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ORIGINAL ARTICLE **OPEN ACCESS**

Development of Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry Method for the Quantification of Saquinavir in Biological Matrices

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ABSTRACT
The main aim of the present study was to develop a sensitive liquid chromatography-electrospray ionization tandem mass spectrometric technique for the quantitation of saquinavirin human plasma. Chromatographic elution was attained thruaPhenominex C18(3.5 µm; 50×4.6 mm) analytical reverse phase column with isocratic mobile phaseofacetonitrile, methanol and pH 4.0 ammonium acetate buffer 10mM in the ratio of 30:50:20 V/V. Liquid-liquid extraction was executed for the drug separation with an ethyl acetate solvent. Parent and product ions were monitored at m/z 671.39/400.29 for Saquinavirand 705.39/144.10for Atazanaviron MRM.Calibration curve of Saquinavir was linear over 167- 6688ng/ml concentration range with regression coefficient (r^2) value of > 0.99. The % RSD values were less than 3.27% for inter-day and intra-day precision and accuracy. The method has excellent recovery and the percentage recovery values of lower quality control (LQC), median quality control (MQC) and higher quality control (HQC) samples were 96.72%, 95.34% and 101.87% respectively.Saquinavirhas more stability for longer time when subjected for different stability environments and the technique was effectively relevant to routine analysis of Saquinavirin biological matrix.
Keywords: *Saquinavir, Protease inhibitor, LC-MS/MS, FDA guidelines and Dilution integrity.*

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INTRODUCTION

Saquinavir (Ro 31-8959, Invirase ®, Fig. l) belongs to a new class of antiretroviral drugs, theprotease inhibitors, and is a potent in vitro and in vivo inhibitor of human immunodeficiency virus (HIV), the causative agent of the acquired immunodeficiency syndrome (AIDS). Proteases are enzymes that cleave protein molecules into smaller fragments. HIV protease is vital for both viral replication within the cell and release of mature viral particles from an infected cell. Saquinavir binds to the active site of the viral protease and prevents cleavage of viral polyproteins, preventing maturation of the virus. Saquinavir inhibits both HIV-1 and HIV-2 proteases[1-4]. Chemically itrepresented as (2*S*)-*N*-[(2*S*,3*R*)-4- [(3*S*)-3-(*tert*-butylcarbamoyl)-decahydroisoquinolin-2-yl]-3-hydroxy-1-phenylbutan-2-yl]-2-(quinolin-2-ylformamido) butanediamide with a molecular formulaand weight of $C_{38}H_{50}N_6O_5$and 670.841.Saquinavir was the first member of its class to be approved by the FDA in the USA in 1995, under its accelerated approval regulations, for use in combination with approved nucleoside analogue reverse transcriptase inhibitors in patients with advanced HIV infection. The pharmacokinetic profile of saquinavir in HIVinfected patients has not yet been thoroughly investigated [5, 6].

Saquinavirliterature review reveals that no single analytical technique was reported for the estimation of it in biological samples individually by LC-MS/MS. Developed methods were exist on RP-HPLC[7-10], ion pair chromatography[11] and spectrophotometry[12]. So, there is a need of highly sensitive and specific LC-MS/MS method for the quantification of saquinavir in biological matrices.

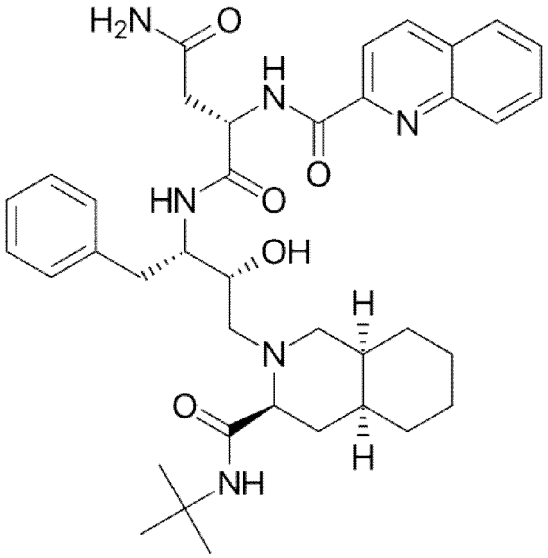

Fig. 1: Structure of Saquinavir

MATERIAL AND METHODS

Chemicals and reagents

Internal standard (Atazanavir) of 99.81% was acquired from Hetero drugs PVT. LTD,Hyderabad, India. Saquinavir(Purity: 99.87%) was obtained from MSN Labs, IndiaAcetonitrile of HPLC-grade and formic acid of analytical grade were bought from J.T.Baker, Hyderabad, India. In the present research work water used from Milli-Q water purification system installed in the lab obtained from Bangalore, India.

