Development and validation of a RP-HPLC method for simultaneous determination of ritonavir and lopinavir in combined dosage form

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ABSTRACT

A reverse phase HPLC method was developed and validated for the simultaneous determination of two antiviral drugs viz. lopinavir and ritonavir. Chromatography was carried out on C18 Column, Inertsil (150 x 4.6 mm, 5 μ) with mobile phase of Buffer: Methanol: Acetonitrile (30:60:10v/v/v) pH 6.5, at a flow rate of 0.6 ml/min and detection was made at 226 nm. The different analytical performance parameters such as linearity, precision, accuracy, specificity, limit of detection (LOD), limit of quantification (LOQ) and robustness were determined according to ICH guidelines. The linearity for each analyte in the desired concentration range was good (r2 >0.9). The recovery of the method was 99.86% and 100.16% for ritonavir and lopinavir respectively. Hence the proposed method was sensitive, precise and accurate and it can be successfully apply to estimate the amount of ritonavir and lopinavir in the formulations by easily available low cost materials.

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INTRODUCTION

In recent years, the treatment of human immunodeficiency virus (HIV)-1 infection and AIDS has been advanced by the development of highly active antiretroviral therapy.

Lopinavir inhibits the HIV viral protease enzyme. This prevents cleavage of the gagpol poly protein and, therefore, improper viral assembly results. This subsequently results in non-infectious, immature viral particles. Combination therapy with the HIV protease inhibitors lopinavir and ritonavir has been shown to be effective against drug-resistant HIV-13. These agents are metabolized by cytochrome P-450 (CYP) 3A in the liver. When lopinavir is administered with ritonavir, ritonavir inhibits the CYP 3A-mediated metabolism of lopinavir, thereby providing increased plasma levels of lopinavir[1].

Lopinavir 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-(1-methylethyl)-2-oxo-1(2H)-pyrimidineacetamide. Its molecular formula is C_{37}H_{48}N_{4}O_{5}, and its molecular weight is 628.801[1].

Ritonavir is chemically designated as 10-Hydroxy-2-methyl-5-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraazatridecan-13-
ionic 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. The structures of Lopinavir and Ritonavir are depicted in figure 1.

![Figure 1: Structures of Lopinavir and Ritonavir](image)

To analyze these drugs, several determination methods have been reported. These included high performance liquid chromatography-mass spectrometry (LC/MS)\(^{[3-5]}\), matrix-assisted laser desorption/ionization, time-of-flight (MALDI-TOF)\(^{[6]}\), high-pressure thin layer chromatography (HPTLC)\(^{[7]}\), Ultra performance liquid chromatography (UPLC)\(^{[8]}\) and immunoassay methods\(^{[9]}\).

Literature also revealed some of the methods by high-performance liquid chromatography (HPLC)\(^{[10-14]}\) in various dosage forms.

The present paper deals with a HPLC assay of lopinavir and ritonavir simultaneously. Benefits of this method are improved sensitivity and recovery compared to all other methods available in the literature. The present HPLC method is well suited for studies generating large number of samples. In addition, much improved sensitivity was achieved with lower limit of quantification (LLOQ) of 0.075 \(\mu g/ml\) for lopinavir and 0.3 \(\mu g/ml\) for ritonavir. Recoveries were about 99.86\% and 100.16\% for ritonavir and lopinavir respectively. The proposed method was subjected to validation according to the ICH guidelines\(^{[15]}\).

**MATERIALS AND METHODS**

**Materials and reagents**

Ritonavir and Lopinavir were kindly supplied as a gift sample by Hetero drug Pvt Ltd. AP. Other reagents such as Acetonitrile, Methanol, Potassium dihydrogen Phosphate and water used were of HPLC and milli-q grade. All other chemicals like glacial acetic acid used were of AR grade from Merck chemicals, Mumbai.

**Chromatography conditions**

Chromatography separation was performed on a HPLC (Younglin with UV detector) at the wavelength of 226 nm. A reverse phase Inertsil C18, (150 X 4.6mm, i.d., 5 \(\mu m\) particle size) column was used. The mobile phase consists of Buffer: Methanol: Acetonitrile (30:60:10\(v/v/v\)) with flow rate 1.2 ml/min. injection volume was 20 \(\mu l\) and the chromatographic runtime of 15 min was used.

**Preparation of buffer solution**

Buffer was prepared by dissolving 2.7218g of Potassium dihydrogen orthophosphate in 1000 mL of water and adjusts the pH 6.5 \(\pm 0.02\) with Ortho Phosphoric acid followed by the degassing of the solution.

**Preparation of mobile phase**

1000 mL of mobile phase was prepared by mixing 300ml of Potassium dihydrogen orthophosphate (0.02M), 250ml of Methanol and 450ml of Acetonitrile.

**Preparation stock solutions**

Accurately weighed and transferred about 10mg of Ritanovir and Lopinavir into a separate 10 mL volumetric flasks. 5 mL of Acetonitrile was added to each volumetric flask and kept in an ultrasonic bath until it dissolved completely. Made the volume up to the mark with methanol and mixed well. This yielded solution of 1000µg/ml concentration.

**Preparation of standard solution**

Spiked accurately about 0.4 mL of Ritanovir and 1.6 mL of Lopinavir stock solution and transferred it into a 10ml volumetric flask. Made the volume up to
the mark with mobile phase and mixed well. This yielded solution of 40 µg/ml and 160 µg/ml concentrations.

