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Research Article

# SIMULTANEOUS HPTLC DETERMINATION OF LOPINAVIR AND RITONAVIR IN COMBINED DOSAGE FORM

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**ABSTRACT**

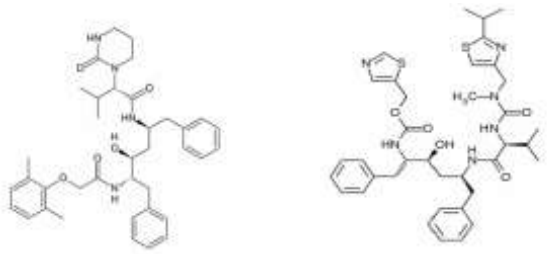
A simple, rapid and accurate High-performance thin-layer chromatography (HPTLC) method has been established and validated for the simultaneous determination of Lopinavir and Ritonavir in tablets. The method is based on HPTLC separation of the two drugs followed by densitometric measurements of their spots at 266 nm. The separation was carried out on Merck TLC aluminium sheets of silica gel 60F-254 using ethyl acetate: ethanol: toluene: diethylamine (7:2.0:0.5:0.5, v/v/v/v) as a mobile phase. Calibration curves were linear in range of 8-20 µg/ml and 2-10 µg/ml for Lopinavir and Ritonavir, respectively. Method was successively applied to tablet formulation. No chromatographic interferences from the tablet excipients were found. The method was validated in accordance with the requirements of ICH guidelines.

**Key words:** Lopinavir; Ritonavir; High-performance thin-layer chromatography; Validation.

## INTRODUCTION

Lopinavir[1] is chemically designated as [1S-[1R*,(R*), 3R*, 4R*]]-N-[4-[[(2,6 dimethylphenoxy)acetyl]amino]-3-hydroxy-5-phenyl-1-(phenylmethyl)pentyl]tetrahydro-alpha-(1methylethyl)-2-oxo 1 (2H) -pyrimidineacetamide. Its molecular formula is $C_{37}H_{48}N_4O_5$, and its molecular weight is 628.80. It is freely soluble in methanol and ethanol, soluble in isopropanol and practically insoluble in water.

Ritonavir[2] is chemically designated as 10-hydroxy-2-methyl-5-(1-methylethyl)-1- [2-(1-methylethyl)-4thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester, [5S-(5R*,8R*,10R*,11R*)]. Its molecular formula is $C_{37}H_{48}N_6O_5S_2$, and its molecular weight is 720.95. It is freely soluble in methanol and ethanol, soluble in isopropanol and practically insoluble in water.



**(a) Lopinavir** **(b)Ritonavir**

**Figure -1:** Structures of lopinavir and ritonavir

Combination therapy with the HIV protease inhibitors lopinavir and ritonavir (combination of lopinavir 200 mg and ritonavir 50 mg is available in market by brand name kaletra®) has been shown to be effective against drug-resistant HIV-1[3]. These agents are metabolized by cytochrome P-450 (CYP) 3A in the liver. 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 validated[7-11] 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 plasma[14-28] 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 tablet, which can be used for its routine analysis in ordinary laboratories.

## EXPERIMENTAL

### Materials and Reagents

Lopinavir and Ritonavir were kindly supplied as a gift sample by Cipla Pharmaceuticals Ltd. Mumbai. Toluene, methanol, ethyl acetate, diethylamine and glacial acetic acid were used as solvents to prepare the mobile phase. All the reagents used were of analytical reagent grade (S.D. Fine Chemicals, Mumbai, India) and used without further purification.

