Original Article

Method Development, Validation and Stability study of Ritonavir in Bulk and Pharmaceutical Dosage Form by Spectrophotometric Method

Abstract

Background: Ritonavir is a protease inhibitor and mostly used as a booster for increasing the bioavailability of other protease inhibitors like Atazanavir Sulfate and Lopinavir. **Aims:** Quality assessment of the new dosage form of Ritonavir i.e. tablets is very essential, so two sensitive, simple and precise methods are developed for quantification of Ritonavir in bulk and tablet dosage forms. **Materials and Methods:** The first method is based on first order derivative method and the second is based on area under curve method. Both the methods are validated according to international conference of harmonization (ICH) guidelines. A stability study of Ritonavir is done in UV – Visible Spectrophotometer under different stress conditions recommended by ICH guidelines. **Results:** The absorption maximum is found to be 239nm in methanol. The absorption maximum in first method is chosen at 253.2nm, and the linearity is found between 4 - 20 µg/ml with coefficient of correlation value 0.9981. In the second method, the range for area under curve selected is 237 – 242nm. The linearity is found between 4 -20 µg/ml with coefficient of correlation value 0.9992. **Conclusion:** The developed methods are validated and found to be simple, rapid, precise and cost-effective. The degradation study in tablet dosage form can be used as a stability indicating assay method.

Key words:
Area under curve, first order derivative, ritonavir, spectrophotometric method, stability study, tablet dosage form

Introduction

Ritonavir (RTV) is a selective, competitive and reversible inhibitor of the human immunodeficiency virus (HIV) protease enzyme. Chemically it is (5*S*,8*S*,10*S*,11*S*)-10-hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11- bis (phenylmethyl)-2,4,7,12-tetraazatridecan-13-oic acid 5- thiazolyl methyl ester [Figure 1]. It is widely used in the treatment against the acquired immune deficiency syndrome (AIDS) and prescribed in combination with other antiretroviral drugs as a booster. RTV is official in Indian Pharmacopeia (IP),[1] British Pharmacopeia,[2] and United States Pharmacopeia (USP).[3] Ritonavir is a selective, competitive inhibitor of liver enzyme Cytochrome P450 (CYP3A)[4] which help to increase the bioavailability of other Protease inhibitors like Atazanavir or Lopinavir in dual protease therapy. Many high performance liquid chromatography (HPLC) methods are reported in biological samples like blood plasma[5,6] and serum,[7] cells[8] and hair.[9] A high performance thin layer chromatography (HPTLC) method is reported in simultaneous estimation of RTV with Lopinavir[10] in capsule dosage form. From the extensive literature review, no analytical methods or stability studies are reported

Access this article online	
Website: http://www.cysonline.org	Quick Response Code
DOI: 10.4103/2229-5186.90894	

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for the tablet dosage form. The challenges for the quality control and bioavailability of RTV in generics produced in India inspired the authors to develop simple methods for quantification of RTV in bulk and stability study in UV – Visible Spectrophotometer. The developed methods were validated according to International conference of harmonization (ICH) guidelines.[11]

Materials and Methods

Instrumentation

A double beam JASCO UV–Visible spectrophotometer, V-630, with spectral band width of 1.5 nm and wavelength accuracy ±0.5 nm, and with a pair of 1 cm matched quartz cells was used for measuring the absorbance of the resulting solution.

Chemicals and reagents

A standard drug of RTV was procured from Matrix Laboratories (Hyderabad, India). Tablets of RTV (Ritomune and Viriton containing 100 mg of RTV, manufactured by Cipla Pvt. Ltd, Goa and Ranbaxy Laboratories, Gurgaon) were purchased from the local market. All the reagents like methanol, hydrochloric acid, sodium hydroxide and hydrogen peroxide were of analytical grade.

Method development

Solubility test

The solubility of the drug was tested in double distilled water, methanol, ethanol, acetonitrile, 1N hydrochloric acid and 1N sodium hydroxide. Methanol was found to be the most suitable solvent for the method development.

Methods

Method A: First order derivative method

In this method, 10 µg/ml solution of standard drug RTV was prepared in methanol and scanned from 400 to 200 nm. The absorption maximum was found at 239 nm [Figure 2]. The absorption spectrum thus obtained was derivatized from first order to fourth order. A first order derivative spectrum was selected for analysis [Figure 3]. From the derivatized spectrum of the drug, the wavelength selected for quantification was 253.2 nm. The calibration curve for RTV was plotted in the concentration range of 4-20 µg/ml at 253.2 nm.

