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METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF LAMIVUDINE AND RALTEGRAVIR BY UV SPECTROPHOTOMETRY AND RP-HPLC

B. Shirisha,

Department of Analysis, Vision College of Pharmacetical Sciences & Research, R.N.S Colony, Boduppal, Uppal, Medchal Dist. Pincode: 500092.

D. Ashlesha,

Department of Analysis, Geethanjali College of Pharmacy, Cheeryal Village, Keesara Mandal, Hyderabad, Telangana 501301

N Mamatha,

Department of Analysis, Jyothishmathi Institute of pharmaceutical sciences, thimmapur, karimnagar 505527

Abstract - The UV-spectroscopic method and RP-HPLC method were developed and validated for the estimation of Lamivudine/Raltegravir per ICH guidelines. Buffer(opa): Acetonitrile (50:50) was used as the solvent. The λ_{max} of Lamivudine/Raltegravir was found to be 302 nm and it was proved linear in the concentration range of Lamivudine 1-8µg/ml and for Raltegravir 2-16µg/ml with a correlation coefficient value of 0.999. Accuracy studies of UV-spectroscopy method was performed at three different levels, i.e., 50%, 100%, and 150% and recovery was found to be in the range of 99.6 to 100.8% for Lamivudine and the range of 98.3 to 101.2% for Raltegravir respectively. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.117 and 0.357 µg/ml for Lamivudine and 0.19 and 0.583 for Raltegravir.

A simple, fast, accurate and precise RP-HPLC method was developed by using Acetonitrile: water, 0.1% ortho phosphoric acid(60:40) .The method was developed by using Kromosil 250 column (250 mm × 4.6 mm, 5µm particle size).and the mobile phase was pumped with Acetonitrile and water (hplc grade water)and PH was adjusted to 3.2 by using ortho phosphoric acid and the mobile phase was pumped at 1ml/min flowrate and the temperature was maintained at 30˚c.the retention time for Lamivudine/raltegravir were found to be 2.110 and 4.617 and the total run time was found to be 8min,the no of Theretical plates and Tailing factor of Lamivudine and Raltegravir were found to be 2584,1.36 and 3448,1.09, RP-HPLC method was found to be Linear in the range of Lamivudine/Raltegravir is 37.5-225µg/ml and 75-450µg/ml with a correlation coeffiecient of 0.999 the accuracy studies of RP-HPLC method was performed

at three different levels i.e.,50%,100%,150% and recovery was found to be 99.24 to 100.13% for Lamivudine and 98.18 to 99.27% for Raltegravir respectively.Repeatability and intermediate results of Lamivudine/Raltegravir were found to be 0.55,1.33 and 0.8,0.4 Robustness of this method was performed by changing the flowrate at 0.9ml/min,1ml/min,1.1ml/min.the organic strength in the mobile phase was changed to 55:45v/v and 65:35 and results were found to be with in the limts.the % RSD was less than 2 the solution stability was determined at 0hr&24hr the %RSD of solutions were less than 2.the Ruggedness values were found to be 0.092 and 0.184 respectively. the %purity of the Dutrebis(label claim 150mg,300mg) was found to be 99.64 for Lamivudine and 100.5 for Raltegravir. The limit of Detection(LOD) and limit of Quantification(LOQ) were found to be 0.441 and 1.336µg/ml for Lamivudine and 0.031 and 0.093µg/ml for Raltegravir for RP-HPLC method. the sample was degraded in acidic,basic,peroxide,heat,photolytic,neutral and the results of Lamivudine/Raltegravir were found to be 5.85,2.6,3.66,1.88,0.83,0.92 and 5.66,2.76,4.89,1.81,0.78,0.69 respectively the above method was a rapid tool for routine analysis of Lamivudine/Raltegravir in the bulk and Pharmaceutical Dosage form.

1. INTRODUCTION

Today, the development of a method of analysis is usually based on prior art or existing literature, using the same or quite similar instrumentation. The development of any new or improved method usually tailors existing approaches and instrumentation to the current analyte, as well as to the final needs or requirements of the method. Method development usually requires selecting the method requirements and deciding on what type of instrumentation to utilize and why.

There are several valid reasons for developing new methods of analysis:

- There may not be a suitable method for a particular analyte in the specific sample matrix.
- Existing methods may be too error, artifact, and/or contamination-prone, or they may be unreliable (have poor accuracy
- y or precision).
- Existing methods may be too expensive, time consuming, or energy intensive, or they may not be easily automated.
- Newer instrumentation and techniques may have evolved that provide opportunities for improved methods, including improved analyte identification or detection limits, greater accuracy or precision, or better return on investment.
- There may be a need for an alternative method to confirm, for legal or scientific reasons, analytical data originally obtained by existing methods.

