



Research Article

ANALYTICAL METHODS DEVELOPMENT & VALIDATION FOR SIMULTANEOUS ESTIMATION OF LOPINAVIR & RITONAVIR IN PHARMACEUTICAL FORMULATION BY SIMULTANEOUS EQUATION METHOD USING UV SPECTROPHOTOMETRY

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ABSTRACT

The present work deals with the simultaneous estimation of Lopinavir and Ritonavir in bulk and pharmaceutical dosage form using the UV method. Shimadzu UV system was used for analysis. The solvent selected for analysis was acetonitrile: water (30:70v/v). The wavelength of Lopinavir and Ritonavir were found 257.5 nm and 240.0 nm respectively. The Linearity for simultaneous equation method was studied by plotting a graph Conc. Vs. absorbance, Linearity was observed in the concentration range 80-180µg/ml for Lopinavir and 10-60 µg/ml of Ritonavir Coefficient of correlation (R^2) was found to be 0.997 and 0.998 for Lopinavir and Ritonavir respectively. The LOD was found to be 2.379µg/ml and 0.66 µg/ml for Lopinavir and Ritonavir respectively. LOQ was found to be 7.21µg/ml and 2 µg/ml for Lopinavir and Ritonavir respectively. The developed method was employed for the analysis of marketing formulation. The amount of drug obtained was in accordance with label claim. The recovery studies were carried out at three levels, i.e.80 %, 100% and 120% by the standard addition method. The precision of the proposed method was also established. The method was found to be accurate and precise.

Keywords: Lopinavir, Ritonavir, Simultaneous Equation Method, UV Spectrophotometer, Validation.

INTRODUCTION

Lopinavir and Ritonavir are antiretroviral drugs from a protease inhibitor class. The drugs have been proved to be effective in anti-HIV treatment. Chemically Lopinavir is (2S)-N-[(2S,4S,5S)-5-[2-(2,6dimethylphenoxyacetamido)-4-hydroxy-1,6-phenylhexan-2-yl]-3-methyl-2-(2-oxo-1,3-diazinan-1-yl)butanamide and its empirical formula is $C_{37}H_{48}N_4O_5$ with a molecular weight of 628.80 (Figure 1 A) ¹⁻³ and Ritonavir (5s, 8s, 10s,11s)-10-hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-is (phenylmethyl)-2,4,7,12-tetraazatridecan-13-oic acid 5-thiazolyl methyl ester of molecular formula $C_{37}H_{48}N_6O_5S_2$ and its molecular weight is 720.95 (Figure 1 B) ¹⁻³. Ritonavir is the most potent protease inhibitor, it has an ability to inhibit CYP-450 and efflux pump-P-glycoprotein as a result the potential for severe drug interaction is quite great because of strong CYP-450 the inhibiting effect of ritonavir. The drug has found value when used in fixed dosage form combination with other Pharmaceutical Ingredients to block their metabolism and acts as a booster for these drugs. In these cases, ritonavir is used in a sub therapeutic dose, but boosts the effectiveness of the co administered drug. ⁴⁻⁷

Literature survey of lopinavir and ritonavir either single or in combination with ritonavir shows that several methods based on UV- spectrophotometry, HPLC and HPTLC were developed and validated. However, there are few UV- spectrophotometric method for simultaneous determination are available which are costly and time consuming. The present method was validated as per ICH guideline.

MATERIALS AND METHODS ⁸⁻¹⁷

Instrument

An UV –visible double beam spectrophotometer of make JASCO, model V-530 with a pair of 1cm matched quartz cell, spectral bandwidth of 2cm and Shimadzu balance, AUX-220 were used for experimental purpose.

Chemicals

Acetonitrile-AR, Distilled water

Method

The stock solutions were prepared as follows-

Preparation of stock solution of Lopinavir

An accurately weighed 100 mg of Lopinavir was transferred to 100ml volumetric flask. Dissolved and made up to the volume with a mixture of acetonitrile: water (30:70v/v) which is previously prepared and sonicated for 10min., obtain the concentration of 1000µg/ml. Stock solution was sonicated for 15 min and filtered it. From this stock solution pipette out 0.8ml, 1ml,1.2ml, 1.4ml, 1.6ml in 10ml volumetric flasks and made the volume with a mixture of Acetonitrile : water (30:70v/v) to get final concentration range 80-160µg/ml.

