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DEVELOPMENT AND VALIDATION OF A RP HPLC METHOD FOR THE SIMULTANEOUS ANALYSIS OF LOPINAVIR AND RITONAVIR IN TABLETS DOSAGE FORM.

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ABSTRACT

The objective of this review study is to analyze simple, accurate, precise and rapid stability indicating HPLC method for simultaneous determination of Lopinavir and Ritonavir in combined dosage forms.

A validated stability indicating reversed phase high-performance liquid chromatographic method was developed for the quantitative determination of two antiviral drugs viz. lopinavir (LPV) and ritonavir (RTV) on Phenomenex – Luna, C18 (250 x 4.6 mm i.d., 5µ) column using mobile phase composition of Buffer: Acetonitrile (55: 45 % V/V) at a flow rate of 1.5 ml/min.

Quantification was achieved with ultraviolet detection at 255nm. The retention time obtained for ritonavir was at 10.92 min and for lopinavir was at 13.23 min. This method has been validated and shown to be specific, sensitive, precise, linear, accurate, rugged, robust and fast.

KEYWORDS: HPLC, method Validation, Lopinavir, ritonavir, antiviral.

INTRODUCTION

Lopinavir is with chemicals named as (2S)-N-[(2S, 4S, 5S)-5-[2-(2,6-dimethylphenoxy)acetamido]-4-hydroxy-1,6-diphenylhexan-2-yl]3-methyl-2-(2-oxo-1,3-diazinan-1-yl)butanamide. It's freely soluble in alcohol and grain alcohol, soluble in isopropyl alcohol and much insoluble in water. Lopinavir inhibits HIV proteolytic enzyme, inflicting the protein incapable of process the polyprotein precursor. This ends up in the assembly of non-infectious and immature HIV particles.

Ritonavir is with chemicals named as one,3-thiazol-5-ylmethylN-[(2S,3S,5S)-3-hydroxy-5-[(2S)-3-methyl-2-)carbamoyl]amino}butanamido]1,6-diphenylhexan-2yl]carbamate. It's freely soluble in alcohol (methanol and ethanol), soluble in isopropyl alcohol and much insoluble in water. Ritonavir, a selection competitive reversible inhibitor of HIV proteolytic enzyme, interferes with the formation of essential proteins and enzymes. At that time the formation of immature and non-infectious viruses follows. It additionally interferes with the assembly of infectious HIV and limits any infectious unfold of the virus. (1, 2)

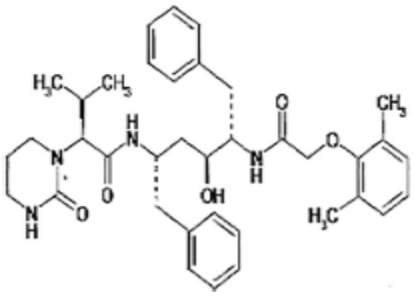

Fig1: structure of lopinavir

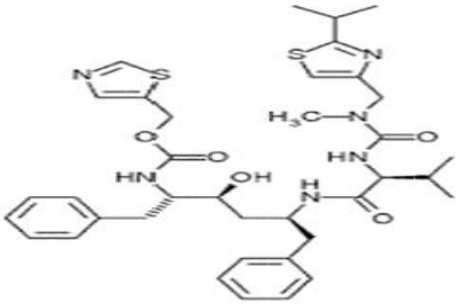

Fig2: structure of Retonavir

Lopinavir and PI (LPV/r) could be a fastened combination for human immunological disorder virus (HIV) proteolytic enzyme inhibitors (PIs). The counseled dose of LPV/r is 400/100 mg taken doubly daily with food, as a part of a mixture medical care with different antiretroviral (ARV) agents. The target of this review

study is to research easy, accurate, precise and fast stability indicating HPLC methodology for synchronous determination of Lopinavir and PI in combined dose forms. (3, 4)

