SCIANALYTICAL STRATEGIES, INC.

Bioanalytical Testing and Consulting for Pharmaceutical and Biotechnology Industries

STUDY TITLE

[redacted] Method Development and Samples Analysis for

[redacted]

CONFIDENTIAL

Date: June 11, 2015

Sponsor: [redacted]

Sponsor Contact: [redacted]

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STATEMENT REGARDING BIOANALYTICAL ASSAY

The experimental work and data described in this report are considered non-GLP. All equipment used for this work was maintained according to manufacturer's specifications and/or SciAnalytical Bioanalytical lab operating procedures. All written and electronic data were stored and archived according to SciAnalytical Bionalytical Services Operating Instructions.

OBJECTIVE

The objective of this study was to develop and qualify two LC/MS/MS methods, one for the quantification of [redacted] and the other being for the quantification of [redacted] in mouse plasma and to use these methods to measure the concentration of the two test articles In mouse plasma following dosing to diseased animals.

MATERIALS AND METHODS

Mouse plasma was received from [redacted] (San Diego, CA) on dry ice on December 14, 2014. Test articles were also received on dry ice on the same day. Plasma and test articles (in powder form) were stored at -80C temperature until initiation of studies.

PREPARATION OF STOCK SOLUTIONS

1.45 mg of [redacted] was dissolved in 1 mL of 50/50 acetonitrile/DMSO to make a 1.45 mg/mL primary stock solution. A reference stock solution of [redacted] at a concentration of 145 ug/ml was made by diluting the primary stock solution 1:10 in acetonitrile. A working stock solution [redacted] was prepared by diluting RSS-A to 40 ug/mL in water containing 0.1% formic acid..

1.80 mg of [redacted] was dissolved in 1 mL of 50/50 acetonitrile/DMSO to make a 1.80 mg/mL primary stock solution. A reference stock solution of [redacted] at a concentration of 180 ug/ml was made by diluting the primary stock solution 1:10 in acetonitrile. A working stock solution [redacted] was prepared by diluting RSS-B to 40 ug/mL in water containing 0.1% formic acid.

METHOD QUALIFICATION OVERVIEW

The LC/MS/MS bioanalytical method for quantitation of [redacted] was evaluated in mouse plasma by processing and analyzing one set of calibration standards on the front end and back end of the plasma samples. Method accuracy was measured by comparing the measured accuracies to the back-calculated concentration values for the calibration standards. The acceptance criteria were an average accuracy between 70% and 130%.

The LC/MS/MS bioanalytical method for quantitation of [redacted] was evaluated in mouse plasma by processing and analyzing one set of calibration standards on the front end and back end of the plasma samples. Method accuracy was measured by comparing the measured accuracies to the back-calculated concentration values for the calibration standards. The acceptance criteria were an average accuracy between 70% and 130%.

PREPARATION OF PLASMA CALIBRATION STANDARDS

9 calibration standards of [redacted] and [redacted] were prepared in plasma for quantification. To prepare the top calibration standard (Standard 9), the 40 ug/mL WSS-A or WSS-B working solution was diluted into blank plasma to a concentration of 2 ug/ml. Subsequently, the remaining 8 standards were prepared by serial dilution (1:1) of Standard 9 into blank rat plasma, followed by addition of 50 uL each standard to 50 uL of blank plasma.

Sample	Volume of Previous Solution	Volume Naïve Plasma	Final Concentration (ng/mL)
Standard 9	5µL	95µL	2,000
Standard 8	50µL	50 µL	1,000
Standard 7	50µL	50 µL	500
Standard 6	50µL	50 µL	250
Standard 5	50µL	50 µL	125
Standard 4	50µL	50 µL	62.5
Standard 3	50µL	50 µL	31.2
Standard 2	50µL	50 µL	15.6
Standard 1	50µL	50 µL	7.8

PREPARATION OF PLASMA QC SAMPLES

Not applicable for this study, as this was a research grade assay

PROCESSING OF [redacted] FOR ANALYSIS

Calibration standards and study samples (plasma) were processed for LC/MS/MS analysis by precipitating 50 µL of each sample with four volumes (200 µL) of ice cold Internal Standard Solution (acetonitrile containing 180 ng/mL of [redacted]). The precipitated samples were centrifuged at 10,000 rpm for 10 minutes. Following centrifugation, 150 µL of each supernatant was transferred to a microtiter plate containing 450 uL of water containing 0.1% formic acid and placed into the autosampler for LC/MS/MS analysis.

