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METHOD VALIDATION FOR QUANTIFICATION OF LOPINAVIR IN HUMAN PLASMA BY USING LC-MS/MS

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ABSTRACT

A satisfactory LC-MS/MS technique was designed and validated in accordance with FDA standards for the quantification of lopinavir in plasma samples employing verapamil as IS. The designing of LC-MS/MS technique utilized a variety of commonly accessible UPLC columns and different mobile phases. Optimal separation was achieved when Waters X bridge C_{18} column (100 mm x 2.1 mm ID, 3.5 µm) was utilized along with mobile phase comprising of 2 mM AFB with 0.1% formic acid: ACN in the proportion of 20:80, v/v at 0.3 mL/min. ESI-MS at +ve ion mode was employed for detecting the sample. For quantitation, the MRM ratios for lopinavir (*m/z* 629.85 to 183.26) and verapamil (*m/z* 455.49 to 165.04) were employed. Verapamil and lopinavir each had retention times of 1.01 and 1.85 minutes, correspondingly. The pearson correlation (r=0.997) was used to confirm linearity for lopinavir around the concentration ranging between 2 ng/mL and 1000 ng/mL, and the total recoveries were 98.28% and 96.47% for lopinavir and verapamil, correspondingly. The presented approach also has a shorter analytic run time than earlier published methods, which is a benefit. As a result, the technique is appropriate for Lopinavir estimation.

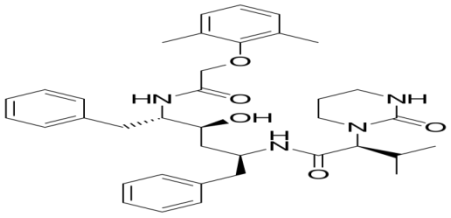
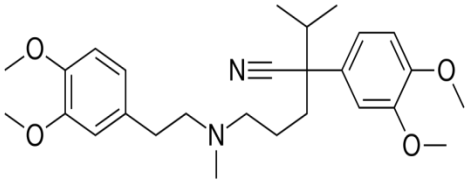
KEY WORDS: Lopinavir, Verapamil, LC-MS/MS, Positive ion mode, internal standard.

INTRODUCTION

Human immunodeficiency virus (HIV) damages the body's immune culminating in acquired immunodeficiency syndrome (AIDS), a persistent and potentially fatal disorder which renders the individual incapable of defending against pathogenic microbes. HIV attacks key immune system components including dendritic cells, helper T cells, and macrophages[1]. In order to solve such issues, an oral treatment of Lopinavir was initiated for the treatment of HIV-1. Clinical studies show that lopinavir treatment method effectively inhibits HIV-1 with tolerability, effectiveness, rapidity, and intensity. Lopinavir has therefore been given clinical approval for AIDS therapy. Employing verapamil as an internal standard (IS), an effort has been undertaken in this paper to establish and verify a new LC-MS/MS technique for the quantification of

lopinavir in plasma samples. According to a survey of the literature, there are relatively limited techniques that have been published for quantifying lopinavir both alone and in combination with other medications by HPLC[2-8], UV-Spectrophotometric [9-13] and IR- Spectrophotometric [14]. Moreover, no research has been conducted for the LC-MS/MS approach to quantify lopinavir in plasma samples. As a result, the researcher has put forth the effort to establish a quick and accurate method in bio-analytical labs to determine the presence of lopinavir in plasma samples. The LC-MS/MS method was effectively used to deliver acceptable levels of selection and sensitivity in a convenient amount of chromatographic run time. Chemical names and structures of Lopinavir and Verapamil (Internal standard) are tabulated in Table 1.

Table 1: Structures of Lopinavir and Verapamil

Official Name	IUPAC Name	Structure
Lopinavir	"(2S)-N-[(2S,4S,5S)-5-[2-(2,6-dimethylphenoxy)acetamido]-4-hydroxy-1,6-diphenylhexan-2-yl]-3-methyl-2-(2-oxo-1,3-diazinan-1-yl)butanamide"	
Verapamil (Internal standard)	"(RS)-2-(3,4-Dimethoxyphenyl)-5-{[2-(3,4-dimethoxyphenyl)ethyl]-(methyl) amino}-2-prop-2-ylpentanenitrile"	

MATERIALS AND METHODS

Instruments used

A LC-MS/MS procedure was carried out using a LC device consisting of a Waters Acquity UPLC device applied to a Waters Quattro Premier XE mass spectroscope employed for measurement and Mass Lynx 4.1 SCN 805 software for computation and information collection. The solid phase is a 100 mm x 2.1 mm ID, 3.5 µm Waters X bridge C18 column. The investigation makes use of a semi-micro-electronic balance (India), Filter paper (Whattman-41), and an ultrasonicator (Frontline FS 4, Mumbai, India).

Chemicals

Lopinavir and Verapamil (Internal Standard) were acquired from Chitra Labs, Hyderabad, India. ACN and MeOH were procured from JT Baker. Milli-Q Water was obtained from Millipore. Ammonium Formate was procured from Fischer chemical and formic acid was procured from Merck, India. Dimethyl Sulfoxide of HPLC grade were procured from Honey well.

