Appendix A6.1.3.37 Validation Summary ZZ25187-02 (Caffeine and Paraxanthine in Plasma)

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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 – Caffeine in Plasma - ZZ25187-02

Information Requested	Data
Validation Summary	(b) (4) Validation Study ZZ25187-02
Analyte	Caffeine
Internal Standard (IS)	d_9-Caffeine
Method Description	Liquid-liquid extraction with analysis/detection by LC-MS/MS
Limit of Quantitation (ng/mL)	20.0 ng/mL
Average Recovery of Drug (% Mean)	81% at 50.0 ng/mL 81% at 500 ng/mL 87% at 4000 ng/mL
Average Recovery of IS (% Mean)	82%
Standard Curve Concentrations (ng/mL)	20.0, 40.0, 100, 250, 500, 1500, 3000, 4250, and 5000 ng/mL
QC Concentrations (ng/mL)	LLOQ QC, 50.0, 500, and 4000 ng/mL
QC Intra-Batch Precision Range (% CV)	1.1 to 8.0%
QC Intra-Batch Accuracy Range (% Bias)	-10.5 to 8.0%
QC Inter-Batch Precision Range (% CV)	4.3 to 7.9%
QC Inter-Batch Accuracy Range (% Bias)	-6.0 to 3.6%
Bench-Top Stability (Hrs)	Short-Term Stability: 24 hours in polypropylene tubes at ambient temperature and in an ice water bath under white light Cumulative Short-Term Stability: 53 hours in polypropylene tubes at ambient temperature and in an ice water bath under white light (total of all thaw cycles)
Stock Stability (Days)	Long-Term Stability for Stock Solutions (Stock): 902 days at approximately 2000 µg/mL in 40:60 methanol:water in a polypropylene container at -20°C
Processed Stability (Hrs)	Post-Preparative Stability: 162 hours in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 148 hours in a polypropylene 96 well plate at 5°C
Freeze-Thaw Stability (Cycles)	6 freeze (-20°C)-thaw (ambient temperature and in an ice water bath) cycles in polypropylene tubes under white light
Long-Term Storage Stability (Days)	Long-Term Stability: 545 days in polypropylene tubes at

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	-20°C
Dilution Integrity	up to 20,000 ng/mL, diluted 10-fold
Selectivity	No significant interference at the retention time and mass transition of caffeine was observed from endogenous components in any of the 6 human plasma (heparin) lots screened or of d_9-caffeine (IS) in any of the 6 human plasma (heparin) lots screened

Additional Information – Caffeine in Plasma - ZZ25187-02

Matrix		Human Plasma	
Anticoagulant		Lithium Heparin	
Bioanalytical Method (BAM) SOP Number		BAM SOP ZZ25187-02	
Detector		AB SCIEX API 4000™	
Assay Volume Required		0.0500 mL	
Regression Type		Weighted linear (1/concentration^2)	
Quantitation Method		Peak area ratio	
Multiple Analytes Measured by Assay		Caffeine at 50.0 ng/mL fortified with paraxanthine at 5000 ng/mL	
Known Metabolite Evaluation		Theophylline (200 ng/mL) Theobromine (600 ng/mL)	
Quality Control Samples		Precision (% CV)	Accuracy (% Bias)
Inter-Batch	**LLOQ**	7.9	-6.0
	Low	4.7	-3.2
	Medium	4.3	3.6
	High	4.6	1.8
Intra-Batch (Batch 10) **Aliquot Method: Manual** **Extraction Method:** **Automated**	**LLOQ**	8.0	-10.5
	Low	3.1	-6.4
	Medium	1.7	0.4
	High	1.1	-1.5
Intra-Batch (Batch 11) **Aliquot Method: Manual** **Extraction Method:** **Automated**	**LLOQ**	2.8	-0.5
	Low	5.1	-1.0
	Medium	3.2	8.0
	High	3.1	6.8
Intra-Batch (Batch 12) **Aliquot Method: Manual** **Extraction Method:** **Automated**	**LLOQ**	5.7	-1.5
	Low	2.1	-1.4
	Medium	2.6	1.8
	High	1.7	-1.8
Matrix Effect		No significant matrix effect was observed in any of the 6 human plasma (heparin) lots that were fortified at the concentration of the LLOQ (20.0 ng/mL) and in any of the 6 lots that were fortified at the concentration of the high QC (4000 ng/mL) sample	
Hemolyzed Sample Integrity		No significant interference for caffeine was observed in any of the 3 hemolyzed human plasma (heparin) lots that were fortified at the concentration of the LLOQ (20.0 ng/mL) and in any of the 3 lots that were fortified at the concentration of the high QC (4000 ng/mL) sample	

