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HPTLC Method for Simultaneous Determination of Lopinavir and Ritonavir in Capsule Dosage Form

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Abstract: A rapid and simple high performance thin layer chromatography (HPTLC) method with densitometry at $\lambda=263$ nm was developed and validated for simultaneous determination of lopinavir and ritonavir from pharmaceutical preparation. Separation was performed on aluminum-backed silica gel 60F₂₅₄ HPTLC plates as stationary phase and using a mobile phase comprising of toluene, ethyl acetate, methanol and glacial acetic acid, in the volume ratio of 7.0:2.0:0.5:0.5 (v/v) respectively. After development, plates were observed under UV light. The detector response was linear in the range of 6.67 to 20.00 $\mu\text{g/spot}$ and 1.67 to 5.00 $\mu\text{g/spot}$ for lopinavir and ritonavir respectively. The validated lowest limit of detection was 21.00 ng/spot and 5.10 ng/spot whereas lowest limit of quantification was 7.00 ng/spot and 21.00 ng/spot for lopinavir and ritonavir respectively. The percentage assay of lopinavir and ritonavir was found between 98.23 to 102.28% and 98.03 to 103.50% 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, Pharmaceutical formulation

Introduction

Lopinavir¹⁻² has the molecular formula C₃₇H₄₈N₄O₅, and molecular weight 628.80 g mol⁻¹. The chemical name of lopinavir is [1S-[1R*, (R*), 3R*, 4R*]]-N-[4 [[(2,6dimethylphenoxy) acetyl] amino]-3-hydroxy-5-phenyl 1(phenyl methyl) pentyl] tetrahydro-alpha-(1-methylethyl)-2-oxo-1(2H) pyrimidineacetamide. Ritonavir¹⁻² has the molecular formula

$C_{37}H_{48}N_6O_5S_2$, and its molecular weight is $720.95 \text{ g mol}^{-1}$. Ritonavir is chemically called as 10-Hydroxy-2-methyl-5- (1-methylethyl) -1- [2-(1-methylethyl) -4-thiazolyl] -3, 6-dioxo-8, 11-bis (phenylmethyl) -2, 4,7,12 -tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester, [5S-(5R*, 8R*, 10R*, 11R*)].

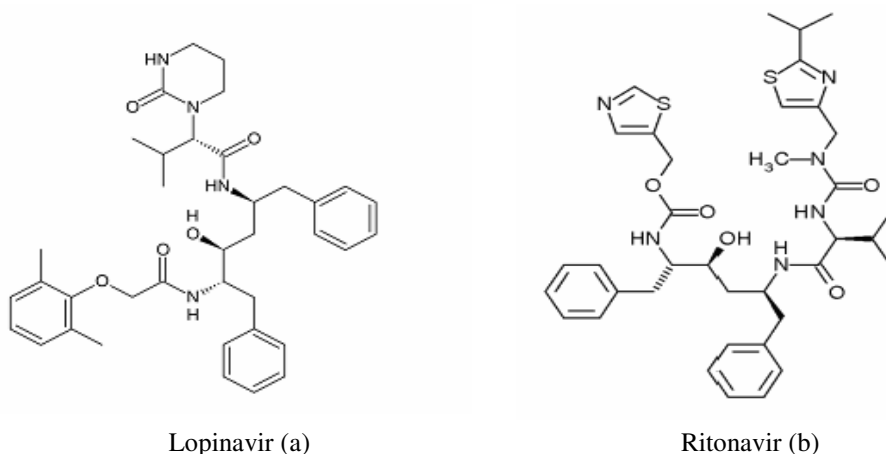


Figure 1. Structures of lopinavir and ritonavir

Combination therapy with the HIV protease inhibitors lopinavir and ritonavir (Sustained release capsule with combination of lopinavir 133.3 mg and ritonavir 33.3 mg is available in market by brand name kaletra[®]) has been shown to be effective against drug-resistant HIV-1³. These agents are metabolized by cytochrome P-450 (CYP) 3A in the liver^{4,6}. When lopinavir is administered with ritonavir as kaletra[®], ritonavir inhibits the CYP 3A-mediated metabolism of lopinavir, thereby providing increased plasma levels of lopinavir^{4,6}. In this work new HPTLC method is developed, optimized and validated⁷⁻¹¹ for the assay of two drugs *viz.*, lopinavir and ritonavir in combined dosage forms.

