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A Liquid Chromatography Positive Ion Tandem Mass Spectrometry Method for the Determination of Saquinavir in Human Plasma

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ABSTRACT

A new, simple, high throughput and accurate LC-ESI-MS has been validated for determination of saquinavir in human plasma by using doxepine as internal standard. The analyte and internal standard were detected with no interference in multiple reaction monitoring (MRM) in positive mode on an electrospray ionization-mass spectrometer on a LUNA 3µ, C_{18} (2), 100A, 100 X 4.60 mm column using a mobile phase of a buffer: Acetonitrile (20:80, V/V). The flow rate was 1 ml/min at the column temperature 40 ±3 ºC. In these chromatographic conditions, the retention times of saquinavir and doxepine were found to be 1.37 min and 1.28 min respectively. The analytes were showed good linearity over a wide concentration range. The analyte and internal standard were stable in standard solution and in plasma samples under storage and processing conditions. The present work was fully developed and validated according to acceptance criteria for bioanalytical method validation.

Keywords: Saquinavir; Doxepine; electrospray ionization; Liquid-chromatography/mass spectrometry.

INTRODUCTION

Chemically, saquinavir is (2*S*)-*N*-[(2*S*,3*R*)-4-[(3*S*)-3-(*tert*-butylcarbamoyl)-decahydroisoquinolin -2-yl]-3-hydroxy-1-phenylbutan-2-yl]-2-(quinolin-2yl formamido) butanediamide is an antiretroviral drug. The structure of saquinavir is shown in Figure 1. It is available in market as brand name invirase [1]. Doses of saquinavir are 400 mg along with ritonavir 400mg twice daily for 28 days. Nausea, vomiting, diarrhea, abdominal discomfort, rhinitis and head ache are few side effects which occur commonly. Because of its low bioavailability 4% it is given in combination with other drugs like ritonavir or lopinavir for treating patients suffering from AIDS through oral dosage form by blocking protease active site and inhibits the activity of the enzyme. This prevents cleavage of the viral polyproteins results in the formation of immature non-infectious viral particles. It is metabolized by CYP3A4 and it is an inhibitor of CYP450. It is excreted through feces (81%) and urine (3%) [2].

The activity of these compounds is usually assayed by UV spectrophotometric method [3], stability-indicating high performance liquid chromatographic method with photodiode array (PDA) detection in presence of degradation products formed from forced degradation was successfully done [4], high performance liquid chromatography (HPLC) method with fluorescence detector with very small sample volume, much sensitive detection limit and at low operating cost in rat plasma [5], high-performance liquid chromatography method with ultraviolet detection in

human plasma [6], The effect of multiple doses of rifabutin on the pharmacokinetics of saquinavir-ritonavir was assessed in 25 healthy subjects [7], simultaneous quantitation of four protease inhibitors (amprenavir, lopinavir, ritonavir and saquinavir) and a non-nucleoside reverse transcriptase inhibitor efavirenz in human peripheral blood mononuclear cells using high-performance liquid chromatography–mass chromatography (LC/MS) [8], determination of the HIV protease inhibitor saquinavir in human plasma, saliva, and urine using liquid–liquid extraction and LC–MS–MS [9-11] has been published until now.

The aim of this present work is to develop and validate a simple, accurate and economically best method for determination of Saquinavir by using LC–MS/MS method in human plasma.