Mobile phase preparation

The necessary buffer content of 2mM Ammonium Formate buffer (AFB) comprising 0.1% formic acid was obtained by mixing together, 126 mg of ammonium formate, 500 ml of Milli-Q

Water, and 1 ml of formic acid. This was followed by adding of more water to bring the volume to 1 liter, degassing in an ultrasonicator, and filtering with the help of a 0.45 µm. The resultant solution was added to ACN in the proportion of 20:80 v/v, followed by filtering and degassing.

Bio-analytical conditions

The separation was conducted by employing the above-prepared mobile phase at a flow rate of 0.3 ml/min. Detection was carried out with the help of atmospheric pressure ESI-MS at +ve ion mode.

MS conditions

The LC-MS/MS analysis was performed using Acquisition duration of 3.0 min, Polarity is Positive, Scan Time was 200 milli seconds (for each MRM), Resolution of Q1Unit and Q3 Unit. Lopinavir Detection of Q1 Mass was 629.85 and Q3 mass was 183.26 and Verapamil Detection of Q1 Mass was 455.49 and Q3 mass was 165.04, Can vary by ± 0.5 Mass Units. Source/Gas parameters (positive mode) API 4000, Gas 1(GS1):45.00 (Psi), Gas 2(GS2):30.00 (Psi), Curtain Gas (CUR): 25.00 (Psi), Collision Gas (CAD):18.00 (Psi), Ion Spray Voltage (IS):4000.00, Temperature (^{0}C):400.00, Interface Heater: Switched on.

Table 2: Variables for MS tuning of Lopinavir

Variables	Lopinavir
De clustering Potential (DP)	70.00 V
Entrance Potential (EP)	20.00 V
Collision Energy (CE)	18.00 V
Collision Cell Exit Potential (CXP)	15.00 V

Plasma samples

Human blood samples were drawn and placed in microfuges comprising K2-EDTA for preparing plasma samples. The supernate from every tube was gathered in another tube after centrifuging them for 15 minutes at 4000 rpm. 1 mL of ACN was dissolved in the supernate, which was subsequently maintained for 10 min to allow precipitation of membrane proteins. The supernate was then retrieved for future application.

Procedure for sample preparation

Step 1: Plasma blank and QC samples were taken out of the refrigerator and left for defrosting. To verify that the samples' components were well mixed, they were vortexed.20 µL of 50%

MeOH in H_2O were added, and the vial was given a blank label. 20 µL of ISTD (Combined ISTD with around 2 µg of Verapamil) to the vials that had already been labelled (excluding the

blank), next100 µL of sample from the allocated samples was shifted to the vials and mixed thoroughly.

Step 2: 0.250 mL of ACN was added to above mixture, agitated, centrifuged at 4000 rpm, 20℃, and the supernate was collected in auto-injector vials with a volume of 150 µL;10 µL of this solution was loaded into the column.

Preparation of calibration curve standards and QC samples

Eight differentconcentrations of lopinavir, varying between2-1000 ng/mL, were used to produce the calibration curve ofstandard. LLOQQCsamples for lopinavir were obtained at2 ng/mL, moderatequality QCsamples for lopinavir were obtained at 480 ng/mL, inferior quality QCsamples at 6 ng/mL, and superiorquality QC samplesat 780 ng/mL. Before usage, thesamples were kept at -70 ±10℃. To test for stability, 12 batches of LQC and HQC samples were kept at -20 °C ± 5 °C.

Chromatographic and Mass spectrometric parameters: Equipment with Waters Acquity UPLC system coupled with a Quattro Premier XE mass spectrophotometer with electrospray ionization (ESI) and Mass Lynx 4.1 SCN 805 software, Column used was Waters X bridge C_{18} column (100 mm x 2.1 mm ID, 3.5 µm), Mobile Phase of 2 mM Ammonium formate buffer with 0.1% formic acid were mixed with HPLC grade Acetonitrile in the proportion of 20:80, v/v, Flow rate of 0.3 mL/min**,** Run time was 3 minutes**,** Splitness of 10:90, Column oven temperature was 30±2ºC, Auto sampler temperature was10ºC, Injection volume of 10 µL, Retention time of Lopinavir was 1.85 minutes, Retention time of Verapamil was 1.01 minutes.

RESULTS AND DISCUSSION

Method optimization

A variety of mobile phase proportionsand factors were examined for the LC-MS/MS technique's optimization. Using a Waters X bridge C18 column (100 mm x 2.1 mm ID, 3.5 m) and solventphase comprisingof 2 mM AFB with 0.1% formic acid:ACNin20:80, v/v, whichwas suppliedat0.3 mL/min by positive ionization(API 4000), optimum separation and excellent peaks were obtained. With aninjection volume of 10 µL, the overall run time was reported to be 3 min.Atmospheric pressure ESI-MSwasused for detecting the drug. Figures 1 and 2 depict the quantification utilized for the precursor to product ion transformations, which are m/z 629.85 to 183.26and m/z 455.49 to 165.04 for lopinavir andverapamil, correspondingly, at RTs of 1.01 and 1.85 minutes, correspondingly. Figure 3 depicts a representative chromatogram of blank plasma, Lopinavir, and Verapamil.