Method B: Area under curve method

In this method, 10 µg/ml solution of standard drug RTV was prepared in methanol and scanned from 400 to 200 nm. From the zero order spectrum of RTV, the wavelength selected for ths study was in a range of 237 to 242 nm [Figure 4]. The calibration curve for RTV was plotted in the concentration range of 4-20 µg/ml with respect to its respective area under the curve.

Assay of ritonavir in tablet dosage form

The tablet dosage forms of two different companies were collected from the local market. 10 mg Equivalent amount of tablet powder was accurately weighed and transferred to a 100 ml volumetric flask and dissolved with methanol and the volume was made up to 100 ml with methanol. The solutions were sonicated for 10 minutes. They were then filtered through Whatman filter paper no. 41. From the filtrate desired concentration of sample solution of 10 µg/ml was prepared for each tablet. The content of the sample solution was quantified from the calibration curve by measuring the absorbance at 253.2 nm for method A, and measuring the area under curve for method B respectively.

Validation of methods

The developed methods were validated according to ICH guidelines. Both the methods were validated in terms of linearity, accuracy, precision, specificity and selectivity, ruggedness, Limit of detection (LOD) and Limit of quantification (LOQ).

Linearity

Linearity of the methods was done by preparing standard stock solution dilutions ranging from 2 µg/ml to 25 µg/ml. When the solution was scanned in UV range of 200 to

