was resolved through the successful reanalysis of the impacted samples.

Multiple linear ranges were employed to measure nicotine, cotinine and *trans*-3′-hydroxycotinine. The assay with the greatest sensitivity, the “trace nicotine assay” (linear ranges of 0.200 to 10.0 ng/mL, 1.00 to 100 ng/mL and 1.00 to 100 ng/mL respectively), was utilized for all of the pK study sample analysis. The biomarker studies however utilized both validated linear ranges. The clinical study samples for subjects that were randomized to the *ad libitum* group which used conventional cigarettes or the test product

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were assayed with the higher concentration linear range (1.00 to 50.0 ng/mL, 5.00 to 500 ng/mL, and 5.00 to 500 ng/mL respectively). Clinical study samples from subjects that were randomized to smoking abstinence were assayed with the trace nicotine assay.

6.1.3.3.1.3.4 Precision, Accuracy, Sensitivity, and Stability

Precision and accuracy, sensitivity and stability results from the validated method for the nicotine, cotinine, and *trans*-3′-hydroxycotinine assays are provided in the bioanalytical method summaries for each study.

6.1.3.3.1.3.5 Specificity

The nicotine assays have been demonstrated to be selectively quantitative in the presence of other nicotine metabolites such as the glucuronides of the target analytes. Additionally selectivity testing was performed with an over-the-counter cocktail covering the major classes of medications.

6.1.3.3.2 Validation of the Nicotine/Cotinine in Plasma Assay (AA33664-06)

The full validation report is archived at (b) (4) Study AA33664-06) and is available on reques (b) (4) .

A summary of the validation parameters tested and the results obtained during the method validation is provided in Appendix A6.1.3.28.

In PMI's studies, this assay was used to assess nicotine and cotinine plasma levels in the SA arms of the reduced exposure studies, as it has a higher sensitivity than the method AA33664-07 or AA33664-08. It was also used to assess plasma nicotine levels in all PK studies, and plasma cotinine levels in the ZRHR-PK-06-US study.

6.1.3.3.2.1 Description of the Analytical Method

The analytical method was developed at (b) (4) (b) (4) and validated according to the standard operating procedures (SOPs) in effect during the conduct of the validation. These SOPs were written based on the good laboratory practices (GLP) principles described in 21 CFR Part 58 and the Guidance for Industry – Bioanalytical Method Validation (FDA (Food and Drug Administration) 2001).The analytical method is documented in BAM SOP AA33664-06. (b) (4) (b) (4)).

An aliquot of human plasma (EDTA) containing the analyte and internal standard was extracted using a solid phase extraction procedure. The extracted samples were analyzed by an HPLC equipped with an AB SCIEX API 4000™ or API 5000™ triple quadrupole mass spectrometers using an ESI source. Positive ions were monitored in the multiple reaction monitoring (MRM) mode. Quantitation was determined using a weighted linear regression analysis ($1/concentration^2$) of peak area ratios of the analyte and internal standard. The reference standard materials for nicotine, cotinine, d_4-nicotine (IS), and d_3-cotinine (IS) are light sensitive. All storage and handling of reference standard material for these analytes should be conducted under UV-shielded light conditions (i.e. protected from white light).

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6.1.3.3.2.2 Biological Matrix

Human plasma, with K_2EDTA as anticoagulant, was purchased from (b) (4) and collected in-house at (b) (4) (b) (4) Human plasma (EDTA), free of significant interference at the retention time and mass transitions of nicotine, cotinine, d_4-nicotine (IS), and d_3-cotinine (IS) was used to prepare quality control (QC) samples. Water was used to prepare calibration standards and used as control matrix.

6.1.3.3.2.3 Validation Components

The minimum requirements for validation included an assessment of accuracy, precision, response function, carryover, sensitivity, selectivity, matrix effect, matrix factor, multiple analytes measured by assay, over-the-counter cocktail testing, known metabolites/isomers/endogenous compound evaluation, hemolyzed sample integrity, lipemic sample evaluation, recovery, stability (sample shipping, long-term, freeze-thaw, short-term, post-preparative, long-term stability for stock solutions, short-term stability for stock solutions, stability of analyte during sample collection and handling, and sample aliquot frozen storage stability), integrity (dilution integrity and processed sample integrity), and stress test.

6.1.3.3.2.3.1 Reference Standards

The reference standards used for the definitely quantitative assays used to measure nicotine and cotinine were purchased from a reputable synthetic laboratory and provided as a solution of known concentration in ampoules. Certification for all reference materials was complete.

6.1.3.3.2.3.2 Internal Standards

The deuterated stable label internal standards utilized for the assays were also purchased from a reputable synthetic laboratory. The solutions of known concentration were provided in ampoules. Each internal standard reference solution was also assessed for the presence of unlabeled analyte.

6.1.3.3.2.3.3 Standard Curve Performance

The establishment of an assay to measure nicotine concentrations below 10 ng/mL requires great diligence in the elimination of sources of contamination. Nicotine is nearly ubiquitous even in the laboratory environment. Nanogram levels of nicotine have been observed on new plastic tubes, bottles, pipette tips, 96-well plates. Double deionized NANOpureTM water was also found to contain small amounts of nicotine which could concentrate during sample extraction.

To eliminate the different sources of contamination the following steps were taken. Only non-smokers processed the clinical study samples, transferring the plasma from centrifuged vacutainers to pre-rinsed storage tubes using pre-rinsed plastic pipettes. The pre-rinsed supplies for the clinical study sample collection were supplied by (b) (4) to the central laboratories responsible for assembling the collection kits. The storage tubes were capped and the pipettes were bagged to avoid the possibility of contamination occurrence after the pre-rinsing.

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All surfaces used to perform the sample extraction including beakers, pipette tips, and 96-well collection plates were pre-rinsed prior to use. All solutions used in the sample extraction process were sub-aliquoted into pre-rinsed bottles and only used on a single occasion. Additional volume, if it remained unused at the end of the day, was discarded to avoid contamination from exposure to nicotine in the air. Sub-aliquots of the solvents were pre-screened prior to use to ensure the absence of nicotine contamination.

Standard calibrator performance was acceptable, though on occasion, small contaminations could result in the loss of the lower limit of quantitation for nicotine. This issue was resolved through the successful reanalysis of the impacted samples.

Multiple linear ranges were employed to measure nicotine and cotinine. The assay with the greatest sensitivity, the "trace nicotine assay" (linear ranges of 0.200 to 10.0 ng/mL, 1.00 to 100 ng/mL and 1.00 to 100 ng/mL respectively), was utilized for all of the pK study sample analysis. The biomarker studies however utilized both validated linear ranges. The clinical study samples for subjects that were randomized to the *ad libitum* group which used conventional cigarettes or the test product were assayed with the higher concentration linear range (1.00 to 50.0 ng/mL, 5.00 to 500 ng/mL, and 5.00 to 500 ng/mL respectively). Clinical study samples from subjects that were randomized to smoking abstinence were assayed with the trace nicotine assay.

6.1.3.3.2.3.4 Precision, Accuracy, Sensitivity, and Stability

Precision and accuracy, sensitivity and stability results from the validated method for the nicotine and cotinine assays are provided in the bioanalytical method summaries for each study.

6.1.3.3.2.3.5 Specificity

The nicotine assays have been demonstrated to be selectively quantitative in the presence of other nicotine metabolites such as the glucuronides of the target analytes. Additionally selectivity testing was performed with an over-the-counter cocktail covering the major classes of medications.

6.1.3.3.3 Validation of the Nicotine/Cotinine in Plasma Assay (AA33664-07)

The full validation report is archived at (b) (4) Study AA33664-07) and is available on request (b) (4)

A summary of the validation parameters tested and the results obtained during the method validation is provided in Appendix A6.1.3.29.

6.1.3.3.3.1 Description of the Analytical Method

The analytical method was developed at (b) (4), and validated according to the standard operating procedures (SOPs) in effect during the conduct of the validation. These SOPs were written based on the good laboratory practices (GLP) principles described in 21 CFR Part 58 and the Guidance for Industry – Bioanalytical Method Validation (FDA (Food and Drug Administration) 2001). The analytical method

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is documented in BAM SOP AA33664-(b) (4) (b) (4) (b) (4) 2014a).

An aliquot of human plasma (EDTA) containing the analyte and internal standard was extracted using a solid phase extraction procedure. The extracted samples were analyzed by an HPLC equipped with an AB SCIEX API 4000™ or API 5000™ triple quadrupole mass spectrometers using an ESI source. Positive ions were monitored in the multiple reaction monitoring (MRM) mode. Quantitation was determined using a weighted linear regression analysis ($1/concentration^2$) of peak area ratios of the analyte and internal standard.

The reference standard materials for nicotine, cotinine, d_4-nicotine (IS), and d_3-cotinine (IS) are light sensitive. All storage and handling of reference standard material for these analytes should be conducted under UV-shielded light conditions (i.e. protected from white light).

6.1.3.3.3.2 Biological Matrix

Human plasma, with K_2EDTA as anticoagulant, was purchased from (b) (4). and collected in-house at (b) (4). Human plasma (EDTA), free of significant interference at the retention time and mass transitions of nicotine, cotinine, d_4-nicotine (IS), and d_3-cotinine (IS) was used to prepare quality control (QC) samples. Water was used to prepare calibration standards and used as control matrix.

6.1.3.3.3.3 Validation Components

The minimum requirements for validation included an assessment of accuracy, precision, response function, carryover, sensitivity, selectivity, matrix effect, matrix factor, multiple analytes measured by assay, over-the-counter cocktail testing, known metabolites/isomers/endogenous compound evaluation, hemolyzed sample integrity, lipemic sample evaluation, recovery, stability (sample shipping, long-term, freeze-thaw, short-term, post-preparative, long-term stability for stock solutions, short-term stability for stock solutions, stability of analyte during sample collection and handling, and sample aliquot frozen storage stability), integrity (dilution integrity and processed sample integrity), and stress test.

6.1.3.3.3.3.1 Reference Standards

The reference standards used for the definitely quantitative assays used to measure nicotine and cotinine were purchased from a reputable synthetic laboratory and provided as a solution of known concentration in ampoules. Certification for all reference materials was complete.

6.1.3.3.3.3.2 Internal Standards

The deuterated stable label internal standards utilized for the assays were also purchased from a reputable synthetic laboratory. The solutions of known concentration were provided in ampoules. Each internal standard reference solution was also assessed for the presence of unlabeled analyte.

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6.1.3.3.3.3.3 Standard Curve Performance

The establishment of an assay to measure nicotine concentrations below 10 ng/mL requires great diligence in the elimination of sources of contamination. Nicotine is nearly ubiquitous even in the laboratory environment. Nanogram levels of nicotine have been observed on new plastic tubes, bottles, pipette tips, 96-well plates. Double deionized NANOpureTM water was also found to contain small amounts of nicotine which could concentrate during sample extraction.

To eliminate the different sources of contamination the following steps were taken. Only non-smokers processed the clinical study samples, transferring the plasma from centrifuged vacutainers to pre-rinsed storage tubes using pre-rinsed plastic pipettes. The pre-rinsed supplies for the clinical study sample collection were supplied by (b) (4) to the central laboratories responsible for assembling the collection kits. The storage tubes were capped and the pipettes were bagged to avoid the possibility of contamination occurrence after the pre-rinsing.

All surfaces used to perform the sample extraction including beakers, pipette tips, and 96-well collection plates were pre-rinsed prior to use. All solutions used in the sample extraction process were sub-aliquoted into pre-rinsed bottles and only used on a single occasion. Additional volume, if it remained unused at the end of the day, was discarded to avoid contamination from exposure to nicotine in the air. Sub-aliquots of the solvents were pre-screened prior to use to ensure the absence of nicotine contamination.

