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Development and Validation of Analytical Method of Zanamivir: An Antiviral Drug

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Abstract:
Zanamivir was examined in a variety of stress conditions, including hydrolytic, oxidative, photolytic, and thermal. Under conditions of alkaline hydrolysis, degradation was seen. Under alkaline degradation circumstances, the main degradation products were suggested. For the purpose of quantifying RAL under forced degradation research, a straight forward, sensitive, and precise HPLC approach was created. The separation uses a combination of toluene, ethylacetate, methanol, and acetic acid (8:1:1:0.1 v/v/v/v) as the mobile phase and aluminum-backed silicagel-G F 254 as the stationary phase. For analysis, the detection wavelength of 299 nm was used. Accuracy and precision (repeatability and intermediate precision levels) were shown by the validated technique within the relevant linear range of RAL (200-700 ng/spot; r = 0.9923, n = 5). The results showed that the limits of quantification and detection were 103.01 ng/spot and 33.99 ng/spot, respectively. The specificity of the results was demonstrated by peak purity indices (>0.9997) and a reasonable resolution between RAL and its degradation products. An analysis of the kinetics of RAL degradation in an alkaline media revealed zero order kinetics with activation energy of 5.99 Kcal/mol.

Keywords: Zanamivir, RAL, HPLC, Quantification, Purity

1. Introduction

A virus is a tiny infectious agent that can only reproduce inside an organism's live cells. A light microscope cannot see most viruses because they are too tiny. All kinds of organisms, including bacteria, plants, animals, and archaea, are infected by viruses. A virion is a viral particle that can live freely (for example, outside of its host) and is made up of segments of nucleic acid (either DNA or RNA) encased in a capsid, a protein coat made up of symmetrically repeating structural units (Figure A) [1-2] The word nucleocapsid refers to the combination of the viral coat and the nucleic acid core. When the nucleocapsid buds through the membranes of the infected cell, certain viruses may have an additional exterior lipoprotein envelope that may be embellished with antigenic viral glycoproteins or phospholipids that were obtained from the host. Enzymes that start the virus's reproduction in the host cell are also present in certain viruses.

Based on whether viruses contain single-stranded or double-stranded nucleic acids and how they work during replication, these two basic groups are often further subdivided into around six subclasses. Viruses may infect almost every living thing. Such infections are widespread in humans, and they are not an exception [3-5].

Human cells are susceptible to viruses, and the immune system will attempt to eradicate the virus from the body when it comes into contact with it. The virus may more readily cling to accessible cells when the immune system is

compromised, which often results in nonspecific symptoms including fever, chills, and muscular pains. Additionally, this facilitates the virus's ability to spread, exacerbating symptoms until the immune system is able to combat it [6].

Viral infections come with a variety of symptoms ranging from mild to severe. Symptoms may vary depending on what part of the body is affected, type of viruses, age, and overall health of the affected person.

Finding medications that are specific to the infections is challenging since viruses take over a lot of the host cell's metabolic functions. Nonetheless, several enzymes are particular to viruses and have been identified as promising targets for medications. The majority of antiviral medications on the market today only work while the virus is reproducing [7-9]. AIDS is caused by HIV infection. HIV is a retrovirus made of RNA. There are two types. The virus that causes AIDS in humans is called HIV-1. Though less virulent than the HIV-1 virus, the HIV-2 organism similarly suppresses the immune system. Although cytotoxic T lymphocytes (CTLs, CD8+ T cells) and CD4+ helper T lymphocytes (CD4 cells) are the primary immune cells involved in HIV's intricate interaction with the host's immune system, other immune cells such macrophages, dendritic cells, and NK cells are also involved. Although the host produces antibodies against different components of HIV, the activity of CD4 cells and CTLs is what first stops HIV from spreading. [9] Like other retroviruses, HIV has a lipid bilayer membrane enclosing the capsid and the enzyme reverse transcriptase. Its surface glycoprotein molecule (gp120) binds strongly to the helper/inducer cells' CD4 receptor protein. The virus releases its genetic material and loses its outer layer after entering the host cell. The reverse transcriptase enzyme transforms the viral RNA into DNA. Transcription and translation of viral DNA allow for the synthesis of new viral proteins. The protease enzyme is responsible for the ultimate maturation of a novel viral protein into an infectious virion. Antiretroviral (ART) medications are necessary for post-exposure prophylaxis, mother-to-child transmission prevention (MTCT), and the treatment and prevention of HIV infection in children. By bringing the viral levels in the body down to very low levels, this therapy helps the immune system regain its vigor. Antiretroviral treatment should be started at a CD4 level of 350 cells/mm3, which usually happens many years after infection, according to current WHO recommendations. Currently, over 20 antiretroviral medications are authorized. [10-12]

