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LCMS/MS METHOD DEVELOPMENT AND VALIDATION FOR THE QUANTIFICATION OF SIMEPREVIR IN HUMAN PLASMA.

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ABSTRACT:
To validate the procedure for estimation of Simeprevir Human K_2EDTA plasma using LC-MS/MS. This study points to build up and validate a simple method logy to quantify the most used drug simeprevir for the treatment of hepatitis C virus (HCV) infection, in human plasma by using Simeprevir and Simeprevir-D6 as an internal Standard (IS) for preclinical studies a validated as per USFDA guidelines the determination of Amprenavir using an Quattro Premier XE LC-MS/MS coupled with 2695 HPLC separation module, a Xterra MS C18 (100 × 4.6 mm, 3.5µm) column and an SPD-M20A PDA detector.
Key words: Acetonitrile,HPLC System,Nitrogen Evaporator, Human PlasmaPoly propylene tubes,Vibramax

INTRODUCTION:

Simeprevir, sold under the trade names **Olysio** among others, is a medication used in combination with other medications for the treatment of hepatitis C.[1] It is specifically used for hepatitis C genotype 1 and 4.[1] Medications it is used with include sofosbuvir or ribavirin and peginterferon-alfa.[1] Cure rates are in 80s to 90s percent.[2][3][4] It may be used in those who also have HIV/AIDS.[1] It is taken by mouth once daily for typically 12 weeks.[1]

Common side effects include feeling tired, headache, rash, itchiness, and sensitivity to sunlight.[1] In those with previous hepatitis B infection, active disease may recur.[1] It is not recommended in those with significant liver problems.[1] During pregnancy when used with ribavirin it may cause harm to the baby while when used with sofosbuvir its safety is unclear.[1][5] Simeprevir is a HCV protease inhibitor.[1]

Simeprevir was developed by Medivir AB and Janssen Pharmaceutica.[6] It was approved for medical use in the United States in 2013.[7] It was removed from the World Health Organization's List of Essential Medicines in 2019.[8][9] It is not available as a generic medication as of 2015.[5] In the United Kingdom a course of treatment with ribavirin and peginterferon-alfa cost about £29,700 in 2015.[10] In the United States a course of treatment with sofosbuvir was more than US$171,000 in 2015 with the simeprevir component costing US$66,360.[11]

MATERIALS and METHOD

Instrumentation

The author had attempted to develop a liquid chromatographic method for the determination of Amprenavir using an Quattro Premier XE LC-MS/MS coupled with 2695 HPLC separation module, a Xterra MS C18 (100 × 4.6 mm, 3.5µm) column and an SPD-M20A PDA detector.Data acquisition was done by using Mass Lynx V 4.1 software. The details of the instruments employed in the study are as follows.

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Table: Instruments used for the present study

HPLC System	Alliance LC
Deep Freezer	(-86) Deg C Deep Forma, Thermo scientific
Microbalance	XP 205, Mettler Toledo
Vibramax	Heidolph
Vacuum pump	Millipore
Refrigerator	Samsung
PH meter	Orion
Micropipettes, Multipette and Micro tips	Brand and Eppendorf
Vortexer	GV lab, Gilson®
Poly propylene tubes	Torson's
Water Purification System	Elix 10 / Milli-Q gradient
Ultra sonicator	Power Sonic510, (Hwashin Technology)
Nitrogen Evaporator	ZymarkTurbovap LV station, Caliper

Drug and Internal standard

The reference samples ofSimeprevir and Simeprevir-D6were gifted by M/s Virchow drug Ltd., Hyderabad.

REAGENTS AND CHEMICALS:

Table: 2.4.2 Reagents and chemicals

Reagent	**Brand**	**Purity/Grade**
Formic acid	Fluka	GR
Ammonium formate	Fluka	AR
Water	Milli Pore	Milli-Q
Acetonitrile	J.T Baker	HPLC
Methanol	Merck	HPLC

Preparation of various solutions:

Diluent:

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Using a calibrated pipette transferred 1.0 mL of formic acid solution into 10 mL volumetric flask and made up the volume with milli-Q grade water. Mixed well and stored at room temperature.

0.1% Formic Acid Buffer:

Measured and transferred1.0 mL of formic acid solution into 1000 mL volumetric flask using measuring cylinder, and made up the volume with milli-Q grade water.

10 mM Ammonium formate pH 4.0 extraction buffer:

Weighed accurately transferred about 630.60 mg of ammonium formate and in to a 1000 mL volumetric flask and made up the volume with milli-Q grade water. Mixed well, adjusted the pH to 4.0 ± 0.05 by adding dilute formic acid solute (0.1% V/V) drop wise.

Mobile phase preparation [Methanol and Formic acid Buffer (80:20%, V/V)]:

Measured and transferred800 mL of methanol and 200 mL of pH 4.0 formic acid buffer to an appropriately sized clean and pre-labeled bottle.

Needle wash [Methanol: Water (90:10%, V/V)]

900 mL of methanol and 100 mL of milli-Q grade water was measured and transferred to an appropriately sized reagent bottle.

Seal wash [Acetonitrile :Water (30:70%, V/V)]

Measured and transferred 300 mL of acetonitrile and 700 mL of milli-Q grade water to an appropriately sized reagent bottle.

Reconstitution solution [Methanol: Mobile Phase Buffer (80:20, V/V)]:

Measured and transferred80 mL of methanol and 20 mL of mobile phase buffer to a 100 mL beaker.

Internal standard solution preparation:

Preparation of ISTD stock solution (about 117µg/mL):

Simeprevirstandard equivalent to 1.16669mg was weighed and transferred into a 10.0mL volumetric flask. The standard was dissolvedwith methanol and made up the volume with the same solvent and mixed well, and stored at 5 ± 3ºC.

Preparation of ISTD Working Solution (about 117µg/mL):

Simeprevir-D6 standard equivalent to 1.16669mg was weighed and transferred into a 10.0mL volumetric flask. The standard was dissolved with methanol and made up the volume with the same solvent and mixed well, and stored at 5 ± 3ºC.

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Preparation of calibration standard:

Preparation of CC Stock Solution (4.666 mcg/mL):

1.16669mg of Simeprevir-standard was weighed and transferred into a 10 mL volumetric flask. It was dissolved in methanol and made up the volume using the same solvent. It was mixed welland stored the solution at 2-8ºC.

Preparation of CC Spiking Solutions:

The calibration curve dilutions were prepared from Amprenavir stock solution as per the table given below in the concentration range of 127.169to 35000.704ng/mL using human Plasma as the diluent. These dilutions (CC spiking solutions) were subsequently used for spiking the screened blank plasma.

Table: 2.4.3 Preparation of Spiking Solution for Calibration Standards

Solution ID	Stock Conc. (ng/mL)	volume taken (mL)	volume diluent (mL)	Final Volume (mL)	Spiking sol. Conc. (ng/mL)	Spiking solution ID
CC Stock	46667.600	3.750	1.250	5.000	35000.074	SS STD 8
SS STD 8	35000.074	4.400	0.600	5.000	30800.062	SS STD 7
SS STD 7	30800.062	3.200	1.800	5.000	19712.394	SS STD 6
SS STD 6	19712.394	3.200	1.800	5.000	12615.932	SS STD 5
SS STD 5	12615.932	2.100	2.900	5.000	5298.692	SS STD 4
SS STD 4	5298.692	1.200	3.800	5.000	1589.607	SS STD 3
SS STD 3	1589.607	1.000	4.000	5.000	317.921	SS STD 2
SS STD 2	317.921	2.000	5.00	5.000	127.169	SS STD 1

Mixed well, store the solution at 2-8ºC

Spiked Calibration Curve Plasma Standards:

The above calibration curve dilutions (CC spiking solutions) were used to spike the screened blank human plasma matrix to prepare the plasma calibration curve standards ranging from 10.173 to 2800.056ng/mL as per the table given below.

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Table: 2.4.4 Preparation of Calibration Standards

Spiking solution ID	Spiking Conc. (ng/mL)	Spiking volume (mL)	Plasma Volume (mL)	Final Volume (mL)	Final Conc. (ng/mL)	Spiked CC ID
SS STD 8	35000.700	0.400	4.600	5.000	2800.056	STD 8
SS STD 7	30800.616	0.400	4.600	5.000	2464.049	STD 7
SS STD 6	19712.394	0.400	4.600	5.000	1576.992	STD 6
SS STD 5	12615.932	0.400	4.600	5.000	1009.275	STD 5
SS STD 4	5298.692	0.400	4.600	5.000	423.895	STD 4
SS STD 3	1589.607	0.400	4.600	5.000	127.169	STD 3
SS STD 2	317.921	0.400	4.600	5.000	25.434	STD 2
SS STD 1	127.169	0.400	4.600	5.000	10.173	STD 1

Storage temperature: Approx. -20°C , Diluent: Human Plasma

0.5 mL aliquots of the above plasma calibration curve standards solutions were taken in pre labeled polypropylene vials which were then capped tightly and stored in a freezer at -20°C

Preparation of Quality Control (QC) Samples:

Preparation o QC Stock Solution (3.0 ng/mL)

Simeprevir standard equivalent to 35.0 mg of Simeprevir was weighed and transferred into a 10mL volumetric flask. It was dissolved in methanol and made up the volume using the same solvent.

Preparation o QC spiking solution:

The quality control dilutions (QC spiking solutions) from Amprenavir stock solution were prepared as per the table given below in the concentration range from 103.15to 35000.00ng/mL using Human Plasma as the diluent. These dilutions (QC spiking solutions) were subsequently used for spiking the screened blank plasma.

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Table: 2.4.5: Preparation of spiking solution for quality control samples

Solution ID	Stock Conc. (ng/mL)	Volume taken (mL)	Volume of diluent (mL)	Final volume (mL)	Final conc. (ng/mL)	Spiking solution ID
QC stock	35000.000	4.000	1.000	5.000	28000.56	SS HQC
SS HQC	28000.560	3.800	1.2	5.000	21280.43	SS MQC1
SS MQC1	21280.43	3.300	3.7	5.000	14045.08	SS MQC2
SS MQC2	14045.08	1.000	4.000	5.000	2809.02	SS INTMD 1
SS INTMD 1	2809.02	1.7	3.300	5.000	955.065	SS LQC
SS LQC	955.065	1.5	3.5	5.000	286.519	LOQ
SS LOQ QC	286.520	1.8	3.2	5.000	103.15	SS LLOQ

Storage temperature: Approx. 5°C

Spiked QC Plasma Samples

The above quality control dilutions (QC spiking solutions) were used to spike the screened blank human plasma to prepare the plasma quality control plasma samples ranging from 10.315to 2800.056ng/mL as per the table given below.

Table: 2.4.6 Preparation of spiked quality control samples

	Stock Conc. (ng/mL)	Volume taken (mL)	Volume of diluent (mL)	Final volume (mL)	Final conc. (ng/mL)	Spiking solution ID
	28000.560	0.5	4.5	5.000	2800.056	SS HQC
SS HQC	Solution ID	0.5	4.5	5.000	2128.043	SS MQC1
SS MQC1	QC stock	0.5	4.5	5.000	1404.508	SS MQC2
SS MQC2	2809.016	0.5	4.5	5.000	280.902	SS INTMD 1
SS LOQ QC	286.520	0.5	4.5	5.000	28.652	LOQ QC
SS LLOQ	103.147	0.5	4.5	5.000	10.315	LLOQ QC

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Storage temperature: -20°C

0.3 mL aliquots of the above plasma quality control samples were taken in pre labeled polypropylene vials which were then capped properly and stored in a freezer at –20°C.

Preparation of standard blank samples:

Prepared the standard blank by spiking the diluent in screened human plasma as described in the following table

Spiking solution ID	Spiking volume (mL)	volume of matrix (mL)	Final Volume (mL)	Spiked CC ID
Diluent	0.200	9.800	10.000	STD Blk

0.300 mL aliquots of the above plasma blank samples were taken in pre labeled polypropylene vials which were then capped properly and stored in a freezer at –20^0C.

METHOD DEVELOPMENT AND OPTIMIZATION OF THECHROMATOGRAPHIC CONDITIONS

For developing the method for the assay of Amprenavir, a systematic study of the effect of various factors were undertaken by varying one parameter at a time and keeping all the other conditions constant. The following studies were conducted for this purpose. Xterra MS C18 (100 × 4.6 mm, 3.5µm)columnwas chosen as the stationary phase for this study.

The mobile phase and the flow rate

In order to get sharp peaks and base line separation of the components, the author has carried out a number of experiments by varying the commonly used solvents, their compositions and flow rate.

To effect ideal separation of the drug under isocratic conditions, mixtures of commonly used solvents likewater, methanol and acetonitrile with or without buffers in different combinations were tested as mobile phases on a Xterra MS C18 (100 × 4.6 mm, 3.5µm), A binary mixture of 0.1% formic acid (1.0 mL of formic acid solution into 1000 mL volumetric flask and make up the volume with Milli-Q water) and acetonitrile in the ratio of 20:80% v/v was proved to be the most suitable of all the combinations since the chromatographic peaks obtained were well defined and resolved and free from tailing.

A mobile phase flow rate of 0.8mL/min. was found to be suitable in the study range of 0.8 -1.0mL/min. Detection was carried out by using mass detectors.

