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NEW SENSITIVE UV SPECTROPHOTOMETRIC METHOD FOR SIMULTANEOUS ESTIMATION OF LOPINAVIR AND RITONAVIR IN FIXED DOSE COMBINATION AS SOFT GELS

Jyothirmayee Devineni*, Vasumathi Rangani, Sravya Nunna

Department of Pharmaceutics, KVSR Siddhartha College of pharmaceutical sciences, Vijayawada, Andhra Pradesh, India.

ABSTRACT

Two new, simple, accurate and sensitive UV Spectrophotometric methods have been developed and subsequently validated for the simultaneous estimation of Lopinavir (LOPI) and Ritonavir (RIT) in a fixed dose combination. Lopinavir and Ritonavir have an absorption maxima at 258nm and 245nm respectively. The first method is based upon the simultaneous equation and second upon the determination of Q value. The simultaneous equation method is based upon the measurements of ratios of absorptivity and absorbance, of both the components at their absorption maxima. The method of Q analysis is based on the measurement of ratios of absorptivity and absorbance, of both components at two selected wavelengths; one is an isoabsorptive point i.e. 237 nm and other being the wavelength maxima of any of the two components, say λ_{max} of Lopinavir i.e. 258 nm. Lopinavir shows linearity over the concentration range of 10-30µg/mL and whereas Ritonavir at 2-10µg/mL at their respective absorption maxima and at isoabsorptive point. The assay and recovery studies from fixed dose combination as liquid fill formulations for soft gels are indicative of accuracy of the proposed methods. The developed methods were validated in accordance to International Conference on Harmonization (ICH) guidelines for linearity, range, accuracy and precision.

Keywords: UV Spectrophotometry, Lopinavir, Ritonavir, Anti-retroviral, Absorption Ratio.

INTRODUCTION

Human immunodeficiency virus (HIV) has been one of the most overwhelming diseases affecting a large pediatrics and adult population of the world. The advent and development of various antiretroviral drugs has led to the significant reduction in the morbidity and mortality rates of the HIV infected population. The standard anti-retroviral (ARV) therapy in treatment of HIV infection necessitates the use of combination drug therapy such as two nucleoside and nucleotide reverse transcriptase inhibitors (NRTIS).Out of various existing ARV drugs currently recommended by the World Health Organization for HIV infection, the combination of Lopinavir and Ritonavir is considered as one of the preferred ARV treatments.

Chemically, Lopinavir is (2S)-N-[(2S,4S,5S)-5-[2-(2,6-dimethylphenoxy)acetyl]amino]-4-hydroxy-1,6-diphen yl hexan-2-yl]-3-methyl-2-(2-oxo-1,3-diazinan-1-yl) butan amide whereas Ritonavir is 1,3-thiazol-5-ylmethyl, N-[(2S,3S,5S)-3-hydroxy-5-[[(2S)-3-methyl-2-[[methyl-[(2-pr o pan-2-yl-1,3-thiazol-4-yl) methyl] carbamoyl] amino] butanoyl]amino]-1,6-diphenylhexan-2-yl]carbamate [10].

Both are protease inhibitors, possessing potent inhibitor activity against HIV viral protease enzyme. This prevents cleavage of the gag-pol polyprotein and, therefore, improper viral assembly results. This subsequently results in non-infectious, immature viral particles. These combination drugs, when given as a fixed dose combination product rather than individual entities have shown to improve therapy in terms of sustained virological suppression and significant reduction in the mortality rates of the HIV/AIDS infected patients.

A literature survey reveals that various analytical methods have been reported for the simultaneous estimation of Lopinavir and Ritonavir in fixed dose combinations, such as RP-HPLC [2-9] dual wavelength spectrophotometric method. The fixed dose combination of Lopinavir and Ritonavir is official in IP 2014, BP 2014 and USP [10].

However, the present work proposed the methodologies (simultaneous equation and Q value) which rely upon the use of simple arithmetic calculations, undemanding instrumentation set up along with the use of cheap reagents. The proposed methodologies provide sensitivity comparable to that achieved by sophisticated and expensive technique like HPLC. The proposed methodologies also surpass the existing spectrophotometric

Corresponding Author :- **Jyothirmayee Devineni** Email:- djyothirmayee9@gmail.com

techniques available. The purpose of this work is to explore new, simple, accurate and sensitive UV spectrophotometric methods for the simultaneous estimation of Lopinavir and Ritonavir in fixed dose combinations. The developed methods were validated in accordance to the international conference on Harmonization (ICH) guidelines for specificity, linearity, range, accuracy and precision.