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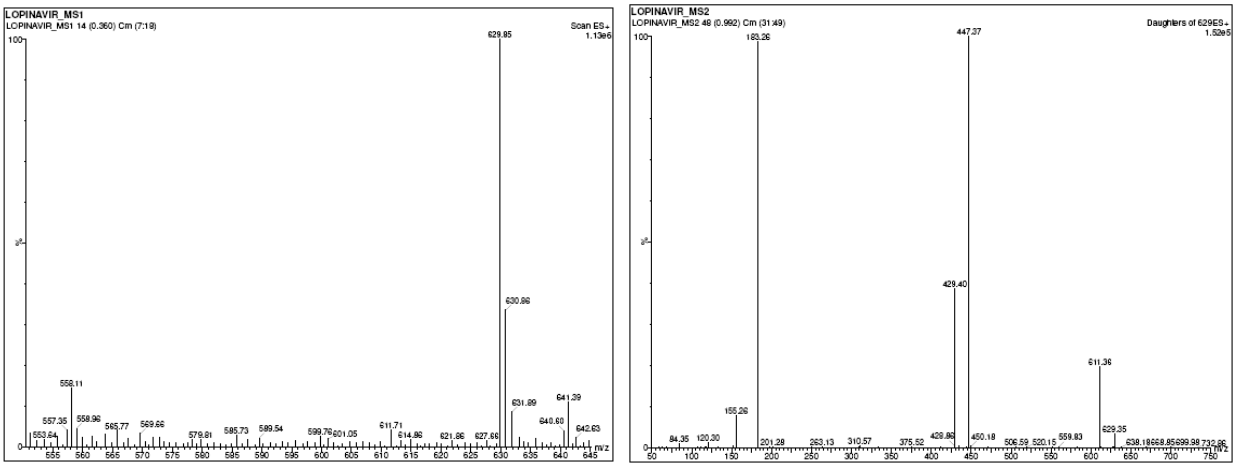

Fig. 1- Mass spectra of Lopinavir for precursor MS1 and product ion masses MS2

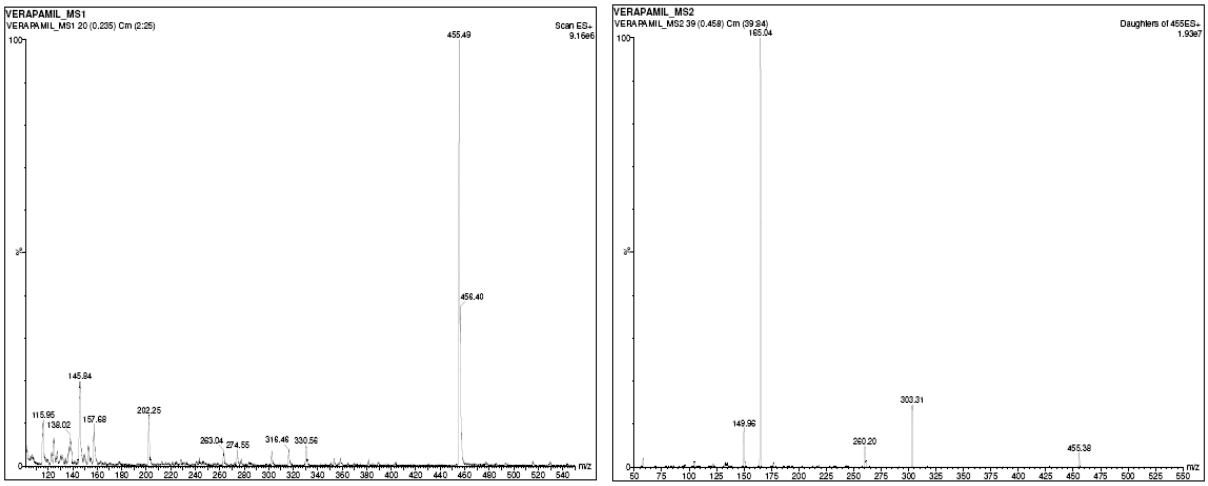

Fig. 2- Mass spectra of Verapamil IS for precursor MS1 and product ion masses MS2