To develop a method, it is necessary to consider the properties of the analyte(s) of interest that may be used to advantage and to establish optimal ranges of analyte parameter values.

Once the instrumentation has been assembled and analyte parameters have been considered, standards should be used for the continued development, optimization, and preliminary evaluation of the method.

It is important that method development be performed using only analytical standards that have been well identified and characterized, and whose purity is already known. Such precautions will prevent problems in the future and will remove variables when one is trying to optimize or improve initial conditions during method development [1].

2. LITERATURE SURVEY

B.SIDDHARTH.et.,al;..(2014) proposed a uv-specrophotometric method for estimation of raltegravir in bulk and tablet dosage form.the solvent used was 0.1N NaoH and the method followed linearity in the range of 10-60µg/ml.This method was validated for various parameters according to ICH guidelinesthe validation parameters were found to be with in the limits.

B.jayakar.et.al;..(2014).proposed a method development and validation of RP-HPLC method for simultaneous determination of lamivudine and

zidovudine.by using Altima c18 5µ (150*4.6mm) with mobile phase ammonium acetate buffer:methanol (80:20) the detection of wavelength in 270nm the method followed linearity in the range of 37.5-112.5mg/ml the proposed method validated for all validation parameters as per ICH guidelines and they are found to be with in the limits.

Rambabu kuchi.et..al;..proposed a new RP-HPLC method development and validation for analysis of anti-viral drug Raltegravir.by using kromosil c_{18} column (250*4.6mm,5µm) with mobile phase 0.01m ammonium dihydrogen phosphate:acetonitrile (50:50) the detection of wavelength in 253nm the method followed linearity in the range of 1-5mg/ml and the retention time was found to be 2.7min the proposed method validated for all validation parameters as per ICH guidelines and they are found to be within the limits.

3. EXPERIMENTAL METHODOLOGY:

3.1 MATERIALS AND METHODS

Table no.1 List of Standard and Sample details

S.NO.	NAME	Sample	Formulation	Company name
1.	Lamivudine API	EPIVIR	DUTREBIS	Merck&co.,inc
2.	Raltegravir API	ISENTRESS		

Table no.2 List of Equipment/Instrument details

S. No.	Name of Instrument	Model	Make
1	Precision balance	CA123	Contech
2	P^H Meter	3 Star	Global
3	HPLC with UV detector	Model 2696with 2996PDA	Waters
4	Column	Kromosil C18(4.6x250mm),5µm	Agilent
5	UV-Spectrophotometer	UV-1800	Shimadzu
6	UV-Spectrophotometer	UV*-3000	Lab India
7	Sonicator	UCB 70	Life care

Table no.3 list of Chemicals and Reagents

No.	Chemicals/Reagents	Make/Grade
1	Acetonitrile	SD Fine Chemicals, (HPLC-Grade)
2	Ortho phosphoric acid	SD Fine Chemicals (GR-Grade)

4. RESULTS AND DISCUSSION:

DISCUSSION ABOUT ANALYSIS OF LAMIVUDINE AND RALTEGRAVIR BY UV AND HPLC METHODS

4.1.1 UV SPECTROPHOTOMETRIC METHOD

4.1.1.2 Simultaneous Estimation Method

Estimation of Lamivudine and Raltegravir was achieved by simultaneous equation method.The normal spectra of Lamivudine and Raltegravir were recorded in Acetonitrile. The linearity was checked in different concentrations at 280 nm for Lamivudine and 302 nm for Raltegravir. The slope, intercept and correlation coefficient values were found to be 0.056, 0.068 and 0.999 for Lamivudine and 0.06, 0.031 and 0.999 for Raltegravir. Precision studies were performed; low % RSD values were obtained which indicate that the proposed method has good precision.LOD and LOQ were found to be 5 µg/ml and 10 µg/ml for Lamivudine, 5µg/ml and 10 µg/ml for Raltegravir respectively. In this method, accuracy was determined by calculation of percentage recovery and average recovery was calculated at 50,100 and 150% levels. The recovery values between prescribed limit of 98-102% shows that method is free from interference of excipients present in formulation. The developed method was validated as per ICH guide lines. The method was successfully used for determination of drugs in tablets. The above discussion proves that the proposed method is simple, rapid and precise. The good recovery values and low relative standard deviation confirm the suitability of the method.

4.1.3 Precision:

Repeatability studies were carried out by taking test concentration and repeating it six times. Interday and intraday precision were done by taking three concentrations and repeating it three times and the values for both system precision and method precision in terms of %RSDwere found to be <2.0%.