Standard calibrator performance was acceptable, though on occasion, small contaminations could result in the loss of the lower limit of quantitation for nicotine. This issue was resolved through the successful reanalysis of the impacted samples.

Multiple linear ranges were employed to measure nicotine and cotinine. The assay with the greatest sensitivity, the "trace nicotine assay" (linear ranges of 0.200 to 10.0 ng/mL, 1.00 to 100 ng/mL and 1.00 to 100 ng/mL respectively), was utilized for all of the pK study sample analysis. The biomarker studies however utilized both validated linear ranges. The clinical study samples for subjects that were randomized to the *ad libitum* group which used conventional cigarettes or the test product were assayed with the higher concentration linear range (1.00 to 50.0 ng/mL, 5.00 to 500 ng/mL, and 5.00 to 500 ng/mL respectively). Clinical study samples from subjects that were randomized to smoking abstinence were assayed with the trace nicotine assay.

6.1.3.3.3.3.4 Precision, Accuracy, Sensitivity, and Stability

Precision and accuracy, sensitivity and stability results from the validated method for the nicotine and cotinine assays are provided in the bioanalytical method summaries for each study.

6.1.3.3.3.3.5 Specificity

The nicotine assays have been demonstrated to be selectively quantitative in the presence of other nicotine metabolites such as the glucuronides of the target analytes. Additionally selectivity testing was performed with an over-the-counter cocktail covering the major classes of medications.

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6.1.3.3.4 *Validation of the Nicotine/Cotinine in Plasma Assay (AA33664-08)*

The full validation report is archived at (b) (4) Study AA33664-08) and is available on reques (b) (4)

A summary of the validation parameters tested and the results obtained during the method validation is provided in Appendix A6.1.3.30.

The method is an updated version of the method AA33664-07, with a pre-column added to the chromatographic set up. This step was added to protect the longevity and consistency of the chromatography for the analytical column used for separation. It was used for the THS and CC arms of the ZRHM-REXA-07-JP study.

6.1.3.3.4.1 *Description of the Analytical Method*

The analytical method was developed at (b) (4) (b) (4) and validated according to the standard operating procedures (SOPs) in effect during the conduct of the validation. These SOPs were written based on the good laboratory practices (GLP) principles described in 21 CFR Part 58 and the Guidance for Industry – Bioanalytical Method Validation (FDA (Food and Drug Administration) 2001). The analytical method is documented in BAM SOP AA33664-08. (b) (4) (b) (4)

An aliquot of human plasma (EDTA) containing the analyte and internal standard was extracted using a solid phase extraction procedure. The extracted samples were analyzed by an HPLC equipped with an AB SCIEX API 4000™ or API 5000™ triple quadrupole mass spectrometers using an ESI source. Positive ions were monitored in the multiple reaction monitoring (MRM) mode. Quantitation was determined using a weighted linear regression analysis ($1/concentration^2$) of peak area ratios of the analyte and internal standard.

The reference standard materials for nicotine, cotinine, d_4-nicotine (IS), and d_3-cotinine (IS) are light sensitive. All storage and handling of reference standard materials for these analytes should be conducted under UV-shielded light conditions (i.e. protected from white light).

6.1.3.3.4.2 *Biological Matrix*

Human plasma, with K_2EDTA as anticoagulant, was purchased from (b) (4) . and collected in-house at (b) (4) . Human plasma (EDTA), free of significant interference at the retention time and mass transitions of nicotine, cotinine, d_4-nicotine (IS), and d_3-cotinine (IS) was used to prepare quality control (QC) samples. A surrogate matrix, water, was used to prepare calibration standard samples and as control matrix.

6.1.3.3.4.3 *Validation Components*

The minimum requirements for validation included an assessment of accuracy, precision, response function, carryover, sensitivity, selectivity, matrix effect, matrix factor, multiple analytes measured by assay, over-the-counter cocktail testing, known metabolites/isomers/endogenous compound evaluation, hemolyzed sample integrity,

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lipemic sample evaluation, recovery, stability (sample shipping, long-term, freeze-thaw, short-term, post-preparative, long-term stability for stock solutions, short-term stability for stock solutions, stability of analyte during sample collection and handling, sample aliquot frozen storage stability, multiple analyte stability evaluation [long-term, freeze-thaw, short-term]), and integrity (dilution integrity and processed sample integrity), and stress test.

6.1.3.3.4.3.1 Reference Standards

The reference standards used for the definitely quantitative assays used to measure nicotine and cotinine were purchased from a reputable synthetic laboratory and provided as a solution of known concentration in ampoules. Certification for all reference materials was complete.

6.1.3.3.4.3.2 Internal Standards

The deuterated stable label internal standards utilized for the assays were also purchased from a reputable synthetic laboratory. The solutions of known concentration were provided in ampoules. Each internal standard reference solution was also assessed for the presence of unlabeled analyte.

6.1.3.3.4.3.3 Standard Curve Performance

The establishment of an assay to measure nicotine concentrations below 10 ng/mL requires great diligence in the elimination of sources of contamination. Nicotine is nearly ubiquitous even in the laboratory environment. Nanogram levels of nicotine have been observed on new plastic tubes, bottles, pipette tips, 96-well plates. Double deionized NANOpure^{TM} water was also found to contain small amounts of nicotine which could concentrate during sample extraction.

To eliminate the different sources of contamination the following steps were taken. Only non-smokers processed the clinical study samples, transferring the plasma from centrifuged vacutainers to pre-rinsed storage tubes using pre-rinsed plastic pipettes. The pre-rinsed supplies for the clinical study sample collection were supplied by (b) (4) to the central laboratories responsible for assembling the collection kits. The storage tubes were capped and the pipettes were bagged to avoid the possibility of contamination occurrence after the pre-rinsing.

All surfaces used to perform the sample extraction including beakers, pipette tips, and 96-well collection plates were pre-rinsed prior to use. All solutions used in the sample extraction process were sub-aliquoted into pre-rinsed bottles and only used on a single occasion. Additional volume, if it remained unused at the end of the day, was discarded to avoid contamination from exposure to nicotine in the air. Sub-aliquots of the solvents were pre-screened prior to use to ensure the absence of nicotine contamination.

Standard calibrator performance was acceptable, though on occasion, small contaminations could result in the loss of the lower limit of quantitation for nicotine. This issue was resolved through the successful reanalysis of the impacted samples.

Multiple linear ranges were employed to measure nicotine and cotinine. The assay with the greatest sensitivity, the “trace nicotine assay” (linear ranges of 0.200 to 10.0 ng/mL,

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1.00 to 100 ng/mL and 1.00 to 100 ng/mL respectively), was utilized for all of the pK study sample analysis. The biomarker studies however utilized both validated linear ranges. The clinical study samples for subjects that were randomized to the *ad libitum* group which used conventional cigarettes or the test product were assayed with the higher concentration linear range (1.00 to 50.0 ng/mL, 5.00 to 500 ng/mL, and 5.00 to 500 ng/mL respectively). Clinical study samples from subjects that were randomized to smoking abstinence were assayed with the trace nicotine assay.

6.1.3.3.4.3.4 Precision, Accuracy, Sensitivity, and Stability

Precision and accuracy, sensitivity and stability results from the validated method for the nicotine and cotinine assays are provided in the bioanalytical method summaries for each study.

6.1.3.3.4.3.5 Specificity

The nicotine assays have been demonstrated to be selectively quantitative in the presence of other nicotine metabolites such as the glucuronides of the target analytes. Additionally selectivity testing was performed with an over-the-counter cocktail covering the major classes of medications.

6.1.3.3.5 Validation of the 3-HPMA/HBMA/CEMA in Human urine (ZZ36536-01)

HBMA in PMI's studies is referred to as 3-HMPMA.

The full validation report is archived at (b) (4) Study ZZ36536-01) and is available on request (b) (4)

A summary of the validation parameters tested and the results obtained during the method validation is provided in Appendix A6.1.3.31.

6.1.3.3.5.1 Description of the Analytical Method

The analytical method was developed at (b) (4), and validated according to the standard operating procedures (SOPs) in effect during the conduct of the validation. These SOPs were written based on the good laboratory practices (GLP) principles described in 21 CFR Part 58 and the Guidance for Industry – Bioanalytical Method Validation (FDA (Food and Drug Administration) 2001). The analytical method is documented in BAM SOP ZZ36536-01. (b) (4) (b) (4)

An aliquot of human urine containing the analyte and internal standard was extracted using a solid phase extraction procedure. The extracted samples were analyzed by an HPLC equipped with an AB SCIEX API 4000™ triple quadrupole mass spectrometer using an ESI source. Negative ions were monitored in the multiple reaction monitoring (MRM) mode. Quantitation was determined using a weighted linear regression analysis ($1/concentration^2$) of peak area ratios of the analyte and internal standard.

3-HPMA, HBMA, CEMA, [$^{15}N^{13}C_3$]3-HPMA (IS), [$^{15}N^{13}C_3$]HBMA (IS), and [$^{15}N^{13}C_3$]CEMA (IS) are light sensitive. All handling of samples, extracts, and solutions

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containing these analytes should be conducted under UV-shielded light conditions (i.e. protected from white light).

6.1.3.3.5.2 Biological Matrix

UriSub®, a urine substitute, was purchased from a commercial supplier. UriSub® was used to prepare calibration standards, LLOQ QC, QC S, and as control matrix.

Human urine was purchased from (b) (4) . and collected in-house at (b) (4) (b) (4) Individual human urine lots were analyzed to evaluate the concentration of individual mercapturic acids. Urine lots collected from non-smokers were used to create a pool to mimic low basal level (QC A, no dilution was needed) and to prepare QC B. The pooled non-smoker urine was also used for preparation of QC A to mimic low basal level by not more than 2.5-fold dilution. Urine lots collected from smokers and non-smokers was used to create a pool to mimic "light smokers" urinary concentrations of mercapturic acids and were used for preparation of QC C – E.

6.1.3.3.5.3 Validation Components

The minimum requirements for validation included an assessment of accuracy, precision, response function, sensitivity, selectivity, matrix effect, multiple analytes measured by assay, known metabolites/isomers/endogenous compound evaluation, additional selectivity, recovery, stability (sample shipping, long-term, freeze-thaw, short-term, post-preparative, long-term stability for stock solutions, short-term stability for stock solutions, stability of analyte during sample collection and handling, sample aliquot frozen storage stability), integrity (dilution integrity and processed sample integrity), and stress test.

6.1.3.3.5.3.1 Reference Standards

The reference materials used for validation and clinical sample analysis were provided either as known concentration solution in ampoules or as a dried salt. The HBMA (HMPMA) reference material was provided as a mixture of diasteriomers ([R, R] + [R,S]).

6.1.3.3.5.3.2 Internal Standards

The internal standards used for both method validation and clinical sample analysis were carbon (C^{13}) and nitrogen (N^{15}) heavy. By placing the labeled atoms on the acetylated cysteine portion of the molecule the strength of the chromatography could be improved using UPLC technology. The selectivity of the mass spectrometric detection also improved as the daughter ion monitored also included the labeled fragment.

6.1.3.3.5.3.3 Standard Curve Performance

The linear ranges for all 3 analytes are appropriately placed to accurately quantitate more than 95% of the clinical samples collected from all treatment types. Commercially available synthetic urine was used as the control matrix. The synthetic urine was also used as a diluent for quality control samples or clinical samples that required dilution for analysis.