Drug profile

Parameters	**Lopinavir**	**Ritonavir**
Molecular formula	C37H48N4O5	C37H48N6O5S2
Molecular weight	628.8008	720.944
Solubility	Acetonitrile, ethanol, DMSO, and dimethyl formamide (DMF).	Acetonitrile, ethanol, DMSO, and dimethyl formamide (DMF).
Mechanism of action	Lopinavir is an inhibitor of the HIV-1 protease enzyme.7 Its design is based on the "peptidomimetic" principle, wherein the molecule contains a hydroxyethylene scaffold which mimics the normal peptide linkage (cleaved by HIV protease) but which itself cannot be cleaved.5 By preventing HIV-1 protease activity, and thus the proteolysis of the Gag polyprotein, lopinavir results in the production of immature, non-infectious viral particles.	Ritonavic inhibits the HIV viral proteinase enzyme that normally cleaves the structural and replicative proteins that arise from major HIV genes, such as gag and pol. Gag encodes proteins involved in the core and the nucleocapsid, while pol encodes the the HIV reverse transcriptase, ribonuclease H, integrase, and protease . Ritonavir prevents the cleavage of the gag-pol polyprotein, which results in noninfectious, immature viral particles. Ritonavir is a potent inhibitor of cytochrome P450 CYP3A4 isoenzyme present both in the intestinal tract and liver
Pharmacokinetic	• When administered alone, lopinavir has exceptionally low oral bioavailability (~25%) - for this reason, it is exclusively co-administered with ritonavir, which dramatically improves bioavailability. • The volume of distribution of lopinavir following oral administration is approximately 16.9 L.	• The absolute bioavailability of ritonavir has not been determined.7 following oral administration, peak concentrations are reached after approximately 2 hours and 4 hours (Tmax) after dosing under fasting and non-fasting conditions, respectively. • The estimated volume of distribution of ritonavir is 0.41 ± 0.25 L/kg. • Ritonavir is highly protein-bound in plasma (~98-99%).

	• Lopinavir is >98% protein-bound in plasma. • Lopinavir undergoes extensive oxidative metabolism, almost exclusively via hepatic CYP3A isozymes. • The elimination half-life of lopinavir is 6.9 ± 2.2 hours. • The estimated apparent clearance following oral administration is approximately 6-7 L/h.	• The isopropylthiazole oxidation metabolite (M-2) is the major metabolite in low plasma concentrations and retains similar antiviral activity to unchanged ritonavir. The cytochrome P450 enzymes CYP3A and CYP2D6 are the enzymes primarily involved in the metabolism of ritonavir. • Ritonavir is primarily eliminated in the feces and urine. • The approximate half-life of ritonavir is 3-5 hours.7 • The apparent oral clearance at steady-state is 8.8 ± 3.2 L/h. Renal clearance is minimal and estimated to be <0.1 L/h.

Table 1: drug profile

MATERIALS AND METHOD

- **Drug samples:** Lopinavir (Assay: 99.80%) and Ritonavir (Assay: 99.65%) were used as standards
- **Tablets used:** Brand: **EMELTRA**; Lopinavir 200mg and Ritonavir 50mg
- **Chemicals and solvents used:**

  All the solvents and reagents used were HPLC grade. Acetonitrile, Ammonium acetate, Acetic acid, and Methanol and Distilled water is used in experiment.
- **Instrumentation :**
  i. HPLC system: Shimadzu Isocratic HPLC system with following configurations, LC-10AT Vp series, Isocratic solvent delivery system (pump).
  ii. Injection system: Rheodyne 7725i injector with 20 µl loop.
  iii. Software: Spinchrome data station.
  iv. Analytical column: Phenomenex – Luna, C18 (250 x 4.6 mm i.d., 5µ).
  v. Detector: UV-Visible – SPD 10AVp series detector.

