Appendix A6.1.3.36 Validation Summary ZZ42666-01 (Hydroxybenzo[a]pyrene in Urine)

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The following validation summary has been prepared by (b) (4) Full method validation report and related SOPs are all available at (b) (4)

(b) (4)

Table 1. Validation Summary – -Hydroxybenzo[a]pyrene in Urine - ZZ42666-01

Information Requested	Data
Validation Summary	(b) (4) Validation Study ZZ42666-01
Analyte	3-Hydroxybenzo[a]pyrene
Internal Standard (IS)	$^{13}C_6$-3-Hydroxybenzo[a]pyrene
Method Description	Solid phase extraction with analysis/detection by LC-MS/MS
Limit of Quantitation (fg/mL)	25.0 fg/mL
Average Recovery of Drug (% Mean)	From dilute human urine: 104% at 50.0 fg/mL 98% at 150 fg/mL 91% at 500 fg/mL
Average Recovery of IS (% Mean)	From dilute human urine (peak area ratio): 73% From human urine (peak area ratio): 95% at 50.0 fg/mL 91% at 150 fg/mL 97% at 500 fg/mL
Standard Curve Concentrations (fg/mL)	25.0, 50.0, 75.0, 100, 150, 250, 500, and 600 fg/mL
QC Concentrations (fg/mL)	LLOQ QC, 58.1, 118, 388, and 448 fg/mL
QC Intra-Batch Precision Range (% CV)	3-Hydroxybenzo[a]pyrene: 1.4 to 17.6% 3-Hydroxybenzo[a]pyrene-O-β-Glucuronide: 3.0 to 16.4%
QC Intra-Batch Accuracy Range (% Bias)	3-Hydroxybenzo[a]pyrene:-20.0 to 11.2% 3-Hydroxybenzo[a]pyrene-O-β-Glucuronide: -7.2 to 11.6%
QC Inter-Batch Precision Range (% CV)	3-Hydroxybenzo[a]pyrene:4.5 to 15.8% 3-Hydroxybenzo[a]pyrene-O-β-Glucuronide: 4.4 to 14.2%
QC Inter-Batch Accuracy Range (% Bias)	3-Hydroxybenzo[a]pyrene:-2.8 to 1.6% 3-Hydroxybenzo[a]pyrene-O-β-Glucuronide: -3.9 to 36
Bench-Top Stability (Hrs)	Short-Term Stability: 24 hours in polypropylene tubes at ambient temperature under UV-shielded light Cumulative Short-Term Stability: 55 hours in polypropylene tubes at ambient temperature under UV-shielded light (total of all thaw cycles)

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Stock Stability (Days)	Long-Term Stability for Stock Solutions (Stock): 1477 days at approximately 150 µg/mL in ethanol in dip- silanized glass screw-cap tubes at -20°C
Processed Stability (Hrs)	Post-Preparative Stability: 156 hours in UPLC vials with silanized glass inserts at 5°C Processed Sample Integrity: 154 hours in UPLC vials with silanized glass inserts at 5°C
Freeze-Thaw Stability (Cycles)	6 freeze (-20°C)-thaw (ambient temperature) cycles in polypropylene tubes under UV-shielded light
Long-Term Storage Stability (Days)	Long-Term Stability: 570 days in polypropylene tubes at -20°C
Dilution Integrity	N/AP
Selectivity	No significant interference at the retention time and mass transition of $^{13}C_6$-3-hydroxybenzo[a]pyrene (IS) was observed from endogenous components in any of the 10 human urine lots screened

Additional Information – -Hydroxybenzo[a]pyrene in Urine - ZZ42666-01

Matrix		Human Urine	
Bioanalytical Method (BAM) SOP Number		BAM SOP ZZ42666-01	
Detector		AB SCIEX Triple Quad™ 6500	
Assay Volume Required		2.70 mL	
Regression Type		Weighted linear (1/concentration)	
Quantitation Method		Peak area ratio	
Quality Control Samples (3-Hydroxybenzo[a]pyrene)		Precision (% CV)	Accuracy (% Bias)
Inter-Batch	**LLOQ**	15.8	-2.8
	Low (Basal)	7.9	-0.7
	Medium	6.1	-0.8
	High (Basal)	6.8	-0.8
	High	4.5	1.6
Intra-Batch (Batch 33) Aliquot Method: Manual Extraction Method: Manual	**LLOQ**	4.6	1.2
	Low (Basal)	2.4	3.3
	Medium	2.8	5.9
	High (Basal)	4.8	-5.7
	High	1.4	5.4
Intra-Batch (Batch 35)[1] Aliquot Method: Manual Extraction Method: Manual	**LLOQ**	17.6	-20.0
	Low (Basal)	8.2	-4.6
	Medium	3.0	-1.7
	High (Basal)	5.2	1.5
	High	3.3	4.0
Intra-Batch (Batch 36) Aliquot Method: Manual	**LLOQ**	16.8	11.2
	Low (Basal)	11.9	1.5