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6.1.3.3.5.3.4 Precision and Accuracy

Quality control samples for these biomarkers of exposure were prepared from two separate basal pools of urine. The lower concentration pool of urine was selected from non-smoker lots. The low concentration pool was diluted with ultrapure water to yield a quality control with a measured concentration near or below 3 times the LLOQ. The low concentration urine pool was supplemented with known concentrations of each analyte to product a mid-low quality control concentration. A second pool of urine was prepared from light smoker lots of urine to provide a high basal pool concentration sample in the concentration ranges of 500 – 2500 ng/mL for 3-HPMA, 150 – 500 ng/mL for HBMA and 27.6 – 82.8 ng/mL for CEMA. The higher concentration urine pool was also supplemented with known concentrations of each analyte to produce a quality control concentration that is approximately 80% of the ULOQ in concentration. Finally, the high concentration pool was also supplemented with higher concentrations of known analyte to produce a quality control sample that would be appropriate for dilution testing if required.

As a measure of precision, all quality controls demonstrated adequate performance. Accuracy may be determined from each of the supplemented quality controls. The spiked additions consistently demonstrated parallelism in quantitation to the calibration standards which were prepared in an artificial urine.

6.1.3.3.5.3.5 Stability

Stability results from the validation method for 3-HPMA, HBMA, and CEMA are provided in the bioanalytical method summaries for each study and summarized in Table 5.

Extended stability testing completed for ambulatory collection coverage was completed with the following results. Stability at each temperature tested (5°C, 20°C and 40°C) was established for the following lengths of time. The test samples were stored in a polyethylene container that is designed to protect the clinical samples from UV light exposure.

Table 5 Stability Results for 3-HPMA, HBMA, and CEMA

Analyte	5°C	20°C	40°C
3-HPMA	96 hours	96 hours	96 hours
HBMA	96 hours	96 hours	48 hours
CEMA	96 hours	96 hours	96 hours

6.1.3.3.5.3.6 Sensitivity, Stability and Specificity

Sensitivity, stability results from the validation testing for 3-HPMA, HBMA, and CEMA are provided in the bioanalytical method summaries for each study.

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6.1.3.3.6 *Validation of Total NNAL and total NNN in Human urine (ZZ34313-03)*

The full validation report is archived at (b) (4) Study ZZ34313-03) and is available on request (b) (4)

A summary of the validation parameters tested and the results obtained during the method validation is provided in Appendix A6.1.3.32.

6.1.3.3.6.1 *Description of the Analytical Method*

The analytical method was developed at (b) (4) (b) (4) and validated according to the standard operating procedures (SOPs) in effect during the conduct of the validation. These SOPs were written based on the good laboratory practices (GLP) principles described in 21 CFR Part 58 and the Guidance for Industry – Bioanalytical Method Validation (FDA (Food and Drug Administration) 2001). The analytical method is documented in BAM SOP ZZ34313-03. (b) (4) (b) (4)

An aliquot of human urine containing the analyte and internal standard was extracted using a solid phase extraction procedure. The extracted samples were analyzed by an HPLC equipped with an AB SCIEX API 4000™ and AB SCIEX Triple Quad™ 6500 triple quadrupole mass spectrometers using an ESI source. Positive ions were monitored in the multiple reaction monitoring (MRM) mode. Quantitation was determined using a weighted linear regression analysis ($1/concentration^2$) of peak area ratios of the analyte and internal standard.

NNAL, NNN, d_4-NNAL (IS), and d_4-NNN (IS) are light sensitive. All handling of samples, extracts, and solutions containing these analytes should be conducted under UV-shielded light conditions (i.e. protected from white light).

6.1.3.3.6.2 *Biological Matrix*

Human urine was collected in-house at (b) (4). Human urine, free of significant interference at the retention time and mass transitions of NNAL, NNN, d_4-NNAL (IS), and d_4-NNN (IS) was used to prepare quality control (QC) samples and for dilution of samples above the analytical range. Ultrapure water was used to prepare calibration standards and as control matrix.

6.1.3.3.6.3 *Validation Components*

The minimum requirements for validation included an assessment of accuracy, precision, response function, carryover, sensitivity, selectivity, matrix effect, matrix factor, multiple analytes measured by assay, co-administered compound evaluation, known metabolites/isomers/endogenous compound evaluation, additional selectivity, recovery, stability (sample shipping, long-term, freeze-thaw, short-term, post-preparative, long-term stability for stock solutions, short-term stability for stock solutions, stability of analyte during sample collection and handling, and co-administered compound stability evaluation [long-term, freeze-thaw, short-term]), and integrity (dilution integrity and processed sample integrity).

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6.1.3.3.6.3.1 Reference Standards

Both NNAL (4-(methylnitrosamino-1-(3-pyridyl)-1-butanol) and NNN (N′-nitrosonornicotine) reference materials were purchased from reputable synthetic laboratories. The reference materials were received as solutions of a known concentration in amber ampoules. At the time of the validation reference materials for all pertinent metabolites of NNAL and NNN were not available commercially. The *N*-glucuronide of NNAL was available for purchase however the naturally occurring *O*-glucuronide of NNAL and the *N*-glucuronide of NNN were not available. For this reason lots of urine collected from consented smokers were used to optimize the assay hydrolysis.

6.1.3.3.6.3.2 Internal Standards

Deuterated internal standards purchased from a reputable synthetic laboratory were used for method validation and the quantitation of clinical samples. It should be noted that the use of ultrapure water as a control matrix for the calibration standards and variability observed in the overall response led to the use of a broader acceptance criteria for internal standard response for this assay. The internal standard response is expected to be within 30% to 180% of the mean internal standard response of the standard calibrators and quality control samples. The standard approach at (b) (4) is a 50% to 180% acceptance criterion.

6.1.3.3.6.3.3 Standard Curve Performance and Sensitivity

The standard calibrators for the total NNAL and NNN assay were prepared in ultrapure water. The initial assay validated to measure both total NNAL and NNN had limits of quantitation of 5.00 pg/mL and 0.750 pg/mL respectively. Though this was the limit that instrument sensitivity allowed at the time, the LLOQ for NNN was not sufficient to measure a large number of clinical samples for subjects that were randomized to certain test products and smoking cessation. This issue was observed in the pilot studies for the P1 program. (b) (4) therefore, with the purchase of more sensitive instrumentation, re-validated the assay with a reduced LLOQ of 0.200 pg/mL. With this modification it became possible to begin observing exposures to nitrosamines that were previously below the limit of quantitation. It is possible that some of the exposures observed were related to diet as many of the urine volunteers were not smokers. To avoid the impact of basal concentrations within the urine control matrix it was necessary to use ultrapure water to prepare the standard calibrators.

6.1.3.3.6.3.4 Precision, Accuracy and Specificity

Quality control samples were prepared in non-smoker urine that had been screened to ensure any observed concentrations were suitably low to eliminate the possibility of bias in the measured concentration.

During the method validation quality control samples were supplemented with N′-nitrosoanatabine, N′-nitrosoanabasine and nornicotine. It has been reported that nornicotine, under an acidic environment may form NNN. To determine the impact of the assay on the formation of NNN from nornicotine quality control samples were fortified to concentrations of 20.0, 40.0 and 100 ng/mL. Supplementation of quality control samples with nornicotine had no impact on the measured results for total NNAL at any of the

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concentrations measured. The impact on NNN quantitation was concentration specific. At the lowest level of nornicotine tested (20 ng/mL) there was no impact on all concentrations measured at and above the low quality control concentration (0.600 ng/mL). At 40.0 and 100 ng/mL of nornicotine a positive bias is observed at the low QC concentration of 22% and 68% respectively. It is expected that at higher concentrations of the minor nicotine metabolite nornicotine typical NNN concentrations around 4 pg/mL are observed. The impact for NNN measurement at higher concentrations with this assay would be within bioanalytical acceptance (~10% positive bias). The conversion rate observed during method qualification was 2.2 ppm and during method validation 4.6 ppm. To demonstrate the analytical selectivity 10 lots of smoker urine were screened for both NNN and nornicotine, providing the conversion impact that may be expected. All positive biases were less than 6% of the measured NNN concentration.

To limit the impact of conversion within the sample processing the steps that include an acidic modifier are required to progress without delay. This specific comment is included within the method instructions.

6.1.3.3.6.3.5 Stability

Stability results from the validation method for total NNAL and NNN are provided in the bioanalytical method summaries for each study and summarized in Table 6. Stability testing included the use of a high concentration smoker quality control to demonstrate stability in the presence of as many of the other tobacco constituents that may be expected to be in the clinical samples.

Extended stability testing completed for ambulatory collection coverage was completed with the following results. Stability at each temperature tested (5°C, 20°C and 40°C) was established for the following lengths of time. The storage conditions were a polyethylene container that is designed to protect the clinical samples from UV light exposure.

Table 6 Stability Results for NNAL and NNN

Analyte	5°C	20°C	40°C
NNAL	96 hours	96 hours	96 hours
NNN	96 hours	96 hours	96 hours

6.1.3.3.7 Validation of HEMA in Human urine (ZZ38073-01)

A summary of the validation parameters tested and the results obtained during the method validation is provided in Appendix A6.1.3.33.

The full validation report is archived at (b) (4) Study ZZ38073-01) and is available on request ((b) (4)

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6.1.3.3.7.1 Description of the Analytical Method

The analytical method was developed at (b) (4) (b) (4) and validated according to the standard operating procedures (SOPs) in effect during the conduct of the validation. These SOPs were written based on the good laboratory practices (GLP) principles described in 21 CFR Part 58 and the Guidance for Industry – Bioanalytical Method Validation (FDA (Food and Drug Administration) 2001). The analytical method is documented in BAM SOP ZZ38073-01. (b) (4) (b) (4)

An aliquot of human urine containing the analyte and internal standard was extracted using a solid phase extraction procedure. The extracted samples were analyzed by an HPLC equipped with an AB SCIEX API 5000™ or QTRAP® 5500 triple quadrupole mass spectrometers using an ESI source. Negative ions were monitored in the multiple reaction monitoring (MRM) mode. Quantitation was determined using a weighted linear regression analysis (1/concentration^2) of peak area ratios of the analyte and internal standard.

HEMA and $^{15}N^{13}C_3$-HEMA (IS) are light sensitive. All handling of samples and extracts containing these analytes should be conducted under UV-shielded light conditions (i.e. protected from white light).

6.1.3.3.7.2 Biological Matrix

Human urine was collected in-house at (b) (4). Human urine was screened for interference at the retention time and mass transitions of HEMA and $^{15}N^{13}C_3$-HEMA (IS). Individual human urine lots were used to prepare quality control (QC) samples. UriSub® (a urine substitute was purchased from a commercial supplier and used to prepare calibration standards, LLOQ QC, and as control matrix.

6.1.3.3.7.3 Validation Components

The minimum requirements for validation included an assessment of accuracy, precision, response function, carryover, sensitivity, selectivity, matrix effect, matrix factor, recovery, stability (sample shipping, long-term, freeze-thaw, short-term, post-preparative, long-term stability for stock solutions, short-term stability for stock solutions, stability of analyte during sample collection and handling, and sample aliquot frozen storage stability), integrity (dilution integrity and processed sample integrity), and stress test.

6.1.3.3.7.3.1 Reference Standards

The reference material used for quantitation is the exact metabolite that is excreted after exposure to ethylene oxide. The reference material was provided as a dicyclohexylamine salt and was weighed as a dry solid to prepare duplicate stock solutions.

6.1.3.3.7.3.2 Internal Standards

The internal standard used for the quantitation of HEMA was a carbon (13C) and nitrogen (15N) heavy stable label isotope. By placing the labeled atoms on the acetylated cysteine portion of the molecule the strength of the chromatography could be improved

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using UPLC technology. The selectivity of the mass spectrometric detection also improved as the daughter ion monitored also included the labeled fragment.

6.1.3.3.7.3.3 Standard Curve Performance

The linear range was appropriately placed to accurately quantitate more than 95% of the clinical samples collected from all treatment types. Commercially available synthetic urine was used as the control matrix. The synthetic urine was also used as a diluent for quality control samples or clinical samples that required dilution for analysis.