A survey of literature reveals that there are two methods reported for the simultaneous determination of lopinavir and ritonavir in pharmaceutical preparations using HPLC^{12,13}. Whereas remaining methods are given for determination of lopinavir and ritonavir in human plasma¹⁴⁻²⁸ using HPLC either by using UV/Visible or Mass Spectroscopy detector. However, no references are reported so far for the simultaneous determination of both drugs in combined dosage form or any such pharmaceutical preparations by HPTLC. In this communication we report a new simple, rapid and precise HPTLC method for simultaneous determination of lopinavir and ritonavir in combination capsule, which can be used for its routine analysis in ordinary laboratories.

Experimental

Chemicals and reagents

The lopinavir and ritonavir working standards were obtained as a gift sample from Cipla Ltd. Mumbai. The formulation, sustained release capsule with combination of lopinavir 133.3 mg and ritonavir 33.3 mg is available in market by brand name KALETRA; toluene, ethyl acetate, methanol and glacial acetic acid were of Qualigens; pre-coated silica gel 60 F₂₅₄ HPTLC plates (Merck # 5548) of E-Merck. All dilutions were performed in standard volumetric flasks.

Instrumentation and chromatographic conditions

Chromatography was performed on pre-coated silica gel 60 F₂₅₄ HPTLC plates (Merck # 5548). Before use they were pre-washed with methanol and dried in an oven at 105°C for 2 h. 10 µL of sample were spotted 8 mm from the edge of the plates by means of a Camag Linomat IV sample applicator. The plates were developed to a distance of 85mm in a Camag twin-trough chamber previously equilibrated 15min with mobile phase *i.e.* toluene:ethyl acetate:methanol:glacial acetic acid [7.0:2.0:0.5:0.5 (v/v)]. The chromatographic conditions had previously been optimized to achieve the best resolution and peak shape. Plates were evaluated by densitometry at $\lambda = 263$ nm with a Camag Scanner II, in conjunction with CATS software for quantitation. The typical chromatogram is shown Figure 2.

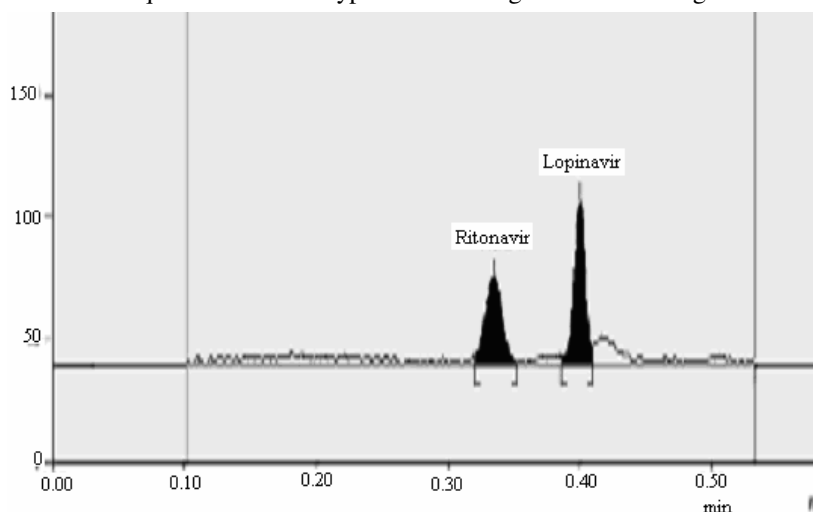


Figure 2. Typical chromatogram showing the separation of (1) Ritonavir and (2) Lopinavir

Preparation of standard stock solution of lopinavir

Accurately weigh 25.6 mg pure standard of lopinavir (97.59 %) and transfer to 10 mL volumetric flask. The drug was dissolved in methanol, diluted up to the mark with methanol and mixed well. This gave a standard stock solution of strength 2500 µg/mL of lopinavir.