Liquid chromatographic-MS/MS system

A modular Liquid Chromatographic (LC) system(Shimadzu, Japan) equipped with a DGU-20-A3 solvent degasser, binary LC-20-AD prominence pump, CTO-ASVP-oven for column and high-throughput SILHTC-autosampler were utilized for present research.

Chromatography was achieved on a PhenominexC18 (3.5 µm; 50×4.6 mm) analytical reverse phase columnwith isocratic mobile phaseofacetonitrile, methanol and pH 4.0 ammonium acetate buffer 10mM in the ratio of 30:50:20 V/V. Chromatographic peaks were resolved by the mobile phase with a flow rate of 0.80 ml/min. Saquinavir and Atazanavir internal standard were separated in the total runtime of 3.5 minutes. The autosampler temperature and analytical Column temperatures were kept at 5 °C and 35 °C respectively.

The eluents of the liquid chromatographic system were infused into the Electro Spray Ionization (ESI) source operated with positive ionization method. Starting 0.5 min eluent was avoided from the chromatographic system to evade unnecessary impurities from the various salts existed in the human plasma samples.

In the mass system following conditions were applied; gas-1, nitrogen (30 psi); gas-2, nitrogen (35 psi); temperature of ion source, 500 °C; curtain-gas, nitrogen (35 psi); voltage of ion spray, 4500 V. Monitoring of transition of m/z 671.39 and 400.29 for Saquinavirand 705.39 and 144.10for Atazanavirwere made on multiple reaction monitoring(MRM). The mass conditions were presented in Table 1.

Table 1: Mass conditions for amprenavir and IS

Component	Precursor Ion (m/z)	Product Ion (m/z)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
Saquinavir	671.39	400.29	45	9	23	45	5
Atazanavir	705.39	144.10	50	9	24	53	5

DP: Declustering potential; EP: Entrance potential; CEP: Collision cell entrance potential; CE: Collisional energy; CXP: Collision cell exit potential**.**

Protocol for Ammonium Acetate Buffer (10 mM)

0.770g of ammonium acetate was weighed and relocated in to 1L volumetric flask. 250mL of HPLC grade water was added and sonicated for 10 min. Finally, the volume was made up to the mark and pH was adjusted with acetic acid to 4.0. The resultant solution was subjected for filtration thru a 0.45µ membrane filter.

Sample Preparation

To 150µl of spiked plasma, 50µl of IS (1µg/ml) was mixed and sonicated for 5min. To the resulting solution, 500 µl of acetonitrile was added and vortexed for 10min, followed by centrifugation at 4500 rpm upto 25.0 min at 5.0°C. The organic phase was dried in alyophiliser. The final residue was solubilized in 500 µl mobile phase and relocated into autosampler vials and infused into an LC-MS/MS system.

Preparation of Standard stock and calibration standards

Saquinavir and IS stock solutions were processed in mobile phase at concentration level of 1.0mg/ml. Quality control(QC) and calibration standard(CC) solutions were processed by spiking blank human plasma sample from thesaquinavir stock solution.CC solutions of eight concentration levels were prepared to produce the final concentrations of 167, 350, 750, 1500, 2650, 4000, 5300 and 6688ng/ml. LQC standard, MQC standard and HQC standards were QC sample solutions and were prepared to produce the concentrations of 468, 3344 and 5016ng/ml respectively. All the stock, QC and CC solutions were maintained at -20℃ till the method of analysis.