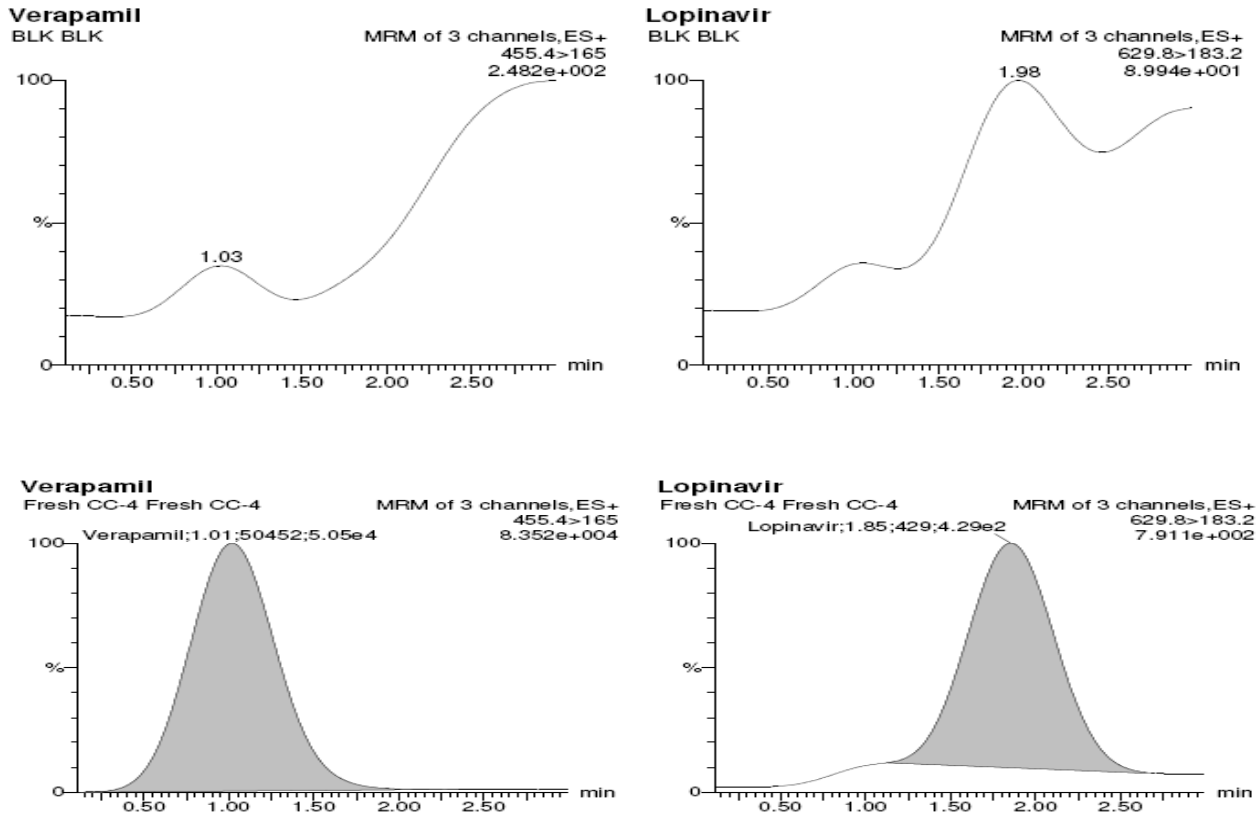

Fig. 3-Chromatogram of blank plasma, Lopinavir and Verapamil

Method validation

The proposed LC-MS technique was verified for selectivity, accuracy, specificity, linearity, recovery, precision, sensitivity, stability, and carry over test in conformity with the guidelines of the FDA recommendations[15].

Sensitivity

The value of 2 ng/mL was chosen as the LLOQ for lopinavir. At this level, lopinavir's precision and accuracy were calculated. The mass transformations of lopinavir and verapamil were not significantly impacted by intrinsic constituents.

Linearity

Seven concentrations (2, 4, 25, 100, 500, 800, and 1000 ng/mL) were used to test the linearity of the drug in human plasma. Peak: area values for every solution was evaluated against their respective concentrations to produce the calibration curve. Figure 4 illustrates the calibration curve, which was linear for Lopinavir in the concentration range from 2 ng/mL - 1000 ng/mL. The least squares method's straight-line fitting over the datasets revealed a consistent relation with little data distribution. According to Table 3, the correlation coefficient (r) for lopinavir was 0.994.

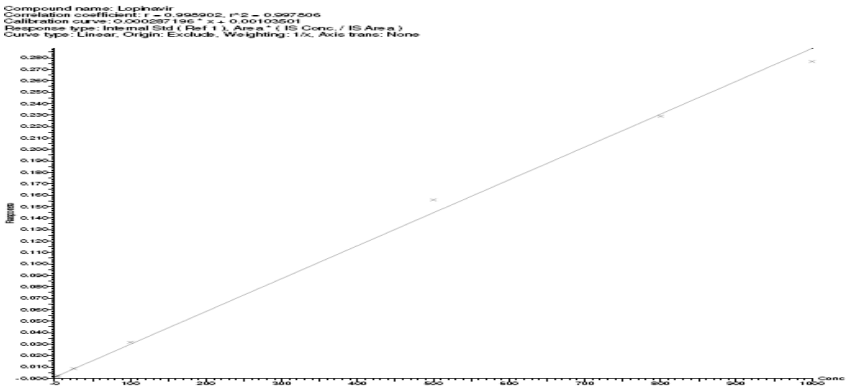

Figure 4: Representative calibration curve for regression analysis of Lopinavir

Table 3: Linearity of Lopinavir

Conc. of Lopinavir (nanogram/mL)	**Peak:Area (Sample area/IS area)**
2	0.002
4	0.002
25	0.009
100	0.032
500	0.156
800	0.229
1000	0.277

Extraction recovery

24 blank samples were processed and 6 batches of each blank sample were diluted with the aqueous QC dilutions at low, middle, and high concentrations lacking internal standards, representing 100% extraction of the analyte(s) (non-extracted samples). IS solution was used for diluting six blanks, representing 100% IS extraction (Non-extracted sample). The samples that weren't removed were injected. Comparative analyses were conducted between the extracted samples of LQC, MQC, and HQC from PA Batch-I and the recovered comparison samples of lopinavir (Precision and accuracy). The IS's response to the recovered comparison samples was contrasted with that of the IS at the MQC level.

$$Extraction\ recovery\ (R\ \%) = \frac{P_b}{P_a}\ x\ 100$$

Where, P_b is the mean peak area responses of plasma samples before extraction and P_a is the mean peak area responses of plasma samples after extraction.