### Instrumentation and chromatographic conditions

The samples were spotted in the form of bands of width 6 mm with 100 µL sample syringe on precoated silica gel aluminium plate 60F-254 (20 cm x 10 cm) with 250 µm thickness; (E MERCK, Darmstadt, Germany) using a Camag Linomat V (Switzerland). The plates were prewashed with methanol and activated at 110 $^0$C for 5 min, prior to chromatography. A constant application rate of 150 nL /sec was employed and space between two bands was 11.6 mm. The slit dimension was kept at 6 mm x 0.45 mm. The mobile phase consists of ethyl acetate: ethanol: toluene: diethylamine (7:2.0:0.5:0.5, *v/v/v/v*). Linear ascending development was carried out in 20 cm x 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland). The optimized chamber saturation time for mobile phase was 25 min, at temperature (25$^0$C ± 2); the length of chromatogram run was 8 cm and TLC plates were air dried. Densitometric scanning was performed on Camag TLC Scanner 3 equipped with winCATS software version 1.3.0 at 266nm. The source of radiation utilized was deuterium lamp. Evaluation was performed using linear regression analysis *via* peak areas.

### Preparation of standard stock solution of lopinavir

Accurately weigh 25.0 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 25.0 mL of lopinavir (2500 µg/mL) and 10.0 mL of ritonavir (1000 µg/mL) standard stock solution in 50.0mL volumetric flask with methanol to get strength of 1000.00 µg/mL of lopinavir and 250.00 µg/mL of ritonavir.

**Preparation of sample solution**

Twenty tablets (KALETRA) were weighed and the average weight was calculated. The tablets were crushed to furnish a homogeneous powder and a quantity equivalent to one tablet was weighed in a 100 mL standard volumetric flask. The powder was 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 procedure**

**Optimization of HPTLC method**

Initially, ethyl acetate and ethanol in the ratio of 1:1 (*v/v*) was tried for both drugs simultaneously. The spots were not developed properly and dragging was observed. Then, toluene, ethanol and ethyl acetate in the ratio of 1:2.5:7 (*v/v/v*) was tried. The developed spots were diffused. To the above mobile phase, 0.2 mL glacial acetic acid was added. Both the peaks were symmetrical in nature and tailing was observed. To improve resolution, 0.5 ml diethylamine was added. Ultimately, mobile phase consisting of ethyl acetate:ethanol:toluene:diethylamine (7:2.5:0.5:0.5 v*/v/v/v*) gave good resolution. Both the peaks were symmetrical in nature and no tailing was observed when plate was scanned at 266 nm. The chamber was saturated with the mobile phase for 25 min at room temperature and plates were activated at 110 °C for 5 min to obtain well-defined spots.

**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 800.00 to 2000.00 µg/mL and 200.00 to 600.0 µg/mL respectively, in methanol to obtain desire linearity range. 10 µL of each solution was applied to a plate (*i.e*. 8.0, 10.0, 12.0, 14.0, 16.00, 18.00 and 20.00 µg/spot for lopinavir and 2.0, 3.0, 4.0, 5.0, 6.0, 8.0 and 10.0 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 8.0 to 20.00 µg/spot and for ritonavir was 2 to 10 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.

**Precision**

Precision of the method was determined in the terms of intra-day and inter-day variation (%RSD). Intra-day precision (%RSD) was assessed by analyzing standard drug solutions within the calibration range, three times on the same day. Inter-day precision (%RSD) was assessed by analyzing drug solutions within the calibration range on three different days over a period of a week. The results are shown in Table 1.

**Sensitivity**

The sensitivity of measurement of Lopinavir and Ritonavir by the use of proposed method was estimated in terms of Limit of Quantitation (LOQ) and Limit of Detection (LOD). The LOQ and LOD were calculated by visual detection. The LOD and LOQ value are 30 ng/spot and 50.0 ng/spot for lopinavir; 10.0 and 20.0 ng/spot for ritonavir.

**Accuracy**

To the pre-analyzed sample a known amount of standard solution of pure drug (lopinavir and ritonavir) was added at three different levels. These solutions were subjected to re-analysis by the proposed method; results of the same are shown in Table 1.

**Specificity**

Specificity of the method was ascertained by analyzing standard drug and sample. The mobile phase resolved both the drugs very efficiently as shown in Fig.2. The spot for lopinavir and ritonavir was confirmed by comparing the $R_f$ and spectra of the spot with that of standard. The wavelength 266 nm for detecting peak purity of lopinavir and ritonavir was assessed by comparing the spectra at three different levels, i.e., peak start (S), peak apex (M) and peak end (E) positions of the spot.