6.1.3.3.7.3.4 Precision and Accuracy

Quality control samples for the biomarker of exposure were prepared from two separate basal pools of urine. The lower concentration pool of urine was selected from non-smoker lots. The low concentration pool was selected to yield a quality control with a measured concentration near or below 3 times the LLOQ. A second pool of urine was prepared from smoker lots of urine to provide a mid-level basal pool of urine to generate mid and high concentration quality control samples through supplementation. The mid-level urine pool was supplemented to achieve concentrations near the geometric mean of the linear range and approximately 80% of the ULOQ concentration. Finally, the mid concentration pool was also supplemented with approximately 49 ng/mL of HEMA to produce a quality control sample that would be appropriate for dilution testing if required.

As a measure of precision, all quality controls demonstrated adequate performance. Accuracy may be determined from each of the supplemented quality controls. The spiked additions consistently demonstrated parallelism in quantitation to the calibration standards which were prepared in artificial urine.

6.1.3.3.7.3.5 Stability

Stability results from the validation method for HEMA are provided in the bioanalytical method summaries for each study and are summarized in Table 7.

Extended stability testing completed for ambulatory collection coverage was completed with the following results. Stability at each temperature tested (5°C, 20°C and 40°C) was established for the following lengths of time. The test samples were stored in a polyethylene container that is designed to protect the clinical samples from UV light exposure.

Table 7 Stability Results for HEMA

Analyte	5°C	20°C	40°C
HEMA	96 hours	96 hours	48 hours

6.1.3.3.7.3.6 Sensitivity and Specificity

Sensitivity and stability results from the validation testing for HEMA are provided in the bioanalytical method summaries for each study.

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6.1.3.3.8 Validation of Nicotine, cotinine, trans-3′-hydroxycotinine, nicotine-n-glucuronide, cotinine-N-glucuronide, trans-3′-hydroxycotinine-o-glucuronide in Human urine (ZZ33881-03)

In PMI's studies, the analytes measured by this method are referred to as "nicotine equivalents" (NEQ).

The full validation report is archived at (b) (4) Study ZZ33881-03) and is available on reques (b) (4)

A summary of the validation parameters tested and the results obtained during the method validation is provided in Appendix A6.1.3.34.

6.1.3.3.8.1 Description of the Analytical Method

The analytical method was developed at (b) (4), and validated according to the standard operating procedures (SOPs) in effect during the conduct of the validation. These SOPs were written based on the good laboratory practices (GLP) principles described in 21 CFR Part 58 and the Guidance for Industry – Bioanalytical Method Validation (FDA (Food and Drug Administration) 2001). The analytical method is documented in BAM SOP ZZ33881-03. All (b) (4) (b) (4)).

An aliquot of human urine containing the analyte and internal standard was extracted using a combined liquid-liquid and solid phase extraction procedure. The extracted samples were analyzed by an HPLC equipped with an AB SCIEX API 4000™ triple quadrupole mass spectrometer using an ESI source. Positive ions were monitored in the multiple reaction monitoring (MRM) mode. Quantitation was determined using a weighted linear regression analysis ($1/concentration^2$) of peak area ratios of the analyte and internal standard.

6.1.3.3.8.2 Biological Matrix

Human urine was collected in-house at (b) (4). Human urine, free of significant interference at the retention time and mass transitions of nicotine, cotinine, *trans*-3′-hydroxycotinine, nicotine-*N*-glucuronide, cotinine-*N*-glucuronide, *trans*-3′-hydroxycotinine-*O*-glucuronide, d_4-nicotine (IS), d_3-cotinine (IS), d_3-*trans*-3′-hydroxycotinine (IS), d_3-nicotine-*N*-glucuronide (IS), d_3-cotinine-*N*-glucuronide (IS), and d_3-*trans*-3′-hydroxycotinine-*O*-glucuronide (IS), was used to prepare calibration standard and quality control (QC) samples and as control matrix.

6.1.3.3.8.3 Validation Components

The minimum requirements for validation included an assessment of accuracy, precision, response function, sensitivity, selectivity, matrix effect, multiple analytes measured by assay, known metabolites/isomers/endogenous compound evaluation, additional selectivity, recovery, stability (sample shipping, long-term, freeze-thaw, short-term, post-preparative, long-term stability for stock solutions, short-term stability for stock solutions, stability of analyte during sample collection and handling, sample aliquot

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frozen storage stability), integrity (dilution integrity and processed sample integrity), and stress test.

6.1.3.3.8.3.1 Reference Standards

The measurement of nicotine equivalents through the direct quantitation of nicotine and 5 major urinary metabolites requires well characterized reference materials. The dry reference materials for the glucuronide metabolites are hygroscopic. For this reason they are either provided as an ampoule of known concentration (trans-3′-hydroxycotinine-*O*-glucuronide) or are weighed in a dry box under an argon atmosphere to avoid moisture exposure. To improve the reproducibility of the weighings and reduce the stickiness of the dry reference materials, the reference materials were provided as organic salts. All reference materials were purchased from a reputable synthetic laboratory and were received with complete certificates of analysis.

6.1.3.3.8.3.2 Internal Standards

The internal standards used with the assays to measure nicotine equivalents are all deuterated stable label analytes.

6.1.3.3.8.3.3 Standard Curve Performance

The linear ranges originally selected for the analysis of nicotine and the 5 major metabolites were sufficient, however, they did result in a significant number of reassays. This was particularly true for *trans*-3′-hydroxycotinine and cotinine-*N*-glucuronide.

The control matrix for the assay was undiluted urine.

Multiple linear ranges were employed to measure nicotine, cotinine and *trans*-3′-hydroxycotinine and their respective glucuronide metabolites. The assay with the greatest sensitivity (linear ranges of 10.0 – 1000 ng/mL for nicotine, cotinine, *trans*-3′-hydroxycotinine and nicotine-*N*-glucuronide ng/mL, 20.0 - 2000 ng/mL for cotinine-*N*-glucuronide, and 50.0 - 5000 ng/mL for *trans*-3′-hydroxycotinine-*O*-glucuronide) was utilized for all clinical study samples from subjects that were randomized to smoking cessation. The clinical study samples for subjects that were randomized to the *ad libitum* group which used conventional cigarettes or the test product were assayed with the higher concentration linear range (50.0 – 5000 ng/mL for nicotine, cotinine, *trans*-3′-hydroxycotinine and nicotine-*N*-glucuronide, 200 – 20,000 ng/mL for cotinine-*N*-glucuronide and *trans*-3′-hydroxycotinine-*O*-glucuronide).

6.1.3.3.8.3.4 Precision, Accuracy, and Sensitivity

Precision and accuracy, and sensitivity results from the validated methods are provided in the bioanalytical method summaries for each study.

6.1.3.3.8.3.5 Stability

Stability results from the validation method for the measurement of nicotine equivalents are provided in the bioanalytical method summaries for each study and summarized in Table 8.

Extended stability testing completed for ambulatory collection coverage was completed with the following results. Stability at each temperature tested (5°C, 20°C and 40°C) was

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established for the following lengths of time. The test samples were stored in a polyethylene container that is designed to protect the clinical samples from UV light exposure.

Table 8 Stability Results for NEQ

Analyte	5°C	20°C	40°C
Nicotine	96 hours	96 hours	96 hours
Cotinine	96 hours	96 hours	96 hours
trans-3´-Hydroxycotinine	96 hours	96 hours	96 hours
Nicotine-*N*-Glucuronide	96 hours	96 hours	96 hours
Cotinine-*N*-Glucuronide	96 hours	96 hours	96 hours
trans-3´-Hydroxycotinine-*O*-Glucuronide	96 hours	96 hours	96 hours

6.1.3.3.8.3.6 Specificity

The selectivite quantitation of the assay was demonstrated in the presence of other metabolites of nicotine. Quality control samples supplemented with high concentrations of nicotine oxide, cotinine oxide, nornicotine and norcotinine were accurately quantitated. Accurate quantitation was also demonstrated in the presence of acetaminophen as this drug is typically allowed on many tobacco study designs.

6.1.3.3.9 Validation of Nicotine, Cotinine, trans-3´-hydroxycotinine, nicotine-n-glucuronide, cotinine-N-glucuronide, trans-3´-hydroxycotinine-O-glucuronide in Human urine (ZZ33881-04)

In PMI's studies, the analytes measured by this method are referred to as "nicotine equivalents" (NEQ).

The full validation report is archived at (b) (4) Study ZZ33881-04) and is available on request (b) (4)

A summary of the validation parameters tested and the results obtained during the method validation is provided in Appendix A6.1.3.35.

6.1.3.3.9.1 Description of the Analytical Method

The analytical method was developed at (b) (4) (b) (4) and validated according to the standard operating procedures (SOPs) in effect during the conduct of the validation. These SOPs were written based on the good laboratory practices (GLP) principles described in 21 CFR Part 58 and the Guidance for Industry – Bioanalytical Method Validation (FDA (Food and Drug Administration) 2001). The analytical method

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is documented in BAM SOP ZZ33881-04. (b) (4) (b) (4)

An aliquot of human urine containing the analyte and internal standard was extracted using a combined liquid-liquid and solid phase extraction procedure. The extracted samples were analyzed by an HPLC equipped with an AB SCIEX API 4000™ triple quadrupole mass spectrometer using an ESI source. Positive ions were monitored in the multiple reaction monitoring (MRM) mode. Quantitation was determined using a weighted linear regression analysis ($1/concentration^2$) of peak area ratios of the analyte and internal standard.

6.1.3.3.9.2 Biological Matrix

Human urine was collected in-house at (b) (4). Human urine, free of significant interference at the retention time and mass transitions of nicotine, cotinine, *trans*-3′-hydroxycotinine , nicotine-*N*-glucuronide , cotinine-*N*-glucuronide , *trans*-3′-hydroxycotinine-*O*-glucuronide, d_4-nicotine (IS), d_3-cotinine (IS), d_3-*trans*-3′-hydroxycotinine (IS), d_3-nicotine-*N*-glucuronide (IS), d_3-cotinine-*N*-glucuronide (IS), and d_3-*trans*-3′-hydroxycotinine-*O*-glucuronide (IS), was used to prepare calibration standard and quality control (QC) samples and as control matrix.

6.1.3.3.9.3 Validation Components

The minimum requirements for validation included an assessment of accuracy, precision, response function, carryover, sensitivity, selectivity, matrix effect, matrix factor, multiple analytes measured by assay, known metabolites/isomers/endogenous compound evaluation, additional selectivity, recovery, stability (sample shipping, long-term, freeze-thaw, short-term, post-preparative, long-term stability for stock solutions, short-term stability for stock solutions, stability of analyte during sample collection and handling, sample aliquot frozen storage stability), integrity (dilution integrity and processed sample integrity), and stress test.

6.1.3.3.9.3.1 Reference Standards

The measurement of nicotine equivalents through the direct quantitation of nicotine and 5 major urinary metabolites requires well characterized reference materials. The dry reference materials for the glucuronide metabolites are hygroscopic. For this reason they are either provided as an ampoule of known concentration (trans-3´-hydroxycotinine-*O*-glucuronide) or are weighed in a dry box under an argon atmosphere to avoid moisture exposure. To improve the reproducibility of the weighings and reduce the stickiness of the dry reference materials, the reference materials were provided as organic salts. All reference materials were purchased from a reputable synthetic laboratory and were received with complete certificates of analysis.

6.1.3.3.9.3.2 Internal Standards

The internal standards used with the assays to measure nicotine equivalents are all deuterated stable label analytes.

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6.1.3.3.9.3.3 Standard Curve Performance

The linear ranges originally selected for the analysis of nicotine and the 5 major metabolites were sufficient, however, they did result in a significant number of reassays. This was particularly true for *trans*-3′-hydroxycotinine and cotinine-*N*-glucuronide.

The control matrix for the assay was undiluted urine.

