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# A Specific LC-ESI-MS/MS Method Development and Validation for the Quantification of Saquinavir in Biological Matrices

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## ABSTRACT

**Introduction:** Saquinavir is a first protease inhibitor. Proteases are enzymes that cleave protein molecules into smaller fragments. No analytical procedure was reported for the estimation of saquinavir by LC-MS/MS.

**Aims:** The objective of the present research was to develop a specific liquid chromatography-electrospray ionization tandem mass spectrometric technique for the quantitation of saquinavir in biological matrices.

**Methodology:** Chromatographic elution was attained thru a Thermo Hypersil ODS stationary column having the dimensions of 50 × 4.6 mm and particle size of 2.4 μm. Isocratic elution was processed with methanol and 0.1%V/V formic acid in the ratio of 90:10 V/V as mobile phase with flow rate of 0.50 ml/min. Liquid-liquid extraction was performed for drug and internal standard isolation with an ethyl acetate solvent. Parent and productions were monitored at m/z 671.3 → 654.3 for saquinavir and 614.3 → 596.3 for indinavir internal standard on multiple reaction monitoring.

**Result:** Linearity graph of drug was rectilinear in concentration over 260.4 to 10416ng/ml having r<sup>2</sup>(correlation coefficient) value more than 0.999. Percentage of RSD findings were ≤5.3% for inter and intraday accuracy and precision. This procedure has good recoveries and %recovery findings of lower quality control (LQC), median quality control (MQC) and higher quality control (HQC) samples were 94.6%, 92.4 and 104.2% respectively.

**Conclusion:** Saquinavir has more stability for longer time when subjected for different stability environments and the technique was effectively relevant to routine analysis of saquinavir in biological matrix.

**Key Words:** Protease inhibitor, Saquinavir, LC-ESI-MS/MS, FDA guidelines, Specificity, Linearity

## INTRODUCTION

Human immunodeficiency virus (HIV) infection and its clinical syndrome acquired immunodeficiency syndrome (AIDS) continue to be a major health problem worldwide. The development of highly effective antiretroviral chemotherapy for AIDS is found very useful in curbing the pandemic of this disease.<sup>1,2</sup> The drug is sold as Fortovase and Invirase brand names and it acts as an antiretroviral agent utilized along with other medicines to prevent or treat HIV/AIDS. Normally it is combined with ritonavir/lopinavir or ritonavir to improve its outcome. Saquinavir is a first protease inhibitor. Proteases are enzymes that cleave protein molecules into smaller fragments.<sup>3</sup>

Bioavailability of saquinavir increases with ritonavir (PI) in dosage forms which was observed clinically. It will benefit to

the patients for taking the less dose of saquinavir and also for maintaining adequate plasma levels of saquinavir to produce effective against the HIV. In this connection it was observed that ritonavir prevents isozyme cytochrome P450 3A4, which metabolize the saquinavir into its inactive metabolite.<sup>4</sup>

Drug binds to effective part of viral protease and inhibits breakdown of viral polyprotein components, inhibiting maturation of virus. Saquinavir inhibits both HIV-1 and HIV-2 proteases. It is a potent inhibitor of HIV replication in vitro and suppresses viral load and increases CD4<sup>+</sup> cell counts in HIV patients *in vivo*. Chemically saquinavir designated 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 weight and formula of 670.841 and C<sub>38</sub>H<sub>50</sub>N<sub>6</sub>O<sub>5</sub> (Figure 1).<sup>5,6</sup>

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Literature on saquinavir revealed that no analytical procedure for the estimation of drug in bulk and formulations was reported on LC-MS/MS. Few methods were reported on spectroscopy,<sup>7</sup> liquid chromatography<sup>8-11</sup> and ion pair chromatography.<sup>12</sup> The development of specific method like LC-MS/MS is highly essential for the quantification of saquinavir in biological matrices.

## MATERIALS AND METHODS

### Chemicals and reagents

Saquinavir (99.91% purity) was acquired from Hetero drugs, Hyderabad, India. Methyl indinavir (Internal standard) of 99.89% was received from MSN Labs, Hyderabad, India. Acetonitrile and methanol of HPLC-grade and formic acid of analytical grade were procured from J.T.Baker, Hyderabad, India. Water of Milli-Q was utilized during the processing of movable phase and washing solvents, acquired from in-built Milli-Q<sup>®</sup>RO system.