Preparation of stock solution of Ritonavir

An accurately weighed 5mg of Lopinavir was transferred to 50ml volumetric flask. Dissolved and made up to the volume with a mixture of Acetonitrile: water (30:70v/v) which is previously prepared and sonicated for 10min., obtain the concentration of 100µg/ml. Stock solution was sonicated for 15 min and filtered it. From this stock solution pipette out 1ml,2ml,3ml,4ml,5ml in 10ml volumetric flasks and made the volume with a mixture of Acetonitrile : water (30:70v/v) to get final concentration range 10-50µg/ml.

Selection of Wavelengths

The stock solution of Lopinavir and Ritonavir were prepared separately in the mixture of Acetonitrile: water (30:70v/v) having concentration 1000µg/ml and 100µg/ml, respectively, diluted with the same to get concentration range 80µg/ml-160µg/ml for Lopinavir and 10-50µg/ml for Ritonavir they were scanned in the wavelength range of 200-400 nm, the λ_{max} of both the drug were found it was 257.5nm for Lopinavir and 240nm for Ritonavir. Figure 2 and Figure 3.

METHOD VALIDATION

Linearity

Preparation of standard calibration curve and selection of analytical linearity ranges

The stock solution of Lopinavir and Ritonavir were prepared separately in the mixture of Acetonitrile: water (30:70v/v) having concentration 1000µg/ml and 100µg/ml, respectively, diluted with the same to get concentration range 80µg/ml-160µg/ml for Lopinavir and 10-50µg/ml for Ritonavir respectively. The absorbances of the different concentration of Lopinavir were measured at 257.5nm and the absorbances of the different concentration of Ritonavir were measured at 240nm respectively. Six replicates analysis was carried out. Absorbance Vs concentration was plotted to obtain the calibration graph. Both the drugs obeyed Beer's law with the above concentration range with the range R² value of 0.997 and 0.998 for Lopinavir and Ritonavir. For simultaneous equation method, Lopinavir was also measured at 240nm and Ritonavir was also measured at 257.5nm respectively. Table 1-4, Figures 4-7.

Optical and regression characteristics

Optical and regression characteristics of the calibration curves are given in table 5.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were separately determined based on the standard deviation of the response of the calibration curve. The standard deviation of the y intercept and slope of calibration curve was used to calculate the LOD and LOQ. The results of the same are shown in the table 6.

LOD calculated from the formula

$$LOD = \frac{3.3 (SD)}{S}$$

where, SD = Standard deviation, S = Slope

LOQ calculated from the formula

$$LOQ = \frac{10 (SD)}{S}$$

where, SD = Standard deviation

S = Slope

Assay

Analysis of standard mixture of Lopinavir and Ritonavir

The stock solution containing a mixture of Lopinavir and Ritonavir in the ratio 4:1 was taken, different dilution from stock solution were prepared having concentration range 50 - 250µg/ml for Lopinavir (12.5-62.5µg/ml for Ritonavir). The concentration range 80 µg/ml for Lopinavir and 20 µg/ml for Ritonavir was selected as a reference standard. The absorbance of the reference standard solution was taken. The results of analysis of reference standard are given in the table 7.

Analysis of tablet formulation

Conventional tablet of Lopinavir (200mg) and Ritonavir (50mg) procured from the local market (Lopimune from Cipla Ltd) the tablet powder equivalent to 10mg of Lopinavir and 2.5mg of Ritonavir was weighed accurately, dissolved in an ACN: H₂O in a 25 ml volumetric flask, Sonicate the solution for 20 min to complete dissolution. The solution was diluted up to the mark to get 400µg/ml of Lopinavir and 100µg/ml of Ritonavir and filtered through Whatman filter paper no. 40. The sample stock solution was suitably diluted with selected solvent to get 80µg/ml of Lopinavir and 20µg/ml of Ritonavir. The resulting stock solution was filtered using Whatman filter paper No.40, the absorbance of the solution was taken, and the amount drug was calculated. The results of analysis of tablet formulation are given in table no. 8.

Precision

A) Repeatability

The repeatability of sample applications and measurement of absorbance were expressed in terms of % R. S. D. and was found to be less than 2% and results of the same are given in the table 10.

B) Intermediate precision

The intermediate precision was determined by six replicate absorbance of the prepared sample solutions. The repeatability of the sample application and measurement of absorbance's for the drug was calculated by repeating the assay six times for each concentration in the same day for intraday precision. The inter-day precision was obtained by assay of six sample sets on different days. The results of the analysis are shown in the table 10.

Accuracy

Recovery studies were carried out by applying the method to drug content present in tablet dosage form to which known amount of the reference standard of Lopinavir and Ritonavir were added at 80%, 100% and 120% levels. The solution for recovery studies at 80%, 100% and 120% levels were prepared according to addition method. The solutions were filtered through Whatman filter paper no. 40 filter paper and analyzed by UV-Spectrophotometric method. At each level of recovery three determinations were performed. The results of same are shown in the table 11 and 12.