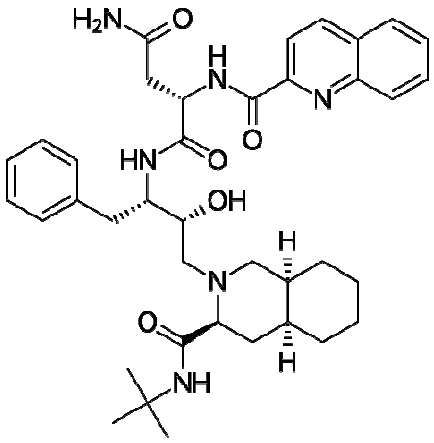

Figure 1: Chemical structure of saquinavir

MATERIALS AND METHODS

Reagents

Saquinavir reference standard and Doxepine internal standard were kindly provided by Varda Biotech Pvt. Ltd., Mumbai, India. HPLC grade Acetonitrile and HPLC grade tertiary butyl methyl ether obtained from Thermo Fisher Scientific India Pvt. Ltd., Mumbai, India. Ammonia solution 25%, Formic acid and Ammonium formate purchased from Merck Specialities India Pvt Ltd., Mumbai, India. HPLC grade water was obtained from Milli-Q water purifier system, Millipore Corporation, Milford, MA, USA.

Standard solutions

Standard solution of 500 ng/ml for both drug and internal standard was prepared in 85:15 V/V of acetonitrile and Water.

FINAL SAMPLE PREPARATION PROCEDURE

Extraction procedure:

100µl of plasma was transferred into pre-labeled sample tubes. To these 50.0 µl of 2 µg/ml ISTD solution was added except the STD Blank vial and vortexed for about 5 sec. 100.0 µl of extraction buffer was added to all the vials and vortex again for 5 sec and 2 ml of TBME was added to the all vials and vortex for a period of 10 min. All the vials were centrifuged at 4500 rpm for 5 min at 4°C. From the above solution, transfer 1.8 ml of supernatant solution into pre-labeled tubes and the solutions were evaporated to dryness under nitrogen at 40 ± 5 °C. 500.0 µl of reconstitution solution was added to all the tubes and vortex for about 1 min. Appropriate volumes of the reconstituted solution were transferred into pre-labeled auto sampler vials and 10 µl was injected into LC-MS/MS.

Procedure for Unextracted Sample Preparation:

20.0µl of respective spiking solutions were taken in pre-labelled tubes. 50.0 µl of 2 µg/ml ISTD solution was added and vortex to mix. 4.480 ml of reconstitution solution was added and vortex to mix. Appropriate volumes were transferred into pre-labeled auto sampler vials and 10µl was injected into LC-MS/MS.

CHROMATOGRAPHIC AND MASS SPECTROMETRY

Quattro Premier XE LC-MS/MS with Mass detector coupled with 2695 HPLC separation module by operating a software masslynx V 4.1 was used for analysis of sample compoud. The analytical column was a Silica based C18 column LUNA 3µ, C_{18} (2), 100A, 100 x 4.60 mm. Mobile Phase was prepared by mixing Ammonium formate buffer (pH 5.0) and Acetonitrile in the ratio of 20:80 V/V. Flow rate was 1 ml min^{-1} at 5 ± 3 ºC autosampler temperature. The retention time for analyte and ISTD were about at 1.37 min and 1.28 min respectively. Electrospray ionization was performed using nitrogen as desolvation gas at 900 flow rate, 50 cone gas flow rate and at desolvation temp 450 ^{o}C. Collision cell gas pressure was 3.5-4.5mbar. Other ion source parameters were: capillary voltage 3 kv; extractor 4.0V; RF lens 0.2V; source temperature 120 ^{o}C. MS analyzer parameters were: resolution 1:- 14 (LM) and 14 (HM); ion energy:- 1 1.0V; collision cell entrance potential 2.0V; collision cell exit potential 2.0V; resolution:- 2 14 (LM) and 14 (HM); ion energy :- 2 1.2V; multiplier 650V; Collision activated dissociation 10; Curtain Gas 25; Ion source gas 1 (Gas 1) 30; Ion source gas 2 (Gas 2) 40; Ion spray Voltage (IS) 5500; Temperature 450 ^{o}C; Pause time 5ms; Collision gas – Nitrogen; for saquinavir;- Dwell time 100ms; Declustering potential 100V; Collision energy 44,53; Collision cell exit potential 21V,15V; Entrance potential 10V respectively; for Doxepin;- Dwell time 100ms; Declustering potential 65V; Collision energy (CE) 37; Collision cell exit potential 6V; Entrance potential 10V respectively. The MRM transitions for analyte and internal standard were 671.5→ 570.3 & 416.1 (Saquinavir), *m/z* 280.4 → 106.9 (Doxepin) respectively.