Validation

The method of analysis was assessed by validation parameters like sensitivity, precision, linearity, recovery, dilution integrity, accuracy, matrix effect and stability.Three QC samples of LQC, MQC and HQCs as well as LLOQ (167ng/ml) were employed and analyzed in method validation.[13-16]

Accuracyand precision

Inter-day and intra-dayaccuracyand precision were examined as a part of precision and accuracy (PA) parameter. Intra-day PA was evaluated by injecting QC solutions (468, 3344 and 5016ng/ml) and LLOQ (167ng/ml) in 5 replicates in a day arbitrarily. Inter-day PA was evaluated by injecting the same QC and LLOQ solutions once in a day for 5different days. The % RSDs for LQC, MQC and HQCs should be ≤20.0% for LLOQQC and ≤15.0% for the remaining control levels.[15]

Linearity

CC standards (Non-zero) of 8 different concentrations at 167, 350, 750, 1500, 2650, 4000, 5300 and 6688ng/ml solutions were prepared andprocessedin3differentruns.Linearity curve (peak area fraction of saquinavir and I Speaks against original concentrations) were plotted by least squares linear regression and reciprocal of the squared concentration ($1/x^2$) utilized as a weighting factor. Deviation should be within ±20.0% for LLOQ and ±15.0% for remaining control levels.

Specificity and selectivity

Method selectivity was analyzed by equating the chromatograms acquired from blank and spiked samples. Method specificity was analyzed by infusing 6 dissimilar lots of blank plasma solutions to confirm no endogenous compounds interfere with saquinavir and IS.

Recovery and Matrix Effect (ME)

Saquinavirrecovery was assessed by paralleling the average peak response of extracted and un-extracted solutions at HQC, MQC and LQC standard levels. At each concentration level percentage recoveries was calculated and finally overall mean recovery was calculated. The ME was analyzed by paralleling the un-extracted samples with post-extracted samples. [18, 19]

$$\%\ \text{Recovery of saquinavir} = \frac{\text{Mean saquinavirpeak response in extracted samples}}{\text{Mean saquinavirpeak response in un} - \text{extracted samples}} \times 100$$

Stability

Stability was analysed at HQC, MQC and LQC quality control levels. It includes bench-top, freeze and thaw, autosampler and long-term stabilities. Bench-top stability was assessed for 5 h at ambient temperature (25℃). Freeze and thaw stability was analyzed by monitoring the quality control solutions at -70℃ for at least 3 h and for thaw cycle keep the solutions at room temperature. Repeat the freeze and thaw cycles for 3 times. The auto sampler stability was analyzed by placing the QC solutions in an autosampler at 10.0 degree centigrade for 8.0 h. Long term stability was assessed by keeping the QC solutionsin a freezer at -70 ℃ for three months.[20,21]

Dilution Integrity

The sample solution more than the upper calibration limit was prepared and evaluated for PA parameters. The percentage nominal concentration must be ±15 %.

RESULTS AND DISCUSSION

The LC-MS/MS peaksofsaquinavirblank, HQC, MQC, LQC and LLOQ concentration levels were shown in Figures 2, and 3.