Multiple linear ranges were employed to measure nicotine, cotinine and *trans*-3′-hydroxycotinine and their respective glucuronide metabolites. The assay with the greatest sensitivity (linear ranges of 10.0 – 1000 ng/mL for nicotine, cotinine, *trans*-3′-hydroxycotinine and nicotine-*N*-glucuronide ng/mL, 20.0 - 2000 ng/mL for cotinine-*N*-glucuronide, and 50.0 - 5000 ng/mL for *trans*-3′-hydroxycotinine-*O*-glucuronide) was utilized for all clinical study samples from subjects that were randomized to smoking cessation. The clinical study samples for subjects that were randomized to the *ad libitum* group which used conventional cigarettes or the test product were assayed with the higher concentration linear range (50.0 – 5000 ng/mL for nicotine, cotinine, *trans*-3′-hydroxycotinine and nicotine-*N*-glucuronide, 200 – 20,000 ng/mL for cotinine-*N*-glucuronide and *trans*-3′-hydroxycotinine-*O*-glucuronide).

For a single study, SA-SCR-01, all subjects were randomized to smoking abstinence. Subjects were allowed to use some nicotine replacement therapy during the first 3 months of smoking abstinence. Samples collected at baseline and 3 months were measured with the higher concentration linear ranges. Samples collected from the 6 month and 12 month time points were measured with the lower concentration linear range assay.

6.1.3.3.9.3.4 Precision, Accuracy, and Sensitivity

Precision and accuracy, and sensitivity results from the validated methods are provided in the bioanalytical method summaries for each study.

6.1.3.3.9.3.5 Stability

Stability results from the validation method for the measurement of nicotine equivalents are provided in the bioanalytical method summaries for each study and is summarized in Table 9.

Extended stability testing completed for ambulatory collection coverage was completed with the following results. Stability at each temperature tested (5°C, 20°C and 40°C) was established for the following lengths of time. The test samples were stored in a polyethylene container that is designed to protect the clinical samples from UV light exposure.

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Table 9 Stability Results for NEQ

Analyte	5°C	20°C	40°C
Nicotine	96 hours	96 hours	96 hours
Cotinine	96 hours	96 hours	96 hours
trans-3′-Hydroxycotinine	96 hours	96 hours	96 hours
Nicotine-*N*-Glucuronide	96 hours	96 hours	96 hours
Cotinine-*N*-Glucuronide	96 hours	96 hours	96 hours
trans-3′-Hydroxycotinine-*O*-Glucuronide	96 hours	96 hours	96 hours

6.1.3.3.9.3.6 Specificity

The selectivite quantitation of the assay was demonstrated in the presence of other metabolites of nicotine. Quality control samples supplemented with high concentrations of nicotine oxide, cotinine oxide, nornicotine and norcotinine were accurately quantitated. Accurate quantitation was also demonstrated in the presence of acetaminophen as this drug is typically allowed on many tobacco study designs.

6.1.3.3.10 Validation of Total 3-hydroxybenzo[a]pyrne in Human Urine (ZZ42666-01)

The full validation report is archived at (b) (4) Study ZZ42666-01) and is available on request (b) (4)

A summary of the validation parameters tested and the results obtained during the method validation is provided in Appendix A6.1.3.36.

6.1.3.3.10.1 Description of the Analytical Method

The analytical method was developed at (b) (4) (b) (4) and validated according to the standard operating procedures (SOPs) in effect during the conduct of the validation. These SOPs were written based on the good laboratory practices (GLP) principles described in 21 CFR Part 58, the Guidance for Industry – Bioanalytical Method Validation (FDA (Food and Drug Administration) 2001), and Guideline on Bioanalytical Method Validation (European Medicines Agency (European Medicines Agency (EMA) 2012). The analytical method is documented in BAM SOP ZZ42666-01. (b) (4) (b) (4)

An aliquot of human urine containing the analyte and internal standard was extracted using a solid phase extraction procedure. The extracted samples were analyzed by an HPLC equipped with an AB SCIEX Triple Quad™ 6500 triple quadrupole mass spectrometer using an ESI source. Positive ions were monitored in the multiple reaction

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monitoring (MRM) mode. Quantitation was determined using a weighted linear regression analysis (1/concentration) of peak area ratios of the analyte and internal standard.

3-Hydroxybenzo[a]pyrene and $^{13}C_6$-3-hydroxybenzo[a]pyrene (IS) are light sensitive. All handling of samples, extracts, and solutions containing these analytes should be conducted under UV-shielded light conditions (i.e. protected from white light).

6.1.3.3.10.2 Biological Matrix

Human urine was purchased from (b) (4). and collected in-house at (b) (4) (b) (4) Canine urine was purchased from (b) (4). Human and/or canine urine, free of significant interference at the retention time and mass transitions of $^{13}C_6$-3-hydroxybenzo[a]pyrene (IS) with a very low basal concentration for 3-hydroxybenzo[a]pyrene, was diluted for use as control matrix on a sample-to-sample basis for the preparation of standards and LLOQ QC during the validation. Individual and/or pooled human urine lots were used for the preparation of low basal and high basal level quality control samples during the validation.

6.1.3.3.10.3 Validation Components

The minimum requirements for validation included an assessment of accuracy, precision, response function, carryover, sensitivity, selectivity, matrix effect, recovery, stability (sample shipping, long-term, freeze-thaw, short-term, post-preparative, long-term stability for stock solutions, short-term stability for stock solutions, stability of analyte during sample collection and handling, and sample aliquot frozen storage stability), and processed sample integrity.

6.1.3.3.10.3.1 Reference Standards

The reference material used for the quantitation of total 3-hydroxybenzo[a]pyrene in human urine was originally purchased from (b) (4). As the assay was further updated an alternate source was needed as the analyte was no longer available from the original source. (b) (4) therefore contracted (b) (4) to produce both 3-hydroxybenzo[a]pyrene and a suitable internal standard. During the method development, validation and initial clinical sample analysis, the most prevalent urinary metabolite 3-hydroxybenzo[a]pyrene-*O*-β-glucuronide was not available for purchase. For this reason the assay was developed and validated with the aglycone only.

Most recently the assay was updated to include the glucuronide metabolite reference material as it became available. Due to solubility issues with the aglycone the glucuronide is currently used in both the standard calibrator spiking solutions and quality control samples.

6.1.3.3.10.3.2 Internal Standards

The originally validated assay to measure 3-hydroxybenzo[a]pyrene utilized a deuterated stable label internal standard. The material was a mixture of various deuterated products. One of the most abundant isotopes (d5-3-hydroxybenzo[a]pyrene) was selected for use as the internal standard. No significant presence of unlabeled analyte was detected at the

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working internal standard concentration. The deuterated internal standard however was separated chromatographically (0.5 – 0.6 min) from the target analyte.

As the assay was further developed, after completion of the pilot study for this program, a carbon heavy internal standard was synthesized at the request of (b) (4) to further improve the analytical performance of the assay. The carbon heavy internal standard (13C6) was not a mixture of isomers but was specifically synthesized with a known structure resulting in a more consistent product for analysis. Additionally the chromatographic separation of the internal standard from the target analyte was also reduced (0.1 – 0.2 min) resulting in more consistent quantitation.

6.1.3.3.10.3.3 Standard Curve Performance

The standard spiking solutions originally validated were prepared with 3-hydroxybenzo[a]pyrene in methanol. Standard spiking solution stability was established up to 58 days in silanized glass when stored at -20°C. It was observed however that the standard performance while acceptable was more variable than preferred with a bioanalytical assay. The analytical concern was that the analyte 3-hydroxybenzo[a]pyrene was poorly soluble even in a methanolic solution in silanized glass.

Once available the 3-hydroxybenzo[a]pyrene-*O*-β-glucuronide reference material was used to prepare standard calibrator spiking solutions in ethanol. The standard performance with the glucuronide reference material has significantly improved. Stability established thus far has also been improved with 85 days currently established for 3-hydroxybenzo[a]pyrene-*O*-β-glucuronide in silanized glass stored at -20°C.

Though 3-hydroxybenzo[a]pyrene is not endogenous, dietary and environmental exposures result in significant concentrations within the urine of non-smoker urine donors. For this reason an alternate control matrix was necessary. The validated approach allows freshly canine urine or diluted human urine to be used as the control matrix. Parallelism was established with both control matrices.

6.1.3.3.10.3.4 Precision and Accuracy

As stated previously the majority of total 3-hydroxybenzo[a]pyrene is excreted as the *O*-glucuronide. Poor solubility of the aglycone in human urine makes it impossible to supplement quality control samples and store them for any period of time. A single freeze/thaw cycle of human urine samples supplemented with 3-hydroxybenzo[a]pyrene resulted in an unacceptable negative bias. The measured basal concentrations made up of the O-glucuronide however were stable out to 6 freeze/thaw cycles. Long-term stability has also been established out to 570 days in polypropylene tubes stored at -20°C.

To ensure coverage of the full linear range two pools of urine were prepared, one from non-smoker consented volunteers and the other from consented volunteers who smoked. Each pool measured as a quality control in replicate both with and without supplementation. This approach therefore incorporated 4 quality control levels spread across the range to demonstrate accuracy, precision and parallelism with each analytical run.

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The current assay has been updated to include the newly purchased 3-hydroxybenzo[a]pyrene-*O*-β-glucuronide reference material in the preparation of the quality controls. The updated approach has been kept the same with a pool of high and low concentration basal pools of urine that are analyzed with and without supplementation on the day of analysis.

6.1.3.3.10.3.5 Sensitivity

As one of the most sensitive assays at (b) (4) sensitivity is a significant concern. The initial validated approach that had a lower limit of quantitation (LLOQ) of 50.0 fg/mL. To achieve this level of sensitivity 3 mL of urine was required for purification, concentration and derivatization. Unfortunately even with this level of sensitivity many clinical samples were not measurable as the concentrations were below the limit of quantitation.

After the pilot study was completed the assay was updated with more sensitive instrumentation, more selective chromatography and a reduced LLOQ. The updated assay achieved more consistent performance with an even lower LLOQ of 25.0 fg/mL.

6.1.3.3.10.3.6 Stability

Stability results from the validation method for total 3-hydroxybenzo[a]pyrene are provided in the bioanalytical method summaries for each study and are summarized in Table 10. For stability testing high concentration and low concentration urine pools with established basal levels were used. These basal levels are composed primarily of the excreted urinary metabolite 3-hydroxybenzo[a]pyrene-*O*-β-glucuronide.

Extended stability testing completed for ambulatory collection coverage was completed with the following results. Stability at each temperature tested (5°C, 20°C and 40°C) was established for the following lengths of time. The test samples were stored in a polyethylene container that is designed to protect the clinical samples from UV light exposure.

Table 10 Stability Results for 3-OH BaP

Analyte	5°C	20°C	40°C
Total 3-OH BaP	96 hours	96 hours	0 hours

6.1.3.3.10.3.7 Specificity

The specificity of the assay has been demonstrated through multiple chromatographic approaches. Optimization of the hydrolysis was achieved by variation of the standard reported hydrolysis using β-glucuronidase (Type H-1 from H. pomatia). An observed plateau of maximal response from high concentration smoker urine samples was used to determine the optimal conditions for hydrolysis. With the updated assay using spiked additions of 3-hydroxybenzo[a]pyrene-*O*-β-glucuronide reference material the quantitative hydrolysis was confirmed.

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Specificity results from the validation testing for the total 3-hydroxybenzo[a]pyrene assay are provided in the bioanalytical method summaries for each study.

6.1.3.3.11 Validation of Caffeine and Paraxanthine in Human Plasma (Heparin) (ZZ25187-02)

The full validation report is archived at (b) (4) Study ZZ25187-02) and is available on request (b) (4)

A summary of the validation parameters tested and the results obtained during the method validation is provided in Appendix A6.1.3.37.