2. Experimental Work

Forced Degradation study of RAL: Stressed Sample Preparation

Stressed samples were chromatographed along with the non-stressed standard sample.

Acid, base, and neutral hydrolysis are examples of hydrolytic degradation.

RAL solutions with 10 mgmL−1 were made using water, 1N HCl, and 1N NaOH. These were exposed to the stressors listed in Table 8.1. Aliquots (1 mL) of the stressed samples were taken out and placed in 10 mL volumetric flasks for HPLC analysis. They were then neutralized with the appropriate acid or base and diluted with acetonitrile to the appropriate level. 2.5 mL aliquots were taken out and diluted with mobile phase to the mark in 10 mL volumetric flasks.

Oxidative Degradation: Induced by Hydrogen Peroxide

RAL Solutions with 10 mgmL−1 were made in water with 6% v/v H2O2 and dark-treated under the conditions listed in Table 8.1. Aliquots (1 mL) of the stressed samples were transferred to 10 mL volumetric flasks and diluted with acetonitrile to the appropriate level for HPLC analysis. A 2.5 mL aliquot was put into a 10 mL volumetric flask and diluted with the appropriate amount of mobile phase.

Thermal Degradation: Induced Dry Heat

To create a uniform layer, the powdered RAL was spread out in a glass tube with a flat bottom and exposed to the circumstances listed in Table 8.1. Following the allotted time, 10 mg of the blend's precisely weighed powder was put into a 10 mL volumetric flask and diluted with acetonitrile until the desired level was reached. A 2.5 mL aliquot was put into a 10 mL volumetric flask and diluted with the appropriate amount of mobile phase.

In a 40 cm × 30 cm × 30 cm chambers equipped with a Phillips UV lamp (8W), photolytic degradation RAL solutions containing 10 mgmL−1 were produced in methanol in a quartz tank and subjected to forced irradiation at a distance of 15 cm from the source. Three hours apart, 1 mL of the sample was taken out and diluted with 10 mL of

acetonitrile for chromatographic analysis. A 2.5 mL aliquot was put into a 10 mL volumetric flask and diluted with the appropriate amount of mobile phase.

Preparation of Solutions

RAL stock solution preparation

A precisely weighed 10 mg of RAL was added to a 10 mL volumetric flask, dissolved, and diluted with 1 mg/mL of HPLC-grade water.

RAL working standard solution preparation

After transferring 0.1 mL of the RAL stock solution into a 10 mL volumetric flask, the flask was diluted with methanol until it reached the 10µg/mL RAL threshold. Aliquots (1, 2, 3, 4, and 5 mL) were put into a series of 10 mL volumetric flasks for the calibration curve, and the mobile phase was added to dilute them to the appropriate level.

System Suitability Tests

System suitability tests are an integral part of liquid chromatography. They are used to verify that resolution and reproducibility of chromatography system are adequate for the analysis to be done. The tests include Resolution (R), Theoretical plate number (N), Tailing factor (T) and Precision of replicate injection.

System suitability parameters were established by injecting 10µL of 10µg/mL RIL working standard solution.

Resolution (RS)

RS is a measure of how well two peaks are separated. For reliable quantitation, well separated peaks are essential for quantitation. The resolution was determined by injecting 10 µL test solution.

Resolution can be calculated using following equations

$RS = 2 (tR2 - tR1)/ (tW1 + tW2)$

Where tR1 and tR2 are retention time of first and second peak respectively, tW1 and tW2 are peak width of first and second respectively and RS is resolution.