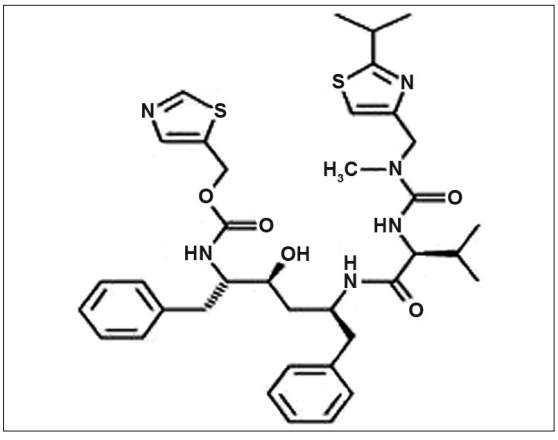

Figure 1: Chemical structure of Ritonavir

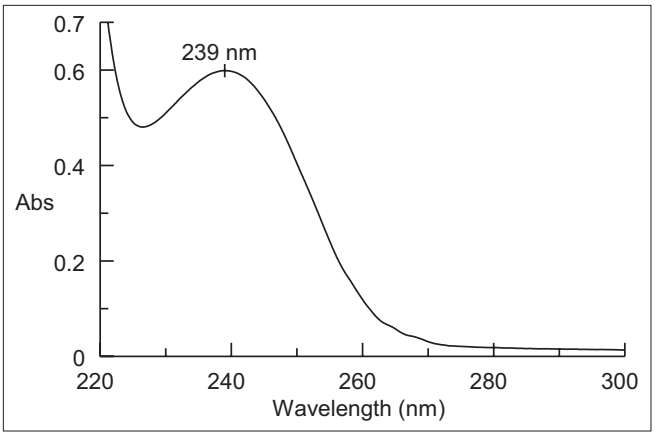

Figure 2: Zero order derivative of Ritonavir in methanol

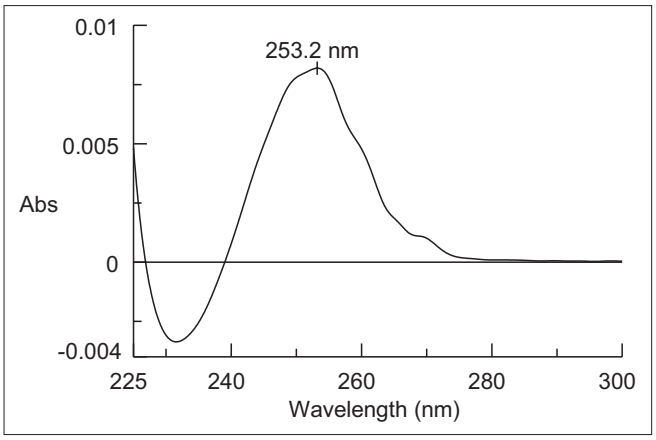

Figure 3: First order derivative of Ritonavir

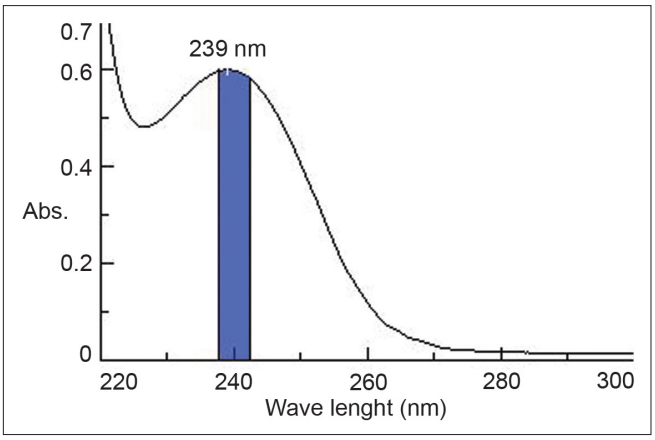

Figure 4: Spectrum of Ritonavir in area under curve method

400 nm, in both the methods the solutions obey the Beer's law from 4 µg/ml to 20 µg/ml.

Accuracy

In both the methods, the accuracy was tested by recovery study by standard addition method. The standard drug was added to the pre-analysed marketed dosage form at three different levels i.e. 25, 50 and 100%.

Precision

The precision of the methods was tested in terms of repeatability and reproducibility. The precision of the proposed methods was performed by intra- and inter-day assay. In intra-day assay the same sample (10 µg/ml) was assayed with time interval for 6 times within the same day. In inter-day assay, the assay of the same sample (10 µg/ml) was done on 3 consecutive days. The precision of the methods was expressed in terms of percent of relative standard deviation (%RSD).

Specificity and selectivity

For both the methods, specificity was checked by interference study. Fixed amount of excipients were added to the sample solution and the assay was done by both the methods. The selectivity of the methods was determined by slight variation of the reaction conditions like change in the solvent and change in the wavelength in both the methods, which altered the results. This signifies the selectivity of the developed methods.

Ruggedness

Ruggedness of both the methods was done by performing the assay of the sample solutions by two different analysts. The results were expressed in terms of %RSD.

Limit of detection and limit of quantification

The LOD and LOQ was determined by using equation (1) and (2) respectively

$LOD = 3.3\ \sigma / S$ ---------- (1)

$LOQ = 10\ \sigma / S$ ---------- (2)

Where 'σ' is the standard deviation of y-intercept and 'S' is the slope of calibration curve.

Degradation study

ICH guideline entitled - stability testing of new drug substances and products, requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on the new formulation of RTV i.e. tablet dosage form. From the literature survey, it was found that the stability study was done by chromatographic method in soft gelatine capsule dosage form. Thus, the authors were inspired to develop a simple, precise and accurate stability indicating method by spectroscopic method. The degradation studies were carried out in various degradation conditions like hydrolysis under acidic medium and basic medium at room temperature, oxidative degradation, thermal degradation in solid state and liquid state.

Stress degradation by hydrolysis under acidic condition

For the stability study of RTV under acidic condition, 1 ml of stock solution of standard drug (100 µg/ml) was taken in a 10 ml volumetric flask and 1 ml of 1N hydrochloric acid was added. The solution was kept aside for 10 minutes at room temperature and the solution was neutralized with 1N sodium hydroxide solution and volume was made up to 10 ml with methanol. Then the absorbance of the resulting solution was measured at time interval of 1, 2, 3, 6, 24, 48 and 72 hours against the blank. The blank was prepared by adding 1 ml of 1N hydrochloric acid with 1 ml 1N sodium hydroxide solution and the volume was made up to 10 ml with methanol.

Stress degradation by hydrolysis under basic condition

For the stability study of RTV under basic condition, 1 ml of stock solution of standard drug (100 µg/ml) was taken in a 10 ml volumetric flask and 1 ml of 1N sodium hydroxide was added. The solution was kept aside for 10 minutes at room temperature and the solution was neutralized with 1N hydrochloric acid solution and volume was made up to 10 ml with methanol. Then the absorbance of the resulting

solution was measured at time interval of 1, 2, 3, 6, 24 and 48 hours against the blank. The blank was prepared by adding 1 ml of 1N hydrochloric acid with 1 ml 1N sodium hydroxide solution and the volume was made up to 10 ml with methanol.