The results show that the %RSD value for repeatability, and interday is 0.411,0.061-1.882 for Lamivudine the results shown in table 4.3,4.5&4.7

and 0.411, 0.294-1.16, for Raltegravir the results shown in table 4.4,4.6&4.8 which indicate that they meet the acceptance criteria and hence the method is said to be precise.

Acceptance criteria

A method is said to be precise if the %RSD value is <2.0%.

4.1.4 Ruggedness:

Ruggedness of the method was performed by assaying the standard drug by two different analysts and in two different instruments. The different instruments used were lab india and shimadz. The results indicate that the %RSD values for different analysts and different instruments was found to be in the range of 0.33-0.35 and 0.50-0.61 for Lamivudine the **results shown in table 4.9** and the %RSD values for different analysts and different instruments was found to be in the range of 0.09-0.10 and 0.18-0.21 for Raltegravir the **results shown in table 4.10** respectively which indicates that they need the acceptance criteria.

Acceptance criteria

A method is said to be robust if the %RSD values is <2%.

Ruggedness results for different analysts and different instrumentation

4.1.5 Accuracy studies of Lamivudine/Raltegravir

Accuracy is the closeness of the results obtained by the method to the true value. Recovery studies were carried out at 50%, 100% and 150% by adding known amount of sample drug solution of Lamivudine/Raltegravir i.e. (1, 2, 13µg/ml), (2,4,6µg/ml) to the standard solution whose concentration is maintained constant for Lamivudine i.e. 5µg/ml and Raltegravir i.e 10µg/ml The %recovery was calculated

Acceptance criteria

A method is said to be accurate if the % recovery studies is in the range of 98-102

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

The LOD and LOQ were calculated by using the slope and SD of response (intercept). The mean slope value and the SD of response were obtained from the calibration curve. The LOD and LOQ results were found to be 0.117, 0.357µg/ml for Lamivudine and 0.19,0.583µg/ml for Raltegravir respectively and are reported in

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF LAMIVUDINE AND RALTEGRAVIR IN THE PHARMACEUTICAL DOSAGE FORM

HPLC METHOD

In this method optimization of different chromatographic parameters like selection of

- Chromatographic method for separation
- Detection wavelength
- Different ionic strengths of mobile phase
- Mobile phase ratio
- Mobile phase pH
- Flow rate etc., were done.

A wavelength of 302nm was selected for the present study. Different mobile phase systems in different proportions were tried. From this a mixture of 0.1 % of orthophasphoric acid adjusted to (pH 3) and Acetonitrile (60:40 v/v) produced symmetric peak shape with good resolution for both the drugs. Next, the drugs were chromatographed under different flow rates from which a flow rate of 1.0 ml/min was selected. The retention times of Lamivudine and Raltegravir were found to be 2.110 and 4.617 , respectively.

TRIALS

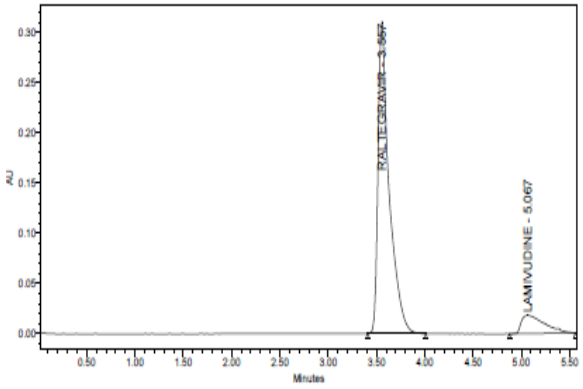
Selection of mobile phase

Trial 1:

Mobile phase: Water P^H adjusted to 3 with OPA and acetonitrile taken in the ratio 55:45A

Chromatographic conditions:

- Flow rate : 1 ml/min
- Column : kromosil 250 column(250mm×4.6mm,5µm particle size).
- Detector wave length: 305nm
- Column temperature: 30°C
- Injection volume: 10 µL
- Run time: 6 min
- Diluent: First dissolved in Methanol and made up with water (50:50).
- Results : lamivudine tailing was not possible



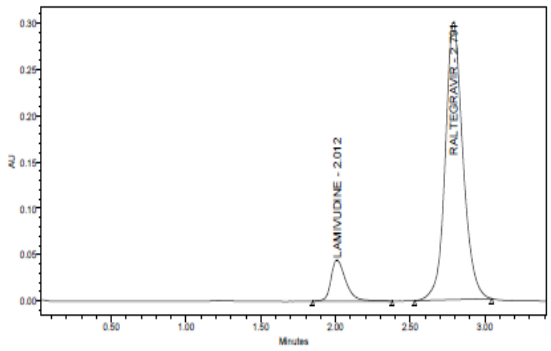
Trial chromatogram 1

Trial 2:

Mobile phase: water P^H adjusted to 3 with OPA and acetonitrile taken in the ratio 55:45A

Chromatographic conditions:

- Flow rate : 1 ml/min
- Column : kromosil 250 column(250mm×4.6mm,5µm particle size).
- Detector wave length: 305nm
- Column temperature: 30°C
- Injection volume: 10 µL
- Run time: 4min
- Diluent: First dissolved in Methanol and made up with water (50:50).
- Results : lamivudine in wide range



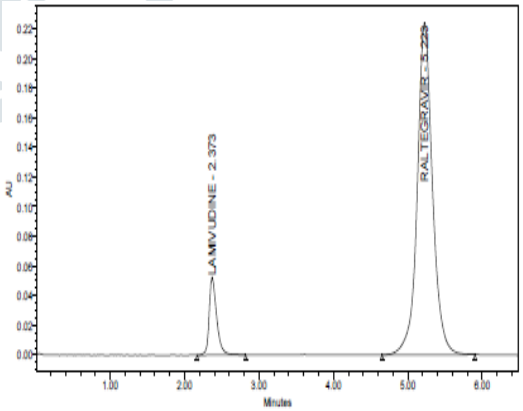
Trial chromatogram 2

Trial 3:

Mobile phase: Water P^H is adjusted to 3 with OPA and acetonitrile taken in the ratio 20:80A

Chromatographic conditions:

- Flow rate : 1 ml/min
- Column : kromosil 250 column(250×4.6mm,5µm particle size)
- Detector wave length: 305nm
- Column temperature: 30°C
- Injection volume: 10 µL
- Run time: 7 min
- Diluent: First dissolved in Methanol and made up with water (50:50).
- Results : RT and resolution is good but will go for another trail for good result



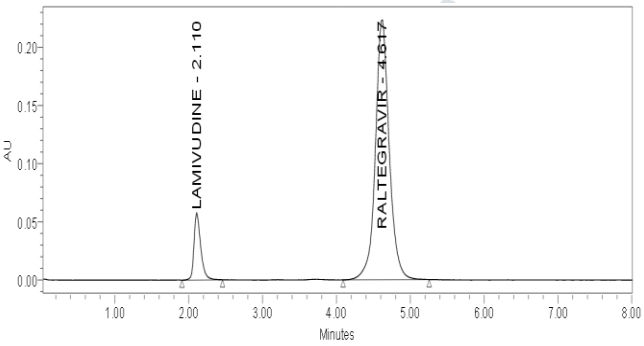
Trial chromatogram 3

Optimized Method: Drugs were eluted with good resolution, retention time all the parameters like Plate count and Tailing factor were within the limits.

Mobile phase: Water P^H is adjusted to 3 with OPA and acetonitrile taken in the ratio 60:40A

Chromatographic conditions:

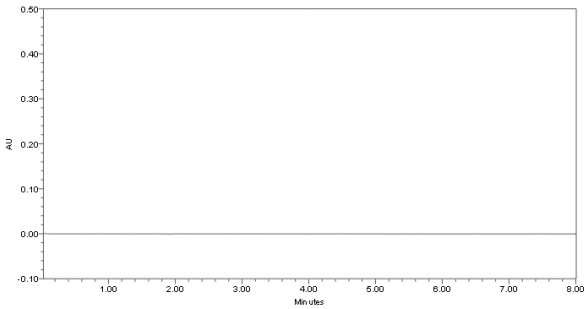
- Flow rate : 1 ml/min
- Column : kromosil 250 column(250×4.6mm,5µm particle size)
- Detector wave length: 305nm
- Column temperature: 30°C
- Injection volume: 10 µL
- Run time: 8 min
- Diluent: First dissolved in Methanol and made up with water (50:50).
- Results : All peaks are having good tailing factor, theoretical plate count and resolution.



Optimized chromatogram of Lamivudine and Raltegravir

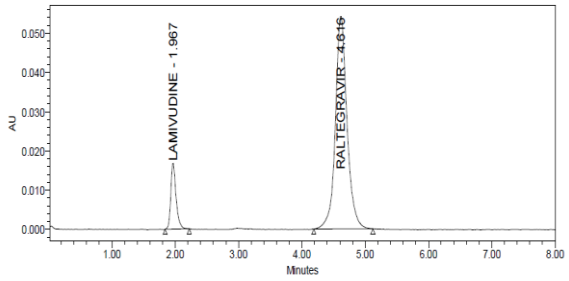
6. RESULTS AND DISCUSSIONS

1. **System Suitability:** All the system suitability parameters are within range and satisfactory as per ICH guidelines.