Preparation of standard stock solution of ritonavir

Accurately weigh 10.0 mg pure standard of ritonavir (100.13%) and transfer to 10mL volumetric flask. The drug was dissolved in methanol, diluted up to the mark with methanol and mixed well. This gave a standard stock solution of strength 1000 µg/mL of ritonavir.

Preparation of working standard solution

Further the mixture of working standard solution was prepared by diluting 26.60 mL of lopinavir (2500 µg/mL) and 16.65mL of ritonavir (1000 µg/mL) standard stock solution in 50.0mL volumetric flask with methanol to get strength of 1333.00 µg/mL of lopinavir and 333.00 µg/mL of ritonavir.

Preparation of sample solution

Twenty capsules (KALETRA) were weighed and the average weight was calculated. The capsules were crushed to furnish a homogeneous powder and a quantity equivalent to one

capsule (431.01 mg) was weighed in a 100 mL standard volumetric flask. The powder dissolved in methanol and diluted up to the mark with methanol. That solution was then sonicated for 30 min. Then cooled to room temperature and diluted with methanol. The solution was filtered through Whatman No. 41 filter paper and the filtrate was used as sample solution.

Validation Procedures

Linearity

Seven different concentrations of mixture of lopinavir and ritonavir were prepared from stock solution of lopinavir (2500 µg/mL) and ritonavir (1000 µg/mL) in the range of 666.50 to 1999.50 µg/mL and 166.50 to 499.50 µg/mL respectively, in methanol to obtain desire linearity range. 10 µL of each solution was applied to a plate (*i.e.* 6.67, 8.66, 10.66, 13.33, 16.00, 18.66 and 20.00 µg/spot for lopinavir and 1.67, 2.16, 2.66, 3.33, 4.00, 4.66 and 5.00 µg/spot for ritonavir) by sample applicator and the plate was developed. The detector response to the different concentrations was measured. The drug peak-area was calculated for each concentration level and a graph was plotted of drug concentration against the peak-area. The plot was linear for lopinavir in the concentration range 6.67 to 20.00 µg/spot and for ritonavir was 1.67 to 5.00 µg/spot. This experiment was carried out thrice and the mean peak area response was used for the calculations. The data were analyzed by linear regression least-squares fitting. The statistical data obtained are given in Table 1.

Table 1. Analytical Performance Data
(Linear Regression Data for Calibration Curve)

	Lopinavir	Ritonavir
Linear working range (LWR), µg/spot	6.67 – 20.00	1.67 – 5.00
Std Err of Y Estimate	296.0132	551.6164
Correlation Coefficient	0.9973	0.9915
X Coefficient(s)	1028.891	4314.1418
Constant	-2359.61	-860.85
Std Err of Coefficient.	23.9161	178.4034

Limit of detection and limit of quantitation

The limit of detection (LOD) was found to be 1.5 ng/spot for lopinavir and 4.6 ng/spot for ritonavir. Limit of quantitation (LOQ) for lopinavir and ritonavir were determined experimentally by spotting six replicates of each drug at LOQ concentration. The LOQ of lopinavir and ritonavir were found to be 21.00 ng/spot and 5.10 ng/spot respectively.

System suitability

A system-suitability experiment was performed before determination of lopinavir and ritonavir in unknown samples. The coefficient of variation (CV) for peak area and R_f value for both the drugs was less than 2.0% for six replicates measurement of the same sample. This shows that the method and the system are suitable for determination of lopinavir and ritonavir.