In PMI's studies, the molar ratio of paraxanthine/caffeine was calculated to assess CYP1A2 activity.

6.1.3.3.11.1 Description of the Analytical Method

The analytical method was developed at (b) (4) (b) (4) according to the current validation SOP 03.01.078 in effect. This SOP was written based on the GLP principles described in 21 CFR part 58 and the Guidance for Industry – Bioanalytical Method Validation (FDA (Food and Drug Administration) 2001). The analytical method is documented in BAM SOP ZZ25187-02. (b) (4) (b) (4)).

An aliquot of human plasma (heparin) containing each analyte and internal standard was extracted using a liquid-liquid extraction procedure. The extracted samples were analyzed by an HPLC equipped with an AB SCIEX API 4000™ mass spectrometer. Positive ions were monitored in the multiple reaction monitoring (MRM) mode. Quantitation was determined using a weighted linear regression analysis ($1/concentration^2$) of peak area ratios of each analyte and internal standard.

The reference standard materials for caffeine, paraxanthine, d_9-caffeine (IS), and d_3-paraxanthine (IS) are light sensitive. All storage and handling of reference standard material for these analytes should be conducted under UV-shielded light conditions (i.e. protected from white light).

6.1.3.3.11.2 Biological Matrix

Human plasma, with lithium heparin as anticoagulant, was purchased from (b) (4) Human plasma (heparin), free of significant interference at the retention time and mass transitions of caffeine, d_9-caffeine (IS), paraxanthine, and d_3-paraxanthine (IS) was used to prepare calibration standard and quality control (QC) samples.

6.1.3.3.11.3 Validation Components

The minimum requirements for validation included an assessment of response function, accuracy, precision, selectivity, matrix effect, multiple analytes measured by assay, known metabolites/isomers/endogenous compounds, hemolyzed sample integrity, lipemic sample evaluation, sensitivity, recovery, stability [sample shipping, long-term (frozen) in matrix, freeze-thaw and short-term in matrix, post-preparative, long-term stability for

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stock solutions, short-term stability for stock solutions, stability of analyte during sample collection and handling, and sample aliquot frozen storage], and integrity (dilution integrity and processed sample integrity), and stress test.

6.1.3.3.11.3.1 Reference Standards

Caffeine and paraxanthine reference materials were either compendial or were purchased from reputable synthetic laboratories. Certifications were complete for all non-compendial reference materials.

6.1.3.3.11.3.2 Internal Standards

Deuterated internal standards were purchased from reputable synthetic laboratories. In all cases the internal standards were appropriately certified.

6.1.3.3.11.3.3 Standard Curve Performance

The standard curve performance for the caffeine and paraxanthine assay met acceptance. The only consistent challenge with the assay performance is the necessity of a large number of human plasma screens required to ensure the selected control blank matrix is suitably clean.

In addition to the presence of caffeine within the control matrices, some lots of solvents and reagents (ethyl acetate and ammonium acetate) were found to include a small portion of caffeine. For this reason the solvents and prepared solutions were screened prior to use to avoid contamination issues.

6.1.3.3.11.3.4 Precision, Accuracy, Sensitivity and Stability

Precision and accuracy, sensitivity, and stability results from the validation method for the caffeine and paraxanthine assays are provided in the bioanalytical method summaries for each study.

6.1.3.3.11.3.5 Specificity

Specificity of the assay quantitation for both caffeine and paraxanthine was confirmed through validation testing for quality control samples supplemented with theophylline and theobromine.

6.1.3.3.12 Validation of 4-Aminobiphenyl, o-Toluidine, 2-Aminonaphthalene, and 1-Aminonaphthalene in Human Urine (ZZ37540-01)

The full validation report is archived at (b) (4) Study ZZ37540-01) and is available on request (b) (4)

A summary of the validation parameters tested and the results obtained during the method validation is provided in Appendix A6.1.3.38.

6.1.3.3.12.1 Description of the Analytical Method

The analytical method was developed at (b) (4) (b) (4) and validated according to the standard operating procedures (SOPs) in effect during the conduct of the validation. These SOPs were written based on the good laboratory practices (GLP) principles described in 21 CFR Part 58, the Guidance for Industry – Bioanalytical

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Method Validation (FDA (Food and Drug Administration) 2001), and Guideline on Bioanalytical Method Validation (European Medicines Agency (European Medicines Agency (EMA) 2012). The analytical method is documented in BAM SOP ZZ37540-01. (b) (4) (b) (4)).

An aliquot of human urine containing the analyte and internal standard was extracted using a liquid-liquid extraction procedure. The extracted samples were analyzed by an HPLC equipped with an AB SCIEX API 4000™ triple quadrupole mass spectrometer using an ESI source. Positive ions were monitored in the multiple reaction monitoring (MRM) mode. Quantitation was determined using a weighted linear regression analysis (1/concentration for 4-aminobiphenyl and 1/concentration^2 for *o*-toluidine, 2-aminonaphthalene, and 1-aminonaphthalene) of peak area ratios of the analyte and internal standard.

4-Aminobiphenyl, *o*-toluidine, 2-aminonaphthalene, 1-aminonaphthalene, [$^{13}C_6$]4-aminobiphenyl (IS), [$^{13}C_6$]*o*-toluidine (IS), [$^{13}C_4$]2-aminonaphthalene (IS), and [$^{13}C_{10}$]1-aminonaphthalene (IS) are light sensitive. All handling of samples, extracts, and solutions containing these analytes should be conducted under UV-shielded light conditions (i.e. protected from white light).

6.1.3.3.12.2 Biological Matrix

Human urine was collected in-house at (b) (4) (b) (4) Human urine was screened for interference at the retention time and mass transitions of [$^{13}C_6$]4-aminobiphenyl (IS), [$^{13}C_6$]*o*-toluidine (IS), [$^{13}C_4$]2-aminonaphthalene (IS), and [$^{13}C_{10}$]1-aminonaphthalene (IS), and to determine approximate concentrations of 4-aminobiphenyl, *o*-toluidine, 2-aminonaphthalene, and 1-aminonaphthalene. Human urine lots with appropriate concentrations were used for the preparation of quality control samples. A 0.1% bovine serum albumin solution was used for the preparation of calibration standards and as control matrix.

6.1.3.3.12.3 Validation Components

The minimum requirements for validation included an assessment of accuracy, precision, response function, carryover, sensitivity, selectivity, matrix effect, multiple analytes measured by assay, known metabolites/isomers/endogenous compound evaluation, recovery, stability (sample shipping, freeze-thaw, short-term, post-preparative, long-term stability for stock solutions, short-term stability for stock solutions, stability of analyte during sample collection and handling, and sample aliquot frozen storage stability), integrity (dilution integrity and processed sample integrity), and stress test.

6.1.3.3.12.3.1 Reference Standards

Reference standards used for the quantitation of 4-aminobiphenyl (4-ABP), *o*-toluidine, 1-aminonaphthalene (1-NA) and 2-aminonaphthalene (2-NA) were all purchased from a reputable synthetic laboratory with complete certification. The reference materials were provided as dried powders.

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Aromatic amines are excreted as many urinary metabolites. The target analytes are excreted without modification, as known metabolites such as aromatic hydroxylamines, acetylated aromatic amines, acetylated *N*-hydroxylamines, aryl-*N*-glucuronides, aryl hydroxyl-*N*-glucuronides, and aryl hydroxyl-*O*-glucuronides. Many of the available urinary metabolites were purchased to use in the optimization of the hydrolysis.

Additionally there are structural isomers of two of the target analytes, 4-ABP and o-toluidine that were purchased for selectivity testing. The structural isomers tested for selectivity are 3-aminobiphenyl, 2-aminobiphenyl, m-toluidine and p-toluidine.

6.1.3.3.12.3.2 Internal Standards

The stable label isotopes purchased to track the analysis of total aromatic amines was carbon heavy ($^{13}C_6$). With a planar molecule it is easy to separate deuterated internal standards from the target analyte. With the UPLC reversed phase chromatography used to achieve separation a deuterated internal standard would not co-elute with the unlabeled analyte. The following internal standards were purchased [13C6]*o*-toluidine, [$^{13}C_4$]2-aminonaphthalene, [$^{13}C_{10}$]1-aminonaphthalene, [$^{13}C_6$]4-aminobiphenyl.

6.1.3.3.12.3.3 Standard Curve Performance

The linear ranges validated for the measurement of 4-ABP, 1-NA, 2-NA and *o*-toluidine were appropriate for the analysis of most of the clinical samples. Based on the biomarker study designs, approximately half of subjects were enrolled either to test products or smoking abstinence. With the lower exposure levels approximately 50% of the o-toluidine and 2-aminonaphthalene samples were near or below the lower limit of quantitation. For the measurements of total 4-ABP and 1-NA, approximately 80% of all samples quantitated within the validated linear ranges.

Due to the presence of some measurable basal concentrations of the aromatic amines in most urine lots an alternate blank control matrix was required. The validated control matrix used was 0.1% bovine serum albumin (BSA) in water. Minimal dilution integrity was established for the validated method with 0.1% BSA used as the diluent.

6.1.3.3.12.3.4 Precision and Accuracy

Due to the complexity of the urinary metabolite profile for each of the targeted aromatic amines it was necessary to use multiple urine lots of high and low concentration to produce suitable quality control samples through pooling various lots. To perform stability experiments and demonstrate precision and accuracy two urine pools of high and low concentration were produced. The targeted concentration for the lower concentration poor is approximately 3 times the LLOQ in concentration. This quality control was predominantly made from non-smoker urine lots. A second mid-high concentration quality control sample was produced by pooling smoker urine lots.

The quality control samples were assayed both with and without supplementation on the day of analysis with the targeted analytes to demonstrate precision, accuracy and parallelism.

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6.1.3.3.12.3.5 Sensitivity

Sensitivity results from the validation testing for the total aromatic amines assay are provided in the bioanalytical method summaries for each study.

6.1.3.3.12.3.6 Stability

Stability results from the validation method for total aromatic amines are provided in the bioanalytical method summaries for each study and summarized in Table 11.

Extended stability testing completed for ambulatory collection coverage was completed with the following results. Stability at each temperature tested (5°C, 20°C and 40°C) was established for the following lengths of time. The test samples were stored in a polyethylene container that is designed to protect the clinical samples from UV light exposure.

Table 11 Stability Results for of *o*-toluidine, 1-NA, 2-NA and 4-ABP

Analyte	5°C	20°C	40°C
o-Toluidine	96 hours	48 hours	48 hours
1-Aminonaphthalene	96 hours	48 hours	48 hours
2-Aminonaphthalene	96 hours	48 hours	96 hours
4-Aminobiphenyl	96 hours	48 hours	0 hours

6.1.3.3.12.3.7 Specificity

Specificity results from the validation testing for the total 4-aminobiphenyl, *o*-toluidine,

1-aminonaphthalene and 2-aminonaphthalene are provided in the bioanalytical method summaries for each study. Structural isomers were added to quality control samples to demonstrate accurate quantitation in their presence at high concentrations (75.0 pg/mL for 2-ABP and 3-ABP; 1000 pg/mL for *m*-toluidine and *p*-toluidine).

The most significant challenge of selectivity with the analysis of aromatic amines is the optimization of the hydrolysis approach. Analytical methods have been published using acidic, alkaline and enzymatic hydrolysis approaches in an attempt to measure comparative aromatic amine exposure. The most common approach utilized either a strong acidic or alkaline modification of the sample for a short period of time (2 hours) maintained at 80°C. Testing with the acetylated metabolites demonstrated that this approach was not optimized specifically for *o*-toluidine and 1-aminonaphthalene. Sample to sample variability could be observed for these two analytes with an incomplete hydrolysis that could confound the results.