Chromatogram of Blank

Linearity: Six Linear concentrations of Lamivudine(37.5-225µg/ml) and Raltegravir (75-450µg/ml) are prepared and injected. Regression equation of the the Lamivudine and Raltegravir are found to be, y =2159 x + 1601, and y = 9701x + 1897and the regression co-efficient was 0.999.

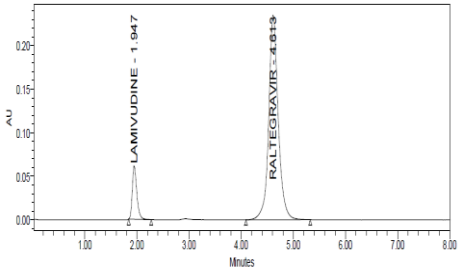


(a) Linearity 25%

2. **Precision:**

Intraday precision (Repeatability):

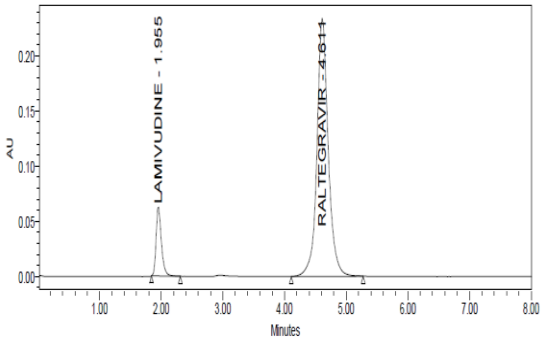
Intraday Precision was performed and % RSD for Lamivudine and Raltegravir were found to be 0.55% and 0.8% respectively



Repeatability Chromatogram of Lamivudine and Raltegravir

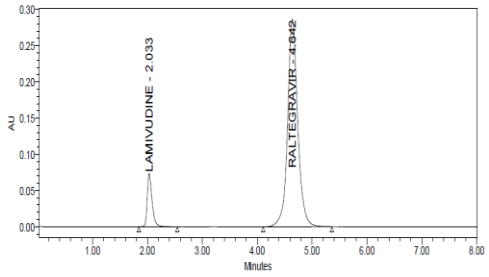
Inter day precision:

Inter day precision was performed with 24 hrs time lag and the %RSD Obtained for Lamivudine and Raltegravir were 1.03% and 0.4%.

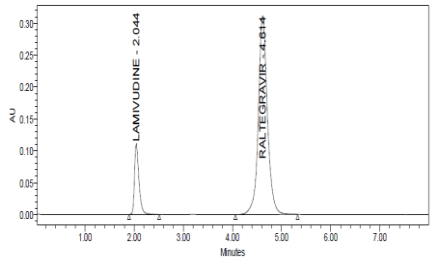


Inter Day precision Chromatogram of Lamivudine and Raltegravir

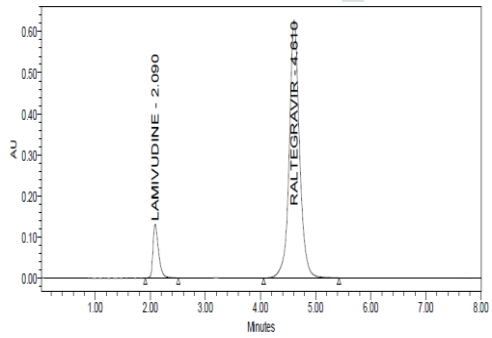
3. **Accuracy:** Three concentrations 50%, 100%, 150%, were injected in a triplicate manner and amount Recovered and % Recovery were displayed in Table 6.5.



Accuracy 50% Chromatogram of Lamivudine and Raltegravir

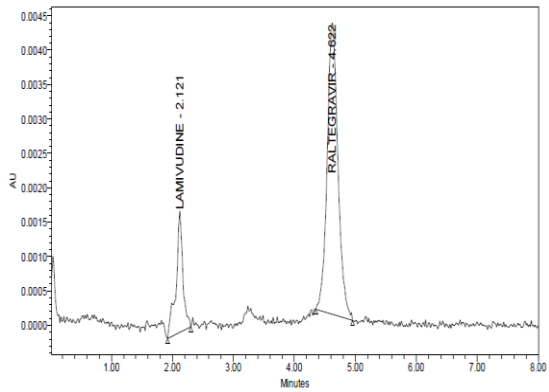


Accuracy 100% Chromatogram of Lamivudine and Raltegravir



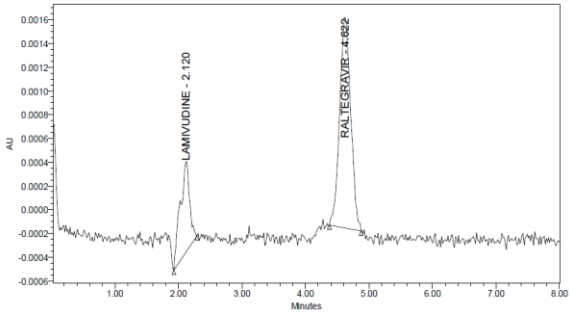
Accuracy 150% Chromatogram of Lamivudine and Raltegravir

4. **LOD:** Limit of detection was calculated by Lamivudine and Raltegravir method and LOD for Lamivudine was found to be 0.441 and Raltegravir was 0.031 respectively.



