RESEARCH ARTICLE

An LC-MS Method Development and Validation for the Estimation of Ritonavir in Plasma Samples

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ABSTRACT

This project aims to create and verify a method using high-performance liquid chromatography-mass spectrometry (HPLC-MS) to measure the amount of ritonavir in human plasma. Saquinavir will be used as the internal standard for this analysis. Chromatographic isolation was processed HYPURITY ADVACE 50 × 4.6 mm, 5 µm (Make: Thermo scientific) analytical column with mobile phase composition of methanol and ammonium acetate 5 mm buffer in the ratio of 85:15% v/v. Detection was processed in a positive ionic approach and the parent and product ion transitions were monitored at 721.30/296.10 for ritonavir and 671.30/570.30 for saquinavir (API 2000). The measurement of the linearity curve for regression analysis The correlation coefficient (r) exhibited a remarkable value of over 0.99 within the concentration range of 8.004 to 1600.001 ng/mL for ritonavir. All eight batches showed no significant matrix effect. It was found that the standardized matrix factor had a precision of 1.90% at the LQC level and 2.38% at the HQC level. It was 0.992 for LQC and 1.005 for HQC when the IS factor was taken into account. Ritonavir had a general recovery rate of 89.07%, with a range of accuracy from 0.85 to 2.55%. The internal standard drug saquinavir had an average recovery rate of 90.18%, with a range of accuracy from 1.89 to 3.40%. The developed method was successfully validated and can be utilized for the assessment of ritonavir in biological matrices in industries, forensic labs, quality control labs, and bioavailability studies.

Keywords: Ritonavir, HIV/AIDS, LC-MS, Method development, Validation.

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INTRODUCTION

The development of highly active antiretroviral therapy (HAART) has greatly improved the clinical treatment of patients with human immunodeficiency virus[-1] (HIV-1) infection. However, there are cases where patients may experience metabolic complications and drug resistance. This unfortunate result may stem from a lack of success in attaining optimal levels of antiretroviral drug concentrations in the bloodstream. Monitoring plasma drug concentration is crucial for ensuring optimal drug efficiency, preventing viral confrontation, managing drug interaction, avoiding adverse effects, and assessing non-adherence. It (Figure 1) is used in combination with other medicines to cure HIV/AIDS.[1,2] This drug, initially created as a suppressor of HIV proteases, is extensively utilized as an enhancer for other inhibitors of proteases. In addition, observation may be used to evaluate drug interactions, such as those with a potent enzyme inhibitor or inducer that has been shown to affect the plasma concentration of the medication. Over the past few years, numerous publications have been on high-performance liquid chromatography (HPLC) methods that enable the simultaneous determination of antiretroviral drugs in plasma.[3-5] Despite their reliability and sensitivity, many of the HPLC methods come with significant drawbacks. Alternative techniques for measuring antiretroviral drugs are available using advanced mass spectrometry methods. However, these methods may not be accessible or affordable for conventional hospital laboratories. Our goal was to create a straightforward, cost-effective, and dependable liquid chromatography–mass spectrometry (LC-MS) method to measure the levels of ritonavir in plasma. We utilized saquinavir as an internal standard for accurate results.[6-9]

MATERIAL AND METHODS

Table 1 represents the different reagents and materials used in the research work.

Chromatographic System and Conditions

A modular LC-MS/MS system from Shimadzu, equipped with a SIL-HTC auto sampler consisting of an LC/20AD solvent delivery system coupled with an API 4000 applied

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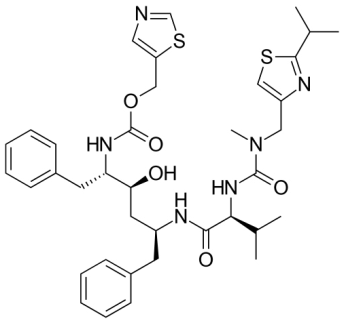

Figure 1: Ritonavir chemical structure

Table 1: Reagents/Materials

S. No	*Materials*	*Manufacturer*	*Grade*
1	Methanol	JT Baker	K15E30
2	Ammonium acetate	Merck	QK1Q612161
3	HPL grade water	Merck	SB2SF62047
4	Milli-Q water	In house	N/AP
5	Formic acid	Merck	AL1A610665
6	Orpheus, 100 mg/1-mL, C18 SPE cartridges	Orochem	DS072210C18EC

Biosystems from Canada, was utilized for the research project. Chromatographic isolation was processed HYPURITY ADVACE 50 × 4.6 mm, 5 µm (Make: Thermo scientific) analytical column with mobile phase composition of methanol and ammonium acetate 5mm buffer in the ratio of 85:15% v/v. Detection was processed in a positive ion approach, and the parent and product ion transitions were monitored at 721.30/296.10 for ritonavir and 671.30/570.30 for saquinavir (API 2000).