The drug molecule was tuned on the mass spectrometer for the detection of the parent ion and the daughter ion (precursor ions) by injecting 0.5µg/mL concentration. The tuning was carried out in both positive and negative modes of ionization, but better sensitivity with more reproducibility was found to be observed in the positive polarity mode. All the optimized potential parameters in the positive polarity mode have been given in the following table. The mass spectrum of the drug molecule is given Figure: 2.5.1.

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Table: 2.5.1 Tuning parameters of Simeprevir and Simeprevir-D6

ES + Source Parameter	Settings	Analyzer Parameter	Settings
Capillary (kV)	3.00	LM Resolution 1	10.0
Cone (V)	30	HM Resolution 1	10.0
Extractor (V)	5	Ion Energy 1	1.0
RF Lens (V).	0.0	Entrance	2
Source Temp (°C)	120	Collision	20
Desolvation Temp (°C)	400	Exit	2
Cone Flow (L/h)	50	LM Resolution 2	10.0
Desolvation Flow (L/h)	800	HM Resolution 2	10.0
Collision gas Pressure	$3.5\times e^{-3}$ -$4.5\times e^{-3}$	Ion Energy 2	1.2
		Multiplier	650

Retention time of Simeprevir

A model chromatogram showing the separation of Simeprevir and Simeprevir-D6(IS) is presented in Figure: 2.5 under the above optimized conditions retention times of 1.10 and 1.09mins respectively.

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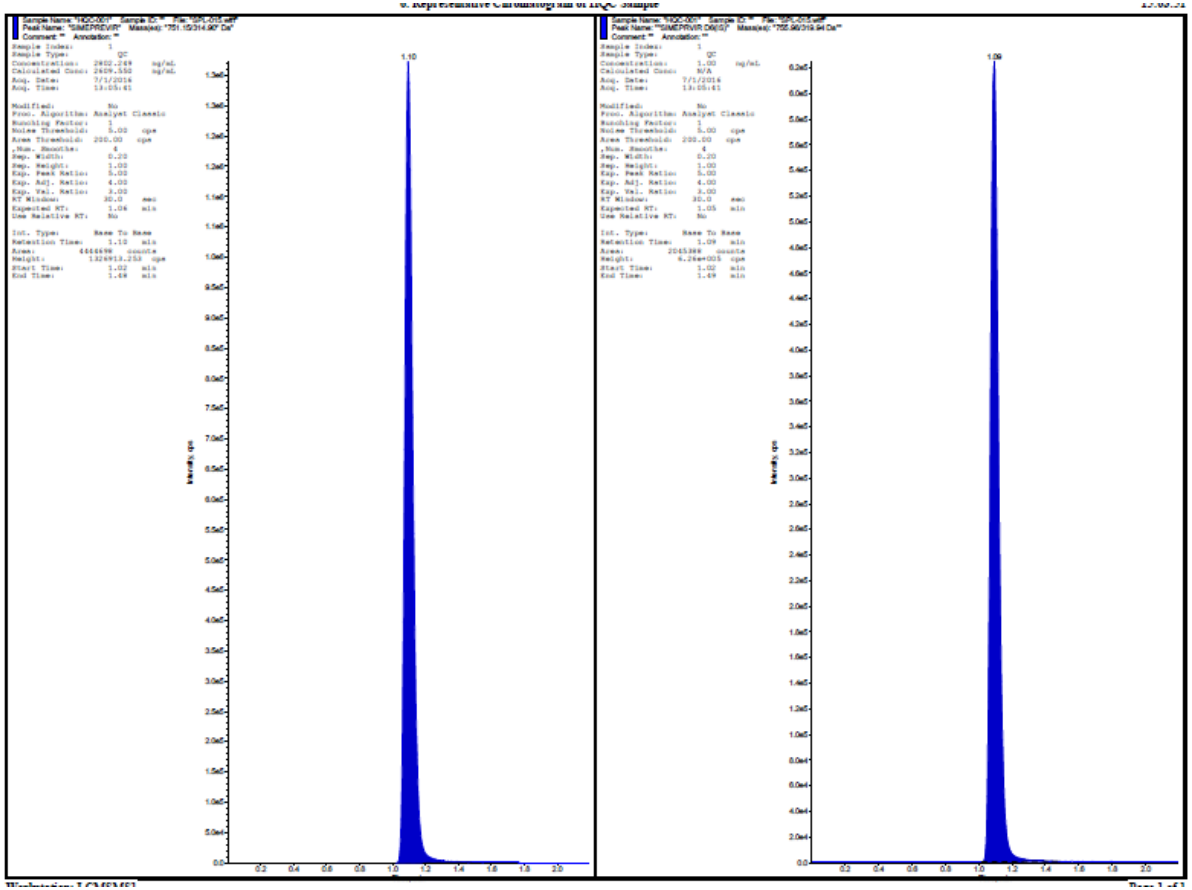

Figure: 2.5

Data acquisition and processing

The chromatograms were obtained and data was processed by the peak area ratio method using the LC solution software. The concentrations of the unknown samples were calculated from the following equation of the regression analysis of the spiked plasma calibration graph using $1\backslash X^2$ as weighing factor.

Y = m X + C

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X = Analyte concentration / Internal standard concentration

Y = Analyte area / Internal standard area (area ratio)

m = Slope of the calibration curve

C = Y intercept value

Table: 2.5.3 Optimized Chromatographic Conditions

Parameter	**Value**
Column	Xterra MS C18 (100 × 4.6 mm, 3.5µm),
Mobile phase	Acetonitrile /Mobile Phase buffer (80/20 V/V)
Flow rate	0.8 mL/min
Run time	2.00min
Column oven temperature	40 ± 2ºC
Auto sampler temperature	5 ± 3ºC
Volume of injection	10µL
Detection	Mass detector
Retention time of Simeprevir	1.10 min.
Retention time of Simeprevir-D6	1.09 min.

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Extraction Process of Plasma Samples and Their Drying

Sample Preparation & Extraction Procedure

The below mentioned sample preparation procedure was followed while processing.

Extraction process of plasma samples and their drying

Sample Preparation & Extraction procedure

The sample preparation procedure was followed while processing.

Step-1: Required number of plasma samples from the deep freezer was retrieved and thawed them at room temperature or in water bath maintained at room temperature and vortexed the tubes to mix. 200 µL of plasma was transferred into pre labeled tubes.

Step-2: 50.0 µL of 700 ng/mL ISTD working solution was added to all the vials except the STD blank and vortexed for about 5 seconds.

Step-3: 100.0 µL of extraction buffer was added to all the vials and vortex for about 5 sec.

Step-4: 2 mL of tertiary butyl methyl ether (TBME) was added to the all vials and vortex for a period of 10 min, interrupting the vortexed for every 1 min.

Step-5: All the vials were centrifuged at 4500rpm, at 4°C for 5 min.

Step-6: About 1.8 mL of supernatant was transferred into pre labeled tubes and the supernatant solutions were evaporated to dryness under nitrogen at 40 ± 5°C.

Step-7: 500.0 µL of reconstitution solution was added to all the tubes and vortexed for about 2 min.

Step-8: Appropriate volumes of the reconstituted solution were transferred into pre-labeled autosampler vials and inject 5 µL was injected into LC-MS/MS.

Procedure for unextracted Sample Preparation:

Step-1: 40.0 µL of respective spiking solutions were taken in pre labeled tubes.

Step-2: 500.0 µL of 700 ng/mL ISTD dilution was added and vortexed to mix.

Step-3: 4.460 mL of reconstitution solution was added and vortexed to mix.

Step-4: Appropriate volumes were transferred into pre-labeled autosampler vials and 5 µL was injected into LC-MS/MS.

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Instrumental Details

LC-MS/MS	:	ABSCIEX API 4000, (CRS-MS-001)
UHPLC	:	SHIMADZUNEXERA X2, (CRS-LC-001)
Software	:	Analyst Version 1.6.2

Analytical Method

Extraction procedure	:	Liquid-Liquid extraction

Chromatographic Conditions

Column	:	Xterra MS C18 (100 × 4.6 mm, 3.5µm)
Flow Rate	:	0.800mL/min

Detection Parameter

Analyte MRM	:	Q1/Q3 – 130.100 /71.100(m/z)
ISTD MRM	:	Q1/Q3 –136.100 /77.100(m/z)
Polarity	:	Positive

Working or Reference Standards

Details	Analyte	Internal Standard
Name	Simeprevir	Simeprevir D6

Preparation of Calibration Curve Standards and Quality Control Samples

Stock solutions of analyte and internal standard were prepared using working standards as per method SOP. Different stock solutions were used for Calibration standards and Quality Control samples.

Preparation of calibration standards and quality control samples was carried as per method SOP using interference free biological Matrix.
Calibration Curve standards and Quality Control Samples Details:

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Storage Temperature	-70±15 ºC, -20±5 ºC
Calibration Curve Standards (CC) Concentrations	STD1-STD8 STD1 –3500.074 ng/mL, STD2 – 3080.065ng/mL, STD3 –1925.040 ng/mL, STD4 –1225.288 ng/mL, STD5 –525.649ng/mL, STD6 –122.739 ng/mL, STD7 – 23.075ng/mL, STD8 – 10.003 ng/mL.
Quality Control (QC) Samples Concentrations	**HQC–**2802.249**ng/mL,** **MQC –** 1423.542**ng/mL,** **LQC**–28.044**ng/mL,** **LLOQ QC –**10.006 ng/mL**.**
Dilution Integrity Quality Control Stock(DIQC)	6300.451**ng/mL**

RESULTS & DISCUSSIONS

System suitability

System suitability was performed by injecting six consecutive injections of AQ STD (equivalent to MQC concentration). The system was found to be sensitive and reproducible and the results are presented in table 2a and 2b. The % CV for retention time should be ≤ 3.0% and the area ratio should be ≤ 5.0%.

Table2: System Suitability

Table 2a: System Suitability

Injection No	Analyte RT (Minutes)	ISTD RT (minutes)	Area Ratio (Analyte/IS)
1	1.08	1.08	1.242
2	1.08	1.08	1.233
3	1.08	1.08	1.227
4	1.08	1.08	1.229
5	1.08	1.08	1.222
6	1.08	1.08	1.222
Mean	1.080	1.080	1.2292
SD	0.0000	0.0000	0.00757
% CV	0.0	0.0	0.6

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Table 2b: System Suitability Performed on Ruggedness Batch

Injection No	Analyte RT (Minutes)	ISTD RT (minutes)	Area Ratio (Analyte/IS)
1	1.07	1.07	1.242
2	1.07	1.07	1.257
3	1.07	1.07	1.267
4	1.07	1.07	1.239
5	1.07	1.07	1.246
6	1.07	1.07	1.219
Mean	1.070	1.070	1.2450
SD	0.0000	0.0000	0.01643
% CV	0.0	0.0	1.3

Auto sampler Carryover

Carry over was assessed by subsequently injecting reconstitution solution after an aqueous standard of highest calibration standard (AQ ULOQ) and blank sample after ULOQ standard along with aqueous and extracted LLOQ samples. Carry over was not observed for the analyte and ISTD for both aqueous and extracted samples. The results are presented in table 3.

Auto-sampler Carryove

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Unextracted/Aqueous			% CARRYOVER	
Sample Name	Analyte Peak Area (counts)	ISTD Peak Area (counts)	ANALYTE	ISTD
RS1-1	300	434	1.3	0.0
AQ ULOQ	5815165	2082283	NA	NA
RS1-2	868	370	3.8	0.0
RS1-3	663	0	2.9	0.0
AQ LLOQ	22622	2379079	NA	NA
Extracted				
STD BL1-1	0	177	0.0	0.0
EXT ULOQ	5059949	1892827	NA	NA
STD BL1-2	575	663	3.2	0.0
STD BL1-3	547	518	3.0	0.0
EXT LLOQ	17987	2148784	NA	NA

Acceptance Criteria:

1. Response (signal) of analyte should be more than or equal to 5.0 times for AQ/Extracted LLOQ sample when compared to 1st acquired RS/STD BL respectively.
2. Reject the experiment for evaluation of above both parameters if % Interference observed in first acquired RS/STD BL (before ULOQ) is >20.0% for analyte and >5.0% for ISTD.
3. Carryover observed in both the RS/ STD BL injected after ULOQ should be ≤20.0% for analyte compare to the analyte response of AQ/Extracted LLOQ sample respectively.
4. Carryover observed in both the RS/ STD BL injected after ULOQ should be ≤5.0% for ISTD response compare to the ISTD response of AQ/Extracted LLOQ sample respectively.

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Selectivity and Specificity

Selectivity

10 lots of plasma BMX-16-075,BMX-16-076,BMX-16-084,BMX-16-085,BMX-16-089, BMX-16-090, BMX-16-054(L), BMX-16-055(L), BMX-16-052(H), and BMX-16-053(H) were evaluated for selectivity. These lots did not show any significant interference at the retention time of analyte and ISTD with respect to LLOQ. The results are presented in table 4a.