Theoretical plate number (N)

Theoretical plate number is a measure of column efficiency, that is, how many peaks can be located per unit run-time of the chromatogram. N is fairly constant for each peak on a chromatogram with a fixed set of operating conditions.

Theoretical plate number can be calculated using the following equation

$N = 16 (Rt/W)2$

Where Rt is retention time, W is width of peak at it base and N is theoretical plate number.

Replica injection accuracy (injection repeatability)

The performance of the HPLC chromatograph, including the plumbing, column, and ambient conditions, at the time the samples are analyzed is indicated by the injection precision, which is given as RSD (relative standard deviation).

The tailing factor (T)

Peak symmetry is measured by the tailing factor, which increases in value as tailing becomes more noticeable. For absolutely symmetrical peaks, the tailing factor equals unity. Because the integrator finds it difficult to determine where or when the peak terminates and, thus, to calculate the area beneath the peak, the quantitation accuracy diminishes as peak tailing increases. Normal Gaussian distribution is not the form of many chromatographic peaks. Consequently, the following formula for chromatographic peaks should be used to determine the tailing factor:

Method Validation

Range and Linearity

By creating standard solutions at five distinct concentrations, linearity was investigated. Three repetitions of each concentration were made. The linearity was measured between 1 and 5 µg/mL. Slope, intercept, and correlation coefficient were used to evaluate linearity. In order to provide accurate, precise, and linear findings, the linearity range was developed by taking into account the realistic range of drug concentrations required in the pharmaceutical product. At least five concentrations must be utilized, per ICH.

Accuracy

The degree of agreement between individual findings when the analytical technique is used for repeated sampling of homogeneous samples is known as the precision of the procedure. It was reported as a percentage of the relative standard deviation and gives a sense of the random error in the data.

Intraday Accuracy

By assaying the reference solution three times during the day for three distinct RIL concentrations, intraday precision was ascertained. (1, 3, and 5 µg/mL)

Interday Accuracy

An experiment using standard solution on three separate days for three different RIL concentrations was used to measure interday precision. (1, 3, and 5 µg/mL)

Limits of quantification and detection

In accordance with ICH recommendations, the following formulas were used to determine the RIL's limits of detection (LOD) and quantitation (LOQ).

$LOD = 3.3 \times \sigma/S$ $LOQ = 10 \times \sigma/S$

Where σ is the standard deviation of the response, and S is the standard deviation of y- intercept of regression lines.

Robustness

Deliberate changes in the method parameters were made in order to assess the alteration in results. Mobile phase composition (±2%), flow rate (±0.1 unit) and pH of the mobile phase (±0.3 unit) were altered and the responses were noted.

Assay of Marketed Formulation of RAL (ISENTRESS® 400mg Tablet by MSD company)

Twenty tablets were taken and there average weight was noted. The powder equivalent to 400mg RAL was weighed and transferred to 100mL volumetric flask. Methanol (25mL) was added and the flask was sonicated for 15min, contents filtered, residue washed with methanol twice (10mL) and was diluted up to the mark. Aliquot (1mL) was transferred into 100mL volumetric flask and was diluted with methanol upto the mark. Aliquot (1mL) was transferred into 10mL volumetric flask and diluted with mobile phase upto the mark and analyzed.

3. Result and Discussion

Development of Stability Indicating Assay Method for RAL

Selection of mobile phase composition

In order to resolve RAL from its degradation products, a variety of mobile phases were investigated. Various compositions of acetonitrile, methanol, and water were attempted, but they failed to distinguish between RAL and the products of its breakdown. One important factor in separating the degradation products from the RAL was changing the mobile phase's pH. The buffer (1g ammonium acetate + 1mL glacial acetic acid in 1L HPLC Grade water) and acetonitrile in a 50:50 v/v ratio made up the optimal mobile phase. RAL's retention time was 6.991 ± 0.100 minutes.

Forced degradation study of RAL

RAL was subjected to various conditions as depicted in Table 1 and the percentage degradation occurred of RAL was calculated.

