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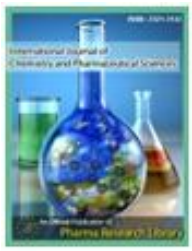

**R E S E A E C H A R T I C L E**

# Method Development and Validation of Elabasvir and Grazoprevir by using RP-HPLC

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**A B S T R A C T**

A new method was established for simultaneous estimation of Elbasvir and Grazopravir by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Elbasvir and Grazopravir by using Symmetry (4.6 x 250mm, 5µm), flow rate was 1.0ml/min, detection wave length was 264nm. The instrument used was WATERS HPLC Auto Sampler, Separation module 2695, PDA Detector, Empower-software version-2.The retention times were found to be 2.8 mins and 4.3 mins. The assay of Elbasvir and Grazopravir was performed with tablets and the % assay was found to be 100.18 and 100.00 which shows that the method is useful for routine analysis. The linearity of Elbosvir and Grazopravir was found to be linear with a correlation coefficient of 0.999 and 0.999, which shows that the method is capable of producing good sensitivity. The acceptance criteria of precision is RSD should be not more than 2.0% and the method show precision 0.5 and 0.1 for Elbasvir and Grazopravir which shows that the method is precise. The acceptance criteria of intermediate precision is RSD should be not more than 2.0% and the method show precision 0.6 and 0.2 for Elbasvir and Grazopravir which shows that the method is repeatable when performed in different days also. The accuracy limit is the percentage recovery should be in the range of 97.0% - 103.0%. The total recovery was found to be 100.11% and 100.38% for Elbasvir and Grazopravir. The validation of developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility. The acceptance criteria for LOD and LOQ is 3 and 10.The LOD and LOQ for Elbosvir was found to be 2.97 and 9.91 and LOD and LOQ for Grazopravir was found to be 3.09 and 10.07. The robustness limit for mobile phase variation and flow rate variation are well within the limit, which shows that the method is having good system suitability and precision under given set of conditions.

**Keywords:** Elbasvir, Grazopravir, HPLC

**A R T I C L E I N F O**

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## 1. Introduction

**Analytical methods:** Methods are developed for new products when no official methods are available. Alternate methods for existing (non-pharmacopoeial) products are developed to reduce the cost and time for better precision and ruggedness [1,2]. Trial runs are conducted, method is optimized and validated. When alternate method proposed is intended to replace the existing procedure comparative laboratory data including merit/demerits are made available

**Description of the Various Analytical Methods**

Titrimetric and gravimetric method of analysis is suitable when the sample is present in pure form or when no interference is observed in the mixture with other materials [3]. Ultraviolet and visible spectrometric method is suitable when no Interference is observed in the mixture [4]. HPLC and GC methods are more advantageous than the above due to their capability in separating organic mixtures and quantitative estimations. AAS is used mainly for quantitative estimation in ppm and ppb levels of elements [5]. Infra-red spectroscopy though mainly used for qualitative analysis can be used for quantitative estimation also. Out of all the above methods, thin layer chromatography plays a very important role in analysis due to its adaptability, flexibility, and cost and time. It can be used both for qualitative and quantitative determination. After separation spots can be scanned with the help of a scanner and quantitative measurement can be made [6].