Table 4: Selectivity & Specificity

Table 4a: Selectivity

S. No.	Biological Matrix Batch No	Response at Analyte RT	Analyte Response In LLOQ	Interference at analyte RT (%)	Response at ISTD RT	ISTD Response In LLOQ	Interference at ISTD RT (%)
1	SIM-16-055	601	17224	3.5	609	2142355	0.0
2	SIM-16-056	0	16018	0.0	0	1955142	0.0
3	SIM -16-057	0	18114	0.0	260	2208310	0.0
4	SIM -16-064	0	17172	0.0	199	2110419	0.0
5	SIM -16-071	0	18277	0.0	223	2226212	0.0
6	SIM -16-081	161	17887	0.9	377	2178879	0.0
7	SIM -16-054 L	0	17631	0.0	158	2228334	0.0
8	SIM -16-055 L	0	18497	0.0	0	2282040	0.0
9	SIM -16-052 H	0	17993	0.0	266	2248618	0.0
10	SIM -16-053 H	0	17625	0.0	0	2188395	0.0

Acceptance Criteria:

1. Response of interfering peaks at the retention time of analyte(s) and/or metabolite(s) should be ≤ 20.0% the response of respective LLOQ sample.
2. Response of interfering peaks at the retention time of ISTD should be ≤ 5.0% of the response of ISTD of respective LLOQ sample.
3. All screened lots including hemolyzed and lipemic matrix lots should meet the acceptance criteria.

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Specificity

Specificity experiment was evaluated by injecting replicate injections of STD Blank, Concomitant Blank (Blank with concomitant medication/other analyte atconcentration equivalent to Cmax respectively), STD ZERO (Blank with IS) at the method parameters of Analyte of interest and compared the percentage of interference with mean response of LLOQ sample. No significant interference of other analyte was observed at retention time of Analyte of interest and ISTD. The results are presented in table 4b.

The concomitant medication evaluated for interference is Cetirizine, Domperidone, Ranitidine, Nicotine, Caffeine, Acetaminophen (Paracetamol), Diclofenac and Ibuprofen.

Table 4b: Specificity

S. No.	Sample ID	Analyte-1				ISTD			
		Area of Interfering peak at analyte RT in presence of other analytes	LLOQ		% Interference	Area of Interfering peak at ISTD RT in presence of other analytes	LLOQ		% Interference
			Area	RT			Area	RT	
1	STD BLANK-1	297	19209	1.09	1.5	0	2332299	1.08	0.0
2	STD BLANK-2	339			1.8	812			0.0
3	CME BlANK-1	0			0.0	0			0.0
4	CME BlANK-2	0			0.0	568			0.0
5	STD ZERO-1	456			2.4	2037573			NA
6	STD ZERO-2	490			2.6	2235221			NA

Concomitant analytes/Other Analytes:
Cetirizine, Domperidone, Ranitidine, Nicotine, Caffeine, Acetaminophen, Diclofenac and Ibuprofen.

Acceptance Criteria:
Response of interfering peaks at the retention time of analyte(s) and/or metabolite(s) should be ≤ 20.0% the response of respective LLOQ sample. Response of interfering peaks at the retention time of ISTD should be ≤ 5.0% of the response of ISTD of respective LLOQ sample.

Matrix Effect

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10 lots of plasma BMX-16-075, BMX-16-076, BMX-16-084, BMX-16-085,BMX-16-089, BMX-16-090, BMX-16-054(L), BMX-16-055(L), BMX-16-052(H), and BMX-16-053(H) were evaluated for matrix effect. Post extracted samples of biological matrix at concentrations equivalent to HQC and LQC were injected along with 6 replicate injections of aqueous samples of HQC and LQC.

The matrix effect calculated by ISTD normalized matrix factor and the %CV of ISTD normalized matrix factor at HQC and LQC levels were 1.3and 0.9which were within the acceptance criteria. The results are presented in the table 5.

Table 5:Matrix Effect

S. No.	Analyte						
	Response of aqueous samples		Response of post extracted samples			Matrix Factor (MF)	
	HQC	LQC	Biological matrix lot No.	HQC	LQC	HQC	LQC
1	4902516	58880	BMX-16-075	4551221	54800	0.93	0.92
2	4947821	60043	BMX-16-076	3905162	47213	0.80	0.79
3	4755433	58186	BMX-16-084	4770117	60550	0.98	1.01
4	4895562	61006	BMX-16-085	4804062	58337	0.99	0.98
5	4889368	59693	BMX-16-089	4835223	57979	0.99	0.97
6	4858530	60570	BMX-16-090	4640457	55635	0.95	0.93
7	NA	NA	BMX-16-054 L	4680056	57812	0.96	0.97
8	NA	NA	BMX-16-055 L	4863635	59208	1.00	0.99
9	NA	NA	BMX-16-052 H	4722603	57924	0.97	0.97
10	NA	NA	BMX-16-053 H	4779574	58020	0.98	0.97
Mean	4874871.7	59729.7	NA	4655211.0	56747.8	0.955	0.950

S. No.	ISTD						
	Response of aqueous samples		Response of post extracted samples			Matrix Factor (MF)	
	HQC	LQC	Biological matrix lot No.	HQC	LQC	HQC	LQC
1	2148977	2407734	BMX-16-075	1977466	2220588	0.92	0.92
2	2174448	2397525	BMX-16-076	1705494	1910024	0.79	0.79
3	2132700	2402557	BMX-16-084	2079548	2422641	0.97	1.00
4	2133552	2462309	BMX-16-085	2080473	2362908	0.97	0.98

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5	2169147	2402258	BMX-16-089	2125008	2313141	0.99	0.96
6	2168884	2436408	BMX-16-090	1974373	2268721	0.92	0.94
7	NA	NA	BMX-16-054 L	1992448	2316302	0.92	0.96
8	NA	NA	BMX-16-055 L	2115385	2363759	0.98	0.98
9	NA	NA	BMX-16-052 H	2071312	2371595	0.96	0.98
10	NA	NA	BMX-16-053 H	2112839	2351512	0.98	0.97
Mean	2154618.0	2418131.8	NA	2023434.6	2290119.1	0.939	0.947

S. No.	Biological Matrix Lot No.	Matrix Factor (MF)		ISTD Normalized MF(HQC)	Matrix Factor (MF)		ISTD Normalized MF(LQC)
		HQC	ISTD		LQC	ISTD	
1	BMX-16-075	0.93	0.92	1.02	0.92	0.92	1.00
2	BMX-16-076	0.80	0.79	1.01	0.79	0.79	1.00
3	BMX-16-084	0.98	0.97	1.01	1.01	1.00	1.01
4	BMX-16-085	0.99	0.97	1.02	0.98	0.98	1.00
5	BMX-16-089	0.99	0.99	1.01	0.97	0.96	1.01
6	BMX-16-090	0.95	0.92	1.04	0.93	0.94	0.99
7	BMX-16-054 L	0.96	0.92	1.04	0.97	0.96	1.01
8	BMX-16-055 L	1.00	0.98	1.02	0.99	0.98	1.01
9	BMX-16-052 H	0.97	0.96	1.01	0.97	0.98	0.99
10	BMX-16-053 H	0.98	0.98	1.00	0.97	0.97	1.00
Mean		0.955	0.939	1.017	0.950	0.947	1.003
SD		NA	NA	0.0128		NA	0.0091
% CV		NA	NA	1.3		NA	0.9

Formula:

$$\text{Matrix Factor} = \frac{\text{Peak Area in Presence of Matrix Ions}}{\text{Mean Peak Area in Absence of Matrix Ions}}$$

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ISTD Normalized Matrix Factor = Matrix Factor of Analyte / Matrix Factor of ISTD

Acceptance Criteria:
The % CV for ISTD normalized matrix factor at both HQC and LQC level should within 15.0%.

Linearity and goodness of fit

An eight point calibration curve was found to be linear over a concentration range from 3500.074ng/mL–10.003ng/mL for analyte. A linear equation was established to provide the best fit for the concentration vs. detector response using $1/X^2$ as weighing factor. The goodness of fit was consistently greater than 0.99 using three P&A batches during the course of validation. The results are presented in table 6.

Table 6: Calculated Concentrations for Calibration Curve Standards

STD ID	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8	Slope	Intercept	rvalue	r^2value
Nominal Concentration (ng/mL)	3500.074	3080.065	1925.040	1225.288	525.649	122.739	23.075	10.003				
Batch ID	Back Calculated Concentrations of Calibration Curve Standards (ng/mL)											
010716-P&A-01	3453.753	2874.413	1891.152	1249.399	548.447	125.507	23.665	9.867	0.0008	0.0007	0.9993	0.9986
% Accuracy	98.7	93.3	98.2	102.0	104.3	102.3	102.6	98.6				
010716-P&A-02,REC-01	3439.536	2925.910	1877.818	1250.183	551.079	123.673	23.765	9.860	0.0008	0.0004	0.9994	0.9988
% Accuracy	98.3	95.0	97.5	102.0	104.8	100.8	103.0	98.6				
020716-P&A-03,SEN-01	3402.064	2880.847	1916.511	1209.337	555.310	127.454	23.852	9.819	0.0008	0.0006	0.9991	0.9982
% Accuracy	97.2	93.5	99.6	98.7	105.6	103.8	103.4	98.2				
Mean	3431.7843	2893.7233	1895.1603	1236.3063	551.6120	125.5447	23.7607	9.8487				
±SD	26.7021	28.0595	19.6555	23.3594	3.4624	1.8908	0.0936	0.0259				
%CV	0.8	1.0	1.0	1.9	0.6	1.5	0.4	0.3				
% Accuracy	98.0	94.0	98.4	100.9	104.9	102.3	103.0	98.5				

Acceptance Criteria:

Accept all the CC standards, when the back calculated concentrations are within ± 15.0% of their respective nominal concentrations except for LLOQ. Accept the LLOQ standard, when the back calculated concentration is within ±20.0% of its nominal concentration.

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Accept the CC, when at least 75% of the total number of CC standards fall within acceptable range including the ULOQ and LLOQ standards.

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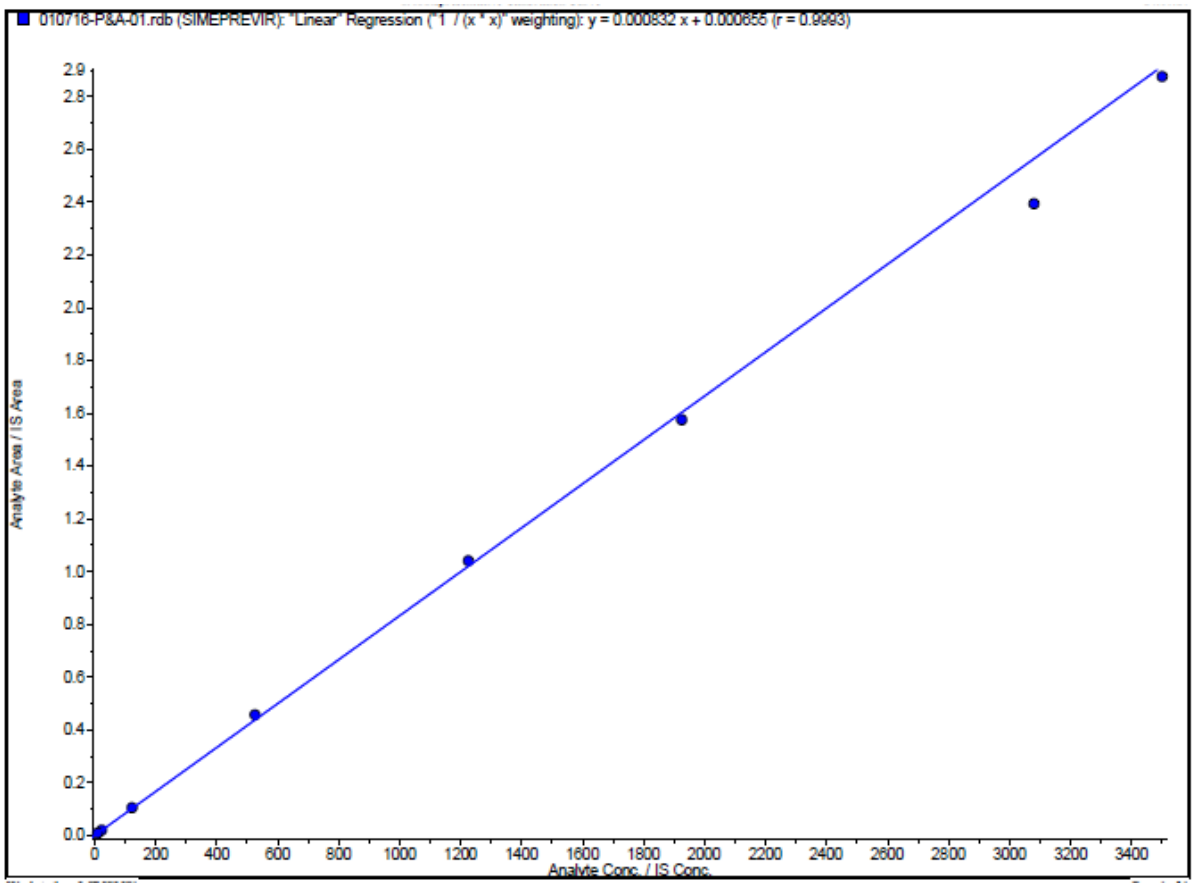

Figure: Calibration Curve of Standards

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Dilution Integrity

Dilution integrity was evaluated as dilution quality control (DQC) along with 3 precision and accuracy batches with diluting 1/5 times of dilution integrity quality control stock (DIQC) prepared at 1.8 times the concentration of ULOQ.