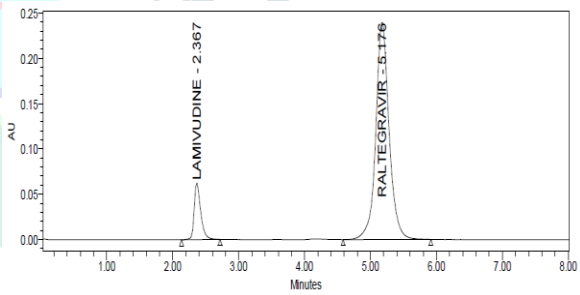
LOD Chromatogram of Lamivudine and Raltegravir

5. **LOQ:** Limit of Quantification was calculated by Lamivudine and Raltegravir method and LOQ for Lamivudine and Raltegravir wre found to be 1.336 and 0.093 respectively.

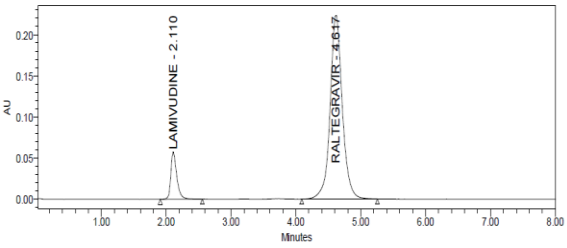


LOQ Chromatogram of Lamivudine and Raltegravir

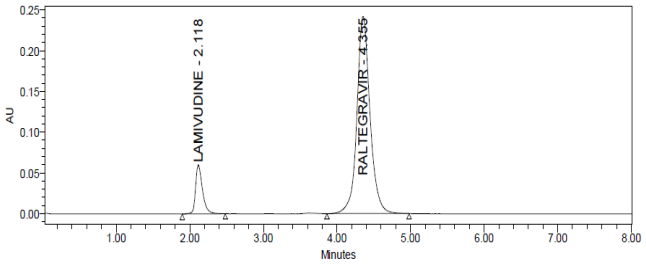
6. **Robustness:** Small deliberate changes in method like Flow rate, mobile phase ratio, and temperature are made but there were no recognized change in the result and are within range as per ICH Guide lines.



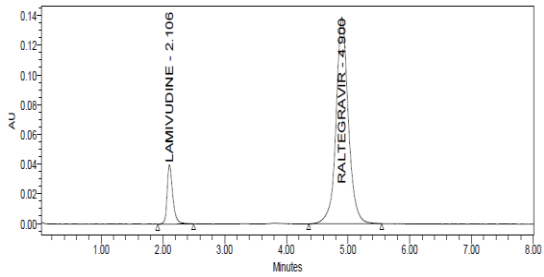
Flow minus Chromatogram of Lamivudine and Raltegravir



Flow plus Chromatogram of Lamivudine and Raltegravir

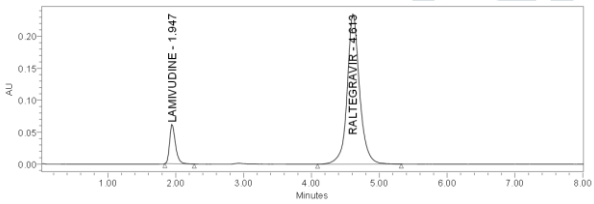


Mobile phase minus Chromatogram ofLamivudine and Raltegravir



Mobile phase Plus Chromatogram ofLamivudine and Raltegravir

Assay: Standard preparations are made from the API and Sample Preparations are from Formulation. Both sample and standards are injected six homogeneous samples. Drug in the formulation was estimated by taking the standard as the reference. The Average %Assay was calculated and found to be 100.22% and 100.9% for Lamivudine and Raltegravir respectively.