Processing of Mobile Phase and Diluent

A volume of 300 mL of ammonium acetate buffer solution with a concentration of 5 mm was carefully transferred into a 2000 mL reagent bottle. Following that, 1700 mL of HPLC-grade methyl alcohol was added to the solution. The mixture was thoroughly combined, undergoing sonication in an ultrasonic device for a duration of 5 minutes. A batch number was given and the 'Solution Preparation' form was filled out. A solution was prepared by combining HPLC-grade methanol and Milli Q water in a volume ratio of 60:40 as a diluent.

Preparation of Ritonavir Stock Solution

Precisely measured, approximately 5 mg of ritonavir hydrochloride working standard was carefully relocated to a 5 mL volumetric flask. It was then dissolved in methyl alcohol and the volume was adjusted with the same solvent to create a solution with a concentration of 1-mg/mL. Adjusted the previously mentioned concentration of the ritonavir solution, taking into consideration its potency and the precise amount that was weighed. The 'Stock Weighing and Solution Preparation' form was completed after receiving a batch number. For no more than six days, the stock solution was kept in the fridge at a temperature between 2 and 8°C.

In order to create CC standards, QC samples, and DIQC samples, the stock solutions were diluted to appropriate proportions using a 60:40 v/v combination of methanol and Milli Q water (Diluent). The solutions were then spiked into plasma. The mobile phase was also used to create all of the other final dilutions, such as the aqueous mixture, system suitability dilutions, and others.

Preparation of Saquinavir Stock Solution (Internal Standard)

To generate a solution with a concentration of 1-mg/mL, about 2,000 mg of saquinavir were relocated to a volumetric flask with a capacity of 2 mL, and the volume was filled with the same amount. The concentration of saquinavir mentioned above has been adjusted to consider its potency, molecular weight, and the quantity that was weighed. The 'Stock Weighing and Solution Preparation' form was filled out, and a batch number was supplied to the individuals involved. For a maximum of six days, the stock solution was kept in the refrigerator at a temperature between 2 and 8°C.

Preparation of Quality Control and Calibration Curve samples

A calibration curve standard was made up of ten different concentrations of ritonavir that were not zero. These concentrations ranged from 8.004 to 1600.001 ng/mL. For ritonavir, quality control levels were made with values of 8.035 ng/mL (LLoQQC), 23.772 ng/mL (LQC), 104.720 ng/mL (MQC 1), 805.542 ng/mL (MQC 2), and 1388.865 ng/mL (HQC). They were kept at –70°C until they were used. The stability of twelve sets of LQC and HQC was tested by placing them in a deep freezer at -20°C. With a concentration of 2702.656 ng/mL, which is about 1.69 times the maximum standard concentration of ritonavir, 24 sets of quality control samples were prepared to ensure dilution integrity. Six rounds of doubling and quadrupling the dilution were performed on these.

Validation

This experimental design aims to show that the test procedure can reliably and consistently deliver repeatable results within the specified tolerances. Every experimental design specifies the quality standards that must be satisfied by the validation parameters.[10-14]

RESULT AND DISCUSSION

Validation Parameters

Selectivity

We obtained plasma (biological matrix) from at least 8 individual batches, including one lipemic and one hemolytic plasma.[15] In order to assess interference, the response in the blank matrix will be compared to the average result of the extracted LLoQ for the drug at the respective retention time (RT) values (Figures 2 to 4). Similarly, the response in the blank matrix will be compared to the average result of the extracted

Table 2: Ritonavir selectivity data

LLoQ QC	*Analyte area*	*Internal standard area*
01	3212	326188
02	3323	323281
03	3346	336218
04	3507	333782
05	3251	329655
06	3321	332817
Mean	3359.0	330256.8
SD	84.04	4883.12
%CV	2.51	1.51

internal standard in LLoQ samples to assess interferences at a retention time of the saquinavir (Table 2).

Sensitivity

The sensitivity of the analysis will be assessed based on the LLoQ. When the analyte(s) and blank matrix are tested at the same retention time or mass transitions, the LLoQ response must be five times greater than that of any interfering compounds. The signal-to-noise ratio also has to be higher than 5:1. The same stock solutions will be used to produce a calibration curve and six samples of the LLoQ.[16,17] The CC and LLoQ samples were processed and analyzed, as shown in Table 3.

