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VALIDATED HPTLC METHOD FOR SIMULTANEOUS DETERMINATION OF LOPINAVIR AND RITONAVIR IN TABLET DOSAGE FORM

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ABSTRACT

A high performance thin layer chromatographic method has been developed for the simultaneous determination of lopinavir and ritonavir from tablet dosage form. Separation was performed on aluminum HPTLC plate (20×10cm) precoated with silica gel F_{254} HPTLC plates as stationary phase and the mobile phase consisting of toluene, ethyl acetate, methanol, formic acid (6:4, 4.5:0.5:0.5v/v/v/v) and wavelength of detection 254nm was used. After development, plates were observed under UV light. The detector response was linear in the range of 2µg/spot - 12µg/spot and 2 µg/spot - 6 µg/spot for lopinavir and ritonavir respectively. The developed method was validated as per ICH guidelines. The validated lowest limit of detection was 0.004827 µg /spot and 0.003369 µg /spot whereas lowest limit of quantification was 0.014627 µg /spot and 0.010208µg /spot for lopinavir and ritonavir respectively. The described method has the advantage of being rapid and easy. Hence it can be applied for routine quality control analysis of lopinavir and ritonavir from pharmaceutical preparation and stability studies.

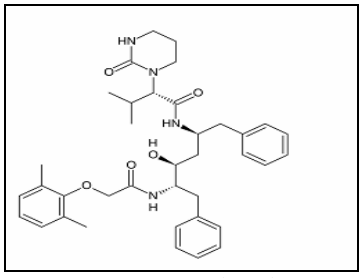
KEYWORDS: Lopinavir, Ritonavir, HPTLC, Validation.

INTRODUCTION

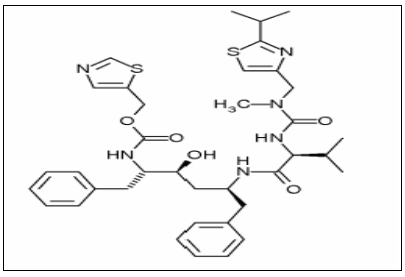
Lopinavir chemically (2S)-N-[(2S,4S,5S)-5-[2-(2,6dimethylphenox acetamido]-4-hydroxy-1,6-iphenylhexan-2-yl]-3-methyl-2-(2-oxo-1,3-diazinan-1-yl)butanamide and its empirical formula is $C_{37}H_{48}N_4O_5$ with a molecular weight of 628.80 (figure 1 A) [1-3]and Ritonavir (5s, 8s, 10s,11s)-10-hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl) -4-thiazolyl]-3,6-dioxo-8,11-is (phenylmethyl)-2,4,7,12-etraazatridecan-13-oic acid 5-thiazolyl methyl ester of molecular formula $C_{37}H_{48}N_6O_5S_2$ and its molecular weight is 720.95(figure 1 B).[1-3] These are antiretroviral drugs from protease inhibitor class. The drugs have been proved to be effective in anti-HIV treatment.

Ritonavir is the most potent protease inhibitor in its ability to inhibit CYP-450 and efflux pump-P-glycoprotein as a result the potential for severe drug interaction is quite great because of strong CYP-450 inhibiting effect of ritonavir. The drug has found value when used in fixed dosage form combination with other PIs to block their metabolism and acts as a booster for these drugs. In these cases ritonavir is used in a sub therapeutic dose but boosts the effectiveness of co administered drug.[4-7]

Literature survey of lopinavir and ritonavir either single or in combination with ritonavir revealed several methods based on HPLC and spectrophotometric methods in pharmaceutical formulation .however there are few HPTLC method for simultaneous determination. The proposed method was validated as per ICH guideline.



A: Lopinavir



B: Ritonavir

Figure 1: Structures of Lopinavir and Ritonavir.

EXPERIMENTAL[8-31]

Material and Method

Reagents (AR Grade)

Methanol, toluene, ethyl acetate, formic acid were produced from Merck India Pvt. Ltd., Mumbai.

Pure Drugs (Working Standards)

Working standards of lopinavir and ritonavir was obtained as a gift sample from Emcure Pvt Ltd and Lopimune tablet (lopinavir-200mg, ritonavir-50 mg) was obtained from local market and analyzed by proposed method.

Instrument

Camag HPTLC system consisting of linomat 5 applicator, camag TLC scanner 3 and Win CATS software V-1.4.4 was used for chromatographic separation. Spotting of samples was done by using Hamilton microliter syringe.