MATERIAL AND METHODS

Lopinavir (Hetero Labs, Hyderabad), Ritonavir (Hetero Labs, Hyderabad) were procured as gift samples. Spectrophotometric studies were carried out using a Schimadzu UV Visible double beam Spectrophotometer. Fixed dose combination as liquid fill formulations for soft gels, [11-13] containing 200mg Lopinavi+50mg Ritonavir(Lopinavir/Ritonavir contains;200mg/50mg) and their respective working standards were prepared in a concentration range of 10-30µg/mL for Lopinavir and 2-10 µg/mL for Ritonavir. Methanol was purchased from Loba chemi pvt. Ltd. (Mumbai, India).

Selection of common solvent

The selection of common solvent was made after assessing the solubility of both the drugs in different solvents. Lopinavir and Ritonavir are freely soluble in methanol and ethanol.

Preparation of Standard Stock Solutions

Standard stock solutions of Lopinavir and Ritonavir were prepared by dissolving 50 mg of Lopinavir and 10 mg of Ritonavir in 10 mL of methanol to obtain the standard stock solutions of 5mg/mL for Lopinavir and 1mg/mL for Ritonavir.

Assessment of Absorption Maxima and Isobestic (Isoabsorptive) point

The dilutions of each Standard stock solution were prepared separately by using a 0.1N Hcl as a buffer medium to obtain the final standard solutions of 10-30µg/mL for Lopinavir and 2-10µg/mL for Ritonavir. The two solutions were scanned separately in the range of 200-400 nm to determine respective wavelength of maximum absorption. Lopinavir and Ritonavir showed wavelengths of absorbance maxima ($\boldsymbol{\lambda}_{max}$) at 258 nm ($\boldsymbol{\lambda}_1$) and 245 nm ($\boldsymbol{\lambda}_2$) respectively. The overlay spectra of Lopinavir and Ritonavir was constructed, considering the dilutions were attuned in such a way that a single point (isoabsorptive point) intersects in between the $\boldsymbol{\lambda}_{max}$ of both the components. An isoabsorptive point was obtained as 237 nm. UV spectra are shown in Fig. 2.and overlay spectra were shown in Fig.3.

Method 1 (Simultaneous Equation Method)

The two equations were constructed based upon the fact that at $\boldsymbol{\lambda}_1$ and $\boldsymbol{\lambda}_2$, the absorbance of the mixture is the sum of individual absorbance of Lopinavir and Ritonavir.

At $\boldsymbol{\lambda}1$, $A1 = ax1bcx + ay1bcy$

At $\boldsymbol{\lambda}2$, $A2 = ax2bcx + ay2bcy$

Where, A1 and A2 are an absorbance of test sample at 258 nm ($\boldsymbol{\lambda}_1$) and 245 nm ($\boldsymbol{\lambda}_2$) respectively.

Rearrange and substitute the above equations,

$Cx = (A2ay1 - \boldsymbol{A1ay2})/$(ax2ay1 - ax1ay2)

$Cy = (A1ax2 - A2ax1)/$(Ax2ay1 - ax1ay2)

Preliminary calculations and assumptions:

1. $ax1$ and ax2 =Absorptivity of Lopinavir at $\boldsymbol{\lambda}_1$ and $\boldsymbol{\lambda}_2$, respectively.
2. $ay1$ and ay2 =Absorptivity of Ritonavir at $\boldsymbol{\lambda}_1$ and $\boldsymbol{\lambda}_2$, respectively.
3. A_1 and A_2 = Absorbance of test sample at $\boldsymbol{\lambda}_1$ and $\boldsymbol{\lambda}_2$, respectively.
4. Cx and Cy are the concentrations of Lopinavir and Ritonavir respectively in test sample.

5. Application of the proposed method for Determination of Lopinavir and Ritonavir in Fixed Dose Combination as Liquid fill formulations for Soft Gels

6. Liquid fill formulations for soft gels (Lopinavir /Ritonavir comprimes; 200mg/50mg) were prepared. The fill formulation which is equivalent to 5mg (20µl) was taken in a 10mL volumetric flask containing few mL of methanol, mixed thoroughly and made up the volume up to the mark with 40% methanol and 0.1 N Hcl(to avoid precipitation). From the prepared solution 10µL was taken and dilutions were made with the 0.1NHcl as a buffer media. Lopinavir and Ritonavir showed absorbance maxima (λ_{max}) at 258 nm ($\boldsymbol{\lambda}_1$) and 245 nm ($\boldsymbol{\lambda}_2$) respectively. The concentration of both Lopinavir and Ritonavir were determined by measuring the absorbance of test sample at selected wavelengths and absorptivity (A 1%, 1cm) for both the drugs at both wavelengths were determined. Values were substituted in the respective formula to obtain concentrations (Table I).