Matrix effect

The matrix impact was assessed by analyzing the analyte and internal standard at two concentration levels (LQC and HQC) in eight replicates each. Two blank plasma samples were derived from 8 different screened plasma batches, which included one lipemic and one hemolytic plasma batch.[18-20] Each replication was treated separately. One set of eight distinct blank matrices was used to introduce the LQC concentration and the internal standard (ISTD). Another set was utilized to

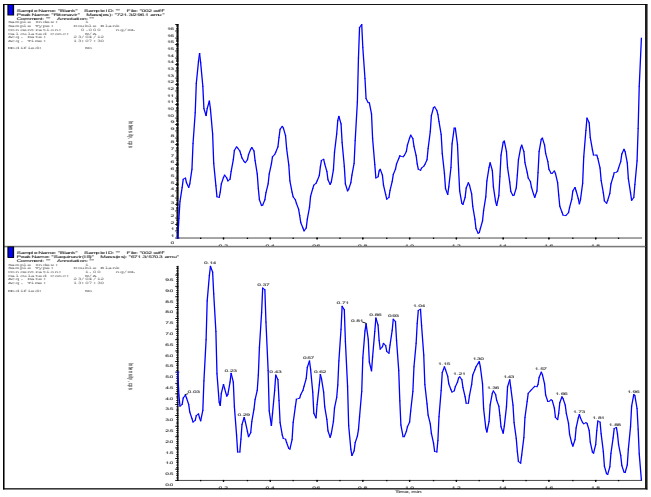

Figure 2: Chromatogram displaying the characteristics of a blank plasma sample of ritonavir

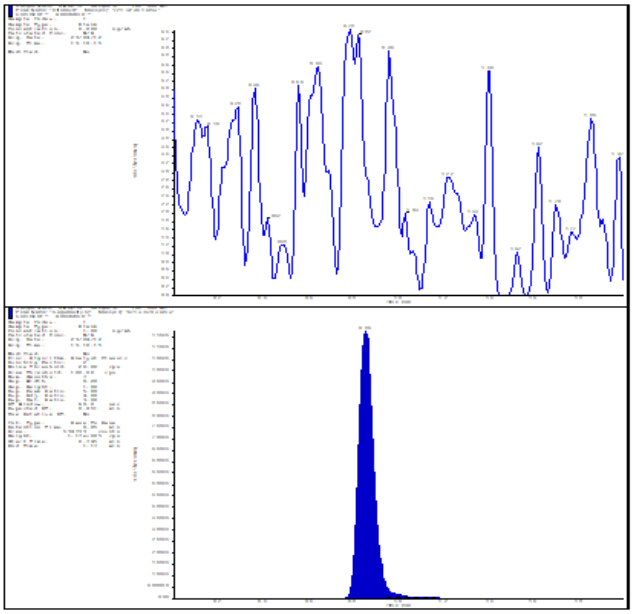

Figure 3: A picture of a chromatogram representing a sample of blank plasma with an internal standard of ritonavir

introduce the HQC concentration, also accompanied by the ISTD. A single batch of liquid samples, with the same quantities as the LQC and HQC, was generated by adding the analyte(s) and ISTD to a reconstitution solution. The samples were then analyzed by injecting them six times each, as shown in Table 4. The formula shown below was used to obtain the normalized MF for both HQC and LQC levels.

$$IS\ normalized\ Matrix\ Factor = \frac{\text{Peak area ratio in presence of matrix ion}}{\text{Mean peak area ratio in absence of matrix ions}}$$

Linearity

Ten concentration levels were created in the biological matrix by adding a specified amount of the drug. The selection of

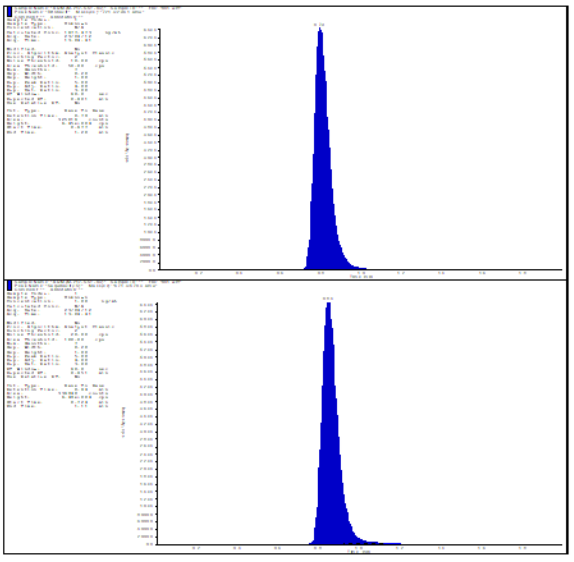

Figure 4: A chromatogram displaying a mixture of ritonavir in an aqueous standard solution, together with an internal standard, is shown