(b) (4) initially qualified an assay that incorporated a strong acidic hydrolysis with samples maintained at 80°C for 24 hours. While the hydrolysis was more complete, it was observed that a plateau of response had not been achieved. Further the samples

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became more difficult to process through a sample clean up achieving a suitable lower limit of quantitation. For this reason a more mild approach was selected to achieve a consistent comparative analysis of a greater portion of the total aromatic amine exposure. The validated approach utilizes a strong acid hydrolysis with samples maintained at 65°C for a period of 16 – 17 hours. The optimized hydrolysis was validated to support the analysis of the aromatic hydroxylamines, acetylated aromatic amines and glucuronide metabolites.

6.1.3.3.13 Validation of Monohydroxy-3-butenyl-mercapturic Acid in Human Urine (VAA98876-02)

The full validation report is archived at (b) (4) and is available on request.

A summary of the validation parameters tested and the results obtained during the method validation is provided in Appendix A6.1.3.39. This method wasvalidated after transfer to (b) (4)). The original validation report number was AA1105_2.

6.1.3.3.13.1 Description of the Analytical Method

The validation was conducted at (b) (4) for a range of 0.100 – 20.0 ng/mL for monohydroxy-3-butenyl-mercapturic acid (MHBMA) in human urine using a urine volume of 250 µL. The method using an LC-MS/MS system with 2 analytical columns is documented in method SOP SM1-383A.

The method using an LC-MS/MS system with a single analytical column, described in details in the Amendment No. 1 to study plan method validation PAA98876-02 for a range of 0.100 – 20.0 ng/mL for monohydroxy-3-butenyl-mercapturic acid (MHBMA) in human urine using a urine volume of 250 µL was additionally validated.

The validation of the assay was performed in accordance with (b) (4) standard procedures, which follow the FDA guidance for the validation of bioanalytical methods (FDA (Food and Drug Administration) 2001) and the EMA guideline on bioanalytical method validation (European Medicines Agency (EMA) 2012).

Briefly, the analytical method can be described as follows:

For stock solution preparation, weighing of MHBMA and MHBMA-^{15}N-$^{13}C_3$ (IS) into weighing boats and subsequent dissolving with methanol in silanized glass vial, as described in (SOP) SM1-383A, was regarded acceptable. Validation samples, calibration standards and QC samples were spiked with IS, acidified with HCl and extracted using a solid phase extraction procedure. Sample extraction was performed on 96 well plates with a maximum of 1 plate per analytical run.

The extracted samples were analysed by an LC-MS/MS method either using a system with 2 analytical columns or by using an LC-MS/MS method with a single analytical column. Negative ions were monitored in the multiple reaction-monitoring (MRM) mode. Quantification was done by peak area ratio and linear regression with a weighting factor of 1/concentration^2.

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Requirements for validation of the LC-MS/MS method using a system with 2 analytical columns, as described in details in study plan method validation PAA98876-02, included an assessment of selectivity, carry-over, stress test, sensitivity, response function, precision and accuracy, dilution integrity, matrix effect, processed sample integrity, solution stability (short-term stability and evaluation of isotope exchange reaction), stability in matrix (storage in UV shielded polypropylene tubes) and performance of BSA- blocked 96-well plates.

Requirements for the validation of the LC-MS/MS method using a system with a single analytical column, as described in detail in amendment no. 1 to study plan method validation PAA98876-02 Am1, included an assessment of selectivity, carry-over, stress test, sensitivity, response function, matrix effect and precision and accuracy.

6.1.3.3.13.2 Biological Matrix

Human urine was collected from employee volunteers in-house. Individual human urine samples were screened to determine the basal concentration of MHBMA. Human urine was pooled and the basal concentration of MHBMA was determined in three separate runs by analysis of 10 replicates within each run. Human urine, free of significant interference at the retention time and mass transitions of the internal standard, was used to prepare quality control (QC) samples at the targeted concentration level of 0.300, 1.50, 15.0 ng/mL and 100 ng/mL (DQC).

Urisub® (a urine substitute) was purchased from (b) (4) – (b) (4) and was used for the preparation of the calibration standards. (b) (4) ® was used for blanks, STD 0, preparation of LLOQ QC samples at 0.100 ng/mL, human urine dilution to prepare QC samples at the targeted concentration level of 0.300 ng/mL (1:10 dilution step only) and for analysis (1:10 dilution) of dilution quality control (DQC) samples.

6.1.3.3.13.3 Validation Components

The purpose of this study was to validate an LC-MS/MS method for the determination of monohydroxy-3-butenyl-mercapturic acid (MHBMA) in human urine. The method using an LC-MS/MS system with 2 analytical columns as described in SOP SM1-383A was validated over a calibration range of 0.100 ng/mL (LLOQ) to 20.0 ng/mL (ULOQ) using stable labeled IS.

The validation was carried out with respect to selectivity, carry-over, stress-test, sensitivity, response function, precision and accuracy, dilution integrity, matrix effect, processed sample integrity, assay performance using BSA-blocked 96-well plates, solution stability and stability in matrix. Selectivity, carry-over, stress-test, sensitivity, precision and accuracy, matrix effect and assay performance using BSA-blocked 96-well plates met acceptance criteria. Dilution integrity, by dilution 1:10 with Urisub®, was demonstrated for up to 100 ng/mL. Processed sample integrity at 5°C for 122 hours was demonstrated.

The method for the determination of monohydroxy-3-butenyl-mercapturic acid (MHBMA) in human urine using an LC-MS/MS system with a single analytical column was additionally validated. The validation using an LC-MS/MS system with a single

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analytical column was repeated with respect to selectivity, carry-over, stress-test, sensitivity, response function, precision and accuracy and matrix effect. Selectivity, carry-over, stress-test, sensitivity, precision and accuracy and matrix effect met acceptance criteria.

6.1.3.3.13.3.1 Reference Standards

The reference standard used to quantify MHBMA was purchased as a crystalline solid from a reputable synthetic laboratory. The reference material was provided as a dicyclyohexylamine salt. The reference material was a mixture of structural isomers 1-(N-acetyl-L-cystein-S-yl)-2-hydroxybut-3-ene and 2-(N-acetyl-L-cystein-S-yl)-1-hydroxybut-3-ene.

6.1.3.3.13.3.2 Internal Standards

The stable label internal standards used for quantitation were both nitrogen (^{15}N) and carbon ($^{13}C_3$) heavy. Isotopic purity testing was also performed for the internal standard materials with no measurable unlabeled analyte detected.

6.1.3.3.13.3.3 Standard Curve Performance

The linear range was appropriately placed to accurately quantitate more than 95% of the clinical samples collected from all treatment types. Due to the common exposure of 1,3-butadiene measurable levels of MHBMA are common in human urine. For this reason commercially available synthetic urine was used as the control matrix. The synthetic urine was also used as a diluent for quality control samples or clinical samples that required dilution for analysis.

6.1.3.3.13.3.4 Precision and Accuracy

Quality control samples were prepared using a human urine pool with a measured basal level of MHBMA As the measured concentrations of MHBMA were significantly higher than the LLOQ, the basal level was diluted with synthetic urine by a factor of 10 to produce a separate low quality control sample near 3 times the LLOQ in concentration. The mid-level and high concentration quality controls were prepared by supplementing the urine basal level to approximately 1.50 ng/mL and 15.0 ng/mL. Additionally, a higher concentration quality control sample was prepared by supplementing the measured basal level to 100 ng/mL to be used to track dilution integrity when needed.

6.1.3.3.13.3.5 Stability

Stability results from the validation method for MHBMA are provided in the bioanalytical method summaries for each study and are summarized in Table 12.

Extended stability testing completed for ambulatory collection coverage was completed with the following results. Stability at each temperature tested (5°C, 20°C and 40°C) was established for the following lengths of time. The test samples were stored in a polyethylene container that is designed to protect the clinical samples from UV light exposure. Extended stability testing was completed under study number CA15081-02.

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Table 12 Stability Results for MHBMA

Analyte	5°C	20°C	40°C
MHBMA	96 hours	96 hours	96 hours

6.1.3.3.13.3.6 Sensitivity and Specificity

Sensitivity and specificity results from the validation testing for S-PMA and S-BMA assay are provided in the bioanalytical method summaries for each study.

6.1.3.3.14 Validation of Total 1-Hydroxypyrene in Human Urine (VAA98876-03)

The full validation report is archived at (b) (4) and is available on request.

A summary of the validation parameters tested and the results obtained during the method validation is provided in Appendix A6.1.3.40.

6.1.3.3.14.1 Description of the Analytical Method

The validation was conducted at (b) (4) for a range of 10.0 – 2000 pg/mL for total 1-hydroxypyrene (1-OHP) in human urine using a urine volume of 500 µL. The validation was initiated following SOP SM1-384A. The supplier of the reference item was changed by amendment and the method as documented in method SOP SM1-384B was applied to obtain validation data. Results, including solution stability data generated according method SOP SM1-384C, are presented in this report.

The validation of the assay was performed in accordance with (b) (4) standard procedures, which follow the FDA guidance for the validation of bioanalytical methods (FDA (Food and Drug Administration) 2001) and the EMA guideline on bioanalytical method validation (European Medicines Agency (EMA) 2012).

Briefly, the analytical method can be described as follows:

To aliquots of 500 µL human matrix or 550 µL of calibration standards in Urisub®, internal standard (IS) and solvent for blank samples were added. After hydrolysis with ß-Glucuronidase, type H-1 from *Helix Pomatia*, samples were extracted by SPE, reconstituted and injected into the LC-MS/MS system.

The extracted samples were analyzed using a UPLC system equipped with an Applied Biosystems/MDS SCIEX API 6500™ triple quadrupole mass spectrometer. Negative ions were monitored in the multiple reaction–monitoring (MRM) mode. Quantification was done by peak area ratio and linear regression with a weighting factor of 1/concentration^2.

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6.1.3.3.14.2 Biological Matrix

Human urine was collected from employee volunteers in-house. Individual human urine samples were screened to determine the basal concentration of 1-OHP. Human urine was pooled and the basal concentration of 1-OHP was determined in three separate runs by analysis of 10 replicates within each run. Human urine, free of significant interference at the retention time and mass transitions of the internal standard, was used to prepare quality control (QC) samples at the targeted concentration level of 30 (basal level), 150, 1600, and 10000 pg/mL (DQC).

Urisub® (a urine substitute) was purchased from (b) (4) (b) (4) and was used for the preparation of the calibration standards. It was also used for blanks, STD 0, the preparation of LLOQ QC samples at 10.0 pg/mL and for analysis (1:10 dilution) of dilution quality control (DQC) samples.

6.1.3.3.14.3 Validation Components

The purpose of this study was to validate an LC-MS/MS method for the determination of 1-hydroxypyrene (1-OHP) in human urine. The method as described in SOP SM1-384B was validated over a calibration range of 10.0 pg/mL (LLOQ) to 2000 pg/mL (ULOQ) using stable labeled IS.

The validation was carried out with respect to selectivity, carry-over, stress-test, sensitivity, response function, precision and accuracy, dilution integrity, matrix effect, processed sample integrity, solution stability and stability in matrix and Urisub®.

Selectivity, carry-over, stress-test, sensitivity, response function, matrix effect, precision and accuracy met acceptance criteria. Dilution integrity and processed sample integrity at 5°C for 116 hours was demonstrated.

6.1.3.3.14.3.1 Reference Standards

The reference materials used to validate and quantify total 1-hydroxypyrene were synthesized and characterized by a reputable laboratory. (b) (4) purchased both the aglycone 1-hydroxypyrene as well as the *O*-glucuronide metabolite of 1-OHP.