The intraday (P&A01) precision (%CV) for DQC samples was 2.0%. The inter day precision for DQC samples was 2.8%, which were within the acceptance criteria ≤15%.

The intraday accuracy for DQC samples was 101.9%. The inter day accuracy for DQC samples was 99.1%, which were within the acceptance criteria of 85-115% to nominal concentration. The results are presented in table 7.

Precision

Precision is measured by coefficient of variation (%CV) over the concentration range of quality control samples during validation. The results are presented in Table 7.

The intraday (P&A01) precision (%CV) for HQC, MQC and LQC samples was1.0,1.6 and 0.8 respectively. The inter day precision for HQC, MQC and LQC samples was 1.1, 1.3, and 1.4 respectively, which were within the acceptance criteria of ≤15%.

The intraday (P&A01) precision (%CV) for LLOQ QC samples was 2.6. The inter day precision for LLOQ QC samples was 3.3, which were within the acceptance criteria of ≤20%.

Accuracy

Accuracy is measured by % difference of back calculated mean concentration of quality control samples to their respective nominal values. The results are presented in Table 7.

The intraday (P&A01) accuracy for HQC, MQC and LQC samples were93.4%,98.1%, and 97.7%,respectively. The inter day accuracy for HQC, MQC and LQC samples was 93.8%, 98.5%, and 98.6% respectively, which were within the acceptance criteria of 85-115% to nominal concentration.

The intraday (P&A01) accuracy for LLOQ QC samples was 91.2%. The inter day accuracy for LLOQ QC samples was 94.4%, which were within the acceptance criteria of 80-120% to **Table 7: Calculated Concentrations for Quality Control Samples**

QC ID	HQC	MQC	DQC	LQC	LLOQQC
Nominal Concentration (ng/mL)	2802.249	1423.542	6300.451	28.044	10.006
BATCH ID	Back Calculated Concentrations of Quality Control Samples (ng/mL)				
P&A-01	2609.550	1363.582	6535.229	27.434	9.304
	2591.253	1424.790	6342.316	27.255	9.329
	2658.370	1413.837	6564.998	27.270	8.865
	2594.133	1385.652	6209.164	27.354	9.386
	2611.816	1401.249	6444.370	27.288	8.898
	2643.320	1387.698	6430.317	27.858	8.977
Mean	2618.0737	1396.1347	6421.0657	27.4098	9.1265
±SD	27.07577	21.91528	130.75342	0.22931	0.23782
%CV	1.0	1.6	2.0	0.8	2.6

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% Accuracy	93.4	98.1	101.9	97.7	91.2
P&A-02,REC-01	2699.324	1398.473	6145.009	27.717	9.633
	2637.849	1382.587	6118.234	27.933	9.556
	2613.033	1402.157	6203.521	27.522	9.789
	2628.048	1406.740	6308.624	27.894	9.739
	2611.544	1409.384	6056.773	26.887	9.818
	2612.546	1407.181	6075.801	27.683	9.600
Mean	2633.7240	1401.0870	6151.3270	27.6060	9.6892
±SD	33.82653	9.88090	93.00135	0.38266	0.10760
%CV	1.3	0.7	1.5	1.4	1.1
% Accuracy	94.0	98.4	97.6	98.4	96.8
P&A-03,SEN-01	2647.052	1440.648	6383.268	28.214	9.743
	2621.086	1416.680	5972.154	27.846	9.302
	2627.350	1395.014	6156.642	27.554	9.510
	2638.985	1420.948	6267.183	27.818	9.174
	2594.164	1379.205	6016.861	27.733	9.832
	2662.450	1410.865	6198.331	28.547	9.497
Mean	2631.8478	1410.5600	6165.7398	27.9520	9.5097
±SD	23.55724	21.33335	153.87953	0.36283	0.25072
%CV	0.9	1.5	2.5	1.3	2.6
% Accuracy	93.9	99.1	97.9	99.7	95.0
Global Statistics					
Mean	2627.8818	1402.5939	6246.0442	27.6559	9.4418
±SD	27.69342	18.48660	175.47231	0.38787	0.31119
%CV	1.1	1.3	2.8	1.4	3.3
% Mean Accuracy	93.8	98.5	99.1	98.6	94.4

Acceptance Criteria:

The % accuracy for each LQC, DQC, MQC and HQC samples should be in the range of ±15.0% (for LLOQ QC ±20.0%) from their nominal concentrations.

At least 67% of overall QC samples (4 out of 6) should be within the acceptance criteria, and at least 50% of QC samples at each level should meet the above mentioned criteria.

The within-run % and between run mean accuracy for LQC, DQC, MQC and HQC samples should be within ±15.0% from their nominal concentration and for the LLOQ QC sample it should be within ±20.0% from its nominal concentration.

The within run and between run precision for LQC, DQC, MQC and HQC samples should be within 15.0% and for the LLOQ QC the precision should be within 20.0%.

Sensitivity

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The Limit of Quantification (LLOQ) of analyte was 10.003 ng/mL. Accuracy and precision at LLOQ levels were 94.9%, 2.9% respectively which were within acceptance criteria of 80-120% of nominal concentration for accuracy and ≤20% for precision .The average signal to noise ratio of LLOQ sample was 1197.83which was within acceptance limit of ≥5. The results are presented in table 8.

Table 8: Sensitivity

Nominal Concentration of LLOQ	10.003ng /mL	
S. No.	Calculated Concentrations of LLOQ Samples (ng /mL)	Signal to Noise Ratio
1	9.435	945.4
2	9.878	1241.1
3	9.395	1666.3
4	9.608	810.9
5	9.603	1294.4
6	9.058	1228.9
Mean	9.4962	1197.83
±SD	0.27411	NA
%CV	2.9	
%Nominal	94.9	

Acceptance criteria:

The precision of LLOQ should be within ±20.0% and Accuracy of LLOQ samples should be within ±20.0% from its nominal concentration.

The average signal to noise ratio should be ≥5.

Recovery

The mean areas of extracted quality control samples of analyte HQC, MQC and LQC were compared against mean areas of post extracted quality control samples of HQC, MQC and LQC. The percent recovery at HQC, MQC and LQC levels were 92.3, 89.5%, and 88.0% respectively. The global recovery for analyte was 89.98%, which was within the acceptance criteria of ≤115%. The results are presented in table 9a.

The mean areas of extracted middle quality control samples of ISTD were compared against mean areas of post extracted middle quality control samples of ISTD. The percent mean recovery for ISTD was 94.6%, which was within the acceptance criteria of ≤115% for global recovery. The results are presented in table 9b.

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Table 9a: Recovery of Analyte

S. No	Analyte Recovery					
	Post Extracted HQC	Extracted HQC	Post Extracted MQC	Extracted MQC	Post Extracted LQC	Extracted LQC
1	5003429	4780519	2844745	2524257	58634	53594
2	4916960	4484681	2845069	2466266	58581	51723
3	4836394	4395342	2836729	2448153	59171	51370
4	4925111	4639926	2807413	2538274	60061	53020
5	4936489	4519439	2734450	2568267	59706	51283
6	4949236	4484083	2816170	2574151	61055	53502
Mean	4927936.5	4550665.0	2814096.0	2519894.7	59534.7	52415.3
SD	54285.22	137613.76	41960.43	52274.97	945.59	1076.10
%CV	1.1	3.0	1.5	2.1	1.6	2.1
% Mean Recovery	92.3		89.5		88.0	
Global Recovery	89.98					
SD	2.184					
%CV	2.4					

Table 9b: Recovery of ISTD

	Post Extracted Area	Extracted Area
MQC	2294502	2195633
	2348815	2169832
	2373138	2123846
	2299179	2194856
	2250337	2216626
	2305975	2225182
Mean	2311991.0	2187662.5
SD	43364.46	36748.73
% CV	1.9	1.7
% Mean Recovery	94.6	

Nominal Calculation formula:
% Mean Recovery of Internal Standard = (Mean Extracted ISTD Peak Area / Mean Post extracted ISTD Peak Area) × 100

Acceptance criteria:
The % CV of mean recovery of ISTD sample should be within 15.0% and recovery should not be more than 115.0%.

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Ruggedness

Ruggedness by Different column and Different analyst:

Ruggedness batch was performed by different column of same make and analysis was performed by different analyst. The results are presented in table 10.

The calibration curve found linear over a concentration range from 3500.074 ng/mL – 10.003 ng/mL for analyte and the goodness of fit was greater than 0.99.

The precision (%CV) for HQC, MQC and LQC samples were2.0, 0.7, and 2.2 respectively, which were within the acceptance criteria of ≤15%. The precision (%CV) for LLOQ QC samples was 2.6, which was within the acceptance criteria of ≤20%.

The accuracy for HQC, MQC and LQC samples was 91.6%, 97.6%, and 99.7% respectively, which were within the acceptance criteria of 85-115% to nominal concentration. The accuracy for LLOQ QC samples was 98.1%, which was within the acceptance criteria of 80-120% to nominal concentration.

Table 10: Ruggedness Different Analyst and Different Column

STD ID	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8
Nominal Concentration (ng/mL)	3500.074	3080.065	1925.040	1225.288	525.649	122.739	23.075	10.003
Calculated Concentration (ng/mL)	3462.327	2925.914	1917.405	1215.316	533.769	127.658	23.918	9.812
%Accuracy	98.9	95.0	99.6	99.2	101.5	104.0	103.7	98.1
%Bias	-1.1	-5.0	-0.4	-0.8	1.5	4.0	3.7	-1.9
Calibration Curve Parameters	Slope	0.0008	Intercept	0.0002	r value	0.9995	r^2 value	0.9990

Quality Control samples		HQC	MQC	LQC	LLOQ QC
Nominal Concentration (ng/mL)		2802.249	1423.542	28.044	10.006
Batch/Run ID	QC ID	Back Calculated Concentration of Quality Control Samples (ng/mL)			
P&A-04(RUG-01)	019	2484.803	1371.136	27.276	9.577
	020	2536.340	1398.611	28.920	9.865
	021	2577.080	1389.968	28.501	9.913
	022	2580.251	1393.347	27.864	10.102
	023	2641.296	1388.737	27.668	9.453
	024	2584.075	1393.097	27.543	10.005
	Mean	2567.3075	1389.1493	27.9620	9.8192
	S.D.	52.50612	9.46623	0.62458	0.25225
	% CV	2.0	0.7	2.2	2.6
	%Mean Accuracy	91.6	97.6	99.7	98.1

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Acceptance criteria:
The % accuracy for each LQC, MQC and HQC samples should be in the range of ±15.0% (for LLOQ QC ±20.0%) from their nominal concentrations.

At least 67% of overall QC samples (4 out of 6) should be within the acceptance criteria, and at least 50% of QC samples at each level should meet the above mentioned criteria.

The % mean accuracy for LQC, MQC and HQC samples should be within ±15.0% from their nominal concentration and for the LLOQ QC sample it should be within ±20.0% from its nominal concentration.

The precision for LQC, MQC and HQC samples should be within 15.0% and for the LLOQ QC the precision should be within 20.0%.

STABILITIES

Short term Stock Solution Stability

Short term stock solution stability of analyte was evaluated by comparing the mean response obtained from 6 replicate injections of aqueous dilution equivalent to ULOQ concentration of drug stock stored at ambient temperature (25±5°C) for 07 hours to that of ULOQ concentration level of drug stock stored at 2-8°C. The short term stock solution stability was 98.0%, which was within acceptance criteria of 90-110%. The results are presented in table 11.
Short term stock solution stability of ISTD was evaluated by comparing the mean response obtained from 6 replicate injections of dilution equivalent to ISTD working solution concentration of ISTD stock stored at ambient temperature (25±5°C) for 06 hours 57 minutes to that of ISTD working solution concentration level of ISTD stock stored at 2-8°C. The short term stock solution stability was 96.3%, which was within acceptance criteria of 90-110%. The results are presented in table 11.

Table 11: Short Term Stock Solution Stability

	Analyte		ISTD	
Nominal Concentration (ng/mL)	Comparison Stock	Stability Stock	Comparison Stock	Stability Stock
	1082042.743	1082042.743	822357.186	822357.186
Replicate No.	Area			
1	8210226	7885263	3305862	3055052
2	8102063	7734510	3485093	3406328
3	8092551	8010197	3493823	3048576
4	7431107	7758058	3332411	3785181
5	7559333	7570818	3272641	2942268
6	7894183	7386914	3263068	3166356
Mean	7881577.2	7724293.3	3358816.3	3233960.2
± SD	318765.62	221935.16	104180.55	312916.97
%CV	4.0	2.9	3.1	9.7
% Stability	98.0		96.3	

Calculation:

$$\% \text{ Mean Stability} = \frac{\text{Mean peak area of stability sample}}{\text{Mean peak area of comparison sample}} \times 100$$

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Acceptance criteria:
The % mean short term stock solution stability for analyte and ISTD at room temperature should be in the range of 90.0% – 110.0% and %CV for stability and comparison should be within 15.0%.