Procedure

Chromatographic condition

Table no.1: Chromatographic condition.

Executed by	**Anchrom test lab pvt ltd**
Plate size	(XxY)20.0x10.0 cm
Material	HPTLC plates silica gel 60F 254
Manufacture	E.MERCK KGaA
Pre-washing	Methanol
Modification	No

Methanol was used as a solvent for solution preparation. Stationary phase was aluminium HPTLC plate (20×10cm) precoated with silica gel F_{254}. Mobile phase consisting of toluene, ethyl acetate, formic acid in the ratio of 6:4.5:0.5:0.5 v/v/v/v was used. Linear ascending development was carried out in a 20×10 cm twin trough glass chamber using mobile phase. Five minutes saturation was required. The development distance was 8 cm which was achieved in 20 min. The TLC plates were removed from chamber and dried at 35^0c for 5 min. The wavelength of detection selected was 253nm since both drugs showed optimum absorbance at that wavelength. The slit dimension was kept 6.00×0.45mm, scanning speed 20 mm/sec and data resolution 100 mcm/step. Atypical densitogram of working standard solution is as shown in Fig.2.

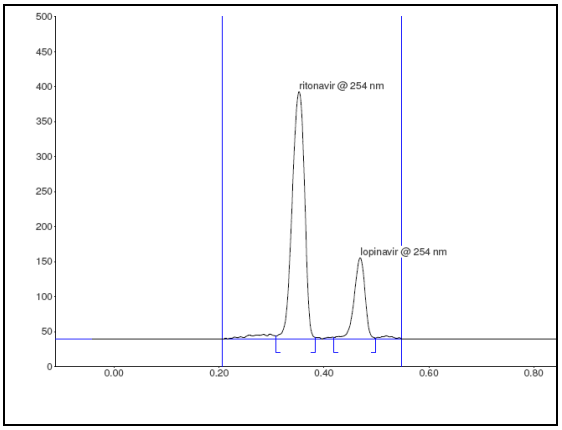


Fig 02: Densitogram of Ritonavir and Lopinavir.

A) Standard Preparation

a) Solution A (Lopinavir)

An accurately weighed quantity of 10 mg of Lopinavir was dissolved in methanol and volume was made up to 10 ml. Pipette out 1ml from this and dissolves in methanol to get 10ml with the same solvent.

b) Solution B (Ritonavir)

An accurately weighed quantity of 10 mg of Ritonavir was dissolved in methanol and volume was made up to 10 ml. Pipette out 1ml from this and dissolves in methanol to get 10ml with the same solvent.

B) Sample preparation

Twenty tablets were weighed and finely powdered. An accurately weighed quantity of tablet powder equivalent to about 10mg of Lopinavir and 2.5mg of Ritonavir was transferred to 25ml volumetric flask, sonicated with 15 ml methanol for 15 min. The volume was made up to the mark with methanol and filtered through whattman filter paper. Further dilutions were carried out to get final concentration 10µg/ml of Lopinavir and 2.5 µg/ml of Ritonavir was used as sample solution.

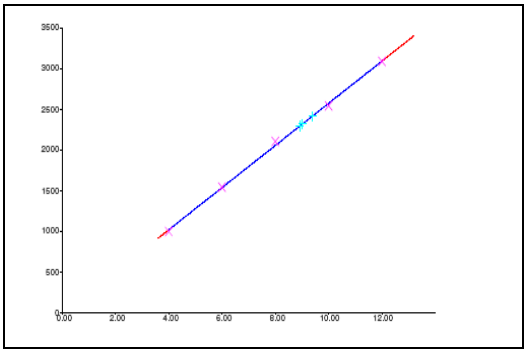
Analysis of standard Laboratory Mixture

Two bands of standard solution and six bands of marketed formulation of equal volume (5µl) were applied on TLC plate and it was developed and scanned as per chromatographic conditions.

Study of Beer's –Lambert's Law for Lopinavir and Ritonavir

The standard solutions of Lopinavir and Ritonavir were applied to the HPTLC plate in the range of 4-12µg/ml for Lopinavir and 2-6 µg/ml of Ritonavir with the help of micro liter syringe using sample applicator. The plate was developed and scanned in above established chromatographic conditions of drugs and curves (Concentrations Vs peak area) were constructed. The results are shown in the table.