6.1.3.3.14.3.2 Internal Standards

The stable label isotope purchased to track the analysis of total 1-OHP was carbon heavy ($^{13}C_6$). With a planar molecule it is easy to separate deuterated internal standards from the target analyte. With the UPLC reversed phase chromatography used to achieve separation a deuterated internal standard would not co-elute with the unlabeled analyte.

6.1.3.3.14.3.3 Standard Curve Performance

The linear range was appropriately placed to accurately quantitate more than 95% of the clinical samples collected from all treatment types. Though 1-hydroxypyrene is not endogenous, dietary and environmental exposures result in significant concentrations within the urine of non-smoker urine donors. For this reason commercially available synthetic urine was used as the control matrix. Synthetic urine was also used as a diluent for quality control samples or clinical samples that required dilution for analysis. The aglycone reference material was used to prepare the calibration standards.

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6.1.3.3.14.3.4 Precision and Accuracy

Quality controls were prepared in human urine with a measured basal level supplemented with 1-OHP-glucuronide. The basal level target for the human urine pool was 30.0 pg/mL. The mid and high concentration quality control samples were supplemented with 1-OHP-glucuronide to obtain 1-hydroxypyrene concentrations of 150 pg/mL and 1600 pg/mL respectively.

The approach of using the glucuronide metabolite in the quality controls was selected because the primary urinary metabolite is 1-OHP-glucuronide. Additionally the approach demonstrates definitively that the hydrolysis is complete and quantitative.

6.1.3.3.14.3.5 Stability

Stability results from the validation method for total 1-hydroxypyrene are provided in the bioanalytical method summaries for each study and are summarized in Table 13.

Extended stability testing completed for ambulatory collection coverage was completed with the following results. Stability at each temperature tested (5°C, 20°C and 40°C) was established for the following lengths of time. The test samples were stored in a polyethylene container that is designed to protect the clinical samples from UV light exposure. Extended stability testing was performed under study number CA15081-03.

Table 13 Stability Results for Total 1-OHP

Analyte	5°C	20°C	40°C
Total 1-OHP	96 hours	96 hours	48 hours

6.1.3.3.14.3.6 Selectivity and Specificity

Sensitivity and specificity results from the validation testing for the total 1-hydroxypyrene assay are provided in the bioanalytical method summaries for each study.

6.1.3.3.15 Validation of S-benzyl Mercparturic Acid and S-phenyl Mercapturic Acid in Human Urine (VAA98876-01)

The full validation report is archived at (b) (4) and is available on request.

A summary of the validation parameters tested and the results obtained during the method validation is provided in Appendix A6.1.3.41.

6.1.3.3.15.1 Description of the Analytical Method

The bioanalytical method has been fully validated at (b) (4) as outlined in the validation report to study AA98877-02 and the method is detailed in the bioanalytical method SOPs AA98877-02 Version No. 1 and AA98877-02 Version No. 2. For the cross-site validation at (b) (4), the bioanalytical method has been detailed in the bioanalytical method SOP SM1-382A, and SOP SM1-382B.

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The validation experiments as part of this study have been performed in accordance with (b) (4) standard procedures, which follow the FDA guidance for the validation of bioanalytical methods (FDA (Food and Drug Administration) 2001) and the EMA guideline on bioanalytical method validation (European Medicines Agency (EMA) 2012).

Briefly, the bioanalytical procedure can be described as follows:

Validation samples, calibration standards and QC samples were spiked with IS and extracted using solid-phase extraction procedure. OASIS® MAX 96-well plates (30 mg) (Waters) were used for solid-phase extraction. Samples extraction was performed on 96-well plates with a maximum of 1 plate per analytical run. The extracted samples were analysed by LC-MS/MS. Negative ions were monitored in the multiple reaction-monitoring (MRM) mode. Quantification is performed using the peak area ratios of analyte versus IS for each pair of analyte and IS. The calibration curve fitting is done by 1/concentration^2-weighted linear regression.

6.1.3.3.15.2 Biological Matrix

The validation was performed using human urine collected from volunteers in-house. Human urine, free of significant interference at the retention time and mass transitions of both internal standards were used to prepare quality control (QC) samples. The working concentrations of SBMA and SPMA in urine quality control samples were determined by adding spiked concentrations of SBMA and SPMA to the mean basal level (BL) concentrations. (b) (4) ®, a synthetic urine substitute was used to prepare calibration standards, LLOQ QC, and as control matrix.

6.1.3.3.15.3 Validation Components

The objective of the present study was to cross-site validate a bioanalytical method for the determination of *S*-Benzyl Mercapturic Acid (SBMA) and *S*-Phenyl Mercapturic Acid (SPMA) in human urine. The bioanalytical method has been fully validated at (b) (4) (b) (4) and was transferred to (b) (4). The cross-site validation study was carried out with respect to inter- and intra-run precision and accuracy, 96-well plate stress test for robotic sample preparation, carry-over and comparison of validation samples prepared and analyzed at (b) (4) for SBMA and SPMA. The cross-site validation was performed for an assay volume of 500 µL over the following calibration ranges:

SBMA: 0.1 – 25 ng/mL

SPMA: 0.025 – 6.25 ng/mL

The bioanalytical method of SBMA and SPMA in human urine met all cross-validation requirements. A data overview is presented in the validation summary below.

6.1.3.3.15.3.1 Reference Standards

The reference standards used to quantify *S*-phenylmercapturic acid and *S*-benzylmercapturic acid were purchased as a crystalline solid from a reputable synthetic laboratory. Both reference materials were provided as dicyclyohexylamine salts.

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6.1.3.3.15.3.2 Internal Standards

The stable label internal standards used for quantitation were both nitrogen (^{15}N) and carbon ($^{13}C_3$) heavy. Isotopic purity testing was also performed for the internal standard materials with no measurable unlabeled analyte detected.

6.1.3.3.15.3.3 Standard Curve Performance

The linear range was appropriately placed to accurately quantitate more than 95% of the clinical samples collected from all treatment types. Due to the common exposure of benzene and toluene measurable levels of both S-PMA and S-BMA are common in human urine. For this reason commercially available synthetic urine was used as the control matrix. The synthetic urine was also used as a diluent for quality control samples or clinical samples that required dilution for analysis.

6.1.3.3.15.3.4 Precision and Accuracy

Quality control samples were prepared using a human urine pool with a measured basal level for both S-PMA and S-BMA. The measured basal level was targeted to be approximately 3 times the LLOQ concentration for S-PMA. As the measured concentrations of S-BMA were significantly higher, the basal level was diluted with synthetic urine by a factor of 4 to produce a separate low quality control sample for S-BMA. The mid-level and high concentration quality controls were prepared by supplementing the urine basal level.

It should be noted that the validated assay could be considered a "total" assay as a strong acid addition is incorporated into the sample processing to convert any "pre-S-PMA" (N-acetyl-*S*(1,2-dihydro-2-hydrophenyl)-L-cysteine into S-PMA prior to detection. Confirmation of the suitability of this approach has been published in peer-reviewed literature (Paci 2007).

6.1.3.3.15.3.5 Stability

Stability results from the validation method for *S*-phenylmercapturic acid and *S*-benzylmercapturic acid are provided in the bioanalytical method summaries for each study and are summarized in Table 14.

Extended stability testing completed for ambulatory collection coverage was completed with the following results. Stability at each temperature tested (5°C, 20°C and 40°C) was established for the following lengths of time. The test samples were stored in a polyethylene container that is designed to protect the clinical samples from UV light exposure. Extended stabilities were performed under study number CA15081-01.

Table 14 Stability Results for S-PMA and S-BMA

Analyte	5°C	20°C	40°C
S-PMA	96 hours	96 hours	48 hours
S-BMA	96 hours	96 hours	96 hours

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6.1.3.3.16 References

(b) (4) Unpublished on file data - Validation of a LC-MS/MS Method for the Determination of 3-HPMA, HBMA, and CEMA in Human Urine. 22 Oct 2015 (b) (4) Study ZZ36536-01. (b) (4) .

(b) (4) Unpublished on file data - Validation of a LC-MS/MS Method for the Determination of Nicotine and Cotinine in Human Plasma (EDTA). 08-Aug-2014. (b) (4) Study AA33664-07. (b) (4) . 2014a.

(b) (4) Unpublished on file data - Validation of a LC-MS/MS Method for the Determination of Nicotine and Cotinine in Human Plasma (EDTA). 08-Aug-2014. (b) (4) Study AA33664-08. (b) (4) . 2014b.

(b) (4) Unpublished on file data - Validation of a LC-MS/MS Method for the Determination of Nicotine and Cotinine in Human Plasma (EDTA). 18-Mar-2016. (b) (4) Study AA33664-06, (b) (4) . 2016a.

(b) (4) Unpublished on file data - Validation of a LC-MS/MS Method for the Determination of Nicotine, Cotinine, and *trans*-3′-Hydroxycotinine in Human Plasma (EDTA). 18-Feb-2016 (b) (4) Study AA33664-01 (b) (4) . 2016b.

(b) (4) Unpublished on file data - Validation of an LC-MS/MS Method for the Determination of 4 Aminobiphenyl, *o*-Toluidine, 2-Aminonaphthalene, and 1-Aminonaphthalene in Human Urine. 06-Nov-2014. (b) (4) Study ZZ37540-01. (b) (4) (b) (4) .

(b) (4) Unpublished on file data - Validation of an LC-MS/MS Method for the Determination of Caffeine and Paraxanthine in Human Plasma (Heparin). 06 Oct-2014. (b) (4) Study ZZ25187-02. (b) (4) . 2014d.

(b) (4) Unpublished on file data - Validation of an LC-MS/MS Method for the Determination of HEMA in Human Urine. 30-Oct-2014. (b) (4) Study ZZ38073-01. (b) (4) .

(b) (4) Unpublished on file data - Validation of an LC-MS/MS Method for the Determination of Monohydroxy-3-butenyl-mercapturic Acid in Human Urine. 03-Feb-2016. (b) (4) Study VAA98876-02. (b) (4) . 2016c.

(b) (4) Unpublished on file data - Validation of an LC-MS/MS Method for the Determination of Nicotine, Cotinine, *trans*-3′-Hydroxycotinine, Nicotine-*N*-Glucuronide, Cotinine-*N*-Glucuronide, *trans*-3′-Hydroxycotinine-*O*-Glucuronide in Human Urine. 08-Mar-2016. (b) (4) Study ZZ33881-03. (b) (4) . 2016d.

(b) (4) Unpublished on file data - Validation of an LC-MS/MS Method for the Determination of Nicotine, Cotinine, *trans*-3′-Hydroxycotinine, Nicotine-*N*-Glucuronide, Cotinine-*N*-Glucuronide, *trans*-3′-Hydroxycotinine-*O*-Glucuronide in Human Urine. 30-Oct-2014. (b) (4) Study ZZ33881-04. (b) (4) . 2014f.

(b) (4) Unpublished on file data - Validation of an LC-MS/MS Method for the Determination of *S*-Benzyl Mercapturic Acid and *S*-Phenyl Mercapturic Acid in Human Urine. 22-Oct-2013. (b) (4) Study VAA98876-01 (b) (4) . 2013.

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(b) (4) Unpublished on file data - Validation of an LC-MS/MS Method for the Determination of Total 1-Hydroxypyrene in Human Urine. 02-Feb-2016. (b) (4) Study VAA98876-03. (b) (4) . 2016e.

(b) (4) Unpublished on file data - Validation of an LC-MS/MS Method for the Determination of Total 3-Hydroxybenzo[a]pyrne in Human Urine. 02 Mar-2016. (b) (4) Study ZZ42666-01 (b) (4) . 2016f.

(b) (4) Unpublished on file data - Validation of an LC-MS/MS Method for the Determination of Total NNAL and Total NNN in Human Urine. 21-Dec-2015 (b) (4) Study ZZ34313-03. (b) (4) . 2015b.

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To the best of my knowledge the above information is accurate. (b) (4)
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