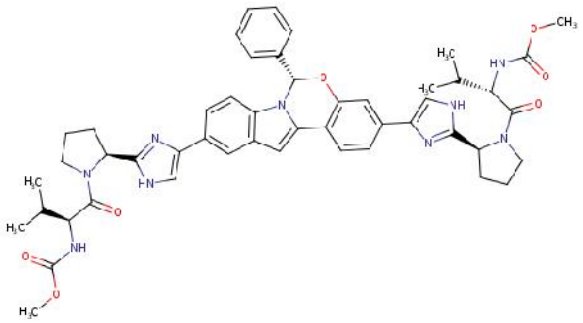

**Figure 1:** Elbasvir

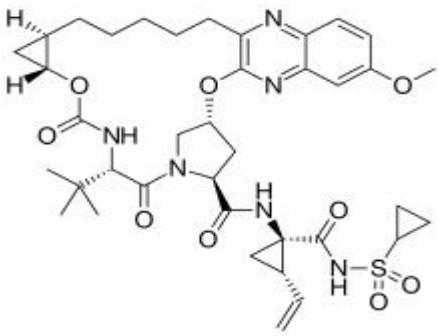

**Figure 2:** Grazopravir

## 2. Materials and Methods

**Apparatus:** The instrument used for the study was WATERS, software: Empower, 2695 separation module, UV detector [10].

**Preparation of mobile phase:**

Accurately measured 450 ml (45%) of above buffer and 550 ml of Acetonitrile HPLC (55%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

**Preparation of Phosphate buffer:**

3.4g of $KH_2PO_4$ in 1000 ml of HPLC water Ph was adjusted with OPA up to 3.0.final solution was filtered through 0.44 µm Membrane filter and sonicate it for 10 mins[11].

**Diluent:** The Mobile phase was used as the diluent.

**Optimization Chromatographic trials for Simultaneous Estimation of Elbasvir and Grazopravir by RP- HPLC.**

**Optimization chromatographic conditions**

Instrument used : Waters HPLC with auto sampler and UV detector.

Temperature : Ambient

Column : Symmetry (4.6 x 250mm, 5µm)

Buffer : 3.4g of $KH_2PO_4$ in 1000 ml of HPLC water Ph was adjusted with OPA up to 3.0.

pH : 3.0

Mobile phase : 45% buffer 55% Acetonitrile

Flow rate : 1 ml per min

Wavelength : 264 nm

Injection volume : 20 µl

Run time : 10 min.

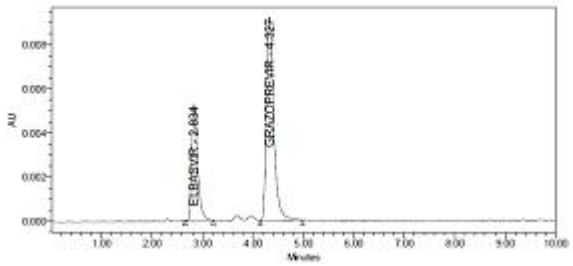

**Figure 3:** Optimization Chromatogram

**Observation:** The separation of two analytical peaks was good. The plate count also above 2000, tailing factor below 2, and the resolution is above 2. The condition is taken as optimized method.

## 3. Results and Discussion

**Method Validation Parameters**

**1. Specificity:** For Specificity Blank and Standard are injected into system, there is no any inteferece of any peak in blank with the retention time of the analytical peaks [12].

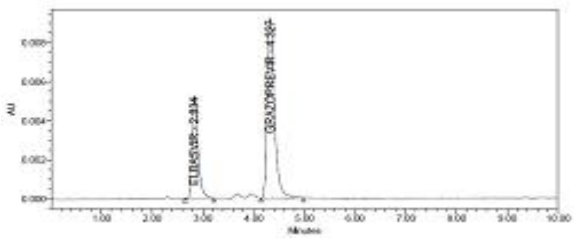

**Figure 3:** Chromatogram for System suitability

**2. Linearity:**

**Preparation of stock solution:** Accurately weigh and transfer 100 mg of Elbasvir and 200 mg of Grazoprevir working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