VALIDATION

System suitability was done by injecting six injections using standard aqueous mixture equivalent to MQC concentration of the calibration curve.

In Carryover effect, checking the solution used to clean the injection needle having the capacity to avoid any carry forward of injected sample in the next runs.

Specificity was determined by comparing chromatograms of ten different samples of human plasma from different sources by using described procedures and conditions to confirm that there are no interfering peaks at retention time at LLOQ of analyte.

The Sensitivity of the method was evaluated by analyzing six LLOQ (1.055 ng/ml) for Saquinavir.

Matrix effect was performed by using six different blank plasma lots at LQC and HQC concentration in triplicate by calculating the % CV of accuracy and precision.

Calibration curves were obtained by plotting the peak area ratios of the saquinavir to the Doxepin versus the nominal concentration using linear regression analysis.

The limit of detection (LOD) was measured by signal to noise ratio (S/N) = 3 and limit of quantitation (LOQ) measured by signal to noise ratio (S/N) = 10.

The precision was evaluated by the % CV at different concentration levels corresponding to LLOQ QC, LQC, MQC2, MQC1 and HQC during the course of validation.

The accuracy of the assay was calculated as the absolute value of the ratio of the calculated mean values of the quality control samples to their respective nominal values, expressed as percentage.

Recovery for analyte, the % mean recoveries were determined by measuring the responses of the extracted plasma quality control samples against unextracted quality control samples at HQC, MQC1, MQC2 and LQC levels.

Recovery for internal standard, the % means recoveries were determined by measuring the responses of internal standard in the extracted samples against unextracted samples respectively.

The dilution integrity of the method was evaluated by diluting the stock solution as spiked standard at concentration 1747.590 ng/ml in the screened plasma.

Ruggedness was performed by using different analyst and using different column.

Reinjection reproducibility was performed by re injecting the previously validated precision and accuracy batch after a period of 22 hours 08 minutes stored at 5 ± 3 °C.

Effect of potential interfering drugs was performed at HQC and LQC concentration levels of saquinavir for paracetamol, ibuprofen, caffeine, diphenhydramine, diclofenac and chlorphenarimine maleate.

Day zero assessment batch was performed using CC standards and 6 replicates of HQC and LQC after bulk spiking of the bulk spiking QC's and the results of this will be used as comparison for long term stability of Analyte in plasma.

Long batch Precision and Accuracy was performed using 24 replicates of LQC, MQC2, MQC1 and HQC in order to simulate the subject sample analysis.

Short term stock solution stability for the analyte was determined by using aqueous standard equivalent to SS HQC concentration, after storage of stock solution over a period of 7 hours 3 minutes at room temperature. Short term stock solution stability for the ISTD at concentration was determined by using ISTD dilution concentration after storage of ISTD stock solution over a period of 6 hours 55 minutes at room temperature. Short term spiking solution stability for the analyte was determined by using aqueous standard equivalent to SS HQC and SS LQC concentration after a storage period of 7 hours 4 minutes at room temperature.

Short term ISTD dilution concentration stability was determined after a storage period of 7 hours 5 minutes at room temperature.

Long term stock solution stability for the analyte at concentration was determined by using aqueous standard equivalent to SS HQC concentration, after a storage period of 8 days 17 hours and 49 minutes at 5 ± 3 °C. Long term stock solution stability for the ISTD at concentration was determined by using ISTD dilution concentration after a storage period 08 days 17 hours and 49 minutes at 5 ± 3 °C.

Long term spiking solution stability for the analyte was determined by using aqueous standard equivalent to SS HQC and SS LQC concentration after a storage period of 08 days 15 hours 57 minutes at 5 ± 3 °C. Long term ISTD dilution concentration (2.012µg/ml) stability was determined after a storage period of 08 days 15 hours 57 minutes at 5 ± 3 °C.