Recoveries for Lopinavir and Verapamil were estimated to span around 97.97%-98.17% and 96.47%, respectively. The overall recovery for Lopinavir was 98.28 %. The findings for recovery studies of Lopinavir and Verapamil tabulated in Table 4 and 5 respectively.

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Table 4: Recovery of Lopinavir from plasma

	LQC Responses		**MQC Responses**		**HQC Responses**	
	Ext. QC	**Non-Ext. QC**	**Ext. QC**	**Non-Ext. QC**	**Ext. QC**	**Non-Ext. QC**
ID	LQC (07-12)	LQC (1-6)	MQC (07-12)	MQC (1-6)	HQC (07-12)	HQC (1-6)
1	180	190	7938	7922	12489	12477
2	179	183	7414	7404	12309	12319
3	178	188	8270	8273	12158	12178
4	176	184	8065	7695	12239	12229
5	179	181	7822	7868	12158	12178
6	181	175	7612	7645	12152	12168
Mean	179	184	7854	7801	12251	12258
SD	1.722	5.320	309.134	294.990	132.099	121.180
CV%	0.963	2.899	3.936	3.781	1.078	0.989
N	6	6	6	6	6	6
Recovery%	97.97		98.71		98.17	
Total recovery%	98.28					

Table 5: Recovery of Verapamil from plasma

Ext. QC ID	**IS Response in Ext. Samples (Area)**	**Non-Ext. QC ID**	**IS Response in Non-Ext. Samples (Area)**
MQC-7	58033	Non Ext.-MQC-1	52281
MQC-8	60428	Non Ext.-MQC-2	52136
MQC-9	53040	Non Ext.-MQC-3	52005
MQC-10	50452	Non Ext.-MQC-4	52095
MQC-11	52728	Non Ext.-MQC-5	53563
MQC-12	48825	Non Ext.-MQC-6	51729
Mean	53918	**Mean**	52302
SD	4459.868	**SD**	644.593
CV%	8.27	**CV%**	1.23
N	6	**N**	6
Recovery%	96.47		

Accuracy and precision

By examining six repetitions at 4 distinct QC levels in two trials on a single day, it was possible to assess the accuracy and precision of the intra assay. The analysis of six repetitions on five distinct runs was used to assess the inter-assay precision and accuracy. With the exception of

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LLOQ QC (20%), the acceptability standards include accuracy below 15% deviation (SD) as well as precision below 15% RSD.

Within-batch precision and accuracy

Intra-batch precision and accuracy for LLOQ QC varied between 1.41%-1.54% and 101.92%-102.5%, whereas precision varied between 0.52% - 6.29% for LQC, MQC, and HQC, and accuracy spanned between 98.31%-104.56%. Table 6 displays the precision and accuracy data for Lopinavir.

Table 6: Within-batch precision and accuracy for Lopinavir

QC	Conc. (ng/mL)			
	LLOQ QC	**LQC**	**MQC**	**HQC**
	2	**6**	**480**	**780**
1	2.05	6.02	505.73	811.50
2	2.07	6.03	535.70	766.09
3	2.08	6.00	543.34	830.22
4	2.04	5.80	472.69	732.33
5	2.03	5.90	478.14	789.60
6	2.00	6.03	475.65	796.70
Mean	2.05	5.96	501.88	787.74
SD	0.03	0.09	31.56	34.62
CV%	1.41	1.57	6.29	4.39
Nominal %	102.5	99.33	104.56	100.99
N	6	6	6	6
7	2.02	6.03	482.52	766.64
8	2.07	5.88	483.73	801.23
9	2.08	6.03	474.11	784.62
10	2.03	5.69	475.62	761.33
11	2.01	5.83	481.34	779.62
12	2.02	5.93	479.53	806.73
Mean	2.04	5.90	479.48	783.36
SD	0.03	0.13	3.86	18.14
CV%	1.44	2.20	0.81	2.32
Nominal %	101.92	98.31	99.89	100.43
N	6	6	6	6
13	2.02	6.07	481.51	782.61
14	2.07	5.98	479.72	782.22
15	2.02	5.96	484.12	774.49
16	2.01	6.02	473.64	783.21
17	2.07	5.97	472.36	780.24
18	2.08	6.04	481.50	786.72
Mean	2.05	6.01	478.81	781.58
SD	0.03	0.04	4.73	4.06
CV%	1.54	0.73	0.99	0.52
Nominal %	102.25	100.11	99.75	100.20
N	6	6	6	6

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Intra-day precision and accuracy

LLOQ QC had an intraday precision of 1.467% and accuracy of 101.88%, whereas LQC, MQC, and HQC had a precision spanning around 0.807% to 2.248% and accuracy ranging from 98.99% to 99.87%. Table 7 displays the outcomes of the intraday precision and accuracy for lopinavir.