Oxidative hydrolysis

For the stability study of RTV in presence of oxygen, 1 ml of stock solution of standard drug (100 µg/ml) was taken in a 10 ml volumetric flask and 1 ml of 0.3% hydrogen peroxide was added. The solution was kept aside for 10 minutes at room temperature and the volume was made up to 10 ml with methanol. Then the absorbance of the resulting solution was measured at time interval of 1 hour and 24 hours against the blank. The blank was prepared by taking 1 ml of 0.3% v/v hydrogen peroxide in a 10 ml volumetric flask and the volume was made up to 10 ml with methanol and kept overnight. The degradation was compared with the spectrum of stock solution (10 µg/ml) before addition of hydrogen peroxide.

Thermal degradation

For the thermal stability of RTV, standard drug was taken in a petriplate and kept in a hot air oven at 75° C for 48 hours. Then the same standard drug was used to prepare a solution of concentration 10 µg/ml. The absorbance of the solution was measured at λmax (239 nm). For the stability in solution state, a stock solution of 10 µg/ml was refluxed at 75° C for 24 hours. The absorbance of the solution was measured at λmax at 0 minutes, 1 hour and 24 hours.

Application of stress degradation study to formulation

As Ritonavir is temperature sensitive, and the stability study was reported in soft gelatine capsule dosage form by liquid chromatography,[12] and in standard drug by Liquid chromatography – Mass Spectroscopy (LC-MS),[13] a simple stability study was developed by applying the same degradation conditions to the tablet dosage form as in case of the previously explained procedure for standard drug.

Results and Discussion

From the literature review, it was found that no method has been developed for the quantification of RTV in new dosage form i.e. tablets formulation. The stability study of ritonavir was done by LC –MS, [12, 13] so a simple method was developed by spectrophotometry study to do a cost effective stability study.

Assay of formulation by method A and B

Both the methods i.e. first derivative method and area under curve method were found to be suitable to quantify RTV in tablet dosage form. In both the methods the content of RTV was found within the IP limit and low value of standard deviation signifies the precision of the methods [Table 1].

Validation of the methods

Linearity

In both the methods, at the selected range of λmax, the drug solutions follow the Beer's Law in the concentration range of 4-20 µg/ml. The values of coefficient of correlation were found to nearly equal to 0.999 [Table 2].

Accuracy

The accuracy of the developed methods was tested by standard addition method at the level of 25, 50 and 100%. The percentage of recovery, lower values of standard deviation and relative standard deviation (<2) indicates the accuracy of the methods [Table 3].

Precision

The intra- and inter-day assay of the formulations by the proposed methods were found to be suitable with very low values of standard deviation. This justifies the reproducibility and repeatability of the proposed methods [Table 4].

Specificity

All the formulations were assayed in presence of the excipients by the proposed methods. It was found that there is no interference of the excipients which justifies the specificity of the drug for the proposed methods.

Ruggedness

Ruggedness of both the methods was checked by performing the assay by both the methods by two different analysts [Table 5].

Limit of detection and Limit of quantification

LOD and LOQ were determined by using the Equation 1 and 2 [Table 2].

Degradation study

Stress degradation by hydrolysis under acidic condition

In stress degradation study under acidic condition, many concentrations of hydrochloric acid were tried from 0.1, 0.5 and 1N, but 1N concentration for degradation study was

Table 1: Assay of formulations used in the study

Name of the formulation	Label claim (mg)	Amount found (mg) Mean* ± SD		% RSD	
		Method A	Method B	Method A	Method B
Ritomune	100	99.97 ± 0.64	99.66 ± 0.38	0.64	0.38
Viriton	100	100.08 ± 0.29	100.03 ± 0.17	0.289	0.169

*Mean of five determinations; SD – Standard deviation; % RSD – Percentage of relative standard deviation

found suitable. The degradation was studied at time interval of 1, 2, 3, 6, 24, 48 and 72 hours against the blank [Figure 5]. The degradation after 72 hours was found to be 24.32%.

Stress degradation by hydrolysis under basic condition

In stress degradation study under basic condition, many concentrations of sodium hydroxide were tried from 0.1, 0.5 and 1N, but 1N concentration for degradation study was found suitable. The degradation was studied at time interval of 1, 2, 3, 6, 24 and 48 hours against the blank [Figure 6]. The degradation after 48 hours was found to be 24.48%.

