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Research Article

A newly validated HPLC method development for simultaneous estimation of ritonavir and lopinavir

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ABSTRACT

The aim of the present work was to develop a isocratict RP-HPLC for simultaneous analysis of ritonavir and lopinavir in tablet dosage form. Method: chromatographic system was optimized using a Agilent XDB C18(150 x 4.6mm,5µm) column with potassium dihydrogen phosphate (pH 4.6) and acetonitrile in the ratio of 45;55, as a mobile phase, at a flow rate of 1.0 ml/min. detection was carried out at 215nm by a photodiode array detector. Result: ritonavir and lopinavir were eluted with retention times of 4.821 and 3.814mins respectively. Beer's lambert's law was obeyed over the concentration ranges of 12.5 to 50µg/ml and 50 to 200µg/ml for ritonavir and lopinavir, respectively. Conclusion: the high recovery and low coefficients of variation confirm the suitability of the method for simultaneous analysis of both drugs in a tablet dosage form. Statistical analysis proves that the method is sensitive and significant for the analysis of ritonavir and lopinavir in pure and in pharmaceutical dosage form without any interference from the excipients. The method was validated in accordance with ICH guidelines. Validation revealed the method is specific, rapid, accurate, precise, reliable, and reproducible.

Keywords: Ritonavir, Lopinavir, HPLC and ICH guidelines.

Introduction

Ritonavir and lopinavir both are protease inhibitor with antiviral activity against HIV. Chemically, Ritonavir is (5S,8S,10S,11S)-10-hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4thiazolyl]-3,6-dioxo 8,11bis (phenyl methyl)- 2,4,7,12- tetraazatridecan-13-oic acid 5-

thiazolyl methyl ester of molecular formula $C_{37}H_{48}N_6O_5S_2$ and molecular weight is 720.95. It is official in Indian Pharmacopoeia and United State Pharmacopoeia [1,2]. Ritonavir is absorbed following oral administration and peak plasma concentrations occur in about 2 to 4 hours. Protein binding is reported to be about 98% and penetration into the CNS is minimal. Ritonavir is extensively metabolized in the liver principally by cytochrome P450 isoenzymes CYP3A and CYP2D6.ritonavir is mainly excreted in the faeces, with a half-life of 3 to 5 hours [3]. The structure of ritonavir was shown in the figure-1.

Lopinavir is Chemically known as (2S)-N-[(2S,4S,5S)-5-[2-(2,6 dimethylphenoxy)acetamido]-4-hydroxy-1,6-iphenylhexan-2-yl]-3-methyl-2-(2-oxo-1,3-diazinan-1-yl) butamide and its empirical formula is $C_{37}H_{48}N_4O_5$ with a molecular weight of 628.80 [4]. Lopinavir inhibits the HIV viral protease enzyme and prevents cleavage of the gagpolpolyprotein. It is given with low dose ritonavir, which acts as a pharmacokinetic enhancer. Lopinavir has been associated with increases in serum cholesterol and triglycerides, and cases of pancreatitis have been reported [5]. The chemical structure was shown in figure 2.

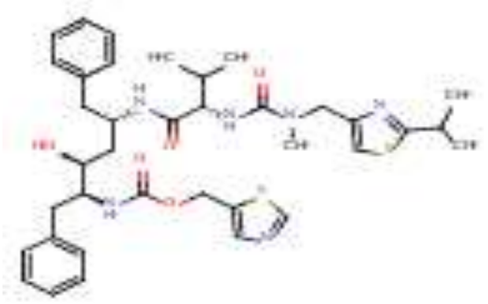

Figure 1; Chemical Structure of Ritonavir.