Table 7: Between-batch precision and accuracy for Lopinavir

QC	Conc. (ng/mL)			
	LLOQ QC	**LQC**	**MQC**	**HQC**
	2	**6**	**480**	**780**
1	2.04	5.80	472.69	732.33
2	2.03	5.90	478.14	789.60
3	2.00	6.03	475.65	796.70
4	2.08	6.03	474.11	784.62
5	2.03	5.69	475.62	761.33
6	2.01	5.83	481.34	779.62
7	2.01	6.02	473.64	783.21
8	2.07	5.97	472.36	780.24
9	2.08	6.04	481.50	786.72
10	2.07	5.98	479.72	782.22
11	2.02	5.96	484.12	774.49
12	2.01	6.02	475.87	796.81
Mean	2.04	5.94	477.06	778.99
SD	0.030	0.112	3.852	17.514
CV%	1.467	1.884	0.807	2.248
Nominal %	101.88	98.99	99.39	99.87
N	12	12	12	12

Between batch/inter-day precision and accuracy

For LLOQ QC, between-batch precision and accuracy were 1.291% and 102.3%, respectively, while for LQC, MQC, and HQC, precision spanned around 1.622% to 3.578%, and accuracy ranged from 99.1% to 100.3%. Table 8 shows the inter batch/day accuracy and precision values for Lopinavir.

Table 8: Inter batch/day precision and accuracy for Lopinavir

QC	Conc. (ng/mL)			
	LLOQ QC	**LQC**	**MQC**	**HQC**
	2	**6**	**480**	**780**
1	2.08	6.04	481.50	786.72
2	2.07	5.98	479.72	782.22
3	2.01	6.02	473.64	783.21
4	2.07	5.97	472.36	780.24
5	2.01	6.02	473.64	783.21
6	2.07	5.97	472.36	780.24
7	2.02	5.98	479.72	782.22
8	2.08	6.03	474.11	784.62
9	2.04	5.80	472.69	732.33
10	2.03	5.90	478.14	789.60
11	2.08	6.00	543.34	830.22
12	2.04	5.80	472.69	732.33
13	2.03	5.90	478.14	789.60
14	2.05	6.02	505.73	811.50
15	2.07	5.98	479.72	782.22
16	2.03	5.69	475.62	761.33
17	2.02	5.93	479.53	806.73
18	2.01	6.02	473.64	783.21
Mean	2.05	5.95	481.46	782.32
SD	0.026	0.096	17.225	23.416
CV%	1.291	1.622	3.578	2.993
Nominal %	102.3	99.1	100.3	100.3
N	18	18	18	18

Stability

6 repetitions of two QC samples, at high and low concentrations, were used to test lopinavir's stability in plasma. Samples were produced by adding the adequate quantities of standard Lopinavir solutions to drug-free plasma. The stability was assessed by taking into account various parameters including freeze-thaw stability, re-injection stability, refrigerated stock/spiking solution stability, wet-extract stability, bench top stability, and room temperature stock/spiking solution stability. In order to assess the analyte's stability in human plasma under various circumstances, stability studies were carried out. By contrasting the area response of samples with that of samples produced from freshly made stock solution, the stock solution stability under ambient temperature and refrigeration (2-8 °C) were determined. Six duplicates were used for each of the following tests: plasma sample stability, processed sample stability, wet extract stability (30 h), bench top stability (6 h), reinjection stability (24 h), and freeze thaw stability (four cycles). If test results fell inside the permitted ranges for accuracy (≤15% SD) and 0precision (≤15% RSD), samples were deemed to be stable. A drug's quality must be sustained

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during the preparation and preservation of clinical samples, or at the very least, pre-analysis variance must be kept to a minimum. Stability testing are crucial in designing a bio-analytical approach because of this fact. In the present investigation, the stability was assessed by taking into account various parameters including freeze-thaw stability, re-injection stability, refrigerated stock/spiking solution stability, wet-extract stability, bench top stability, and room temperature stock/spiking solution stability. Because the acceptability standards (variance readings for area below 15%) were always fulfilled in all circumstances, the findings demonstrate that lopinavir is robust under the investigated conditions.

Room temperature stock solution stability

With a precision ranging between 2.37% and 3.13%, Lopinavir's stability was estimated to be 99.43%, while that of Verapamil's was 101.1% with a precision ranging between 2.24% - 5.78%. Table 9 displays the stock solution stability data at ambient temperature.

Table 9: Room temperature stock solution stability of Lopinavir and Verapamil

S. No.	**Lopinavir Peak Area**		**Verapamil Peak Area**	
	0 hr	**6 hr**	**0 hr**	**6 hr**
1	7922	8153	57661	60428
2	8065	8016	55057	59673
3	8270	8288	57536	61759
4	7822	7935	62136	59714
5	7612	8174	63473	63185
6	7916	7808	62733	61474
Mean	7937	8044	59766	61039
SD	248.28	190	3456.60	1364.39
CV %	3.13	2.37	5.78	2.24
Stability %	99.43		101.1	

Refrigerated stock solution stability (at 2-8 °C)

It was discovered that the stock solution remained stable for 4 days. Table 10 displays the stock solution stability of lopinavir and verapamil, which were determined to be 101.3% and 101.2%, correspondingly.