Long term Stock Solution Stability

Long term stock solution stability of analyte was evaluated by comparing the mean response obtained from 6 replicate injections of aqueous dilution equivalent to ULOQ concentration of drug stock stored in refrigerator (2-8°C) for 06 days 19 hours to that of ULOQ concentration level of drug stock prepared freshly. The long term stock solution stability was 103.6%, which was within acceptance criteria of 90-110%. The result is presented in table 12.

Long term stock solution stability of ISTD was evaluated by comparing the mean response obtained from 6 replicate injections of dilution equivalent to ISTD working solution concentration of ISTD stock stored in refrigerator temperature (2-8°C) for 06 days 19 hours to that of ISTD working solution concentration level of ISTD stock prepared freshly. The long term stock solution stability was 96.1%, which was within acceptance criteria of 90-110%. The result is presented in table 12.

Table 12: Long Term Stock Solution Stability

Long term stock solution stability of analyte was evaluated by comparing the mean response obtained from 6 replicate injections of aqueous dilution equivalent to ULOQ concentration of drug stock stored in refrigerator (2-8°C) for 06 days 19 hours to that of ULOQ concentration level of drug stock prepared freshly. The long term stock solution stability was 103.6%, which was within acceptance criteria of 90-110%. The result is presented in table 12.

Long term stock solution stability of ISTD was evaluated by comparing the mean response obtained from 6 replicate injections of dilution equivalent to ISTD working solution concentration of ISTD stock stored in refrigerator temperature (2-8°C) for 06 days 19 hours to that of ISTD working solution concentration level of ISTD stock prepared freshly. The long term stock solution stability was 96.1%, which was within acceptance criteria of 90-110%. The result is presented in table 12.

Table 12: Long Term Stock Solution Stability

	Analyte		ISTD	
Nominal Concentration (ng/mL)	comparison Stock	Stability Stock	comparison Stock	Stability Stock
	1082042.743	1052970.373	822357.186	822357.186
Correction Factor	1.03		1.00	
Replicate No.	Area/Area Ratio			
1	5574687	5547720	2598012	2368741
2	5763758	5689421	2517278	2319507
3	5622196	5567620	2497995	2417335
4	5611000	5484329	2630768	2456628
5	5653160	5761518	2475577	2473190
6	5732238	6174677	2326954	2421034
Mean	5659506.5	5704214.2	2507764.0	2409405.8
± SD	73672.01	251529.61	106947.55	56976.96
%CV	1.3	4.4	4.3	2.4

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%Stability	103.6	96.1

Short term Working Solution Stability

Short term working solution stability of analyte was evaluated by comparing the mean response obtained from 6 replicate injections of ULOQ and LLOQ working solutions stored at ambient temperature (25±5°C) for 06 hours 06minutes and 06 hours 03 minutes respectively to that of ULOQ and LLOQ working solutions prepared freshly from drug stock stored at 2-8°C. The short term working solution stability was 100.5% and 98.6% which was within acceptance criteria of 90-110%. The results are presented in table 13.

Short term working solution stability of ISTD was evaluated by comparing the mean response obtained from 6 replicate injections of ISTD working solution stored at ambient temperature (25±5°C) for 06 hours 46 minutes to that of ISTD dilution prepared freshly from ISTD stock stored at 2-8°C. The short term working solution stability was 91.2%, which was within acceptance criteria of 90-110%. The results are presented in table 13.

Table 13: Short Term Working Solution Stability

	Analyte at ULOQ		Analyte at LLOQ		ISTD	
Nominal Concentration (ng/mL)	comparison Solution	Stability Solution	comparison Solution	Stability Solution	comparison Solution	Stability Solution
	175055.503	175055.503	507.661	507.661	2055.893	2055.893
ReplicateNo.	Area/Area Ratio					
1	4444771	4692039	15893	15324	3305862	3279615
2	4798400	5038654	15927	16077	3485093	2966338
3	4641235	4857422	16490	15313	3493823	3167353
4	4830702	4685332	16367	16475	3332411	3144112
5	4834638	4645795	16244	16468	3272641	2914628
6	4937103	4723555	15894	15795	3263068	2897643
Mean	4747808.2	4773799.5	16135.8	15908.7	3358816.3	3061614.8
± SD	176630.82	148702.10	265.19	523.74	104180.55	156894.86
%CV	3.7	3.1	1.6	3.3	3.1	5.1
%Stability	100.5		98.6		91.2	

Long term Working Solution Stability

Long term working solution stability of analyte was evaluated by comparing the mean response obtained from 6 replicate injections of ULOQ and LLOQ working solutions stored in refrigerator (2-8°C) for 06 days 17 hours,to that of ULOQ and LLOQ working solutions prepared freshly. The long term working solution stability for analyte for ULOQ and LLOQ was 99.3% and 102.8%respectively, which was within acceptance criteria of 90-110%. The results are presented in table 14.

Long term working solution stability of ISTD was evaluated by comparing the mean response obtained from 6 replicate injections of ISTD working solution stored in refrigerator (2-8°C) for 06 days 17 hours,to that of ISTD dilution prepared freshly. The short term working solution stability was 96.7%, which was within acceptance criteria of 90-110%. The result is presented in table 14.

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Table 14: Long Term Working Solution Stability

	Analyte at ULOQ		Analyte at LLOQ		ISTD	
S.No	comparison Solution	Stability Solution	comparison Solution	Stability Solution	comparison Solution	Stability Solution
Nominal Concentration (ng/mL)	179619.095	175003.676	502.933	500.149	2055.893	2055.893
Correction factor	1.03		1.01		1.00	
Replicate No.	Area/Area Ratio					
1	5574687	5429456	16129	16857	2598012	2393472
2	5763758	5219899	16860	17264	2517278	2390429
3	5622196	5460521	16721	16954	2497995	2426745
4	5611000	5487688	16953	17172	2630768	2459208
5	5653160	5328394	16904	17008	2475577	2443036
6	5732238	5873995	16643	17206	2326954	2430484
Mean	5659506.5	5466658.8	16701.7	17076.8	2507764.0	2423895.7
± SD	73672.01	222618.19	303.62	160.58	106947.55	27241.35
%CV	1.3	4.1	1.8	0.9	4.3	1.1
%Stability	99.3		102.8		96.7	

Bench top Stability

Note: Freshly prepared calibration curve standards used for the assessment of stability experiments Bench top Stability, Freeze thaw stability, Stability In Extract –Ambient, Stability In Extract – Auto sampler, Dry Extract Stability.

The calibration curve found linear from 3499.155ng/mL–10.005ng/mL for analyte and the goodness of fit was greater than 0.99. The results are presented in table 15.

The bench top stability at high and low quality control levels were determined by comparing the mean back calculated concentrations of the replicate samples kept on bench at ambient temperature (25±5°C) for about 15 hours 29 minutes to that of nominal concentrations. The % bench top stability for HQC and LQC sample were 93.3% and 97.8% respectively which were within the acceptance of 85-115%. The precision for HQC and LQC was 1.0% and 2.4% respectively, which were within the acceptance criteria of ≤15%. The results are presented in table 16.

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Table15: Calculated Concentrations for Freshly spiked Calibration Curve Standards on Date 05 Jul 2016

Acquisition Batch ID: 050716-STABILITIES-01					Date : 05 Jul 2016			
STD ID	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8
Nominal Concentration (ng/mL)	3499.155	3079.256	1924.535	1224.967	525.511	122.707	23.069	10.005
Calculated Concentration (ng/mL)	3504.273	2927.117	1926.193	1218.087	550.872	120.199	24.044	9.831
%Accuracy	100.1	95.1	100.1	99.4	104.8	98.0	104.2	98.3
Calibration Curve Parameters	Slope	0.0008	Intercept	0.0001	r value	0.9994	r^2 value	0.9988

Table 16: Bench Top Stability

Nominal Concentration (ng/mL)		HQC	LQC
		2802.249	28.044
S. No.	QC ID	Calculated Concentrations of Quality Control Samples (ng/mL)	
1	169	2565.365	27.892
2	170	2624.574	27.878
3	171	2625.131	27.820
4	172	2638.463	27.840
5	173	2610.377	26.585
6	174	2624.678	26.535
Mean		2614.7647	27.4250
±SD		25.78041	0.67071
%CV		1.0	2.4
% Mean Accuracy		93.3	97.8

Freeze thaw stability

The freeze thaw stability at high and low quality control levels were determined after completing five freeze thaw cycles by comparing the mean back calculated concentrations of the replicate samples that complete five freeze thaw cycles(at -70±15°C) with that of nominal concentrations. The % freeze thaw stability for HQC and LQC sample were 90.8% and 97.4%respectively which were within the acceptance of 85-115%. The precision for HQC and LQC was.3.1% and 1.1% respectively, which were within the acceptance criteria of ≤15%. The results are presented in table 17a.

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Table 17: **Freeze thaw Stability**
Table 17a: Freeze Thaw Stability (-70± 15ºc)

Nominal Concentration (ng/mL)		HQC	LQC
		2802.249	28.044
S. No.	QC ID	Calculated Concentrations of Quality Control Samples (ng/mL)	
1	181	2625.255	27.355
2	182	2495.297	27.515
3	183	2457.807	27.345
4	184	2489.982	27.194
5	185	2546.295	26.808
6	186	2653.450	27.734
Mean		104.7658	2544.6810
±SD		2.30837	79.12436
%CV		2.2	3.1
% Mean Accuracy		103.8	90.8

The freeze thaw stability at high and low quality control levels were determined after completing five freeze thaw cycles by comparing the mean back calculated concentrations of the replicate samples that complete five freeze thaw cycles(at -20±5°C) with that of nominal concentrations. The % freeze thaw stability for HQC and LQC sample were 93.1% and 99.2% respectively, which were within the acceptance of 85-115%. The precision for HQC and LQC was 1.2% and 2.7% respectively, which were within the acceptance criteria of ≤15%. The results are presented in table 17b.

Table 17b: **Freeze Thaw Stability (-20±5ºc)**

Nominal Concentration (ng/mL)		HQC	LQC
		2802.249	28.044
S. No.	QC ID	Calculated Concentrations of Quality Control Samples (ng/mL)	
1	241	2599.418	28.63
2	242	2670.116	28.539
3	243	2612.115	27.443
4	244	2596.940	28.080
5	245	2580.521	27.597
6	246	2596.664	26.613
Mean		103.0330	2609.2957
±SD		2.01701	31.44784
%CV		2.0	1.2

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% Mean Accuracy	102.1	93.1

Stability In Extract – Ambient

The stability in extract at high and low quality control levels were determined by comparing the mean back calculated concentrations of the replicate samples kept on bench at ambient temperature (25±5°C) after reconstitution for about 06 hours 31 minutes to that of nominal concentrations. The % stability in extract at ambient temperature for HQC and LQC sample were 92.3% and 96.8% respectively, which were within the acceptance of 85-115%. The precision for HQC and LQC was 2.6% and 2.1% respectively, which were within the acceptance criteria of ≤15%. The results are presented in table 18.

Table 18: Stability of Extract – Ambient

Nominal Concentration (ng/mL)		HQC	LQC
		2802.249	28.044
S. No.	QC ID	Calculated Concentrations of Quality Control Samples (ng/mL)	
1	145	2518.108	26.434
2	146	2527.678	28.142
3	147	2679.608	27.229
4	148	2640.152	27.19
5	149	2608.221	27.022
6	150	2545.550	26.893
Mean		105.8583	2586.5528
±SD		2.51634	66.08149
%CV		2.4	2.6
% Mean Accuracy		104.9	92.3

Stability In Extract –Autosampler

The stability in extract at high and low quality control levels were determined by comparing the mean back calculated concentrations of the replicate samplesafterreconstitutionfor about 35 hours 35 minutes to that of nominal concentrations. The % stability in extract at autosampler temperature(10±1°c) for HQC and LQC samples were 92.4% and 97.4% respectively, which were within the acceptance of 85-115%. The precision for HQC and LQC was 1.8%and 2.8% respectively, which were within the acceptance criteria of ≤15%. The results are presented in table 19.

Table 19: Stability of Extract – Autosampler

Nominal Concentration (ng/mL)		HQC	LQC
		2802.249	28.044
S. No.	QC ID	Calculated Concentrations of Quality Control Samples (ng/mL)	

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1	133	2610.765	27.72
2	134	2638.013	26.128
3	135	2516.305	28.474
4	136	2593.283	27.091
5	137	2556.952	27.301
6	138	2628.242	27.244
Mean		105.8103	2590.5933
±SD		1.55282	46.32639
%CV		1.5	1.8
% Mean Accuracy		104.9	92.4

Dry Extract Stability

The dry extract stability at high and low quality control levels were determined by comparing the mean back calculated concentrations of the replicate samples stored in refrigerator (2-8°C) for about 29 hours 54 minutes to that of nominal concentrations. The % dry extract stability for HQC and LQC sample were 92.3% and 96.7% respectively, which were within the acceptance of 85-115%. The precision for HQC and LQC was 1.6% and 2.2% respectively, which was within the acceptance criteria of ≤15%. The results are presented in table 20.