### Liquid chromatographic-MS/MS system

An LC-MS/MS SCIEX API4000 instrument furnished with a positive Electro-spray ionization (ESI) source and Shimadzu prominence HPLC comprising of SIL-HTC autosampler, binary pumps and column oven was utilized in the present work. Analyte quantitation, acquisition of data and its integration were processed by utilizing analyst software-1.6.3 version.

### Liquid chromatography

Chromatographic elution was attained on a Thermo Hypersil ODS stationary column having the dimensions of 50 × 4.6 mm and particle size of 2.4 μm. Column oven temperature was monitored at 30.0 °C and 0.5 mL/min flow rate. Injection volume was 5 μL. Isocratic elution was processed with methanol and 0.1%V/V formic acid in the ratio of 90:10 V/V as mobile phase. Saquinavir and indinavir internal standard were separated in the total runtime of 6 minutes. The autosampler temperature and analytical Column temperatures were kept at 5.0 °C and 30.0 °C respectively.

### Mass system conditions

The mass spectrometer was operated under multiple reactions monitoring (MRM) mode in positive ion mode for both saquinavir and internal standard (IS). The adjusted mass system parameters for saquinavir and IS were: both of drying gas and sheath gas temperatures were 350 °C. Nebulizer pressure was monitored at 25.0 psi. The sheath gas and drying gas flow rates were 10.0 L/min and 5.0 L/min, respectively. Capillary voltage was set at 3.0 kV. Collision energy and fragment or voltage were 15 eV and 120 V for saquinavir, 15 eV and 100 V for IS. The transitions (precursor to

product) monitored were m/z 671.3 → 654.3 for saquinavir and 614.3 → 596.3 for IS. The dwell time was 200 ms for each transition.

### Calibration standards

A stock solution of 1mg/mL solution of saquinavir was prepared freshly by dissolving 10mg of drug in 10mL of 70% methanol. Calibration standards of eight different concentration levels were processed by spiking blank plasma with saquinavir standard solution to get the concentrations of 260.4, 520.8, 1302, 3100, 5100, 6950, 8750 and 10416 ng/mL.

### Quality Control Standards

These solutions were prepared at three different levels of lowest quality control(LQC), standards, median quality control(MQC) standards and highest quality control(HQC) standards. These quality control(QC) samples were prepared according to calibration standards to get the concentration of 729.1, 5208 and 7812 ng/mL for LQC, MQC and HQC respectively. Prepared solutions were store at -20°C till the time of analysis.

### Sample Preparation Method

Sample solution was prepared by transferring 300 μl of plasma and 50μl of indinavir (1μg/mL) into a polypropylene tube and vortexed for 2 min. Saquinavir and IS were extracted with 4mL of ethyl acetate as solvent followed by centrifugation of the solution at 4500 rpm/min for 20min. After centrifugation, organic phase was separated and it was dried using lyophiliser. The resulting product was made solubilize in 250μl of mobile phase and then it transferred into a pre-labeled vials. The vials were kept in an auto-sampler and infused into LC-MS/MS system.

### Method validation

The developed method was validated for the parameters: specificity, selectivity, linearity, stability, matrix effect, recovery, precision and accuracy.<sup>13, 14</sup>

## RESULTS

### Mass system conditions

For the mass determination, we applied the MRM in positive mode with saquinavir to achieve higher sensitivity and better specificity. ESI was expected to be the best source of ions for LC-MS/MS and the precursor ions and product ions were ascertained by infusing the standard solutions into the mass instrument through a syringe pump. The product ions mass spectra of saquinavir and IS were obtained at m/z 654.3 and 596.3 which were selected as detection ions. Meanwhile, the parameters of mass spectrum environments (capillary voltage,

ION-spray voltage and temperature, heater gas, nebulizer gas, collision gas and curtain gas and so on) were optimized to acquire higher mass response.

### Internal standard selection

In the present study, indinavir was selected as the IS because it had similar chromatographic behaviors, ionization, extraction efficiency and retention action as the saquinavir and there was no obvious interferences found at the retention times of analytes and IS according to the method validation results.