Ruggedness

The same experiment was carried out by different analyst. Limit: Overall RSD for twelve results obtained from two different systems should not be more than 2.0 %. Overall RSD for twelve results obtained from two different analysts should not be more than 2.0 %. Overall RSD for twelve results obtained from two different analysts were found to be within limits. Therefore, the UV Spectroscopic method for the determination of both drugs is rugged. Table 13.

RESULT

A simple, precise and accurate UV-Spectrophotometric method was developed for Simultaneous estimation of Lopinavir and Ritonavir in bulk and marketed tablet formulation, using solvent of Acetonitrile: water (AR grade), in the ratio 30: 70. The absorbances were taken at 257.5 nm and 240 nm for Lopinavir

and Ritonavir respectively. The stock solution of Lopinavir and Ritonavir were prepared separately in the mixture of Acetonitrile: water (30:70v/v) having concentration 1000µg/ml and 100µg/ml respectively, diluted with the same to get concentration range 80µg/ml-160µg/ml for Lopinavir and 10-50µg/ml for Ritonavir respectively. The absorbances of the different concentration of Lopinavir were measured at 257.5nm and the absorbances of the different concentration of Ritonavir were measured at 240nm respectively six replicate analyses were carried out. Absorbance Vs concentration was plotted to obtain the calibration graph. Both the drugs obeyed Beer's law with the above concentration range with the range R²-value of 0.997 and 0.998 for Lopinavir and Ritonavir. Optical and regression characteristics of the calibration curves were carried out. The LOD and LOQ values for Lopinavir was 2.37 µg/ml and 7.21 µg/ml and for Ritonavir 0.66 µg/ml and 2 µg/ml respectively. 91.37 % w/w and 93.31 %w/w Assay values were for marketed formulation with respect to Lopinavir and Ritonavir respectively. % R.S.D. of method was less than one.

Table 1: Standard calibration data of ritonavir at 257.5 nm

Concentration (µg/ml)	Absorbance (nm)
0	0
10	0.237
20	0.414
30	0.632
40	0.843
50	1.03
60	1.273

Table 2: Standard calibration data of ritonavir at 257.5nm

Concentration (µg/ml)	Absorbance (nm)
0	0
10	0.073
20	0.123
30	0.178
40	0.228
50	0.29
60	0.338

Table 3: standard calibration data of lopinavir at 257.5nm

Concentration (µg/ml)	Absorbance (nm)
0	0
80	0.1201
100	0.15
120	0.1865
140	0.2144
160	0.2555
180	0.2876

Table 4: standard calibration data of lopinavir at 240.0nm

Concentration (µg/ml)	Absorbance (nm)
0	0
80	0.0768
100	0.109
120	0.125
140	0.1504
160	0.1733
180	0.1988

Table 5: optical and regression characteristics

Parameters	Lopinavir		Ritonavir	
	257.5	240	240	257.5
Beer's law limits(µg/ml)	80-180	80-180	10-60	10-60
Coefficient of correlation (R ²)	0.995	0.995	0.998	0.999
Regression equation, Y=mx+c	Y=0.001x(-0.007)	Y=0.001x(-0.014)	Y=0.0206x(+0.014)	Y=0.0053x(+0.017)
Slope(m)	0.001	0.001	0.0206	0.0053
Intercept(c)	-0.007	-0.014	0.014	0.017

Table 6: LOD and LOQ

Parameters	Lopinavir (µg/ml)		Ritonavir (µg/ml)	
	LOD	2.379	6.6	0.66
LOQ	7.21	20.82	2.00	11.15

Table 7: Analysis of reference standard for lopinavir and ritonavir

Sr. No.	Name of the drug	Concentration Taken (µg/ml)	Concentration Found (µg/ml)	% Obtained	SD	%RSD
1	Lopinavir	80	73.66	95.47333	0.582266	0.609873
2	Ritonavir	20	18.26	98.06333	0.411015	0.419132

*Average of six determinations

Table 8: Analysis of tablet formulation

Sr. No.	Amount Taken ($\mu\text{g}/\text{tab}$)		Amount found ($\mu\text{g}/\text{tab}$)		%of label claim	
	Lopinavir	Ritonavir	Lopinavir	Ritonavir	Lopinavir	Ritonavir
1	80	20	72.98	18.52	91.22	92.60
2	80	20	73.24	18.63	90.30	93.15
3	80	20	72.65	19.02	90.81	95.10
4	80	20	72.86	18.46	91.07	92.30
5	80	20	74.01	18.24	92.51	91.21
6	80	20	73.85	19.13	92.31	95.55