There are many methods reported for the determination of determination of LPV and RTV in pharmaceutical preparations and in human plasma individually or in combination with other antiviral drugs using HPLC [6-17] UV/Visible or Mass Spectroscopy detector. These included matrix-assisted laser desorption/ionization, tandem time of-flight (MALDI-TOF) [18], high-pressure thin layer chromatography (HPTLC) [19] and immunoassay methods [20].However, no references are reported so far for the simultaneous determination of both drugs in combined dosage form or any such pharmaceutical preparations by HPLC. In this communication we report a new simple, rapid and precise RP-HPLC method for the simultaneous determination of LPV and RTV in combination dosage form, and also used for the API content, which can be used for its routine analysis in ordinary laboratories. In the present study we are optimizing the method using fewer amount of organic solvent. Due to this reducing cost of analysis, so that formulation becomes economic. It is necessary to develop a method to determine the combination both in pure and formulation.

All chemicals were of AR-grade. All reagents were of HPLC grade and purchased from Merck pharmaceuticals. The formulation was purchased from the local pharmacy.

Figure 2: Chemical structure of Lopinavir.

INSTRUMENTATION

The analysis was performed on Waters2695 HPLC system with Waters2996 Photodiode Array detector. Data acquisition was performed by using Empower 2 software. Agilent XDB, C18 column (150 x 4.6mm, 5µ) was used as stationary phase. Injections were performed by the manual injector with 10µl. Different mobile phases were tested in order of their polarity to find out the best conditions for the separation of ritonavir and lopinavir. The selected mobile phase Potassium Dihydrogen Ortho Phosphate buffer (pH 4.6) and acetonitrile in the ratio of 55:45%v/v gave acceptable retention time (RT) and good resolution between ritonavir and lopinavir. The flow rate was maintained at 1.0 mL min^{-1}, with a run time of 10 min. the mobile phase was filtered by using 0.45µ filter and it was degassed by sonication prior to use. All determinations were made at ambient temperature.

Standard solution and calibration curve preparation

Ritonavir (2.5mg) and lopinavir (10mg) were weighed and transferred separately to 10ml volumetric flask. Both the drugs were dissolved in 7ml of HPLC grade methanol and the volume was made up with the same to prepare 1000µg/ml. Pipette out 0.5ml of above and transfer in 10ml volumetric flask and make up the volume till the mark with methanol to make 50µg/ml standard solutions. Calibration standards were prepared taking aliquots and further diluted stock solutions in the concentration ranges of 12.5 to 50µg/ml and 50 to 200µg/ml for ritonavir and lopinavir, respectively and peak areas were plotted against the corresponding concentrations to obtain the calibration graphs. [21,22]

SAMPLE PREPARATION

For the analysis of a tablet dosage form, 20 tablets were weighed individually and their average mass was determined. Then the tablets were crushed to fine powder. Transferred an accurately weighed portion of the powder, equivalent to 2.5mg of ritonavir and 10mg of lopinavir to a 100ml volumetric flask and diluted with methanol till the mark and sonicate for 25 minutes. The solution was filtered through a Whatmann filter paper no.1.Filterate was then appropriately diluted with mobile phase to get a final concentration. Before the assay of the tablet formulations, 3 replicate aliquot of the appropriately diluted tablet stock solution were sonicated for 15minutes, then injected into the chromatographic system and analyzed quantitatively.

OPTIMISATION OF HPLC METHOD

The HPLC procedure was optimized with a view to develop a simultaneous assay method for ritonavir and lopinavir. Preliminary experiments were carried out to optimize the parameters affecting simultaneous estimation of two drugs. Reverse phase column [Agilent XDB C18 (150 x 4.6mm, 5µ) column] was selected on the basis of the polarity of drugs for analysis. Following parameters were optimized for the development of method i.e. column, wavelength, mobile phase concentration, solvent, flow rate, concentration of buffer. The solvent type, solvent strength, detection wavelength and flow rate were varied to determine the best chromatographic conditions for the separation of ritonavir and lopinavir in chromatogram. The mobile phase conditions were optimized to avoid interference from solvent and formulation excipients. Other criteria, for example, time required for analysis, flow rate of mobile phase, symmetry of eluted peaks, assay sensitivity and solvent noise during drug analysis were also considered. The spectra of the analytes were determined independently and in combination. It was observed that at wavelength 215nm both the drugs could be detected simultaneously with no mobile phase interference, good separation, sensitivity and consistent baseline. The feasibility of various combinations of solvents such as acetonitrile, methanol, buffer and water with altered flow rate (in the range 0.8 – 1.2 ml/min), was investigated for complete chromatographic resolution of above drugs with best sensitivity, efficiency, and peak shape.