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Lipemic Sample Evaluation	No significant interference for caffeine was observed in any of the 3 lipemic human plasma (heparin) lots that were fortified near the concentration of the low QC (40.0 ng/mL) and in any of the 3 lots that were fortified near the concentration of the high QC (4250 ng/mL) sample
Sample Shipping Stability (Days)	3 days in polypropylene tubes at -80°C
Long-Term Stability for Stock Solutions (Substock)	155 days at 100 µg/mL in 40:60 methanol:water in a polypropylene container at -20°C 154 days at 20.0 ng/mL in 40:60 methanol:water in a polypropylene container at -20°C
Long-Term Stability for Stock Solutions (Internal Standard)	refer to long-term stability for stock solutions data collected from unlabeled caffeine for labeled internal standard stability
Short-Term Stability for Stock Solutions (Stock)	8 hours at approximately 2000 µg/mL in 40:60 methanol:water in a polypropylene container at ambient temperature under white light
Short-Term Stability for Stock Solutions (Substock)	8 hours at 20.0 ng/mL in 40:60 methanol:water in a polypropylene container at ambient temperature under white light
Short-Term Stability for Stock Solutions (Internal Standard)	refer to short-term stability for stock solutions data collected from unlabeled caffeine for labeled internal standard stability
Stability of Analyte During Sample Collections and Handling	up to 120 minutes in human whole blood (heparin) in polypropylene tubes at ambient temperature under white light
Sample Aliquot Frozen Storage Stability	samples aliquoted manually at a volume of 0.0500 mL, stored for 72 hours in a polypropylene 96 well plate at -20°C prior to extraction
Batch Size	192 injections

Table 2. Validation Summary – Paraxanthine in Plasma - ZZ25187-02

Information Requested	Data
Validation Summary	(b) (4) Validation Study ZZ25187-02
Analyte	Paraxanthine
Internal Standard (IS)	d_3-Paraxanthine
Method Description	Liquid-liquid extraction with analysis/detection by LC-MS/MS
Limit of Quantitation (ng/mL)	20.0 ng/mL
Average Recovery of Drug (% Mean)	59% at 50.0 ng/mL 61% at 500 ng/mL 63% at 4000 ng/mL
Average Recovery of IS (% Mean)	60%
Standard Curve Concentrations (ng/mL)	20.0, 40.0, 100, 250, 500, 1500, 3000, 4250, and 5000 ng/mL

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QC Concentrations (ng/mL)	LLOQ QC, 50.0, 500, and 4000 ng/mL
QC Intra-Batch Precision Range (% CV)	0.7 to 4.7%
QC Intra-Batch Accuracy Range (% Bias)	-9.5 to 4.0%
QC Inter-Batch Precision Range (% CV)	1.9 to 6.5%
QC Inter-Batch Accuracy Range (% Bias)	-3.3 to -0.6%
Bench-Top Stability (Hrs)	Short-Term Stability: 24 hours in polypropylene tubes at ambient temperature and in an ice water bath under white light Cumulative Short-Term Stability: 53 hours in polypropylene tubes at ambient temperature and in an ice water bath under white light (total of all thaw cycles)
Processed Stability (Hrs)	Post-Preparative Stability: 162 hours in a polypropylene 96 well plate at 5°C Processed Sample Integrity: 148 hours in a polypropylene 96 well plate at 5°C
Freeze-Thaw Stability (Cycles)	6 freeze (-20°C)-thaw (ambient temperature and in an ice water bath) cycles in polypropylene tubes under white light
Long-Term Storage Stability (Days)	Long-Term Stability: 545 days in polypropylene tubes at -20°C
Dilution Integrity	up to 10,000 ng/mL, diluted 10-fold
Selectivity	No significant interference at the retention time and mass transition of paraxanthine was observed from endogenous components in any of the 6 human plasma (heparin) lots screened or of d_3-paraxanthine (IS) in any of the 6 human plasma (heparin) lots screened