Table 3: Response-concentration relationships a linear model Information about ritonavir's sensitivity

	ST-A	*ST-B*	*ST-C*	*ST-D*	*ST-E*	*ST-F*	*ST-G*	*ST-H*	*ST-I*	*ST-J*
CC	8.004	16.008	40.020	80.040	160.080	320.160	640.320	960.001	1280.001	1600.001
Lower Limit	6.403	13.607	34.017	68.034	136.068	272.136	544.272	816.001	1088.001	1360.001
Upper Limit	9.605	18.409	46.023	92.046	184.092	368.184	736.368	1104.001	1472.001	1840.001
1	8.304	14.483	41.798	80.301	162.816	321.171	637.927	975.323	1247.954	1604.145
% Nominal	103.75	90.47	104.44	100.33	101.71	100.32	99.63	101.60	97.50	100.26

Table 4: Matrix effect of ritonavir.

J-0262014	*Low QC*				*High QC*			
Matrix Lot. No.	*Analyte area*	*Internal Standard area*	*Ratio*	*IS Normalized Matrix Factor*	*Analyte area*	*Internal Standard area*	*Ratio*	*IS Normalized Matrix Factor*
BLANK (CD2-P040412-658)-01	9361	378571	0.02	0.97	546496	377220	1.45	0.97
BLANK (CD2-P040412-663)-02	9959	381151	0.03	1.03	571080	363232	1.57	1.05
BLANK (CD2-P040412-664)-3	9833	384834	0.03	1.01	570178	380073	1.50	1.00
BLANK (CD2-P040412-665)-4	9593	379239	0.03	1.00	558535	367635	1.52	1.01
BLANK (CD2-P040412-666)-5	9674	385346	0.03	0.99	567658	379097	1.50	1.00
BLANK (CD2-P040412-667)-6	9580	388514	0.02	0.97	565793	371408	1.52	1.02
BLANK (CD2-P060911527(Lipemic)	9332	372907	0.03	0.99	552759	364355	1.52	1.01
BLANK (CD2-P060911-528(Hemolysed)-8	9786	391728	0.02	0.98	560626	378688	1.48	0.99
	Mean			0.992	Mean			1.005
	SD			0.0188	SD			0.0239
	% CV			1.90	% CV			2.38
	N			8	N			8

standards' concentration will be based on the estimated concentration range in the research and will be displayed on the calibration curve. To get the optimal fit for the concentrations/responses association, a weighting factor of 1/X2 will be chosen for the linear equation. The calibration curve that represents the regression analysis. The correlation coefficient for ritonavir was higher than 0.99 (Figure 5), within the range of 8.004 to 1600.001 ng/mL.

Accuracy and precision

Precision and accuracy within and between batches were determined by examining at least three bioanalytical batches. The concentrations of the QC samples will be calculated based on the relevant calibration curve. The precision and accuracy within a batch were assessed by calculating the coefficient of variation (CV%) and the percentage of nominal value (%Nominal) at each concentration level of QC samples in a bioanalytical batch. The assessment of accuracy and precision across batches and numerous days will be conducted by computing the coefficient of variation (CV%) and the percentage of nominal value (%Nominal), respectively. This analysis will be performed for each quality control (QC) concentration level in all bioanalytical batches conducted on different days, as shown in Tables 5 and 6.