Table 2: Optical characteristics of Method A and B in the study

Parameters	Method A	Method B
Wavelength (nm)	253.2	237–242
Beer - Lambert's Law (μg/ml)	4–20	4–20
LOD (μg/ml)	0.16	0.075
LOQ (μg/ml)	0.48	0.228
Molar absorptivity (l mol^{-1} cm^{-1})	73×10^4	65×10^4
Sandell's sensitivity (μg/cm^2/0.001 absorbance unit)	0.099	0.111
Regression equation [Y = mX + c]		
Slope (m)	0.062	0.091
Intercept (c)	0.028	0.022
Correlation coefficient (r^2)	0.998	0.999

LOD – Limit of detection; LOQ – Limit of quantification

Oxidative hydrolysis

For oxidative hydrolytic study, various concentrations of hydrogen peroxide were tried like 0.3, 3 and 30% v/v. But at 0.3% v/v hydrogen peroxide, suitable degradation resulted [Figure 7]. After 24hrs degradation study, not only the drug concentration reduced but also the λmax shifted from 238.9 to 243.2 nm. The degradation after 24 hrs was found to be 63.08%.

Thermal degradation

Stress degradation studies under thermal condition were done in two conditions like dry heat degradation and in solution phase. No degradation was found in dry heat state but marked degradation was found in solution phase. The solution of RTV in methanol was refluxed at 75° C for 24 hours and the degradation was found to be 100% [Figure 8].

Application of stress degradation study to tablet dosage form

The degradation study was applied to tablet dosage form. The percentage of degradation of tablet under different stress condition is represented in [Table 6]. The tablet under acidic hydrolysis degradation condition degrades by 24.32%; in basic hydrolysis degrades by 24.48%, in oxidative hydrolysis degrades by 63.08%. Under thermal degradation, there is no degradation in dry condition; however, in solution phase the degradation was 100%. The degradation study was carried

Table 3: Accuracy of the methods (Recovery study) used in the study

Name of the formulation	Amount of sample taken (μg/ ml) + amount of standard added (μg/ ml)	Mean*(%)±SD		% RSD	
		Method A	Method B	Method A	Method B
Ritomune	8+2	100.05±0.55	100.08±0.63	0.549	0.629
	8+4				
	8+8				
Viriton	8+2	100.32±0.62	100.22±0.7	0.618	0.698
	8+4				
	8+8				

*Mean of five determinations; SD – Standard deviation; % RSD – Percentage of relative standard deviation

Table 4: Precision of the methods used in the study

Name of the formulation	Intra-day precision (n=6) Mean*(%)±SD, % RSD		Inter-day precision (n=3) Mean*(%)±SD, % RSD	
	Method A	Method B	Method A	Method B
Ritomune	100.54±0.02, 0.019	100.22±0.02, 0.019	99.64±0.27, 0.27	100.08±0.62, 0.619
Viriton	100.01±0.46, 0.459	99.77±0.32, 0.32	100.14±0.47, 0.469	99.83±0.61, 0.61

*Mean of five determinations; SD – Standard deviation; % RSD – Percentage of relative standard deviation

Table 5: Ruggedness of the methods used in the study

Name of the formulation	Analyst 1 [Mean* (%)±SD], % RSD		Analyst 2 [Mean* (%)±SD], % RSD	
	Method A	Method B	Method A	Method B
Ritomune	99.58±0.2, 0.20	99.65±0.16, 0.16	99.03±0.34, 0.34	100.01±0.6, 0.59
Viriton	99.95±0.4, 0.40	99.42±0.36, 0.36	99.6±0.38, 0.38	99.5±0.3, 0.30

*Mean of five determinations; SD – Standard deviation; % RSD – Percentage of relative standard deviation