Additional Information – Paraxanthine in Plasma - ZZ25187-02

Matrix		Human Plasma	
Anticoagulant		Lithium Heparin	
Bioanalytical Method (BAM) SOP Number		BAM SOP ZZ25187-02	
Detector		AB SCIEX API 4000™	
Assay Volume Required		0.0500 mL	
Regression Type		Weighted linear (1/concentration^2)	
Quantitation Method		Peak area ratio	
Multiple Analytes Measured by Assay		Paraxanthine at 50.0 ng/mL fortified with caffeine at 5000 ng/mL	
Known Metabolite Evaluation		Theophylline (200 ng/mL) Theobromine (600 ng/mL)	
Quality Control Samples		Precision (% CV)	Accuracy (% Bias)
Inter-Batch	**LLOQ**	6.5	-3.0
	Low	4.1	-1.8
	Medium	1.9	-0.6
	High	2.1	-3.3
Intra-Batch (Batch 10)	**LLOQ**	4.7	-3.0
Aliquot Method: Manual	**Low**	4.3	-3.0

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Extraction Method: Automated	**Medium**	1.9	-1.8
	High	2.2	-4.8
Intra-Batch (Batch 11) Aliquot Method: Manual Extraction Method: Automated	**LLOQ**	4.6	4.0
	Low	3.5	0.2
	Medium	1.9	0.0
	High	1.5	-2.5
Intra-Batch (Batch 12) Aliquot Method: Manual Extraction Method: Automated	**LLOQ**	2.7	-9.5
	Low	3.5	-3.6
	Medium	0.7	0.0
	High	1.3	-2.3
Matrix Effect		No significant matrix effect was observed in any of the 6 human plasma (heparin) lots that were fortified at the concentration of the LLOQ (20.0 ng/mL) and in any of the 6 lots that were fortified at the concentration of the high QC (4000 ng/mL) sample	
Hemolyzed Sample Integrity		No significant interference for paraxanthine was observed in any of the 3 hemolyzed human plasma (heparin) lots that were fortified at the concentration of the LLOQ (20.0 ng/mL) and in any of the 3 lots that were fortified at the concentration of the high QC (4000 ng/mL) sample	
Lipemic Sample Evaluation		No significant interference for caffeine was observed in any of the 3 lipemic human plasma (heparin) lots that were fortified at a low concentration (40.0 ng/mL) and in any of the 3 lots that were fortified near the concentration of the high QC (4250 ng/mL) sample	
Sample Shipping Stability (Days)		3 days in polypropylene tubes at -80°C	
Long-Term Stability for Stock Solutions (Substock)		155 days at 100 µg/mL in 40:60 methanol:water in a polypropylene container at -20°C 154 days at 20.0 ng/mL in 40:60 methanol:water in a polypropylene container at -20°C	
Long-Term Stability for Stock Solutions (Internal Standard)		refer to long-term stability for stock solutions data collected from unlabeled paraxanthine for labeled internal standard stability	
Short-Term Stability for Stock Solutions (Substock)		18 hours at 100 µg/mL in 40:60 methanol:water in a polypropylene container at ambient temperature under white light 8 hours at 20.0 ng/mL in 40:60 methanol:water in a polypropylene container at ambient temperature under white light	
Short-Term Stability for Stock Solutions (Internal Standard)		refer to short-term stability for stock solutions data collected from unlabeled paraxanthine for labeled internal standard stability	
Stability of Analyte During Sample Collections and Handling		up to 120 minutes in human whole blood (heparin) in polypropylene tubes at ambient temperature under white light	
Sample Aliquot Frozen Storage Stability		samples aliquoted manually at a volume of 0.0500 mL, stored for 72 hours in a polypropylene 96 well plate at -20°C prior to extraction	
Batch Size		192 injections	

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