PROCESSING OF CCP-100010-4 FOR ANALYSIS

Calibration standards and study samples (plasma) were processed for LC/MS/MS analysis by precipitating 50 µL of each sample with four volumes (200 µL) of ice cold Internal Standard Solution (acetonitrile containing 145 ng/mL of [redacted]). The precipitated samples were centrifuged at 10,000 rpm for 10 minutes. Following centrifugation, 150 µL of each supernatant was transferred to a microtiter plate containing 450 uL of water containing 0.1% formic acid and placed into the autosampler for LC/MS/MS analysis.

LC/MS/MS ANALYSIS

Processed plasma samples were analyzed using the following LC/MS/MS conditions:

HPLC and Autosampler:	**Spark Holland Symbiosis**
Mobile Phase:	**A-0.1% formic acid in water**
	B-0.1% formic acid in acetonitrile
Column:	**Column: 2.1 x 50 mm Phenomenex Kinetex 5um C18 Column**
Injection Volume:	**10 µL**
Gradient:	**10% B for 0.5 minute followed by 10%-90% B in 2.5 minutes**
Flow Rate:	**400 µL/min**
Mass Spectrometer:	**AB SCIEX API4000Qtrap**
Interface:	**Electrospray at 500º C**
Software:	**Analyst v1.6.1**
Polarity:	**Positive Ion**
Q1/Q3 Ions:	[redacted]

[redacted] METHOD QUALIFICATION

The method qualification samples for plasma was processed and analyzed on December 21st, 2014. [redacted] was used as the internal standard and the calibration curves were fit using power regression. Results of the analysis are presented below. Assay performance data are summarized in Appendix I. The chromatograms from the lowest calibration standard and the calibration curve are presented in Appendix II.

[redacted] Calibration Standards – Plasma (front-end)

Sample Name	Sample Type	Analyte Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy (%)
Standard 1	Standard	7.8	9.26	119
Standard 2	Standard	15.6	12.5	80.2
Standard 3	Standard	31.2	27.5	88.2
Standard 4	Standard	62.5	64.9	104
Standard 5	Standard	125	144	115
Standard 6	Standard	250	264	106
Standard 7	Standard	500	501	100
Standard 8	Standard	1000	968	96.8
Standard 9	Standard	2000	2040	102

[redacted] Calibration Standards – Plasma (back-end)

Sample Name	Sample Type	Analyte Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy (%)
Standard 1	Standard	7.8	9.45	121.
Standard 2	Standard	15.6	11.5	73.7
Standard 3	Standard	31.2	30.3	97.0
Standard 4	Standard	62.5	62.4	99.8
Standard 5	Standard	125	132.	106.
Standard 6	Standard	250	256.	102.
Standard 7	Standard	500	484.	96.9
Standard 8	Standard	1000	884.	88.4
Standard 9	Standard	2000	2080.	104.

The average accuracy and precision for the calibration standards in each matrix met the acceptance criteria of 70-130% for average accuracy.

[redacted] METHOD QUALIFICATION

The method qualification samples for plasma was processed and analyzed on December 21st, 2014. CCP-100016-3 was used as the internal standard and the calibration curves were fit using power regression. Results of the analysis are presented below. Assay performance data are summarized in Appendix I. The chromatograms from the lowest calibration standard and the calibration curve are presented in Appendix II.

[redacted] Calibration Standards – Plasma (front-end)

Sample Name	Sample Type	Analyte Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy (%)
Standard 1	Standard	7.8	6.72	86.1
Standard 2	Standard	15.6	15.4	98.9
Standard 3	Standard	31.2	31.4	101.
Standard 4	Standard	62.5	67.2	108.
Standard 5	Standard	125	129.	103.
Standard 6	Standard	250	244.	97.7
Standard 7	Standard	500	489.	97.9
Standard 8	Standard	1000	932.	93.2
Standard 9	Standard	2000	1910.	95.7

[redacted] Calibration Standards – Plasma (back-end)

Sample Name	Sample Type	Analyte Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy (%)
Standard 1	Standard	7.8	6.34	81.2
Standard 2	Standard	15.6	14.8	95.2
Standard 3	Standard	31.2	35.2	113.
Standard 4	Standard	62.5	70.1	112.
Standard 5	Standard	125	131.	105.
Standard 6	Standard	250	254.	102.
Standard 7	Standard	500	534.	107.
Standard 8	Standard	1000	978.	97.8
Standard 9	Standard	2000	2140.	107.

The average accuracy and precision for the calibration standards in each matrix met the acceptance criteria of 70-130% for average accuracy.