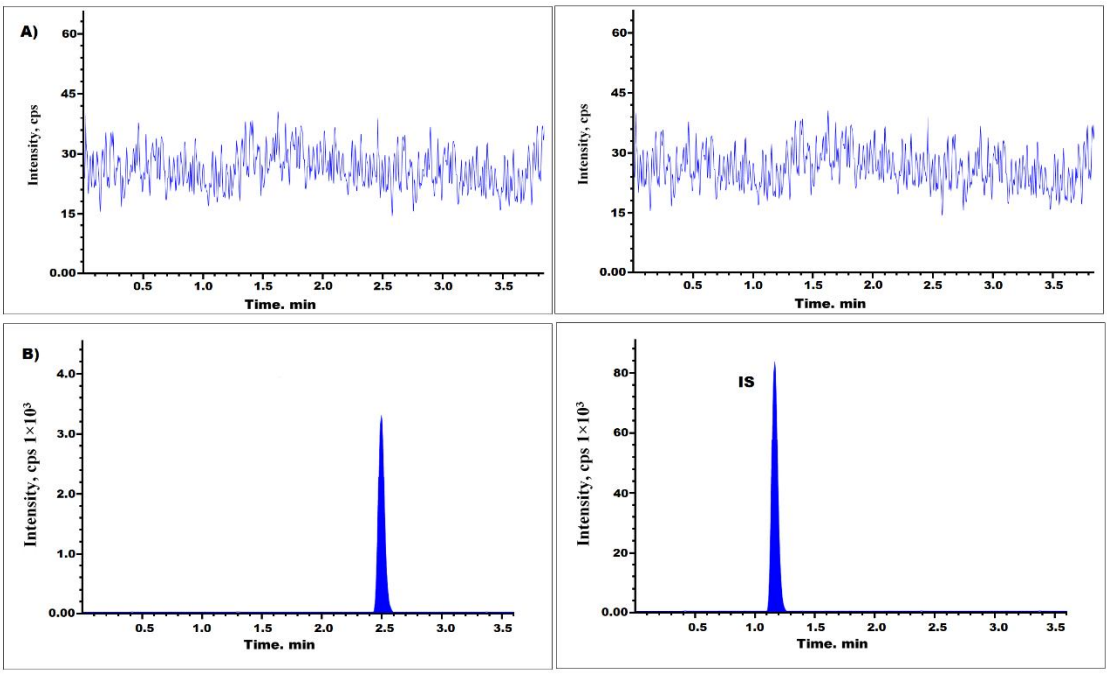

Fig. 2: Saquinavirchromatograms (A) blank and (B) spiked LLOQ-samples.

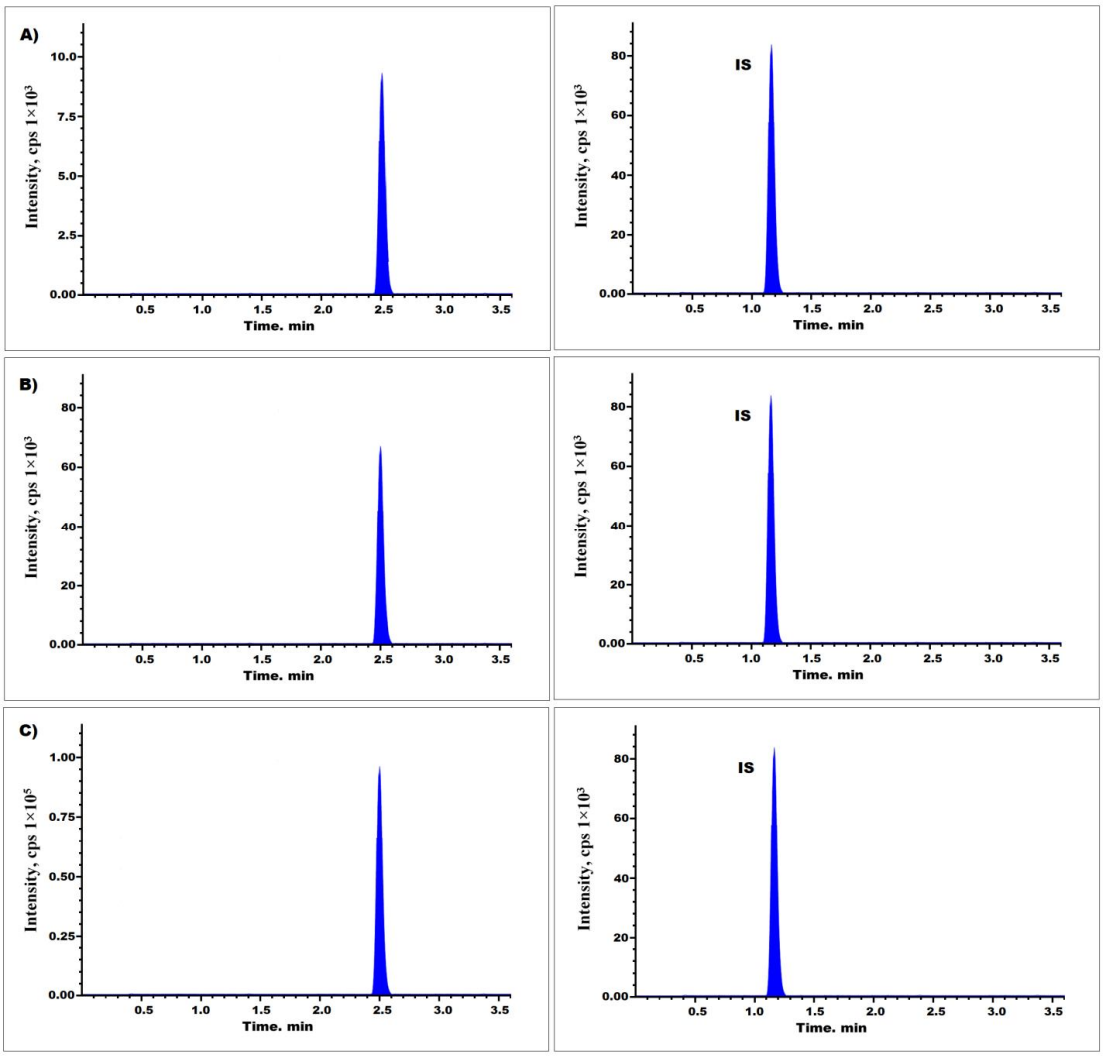

Fig. 3: Chromatograms of saquinavirspiked at (A) LQC, (B) MQC and (C) HQC samples

Method validation

Specificity

From the Figure 2, system chromatographic conditions were clearly separating saquinavir and internal standard from endogenous and other plasma substances. The saquinavir-LLOQ peak response is more than 20% the interference peak response andIS peak response is more than 5% from the interference peak response.