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

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Additional Information – -Hydroxybenzo[a]pyrene in Urine - ZZ42666-01			
Extraction Method: Manual	**Medium**	5.6	-0.8
	High (Basal)	3.0	4.1
	High	2.7	1.6
Intra-Batch (Batch 41) Aliquot Method: Manual Extraction Method: Manual	**LLOQ**	12.0	-6.4
	Low (Basal)	3.5	-5.7
	Medium	5.8	-6.8
	High (Basal)	9.5	-2.6
	High	3.9	-4.7
Intra-Batch (Batch 42) Aliquot Method: Manual Extraction Method: Manual	**LLOQ**	8.1	-0.8
	Low (Basal)	7.1	1.7
	Medium	6.7	0.0
	High (Basal)	7.0	-2.1
	High	3.5	1.8
Quality Control Samples (3-Hydroxybenzo[a]pyrene-O-β-Glucuronide)		Precision (% CV)	Accuracy (% Bias)
Inter-Batch	**LLOQ**	14.2	3.6
	Low (Basal)	8.6	0.0
	Medium	4.4	-3.9
	High (Basal)	5.9	-0.3
	High	4.9	-1.3
Intra-Batch (Batch 157) Aliquot Method: Manual Extraction Method: Manual	**LLOQ**	16.4	-7.2
	Low (Basal)	6.2	-2.9
	Medium	3.0	-6.2
	High (Basal)	4.0	-4.0
	High	3.4	-5.2
Intra-Batch (Batch 158) Aliquot Method: Manual Extraction Method: Manual	**LLOQ**	11.3	6.8
	Low (Basal)	11.1	5.2
	Medium	4.5	-3.1
	High (Basal)	4.7	6.2
	High	4.8	1.3
Intra-Batch (Batch 160) Aliquot Method: Manual Extraction Method: Manual	**LLOQ**	10.2	11.6
	Low (Basal)	7.7	-0.1
	Medium	4.7	-1.6
	High (Basal)	4.0	-0.6
	High	4.1	0.0
Matrix Effect		No significant matrix effect was observed in 9 of the 10 human urine lots that were fortified with 3-hydroxybenzo[a]pyrene at a low concentration or in any of the 10 human urine lots that were fortified with 3-hydroxybenzo[a]pyrene at a high concentration	
Sample Shipping Stability		12 days in polypropylene tubes at -80°C	
Long-Term Stability for Stock Solutions (Substock)		57 days at 1000 ng/mL in ethanol in a silanized glass container at -20°C 85 days at 18.0 pg/mL in ethanol in a silanized glass container at -20°C 85 days at 0.750 pg/mL in ethanol in a silanized glass container at -20°C	

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Additional Information – -Hydroxybenzo[a]pyrene in Urine - ZZ42666-01	
Long-Term Stability for Stock Solutions (Internal Standard Stock)	Refer to long-term stability for stock solutions data collected from unlabeled 3-hydroxybenzo[a]pyrene for labeled internal standard stability
Short-Term Stability for Stock Solutions (Stock)	6 hours at approximately 150 µg/mL in ethanol in a silanized glass container at ambient temperature under UV-shielded light
Short-Term Stability for Stock Solutions (Substock)	15 hours at 1000 ng/mL in ethanol in a silanized glass container at ambient temperature under UV-shielded light 15 hours at 0.750 pg/mL in ethanol in a silanized glass container at ambient temperature under UV-shielded light
Short-Term Stability for Stock Solutions (Internal Standard Stock)	Refer to short-term stability for stock solutions data collected from unlabeled 3-hydroxybenzo[a]pyrene for labeled internal standard stability
Short-Term Stability for Stock Solutions (Internal Standard Substock)	6 hours at 25.0 pg/mL in methanol in a silanized glass container at ambient temperature under UV-shielded light
Stability of Analyte During Sample Collection and Handling	Up to 24 hours in a high-density polyethylene container at 5°C, then up to 120 minutes in polypropylene tubes at ambient temperature under white light conditions Up to 96 hours in non-smoker human urine in high-density polyethylene containers at 5°C, 20°C, and 40°C under UV-shielded light, up to 96 hours in smoker human urine in high-density polyethylene containers at 5°C and 20°C under UV-shielded light
Sample Aliquot Frozen Storage Stability	Samples aliquoted manually at a volume of 2.70 mL, stored for 170 hours in 16 x 100 mm silanized glass screw-cap tubes at -20°C prior to extraction
Batch Size	96 injections

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