Table 9: Statistical validation of tablet formulation

Drug	Mean	S.D.	% R.S.D.
Lopinavir	91.37	0.631243	0.689589
Ritonavir	93.31	1.785759	1.915258

*Average of six determinations

Table 10: Intraday and inter day precision

Drug	Amount Taken ($\mu\text{g}/\text{ml}$)	Inter-day			Intra-day		
		Mean ($\mu\text{g}/\text{ml}$)	S.D	% RSD	Mean ($\mu\text{g}/\text{ml}$)	S.D	% RSD
Lopinavir	80	78.59667	0.456326	0.580592	79.02333	0.719884	0.910977
Ritonavir	20	19.27333	0.300888	1.56116	19.31667	0.296704	1.536001

*Average of six determinations

Table 11: Recovery study at different levels for lopinavir and ritonavir

Level of % Recovery	Amount present (mg)		Amount of standard added (mg)		% recovery	
	Lopinavir	Ritonavir	Lopinavir	Ritonavir	Lopinavir	Ritonavir
80	80	20	64	16	87.23	86.01
	80	20	64	16	86.21	86.53
	80	20	64	16	87.63	85.96
100	80	20	80	20	91.56	91.56
	80	20	80	20	90.23	91.46
	80	20	80	20	91.26	90.75
120	80	20	96	24	91.85	90.97
	80	20	96	24	91.48	90.68
	80	20	96	24	92.14	91.21

*Average of three at each level of recovery

Table 12: Statistical validation of recovery studies

Level of % recovery	% mean recovery		Standard deviation		% R.S.D.	
	Lopinavir	Ritonavir	Lopinavir	Ritonavir	Lopinavir	Ritonavir
80	87.02	86.16	0.7322	0.3156	0.8413	0.3663
100	91.01	91.25	0.6975	0.4416	0.7664	0.4839
120	91.82	90.95	0.3308	0.2653	0.3602	0.2919

Table 13: Ruggedness-analysis of formulation

Sr.no.	Amount taken ($\mu\text{g}/\text{ml}$)		Amount found ($\mu\text{g}/\text{ml}$)		% Amount Found	
	Lopinavir	Ritonavir	Lopinavir	Ritonavir	Lopinavir	Ritonavir
1	80	20	72.95	18.60	91.18	93.01
2	80	20	73.23	18.41	91.53	92.05
3	80	20	73.64	19.13	92.05	95.65
4	80	20	72.86	18.34	91.07	91.70
5	80	20	73.91	18.35	92.38	91.75
6	80	20	73.15	19.10	91.43	95.50

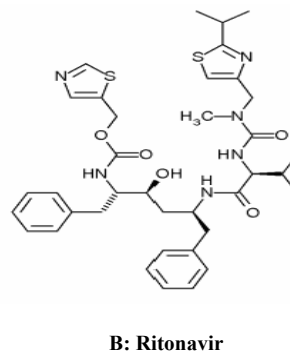
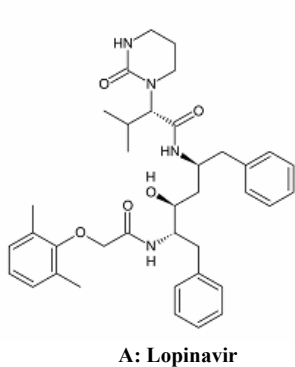