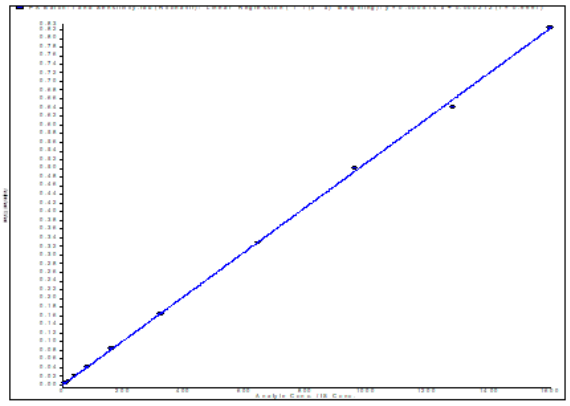

Figure 5: Ritonavir calibration curve

Recovery

Six sets of quality control samples (LQC, MQC2, and HQC) were either taken out from the deep freezer and thawed or prepared afresh. The extracted samples underwent processing

Table 5: Ritonavir within batch accuracy and precision

	Nominal concentrations(ng/ml)									
	LLOQQC		*LQC*		*MQC 1*		*MQC 2*		*HQC*	
QC	*8.035*	*%Accuracy*	*23.772*	*%Accuracy*	*104.72*	*%Accuracy*	*805.542*	*%Accuracy*	*1388.865*	*%Accuracy*
1	7.412	92.24	21.331	89.73	103.324	98.67	777.048	96.46	1370.606	98.69
2	6.710	83.51	21.012	88.39	107.105	102.28	794.689	98.65	1354.029	97.49
3	7.576	94.29	21.727	91.40	97.491	93.10	746.078	92.62	1331.143	95.84
4	7.362	91.62	21.170	89.05	98.006	93.59	772.439	95.89	1334.352	96.07
5	7.422	92.37	20.670	86.95	100.470	95.94	748.573	92.93	1358.013	97.78
6	7.284	90.65	21.923	92.22	102.058	97.46	760.992	94.47	1361.645	98.04
Mean	7.2943		21.3055		101.4090		766.6365		1351.6313	
S.D.	0.30186		0.46216		3.58736		18.49223		15.65333	
C.V.%	4.14		2.17		3.54		2.41		1.16	
% Nominal	90.78		89.62		96.84		95.17		97.32	
N	6		6		6		6		6	
7	7.939	98.81	21.936	92.28	97.399	93.01	773.346	96.00	1381.508	99.47
8	6.838	85.10	21.207	89.21	100.228	95.71	780.188	96.85	1317.058	94.83
9	7.411	92.24	21.533	90.58	99.895	95.39	795.940	98.81	1318.894	94.96
10	7.015	87.31	20.226	85.08	102.146	97.54	791.703	98.28	1342.891	96.69
11	6.926	86.20	21.570	90.74	99.860	95.36	774.108	96.10	1337.445	96.30
12	7.465	92.91	20.726	87.19	98.439	94.00	756.901	93.96	1406.484	101.27
Mean	7.2657		21.1997		99.6612		778.6977		1350.7133	
S.D.	0.45488		0.62656		1.62487		14.09580		35.89169	
C.V.%	6.26		2.96		1.63		1.81		2.66	
% Nominal	90.43		89.18		95.17		96.67		97.25	
N	6		6		6		6		6	
13	7.365	91.66	22.250	93.60	100.217	95.70	756.925	93.96	1331.617	95.88
14	7.447	92.68	20.518	86.31	98.465	94.03	783.925	97.32	1339.256	96.43
15	6.984	86.92	21.011	88.38	99.931	95.43	766.844	95.20	1307.895	94.17
16	7.569	94.20	21.449	90.23	98.767	94.32	785.477	97.51	1302.359	93.77
17	7.583	94.38	21.226	89.29	98.907	94.45	791.338	98.24	1315.442	94.71
18	8.183	101.85	20.072	84.44*	99.221	94.75	751.403	93.28	1301.500	93.71
Mean	7.5218		21.0877		99.2513		772.6520		1316.3448	
S.D.	0.39053		0.75666		0.68802		16.57298		15.78827	
C.V.%	5.19		3.59		0.69		2.14		1.20	
% Nominal	93.61		88.71		94.78		95.92		94.78	
N	6		6		6		6		6	

by including the internal standard and then injecting them. About 18 matrix samples, obtained from a single lot and devoid of any substances, were subjected to processing. These samples were then mixed with six sets of QC dilutions at low, middle, and high concentrations, as well as an internal standard. This was done to simulate the complete extraction of the analyte(s) in the samples, resulting in non-extracted samples. All duplicates of unprocessed material were injected. The mean answer, standard deviation (SD), and coefficient of variation (CV%) were computed.

