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A Validated RP-HPLC Stability Indicating Assay Method For Simultaneous Estimation of Lopinavir And Ritonavir – Application to Bulk Drugs

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ABSTRACT

A simple, precise and stability-indicating HPLC method was developed and validated for the simultaneous estimation of anti-retroviral drugs Lopinavir and Ritonavir. The separation was achieved on AgilentC8, 150mm* 4.6 ,5µ column with isocratic flow. The mobile phase at a flow rate of 1.5ml consisted of 0.05M Potassium Dihydrogen Orthophosphate buffer and Acetonitrile: MeOH in the ratio of (80:20).The ratio of buffer: organic is (45:55).The UV detection was carried out at 210nm. The method was successfully validated in accordance to ICH guidelines. This method was then used to study the stability aspects of both the drugs when subjected to acidic, alkaline, thermal and photo degradation condition.

Keywords: Lopinavir, Ritonavir, Stability Indicating Assay Method, RP-HPLC.

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INTRODUCTION

Lopinavir, is chemically known as (2H) – pyrimidine acetamide N- [[4-(2,6- methyl phenoxy)acetyl]amino]-3-hydroxy 5- phenyl- 1- (phenyl methyl) pentyl, tetrahydro-α- (1- methyl ethyl)- 2- oxo and its empirical formula is C37H48N4O5, having a molecular weight of 628.80[1] Ritonavir, is chemically known as 2,4,7,12- tetra azatridecan- 13oicacid, 10 hydroxy- 2- methyl- 5- (1- methyl ethyl)- 1- [2- (1- methyl ethyl)- 4- thiazolyl]- 3,6- dioxo- 8,11- bis(phenyl methyl)- 5- thiazolmethyl ester and its empirical formula is C37H48N6O5S2 with a molecular weight of 720.90.[2] Lopinavir was developed by Abbott in an attempt to improve HIV resistance and serum protein-binding properties of the company's earlier protease inhibitor, ritonavir. When administered alone, lopinavir has insufficient bioavailability; however, like several HIV protease inhibitors, its blood levels are greatly increased by low doses of ritonavir, a potent inhibitor of cytochrome P450 3A4.[3] Abbott therefore pursued a strategy of co-administering lopinavir with sub-therapeutic doses of ritonavir, and lopinavir is only marketed as a co-formulation with ritonavir.

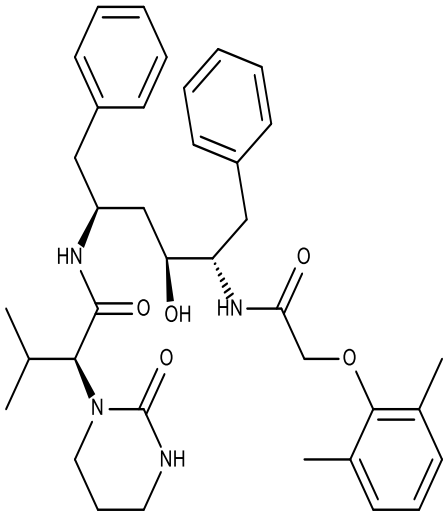

Figure 1: Structure of Lopinavir

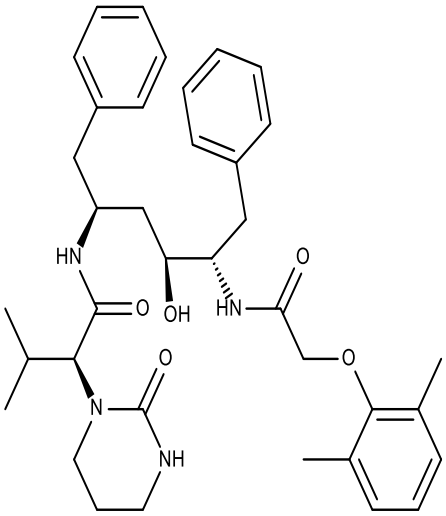

Figure 2: Structure of Ritonavir

Various analytical methods have been reported for the assay of lopinavir and ritonavir individually or combination with other drugs in biological samples/formulations. They include HPLC,[3-6] high performance thin layer chromatography, [7] derivative UV Spectrophotometry. [8] There are also methods for simultaneous estimation of both the drugs official in Indian British and US pharmacopeia. [9,10] However an extensive literature search didn't reveal stability indicating method for both the drugs in bulk form. Therefore, attempts were made to develop and validate simple, precise, and sensitive, isocratic reverse phase stability indicating high performance liquid