STUDY SAMPLE ANALYSIS

Study plasma samples were prepared and analyzed on December 21st, 2014. Results shown below:

Group 6 Plasma Concentrations for [redacted] (50 mg/kg dose)

Sample Name	Time-Point	Sample Type	Calculated Concentration (ng/mL)
Plasma Gr6An1	1hr	Unknown	65.4
Plasma Gr6An2	1hr	Unknown	36.3
Plasma Gr6An3	1hr	Unknown	72.9
Plasma Gr6An4	1hr	Unknown	38.1
Plasma Gr6An5	2hr	Unknown	48.2
Plasma Gr6An6	2hr	Unknown	57.4
Plasma Gr6An7	2hr	Unknown	39.2
Plasma Gr6An8	2hr	Unknown	66.1
Plasma Gr6An1	4hr	Unknown	17.1
Plasma Gr6An2	4hr	Unknown	1.36
Plasma Gr6An3	4hr	Unknown	4.39
Plasma Gr6An4	4hr	Unknown	8.82
Plasma Gr6An5	8hr	Unknown	2.61
Plasma Gr6An6	8hr	Unknown	3.03
Plasma Gr6An7	8hr	Unknown	< 0
Plasma Gr6An8	8hr	Unknown	16.8

$AUC_{0\text{->}inf}$ = 183 ng*h/ml (using average concentrations of 4 animals at each time-point)

Group 7 Plasma Concentrations for [redacted] ([redacted] mg/kg dose)

Sample Name	Time-Point	Sample Type	Calculated Concentration (ng/mL)
Plasma Gr7An1	1hr	Unknown	31.2
Plasma Gr7An2	1hr	Unknown	93.2
Plasma Gr7An3	1hr	Unknown	63.2
Plasma Gr7An4	1hr	Unknown	259.
Plasma Gr7An5	2hr	Unknown	85.7
Plasma Gr7An6	2hr	Unknown	114.
Plasma Gr7An7	2hr	Unknown	142.
Plasma Gr7An8	2hr	Unknown	89.7
Plasma Gr7An1	4hr	Unknown	10.3
Plasma Gr7An2	4hr	Unknown	34.9
Plasma Gr7An3	4hr	Unknown	16.0
Plasma Gr7An4	4hr	Unknown	24.4
Plasma Gr7An5	8hr	Unknown	4.87
Plasma Gr7An6	8hr	Unknown	9.30
Plasma Gr7An7	8hr	Unknown	4.51
Plasma Gr7An8	8hr	Unknown	8.32

$AUC_{0\text{->}inf}$ = 367 ng*h/ml (using average concentrations of 4 animals at each time-point)

Group 8 Plasma Concentrations for [redacted] ([redacted] mg/kg dose)

Sample Name	Time-Point	Sample Type	Calculated Concentration (ng/mL)
Plasma Gr8An1	1hr	Unknown	89.6
Plasma Gr8An2	1hr	Unknown	84.4
Plasma Gr8An3	1hr	Unknown	247.
Plasma Gr8An4	1hr	Unknown	230.
Plasma Gr8An5	2hr	Unknown	100.
Plasma Gr8An6	2hr	Unknown	97.2
Plasma Gr8An7	2hr	Unknown	110.
Plasma Gr8An8	2hr	Unknown	134.
Plasma Gr8An1	4hr	Unknown	14.3
Plasma Gr8An2	4hr	Unknown	13.8
Plasma Gr8An3	4hr	Unknown	15.9
Plasma Gr8An4	4hr	Unknown	15.2
Plasma Gr8An5	8hr	Unknown	3.37
Plasma Gr8An6	8hr	Unknown	6.86
Plasma Gr8An7	8hr	Unknown	8.08
Plasma Gr8An8	8hr	Unknown	36.8

$AUC_{0\text{->}inf}$ = 439 ng*h/ml (using average concentrations of 4 animals at each time-point)

Group 9 Plasma Concentrations for [redacted] ([redacted] mg/kg dose)

Sample Name	Time-Point	Sample Type	Calculated Concentration (ng/mL)
Plasma Gr9An1	1hr	Unknown	634.
Plasma Gr9An2	1hr	Unknown	406.
Plasma Gr9An3	1hr	Unknown	263.
Plasma Gr9An4	1hr	Unknown	327.
Plasma Gr9An5	2hr	Unknown	206.
Plasma Gr9An6	2hr	Unknown	228.
Plasma Gr9An7	2hr	Unknown	406.
Plasma Gr9An8	2hr	Unknown	147.
Plasma Gr9An1	4hr	Unknown	81.0
Plasma Gr9An2	4hr	Unknown	42.8
Plasma Gr9An3	4hr	Unknown	121.
Plasma Gr9An4	4hr	Unknown	64.8
Plasma Gr9An5	8hr	Unknown	152.
Plasma Gr9An6	8hr	Unknown	26.9
Plasma Gr9An7	8hr	Unknown	114.
Plasma Gr9An8	8hr	Unknown	104.