Method 2 (Absorbance Ratio / Q Value Method)

The overlay spectrum of the two candidate drugs was obtained and isoabsorptive point was obtained as 237 nm. The two wavelengths were selected, one as 237 nm (isoabsorptive point) and other being the wave length maxima of any of the two components, i.e. 258 nm (wavelength of maximum absorption of Lopinavir) in the present case. The serial dilutions were prepared and absorbance and absorptivity for both the drugs , were measured at selected wavelengths and were also calculated. The Q value is used for the estimation of concentrations of drugs in sample solutions. The following formulas are used in this method.

$$Q0 = \frac{\text{Absorptivity of test sample at 237 nm}}{\text{Absorptivity of test sample at 258 nm}}$$

$$Q1 = \frac{\text{Absorptivity of Lopinavir at 237 nm}}{\text{Absorptivity of Lopinavir at 258 nm}}$$

$$Q2 = \frac{\text{Absorptivity of Ritonavir at 237 nm}}{\text{Absorptivity of Ritonavir at 258 nm}}$$

$$C1 = \frac{(Q0-Q2)}{(Q1-Q2)} X \frac{A}{a1}$$

$$C2 = \frac{(Q0-Q1)}{(Q2-Q1)} X \frac{A}{a2}$$

Preliminary calculations and assumptions:

1. A = Absorbance of test sample at isoabsorptive point.
2. a1 and a2 = Absorptivity of Lopinavir and Ritonavir respectively at isoabsorptive point.
3. C1 and C2 are the concentrations of Lopinavir and Ritonavir respectively in test sample.

Application of the proposed method for Determination of Lopinavir and Ritonavir in Fixed Dose Combination as Liquid fill formulations for Soft Gels

The concentrations of both Lopinavir and Ritonavir was determined by measuring the absorbance of the test sample at 258 nm and 237 nm and absorptivity of Lopinavir and Ritonavir at 258 nm and 237 nm. Values were substituted in the respective formula to obtain concentrations (Table I).

Method validation

The developed UV spectrophotometric methods were validated to confirm that they were suitable for their intended purpose as described in International Conference on Harmonization Q2 (R1) guidelines.

Linearity and Range

The linearity of measurement was evaluated by analyzing different concentration of standard solution of Lopinavir and Ritonavir at their respective wavelength of absorption maxima and at isoabsorptive point. The Beer-Lambert concentration range was found to be 10-30µg/mL for Lopinavir and 2-10µg/mL for Ritonavir at their respective absorption maxima and at isoabsorptive point. Calibration plot of Lopinavir and Ritonavir are shown in Fig.4 and Fig.5 respectively. Linear regression data for the calibration curves of Lopinavir and Ritonavir were shown in Table II.

Accuracy

To ascertain accuracy of the proposed methods, recovery studies were carried out by standard addition method at three levels (50%, 100% and 150%).Percent recoveries for both Lopinavir and Ritonavir by both the methods were calculated and found within the range (Table III).

Precision

Precision of the methods were estimated with respect to both repeatability (intra-assay) and intermediate precision (inter-day). It was performed by measuring the absorbance of a concentration range of 20, 25 and 30 µg/mL of Lopinavir and 6, 8, 10 µg/mL of Ritonavir at their respective wavelengths of absorption maxima (λ_{max}). Intra-assay precision was assessed by measuring the absorbance of the same sample concentration in triplicate and inter-day precision was assessed by measuring absorbance of the same sample concentration in triplicate on three different days over a period of one week.

For intra-assay precision (repeatability), the values of RSD of three concentrations 20, 25 and 30 µg/mL of Lopinavir and 6, 8, 10 µg/mL of Ritonavir level were obtained as 0.008, 0.005, 0.006% for Lopinavir and 0.007, 0.005, 0.007 % for Ritonavir respectively. The developed method was found to be precise as shown in Table IV.