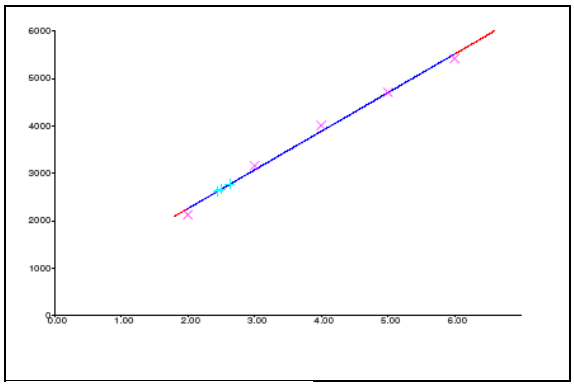
The calibration curves were shown in fig.03.



Sr. No.	Concentration of Lopinavir	Area Mean
1	4	992.62
2	6	1538.93
3	8	2099.89
4	10	2535.60
5	12	3085.21

Y = -22.28 + 259.1 * X r = 0.99925 sdv = 1.79

Fig. No. 03: Calibration Curve for Lopinavir.



Sr. No.	Concentration of Ritoinavir	Area Mean
1	2	2114.12
2	3	2148.22
3	4	3999.26
4	5	4696.95
5	6	5406.89

Y = 619.4 + 813.4 * X r = 0.99624 sdv = 3.33

Fig. No.: 04 Calibration Curve of Ritonavir.

Application of proposed method for estimation of Lopinavir and Ritonavir in laboratory mixture

Accurately weighed quantity if 10mg of Lopinavir and 2.5 mg Ritonavir were transferred to 25 ml volumetric flask, dissolve in methanol and volume was made up to the mark .Resulting solution has 400µg/ml of Lopinavir and 100µg/ml of Ritonavir. The solution was diluted to get the concentration 10µg/ml of Lopinavir and 2.5 µg/ml of Ritonavir, the solution was spotted for assay of Lopinavir and Ritonavir. The concentrations were calculated by regression equation and the results are shown in the table 34. The peak areas of standard and sample bands were compared to obtain a concentration of laboratory mixture. And % estimation was calculated using following formula,

% Estimated = Amt. Estimated / Amt. applied x 100

Table No: 2 Estimation of Lopinavir and Ritonavir in mixture.

Component	Amount taken (µg/ml)	Amount found (µg/ml)	Amount found %	%RSD
Lopinavir	10	9.33	93.31	0.5859
Ritonavir	2.5	2.52	100.8	0.4131

Application of proposed method for estimation of Lopinavir and Ritonavir in Marketed formulation

Table No: 3 Estimation of Lopinavir and Ritonavir in Marketed formulation.

Component	Amount taken (µg/ml)	Amount found (µg/ml)	Amount found %	%RSD
Lopinavir	10	9.43	94.30	0.5127
Ritonavir	2.5	2.49	100.0	0.7573

Validation

Validation of the proposed method was validated in terms of linearity, accuracy, precision, limit of detection, limit of quantification, ruggedness.

Linearity

Linearity evaluate the analytical method's ability (within the given range) to obtain a response that directly proportional to concentration (amount of analyte in the sample. A solvent system that would give dense and compact spot with significant R_f values were desire for

quantification of Lopinavir and Ritonavir in marketed formulation. The mobile phase consisting of toluene, ethyl acetate, methanol and formic acid in the ratio 6:4.5:0.5:0.5 gave Rf value 0.36±0.02 and 0.23±0.01 for Lopinavir and Ritonavir respectively. The linear regression data n=5 showed a good relationship over the concentration range of 2-8 µg/ml for Lopinavir and 2-6 µg/ml of Ritonavir respectively as shown in fig.3 and 4.

Selectivity

The sensitivity of measurements of Lopinavir and Ritonavir by proposing a method by use of the proposed method was estimated in term of the limit of Quantitation (LOQ) and the lowest concentration detected under the chromatographic conditions as the Limit of detection (LOD).

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were separately determined based on the standard deviation of the response of the calibration curve. The standard deviation of the y intercept and slope of calibration curve was used to calculate the LOD and LOQ. The results of the same are shown in the table no.49.

LOD calculated from the formula

$$\text{LOD} = \frac{3.3 \times SD}{S}$$

where, SD = Standard deviation, S = Slope

LOQ calculated from the formula

$$\text{LOQ} = \frac{10 \times SD}{S}$$

where, SD = Standard deviation, S = Slope.