$AUC_{0\text{->}inf}$ = 1720 ng*h/ml (using average concentrations of 4 animals at each time-point)

APPENDIX I - ASSAY PERFORMANCE

Plasma Assay Qualification

I. Carryover

Sample Name	Analyte Peak Area (counts)	% Carryover
Standard 9	2.59+06	
Carryover 1	2.04+03	0.09

II. Standard Curve information

	Assay info	Acceptance Criteria
LLOQ	7.8 ng/ml	N/A
ULOQ	2000 ng/mL	N/A
No. of Points Spanning Cal. Curve	9	≥5
Accepted No. of Stds within Curve- must include both LLOQ's & ULOQ's	9 of 9 accepted	100% of Standards within Cal. Range
Curve Fitting	Power	Power→ Linear 1/x

Suggested accuracy acceptance criteria: ≤ 25% except ≤ 30% for LLOQ.

APPENDIX II- LLOQ CHROMATOGRAMS AND CALIBRATION CURVES

Figure Legend 1. [redacted] Calibrator 1 Analyte (left image); Internal Standard (right image)

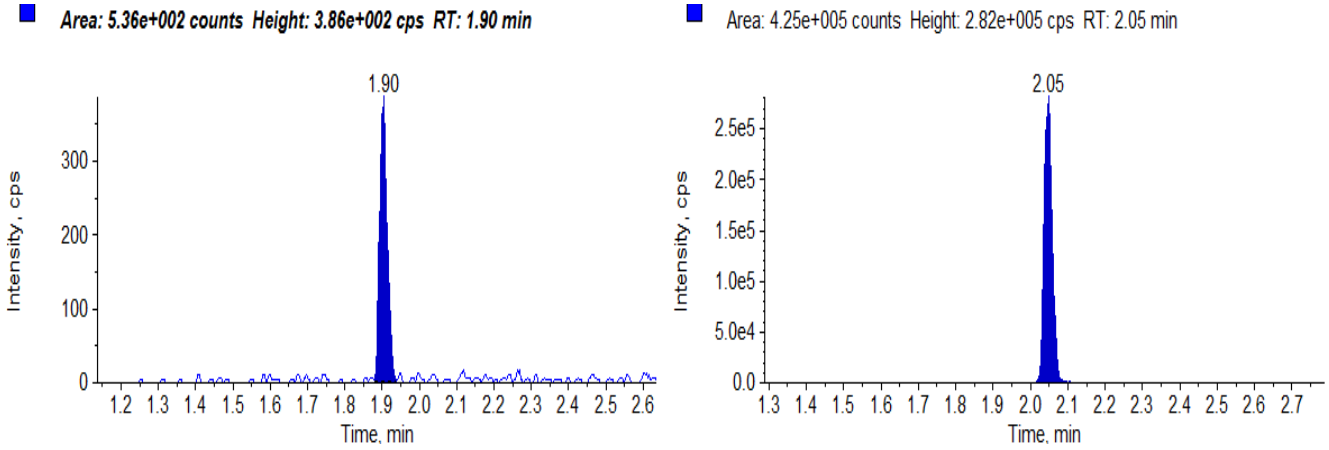
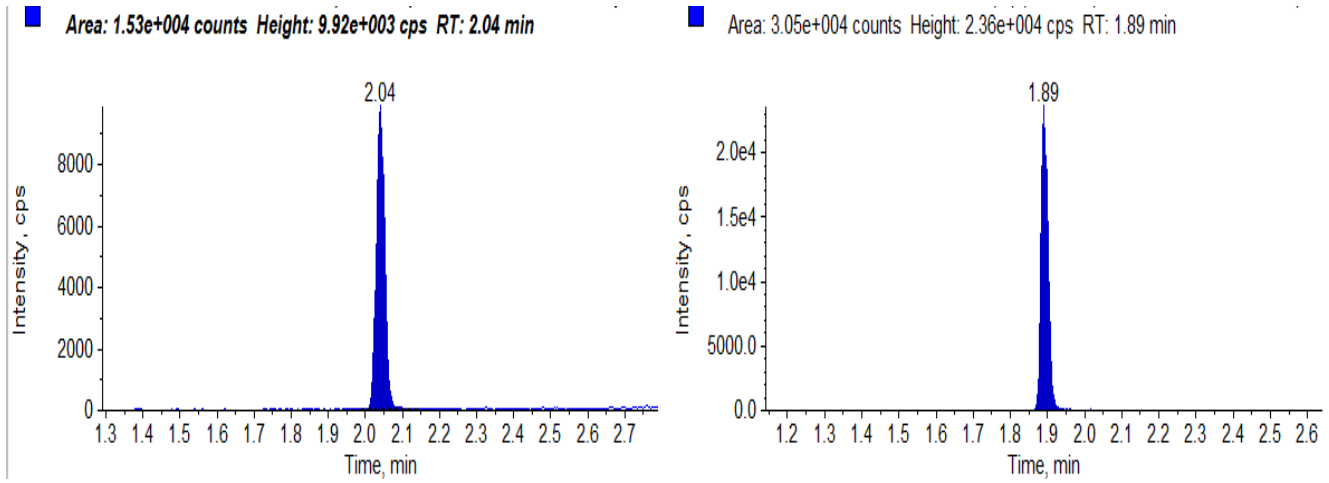
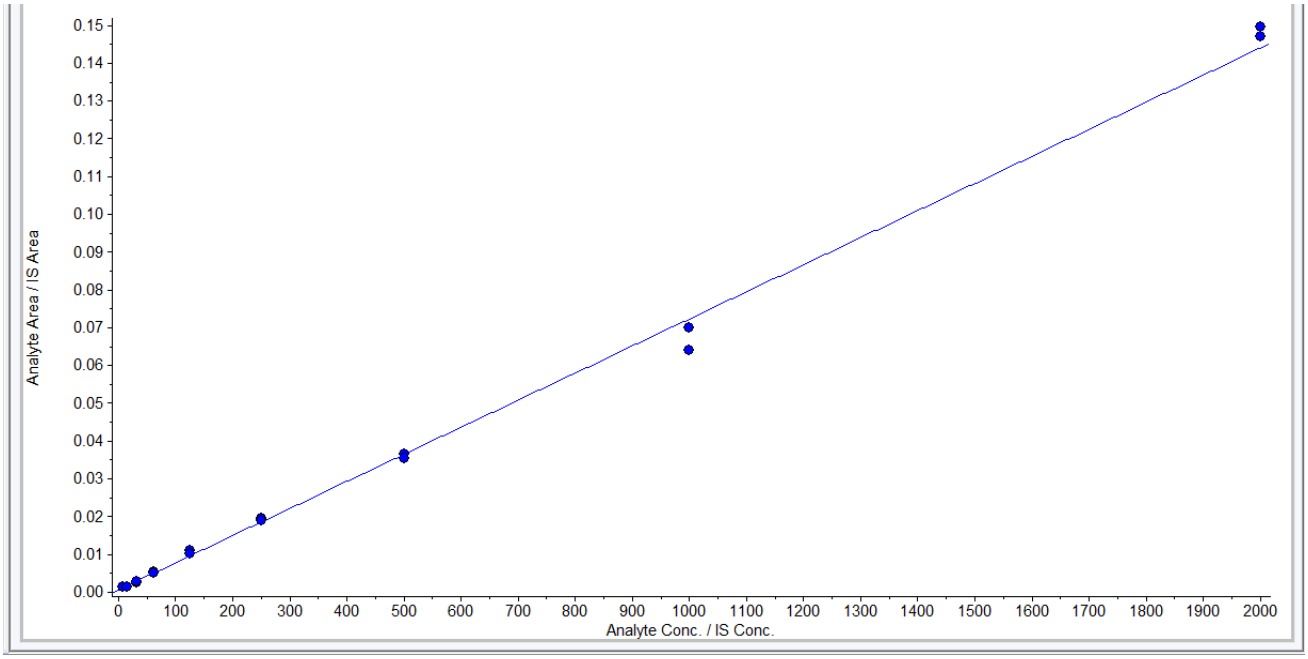


Figure Legend 2. [redacted] Calibrator 1 Analyte (left image); Internal Standard (right image)



[redacted] **Plasma Calibration Curve (overlay of front-end and back end curves)**



[redacted] **Plasma Calibration Curve (overlay of front-end and back end curves)**