chromatographic method for simultaneous determination of both the drugs and their degradation products in bulk drugs. The parent guideline on drug stability testing Q1A (R2) issued by international conference on harmonization stipulates stress studies be carried out on a drug in order to establish the drug's inherent stability characteristics [11]. Literature studies show various analytical methods reported for the estimation of individual, binary or tertiary combination of anti-retroviral drugs or in combination with oral contraceptives. [12,13,14]

MATERIALS AND METHOD

Chemicals and Solvents

Lopinavir and Ritonavir were procured from Hetero Drugs Pvt. Ltd. All the chemicals and reagents such as sodium hydroxide, hydrochloric acid, hydrogen peroxide (30%) used, were of analytical grade while, acetonitrile and methanol procured from S.D Fine Chemical were of HPLC grade. A MilliQ plus water purification system (Miliford, USA), was used to prepare distilled water (>18 μΩ).

Instruments and software

Agilent HPLC system was used for method development, degradation studies, and validation. The output signal was monitored and processed using LCchrom software. C8 (150 mm × 4.6 mm i.d, particle size 5μ) column (Agilent ODS 3 V) was used for liquid chromatography studies. The analysis was carried out at ambient temperature. A water bath equipped with temperature controller was used to carry out degradation studies for all solutions. A controlled temperature dry air oven (META-LAB, GMP Model, MSI-66) was used for solid-state thermal stress studies. All pH measurements were carried out using pH meter (LABINDIA, PICO pH meter).All the weighing was carried out on a balance (SHIMADZU, AUX-220).

Method Development

Method development trials involved optimizing the mobile phase, columns and chromatographic conditions. The trials included acetonitrile: water, methanol: water, phosphate buffer: acetonitrile and phosphate buffer: methanol in various proportions. Several columns namely C18 250mm×4.6mm, C18 150mm×4.6mm, C8 250mm ×4.6mm and C8 150mm×4.6mm were evaluated. After several permutations and combinations of different mobile phases, columns and chromatographic conditions, optimized method was developed. Representative chromatogram is shown in Figure 1.

Mobile Phase

Buffer was made of 0.05M Potassium dihydrogen orthophosphate (KH2PO4), pH adjusted to 3

with ortho-phosphoric acid(OPA). The mobile phase comprised of buffer (55):Acetonitrile and Methanol (45) in the proportion of 80:20 respectively.

Preparation of Standard Solution

Two different standard stock solutions of lopinavir and ritonavir were made by dissolving 100mg of the drug in 100ml of methanol. The concentration of these solutions was 1000µg/mL. 1mL was withdrawn from each solution and added to a 10 mL volumetric flask and the volume was made up using the mobile phase. The concentration of the standard solution was 100µg/mL. The representative standard chromatogram is shown in Figure 3.

Table 1: Validation Parameters

Validation Parameters	R.S.D
Linearity	Regression Co-efficient –0.991 (L) and 0.996(R)
Specificity	No interference
Precision	Less than 2% for both intra-day and inter-day (n=6)
Accuracy	Less than 2% for n=3
Limit of Detection (LOD)	Lopinavir (1.79µg/ml) and Ritonavir (3.04µg/ml)
Limit of Quantification (LOQ)	Lopinavir (5.43µg/ml) and Ritonavir (9.21µg/ml)

Forced Degradation Studies

Forced Degradation Studies of the drugs, in combination, were carried out under different stress conditions as mentioned in ICH Q1A (R2)[3]. The standard solution containing both the drugs in the concentration of 100µg/mL was subjected to acidic, alkaline and oxidative stress condition. The subjected degradation condition varied from 0.1N to 5N. Finally, solutions were neutralized to pH7.Oxidative stress studies were carried out for 5hrs in 30% H2O2.The active pharmaceutical ingredients i.e. both lopinavir and ritonavir were subjected to thermal and photo degradation as explained in detail below.