Table No 4: LOD and LOQ For HPTLC.

Parameters	Lopinavir	Ritonavir
LOD	0.004827	0.003369
LOQ	0.014627	0.010208

Specificity

Specificity is the ability to find and quantify the compound of interest also in the presence of other compounds. This means for chromatographic methods, that the analyte can be separated with sufficient resolution and that it can be directed with suitable instruments. The specificity of method was checked by comparing the peak of pure Lopinavir and Ritonavir with that of Tablet formulation containing other excipients along with pure drugs. A baseline should not give any peak at corresponding retention factor, also should not produce noise.

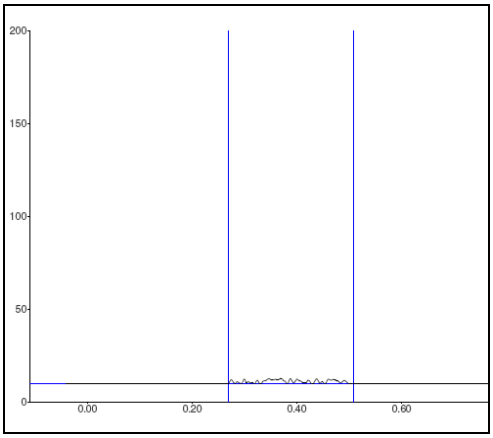


Fig.No:5 Chromatogram of mobile phase.

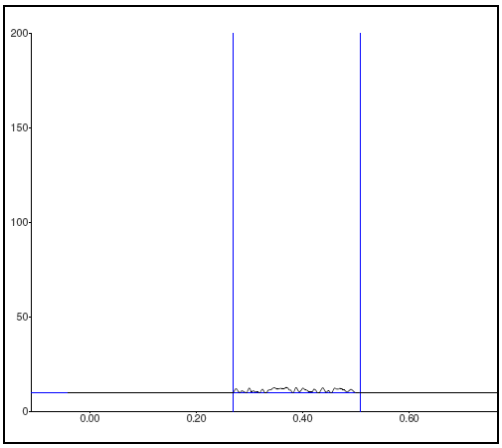


Fig. No: 6 Chromatogram of solvent.

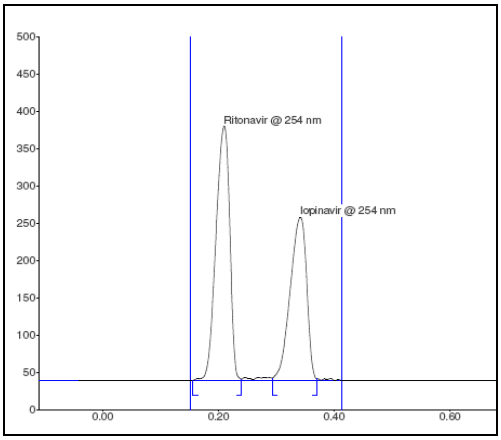


Fig. No: 7 Chromatogram of Drug

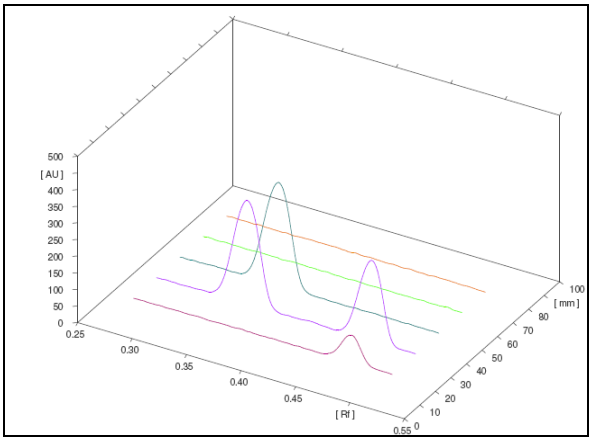


Fig. No: 8 3D diagram of Lopinavir, and Ritonavir, mixture & mobile phase mixture.

Repeatability

Repeatability of sample application was assessed by spotting µl of drug solution 5 times on the TLC plate followed by development of plate and recording peak area for 5 spots and %RSD determined. Repeatability of measurement of peak area was determined by spotting 10µl of Lopinavir and 2.5 µl of Ritonavir on a plate and developed the plate. The separate spot was scanned 5 times without changing the position of the plate and %RSD for measurement of peak areas of Lopinavir and Ritonavir were found to be 1.6402 % and 1.3102 % respectively.