### Method validation

#### Specificity

Blank plasma samples obtained from six different batches of human plasma was spiked with the saquinavir at LLOQ and IS to assess the specificity. As Figure 2 showed, the retention times of saquinavir and IS were found to be 5.0 and 3.2 min, respectively. There was no noticeable intrusion of matrix substance and internal standard on saquinavir analytical data, and the response of all interfering peaks were less than 20% of LLOQ samples.<sup>14, 15</sup>

#### Sensitivity and linearity

The method linearity was established for saquinavir and excellent linearity was exhibited in the concentration range of 260.4 to 10416 ng/ml (Table 1). The linearity graphs were produced by the peak area ratios (y) of the saquinavir to the IS versus the concentrations (x) with the  $1/C^2$  weighting factor. The developed method linearity equation of the calibration graphs was  $y = 0.00843x + 0.00249$  with a correlation coefficient ( $r^2$ ) value of 0.9991. The LLOQ of saquinavir was 260.4 ng/ml (signal-to-noise ratio > 5.0) assessed by 5 replicate sample solutions which were adequate for reliable quantitation of saquinavir in the plasma samples study.<sup>16</sup>

#### Accuracy and precision

The intra-batch and inter-batch precision and accuracy were evaluated by six spiked samples of plasma with saquinavir at LLOQ, LQC, MQC and HQC levels in a batch and in 3 succeeding batches, respectively. The results of precision and accuracy for quantitation of saquinavir were represented in the Table 2. The percentage relative standard deviation values of inter and intra-batch precision were present in the range of 2.8 to 5.3. The deviation of relative error in the accuracy of inter and intra-batch values were present in between -4.52 to 5.88.<sup>16, 17-21</sup>

#### Extraction recovery

The biological samples were effectively pretreated before the analysis. The extraction recovery was evaluated by calculating the peak area ratio of LQC, MQC and HQC level solutions (n=6) of saquinavir to the extracted spiked samples at

respective concentration levels. In same manner, extraction recovery of indinavir was assessed by calculating the peak area ratio of quality control plasma sample solutions (n=6) to spiked human plasma samples at respective concentration levels. The mean extraction recovery of saquinavir was 94.6%, 92.4 and 104.2% at low, medium and high QC levels respectively. The mean extraction recovery of indinavir was 94.8% at the concentration of 100 ng/L. The results were represented in the Table 3 and Figures 3 to 5.<sup>15, 18-21</sup>

#### Matrix effect

Co-eluting matrix constituents can reduce or increase ionization process in mass system, which may not produce a detectable response in blank matrix due to accuracy of the method. Hence, the different matrix related component ions dominance was assessed by estimating the IS normalized matrix factor in eight individual sources (consisting 2-hemolytic and 2-lipemic batches) of human plasma. The average IS normalized matrix factor for all the analytes were present in between 0.95 and 1.06 with a %RSD of  $\leq 4.99$  and the results were shown in the Table 4.<sup>16</sup>

#### Dilution integrity

The dilution integrity was processed at twice the concentration ULOQ (upper limit of quantification) for saquinavir. After the 1:4 dilution the average back-calculated drug content for dilution quality control samples were within 85–115% of the nominal value with a %RSD of  $\leq 4.5$ .

#### Stability

Stability of saquinavir was evaluated in both matrix and aqueous based samples. Saquinavir and indinavir in-stock solutions were not affected at 1–10°C for 70 days and stock solutions in diluent were not affected at 1–10°C for 48 h. Matrix stability was assessed at -70 and -20°C for 60 days. Matrix stability was analysed against freshly prepared spiked linearity standards. The findings of stability data were represented in Table 5. Drug was not degraded upto 20 h at the bench-top at a temperature of below 10°C and after six freeze-thaw cycles. The prepared sample solutions were stable at 10°C for 72 h in the auto-sampler.<sup>17</sup>

## DISCUSSION

Method development was processed with different mobile phases and finally optimized with methanol and 0.1%V/V formic acid in the ratio of 90:10 V/V thru Thermo Hypersil ODS (50 × 4.6 mm, 2.4 μm) column. Optimized method was validated as per the FDA and EMA guidelines for the specificity, selectivity, linearity, stability, matrix effect, recovery, precision and accuracy parameters. The LLOQQC was analysed with satisfactory accuracy, < 20% of precision and > 5 of signal-to-noise (S/N) ratio. To evaluate the carry-over

effect, the blank plasma solution was infused into chromatographic system after analyzing the highest concentration level of calibration standard in analytical run and there was no visible carry-over effect was found. The method has linearity in the concentration range of 260.4 to 10416 ng/ml. These findings of precision and accuracy proved that they were reliable for the estimation of saquinavir in human plasma. Method has good extraction recovery with mean recovery of 97.07%. Method was subjected for freeze-thaw, refrigerator, bench-top, long term and in-injector stability and has high degree of stability.