Acid catalyzed degradation

The solution containing each of lopinavir and ritonavir (100µg/mL) was subjected to different strengths of Hydrochloric acid (HCl) like 0.1N, 1N and 5N HCl at room temperature for 2,4,6,8 and 24 hours respectively. The solution was neutralised and volume was made up using the mobile phase. The maximum degradation was obtained with 5N HCl after 24 hours. The Representative Chromatogram is shown in Fig 6 and the results are shown in Table 2.

Alkali catalysed degradation

The solution containing each of lopinavir and ritonavir (100µg/mL) was subjected to different strengths of Sodium Hydroxide (NaOH) like 0.1N, 1N and 5N NaOH at room temperature for 2,4,6,8 and 24 hours respectively The solution was neutralised and volume was made up using the

mobile phase. The maximum degradation was obtained with 5N NaOH after 24hours.Representative Chromatogram is shown in Fig 7and the results are shown in Table 2.

Peroxide degradation

The solution containing each of lopinavir and ritonavir (100µg/mL) was subjected to 30% Hydrogen Peroxide (H2O2) at room temperature for 2,4,6,8 and 24 hours respectively. The solution was neutralised and volume was made up using the mobile phase. Representative Chromatogram is shown in Fig 8 and the results are shown in Table 2.

Photo degradation

The active pharmaceutical ingredients i.e. both lopinavir and ritonavir were exposed to UV chamber at both 254nm and 360nm for 24 hours and the solution of the same containing a mixture of the two (100µg/mL) each in mobile phase was made analysed. Representative Chromatogram is shown in Fig 9 and the results are shown in Table 2.

Thermal degradation

The active pharmaceutical ingredients were exposed to a temperature of 105^{O}C for 24hrs.The solution containing each drug in the concentration of (100µg/mL) was made using the mobile phase. Representative Chromatogram is shown in Fig 10 and the results are shown in Table 2.

RESULTS AND DISCUSSION

Precision

The method was validated for intra-day as well as inter-day precision. The solution containing 15µg/ml of both lopinavir and ritonavir was injected (n=6). The RSD for both intra as well as inter day was less than 2%.

Linearity

The drugs were found to be linear over the range of 5µg/ml to 30µg/ml. Figure 4 shows the linearity graphs of both the drug.