Table 20: Dry Extract Stability

Nominal Concentration (ng/mL)		HQC	LQC
		2802.249	28.044
S. No.	QC ID	Calculated Concentrations of Quality Control Samples (ng/mL)	
1	157	2568.701	27.074
2	158	2593.201	27.180
3	159	2580.637	26.158
4	160	2654.951	27.147
5	161	2529.472	27.133
6	162	2599.215	28.040
Mean		105.1908	2587.6962
±SD		2.12051	41.19927
%CV		2.0	1.6
% Mean Accuracy		104.3	92.3

Calibration Curve Stability

The Calibration curve stability at high and low calibration curve standard levels were determined by comparing the mean back calculated concentrations of the replicate samples for about 05 Days to that of nominal concentrations. The % calibration curve stability for ULOQ and LLOQ sample were 97.6% and 97.8% respectively, which were within the acceptance of 85-115% for ULOQ and 80-120% for LLOQ. The precision for ULOQ and LLOQ was

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3.8% and 2.5% respectively, which was within the acceptance criteria of ≤15% for ULOQ and ≤20% for LLOQ. The results are presented in table 21.

Table 21: Calibration Curve Stability

Nominal Concentration (ng/mL)		ULOQ	LLOQ
		3500.074	10.003
S. No.	QC ID	Calculated Concentrations of Quality Control Samples (ng/mL)	
1	001	3160.333	10.097
2	002	3457.777	9.452
3	003	3501.693	9.602
4	004	3489.732	9.708
5	005	3406.650	9.834
6	006	3483.903	9.992
Mean		135.4912	3416.6813
±SD		0.52170	130.06620
%CV		0.4	3.8
% Mean Accuracy		107.9	97.6
% Bias		7.9	-2.4

Autosampler Reinjection Reproducibility

Calibration curve standards and quality control samples of P&A01 were stored in autosampler temperature (10±1°c) for 29 hours 38Minutes and reinjected. The results are presented in table 22.

The calibration curve found linear from 3500.074ng/mL – 10.003ng/mL for analyte and the goodness of fit was greater than 0.99.

The precision for HQC, MQC, DQC and LQC samples was 1.5%, 1.2%, 2.0% and1.5% respectively, which was within the acceptance criteria ≤15%. The precision for LLOQ QC samples was 1.4%, which was within the acceptance criteria ≤20%.

The accuracy for HQC, MQC, DQC and LQC samples was 92.9%, 98.5%, 102.1% and 98.1% respectively, which were within the acceptance criteria of 85-115% to nominal concentration. The accuracy for LLOQ QC samples was 96.0%, which was within the acceptance criteria of 80-120% to nominal concentration.

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QC ID	HQC	MQC	DQC	LQC	LLOQQC
Nominal Concentration (ng/mL)	2802.249	1423.542	6300.451	28.044	10.006
BATCH ID	Back Calculated Concentrations of Quality Control Samples (ng/mL)				
010716-P&A-01	2599.55	1393.582	6535.229	27.834	9.514
	2592.253	1429.79	6392.316	27.355	9.529
	2568.37	1413.837	6564.998	27.427	9.502
	2596.133	1385.652	6210.164	27.354	9.596
	2608.816	1401.249	6454.37	27.288	9.898
	2653.32	1387.698	6430.317	27.858	9.577
Mean	2603.07367	1401.968	6431.232	27.51933	9.602667
±SD	28.0702	17.0624	126.1986	0.2569	0.1492
%CV	1.08	1.22	1.96	0.93	1.55
% Accuracy	92.9	98.5	102.1	98.1	96.0

Stability of Analyte in Blood

The stability of analyte in Blood at high and low quality control levels were determined by comparing the mean area ratio of the replicate samples kept on bench at ambient temperature (25±5°C) for about 02 Hours, 21 Minutes and ice bath is 02 Hours, 18 Minutes to that of mean area ratio of freshly prepared samples. After completion of the stability period plasma samples were separated by centrifugation of both stability and comparison blood samples. The plasma samples were processed and injected as per method SOP.

The % stability of analyte in blood at ambient for HQC and LQC sample were 97.9% and 98.3% respectively, which were within the acceptance of 85-115%. The precision for HQC and LQC was 0.9% and 2.1% respectively, which were within the acceptance criteria of ≤15%. The results are presented in table 23a.

The % stability of analyte in blood at Ice bath for HQC and LQC sample were 97.6% and 105.0% respectively, which were within the acceptance of 85-115%. The precision for HQC and LQC was 1.7% and 10.6% respectively, which were within the acceptance criteria of ≤15%. The results are presented in table 23b.

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Table 23: **Analyte Stability in Blood**
Table 23a: Analyte Stability in Blood – Ambient

Nominal Concentration (ng/mL)	HQC		LQC	
	2802.249		28.044	
	COMPARISON	STABILITY	COMPARISON	STABILITY
S. No.	AREA RATIO			
1	3.759	3.624	0.040	0.039
2	3.702	3.537	0.041	0.039
3	3.590	3.614	0.041	0.039
4	3.777	3.583	0.040	0.041
5	3.589	3.626	0.038	0.039
6	3.626	3.606	0.040	0.039
Mean	3.6738	3.5983	0.0400	0.0393
±SD	0.08391	0.03384	0.00110	0.00082
%CV	2.3	0.9	2.7	2.1
% Mean Accuracy	97.9		98.3	

Table 23b: **Analyte Stability in blood –Ice bath**

Nominal Concentration (ng/mL)	HQC		LQC	
	2802.249		28.044	
	COMPARISON	STABILITY	COMPARISON	STABILITY
S. No.	AREA RATIO			
1	3.759	3.515	0.040	0.040
2	3.702	3.599	0.041	0.041
3	3.590	3.637	0.041	0.040
4	3.777	3.547	0.040	0.051
5	3.589	3.542	0.038	0.039
6	3.626	3.676	0.040	0.041

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Mean	3.6738	3.5860	0.0400	0.0420
±SD	0.08391	0.06224	0.00110	0.00447
%CV	2.3	1.7	2.7	10.6
% Mean Accuracy	97.6		105.0	

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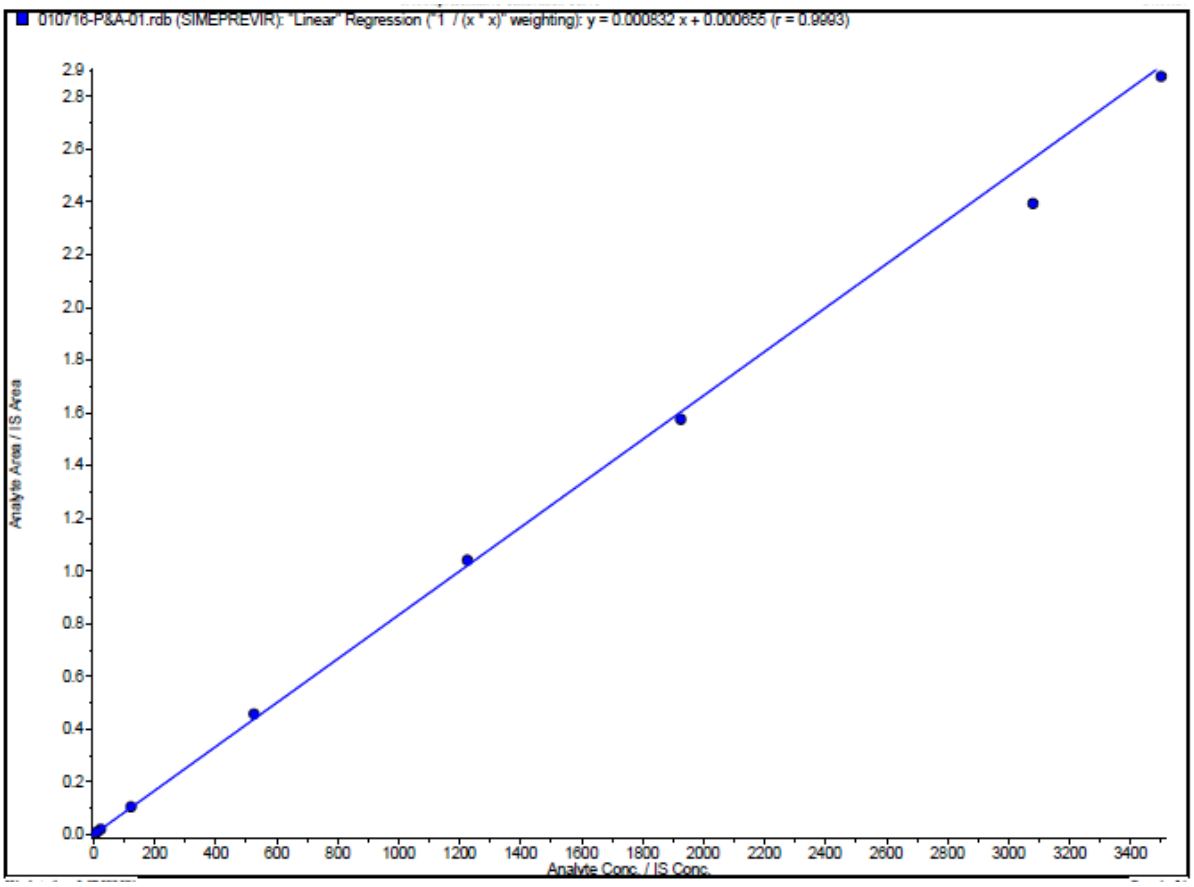
Appendix 1: Chromatograms

Representative chromatograms of P&A01 were listed as below:



1. A Representative Calibration Curve

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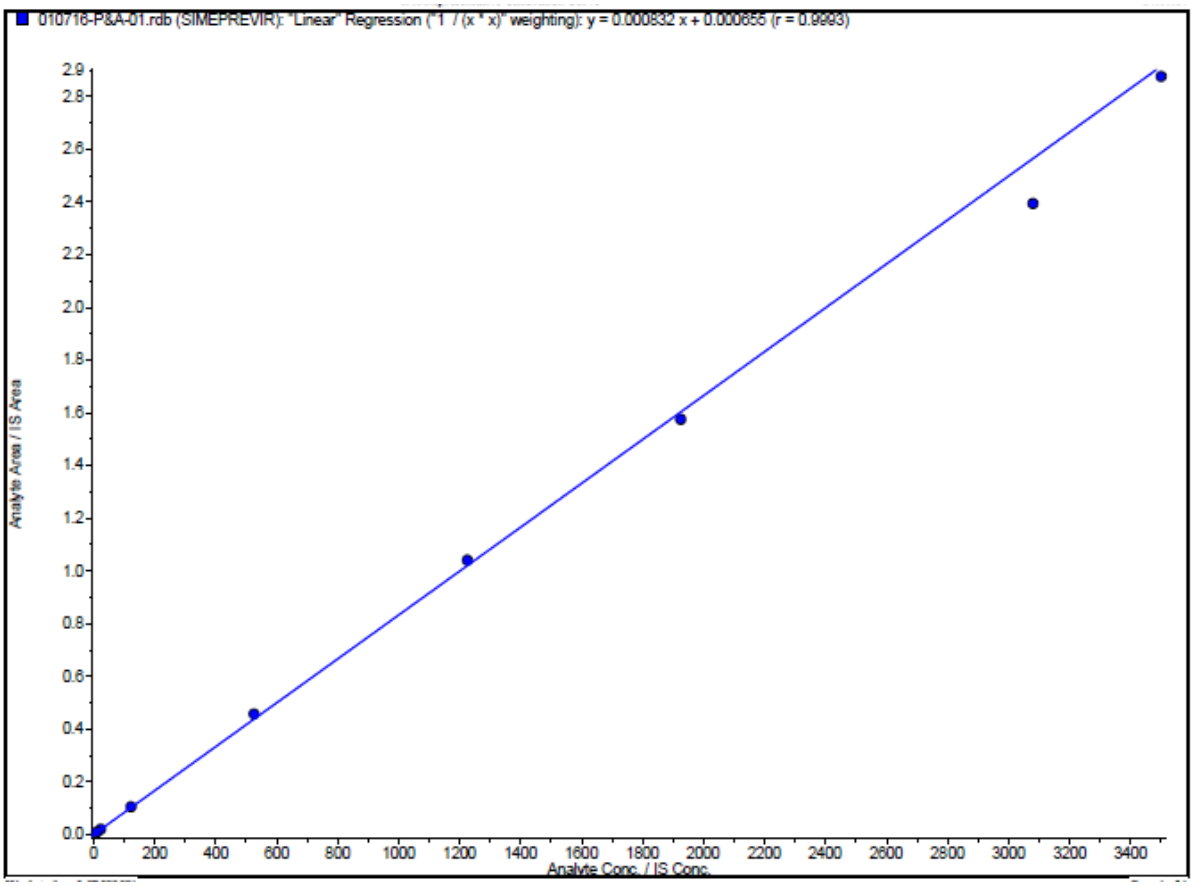
Appendix 1: Chromatograms

Representative chromatograms of P&A01 were listed as below:



2. A Representative Calibration Curve

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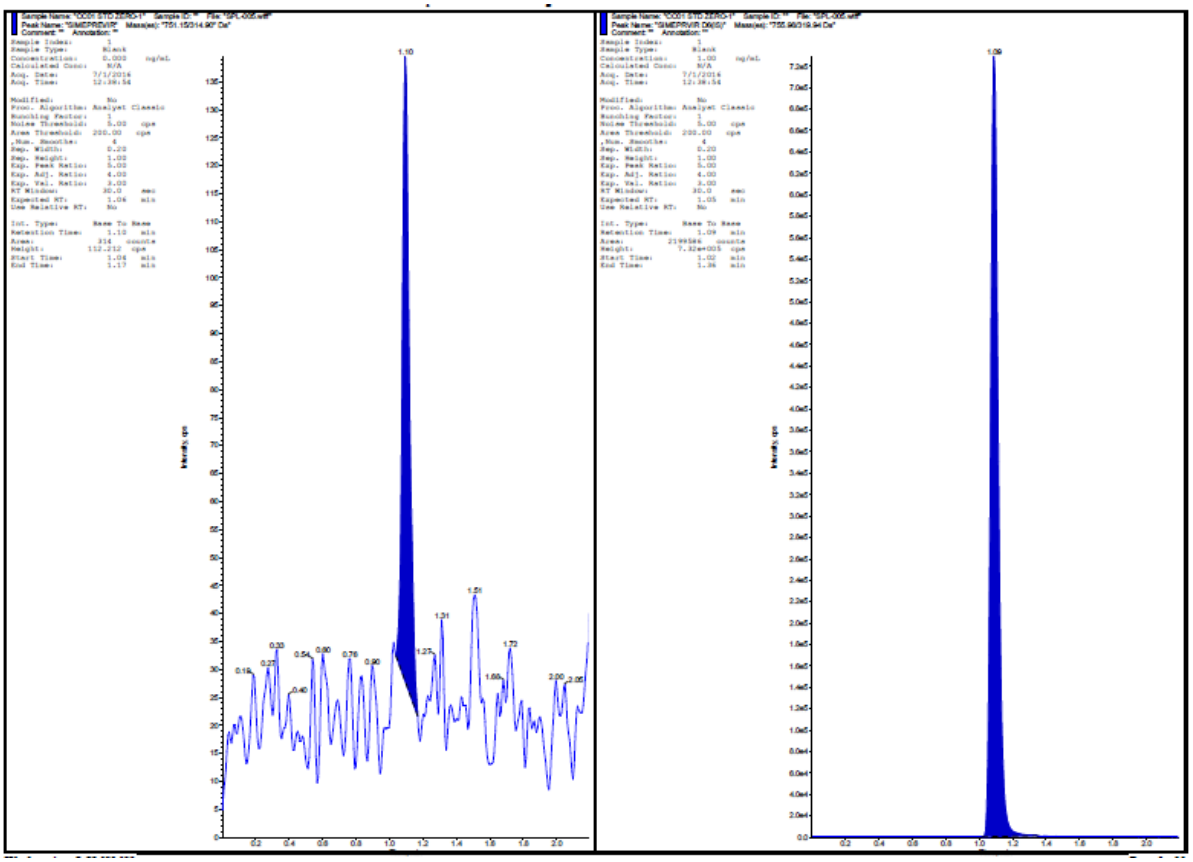

3. Representative Chromatogram of Standard Blank

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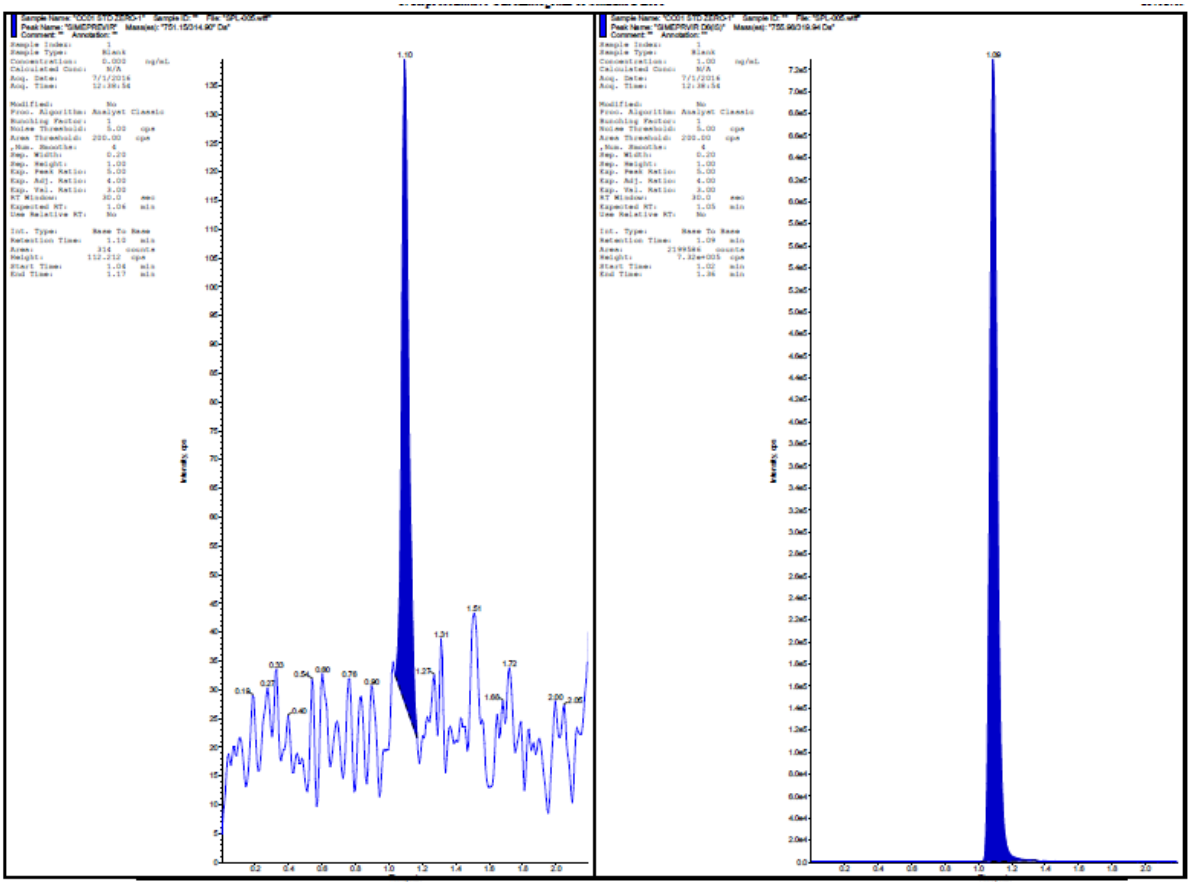

4. Representative Chromatogram of Standard Zero

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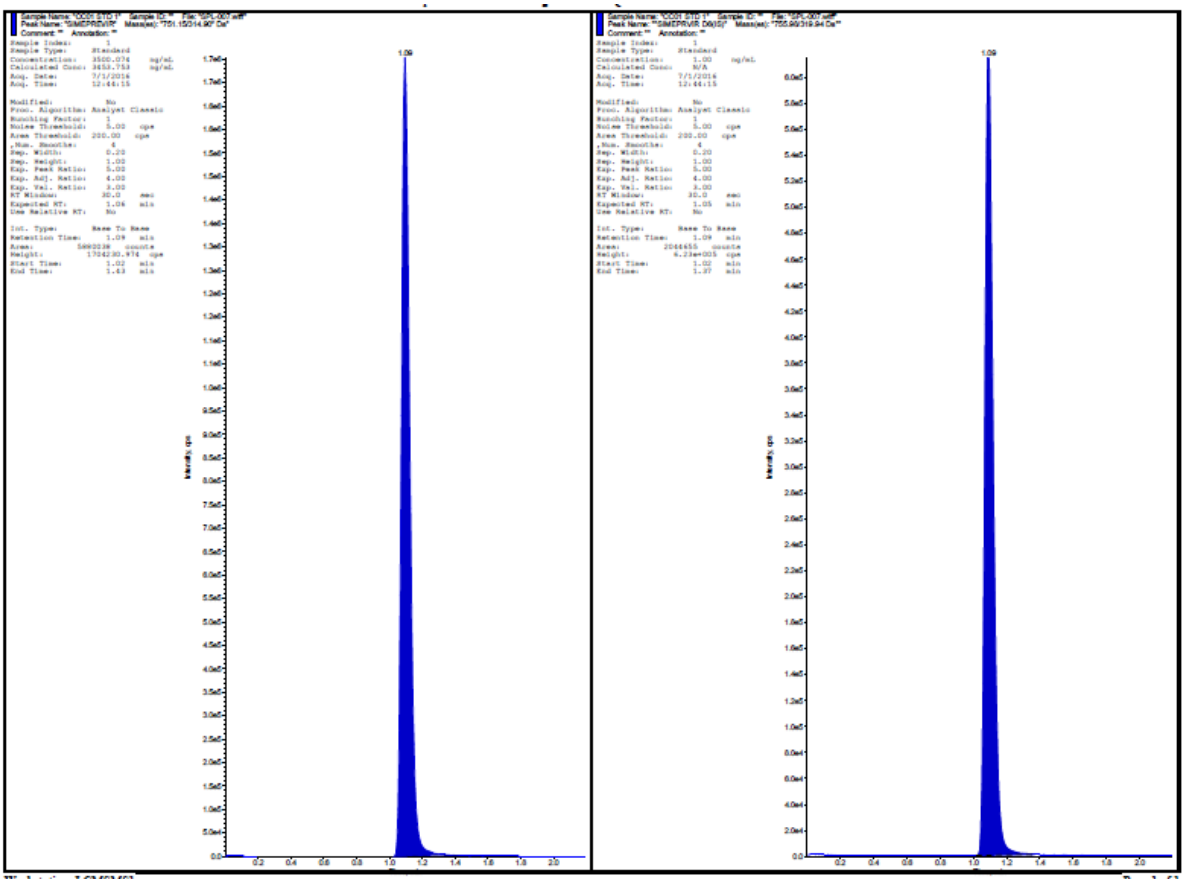

5. Representative Chromatogram of LLOQ Standard

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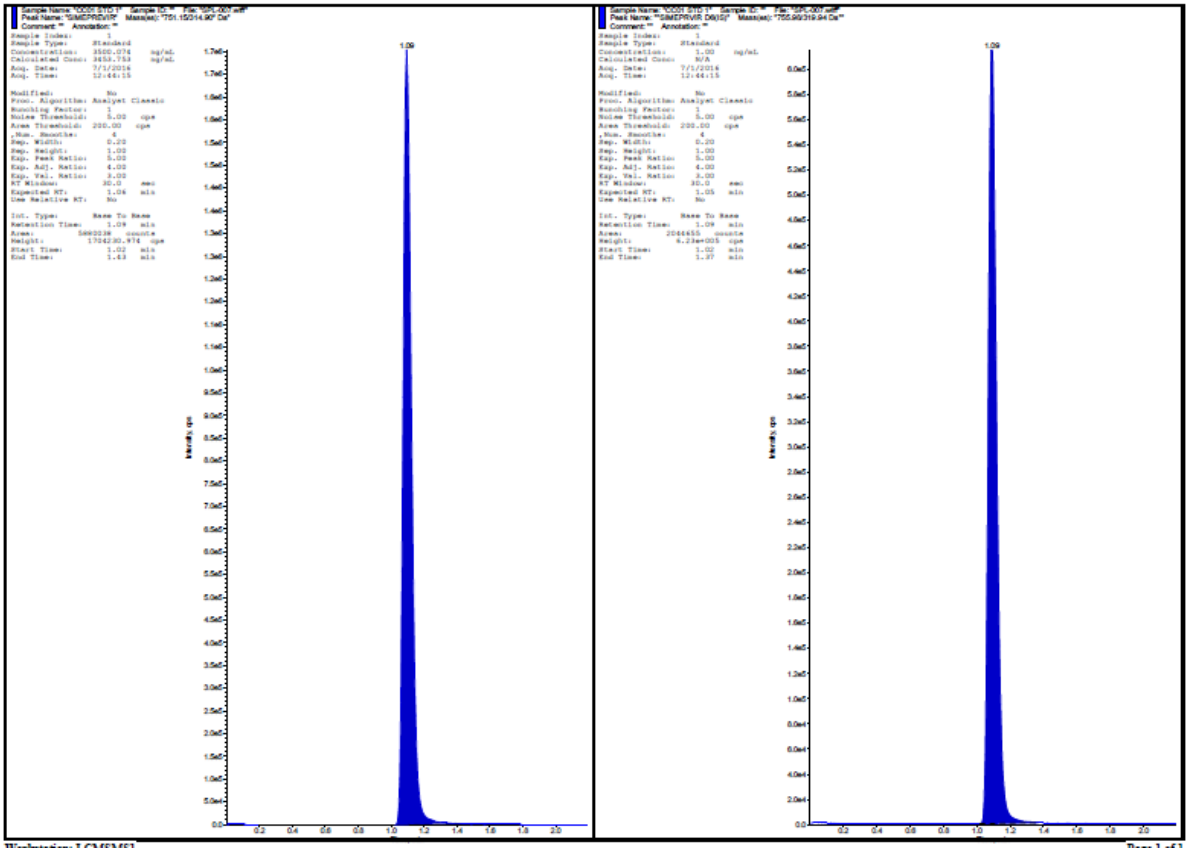