Assay (from the pharmaceutical preparation)

10 µL working standard solution (13.33 µg/spot of lopinavir and 3.33 µg/spot of ritonavir) and sample solutions were spotted on the plate and the plate was developed and evaluated as described above. The procedure was repeated five times, individually weighing the capsule powder each time. The densitometric responses from the standard and sample were used to calculate the amounts of the drug in the capsule. The results obtained is as shown in Table 2

Table 2. Results of HPTLC Assay Studies (from pharmaceutical preparations) (n=5)

S. No.	Weight of sample taken, mg	Lopinavir		Ritonavir	
		% Assay	Amount found, mg	% Assay	Amount found, mg
1	432	98.23	130.94	103.50	34.46
2	435	98.46	131.24	98.03	32.64
3	425	102.28	136.34	101.76	33.89
4	439	99.80	133.04	99.78	33.23
5	433	100.73	134.27	103.37	34.42
Mean		99.90	133.17	101.29	33.73
Standard Deviation		1.5000	2.0000	2.1130	0.7040
% CV		1.50	1.50	2.09	2.09

Recovery studies

The accuracy of the experiment was established by spiking pre-analyzed sample with known amounts of the corresponding drugs at three different concentration levels *i.e.* 20, 40 and 60 % of the drug in the capsule (the external standard addition technique). The spiked samples were then analyzed for five times. The results from recovery analysis are given in Table 3 and 4 the mean recovery is within acceptable limits, indicating the method are accurate.

Table 3. Results from recovery analysis for lopinavir (n=5)

Level, %	Amount of lopinavir added, mg	Total amount of lopinavir found, mg	SD	% CV	% Recovery
0.0	(133.30)+0.00	135.28	4.1349	3.06	101.49
20	(133.30)+26.66	160.84	12.4488	7.74	100.55
40	(133.30)+53.32	186.28	9.3409	5.01	99.82
60	(133.30)+79.98	212.38	8.6555	4.08	99.58
				Average % Recovery	100.36

Table 4. Results from recovery analysis for ritonavir (n=5)

Level, %	Amount of ritonavir added, mg	Total amount of ritonavir found, mg	SD	% CV	% Recovery
0.0	(33.30)+0.00	34.01	0.9672	2.84	102.14
20	(33.30)+6.66	38.54	2.2264	5.78	96.45
40	(33.30)+13.32	46.84	1.2054	2.57	100.47
60	(33.30)+19.98	54.36	1.1327	2.08	102.03
				Average % Recovery	100.27

Results and Discussion

Use of pre-coated silica gel HPTLC plates with toluene:ethyl acetate:methanol:glacial acetic acid, in the volume ratio of 7.0:2.0:0.5:0.5 resulted in good separation of the drug.

Figure 2 shows a typical densitogram obtained from lopinavir and ritonavir. Regression analysis of the calibration data for lopinavir and ritonavir showed that the dependent variable (peak area) and the independent variable (concentration) were represented by the equations

$$Y=1028.89X+ (-2359.61) \text{ for lopinavir and } Y= 4314.14X+ (-860.85) \text{ for ritonavir.}$$

The correlation of coefficient (r^2) obtained was 0.9973 for lopinavir and that for ritonavir is 0.9915. That means a good linear relationship was observed between the concentration range 6.67 to 20.00 $\mu\text{g/spot}$ and 1.67 to 5.00 $\mu\text{g/spot}$ for lopinavir and ritonavir respectively. The system suitability experiment was carried out before the determination of lopinavir and ritonavir in unknown samples. The coefficient of variation was less than 2% for replicate measurements of the same sample. This shows that the method and the system both are suitable for the determination of unknown samples. The assay of lopinavir and ritonavir was found to be 99.90% and 101.29%. From the recovery studies it was found that about 100.36% and 100.27% of lopinavir and ritonavir respectively which indicates high accuracy of the method. The absence of additional peaks in chromatogram indicates non- interference of the common excipients used in capsules.

Method application

The validated HPTLC method was used for to lopinavir and ritonavir in their combined dosage form. The mean assay results, expressed as a percentage in the label claim, are shown in Table 2. The results indicated that the amount of each drug in the capsules is within the requirements of 80 to 110% of the label claim

Conclusion

As the proposed method is highly accurate, selective and precise hence can be used for a routine quality-control analysis and quantitative simultaneous determination of lopinavir and ritonavir in pharmaceutical preparations. The method is also fast and requires approximately 45 min for analysis.

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