Freeze thaw stability of the spiked quality control samples was determined after four freeze thaw cycles stored at -28 °C ± 5 °C.

Bench top stability of the spiked quality control samples was determined for a period of 6 hours 35 min stored at room temperature.

Autosampler stability of the processed quality control samples was determined for a period of 55 hours 37 minutes by storing them in autosampler maintained at temperature 5 ±3 °C.

Wet extract stability of the spiked quality control samples was determined for a period of 07 hours 47 min by storing them at room temperature. Wet extract stability of the spiked quality control samples was determined for a period of 47 hours 11 minutes by storing them at 5 ±3 °C. Dry extract stability of the spiked quality control samples was determined for a period of 27 hours 18 min by storing them at -28 ±5 °C.

Stability of analyte in blood was determined at room temperature for a period of 3 hours and 20 minutes. Stability of analyte in blood was determined at 5 ±3 °C for a period of 3 hours 02 minutes

RESULTS AND DISCUSSION

System suitability

The % CV of the retention times of MQC1 was found to be ≤ 0.28 and ≤ 0.27 % for the saquinavir and doxepine, respectively which were within the limit (≤ 2.00%). The % CV of the peak area ratio was found to be ≤ 2.01which was within the specifications (≤ 5.00 %).

Carryover effect
The observed % CV for peak area ratio (peak are of the saquinavir divided by peak area of the doxepine) was found to be zero (limit of maximum allowable % is 20.00), which indicates there was no carry over throughout the validation.

Sensitivity
The precision and accuracy for Saquinavir at LLOQ level were found to be 3.29 % and 104.64 % respectively (limit of acceptance is within 80.00-120.00).

Matrix Effect
The % CV of back calculated concentrations for the HQC and LQC samples of all the lots was found to be 1.96 and 5.67 respectively (acceptance limit 15.00 %). The overall % accuracy of back calculated concentrations for the HQC and LQC samples of all the lots was found to be 103.70 and 104.12 respectively (acceptance limit 85.00 - 115.00 %).

Linearity
All the four calibration curves analyzed during the course of validation were found to be linear for the standards concentration ranging from 1.055 – 703.054 ng/ml. The correlation coefficient (r) was observed to be ≥ 0.9988. The overall % mean accuracy for the CC standards was found to be in between 94.79– 104.07 % and the overall precision was ≤ 3.27 %. The chromatography observed during the validation of the method was represented in the figure 2.

Precision
Within Batch Precision and Between Batch Precision
The % CV of back calculated concentrations for all quality control samples of LQC, MQC2, MQC1, HQC concentration levels were ranged from 0.79 to 9.57 (acceptance limit 15.00).

Table 1: Within Batch Precision and Accuracy

Acquisition Batch ID		HQC	MQC1	MQC2	LQC	LLOQ QC
		Nominal Concentration (ng/mL)				
		558.425	301.550	66.341	3.052	1.099
		Nominal Concentration Range (ng/mL)				
		(474.661-642.189)	(256.318-346.783)	(56.390-76.292)	(2.594-3.510)	(0.879-1.319)
		Calculated Concentration (ng/mL)				
P and A Batch I	**n**	6	6	6	6	6
	Mean	544.8668	288.6475	66.2317	3.3143	1.0863
	SD	14.85947	5.37302	1.77998	0.09153	0.01409
	%CV	**2.73**	**1.86**	**2.69**	**2.76**	**1.30**
% Mean Accuracy		**97.57**	**95.72**	**99.84**	**108.60**	**98.85**
P and A Batch II	**n**	6	6	6	6	6
	Mean	493.5858	276.8938	62.8512	2.8343	0.9560
	SD	3.88808	7.10376	4.42226	0.12938	0.04997
	%CV	**0.79**	**2.57**	**7.04**	**4.56**	**5.23**
% Mean Accuracy		**88.39**	**91.82**	**94.74**	**92.87**	**86.99**
P and A Batch III	**n**	6	6	6	6	6
	Mean	503.6208	272.3048	58.4010	2.8543	0.9605
	SD	8.57155	26.07109	1.04306	0.07919	0.08355
	%CV	**1.70**	**9.57**	**1.79**	**2.77**	**8.70**
% Mean Accuracy		**90.19**	**90.30**	**88.03**	**93.52**	**87.40**