Accuracyand precision

Saquinavir inter-day and intra-day accuracyandprecision were analyzed and the %RSD values were calculated for the same and were tabulated in the Table 2.

Table 2: Saquinavirprecision and accuracy data

Nominal concentration (ng/ml)	Intra-day		Inter-day	
	Accuracy (%)	%RSD	Accuracy (%)	%RSD
167	96.24	2.54	97.84	2.64
468	102.65	1.85	93.82	3.21
3344	97.08	3.27	96.34	1.83
5016	95.87	2.91	102.42	2.95

Linearity

Saquinavir calibration graph was rectilinear in concentration over167 to 6688ng/ml with regression equation of Y = 0.1864 X + 4.675. The regression coefficient (r^2) value is more than 0.99 which was acceptable as per the FDA regulatory guidelines.[19]

Recovery and Matrix Effect (ME)

The developed technique has nice recovery and the recovery findings were 96.72%, 95.34% and 101.87% for LQC, MQC and HQC quality control samples respectively. The data for saquinavir recovery were tabulated in Table 3. The matrix effect was evaluated at HQC, MQC and LQC level and the calculated %CV values were 2.42%, 1.84% and 4.19%, respectively.

Table 3: Extraction recovery rates of analytes

Concentration level	X	Y	% Recovery	% Mean recovery	%RSD
LQC	9018	8722	96.72	97.97	2.87
MQC	64328	61330	95.34		
HQC	96813	98623	101.87		
IS	84267	83120	98.64		

X, mean response of unextracted samples; Y, mean response of extracted samples.

Dilution Integrity

Dilution integrity of saquinavir was performed and evaluated. The percentage nominal was within the limit (± 15%) and the estimated precision was less than or equals to 15%. It shows that the drug can be dilute to twenty times and the results will be reproducible.

Stability

All the QC standards were exposed to different stability conditions and evaluated to analyse the stability of saquinavir. From evaluated %CV stability data, saquinavir was more stable at different environments likebench-top stability (<5.26%), freeze-thaw stability (<6.95%), autosampler stability (<2.05%) and long term stability (<3.12%) and the values were represented in Table 4.

Table 4: Stability Results

Parameter	QC level	X	Y	%RSD	%Stability
Freeze–thaw stability	LQC	468	435.49	3.51	93.055
	HQC	5016	5101.08	2.54	101.69
Autosampler stability	LQC	468	456.58	3.4	97.56
	HQC	5016	4938.70	1.9	98.45
Benchtop stability	LQC	468	458.42	2.5	97.95
	HQC	5016	4752.04	3.7	94.74
Long-term stability	LQC	468	453.44	6.2	96.89
	HQC	5016	5101.45	1.61	101.70

X, nominal concentration (ng/mL); Y, mean concentrations (ng/mL) of analytes

CONCLUSION

A simple and specific LC-MS/MS technique for the saquinavir was developed and validated by utilizing atazanavir as IS. This method has excellent recovery, accuracy and precision compared with existed methods for the analysis of drug in plasma samples. The drug was subjected for extraction with ethyl acetate from human plasma samples by LLE. The drug was eluted within 3.5 min using a Phenominex C18 (3.5 µm; 50×4.6 mm) analytical reverse phase column with isocratic mobile phaseofacetonitrile, methanol and pH 4.0 ammonium acetate buffer 10mM in the ratio of 30:50:20 V/Vwith flowrate of 0.80 ml/min. The developed technique was validated as per the FDA regulatory guidelines and all the parameters of validation were within standard limit. The developed method was successfully applicable for the routine analysis of saquinavir in biological matrices.

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CONTRIBUTION OF AUTHOR'S

All are equally contributed.

CONFLICTS OF INTEREST

None.

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