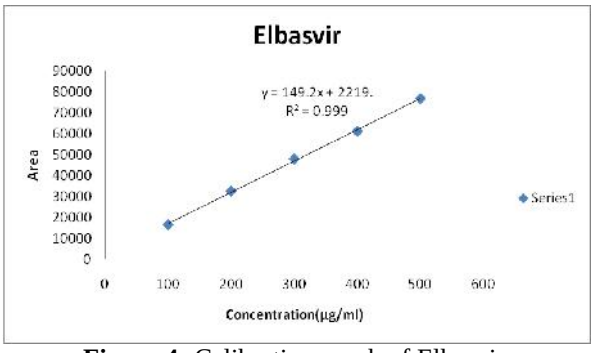


**Figure 4:** Calibration graph of Elbasvir

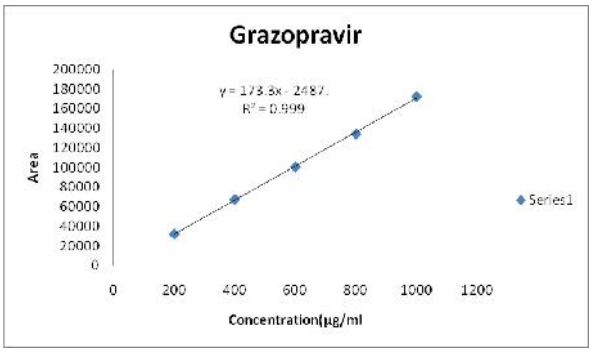


**Figure 5:** Calibration graph of Grazopravir

**Preparation of Level – I (100 ppm of Elbasvir &200ppm of Grazoprevir):** 0.1 ml of above stock solutions has taken in 10ml of volumetric flask, dilute up to the mark with diluent.

**Preparation of Level – II (200ppm of Elbasvir &400ppm of Grazoprevir):**

0.2 ml of above stock solutions has taken in 10ml of volumetric flask, dilute up to the mark with diluent.

**Preparation of Level – III (300 ppm of Elbasvir & 600ppm of Grazoprevir):**

0.3 ml of above stock solutions has taken in 10ml of volumetric flask, dilute up to the mark with diluent.

**Preparation of Level – IV (400ppm of Elbasvir & 800ppm of Grazoprevir):**

0.4 ml of above stock solutions has taken in 10ml of volumetric flask, dilute up to the mark with diluent

**Preparation of Level – V (500ppm of Elbasvir & 1000ppm of Grazoprevir):**

**0.5** ml of above stock solutions has taken in 10ml of volumetric flask, dilute up to the mark with diluents[13]t

**Procedure:**

Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

**3. Precision**

**Preparation of stock solution:**

Accurately weigh and transfer 100 mg of Elbasvir and 200 mg of Grazoprevir working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

**Procedure:**

The standard solution was injected for six times and measured the area for all six. Injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits [14].

**4. Intermediate Precision/Ruggedness**

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day.

**Preparation of stock solution:**

Accurately weigh and transfer 100 mg of Elbasvir and 200 mg of Grazoprevir working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents [15].

**Procedure:**

The standard solutions prepared in the precision was injected on the other day, for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

**5. Accuracy**

**Preparation of Standard stock solution:**

Accurately weigh and transfer 100 mg of Elbasvir and 200 mg of Grazoprevir working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)**.** Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents [16].

**Preparation Sample solutions:**

**For preparation of 50% solution (With respect to target Assay concentration):** Accurately weigh and transfer 50 mg of Elbasvir and 100 mg of Grazoprevir working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents [17].

**For preparation of 100% solution (With respect to target Assay concentration):**

Accurately weigh and transfer 100 mg of Elbasvir and 200 mg of Grazoprevir working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

**For preparation of 150% solution (With respect to target Assay concentration):**

Accurately weigh and transfer 150 mg of Elbasvir and 300 mg of Grazoprevir working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents [18].

**Procedure:**

Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions.

Calculate the Amount found and Amount added for Elbasvir & Grazoprevir and calculate the individual recovery and mean recovery values.

**6. Limit of Detection: (for Elbasvir)**

**Preparation of 300µg/ml solution:**

Accurately weigh and transfer 100 mg of Elbasvir working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents [19].

**Preparation of 0.99 µg/ml solution:**

Further pipette 0.33ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

**Limit of Detection: (for Grazoprevir)**

**Preparation of 600 µg/ml solution:**

Accurately weigh and transfer 200 mg of Grazoprevir working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents [20].