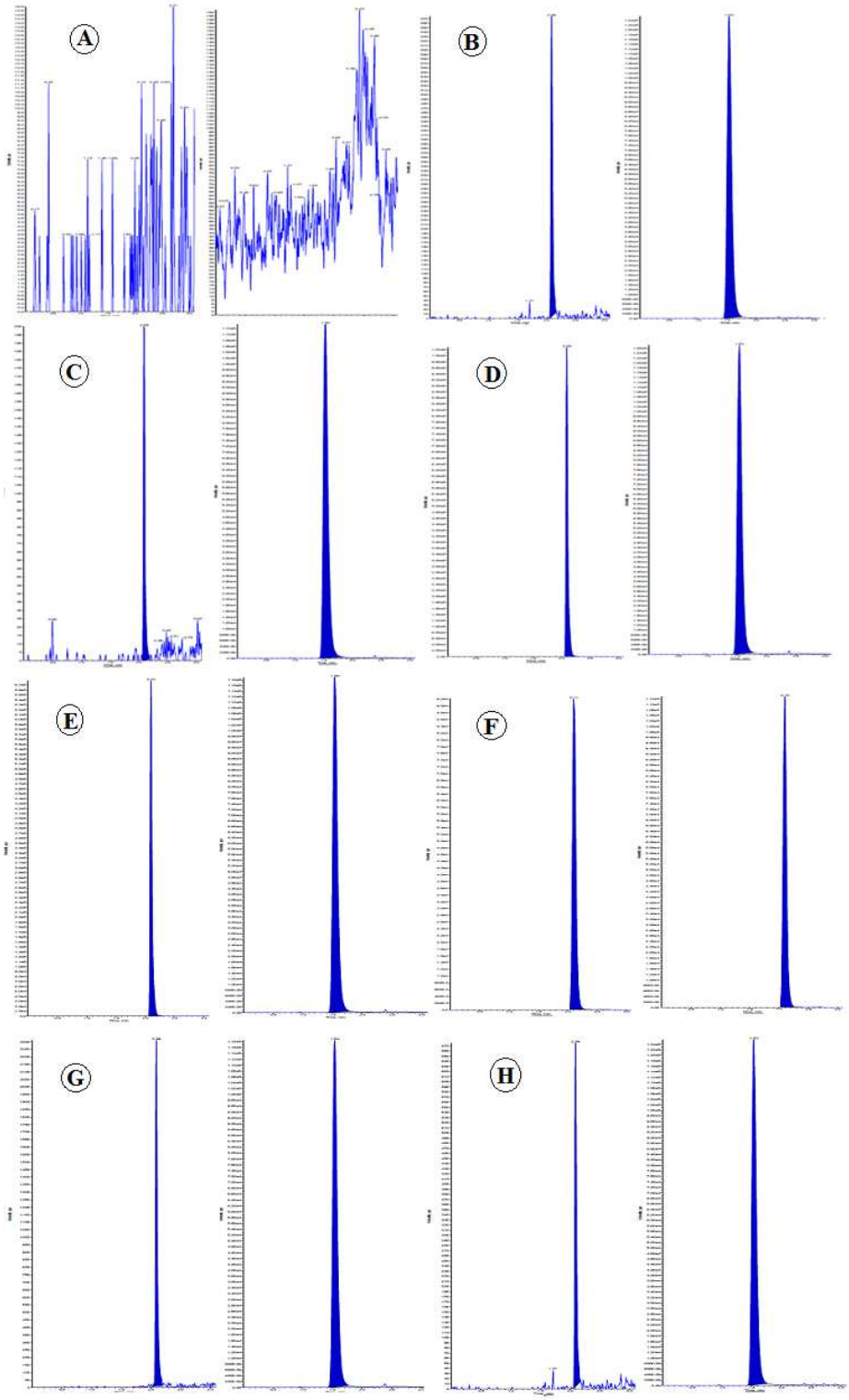

Figure 2: A Representative Chromatogram of A) Standard Blank B) LLOQ Standard C) Standard Zero D) ULOQ Standard E) HQC sample F) MQC sample G) LQC sample H) LLOQ QC sample