Table 1. Result of analysis of Test sample (Lopinavir/Ritonavir comprises; 200mg/50mg)

% Conc.Estimated*	Method I		Method II	
	Lopinavir	Ritonavir	Lopinavir	Ritonavir
(Mean ± R.S.D)	101.1 ± 0.21	98.24 ± 0.09	100 ± 0.11	99.14 ± 0.18

*Average of three determinations; R.S.D. = Relative standard deviation

Table 2. Linear Regression Data for the calibration curves

Parameters	Lopinavir		Ritonavir	
	258 nm (λ_{max})	237 nm (isoabsorptive point)	245 nm(λ_{max})	237 nm (isoabsorptive point)
Regression equation	y=0.003x-0.035	y=0.004x-0.031	y=0.011x+0.007	y=0.009x+0.007
Regression coefficient(R^2)	0.915	0.774	0.996	0.995
Slope	0.003	0.004	0.011	0.009
Intercept	0.035	0.031	0.007	0.007

Table 3. Recovery Studies

Method	Drug	Level (%)	Recovery (%)	Mean (%) ± R.S.D
I	Lopinavir	50	98.93	99.187± 0.74
		100	100.02	
		150	98.60	
	Ritonavir	50	98.82	98.90 ± 0.40
		100	98.55	
		150	99.34	
II	Lopinavir	50	99.12	99.45 ±0.24
		100	100.33	
		150	98.92	
	Ritonavir	50	98.22	98.95 ± 0.52
		100	99.01	
		150	99.62	

R.S.D = Relative Standard Deviation

Table 4. Precision of the proposed methods

Drug	Conc.(µg/mL)	Repeatability(intra-assay precision)		Inter-day precision	
		Average Absorbance*	%RSD	Average Absorbance**	%RSD
Lopinavir	20	0.254	0.32	0.2537	0.36
	25	0.3244	0.37	0.3271	0.73
	30	0.4213	0.10	0.4229	0.22
Ritonavir	6	0.2163	0.25	0.2156	0.71
	8	0.2844	0.23	0.2880	1.21
	10	0.362	0.99	0.3602	0.49

SD= Standard Deviation; RSD =Relative Standard Deviation,*Average of triplicate determinations, ** Average of triplicate determinations in three different days.

Table 5. Statistical comparison of the results obtained by proposed methods

Methods	F-test	t-test
Method I and II	0.06	0.32

Fig 1. Chemical Structure of Lopinavir (a) and Ritonavir (b)