Table 1: Stress conditions for RAL

Condition	Solvent	Temperature (˚C)	Time (h)	% Degradation
Hydrolytic				
Acid	1N HCl	100	1	89.33
Base	1N NaOH	100	4	99.75
Neutral	Water	100	12	72.35
Oxidation	6% H_2O_2	Room Temperature	48	49.21
Thermal	-	100	12	2.98
Photolytic	Methanol	Room Temperature	6	11.34

Hydrolytic Degradation: Acid, Base and Neutral Hydrolysis

RAL is found to be highly labile in almost every condition and undergoes severe degradation in short period of time. The chromatograms are in figure 1-3.

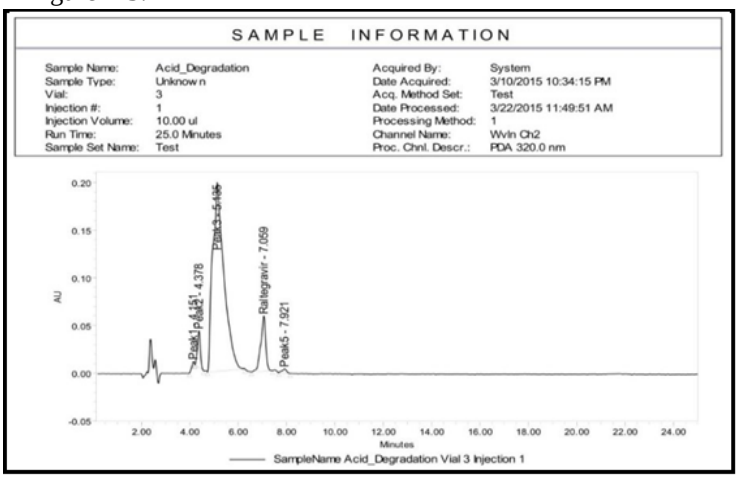

Figure 1: HPLC chromatogram of acidic degradation of RAL

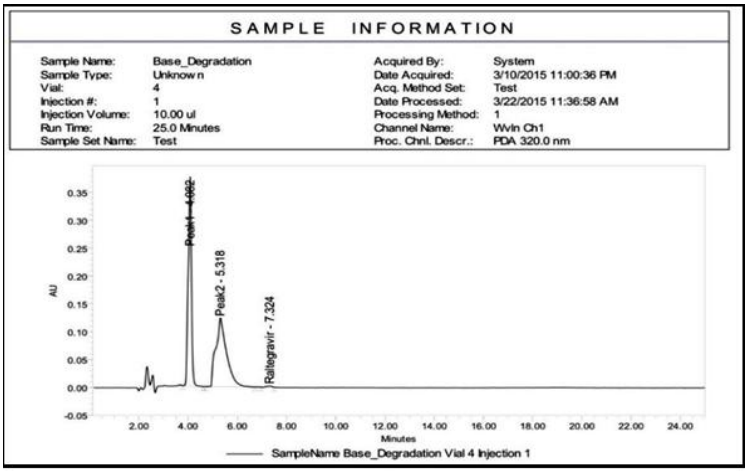

Figure 2: HPLC chromatogram of alkaline degradation of RAL

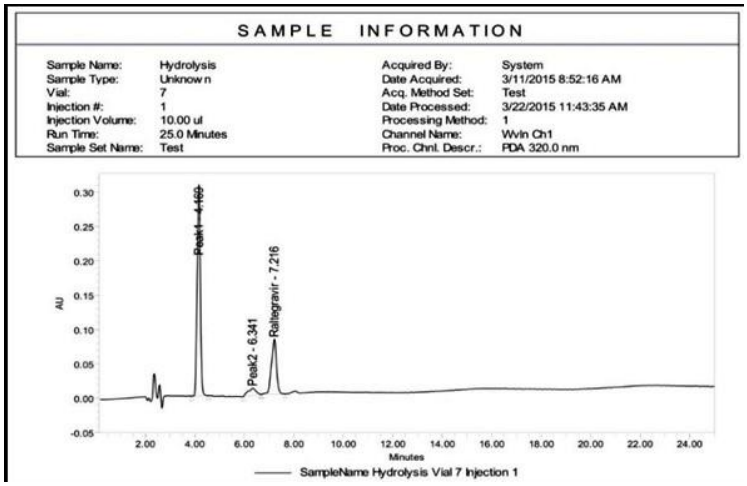

Figure 3: HPLC Chromatogram of neutral hydrolytic degradation of RAL

Oxidative Degradation: Hydrogen Peroxide Induced

RAL is found to be undergoing oxidative degradation as shown in figure 4.