## CONCLUSION

In the present research work, a sensitive and accurate LC-MS/MS technique was developed and validated for the successful determination of saquinavir in human plasma. This method exhibited excellent specificity, linearity, accuracy, precision and stability. The linearity equation and correlation coefficient ( $r^2$ ) findings were  $y = 0.00843x + 0.00249$  and 0.9991 respectively. The %RSD of intra and inter-day precision of the developed technique was found between 2.8 to 5.3% for the QC-samples (260.4, 729.1, 5208 and 7812 ng/ml). Hence, the validated method can be applicable for the pharmacokinetic and toxicokinetic studies in the clinical and forensic analysis of saquinavir in different kinds of biological matrices successfully.

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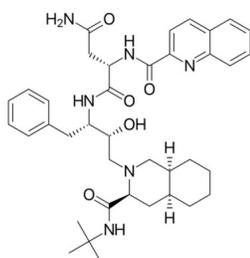


Figure 1: Structure of Saquinavir.

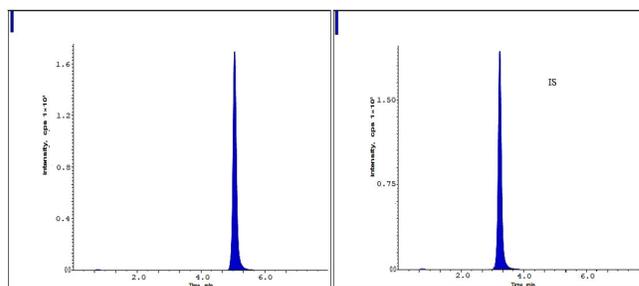


Figure 4: Chromatograms of saquinavir at MQC level.

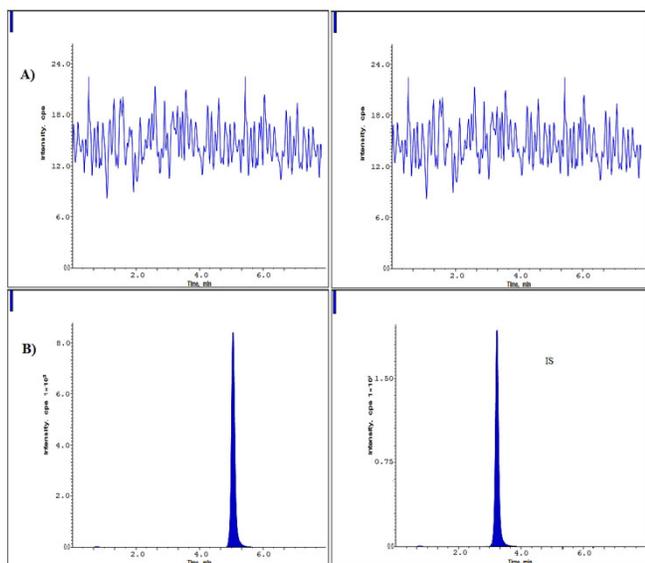


Figure 2: Chromatograms of blank plasma (A) and LLOQ (B) samples.

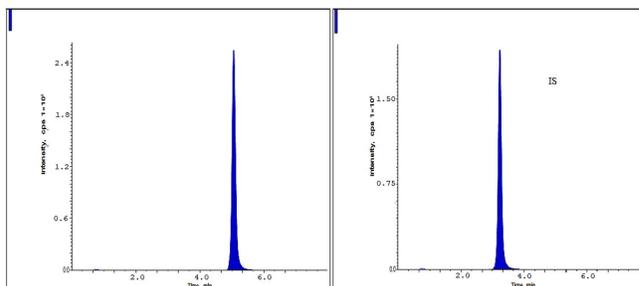


Figure 5: Chromatograms of saquinavir at HQC level.