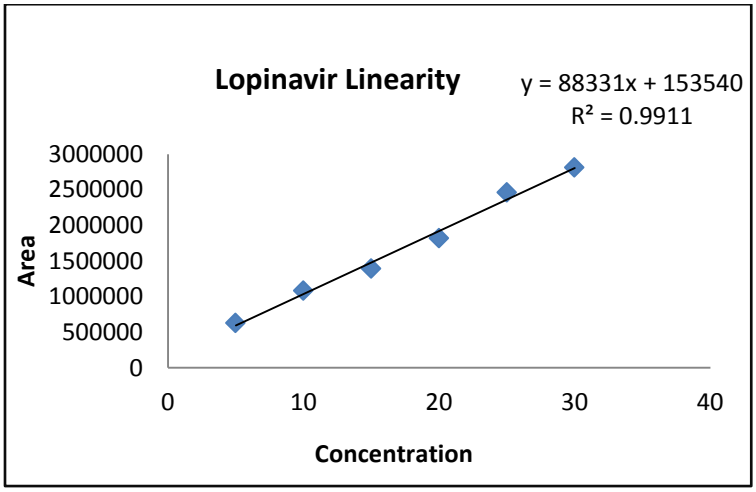

Figure 4.1: Lopinavir linearity graph

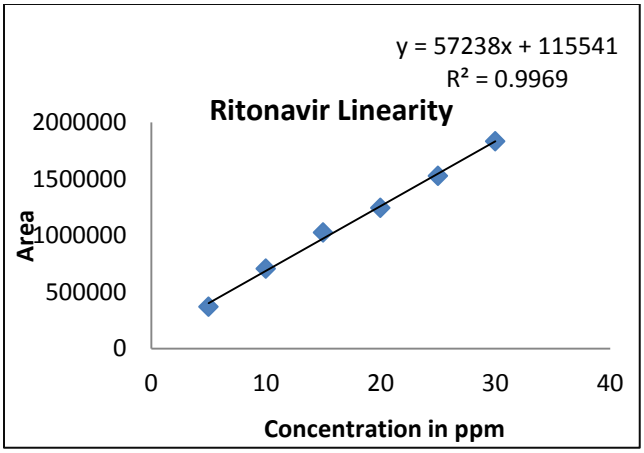

Figure 4.2: Ritonavir linearity graph

LOD

Limit of detection for lopinavir was found to be 1.79µg/ml. The limit of detection for ritonavir was found to be 3.04µg/ml.

LOQ

Limit of quantification was found to be 5.43µg/ml for lopinavir and 9.21µg/ml for ritonavir.

Specificity

The developed method was found to be specific as there was no interference observed.

Robustness

In order to demonstrate the robustness of the method, system suitability parameters were verified by making deliberate changes in the chromatographic conditions i.e. change in the flow rate by ±0.05ml/min, change in pH by ±0.1 unit and change in the ratio of mobile phase (± 2%). The method was found to be robust over and acceptable range of HPLC parameters.

Forced Degradation Studies

The above mentioned percent degradation of both lopinavir and ritonavir is with respect to their decrease in the areas. Peak purity of the drug is not affected. There are no impurities seen as well. However, it is only in thermal and photo degradation undesired peaks are seen which could be impurities as lopinavir is known to degrade under U.V light. Thus, the conditions subjected to the drugs makes them undergo forced degradation thereby being able to detect any difference in the response in terms of their areas and impurities if any as seen in thermal and photo degradation.

Table 2: Percent Degradation of Both Lopinavir And Ritonavir

Conditions	Percent Degradation Lopinavir	Percent Degradation R itonavir
Acid(5N HCl) 24hr at Room Temperature (RT)	22.03%	50.06%
Base(5N NaOH) 24 hr at Room Temperature (RT)	43.65%	49.32%
Hydrogen Peroxide H_2O_2	40.58%	9%
Thermal Degradation(6hr at 60^{O}C)	49.2%	50%
Photo degradation(254 nm for 24 hrs)	30.94%	50.25%

Table 3: System Suitability Parameters

System-Suitability Parameters	Lopinavir	Ritonavir
Retention Time	6.433	5.433
Theoretical Plates	5276	4622
Tailing factor/Asymmetry	1.52	1.61
Resolution	2.96	-