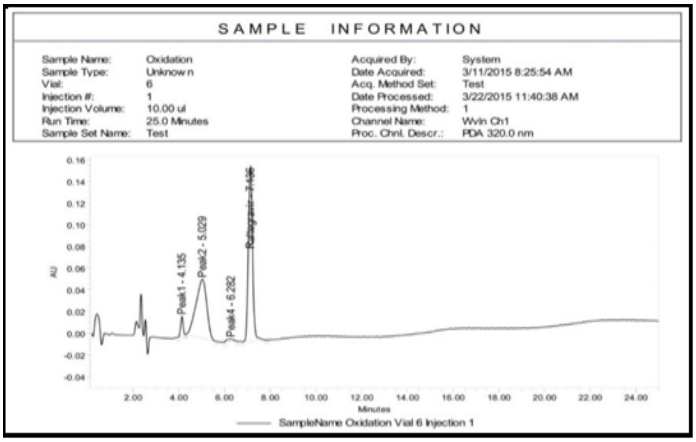

Figure 4: HPLC chromatogram of oxidative degradation of RAL

Thermal Degradation: Dry Heat Induced

RAL is found to be stable under thermal (dry heat) stress as shown in figure 5.

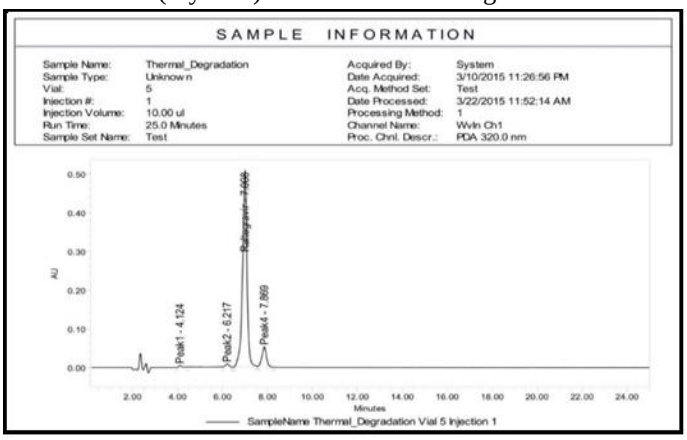

Figure 5: HPLC chromatogram of thermal degradation of RAL

Photolytic Degradation

RAL undergoes degradation under UV light. The chromatogram for UV stress study is shown in figure 6.

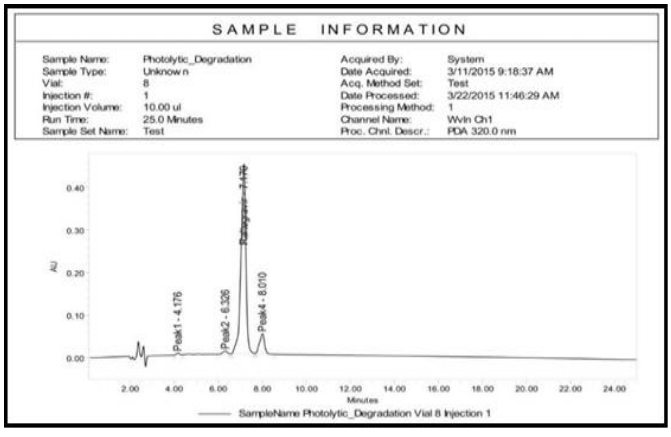

Figure 6: HPLC chromatogram of Photolytic degradation of RAL

System Suitability Results

The results for various parameters ascertained for the method are reported in Table 2.

Table 2: System suitability parameters for the developed HPLC method

Parameter	RAL
Retention time (min)	6.991 ± 0.100
Capacity factor (K)	
Asymmetry factor	0.87
Injection Repeatability (%RSD, n=6)	0.91
Number of theoretical plates	7332
Height equivalent to theoretical plates (HETP)	3.40 × 10-2

Validation of the developed HPLC method

Linearity and Range

Calibration curve was obtained by plotting average peak area vs concentration of RAL (n=3). Linear relation was obtained between mean peak area and the concentration of RAL over the range of 1-5µg/mL.