6. Representative Chromatogram of ULOQ Standard

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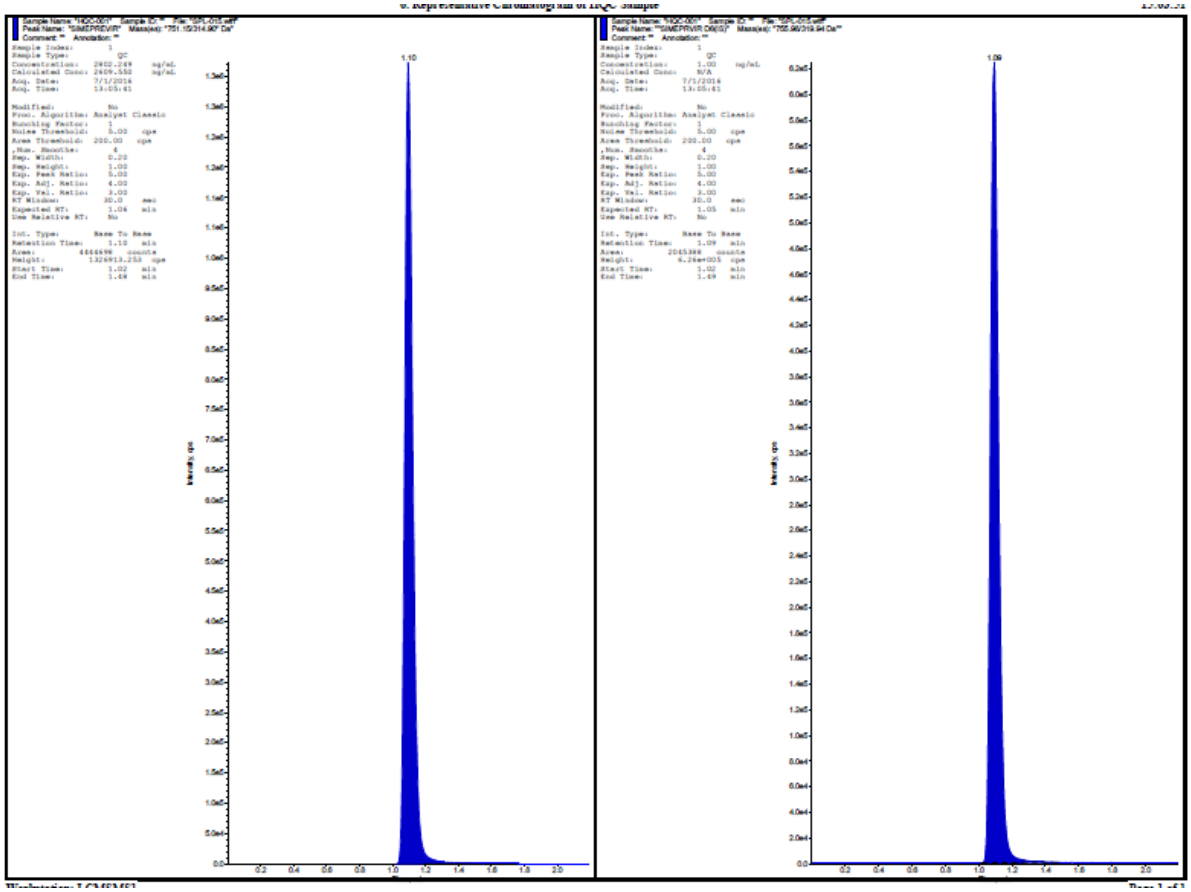

7. Representative Chromatogram of HQC Samples

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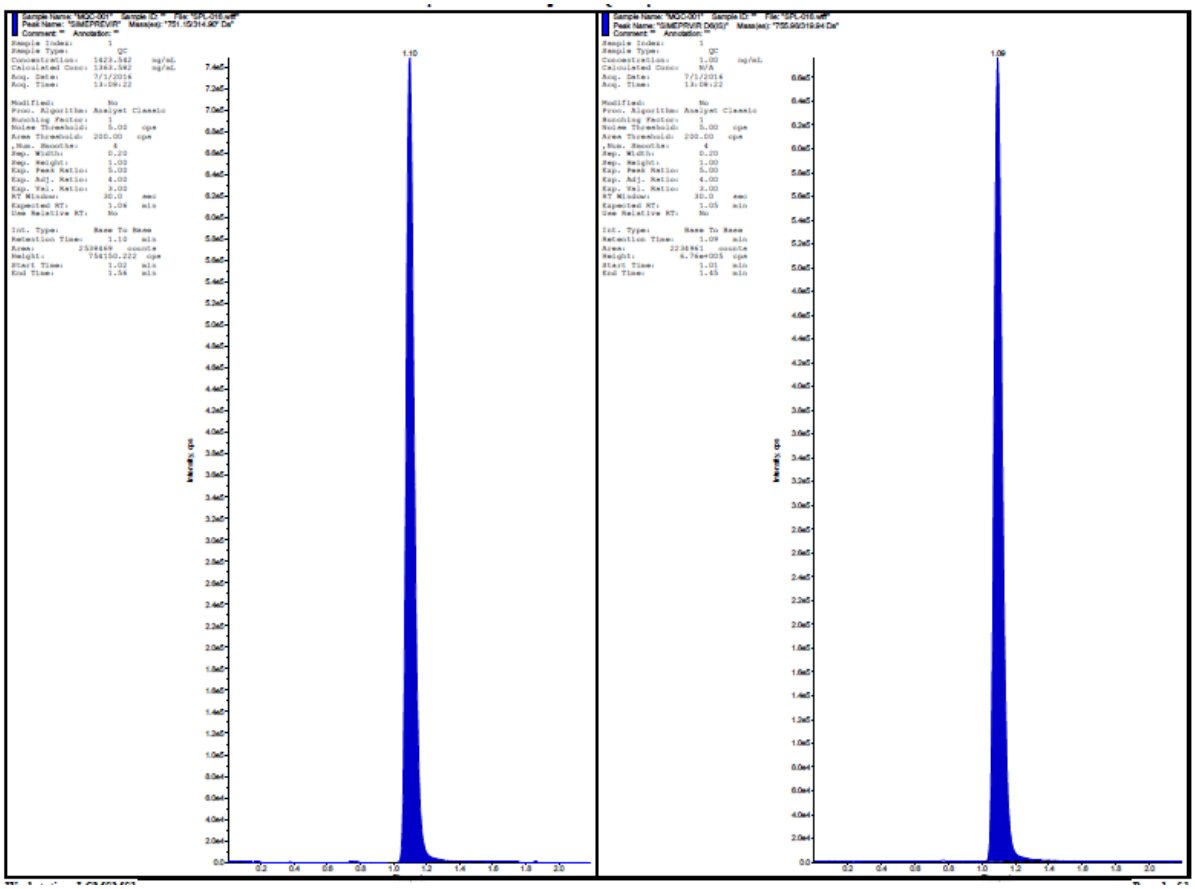

8. Representative Chromatogram of MQC Samples

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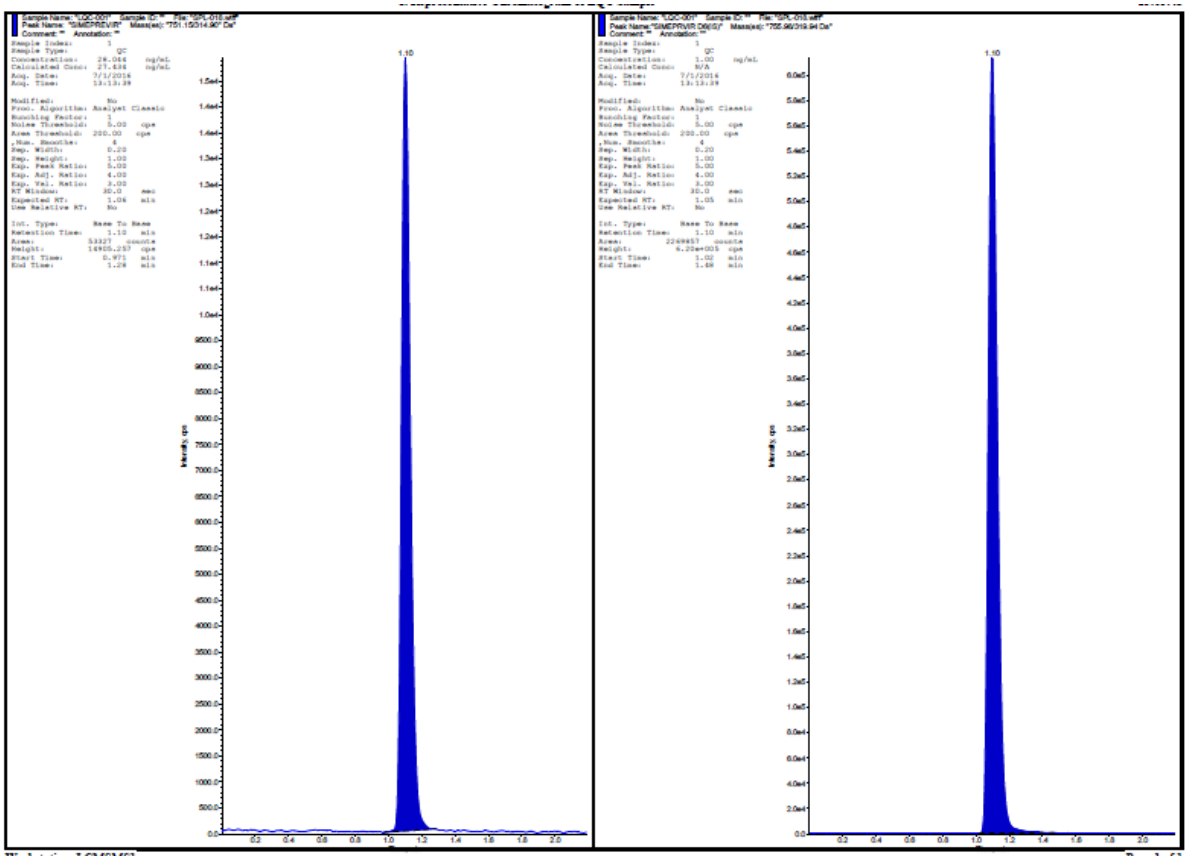

9. Representative Chromatogram of LQC Samples

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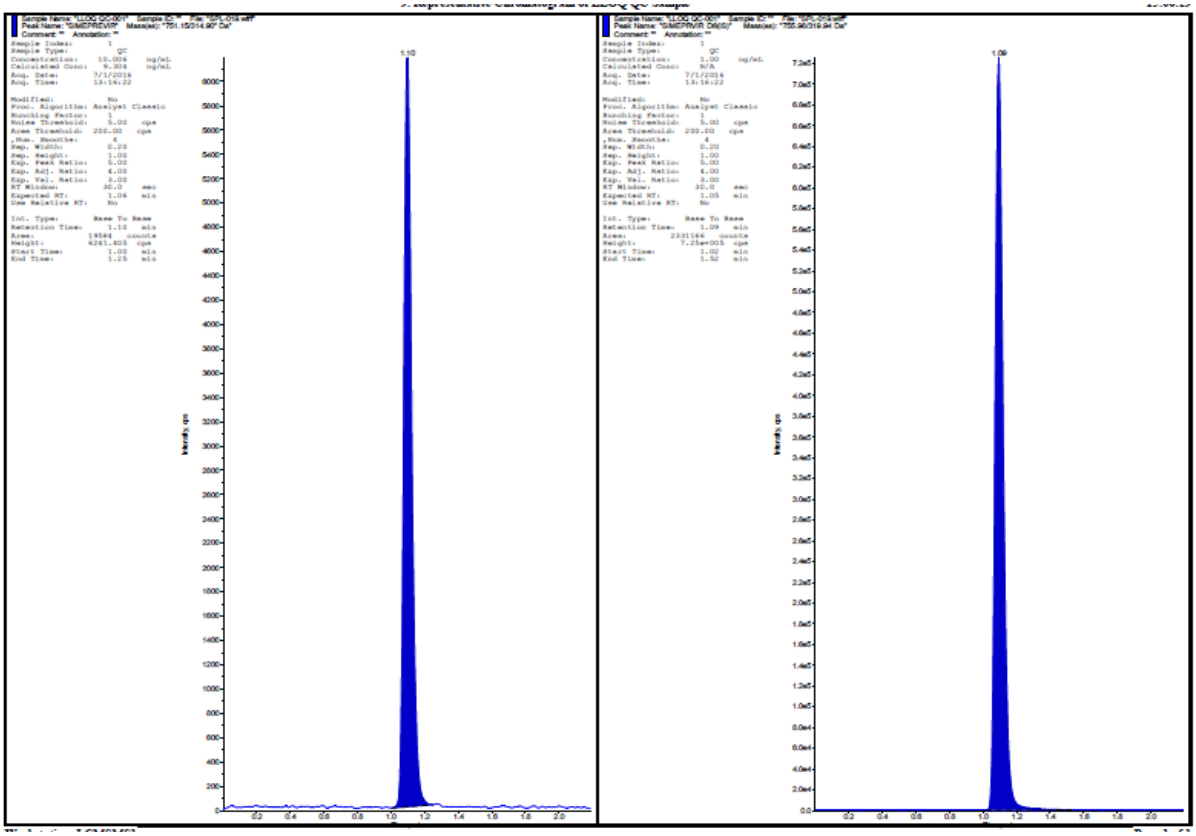

10. sentative Chromatogram of LLOQ QC

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