Method Validation

The method was validated according to the ICH guidelines. The accuracy, precision, Linearity, Specificity, ruggedness and robustness were determined by analyzing 12.5 - 50 µg/ml and 50 -200 µg/ml of RTV and LPV drugs respectively. [23-26].

System Suitability

System suitability parameter was calculated before starting validation parameters. It was determined by taking the Coefficient of variation of peak area, peak asymmetry and theoretical plate of the six standards injections by using the same standard method which given assay method.

Linearity and Range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of the analyte in the sample.

The range of the analytical procedure is the interval between the upper and lower concentration of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. Linearity established across the range of the analytical procedure. It was determined at five levels over the range of 25% to 150% of test concentrations. A standard linearity solution was prepared to attain concentration of 25%, 50%, 100%, 125%, and 150%

of the test concentration. The area at each level is calculated and a graph area versus concentration is plotted. The correlation co-efficient (r^2) was calculated and recorded.

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. The precision of an analytical method was determined by assaying a sufficient number of aliquots of a homogenous sample to be able to calculate statistically valid estimates of standard deviation and relative standard deviation.

Repeatability

Repeatability expresses the precision under the same operating conditions. It was assessed by performing the determination of single concentrations and six replicates of working standard solution in intraday and inter day study.

Reproducibility

Reproducibility expresses the precision between laboratories. The reproducibility of an analytical method was determined by analysis of aliquots from homogenous lots in different laboratory. It was assayed by performing six determination and two replicator of each concentration in two laboratories.

Robustness

Robustness of analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The robustness was studied by evaluating the effect of small but deliberate variations in chromatographic conditions. The conditions studied were flow rate (altered by ± 0.2ml/min).

Accuracy and recovery study

The accuracy of an analytical procedure expresses the closeness of the agreement between value which is accepted either as conventional true value or an accepted reference value and value found. Accuracy is calculated as the percentage of recovery by the assay of the known added amount of analyte (50%, 100% and 150%) in the sample. Accuracy assayed by using nine determinations over a minimum of three concentration levels, covering the specified range (i.e. three concentrations and three replicates of each concentration.)

Specificity

In case of the assay, demonstration of specificity requires that it can be shown by the presence of impurities or excipients. It was done by spiking the drug substance or product with the appropriate levels of impurities or excipients and demonstrating that the assay result is unaffected by the presence of these extraneous materials. Placebo (sample without analyte) was prepared in the same way as the sample under the conditions prescribed in the assay method and duplicate injection was taken and observed any significant peak area (not more than 1%) at the analyte RT.

RESULTS

Development and optimization of HPLC method

The proposed method was optimized with a view to develop a suitable analytical method for the analysis of doxycycline and tinidazole in combined pharmaceutical dosage form. It was found that the mobile phase containing Potassium Dihydrogen Ortho Phosphate buffer and acetonitrile (pH 4.6) in the ratio of 55:45%v/v in gradient elution mode at a flow rate of 1.0ml/min gave optimum and adequate peak separation, with less tailing and resulted in the best resolution. All experiments were performed at ambient temperature. Run time was taken 10min for each run. Under the optimum chromatographic conditions, the retention times obtained.

Validation

System suitability

System suitable parameters such as retention time, theoretical plates, peak area, resolution, and peak asymmetry were determined. The results obtained were statistically analyzed and found within the range (table-1).

Linearity and range

The statistical data obtained are represented in table-2. The result shows that within the concentration range 12.5 to 250µg/ml and 50 to 200µg/ml for ritonavir and lopinavir, respectively. There was an excellent correlation between peak area and concentration of each drug.