The linear equation and regression obtained for calibration curve are as follows Y = 2E+06X + 2E+06, r2 = 0.999

Where, Y = Area of the chromatographic peak of RAL X = Concentration of RAL

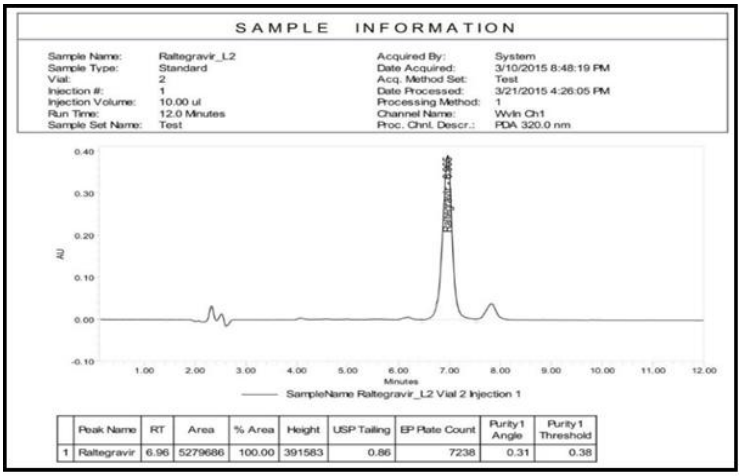

Figure 7: HPLC Chromatogram of Standard Level 2 of RAL

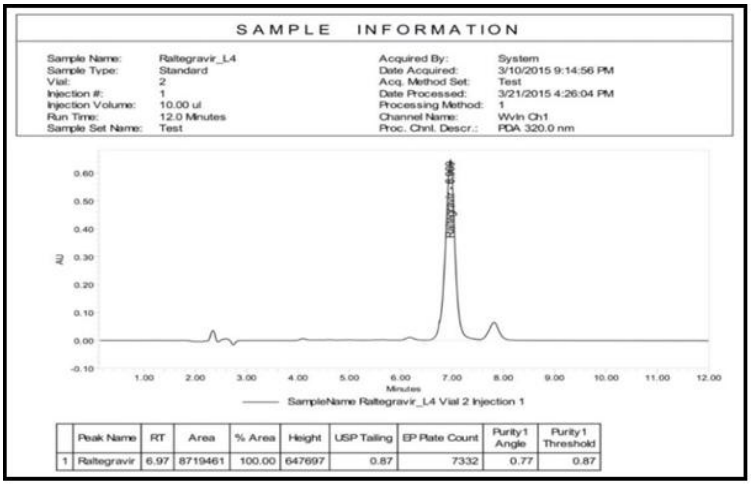

Figure 8: HPLC Chromatogram of Standard Level 3 of RAL

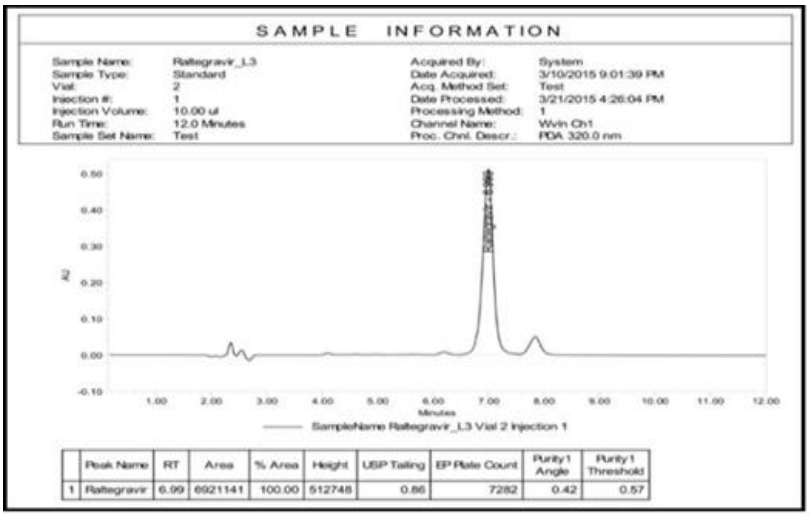

Figure 9: HPLC Chromatogram of Standard Level 4 of RAL

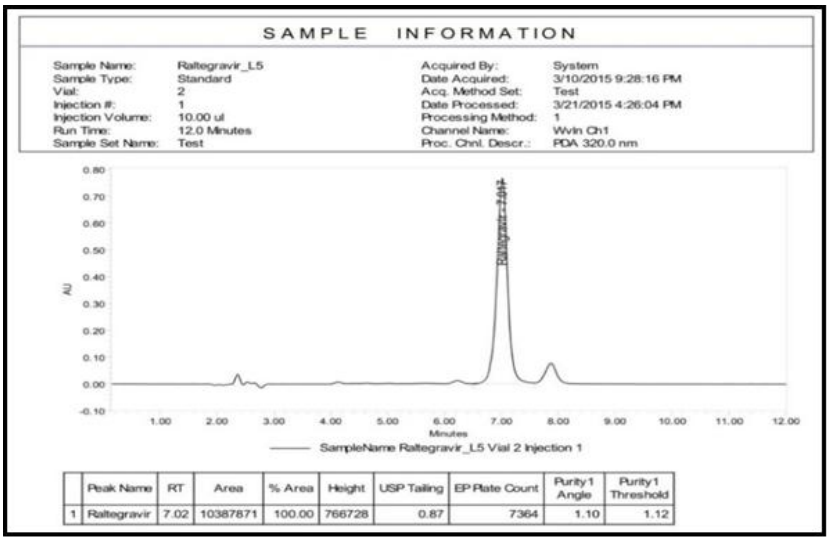

Figure 10: HPLC Chromatogram of Standard Level 5 of RAL

Table 3: HPLC Linearity data for RAL

Conc. (µg/mL)	Area 1	Area 2	Area 3	Average Area (n=3)	SD	CV
1	3430205	3398423	3429564	3419397.3	18167.13	0.53
2	5279686	5242384	5342384	5288151.3	50534.61	0.96
3	6921141	7012344	6898429	6943971.3	60291.58	0.87
4	8719461	8639456	8774932	8711283.0	68107.24	0.78
5	10387871	10239512	10453133	10360172.0	109471.00	1.06

Precision

Intraday Precision

Intraday precision was performed by analyzing three different concentrations (1-3- 5µg/mL) within linearity range, three times in a day (3*3 determinations). Intraday Precision data are summarized in Table 4 CV for Intraday Precision was found to be within 0.52-0.83.