Table No5: Repeatability.

Drug	Applied amount (µg/ml)	Amount found (µg/ml)	% RSD
Lopinavir	**10**	**9.2033**	**1.7699**
Ritonavir	**2.5**	**2.4533**	**1.3102**

Precision

The intra-day precision was determined by analyzing standard solution in the concentration range of 2-12µg/spot for Lopinavir and 2-6µg/spot for Ritonavir for three times on the same day while inter-day precision was determined by analyzing corresponding standard daily for 3 days over a period of 1 week. The intra-day and inter-day % RSD for both drugs were found to be linear in the range of 1.39-1.84% and 1.14-1.86 % for Lopinavir and 0.92-1.13 % and 1.14-1.79 % of Ritonavir respectively, shown in table -6.

These values indicate that the method is précised.

Table No 6: Intraday and Interday Precision.

Intraday study			Interday study		
Drug	**Concentration**	**% RSD**	**Drug**	**Concentration**	**% RSD**
Lopinavir	6 µg/spot	1.6339	**Lopinavir**	6µg/spot	1.8699
	8 µg/spot	1.3949		8µg/spot	1.1408
	10 µg/spot	1.8409		10µg/spot	1.2232
Ritonavir	3µg/spot	0.9226	**Ritonavir**	3µg/spot	1.7925
	4µg/spot	1.0661		4µg/spot	1.1456
	5µg/spot	1.1301		5µg/spot	1.7513

**Mean of six determinations*

Accuracy

Accuracy of the proposed method was ascertained on the basis of recovery studies performed by the standard addition method at different levels of standard taken (i.e. 80to120%) A sample solution having concentration 10µg/ml of Lopinavir and 2.5µg/ml of Ritonavir were taken from stock solution of marketed formulation. To the above prepared solutions 80%, 100%, and 120% of the standard drug solutions were spiked and the percentage recoveries were found to be within the limits are given in the table 7.

Recovery is calculated by following formula,

% Recovery = A-B/C x 100

Where,

A= total amount of drug estimated, µg/ml

B= Amount of the drug contributed by tablet powder, µg/ml

C= Amount of pure drug added µg/ml.

Table No 7: Recovery studies of Lopinavir and Ritonavir for HPTLC.

Level of recovery	Drug	Amount of drug taken µg/spot	Amount of drug added µg/spot	% Recovery	S.D.	% RSD
80	Lopinavir	10	8	91.8	0.864947	0.938053
	Ritonavir	2.5	2	87.8	0.979864	1.122067
100	Lopinavir	10	10	92.0	0.84388	0.916331
	Ritonavir	2.5	2.5	86.2	1.398714	1.59853
120	Lopinavir	10	12	92.7	0.679117	0.735134
	Ritonavir	2.5	3	88.1	1.238238	1.41486

**Mean of six determinations*

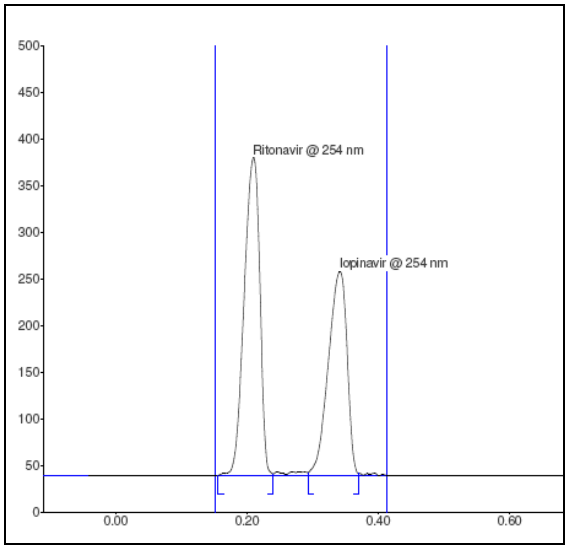


Fig. No.9: Chromatogram of Lopinavir, and Ritonavir.

Ruggedness

The Ruggedness studies were carried out for different parameters i.e. different elapsed times (Intra-day and Inter-day) and different analysts.