**Preparation of 12.0 µg/ml solution:**

Further pipette 0.2ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent

**7. Limit of Quantification**

**Preparation of 300 µg/ml solution:**

Accurately weigh and transfer 100 mg of Elbasvir working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

**Preparation of 33.0 µg/ml solution:** Further pipette 1.1 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

**Preparation of 600µg/ml solution:**

Accurately weigh and transfer 200 mg of Grazoprevir working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

**Preparation of 37.8 µg/ml solution:**

Further pipette 0.63ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

**8. Robustness**

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

A. The flow rate was varied at 0.9 ml/min to 1.1ml/min.

Standard solution 300 ppm of Elbasvir & 600ppm of Grazoprevir was prepared and analysed using the varied flow rates along with method flow rate. On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%.

B. The Organic composition in the Mobile phase was varied from ±10%.

Standard solution 300ppm of Elbasvir & 600 ppm of Grazoprevir was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method. On evaluation of the above results, it can be concluded that the variation in 10%. Organic composition in the mobile phase affected the method significantly. Hence it

Indicates that the method is robust even by change in the Mobile phase ±10

**9. Degradation Studies**

The International Conference on Harmonization (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 Elbasvir and Grazoprevir using the proposed method.

**Preparation of stock:**

Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 100 mg of Elbasvir and 200 mg Grazoprevir in sample into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate it up to 30 mins to dissolve it completely and make volume up to the mark with the same solvent. Then it is Filtered through 0.44 micron Injection filter. (Stock solution).

**Hydrolytic degradation under acidic condition**

Pipette 0.3 ml of above solution into a 10ml volumetric flask and 3 ml of 0.1N HCl was added. Then, the volumetric flask was kept at 60ºC for 24 hours and then neutralized with 0.1 N NaOH and make up to 10ml with diluent. Filter the solution with 0.44 microns syringe filters and place in vials.

**Hydrolytic degradation under alkaline condition**

Pipette 0.3 ml of above solution into a 10ml volumetric and add 3ml of 0.1N NaOH was added in 10ml of volumetric flask. Then, the volumetric flask was kept at 60ºC for 24 hours and then neutralized with 0.1N HCl and make up to 10ml with diluent. Filter the solution with 0.44 microns syringe filters and place in vials [21].

**Thermal induced degradation**

Elbasvir and Grazoprevire sample was taken in petridish and kept in Hot air oven at $110^0$ C fo 3 hours. Then the

sample was taken and diluted with diluents and injected into HPLC and analyzed.

**Oxidative degradation**

Pipette 0.3 ml above stock solution into a 10ml volumetric flask and 1ml of 30% w/v of hydrogen peroxide added in 10 ml of volumetric flask and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min. Filter the solution with 0.45 microns syringe filters and place in vials.

**Photo degradation:**

Pipette 0.3 ml above stock solution into a 10ml volumetric flask and expose to sunlight for 24hrs and the volume was made up to the mark with diluent. Filter the solution with 0.45 microns syringe filters and place in vials

**Table 1:** Area of different concentration of Elbasvir

| S. No | Linearity Level | Concentration | Area |
|---|---|---|---|
| 1 | I | 100 | 16472 |
| 2 | II | 200 | 32577 |
| 3 | III | 300 | 47931 |
| 4 | IV | 400 | 61145 |
| 5 | V | 500 | 76795 |
| Correlation Coefficient | | | 0.999 |

**Table 2:** Area of different concentration of Grazopravir

| S. No | Linearity Level | Concentration | Area |
|---|---|---|---|
| 1 | I | 200 | 32441 |
| 2 | II | 400 | 67728 |
| 3 | III | 600 | 100630 |
| 4 | IV | 800 | 134448 |
| 5 | V | 1000 | 172463 |
| Correlation Coefficient | | | 0.999 |