Table 4: Intraday Precision data for RAL

Conc. (µg/mL)	Area 1	Area 2	Area 3	Average Area (n=3)	SD	CV
1	3430205	3464974	3456387	3450522.0	18111.31	0.52
3	6921141	7029126	7010370	6986879.0	57698.01	0.83
5	10387871	10423653	10293563	10368362.3	67203.37	0.65

Interday Precision

Interday precision was performed by analyzing three different concentrations (1-3- 5µg/mL) within linearity range, on three consecutive days. Interday Precision data are summarized in Table 5 CV for Interday Precision was found to be within 0.77-1.28.

Table 5: Interday Precision data for RAL

Conc. (µg/mL)	Area 1	Area 2	Area 3	Average Area (n=3)	SD	CV
1	3430205	3388952	3415687	3411614.6	20925.83	0.61
3	6921141	6753132	6879424	6851232.3	87480.48	1.28
5	10387871	10327765	10486634	10400756.6	80214.53	0.77

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Limit of detection and quantitation were computed using equations suggested by ICH guidelines. The values obtained are summarized in below table 6.

Table 6: LOD and LOQ data for RAL

LOD (µg/mL)	0.29
LOQ (µg/mL)	0.95

Robustness

The studies clearly indicated that the deliberate changes made in parameters did not alter the responses to more than 2%.