RESULTS AND DISCUSSION

For HPTLC method development, both pure drugs were spotted on TLC plates and run in mobile phase consisting of toluene, ethyl acetate and methanol was tried in different ratio like 6:2:2 (v/v/v) & 6:3:1 (v/v/v) was tried for both the drugs simultaneously. The mixture of acetonitrile, water and methanol in the ratio of 5.5:3:1.5 was also tried no proper spot were observed.

Then finally in toluene, ethyl acetate, methanol and formic acid in the ratio 6:4.5:0.5:0.5 was tried and this gave better result, both peaks were symmetrical in size, Rf value 0.36±0.02 and 0.23±0.01 for Lopinavir and Ritonavir respectively. The optimum wavelength selected was 254nm. The spot pure drugs were observed in the chromatogram of the drug sample extracted from the marketed tablets. There was no interference from the excipients present in tablets. The drug content was found to be 94.30 and 100.0 for Lopinavir and Ritonavir respectively.

The polynomial regression data for calibration poll showed a good linear relationship over concentration range 2µg/spot -12µg/spot and 2 µg/spot - 6 µg/spot for Lopinavir and Ritonavir respectively. Lopinavir and Ritonavir show good correlation coefficient (R^2= 0. 9962 and R^2=0. 9992 for Lopinavir and Ritonavir respectively) in giving concentration range. Limit of detection (LOD) was found to be 0.0048 µg/spot and limit of quantification (LOQ) was found to be 0.0146 µg/spot for Lopinavir and 0. 0033 µg/spot and 0. 01020 µg/spot for Ritonavir respectively.

The result in the table revealed the excellent accuracy and precision of assay method. The proposed method used for simultaneous determination of a drug in combination from a pharmaceutical dosage form after spiking to 80%, 100%, and 120% of additional drug afforded recover 87.8% - 92.7%. The ruggedness of the method was determined by Intraday, inter day and different analyst studies.

Table No 8: Summary of validation parameter for HPTLC.

Parameter	Lopinavir	Ritonavir
Linearity range	2µg/spot -12µg/spot	2 µg/spot - 6 µg/spot
Regression coefficient	0.9962	0.9992
Slope(m)	259.1	813.4
Intercept(c)	-22.28	619.4
LOD(µg/spot)	0.004827	0.003369
LOQ(µg/spot)	0.014627	0.010208
Precision (%RSD)	1.39-1.84%	0.92-1.13 %
Intra day **Interday**	1.14-1.86 %	1.14-1.79 %
% Recovery	92.1666	87.5666
Assay (%)	94.30	100.0
Repeatability	1.7699	1.3102

SUMMARY

The present work deals with the simultaneous estimation of Lopinavir and Ritonavir in bulk and pharmaceutical dosage form using the HPTLC method .Study was performed in Anchrome lab. Pvt. Ltd. CAMAG Automatic TLC Sampler 4 (ATS4) "ats4_070618" S/N 070618 instrument was used. HPTLC plates silica gel 60F 254 was used as the stationary phase. The optimal composition of the mobile phase selected for analysis was toluene, ethyl acetate, methanol and formic acid in the ratio 6:4.5:0.5:0.5. Solution of Lopinavir and Ritonavir in appropriate dilution was scanned using double beam UV visible spectrophotometer in the spectrum mode between the wavelength range of 400.0nm to 200nm and their spectra were run thus the wavelength selected was 254.0nm .Both the drugs were found to have significant absorbance at this wavelength. The retention factor for Lopinavir and Ritonavir was found to be0. 36±0.02 and 0.23±0.01 respectively. Linearity of detector response was studied by plotting a

graph of concentration vs. mean peak area. Linearity was observed in the concentration range 2-12µg/ml for Lopinavir and 2-6µg/ml of Ritonavir. Coefficient of correlation was found to be 0.999and 0.996 for Lopinavir and Ritonavir respectively. The developed method was employed for the analysis of marketed formulation. The amount of drug obtained was in accordance with label claim. The recovery studies were carried out at three levels, i.e.80 %, 100% and120% by the standard addition method. The precision of the proposed method was also established. The method was found to be accurate and precise.

CONCLUSION

The HPTLC method was successfully developed and validated. The standard deviation and %RSD calculated for the proposed HPTLC method .The method is now, indicating a high degree of precision of the method. The result of the recovery studies performed show a high degree of accuracy of the proposed method. Hence, it can be concluded that the developed HPTLC method is simple, accurate, Précised and economical and can be employed successfully in the estimation of Lopinavir and Ritonavir in bulk and pharmaceutical dosage form.

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