Stability studies

An internal standard and standard analyte solution was made. For the purpose of refrigerated stock solution stability, appropriate portions of the solutions were chilled to between 2 and 8°C and then kept in the fridge. 1) To determine how

Table 6: Ritonavir intraday batch accuracy and precision

	Nominal concentrations(ng/ml)									
	LLOQQC		*LQC*		*MQC 1*		*MQC 2*		*HQC*	
QC	*8.035*	*% Accuracy*	*23.772*	*% Accuracy*	*104.72*	*% Accuracy*	*805.542*	*%Accuracy*	*1388.865*	*%Accuracy*
1	7.412	92.24	21.331	89.73	103.324	98.67	777.048	96.46	1370.606	98.69
2	6.710	83.51	21.012	88.39	107.105	102.28	794.689	98.65	1354.029	97.49
3	7.576	94.29	21.727	91.40	97.491	93.10	746.078	92.62	1331.143	95.84
4	7.362	91.62	21.170	89.05	98.006	93.59	772.439	95.89	1334.352	96.07
5	7.422	92.37	20.670	86.95	100.470	95.94	748.573	92.93	1358.013	97.78
6	7.284	90.65	21.923	92.22	102.058	97.46	760.992	94.47	1361.645	98.04
7	7.939	98.81	21.936	92.28	97.399	93.01	773.346	96.00	1381.508	99.47
8	6.838	85.10	21.207	89.21	100.228	95.71	780.188	96.85	1317.058	94.83
9	7.411	92.24	21.533	90.58	99.895	95.39	795.940	98.81	1318.894	94.96
10	7.015	87.31	20.226	85.08	102.146	97.54	791.703	98.28	1342.891	96.69
11	6.926	86.20	21.570	90.74	99.860	95.36	774.108	96.10	1337.445	96.30
12	7.465	92.91	20.726	87.19	98.439	94.00	756.901	93.96	1406.484	101.27
Mean	7.2800		21.256		100.531		772.661		1351.173	
S.D.	0.3487		0.5271		2.80764		16.8946		26.40373	
C.V.%	4.78		2.48		2.79		2.19		1.95	
% Nominal	90.60		89.40		96.00		95.92		97.29	

Table 7: Recovery of ritonavir from human plasma

J-0262014	*LQC*		*MQC2*		*HQC*	
	Extract LQC	*Non-extract LQC*	*Extract MQC2*	*Non-extract MQC2*	*Extract HQC*	*Non-extract HQC*
1	8010	9324	288412	325832	490730	551639
2	8312	9434	289804	320983	501094	544362
3	8122	9379	293019	325407	477136	539269
4	8349	9689	283413	321820	476223	545032
5	8529	9288	297922	319163	497512	561276
6	8531	9418	281209	324972	488938	531205
Mean	8308.8	9422.0	288963.2	323029.5	488605.5	545463.8
SD.	211.50	142.03	6142.52	2752.48	10244.88	10307.75
C.V.(%)	2.55	1.51	2.13	0.85	2.10	1.89
N	6	6	6	6	6	6
% Recovery	88.19		89.45		89.58	

stable the solution was after being placed on the bench for 6 hours while making the stock dilution, we tested the stability of room temperature stock solutions using the remaining volume of the stock solution. 2) After six sets of LQC and HQC were removed from the deep freezer, they were stored at the right conditions for 12 hours without processing. Newly spiked standards for the calibration curve and quality control samples (Low, Middle, and High QC) were processed and analyzed with the bench-top stability samples. The injection during the suggested stability period was made possible by processing six sets of LQC and HQC samples. The autosampler was set to the appropriate temperature to hold the processed samples. Along with newly spiked calibration curve standards and quality control samples, the stability QC samples were injected on the

day of stability. 4) Along with newly spiked calibration curve standards and quality control samples, six sets of long-term stored quality control samples (LQC and HQC) were taken out of the deep freezer and processed on the day of assessment. Five, six sets of QCs (LQC and HQC) and one set of calibration curves were processed and injected. Data from the calibration curve was used to recalculate the QC concentration. After the first injection of the QC (LQC and HQC) sample, it took some time for the quality control samples to be re-injected. The concentration of the re-injected QCs was determined by referring back to the data from the original calibration curve. The stability statistics were all within the specified range.

CONCLUSION

All of the tests included in this report including those for selectivity, matrix effect, linearity, precision and accuracy, stability, recovery, dilution integrity, and concomitant drug effect fell within the acceptable range for the bio-analytical batches specified by Piramal Clinical Research. Ritonavir (parent) and internal standard saquinavir (product) may be detected with m/z - 721.30 and 296.10, respectively, in human plasma within the concentration range of 8.004 to 1600.001 ng/mL, according to the aforementioned analytical procedure.

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