The % CV of the back calculated concentrations for LLOQ QC was ranged from 1.30 to 8.70 (acceptance limit 20.00). The results are summarized in the Table 1. The % CV of back calculated concentrations for all quality control samples at LQC, MQC2, MQC1, HQC concentration levels were ranged from 4.81 to 8.25 (acceptance limit 15.00) and the % CV of back calculated LLOQ QC was 8.18 (acceptance limit of 20.00). The results are summarized in the Table 1

Accuracy

Within Batch Accuracy

The % mean accuracy of back calculated concentrations for all quality control samples at LQC, MQC2, MQC1 and HQC concentration levels were ranged from 88.03 % to 108.60 %, which is within acceptance limit 85.00 - 15.00. The % mean accuracy of back calculated concentrations for all the samples of LLOQ QC were ranged from 86.99 to 98.85, which is within the acceptance limit of 80.00 - 120.00. The results are summarized in the Table 1.

Between Batch Accuracy

The % mean accuracy of back calculated concentrations for all quality control samples at LQC, MQC2, MQC1 and HQC concentration levels were ranged from 92.05 to 98.33 (acceptance limit is 85.00 - 115.00). The % mean accuracy of back calculated concentrations for LLOQ QC was 91.08 (acceptance limit is 80.00). The results are summarized in the Table 2

Table 2: Between Batch Precision and Accuracy

n	18	18	18	18	18
Mean	514.0245	279.2821	62.4946	3.0010	1.0009
SD	24.7461	16.5347	4.2299	0.2475	0.0819
%CV	**4.81**	**5.92**	**6.77**	**8.25**	**8.18**
% Mean Accuracy	**92.05**	**92.62**	**94.20**	**98.33**	**91.08**

Recovery for analyte and internal standard

The % mean recovery for Saquinavir at HQC, MQC1, MQC2 and LQC levels was found to be 90.16, 86.09, 84.98 and 89.14 % respectively. Over all % CV at all QC levels was 2.80 which are within the acceptance limit of 20.00.
The % CV of recovery of internal standard at each QC samples of LQC, MQC1, MQC2 and HQC was in the range of 1.07-3.46, which were within the acceptance limit ≤ 15.00. The overall % mean recovery for internal standard was found to be 106.21 % with % CV of 2.80, which is within the acceptance limit ≤ 20.00.

Dilution Integrity

The precision for dilution integrity of 1/5 and 1/10 was found to be 0.48 and 1.40 % respectively. The % mean accuracy for dilution integrity of 1/5 and 1/10 was found to be 99.76 and 102.17 % respectively (acceptance limit 85.00 - 115.00 %).

Table 3: Ruggedness Precision and Accuracy

Saquinavir	**HQC**	**MQC1**	**MQC2**	**LQC**	**LLOQ QC**
	Nominal Concentration (ng/mL)				
	558.425	301.550	66.341	3.052	1.099
Different Column					
Mean	488.0622	275.2010	57.0388	2.6568	0.9233
SD	25.02732	12.65914	2.00751	0.13775	0.03357
% CV	5.13	4.60	3.52	5.18	3.64
% Mean Accuracy	87.40	91.26	85.98	87.05	84.02
Different Analyst					
Mean	542.2595	276.1082	63.4048	3.1020	1.1620
SD	10.80143	6.28652	1.05924	0.13098	0.03911
% CV	1.99	2.28	1.67	4.22	3.37
% Mean Accuracy	97.11	91.56	95.57	101.64	105.73