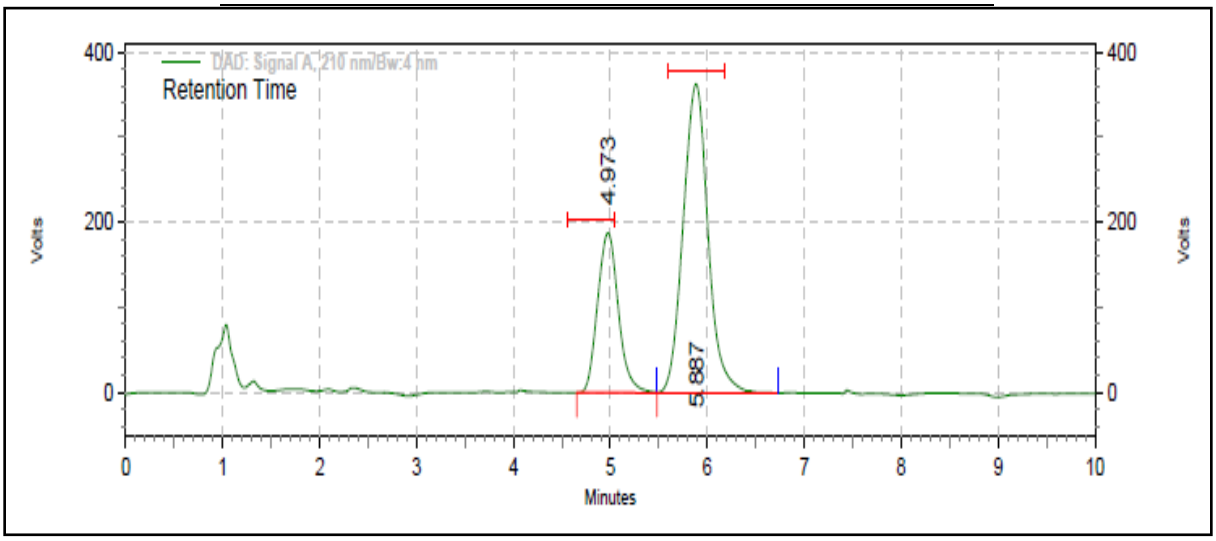

Figure 3: HPLC Chromatogram of lopinavir and ritonavir

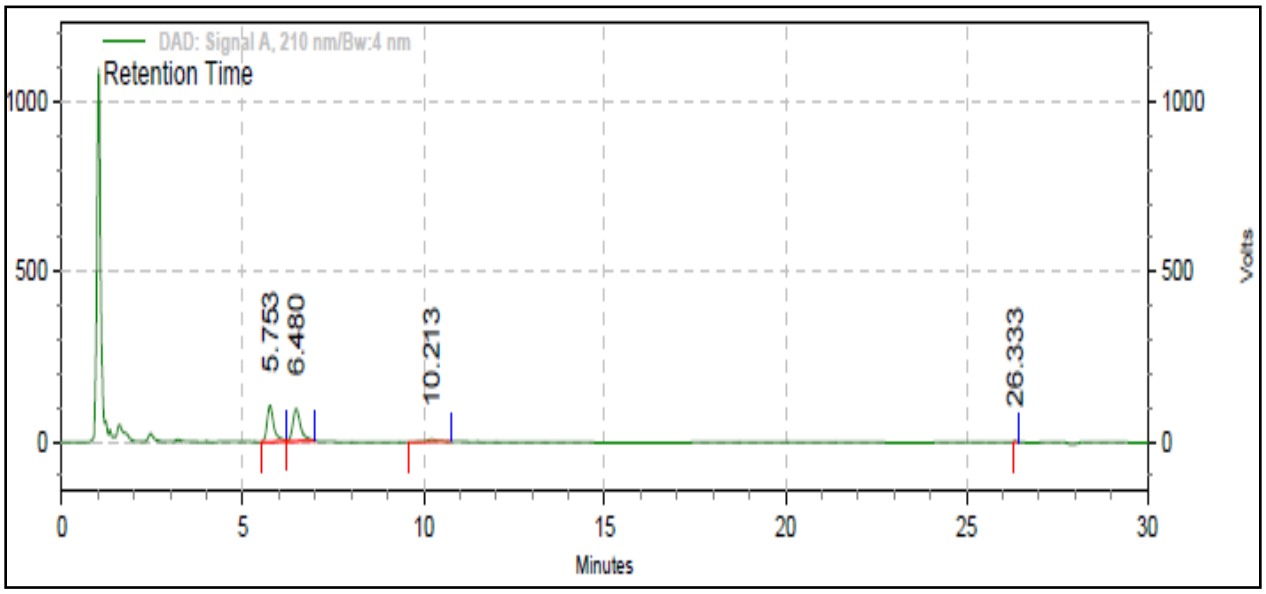

Figure 5: 5N HCl RT 24hr

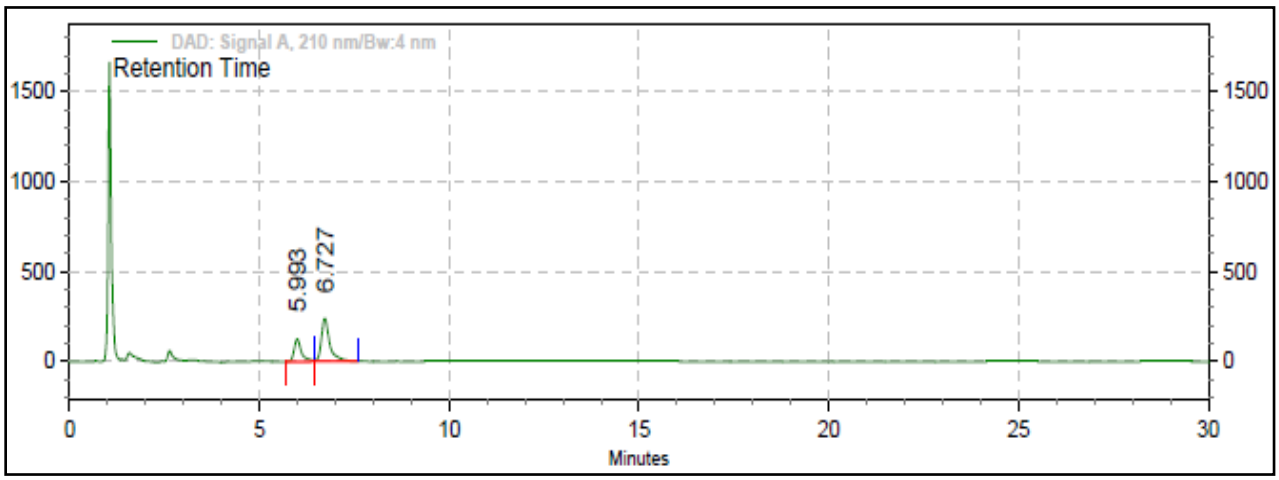

Figure 6: 5N NaOH RT 24hr

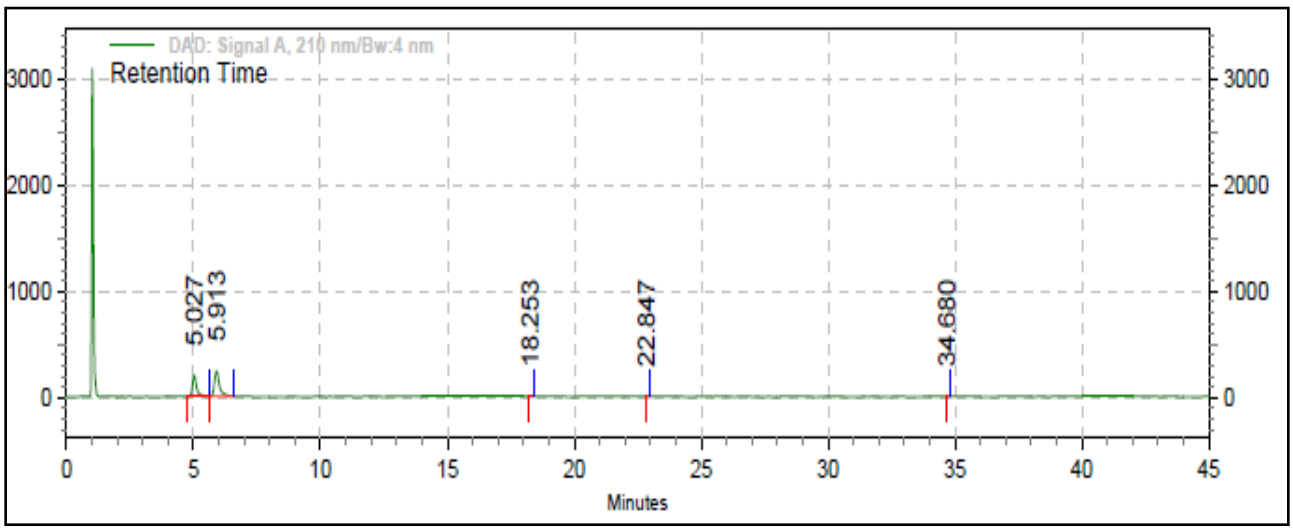