Limit of detection (LOD) and Limit of Quantification (LOQ)

The limit of detection and limit of quantification for ritonavir and lopinavir were calculated from the linearity data using relative standard deviation of the response and the slope of the calibration curve. The limit of detection of a compound is defined as the lowest concentration of analyte that can be detected. LOD value of ritonavir and lopinavir was found to be 0.132 and 0.685µg/mL respectively. The limit of quantification is the lowest concentration of a compound that can be quantified with acceptable precision and accuracy. LOQ value of ritonavir and lopinavir was found to be 0.401 and 2.076µg/mL respectively.

Repeatability

The results of the intraday and inter day precision experiments are shown in table – 3. Separation of the drugs was found to be similar when analysis was performed on different time (intraday) and on different days (inter day). The developed method was found to be precise, with relative standard deviation (RSD) values less than 2%.

Reproducibility

The results of the reproducibility experiments (performed in different laboratories) are shown in table -4. The developed method was found to be precise, with RSD values less than 2%

Robustness

Minor change in chromatographic condition (change in flow rate, altered by 0.2ml/min) did not cause a significant change in, peak area, theoretical plates and RT of ritonavir and lopinavir (table - 5).

Accuracy and recovery study

Good recoveries of the ritonavir (98.2 - 101.469) and lopinavir (98.4 – 100.78) were obtained at various added concentrations for the ritonavir and lopinavir (table - 6).

Specificity

Injections of placebo (sample without analyte) were performed to confirm specificity of method. Obtained results show that excipients mixture of the tablet shows no specific peak at the RT of the analyte peak. This shows that the excipients do not interfere with the analyte peak and the assay is specific for the simultaneous estimation of ritonavir and lopinavir tablets.

DISCUSSION

A suitable analytical procedure refers to the way of performing the analysis with accuracy and precision. This developed method is describes in detail the steps necessary to perform each parameter for validation. The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. The quality control laboratory requires analytical methods which are simple, robust, and rugged, interpretation of results of method is directly proportional to the concentration of analyte within a given range shows linearity of method. Different environmental condition doesn't cause any significant change in results shows stability and reproducibility of developed method. There was no interference by excipients with analyte peak shows proposed method is specific for analyte. As well as recovery study shows the developed method is highly accurate. Hence the proposed HPLC method has been evaluated and validated for the accuracy, precision, and linearity and found to be convenient, sensitive and specific for the quality control of ritonavir and lopinavir in tablet dosage form.

Table 1:
System suitability tests (S.D – Standard Deviation, R.S.D – Relative Standard Deviation)

Parameter	**Compound**	
	Ritonavir	**Lopinavir**
Retention Time	S.D – 0.0295	S.D - 0.0161
	R.S.D – 0.685	R.S.D – 0.469
Peak Area	S.D – 5753.9	S.D - 44266.7
	R.S.D – 1.0	R.S.D – 1.5
Peak Asymetry	S.D – 0.0287	S.D – 0.0209
	R.S.D – 1.6628	R.S.D – 1.357
Theortical Plates	S.D – 79.72	S.D – 18.715
	R.S.D – 2.972	R.S.D – 0.8881

Table 2:
Linearity parameters for calibration curves of RIT and LOP

Compound	**Level of conc. In µg/ml**	**Slope**	**Intercept**	**Correlation coefficient (r^2)**
		Mean ± S.D	**Mean ± S.D**	
RIT	6.25 - 37.5	19604.6 ± 174.4	6914.6 ± 786.5	0.999
LOP	25 - 150	26371.33 ± 151.38	21.847.3 ± 5475.8	0.999

Table 3:
Precision of method. (S.D – Standard Deviation, R.S.D – Relative Standard Deviation)

Compound	**Interday**		**Intraday**	
	Mean area ± S.D	**R.S.D**	**Mean area ± S.D**	**R.S.D**
RIT	513178 ± 2539.7	0.4	528530 ± 6941.6	1.3
LOP	2752412 ± 37793	1.3	2888800 ± 25332	0.8

Table 4:
Ruggudness study. (S.D – Standard Deviation, R.S.D – Relative Standard Deviation)