**Table 3:** Accuracy (recovery) data for Elbasvir

| %Concentration (at specification Level) | Area | Amount Added (mg) | Amount Found (mg) | % Recovery | Mean Recovery |
|---|---|---|---|---|---|
| 50% | 24492 | 50 | 49.95 | 99.89 | 100.11 |
| 100% | 48488 | 100 | 99.70 | 99.70 | |
| 150% | 73486 | 150 | 151.10 | 100.73 | |

**Table 4:** Accuracy (recovery) data for Grazopravir

| %Concentration (at specification Level) | Area | Amount Added (mg) | Amount Found (mg) | % Recovery | Mean Recovery |
|---|---|---|---|---|---|
| 50% | 49702 | 100 | 100.52 | 100.52 | 100.38 |
| 100% | 99405 | 200 | 201.04 | 100.52 | |
| 150% | 148499 | 300 | 300.34 | 100.11 | |

**Table 5:** Results of Precision for Elbasvir and Grazopravir

| Injection | Area for Elbasvir | Area for Grazoprevir |
|---|---|---|
| Injection-1 | 48997 | 98709 |
| Injection-2 | 48348 | 98962 |
| Injection-3 | 48957 | 98700 |
| Injection-4 | 48487 | 98687 |
| Injection-5 | 48674 | 98901 |
| Injection-6 | 48691 | 98960 |
| **Average** | 48692.3 | 98819.8 |
| **Standard Deviation** | 254.5 | 134.7 |
| **%RSD** | 0.5 | 0.1 |

**Table 6:** Results of Intermediate precision for Elbasvir and Grazopravir

| Injection | Area for Elbasvir | Area for Grazoprevir |
|---|---|---|
| Injection-1 | 48673 | 98783 |
| Injection-2 | 48720 | 98674 |
| Injection-3 | 48793 | 98647 |
| Injection-4 | 48657 | 98359 |

| | | |
|---|---|---|
| Injection-5 | 48082 | 98747 |
| Injection-6 | 48956 | 98911 |
| **Average** | 48646.8 | 98686.8 |
| **Standard Deviation** | 297.4 | 185.7 |
| **%RSD** | 0.6 | 0.2 |

**Table 7:** Results for variation in flow for Grazopravir

| S. No | Flow Rate (ml/min) | System Suitability Results | |
|---|---|---|---|
| | | USP Plate Count | USP Tailing |
| 1 | 0.9 | 3013.80 | 1.4 |
| 2 | 1.0 | 2935.56 | 1.42 |
| 3 | 1.1 | 2845.18 | 1.43 |

**Table 8:** Results for variation in flow for Grazopravir

| S. No | Flow Rate (ml/min) | System Suitability Results | | |
|---|---|---|---|---|
| | | USP Plate Count | USP Tailing | USP Resolution |
| 1 | 0.9 | 4951.17 | 1.46 | 6.64 |
| 2 | 1.0 | 4800.53 | 1.46 | 6.50 |
| 3 | 1.1 | 4596.34 | 1.42 | 6.34 |

**Table 9:** System suitability results for Elbasvir

| S. No | Change in Organic Composition in the Mobile Phase | System Suitability Results | |
|---|---|---|---|
| | | USP Plate Count | USP Tailing |
| 1 | 10% less | 3013 | 1.1 |
| 2 | *Actual | 2935.56 | 1.42 |
| 3 | 10% more | 2841.98 | 1.44 |

**Table 10:** System suitability results for Grazoprevir

| S. No | Change in Organic Composition in the Mobile Phase | System Suitability Results | | |
|---|---|---|---|---|
| | | USP Plate Count | USP Tailing | USP Resolution |
| 1 | 10% less | 4751 | 1.45 | 6.64 |
| 2 | *Actual | 4800.53 | 1.46 | 6.50 |
| 3 | 10% more | 4160.01 | 1.42 | 4.04 |