**Method validation**

Validation experiments were performed to demonstrate System suitability, precision, linearity, Accuracy, Limit of detection and Limit of quantification.

**Precision**

The precision of the method was evaluated by carrying out six independent asses of test sample against a qualified reference standard and the %RSD of assay was calculated (% RSD should not be more than 2%).

**Accuracy**

Accuracy for the assay of Ritanovir and Lopinavir determined by applying the method in triplicate samples to which known amount of Ritanovir and Lopinavir standard is added at different levels (50%, 100%, and 150%). Each solution was injected thrice (n=3) into HPLC system and the average peak area was calculated from which Percentage recoveries were calculated. (% Recovery should be between 98.0 to 102.0%).

**Linearity**

The Linearity of detector response was established by plotting a graph to concentration versus area of Ritanovir and Lopinavir standard and determining the correlation coefficient. A series of solution of Ritanovir and Lopinavir standard solution in the concentration ranging from about 5 - 60 µg/ml of Ritanovir and 20 - 240 µg/ml of Lopinavir respective levels of the target concentration were prepared and injected into the HPLC system.(Correlation coefficient should be not less than 0.999.)

**Limit of Detection (LOD) Limit of Quantification (LOQ)**

LOD and LOQ for the were determined at signal to noise ratios of 3:1 and 10:1, respectively by injecting series of dilute solutions with known concentrations.

**RESULTS AND DISCUSSION**

**Method development**

Different chromatographic conditions were experimented to achieve better efficiency of the chromatographic system. Parameters such as mobile phase composition, wavelength of detection, column, column temperature, and pH of mobile phase were optimized. Several proportions of buffer, and solvents (water, methanol and acetonitrile) were evaluated in order to obtain suitable composition of the mobile phase. Choice of retention time, tailing, theoretical plates, and run time were the major tasks while developing the method.Buffers like sodium dihydrogen orthophosphate and disodium hydrogen orthophosphate did not yield desired results. The composition of mobile phase Acetonitrile: Methanol: Buffer (50:30:20 v/v/v) of pH 3.5 with flow rate of 0.8 ml/min and detection at 210 nm of runtime of 6 min shown merging of two drugs. The composition of mobile phase Acetonitrile: Methanol: Buffer (60:30:20 v/v/v) of pH 3.5 with flow rate of 1 ml/min and detection at 226nm of runtime of 15 min yielded peaks with non-sink in the base line with unstable retention times.

At Acetonitrile: Methanol: Buffer (45:25:30 v/v/v) of pH 6.5 with flow rate of 1.2 ml/min and detection at 226nm of runtime of 12 min, a perfect chromatogram was eluted. The typical chromatogram obtained from final HPLC conditions are depicted in Figure 2.

![Figure 2: typical chromatogram of Ritonavir and Lopinavir by proposed method](image-url)

**Figure 2: typical chromatogram of Ritonavir and Lopinavir by proposed method**

**Method validation**

Based on International Conference on Harmonization (ICH) guidelines, the method is validated with regard to system suitability, linearity, accuracy, precision, LOD and LOQ as follows.

**(I) system suitability**

The system suitability parameter tailing factor for the proposed HPLC method from the standard injection of Ritonavir and Lopinavir are 1.03 and 1.02 re-
spectively. Theoretical Plates Obtained from the standard injection of Ritonavir and Lopinavir are 5350.4 and 6140.8 respectively. The results proved that the optimized HPLC method fulfils these requirements within the USP accepted limits.

(II) Precision

The % R.S.D. of Ritonavir and Lopinavir assay during the method precision was found to be 0.052948% and 0.089858% respectively, indicating excellent precision of the method. The results are summarized in TABLE 1.

(III) Accuracy

Percent recovery of Ritonavir samples ranged from 98.0% to 101.5%, and the Percent recovery of Lopinavir samples ranged from 98.0% to 102.0% showing the good accuracy of the method. The result is shown in TABLE 2.

(IV) Limit of Detection (LOD) Limit of Quantification (LOQ)

The LOD of Ritonavir and Lopinavir were found to be 0.02 µg/ml and 2 µg/ml respectively. The LOQ was 0.05 µg/ml and 1 µg/ml for Ritonavir and Lopinavir respectively. Since the LOQ and LOD values of Ritonavir and Lopinavir achieved at a very low level, this method can be suitable for cleaning validation in the pharmaceutical industry.

(V) Linearity

The linearity of the calibration plot for the method was obtained over the calibration ranges tested, i.e., 5-60 µg/ml for Ritonavir and 20-240 µg/ml for Lopinavir three times, and the correlation coefficient obtained was
0.9998 and 1 for Ritonavir and Lopinavir respectively, thus indicating excellent correlation between peak areas and concentrations of the analytes.

(VI) Robustness

In all the deliberately varied chromatographic conditions in the concentration range for the evaluation of robustness 5-60 μg/ml for Ritonavir and 20-240 μg/ml for Lopinavir (n=3). It can be concluded that the variation in flow rate and the variation in 10% Organic composition do not affect the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10% and change in the Mobile phase ±10%. The results are summarized in TABLE 3.

CONCLUSION

The new, simultaneous RP-HPLC method proved to be simple, linear, precise, accurate, robust, rugged and rapid. The developed method was capable of giving faster elution, maintaining good separation more than that achieved with other available HPLC methods. The short retention times allows the analysis of a large number of samples in a short period of time and is therefore more cost-effective for routine analysis in the pharmaceutical industries. It is suitable for rapid and accurate quality control of ritonavir and lopinavir in combined dosage forms.

REFERENCES