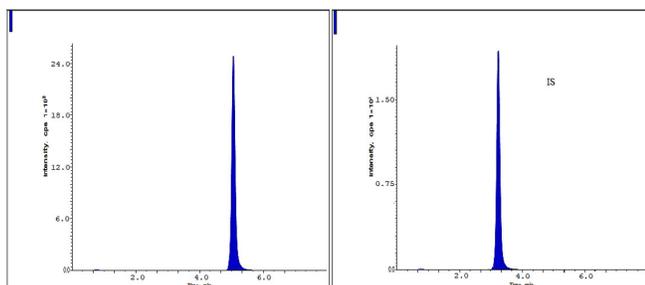


Figure 3: Chromatograms of saquinavir at LQC level.

Table 1: Calibration standards for saquinavir.

CS-ID	Concentration (ng/ml)	Mean <sup>a</sup> (ng/ml)	%RSD	%RE
CS-1	260.4	255.4	3.8	1.92
CS-2	520.8	512.6	2.7	1.57
CS-3	1302	1351	2.1	-3.76
CS-4	3100	2995	4.2	3.39
CS-5	5100	5236	3.5	-2.67
CS-6	6950	7024	1.9	-1.06
CS-7	8750	8513	2.9	2.71
CS-8	10416	9976	3.7	4.22

CS- Calibration standard; RSD- Relative standard deviation; RE- Relative error

Table 2: Intra-batch and Inter-batch Accuracy and Precision

Concentration level	Nominal concentration (ng/ml)	Intra-batch			Inter-batch		
		Amount found <sup>a</sup> (ng/ml)	%RSD	%RE	Amount found <sup>b</sup> (ng/ml)	%RSD	%RE
LLOQ	260.4	248.8523	4.1	-4.52	254.63	2.8	-2.21
LQC	729.1	755.4857	3.1	3.62	773.96	5.2	6.15
MQC	5208	5352.346	2.9	2.77	5415.85	3.7	3.99
HQC	7812	7857.036	5.3	0.58	8271.02	2.4	5.88

RSD: Relative standard deviation; RE: Relative error;

**Table 3: Extraction Recovery of saquinavir and IS.**

Concentration level	A	B	% Recovery	% Mean recovery	%RSD
LQC	24430	23110	94.6		
MQC	174500	161238	92.4	97.07	5.28
HQC	261750	272743	104.2		
IS	191216	181272	94.8		

RSD- Relative standard deviation; A, mean recoveries of un-extracted samples; B, average recovery of extracted samples.

**Table 4: Matrix Effect Results.**

Saquinavir	LQC level			HQC level		
	Analyte MF	IS MF	IS normalized MF	Analyte MF	IS MF	IS normalized MF
L-1	1.09	1.13	0.96	1.12	1.08	1.04
L -2	1.05	1.09	0.96	1.09	1.1	0.99
L -3	1.11	1.03	1.077	1.07	1.02	1.05
L -4	1.12	1.11	1.00	1.03	1.1	0.95
L -5 <sup>a</sup>	1.11	1.08	1.02	1.08	1.11	0.97
L -6 <sup>a</sup>	1.13	1.07	1.05	1.12	1.02	1.09
L -7 <sup>b</sup>	1.08	1.12	0.96	1.09	1.11	0.98
L -8 <sup>b</sup>	1.02	1.09	0.93	1.16	1.09	1.06
Mean		0.99			1.016	
SD		0.047			0.05	
%RSD		4.745			4.98	

a, Hemolyzed lot; b, Lipemic lot; MF, matrix factor; RSD, Relative standard deviation

**Table 5: Stability Results of Saquinavir**

Parameter	QC level	X	Y	%RSD	%Stability
Freeze and thaw stability	LQC	260.4	248.5121	4.2	95.43475
	HQC	7812	7976.19	2.9	102.1018
Stability in refrigerator (1–10°C for 48 h)	LQC	260.4	268.4834	3.4	103.1042
	HQC	7812	8101.558	1.9	103.7066
Bench-top stability (at <10°C for 20 h)	LQC	260.4	272.5972	2.5	104.684
	HQC	7812	7458.641	3.7	95.47672
Long term stability (60 days at –20°C)	LQC	260.4	277.7627	5.8	106.6677
	HQC	7812	7627.526	2.7	97.63858
In-injector stability (at 10°C for 72 h)	LQC	260.4	267.7616	6.2	102.8271
	HQC	7812	8118.302	1.61	103.9209
Long-term stability (60 days at –70°C)	LQC	260.4	248.7905	2.4	95.54165
	HQC	7812	7801.318	4.6	99.86327

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