**Repeatability**

Repeatability of sample application was assessed by spotting (0.1 µg µL-1) of drug solution seven times on a TLC, followed by development of plate and recording the peak area for seven spots. The % RSD for peak area values of lopinavir and ritonavir was found to be 0.13 and 0.86, respectively.

**Assay (from the pharmaceutical preparation)**

10 µL working standard solution (10.00 µg/spot of lopinavir and 2.5 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 are as shown in Table 2.

**RESULTS AND DISCUSSION**

Use of pre-coated silica gel HPTLC plates with ethyl acetate: ethanol: toluene: diethylamine (7:2.0:0.5:0.5, *v/v/v/v*) 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=1428.89X+ (-4455.61) for lopinavir and Y= 6541.14X+ (-610.85) for ritonavir.

The correlation of coefficient (r2) obtained was 0.9998 for lopinavir and that for ritonavir is 0.9945. That means a good linear relationship was observed between the concentration range 8.0 to 20.00 µg/spot and 2.0 to 10.00 µg/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 99.98 ± 0.16 % and 101.29 ± 0.10 %, respectively. From the recovery studies it was found that about 98.58 ± 0.12 % and 100.3 ± 0.11 % 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 tablets.

**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 tablet 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 aroutine quality-control analysis and quantitative simultaneous determination of lopinavir and ritonavir in pharmaceutical preparations. The method is also fast and requires approximately 50 min for analysis.

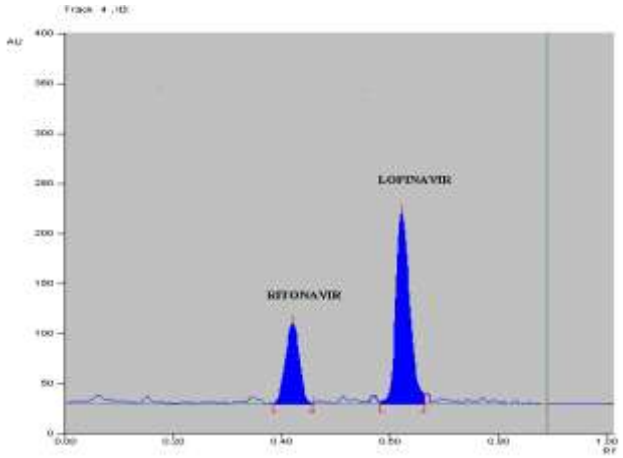



**Figure -2:** Densitogram of Ritonavir and Lopinavir

**Table -1: Summary of Validation Parameters Of Proposed HPTLC Method**

| Parameters | Lopinavir | Ritonavir |
|---|---|---|
| Range ( µg/spot) | 8-20 | 2-10 |
| Regression equation y=mx+c | Y=1428.89X + (4455.61) | Y=6541.14X + (-610.85) |
| Slope | 1428.89 | 6541.14 |
| Intercept | 4455.61 | 610.85 |
| Correlation coefficient ($r^2$) | 0.9998 | 0.9945 |
| LOD (ng per spot) | 30.0 | 10.0 |
| LOQ (ng per spot) | 50.0 | 20.0 |
| %Recovery ± SD,(n=3) | 99.58 ±0.12 | 100.3 ±0.11 |
| Repeatability (%RSD, n=6), | 0.13 | 0.86 |
| Interday precision (%RSD) (n = 3) at 3 range | 0.15-0.91 | 0.19-0.45 |
| Intraday precision (%RSD) (n = 3) at 3 range | 0.19-0.25 | 0.25-0.78 |

[a]SD = Standard deviation, [b]RSD = Relative standard deviation

**Table 2: Analysis of lopinavir and ritonavir in formulations by proposed method (n=6)**

| Formulation | Label Amount(mg) | | Amount found(mg) | | % Assay ± SD | |
|---|---|---|---|---|---|---|
| | Lopinavir | Ritonavir | Lopinavir | Ritonavir | Lopinavir | Ritonavir |
| Tablet | 100 | 25 | 99.98 | 24.98 | 99.98 ± 0.16 | 99.92 ± 0.10 |

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