Ruggedness

The precision for the quality control samples at all concentration levels for different column and different analyst were ranged from 3.52 to 5.18 and 1.67 to 4.22, respectively (acceptance limit 15.00 %). The % CV of back

calculated concentrations for all the samples of LLOQ QC for different column and different analyst were found to be 3.64 and 3.37 respectively (acceptance limit 20.00). The results are summarized in the Table 3

The % mean accuracy for the quality control samples at all concentration levels for different column and different analyst were ranged from 85.98 to 91.26 and 91.56 to 101.64 respectively (acceptance limit 85.00-115.00). LLOQ QC % accuracy was found to be 84.02 and 105.73 respectively (acceptance limit 80.00-120.00). The results of quality control samples of different Analyst and by using different column are summarized in the Table 3.

Reinjection reproducibility

The % CV of back calculated concentrations for all quality control samples of LQC, MQC2, MQC1, HQC and LLOQ concentration levels were ranged from 0.71 to 8.45 (acceptance limit of 15.00) and 11.30% (acceptance limit of 15.00 %), respectively. The % mean accuracy of back calculated concentrations for all quality control samples at LQC, MQC2, MQC1, HQC (acceptance limit 85.00 - 115.00) and LLOQ concentration levels were ranged from 89.79 to 95.14 and 93.04 % (acceptance limit of 80.00 - 120.00), respectively.

Effect of potential interfering drugs

Effect of potential interfering drugs was performed at HQC and LQC concentration levels of Saquinavir for Paracetamol, Ibuprofen, Caffeine, Diphenhydramine, Diclofenac and Chlorphenarimine Maleate. At least 67 % (2 out of 3) of samples at each level (HQC and LQC) were within 85.00-115.00 % of potential interfering drugs (i.e. Paracetamol, Ibuprofen, Caffeine, Diphenhydramine, Diclofenac and Chlorphenarimin Maleate). The results of quality control samples are summarized in the Table 4.

Day Zero Assessment Batch

The % CV of back calculated concentrations for quality control samples at HQC and LQC concentration levels was found to be 3.22 and 3.65 respectively (acceptance limit 15.00). The % mean accuracy of back calculated concentrations for quality control samples at HQC and LQC concentration levels was found to be 92.60 and 94.61 respectively (acceptance limit 85.00 - 115.00). The % mean accuracy of back calculated concentrations for quality control samples at HQC and LQC concentration levels was found to be 92.60 and 94.61 respectively, which are within acceptance limit 85.00 - 115.00.

Table 4: Effect of potential interfering drugs

	Saquinavir		
S. No.	**Compounds**	**HQC**	**LQC**
1	**Paracetamol**		
	Mean	3221.4847	30.2980
	% Mean Accuracy	100.20	105.61
2	**Ibuprofen**		
	Mean	3238.3193	29.4073
	% Mean Accuracy	100.73	102.51
3	**Caffeine**		
	Mean	3251.1237	30.7207
	% Mean Accuracy	101.12	107.09
4	**Diphenhydramine**		
	Mean	3356.6510	31.9520
	% Mean Accuracy	104.41	111.38
5	**Diclofenac**		
	Mean	3328.4113	30.9557
	% Mean Accuracy	103.53	107.90
6	**Chlorphenarimin Maleate**		
	Mean	3247.1483	30.9763
	% Mean Accuracy	101.00	107.98

Long batch Precision and Accuracy

The % CV of back calculated concentrations for all quality control samples at LQC, MQC2, MQC1, HQC and LLOQ concentration levels were 7.67, 3.09, 3.35, 4.14 (acceptance limit ≤ 15) and 14.63 (acceptance limit ≤ 20) respectively.

The % mean accuracy of back calculated concentrations for all quality control samples at LQC, MQC2, MQC1, HQC and LLOQ concentration levels were 95.13, 95.10, 95.86, 92.25 (acceptance limit 85-115) and 103.05 (acceptance limit is 80.00-120.00), respectively.