Table 10: Refrigerated stock solution stability of Lopinavir and Verapamil

S. No.	Lopinavir		Verapamil	
	Stability standard stock	Comparison standard stock	Stability standard stock	Comparison standard stock
	Peak Area	Peak Area	Peak Area	Peak Area
1	262	267	57321	53435
2	250	243	55257	54611
3	249	251	57536	54366
4	257	285	53673	53252
5	211	244	52307	52095
6	241	267	51399	53563
Mean	245	260	54582	53554
SD	18.238	16.416	2561.923	895.625
CV %	7.4	6.3	4.7	1.7
N	6	6	6	6
Mean response of standard stock	252.236		54067.917	
Mean standard response	249		53435	
Response %	101.3		101.2	

Room temperature spiking solution stability

Lopinavir's stability was determined to be 100.1%, with a precision ranging between 8.13%–9%, while that of Verapamil was 99.45% with a precision ranging between 3.5%-5.4%. The findings of spiking solution stability at ambient temperature are displayed in Table 11.

Table 11: Room temperature spiking solution stability of Lopinavir and Verapamil

S. No.	Lopinavir Peak Area		Verapamil Peak Area	
	0 hr	6 hr	0 hr	6 hr
1	249	236	53040	50789
2	255	290	50452	54958
3	284	243	52728	51139
4	245	256	48825	57321
5	217	244	52095	55257
6	235	237	53563	57536
Mean	248	251	51784	54500
SD	22.287	20.396	1803.338	2933.462
CV %	9	8.13	3.5	5.4

Stability %	100.1	99.45

Refrigerated spiking solution stability of Lopinavir (at 2-8 °C)

It was discovered that the spiking solutions remained stable for 3 days. Table 12 displays the stability of Lopinavir at LQC concentration, which was determined to be 100.3%.

Table 12: Refrigerated spiking solution stability of Lopinavir at LQC level for 3 days

S. No.	**Stability standard spiking solution (LQC)**	**Comparison standard spiking solution (LQC)**
	6 ng/mL	**6 ng/mL**
1	6.02	5.21
2	6.03	5.98
3	6.61	6.00
4	6.56	5.97
5	6.13	5.98
6	5.70	6.03
Mean	6.17	5.86
SD	0.35	0.32
CV %	5.67	5.47
N	6	6
Response %	100.3	

Bench top stability

According to the established standards, lopinavir was demonstrated to be stable for a maximum of six hours. The precision varied between 5.47% - 8.01%, while the % mean nominal spanned around 97.69% - 101.8%. Table 13 shows the outcomes of the stability of the bench top.

Table 13: Bench top stability of Lopinavir

S. No.	**Conc. (ng/mL)**	
	LQC	**HQC**
	6	**780**
1	5.21	748.25
2	5.98	853.52
3	6.00	894.28
4	6.04	747.00
5	5.98	754.00
6	5.96	767.00
Mean	5.86	794
SD	0.32	63.61
CV %	5.47	8.01
Nominal %	97.69	101.8
N	6	6

Auto sampler stability

The findings show that the treated samples remained unchanged for about 32 hours. The precision varied between 0.69% - 4.97%, while the nominal % spanned around 99.3% - 102.6%. Table 14 displays the auto sampler stability findings.

Table 14: Auto sampler stability of Lopinavir

S. No.	Conc. (ng/mL)	
	LQC	**HQC**
	6	**780**
1	6.72	774.10
2	6.10	778.10
3	6.27	777.40
4	5.92	778.00
5	5.90	774.49
6	6.02	764.10
Mean	6.16	774.36
SD	0.31	5.33
CV %	4.97	0.69
Nominal %	102.6	99.3
N	6	6

Freeze-thaw stability

Table 15 displays the stability of lopinavir against freezing and thawing. During 4 freeze-thaw sessions, the nominal % varied between 98.8% - 99.3%, while the precision spanned between 1.64% - 2.82%.

Table 15: Freeze-thaw stability of Lopinavir

S. No.	**Conc. (ng/mL)**	
	LQC	**HQC**
	6	**780**
1	6.03	784.62
2	5.80	732.33
3	5.90	789.60
4	5.83	787.1
5	6.02	784.1
6	5.97	769.2
Mean	5.93	774.49
SD	0.10	21.85
CV %	1.64	2.82
Nominal %	98.8	99.3

N	6	6

Re-injection stability

The outcomes show that the samples that have been pumped back were stable for 24hrs. The accuracy spanned between 1.52% through 6.18% during that period, while the % stability extended between 99.84% through 100.47%. Findings of Lopinavir's re-injection stability are displayed in Table 16.