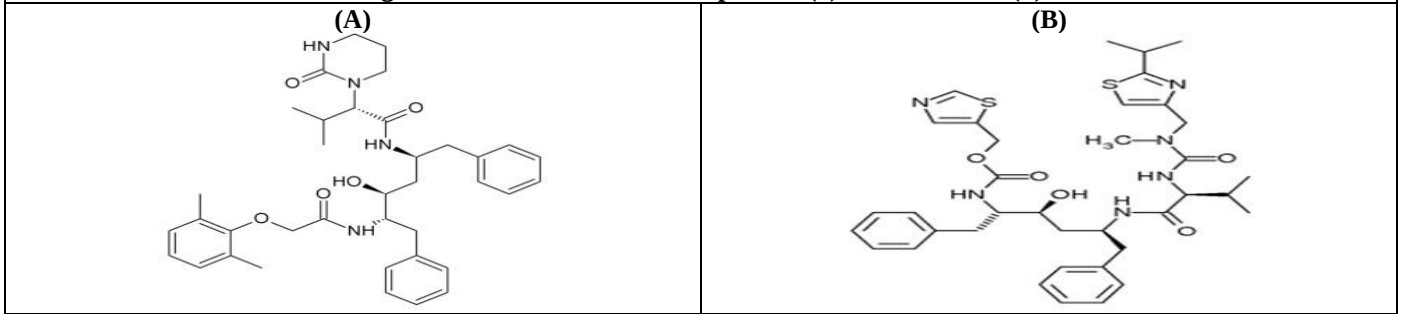


Fig 2. UV spectra of Lopinavir, Ritonavir

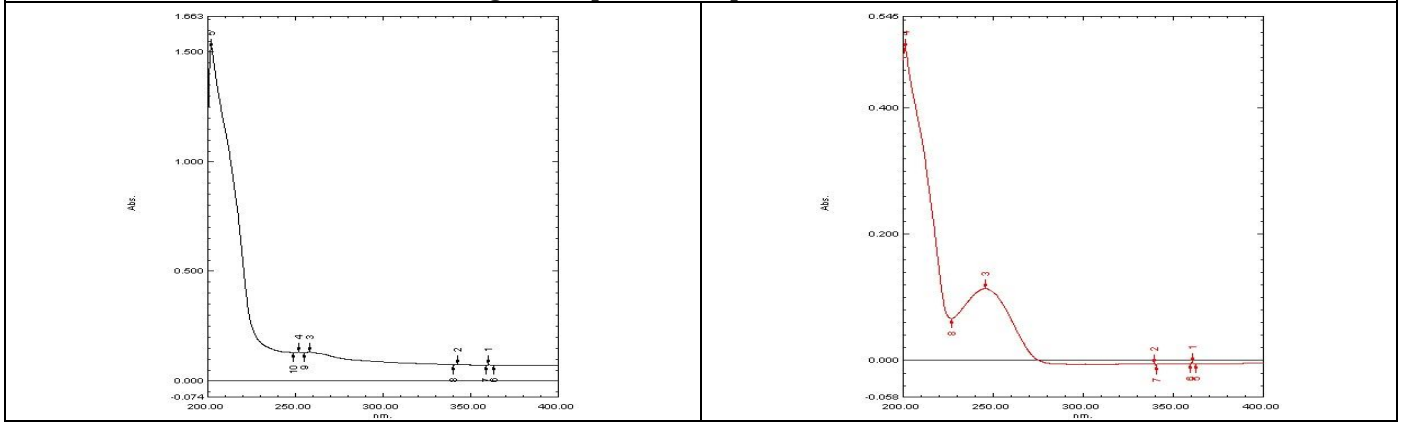


Fig 3. overlay spectrum

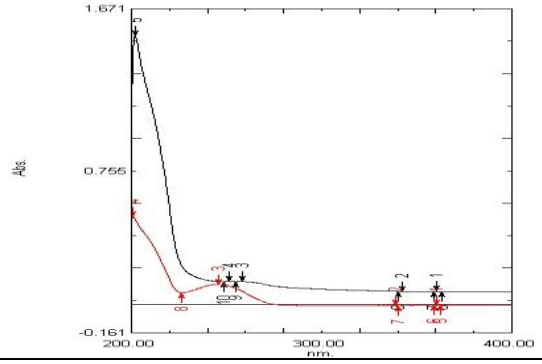


Fig 4. calibration plot of lopinavir

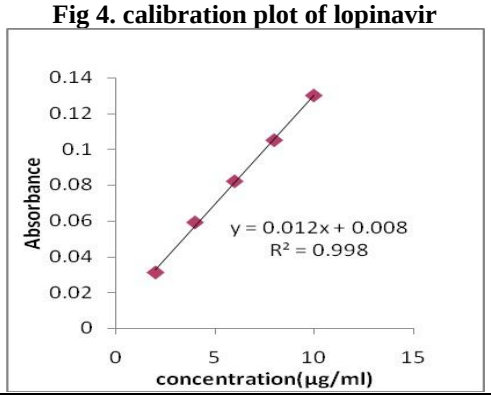
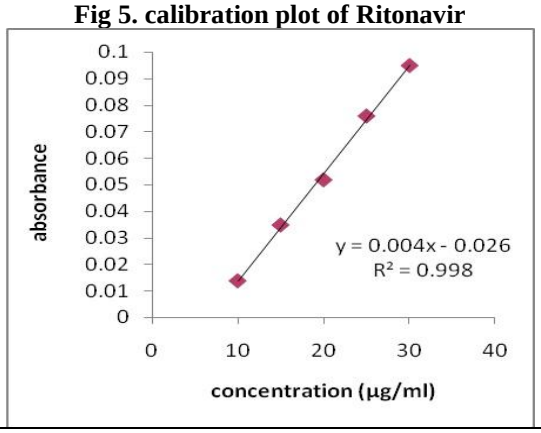


Fig 5. calibration plot of Ritonavir



RESULTS AND DISCUSSION

The two methods selected for multi-component analysis were given the satisfactory results. The UV spectra of Lopinavir and Ritonavir exhibit λ_{max} of 258 nm and 245 nm respectively. Additionally an isoabsorptive point was observed at 237 nm .These wavelengths were selected for simultaneous estimation and Q analysis of Lopinavir and Ritonavir and are assumed to be sensitive wavelengths. Standard calibration curves for Lopinavir and Ritonavir were found to be linear over the concentration range of 10-

30 µg/mL and 2-10 µg/mL at their respective absorption maxima and at an isoabsorptive point. The accuracy of the methods were confirmed by recovery studies from fixed dose combination as a liquid fill formulation for soft gels at three different levels of standard additions, recovery in the range of 99.18 - 99.45 % for Lopinavir and 98.90 - 98.95 % for Ritonavir by method I and method II respectively justifies the accuracy of method.

COMPARISON

The statistical comparison of the results of both the proposed methods were carried out and it was found that there was no significant difference between Method I (Simultaneous equation method) and Method II(absorbance ratio/Q value method) since the calculated and F-tests did not exceed the theoretical values at the 95% confidence level (Table v**).**

CONCLUSION

The developed methods were simple, accurate, and sensitive, subsequently validated and can be used for the outline analysis of fixed dose combinations of Lopinavir and Ritonavir.

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CONFLICTS OF INTEREST

The authors have none to declare.

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