STABILITY OF ANALYTES

For stock solution stability in short term stock solution stability for analyte and internal standard stability was assessed by comparing against the freshly weighed stock concentration and prepared aqueous standard equivalent to SS HQC concentration and for prepared internal standard dilution concentration. The % mean stability for analyte and internal standard was found to be 101.91 and 97.92 respectively. For Short Term Spiking Solution Stability for analyte and working Solution stability for internal standard, the % mean stability for SS HQC and SS LQC was found to be 99.79 and 101.32 for HQC and LQC concentrations respectively. For prepared internal standard dilution concentration the % mean stability was found to be 100.39. For long term stock solution stability for analyte and internal standard the % mean stability for prepared aqueous standard equivalent to SS HQC concentration and by using internal standard dilution concentration was found to be 100.42 and 99.50 respectively. For long term spiking solution stability for analyte and working solution stability for internal standard the % mean stability for SS HQC and SS LQC was found to be 99.43 and 98.42 respectively. The % mean stability for internal standard dilution concentration was found to be 100.46. In Freeze Thaw Stability the % mean stability for HQC and LQC was found to be 97.58 and 99.18 respectively.

Table 5: Stability study conditions and % mean stability results

Stability Study	Condition	N	% Mean stability	
			HOQ	LOQ
Short Term Stock Solution Stability for Analyte and Internal Standard	7 hours 3 minutes at room temperature	6	101.91	97.2
Short Term Spiking Solution Stability for Analyte and Working Solution Stability for Internal Standard	7 hours 4 minutes at room temperature		99.79	101.32
Long Term Stock Solution Stability for Analyte and Internal Standard	8 days 17 hours and 49 minutes at 5 ± 3 °C		100.42	99.50
Long Term Spiking Solution Stability for Analyte and Working Solution Stability for Internal Standard	08 days 15 hours 57 minutes at 5 ± 3 °C		99.43	98.42
Freeze thaw stability	Four freeze thaw stored at -28±5ºC		97.58	99.18
Bench top stability	6 hours 35 min stored at room temperature		98.34	101.30
Wet extract stability at room temperature	07 hours 47 min by storing them at room temperature		97.33	100.52
Wet extract stability at refrigerated temperature	47 hours 11 minutes by storing them at 5 ±3 °C		99.08	106.84
Dry extract stability	27 hours 18 min by storing them at -28 ±5 °C		101.86	103.05
Auto sampler stability	55 hours 37 minutes by storing them in autosampler maintained at temperature 5 ±3 °C		99.62	98.68
Stability of analyte in blood at room temperature	03 hours and 20 minutes		99.15	94.29
Stability of analyte in blood at refrigerated	5 ±3 °C for a period of 3 hours		104.68	93.14

In Bench Top Stability the % mean stability for HQC and LQC was found to be 98.94 and 101.30 respectively. In autosampler stability the % mean stability for HQC and LQC was found to be 96.62 and 98.68, respectively. In Wet Extract Stability at room temperature the % mean stability for HQC and LQC was found to be 97.33 and 100.52 respectively. In wet extract

stability at refrigerated temperature the % mean stability for HQC and LQC was found to be 99.08 and 106.64 respectively. In dry extract stability the % mean stability for HQC and LQC was found to be 101.86 and 103.05 respectively. The stability of analyte in blood at room temperature, the % mean stability for HQC and LQC was found to be 99.15 and 94.29 % respectively at room temperature. In stability of analyte in blood at refrigerated temperature the % mean stability for HQC and LQC was found to be 104.68 and 93.14 respectively at 5 ±3 °C. The acceptance limit for % mean solution stability of drug and internal standard for all stability parameters is in the range of 90.00-110.00 %. The % CV of response ratio at each level for all stability parameters within the acceptance limits (≤ 10.00). All the conditions and results for all stability parameters were represented in Table 5.

CONCLUSION

A new, simple, high throughput, and accurate LC–MS/MS method for the determination of saquinavir in human plasma according to acceptance criteria for bioanalytical method validation was successfully developed and

validated. The current method could be useful for the estimation of saquinavir in human blood samples of different pharmacokinetic studies.

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