Assay chromatogram of Lamivudine/Raltegravir

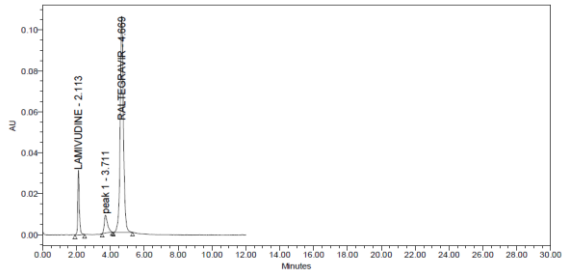
Degradation Studies: Degradation studies were performed with the formulation and the degraded samples were injected. Assay of the injected samples was calculated and all the samples passed the limits of degradation

Degradation studies for Lamivudine/Raltegravir

Acid degradation:

One millilitre of topical solution of Lamivudine and Raltegravir was taken from the stock solution into a clean and dry volumetric flask and add 1 mL of diluent and sonicated, then the solution was sonicated and treated with 1 mL of 2Nhydrochloric acid and the sample was left undisturbed for6 hrs on a bench top and after the specific period of time,The solution was neutralized by adding 1 mL of alkali and the solution was made up to 10 mL with methanol and water(80:20) ratio as diluent and injected.

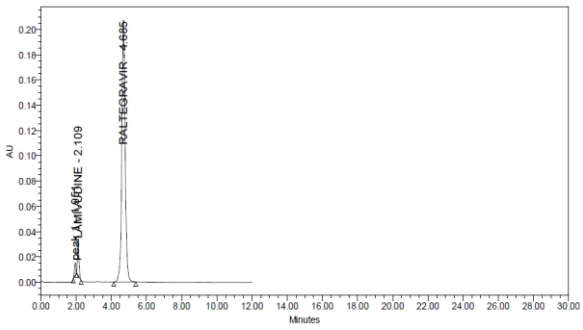
(a).Acid degradation



Base degradation:

One millilitre of topical solution of Lamivudine and Raltegravir was taken from the stock solution into a clean and dry volumetric flask and add 1 mL of diluent and sonicated, then was treated with 1 mL of 2 M sodium hydroxide and the sample was left undisturbed for 6 hrs on a bench top and after the specific period of time, the solution was neutralized with acid and the volume was made up to 10 mL with acetonitrile and water (80:20) ratio as diluent and injected.

(b).Base degradation

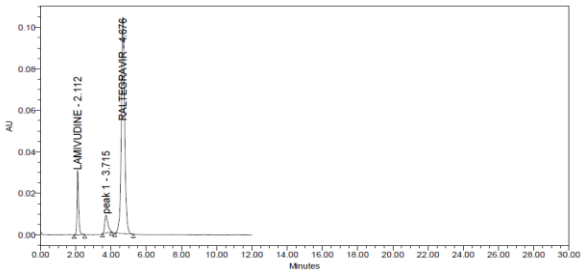


Peroxidase Degradation:

One millilitre of topical solution of Lamivudine and Raltegravir was taken from the stock solution into a clean and add 1 mlof diluent in a clean and dry volumetric flask and then the solution was sonicated and treated with 1 mL of 30% v/v hydrogen peroxide

and the sample was left undisturbed for6 hrs on a bench top and after the specific period of time, the volume was made up to 10 mL with methanol and water (80:20) ratio as diluent and injected.sss

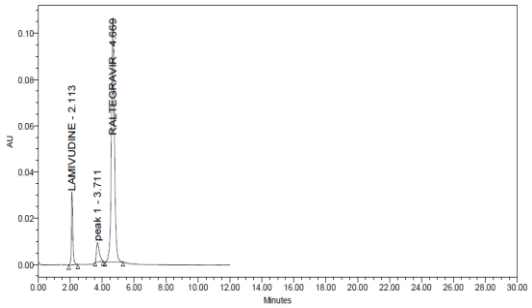
(c) Peroxidase degradation



Heat degradation:

One millilitre of topical solution of Lamivudine and Raltegravir was taken from the stock solution into a clean and dry volumetric flask and 1 mL of diluent, the solution was kept in hot air oven at 105◦C for 6 hrs and after the specific period of time the volume was made up to 10 mL with methanol and water (80:20) ratio as diluent and injected.

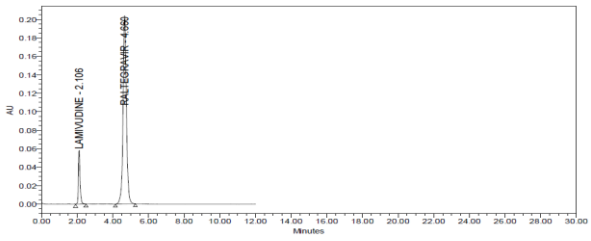
(d) Heat degradation



Photolytic degradation:

One millilitre of topical solution of Lamivudine and Raltegravir was taken from the stock solution into a clean and dry volu-metric flask and the solution was subjected to UV light up to200 watt hours/square metre and immediately cooled after the specific period of time and the volume was made up to10 mL with methanol and water (80:20) ratio as diluent and injected.