Figure 1: Structure of lopinavir and ritonavir

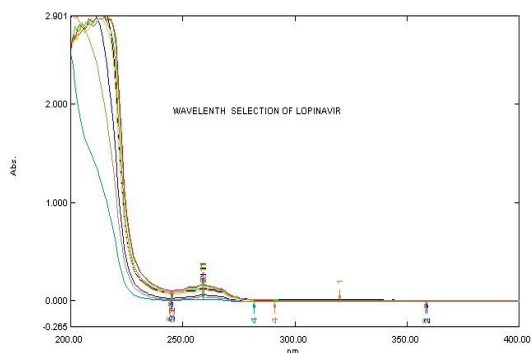


Figure 2: Overlay spectra of lopinavir

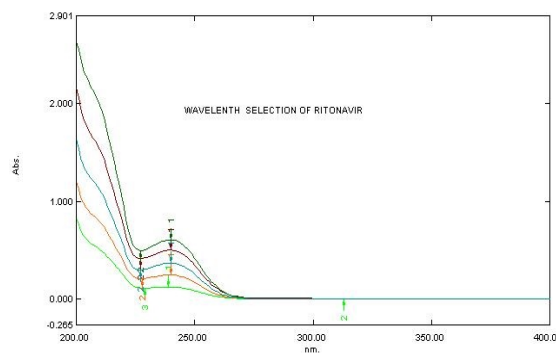


Figure 3: Overlay spectra of ritonavir

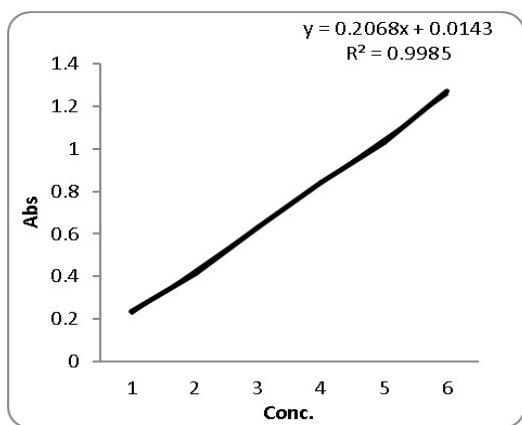


Figure 4: Calibration curve of ritonavir at 257.5nm

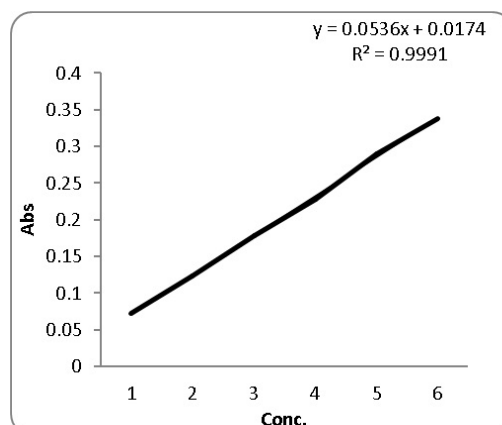


Figure 5: Calibration curve of ritonavir at 240nm

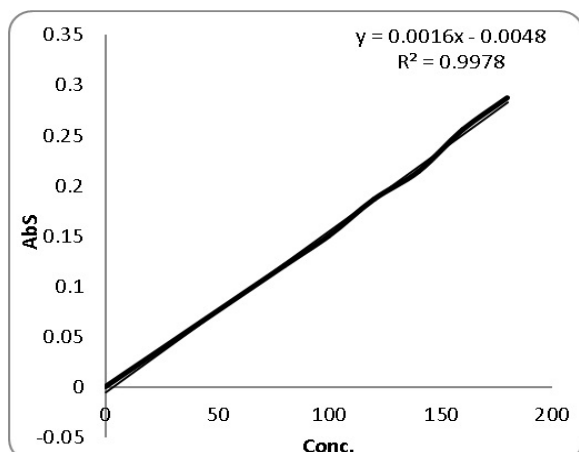


Figure 6: Calibration curve of lopinavir at 257.5 nm

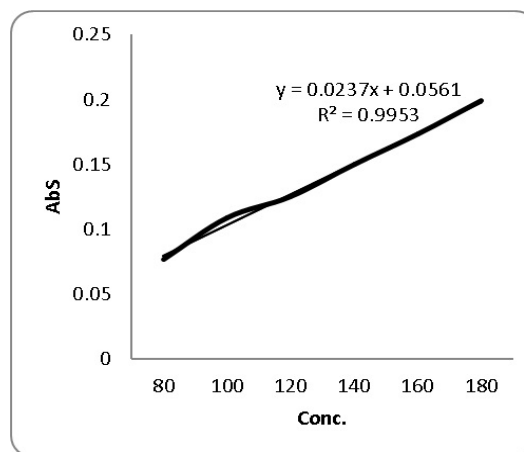


Figure 7: Calibration curve of lopinavir at 240 nm

DISCUSSION

The given methods were validated as per the ICH guidelines. The given method followed the Beers Lambert's law (linearity) in the range of 80µg/ml-160µg/ml for Lopinavir and 10-50µg/ml for Ritonavir respectively. Optical and regression characteristics of the calibration curves showed Coefficient of correlation was within the limit. From LOD and LOQ method showed the sensitivity. Assay value showed the accuracy of method. Precision and recovery study were carried out which shows the method is precise. Ruggedness study shows that the given method was stable.

CONCLUSION

The developed method was validated as per ICH guidelines. The method follows all validation parameters and the developed method is easy, simple, follows accuracy and precision. The ruggedness indicates the developed method was stable.

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