- **Preparation of mobile phase:**
  i. **Preparation of buffer solution:** Weighed & transferred about 0.77g of Ammonium acetate into a beaker containing 1000ml of Water and dissolved completely. The pH of the Solution was adjusted to 6.5±0.05 with glacial acetic acid and then filtered through 0.45µm membrane filter
  ii. **Preparation of Mobile Phase:** Mobile phase is prepared by mixing 550 ml of buffer and 450 ml of acetonitrile
  iii. **Mobile-Phase Ratio**: Buffer: Acetonitrile (55: 45 % V/V).
- **Method for the estimation**

With the optimized chromatographic conditions, a steady baseline was recorded. After stabilization of the baseline for about 30 minutes, successive aliquots of the standard solution of the same concentration were injected and chromatogram was recorded until the reproducibility of the peak areas was satisfactory. This procedure was repeated using the sample solution so that duplicate injection of the sample solution was bracketed by injection of the standard solution

- **VALIDATION OF METHOD**

The developed method was validated for as per ICH Q2 (R1) guidelines for various parameters such as accuracy, precision, linearity, robustness, limit of detection (LOD), limit of quantitation (LOQ), and stability.

i. Accuracy

The accuracy of the RP-HPLC method was evaluated by selecting three different concentrations lower quantitation limit (LQC), medium quantitation limit (MQC), and higher quantitation limit (HQC). In each concentration, a minimum of six injections were given and the amount of the drugs present, percentage recovery, and related standard deviation were calculated. The percentage recovery was calculated using the formula

$$X = (Y- C/m) \times 100$$

Where, X is % recovery

Y is area of test sample occurred

C is y intersept

M is the slope

ii. Precision

The precision of the developed method was studied by performing interday and intraday variations. The precision of the developed method was studied by performing interday and intraday variations in which three replicate solution of standard and test are run in system at different time. The amount of

each drug, percentage content, standard deviation, and percentage relative standard deviation were calculated

iii. Linearity and range

The six series of standard solutions were selected for assessing linearity range. The calibration curve was plotted using peak area versus concentration of the standard solution and the regression equations were calculated. The least squares method was used to calculate the slope, intercept and correlation coefficient

iv. LOD and LOQ

The LOD and LOQ of RP, PP, and IP were determined by injecting progressively lower concentrations of the standard solutions into the HPLC column using the optimized chromatographic conditions in accordance with 3.3 s/n and 10 s/n criteria, respectively, where, s/n indicates signal-to-noise ratio.

v. Robustness

For the demonstrating the robustness of method, slight variations in the optimized conditions were done and the standard solution was injected.

Flow rate ± 1 ml/min.

Wavelength ± 1nm

RESULT AND DISCUSSION

- **Method development**

Chromatographic conditions

	Parameters	Specification
1	HPLC	Shimadzu Isocratic HPLC system
2	Software	Spinchrome data station
3	Column	C18 column,
4	Mobile Phase	Buffer: Acetonitrile (55: 45 % V/V).
5	Diluents	Mobile phase
6	Detection Wavelength	255nm
7	Flow rate	1.5mL/min
8	Run Time	20 min
9	Volume of injection	20µl

Table 2: optimized chromatographic condition lopinavir and ritonavir

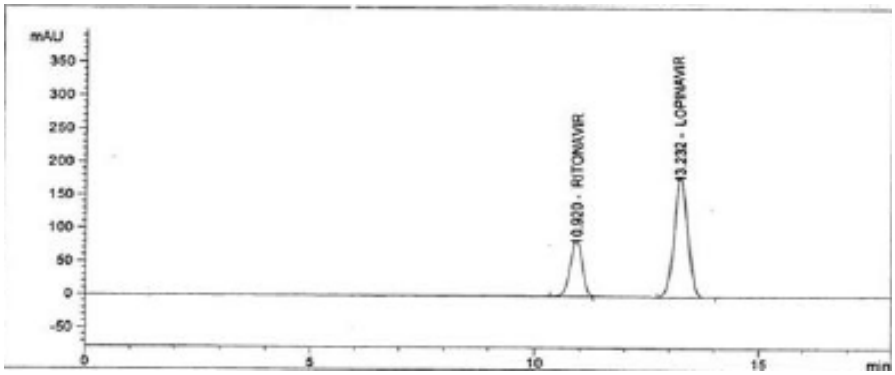

Fig 3: **chromatogram for lopinavir and ritonavir**

Determination of Lopinavir and Ritonavir in dosage forms by the developed RP-HPLC method was carried out. The standard and sample solutions were prepared and the chromatograms were recorded.