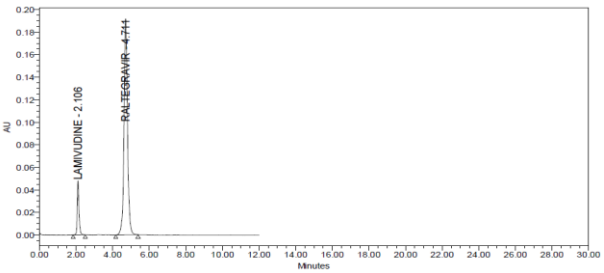
(e) Photolytic degradation



Neutral degradation:

One millilitre of topical solution of Lamivudine and Raltegravir taken from the stock solution into a clean and dry volumetric flask and the solution was treated with 1 mL of HPLCgrade water and heated on a water bath for 30 mins and then the sample was cooled and the volume was made up 10ml with methanol and water in ratio.

(f) Neutral degradation



5. CONCLUSION

UV-VISIBLE SPECTROPHOTOMETRIC METHOD:

Estimation of Lamivudine and Raltegravir was achieved by simultaneous equation method. The normal spectra of Lamivudine and Raltegravir were recorded in Acetonitrile. The linearity was checked in different concentrations at 280 nm for Lamivudine and 302 nm for Raltegravir. The slope, intercept and correlation coefficient values were found to be 0.056, 0.068 and 0.999 for Lamivudine and 0.06, 0.031 and 0.999 for Raltegravir. Precision studies were performed; low % RSD values were obtained which indicate that the proposed method has good precision.LOD and LOQ were found to be 5 µg/ml and 10 µg/ml for Lamivudine, 5µg/ml and 10 µg/ml for Raltegravir respectively. In this method, accuracy was determined by calculation of percentage recovery and average recovery was calculated at 50,100 and

150% levels. The recovery values between prescribed limit of 98-102% shows that method is free from interference of excipients present in formulation. The developed method was validated as per ICH guide lines. The method was successfully used for determination of drugs in tablets. The above discussion proves that the proposed method is simple, rapid and precise. The good recovery values and low relative standard deviation confirm the suitability of the method.

RP-HPLC METHOD: A simple, fast, accurate and precise RP-HPLC method was developed by using Acetonitrile: water, 0.1% ortho phosphoric acid(60:40) .The method was developed by using Kromosil 250 column (250 mm × 4.6 mm, 5µm particle size).and the mobile phase was pumped with Acetonitrile and water (hplc grade water)and PH was adjusted to 3.2 by using ortho phosphoric acid and the mobile phase was pumped at 1ml/min flowrate and the temperature was maintained at 30˚c.the retention time for Lamivudine/raltegravir were found to be 2.110 and 4.617 and the total run time was found to be 8min,the no of Theretical plates and Tailing factor of Lamivudine and Raltegravir were found to be 2584,1.36 and 3448,1.09, RP-HPLC method was found to be Linear in the range of Lamivudine/Raltegravir is 37.5-225µg/ml and 75-450µg/ml with a correlation coeffiecient of 0.999 the accuracy studies of RP-HPLC method was performed at three different levels i.e.,50%,100%,150% and recovery was found to be 99.24 to 100.13% for Lamivudine and 98.18 to 99.27% for Raltegravir respectively. Repeatability and intermediate results of Lamivudine/Raltegravir were found to be 0.55,1.33 and 0.8,0.4 Robustness of this method was performed by changing the flowrate at 0.9ml/min,1ml/min,1.1ml/min.the organic strength in the mobile phase was changed to 55:45v/v and 65:35 and results were found to be with in the limts.the % RSD was less than 2 the solution stability was determined at 0hr&24hr the %RSD of solutions were less than 2.the Ruggedness values were found to be 0.092 and 0.184 respectively.the %purity of the Dutrebis(label claim 150mg,300mg) was found to be 99.64 for Lamivudine and 100.5 for Raltegravir. The limit of Detection(LOD) and limit of Quantification(LOQ) were found to be 0.441 and 1.336µg/ml for Lamivudine and 0.031 and 0.093µg/ml for Raltegravir for RP-HPLC method.the sample was degraded in acidic,basic,peroxide,heat,photolytic,neutral and the results of Lamivudine/Raltegravir were found to be 5.85,2.6,3.66,1.88,0.83,0.92 and 5.66,2.76,4.89,1.81,0.78,0.69 respectively the above method was a rapid tool for routine analysis of Lamivudine/Raltegravir in the bulk and Pharmaceutical Dosage form.

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