Table 7: Robustness data for RAL

S. No.	Parameters	Retention time	Asymmetry factor	% RSD of response
1.	Organic Phase: Aqueous Phase (50:50 v/v)	6.973	0.87	-
2.	Organic Phase: Aqueous Phase (48:52 v/v)	6.821	0.92	0.68
3.	Organic Phase: Aqueous Phase (52:48 v/v)	7.102	0.81	0.93
4.	Flow rate (1mL/min)	6.973	0.87	-
5.	Flow rate (0.9mL/min)	7.075	0.95	0.78
6.	Flow rate (1.1mL/min)	6.701	0.62	1.07

Assay of Marketed Formulation of RAL (ISENTRESS® 400mg Tablet by MSD company)

Available marketed formulations was assayed using developed method and the results are depicted in table and chromatogram in figure 11.

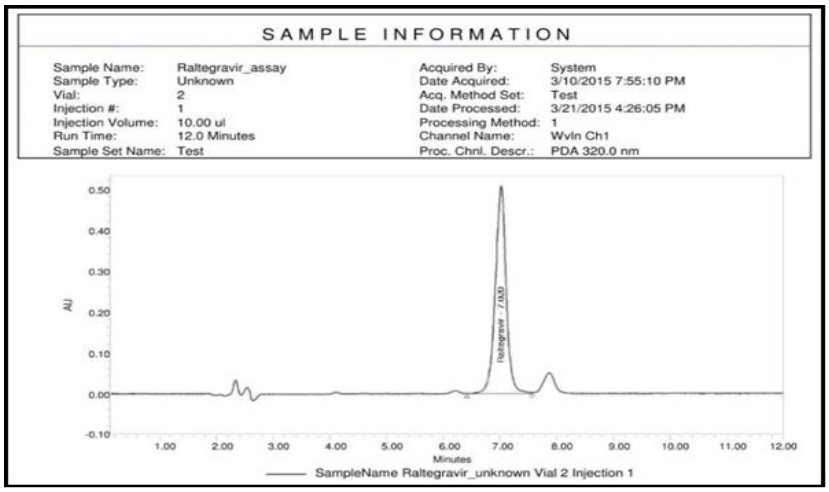

Figure 11: HPLC Chromatogram of assay of marketed formulation of RAL

Table 8: Assay result of marketed formulation of RAL

Marketed Formulation	Company Name	Lable Claim (mg)	Amount Found mg (Mean ± S.D.)	% Recovery (Mean ± S.D.)
Isentress 400 mg Tablet	MSD	400	399.84 ± 3.40	99.96 ± 0.85

Table 9: Summary of Validation Parameters of developed HPLC method

Parameter	RAL
Linearity (µg/mL)	1-5
r2	0.999
Intraday Precision (CV)	0.52-0.83
Interday Precision (CV)	0.77-1.28
LOD (µg/mL)	0.29
LOQ (µg/mL)	0.95
Robustness	Robust

4. Conclusion

A stability indicating HPLC method for determination of RAL was developed and validated as per ICH guidelines. The method could resolve all the degradation products generated during stressed conditions. The developed HPLC method is simple, precise, accurate and cost-effective and can be adopted for routine analysis of RAL in bulk and its available marketed formulations without any interference from the excipients or degradation impurities. The HPLC method for determination of RAL. System suitability test was carried out for Resolution (RS), Theoretical plate number (N), Precision of replicate injection and tailing factor for developed method and obtained results were shown in Table 8.2. In validation, Linearity was established for HPLC method in range of 1-5 µg/mL with corelation coefficient 0.999. CV for Intraday and Interday precision for method were in range of 0.52-0.83 and 0.77-1.28 respectively. LOD and LOQ for the method were found to be 0.29 and 0.95 µg/mL respectively. Robustness for the method was studied as per Table 8.7 and indicated that the changes in composition and Flow rate of mobile phase as shown in table did not affect the responses to more than 2% and suggested that developed HPLC method was Robust. The developed and validated method was successfully applied for assay of RAL in available marketed formulation and % Recovery for RAL (Mean ± S.D.) was found to be 99.96 ± 0.85. Forced degradation study of

RAL was carried out in bulk as per ICH guideline and obtained detailed results were shown in Table 8.1 indicate that RAL was found to be labile in all condition of stressed applied as in Table 8.1 except thermal condition of stress.

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