Figure 7: H_2O_2 (30%) 4hr RT

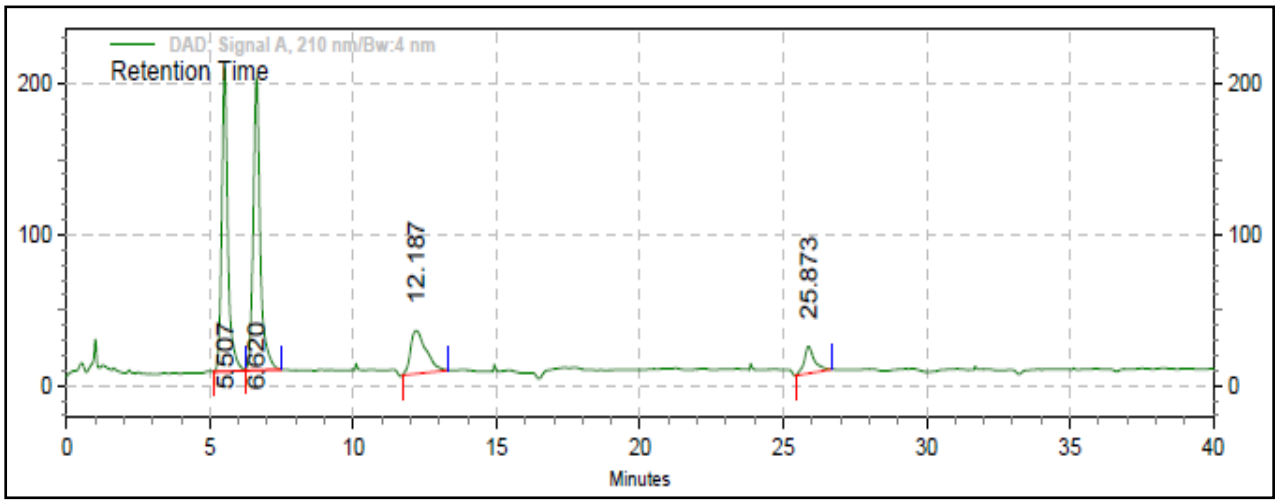

Figure 8: Thermal Degradation

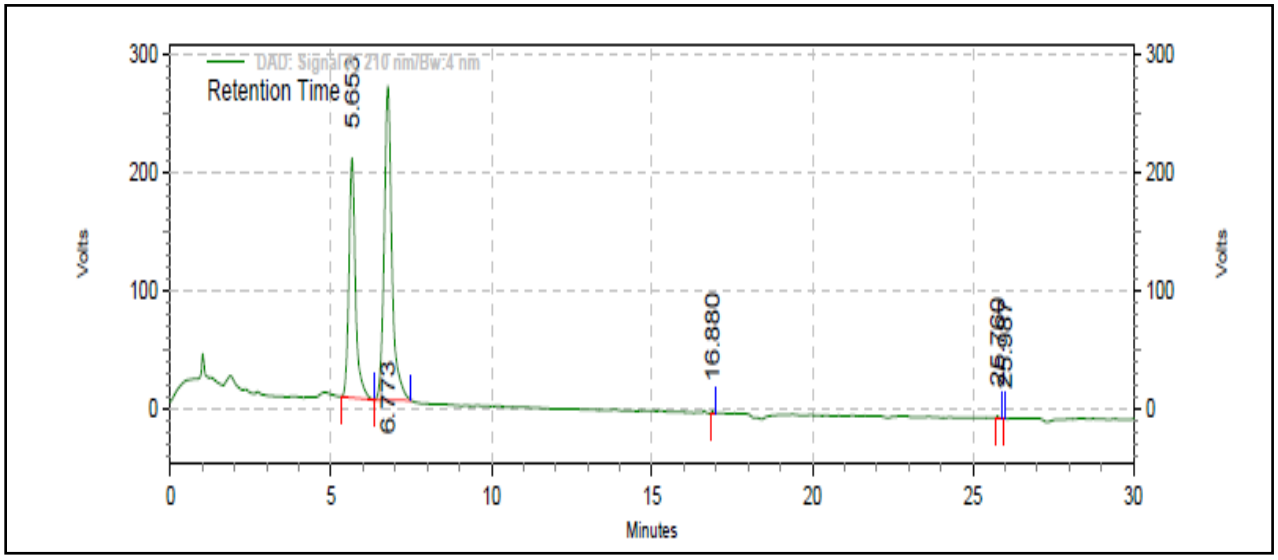

Figure 9: Photo Degradation

CONCLUSION

The optimized method was simple, precise, linear, robust and also stability indicating. The experimental conditions mentioned were found to be the most appropriate when subjected the bulk drugs to the degradation conditions. Thus, the above proposed method can be used for analysis of lopinavir and ritonavir when exposed to different degradation conditions.

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