Appendix A6.1.3.33 Validation Summary ZZ38073-01 (HEMA 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 – HEMA in Urine - ZZ38073-01

Information Requested	Data
Validation Summary	(b) (4) Validation Study ZZ38073-01
Analyte	HEMA
Internal Standard (IS)	$^{15}N^{13}C_3$-HEMA
Method Description	Solid phase extraction with analysis/detection by LC-MS/MS
Limit of Quantitation (ng/mL)	0.100 ng/mL
Average Recovery of Drug (% Mean)	64% at 0.200 ng/mL 74% at 2.00 ng/mL 79% at 16.0 ng/mL
Average Recovery of IS (% Mean)	From UriSub®: 72% From human urine: 87% at 0.200 ng/mL 88% at 2.00 ng/mL 89% at 16.0 ng/mL
Standard Curve Concentrations (ng/mL)	0.100, 0.200, 0.400, 1.00, 2.00, 5.00, 10.0, 16.0, and 20.0 ng/mL
QC Concentrations (ng/mL)	LLOQ QC, 0.225, 1.40, and 14.9 ng/mL
QC Intra-Batch Precision Range (% CV)	2.2 to 5.8%
QC Intra-Batch Accuracy Range (% Bias)	-5.4 to 9.8%
QC Inter-Batch Precision Range (% CV)	3.2 to 6.1%
QC Inter-Batch Accuracy Range (% Bias)	-2.0 to 4.9%
Bench-Top Stability (Hrs)	Short-Term Stability: 27 hours in clear polypropylene tubes at ambient temperature under white light Cumulative Short-Term Stability: 53 hours in brown polypropylene tubes at ambient temperature under white light (total of all thaw cycles) Short-Term Stability: 4 hours in polypropylene tubes at ambient temperature under UV-shielded light
Stock Stability (Days)	Long-Term Stability for Stock Solutions (Stock): 258 days at approximately 100 µg/mL in methanol in a polypropylene container at -20°C

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Information Requested	Data
Processed Stability (Hrs)	Post-Preparative Stability: 205 hours in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 171 hours in a polypropylene 96 well plate at 5°C
Freeze-Thaw Stability (Cycles)	6 freeze (-20°C)-thaw (ambient temperature) cycles in brown polypropylene tubes under white light
Long-Term Storage Stability (Days)	Long-Term Stability: 406 days in brown polypropylene tubes at -20°C
Dilution Integrity	Up to 49.9 ng/mL, diluted 5-fold
Selectivity	AB SCIEX API 5000™ Mass Spectrometer: No significant interference at the retention time and mass transition of $^{15}N^{13}C_3$-HEMA (IS) in any of the 10 human urine lots screened AB SCIEX QTRAP® 5500 Mass Spectrometer: No significant interference at the retention time and mass transition of $^{15}N^{13}C_3$-HEMA (IS) in any of the 10 human urine lots screened

Additional Information – HEMA in Urine - ZZ38073-01

Matrix		Human Urine	
Bioanalytical Method (BAM) SOP Number		BAM SOP ZZ38073-01	
Detector		AB SCIEX API 5000™ or QTRAP® 5500	
Assay Volume Required		0.300 mL	
Regression Type		Weighted linear (1/concentration^2)	
Quantitation Method		Peak area ratio	
Quality Control Samples		Precision (% CV)	Accuracy (% Bias)
Inter-Batch	**LLOQ**	5.1	2.0
	Low	6.1	4.9
	Medium	4.0	0.7
	High	3.2	-2.0
Intra-Batch (Batch 28) **Aliquot Method: Manual** **Extraction Method:** **Automated**	**LLOQ**	3.7	4.0
	Low	4.5	9.8
	Medium	3.3	2.1
	High	2.2	-2.0
Intra-Batch (Batch 29) **Aliquot Method: Manual** **Extraction Method:** **Automated**	**LLOQ**	3.2	2.0
	Low	2.7	2.7
	Medium	4.0	2.1
	High	2.7	-1.3
Intra-Batch (Batch 31) **Aliquot Method: Manual** **Extraction Method:** **Automated**	**LLOQ**	2.5	-5.4
	Low	3.5	-2.2
	Medium	3.2	-3.6
	High	2.8	-4.7
Intra-Batch (Batch 34) **Aliquot Method: Manual** **Extraction Method:** **Automated**	**LLOQ**	4.1	5.0
	Low	5.8	6.2
	Medium	4.0	-0.7
	High	3.1	1.3

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Additional Information – HEMA in Urine - ZZ38073-01

Matrix Effect	AB SCIEX API 5000™ Mass Spectrometer: No significant matrix effect was observed in any of the 10 human urine lots that were fortified with HEMA at a low concentration or in any of the 10 human urine lots that were fortified with HEMA at a high concentration AB SCIEX QTRAP® 5500 Mass Spectrometer: No significant matrix effect was observed in any of the 10 human urine lots that were fortified with HEMA at a low concentration or in any of the 10 human urine lots that were fortified with HEMA at a high concentration
Sample Shipping Stability	8 days in brown polypropylene tubes at -80°C
Long-Term Stability for Stock Solutions (Substock)	177 days at 1.20 ng/mL in methanol in a polypropylene container at -20°C
Long-Term Stability for Stock Solutions (Internal Standard Stock)	Refer to long-term stability for stock solutions data collected from unlabeled HEMA for labeled internal standard stability
Long-Term Stability for Stock Solutions (Internal Standard Substock)	10 days at 18.0 ng/mL in methanol in a polypropylene container at -20°C
Short-Term Stability for Stock Solutions (Stock)	23 hours at approximately 100 µg/mL in methanol in a polypropylene container at ambient temperature under white light
Short-Term Stability for Stock Solutions (Substock)	23 hours at 1.20 ng/mL in methanol in a polypropylene container at ambient temperature under white light
Short-Term Stability for Stock Solutions (Internal Standard Stock)	Refer to short-term stability for stock solutions data collected from unlabeled HEMA for labeled internal standard stability
Short-Term Stability for Stock Solutions (Internal Standard Substock)	23 hours at 18.0 ng/mL in methanol in a polypropylene container at ambient temperature under white light
Stability of Analyte During Sample Collection and Handling	2 days in human urine in amber high-density polyethylene containers at 5°C in a refrigerator, 4 hours in human urine in brown polypropylene tubes at ambient temperature under white light Up to 96 hours in smoker and non-smoker human urine in high-density polyethylene containers at 5°C and 20°C under UV-shielded light, up to 48 hours in smoker and non-smoker human urine in high-density polyethylene containers at 40°C under UV-shielded light
Sample Aliquot Frozen Storage Stability	Samples aliquoted manually at a volume of 0.300 mL, stored for 94 hours in a polypropylene 96 well plate at -20°C prior to extraction
Batch Size	192 injections

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