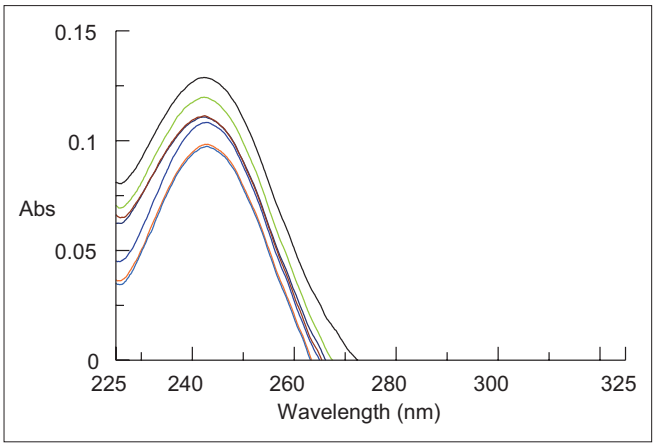

Figure 5: Spectra of degradation of Ritonavir under acidic hydrolysis

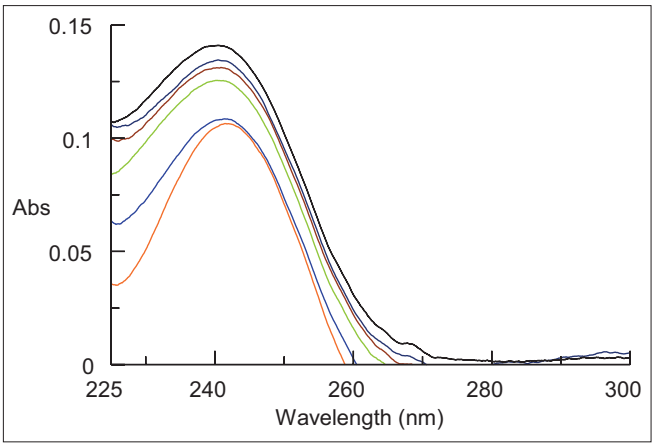

Figure 6: Spectra of degradation of Ritonavir under basic hydrolysis

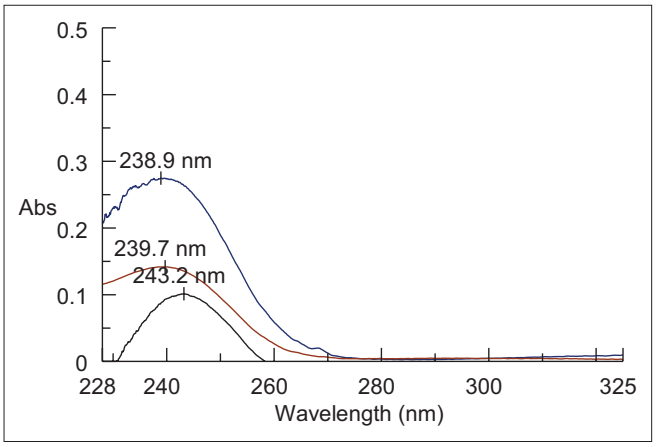

Figure 7: Spectra of degradation of Ritonavir under oxidative hydrolysis

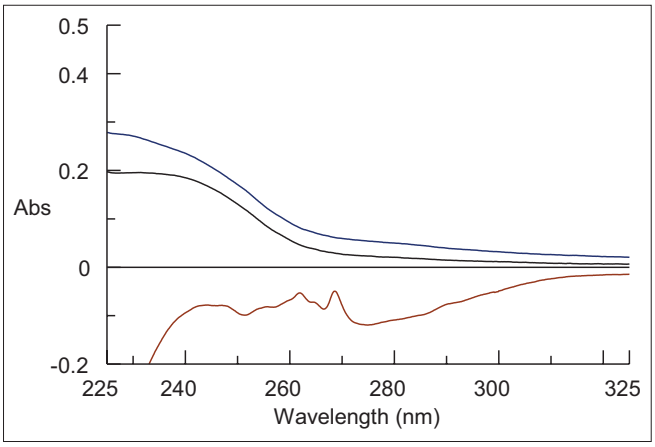

Figure 8: Spectra of degradation of Ritonavir under thermal condition in solution state

Table 6: Stress degradation study in tablet formulation in the study

Stress conditions	% of degradation in tablets
Acidic hydrolysis – 1.0 N Hydrochloric acid solution	24.32
Basic Hydrolysis – 1.0 N Sodium Hydroxide solution	24.48
Oxidative Hydrolysis – 0.3% v/v Hydrogen Peroxide	63.08
Thermal degradation – dry heat degradation	No degradation
Thermal degradation – solution phase degradation	100

out in presence of common excipients present in a tablet, however, showed no interference of excipients.

Conclusion

The developed methods are validated and found to be simple, rapid, precise and cost-effective. These methods can be used for not only quantification, but also quality control of RTV in bulk and dosage form. The degradation study can be carried out in small laboratories in a cost effective way. The degradation study in tablet dosage form can be used as a stability indicating assay method.

Acknowledgment

The authors are thankful to Siksha 'O' Anusandhan University for providing the necessary research facilities to carry out the present work. The authors are thankful to Matrix Laboratories (Hyderabad, India) for providing the standard drug of Ritonavir as a gift sample to carry out the present work.

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How to cite this article: Behera A, Moitra SK, Si SC, Meher AK, Gowri Sankar AP. Method development, validation and stability study of ritonavir in bulk and pharmaceutical dosage form by spectrophotometric method. Chron Young Sci 2011;2:161-7.

Source of Support: Nil, **Conflict of Interest:** None declared