Table 16: Re-injection stability of Lopinavir

S. No.	**0 hours**		**24 hours**	
	Conc. (ng/mL)			
	LQC	**HQC**	**LQC**	**HQC**
	6	**780**	**6**	**780**
1	6.13	801.6	6.03	732.33
2	5.70	747.1	5.80	789.60
3	6.82	754.1	5.90	811.50
4	6.04	846.6	5.97	779.62
5	5.98	757.1	6.04	783.21
6	5.96	762.1	5.98	780.24
Mean	6.10	778.10	5.95	779.42
SD	0.38	38.67	0.09	25.94
CV %	6.18	4.97	1.52	3.33
Nominal %	101.7	99.8	99.2	99.9
N	6	6	6	6
Stability %			100.47	99.84

Wet-extract stability

Table 17 displays the findings for wet extract stability. The findings show that the treated samples remained stable around 30 hours. The accuracy varied between 1.54% through 3%, while the nominal % spanned between 98.7% through 99.7% during that period.

Table 17: Wet-extract stability of Lopinavir

S. No.	**Conc. (ng/mL)**	
	LQC	**HQC**
	6	**780**
1	5.83	784.62
2	6.02	732.33
3	5.97	789.60
4	6.00	798.6
5	5.80	779.62
6	5.90	783.21

Mean	5.92	778.00
SD	0.09	23.31
CV %	1.54	3.00
Nominal %	98.7	99.7
N	6	6

Matrix effect of Lopinavir

The plasma was drawn from twelve separate lots and is divided into two subsets of extracted blank samples, each having 6 tubes. Corresponding aqueous concentrations of LQC are used in one set, and corresponding aqueous concentrations of HQC in the other. The above samples were referred to as post-spiked samples. Alongside similar aqueous LQC and HQC samples, these samples are examined. The following equation is used to calculate the percent response ratio, which is used to assess the matrix effect.

$$Response\ ratio\ (\%) = \frac{\text{Mean area ratio of post spiked samples}}{\text{Mean area ratio of equivalent aqueous samples}}\ x\ 100$$

Both low (LQC) and high (HQC) levels of lopinavir did not cause any detectable matrix effects in any of the 8 sets, including those containing haemolysis and lipemic plasma. In Table 18, it was determined that lopinavir's precision and accuracy at LQC level were 0.59% and 101.8%, while at HQC concentration, they were 1.12% and 100.6%, correspondingly.

Table 18: Matrix effect of Lopinavir

Plasma	**LQC (6 ng/mL)**			**Mean**	**HQC (780 ng/mL)**			**Mean**
(Batch No.)	**1**	**2**	**3**		**1**	**2**	**3**	
1	6.09	6.08	6.02	6.06	781.6	772.6	776.6	776.9
2	6.17	6.07	6.19	6.14	781.1	761.1	769.1	770.4
3	6.13	6.09	6.17	6.13	791.6	799.6	796.6	795.9
4	6.07	6.18	6.18	6.14	783.2	772.1	787.1	780.8
5	6.09	6.03	6.22	6.11	794.1	774.1	799.1	789.1
6	6.31	5.99	6.09	6.13	806.6	771.6	786.6	788.3
(Lipemic)	6.01	6.11	6.17	6.10	797.1	787.1	797.1	793.8
Hemolytic)	5.98	6.09	6.08	6.05	782.1	774.1	784.1	780.1
Mean				6.11	**Mean**			784.42
SD				0.04	**SD**			8.78
CV %				0.59	**CV %**			1.12
Nominal %				101.8	**Nominal %**			100.6
N				8	**N**			8

CONCLUSION

The current LC-MS/MS technique was designed and validated in accordance with FDA standards for the quantification of lopinavir in plasma samples employing verapamil as IS. An experiment considered suitable for real-time assessment was designed adopting a quick and low-cost LLE technique and a gradient chromatographic setup which utilizes a RP column. The technique was designed to estimate Lopinavir levels in human plasma quickly and precisely.

Interference peaks were not discovered, and also the chromatographic peaks were well separated. The designing of LC-MS/MS technique utilized a variety of commonly accessible UPLC columns and different mobile phases. Optimal separation was achieved when Waters X bridge

C_{18} column (100 mm x 2.1 mm ID, 3.5 µm) was utilized along with mobile phase comprising of 2 mM AFB with 0.1% formic acid: ACN in the proportion of 20:80, v/v at 0.3 mL/min. ESI-MS at +ve ion mode was employed for detecting the sample. For quantitation, the MRM ratios for lopinavir (*m/z* 629.85 to 183.26) and verapamil (*m/z* 455.49 to 165.04) were employed. Verapamil and lopinavir each had retention times of 1.01 and 1.85 minutes, correspondingly. The pearson correlation (r=0.997) was used to confirm linearity for lopinavir around the concentration ranging between 2 ng/mL and 1000 ng/mL, and the total recoveries were 98.28% and 96.47% for lopinavir and verapamil, correspondingly. It was discovered that the CV% estimates for lopinavir were ≤ 15%, indicating the accuracy, stability, and precision of the suggested approach. Regarding selectivity, precision, linearity, recovery, accuracy, stability, and matrix effect assays, the LC-MS/MS technique for estimating lopinavir in plasma samples performed exceptionally well. The presented approach also has a shorter analytic run time than earlier published methods, which is a benefit. As a result, the technique is appropriate for Lopinavir estimation.

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