Validation of Method:

- **System suitability test :**

  The suitability of the system was evaluated by the values obtained for Theoretical plate, Resolution and tailing factor of the chromatogram of standard drugs. The selectivity of the method was revealed by the repeated injection of mobile phase and no interference was found.

Sr. no	Retention time		Theoretical plate		Asymmetric factor	
	Lopinavir	Ritonavir	Lopinavir	Ritonavir	Lopinavir	Ritonavir
1	13.23min	10.92 min	7599	6782	1.012	1.001

Table 3: system suitability test for lopinavir and ritonavir

- **Accuracy :**

  The accuracy of the method was determined by %recovery experiments. The recovery studies were carried out by preparing 6 individual samples with same procedure from the formulation and injecting. The percentage recovery was calculated. From the data obtained, added recoveries of standard drugs were found to be accurate.

  The % recovery range for lopinavir and ritonavir range is found between 99.88-99.90 and 99.86-99.99.

- **Precision :**

  The system and method precision of the method were demonstrated by inter day, intraday and repeatability of injection studies. All the solutions were injected into the chromatographic system. Precision is calculated in terms of systemic and method precision. The systemic and method precision is found

Sr. no	System precision		method precision	
	Lopinavir	Ritonavir	Lopinavir	Ritonavir
1	0.06	0.20	0.062	0 .53

Table 4: precision for lopinavir and ritonavir

The peak area and percentage relative standard deviation were calculated. From the data obtained, the developed HPLC method was found to be precise.

- **Linearity :**

The standard drug solutions of varying concentrations ranging from 20% to 120% of the targeted level of the assay concentration (i.e.) 5 µg/ml to 30 µg/ml of Ritonavir and 20µg/ml to 120µg/ml of Lopinavir, were examined by the proposed method. The response factor, slope, intercept, correlation co-efficient and Residual sum of squares values were calculated.

The equation was found to be

For lopinavir

$$Y = 44.77x + 134.3$$

For ritonavir

$$Y = 6.004x + 66.80$$

Coefficient of correlation for both drugs was found to be 0.999.

- **Limit of detection (LOD) and limit of quantitation (LOQ)**

For this study, three replicates of sample at lowest concentrations were measured and quantified. The equations are LOD=3.3×σ/S and LOQ=10×σ/S where, 'σ' is standard deviation of lowest three concentrations and 'S' is slope of linearity equation. The LOD and LOQ is find out in terms of signal to noise ratio

		Lopinavir	Ritonavir
Limit of Detection	Signal noise ratio should be more than 3:1	0.153	0.043
Limit of Quantitation	Signal noise ratio should be more than 10:1	0.462	0.132

Table 5: LOD and LOQ for lopinavir and ritonavir

- **Robustness :**

  The robustness of the method was studied by carrying out experiments by changing. The response factors for these changed chromatographic parameters were almost same as that of the fixed chromatographic parameters and hence developed method is said to be robust

Summary of results:

Parameter	Observation		Limit
	Lopinavir	Ritonavir	
Retention time	13.23 min	10.92 min	-
Theoretical plates	7599	6782	-
Asymmetric factor	1.012	1.001	-
Coefficient of correlation	0.999	0.999	Correlation coefficient NMT – 0.999
Accuracy (% recovery)	99.88-99.90	99.86-99.99	% Recovery range 98 –102 %
System precision	0.06	0.20	RSD NMT 2.0%
Method precision	0.062	0 .53	RSD NMT 2.0%
LOD	0.153	0.043	Signal noise ratio should be more than 3:1
LOQ	0.462	0.132	Signal noise ratio should be more than 10:1

Table 6: Summary of results

CONCLUSION:

The developed HPLC method for the estimation of lopinavir and ritonavir was performed in mobile phase Buffer: Acetonitrile (55: 45 % V/V). This method is successfully validated as per ICH guidelines. It is indicate that this newly developed method was found to be accurate, simple, precise, and reproducible. This method can be easily applied for quality control analysis in industry. The retention time of this method will significantly reduce the analysis time and cost.

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