Compound	**Analyst - 1**		**Analyst – 2**	
	Mean area ± S.D	**R.S.D**	**Mean area ± S.D**	**R.S.D**
RIT	532531 ± 6368.7	1.2	554426 ± 29626.2	5.3
LOP	3065266 ± 17685.6	0.6	2937927 ± 26404.7	0.9

Table 5:
Robustness study. (S.D – Standard Deviation, R.S.D – Relative Standard Deviation)

Parameter		**Ritonavir**		**Lopinavir**	
		Mean area ± S.D	**R.S.D**	**Mean area ± S.D**	**R.S.D**
Flow Rate	0.8ml/min	586278 ± 4497.9	0.7	3228742 ± 21410.5	0.7
	1.2ml/min	582896 ± 3449.9	0.5	2723666 ± 20148	0.7
Mobile Phase	50:50	501583 ± 1738	0.3	2769461 ± 2569	0.9
	60:40	541857 ± 1378.6	0.3	2980612 ± 26833.7	0.9
Temperature	- 5˚C	528530 ± 6941.6	1.3	2888800 ± 25332	0.8
	+ 5˚C	501463 ±1230.3	0.2	2681923 ± 19482.6	0.7

Table 6:
Accuracy and recovery study. (S.D – Standard Deviation, R.S.D – Relative Standard Deviation)

Compound	Amount of drug added (%)	Theoretical cotent (µg/ml)	Concentration found (µg/ml) ±SD	RSD (%)	Recovery (%)
Ritonavir	50	12.5	12.677 ± 0.095	0.7549	101.42
	100	25	25.025 ± 0.146	0.5834	100.1
	150	37.5	37.27 ± 0 .658	1.7657	99.386
Lopinavir	50	50	50.126 ± 0.283	0.56	100.26
	100	100	99.692 ± 0.233	0.2343	99.69
	150	150	150.8 ± 1.75	1.16	100.535

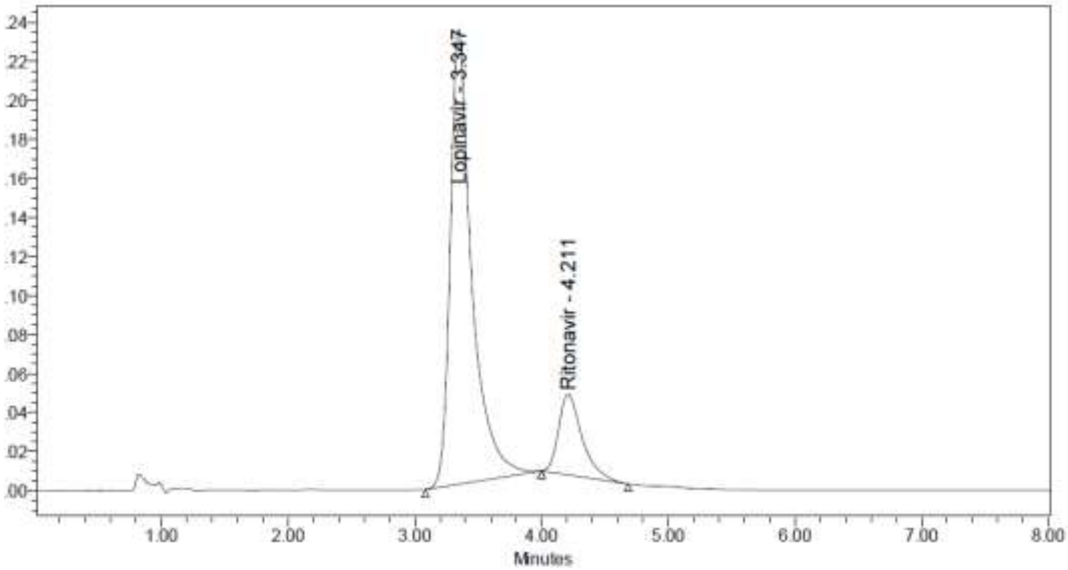

Figure 3: Chromatogram of assay of ritonavir and lopinavir

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