**Table 11:** Limit of Detection for Elbasvir and Grazopravir

| Drug name | Baseline noise(µV) | Signal obtained (µV) | S/N ratio |
|---|---|---|---|
| Elbasvir | 58 | 172 | 2.97 |
| Grazopravir | 558 | 179 | 3.09 |

**Table 12:** Limit of Quantification for Elbasvir and Grazopravir

| Drug name | Baseline noise(µV) | Signal obtained (µV) | S/N ratio |
|---|---|---|---|
| Elbasvir | 58 | 575 | 9.91 |
| Grazopravir | 58 | 584 | 10.07 |

**Table 13:** Results of degradation parameters

| Sample Name | Elbasvir | |
|---|---|---|
| | Area | % Degraded |
| **Standard** | 48538 | |
| **Acid** | 44953 | 7.39 |
| **Base** | 47130 | 2.90 |
| **Peroxide** | 45836 | 5.57 |
| **Thermal** | 44789 | 7.72 |
| **Photo** | 46184 | 4.85 |
| **Sample Name** | Grazoprevir | |
| | **Area** | **% Degraded** |
| **Standard** | 98691 | |
| **Acid** | 95659 | 3.07 |

| | | |
|---|---|---|
| **Base** | 92821 | 5.95 |
| **Peroxide** | 97498 | 1.21 |
| **Thermal** | 93151 | 5.61 |
| **Photo** | 96389 | 2.33 |

## 4. Conclusion

The estimation of Elbosvir and Grazopravir was done by RP-HPLC. The assay of Elbosvir and Grazopravir was performed with tablets and the % assay was found to be 100.18 and 100.00 which shows that the method is useful for routine analysis. The linearity of Elbosvir and Grazopravir was found to be linear with a correlation coefficient of 0.999 and 0.999, which shows that the method is capable of producing good sensitivity. The acceptance criteria of precision is RSD should be not more than 2.0% and the method show precision 0.5 and 0.1 for Elbosvir and Grazopravir which shows that the method is precise. The acceptance criteria of intermediate precision is RSD should be not more than 2.0% and the method show precision 0.6 and 0.2 for Elbosvir and Grazopravir which shows that the method is repeatable when performed in different days also. The accuracy limit is the percentage recovery should be in the range of 97.0% - 103.0%. The total recovery was found to be 100.11% and 100.38% for Elbosvir and Grazopravir. The validation of developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility. The acceptance criteria for LOD and LOQ is 3 and 10.The LOD and LOQ for Elbosvir was found to be 2.97 and 9.91 and LOD and LOQ for Grazopravir was found to be 3.09 and 10.07. The robustness limit for mobile phase variation and flow rate variation are well within the limit, which shows that the method is having good system suitability and precision under given set of conditions.

## 5. References


[1] Becket and Stenlake, Practical pharmaceutical chemistry, part 24th edition CBS publications and distributors, 2005.

[2] P.D. Sethi, HPLC quantitative analysis of pharmaceutical formulations CBS publications and distributors, 1st edition, 2001.

[3] B.K Sharma, instrumental method of chemical analysis, 23rd edition, goal publishers 2004.

[4] Validating chromatographic methods, David M.Bliesner. International conference on harmonization**:** ICH Q 2 (R1) Validation of Analytical Procedures: Text and Methodology 1995.

[5] Skoog D A, West D M, Holler FJ: Introduction of analytical chemistry. Sounder college of publishing, Harcourt Brace college publishers. (1994), PP 1-5.

[6] ZEPATIER™ (elbasvir and grazoprevir) Tablets, for Oral Use. Full Prescribing Information" (PDF). Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc. Retrieved 31 January 2016.

[7] FDA approves Zepatier for treatment of chronic hepatitis C genotypes 1 and 4" U.S. Food and Drug Administration. Retrieved 31 January 2016.

[8] Lindsay Sandy, HPLC by open learning, (1991) 30-45.

[9] Lough WJ, Wainer IWW. HPLC fundamental principles and practices, (1991) 52- 67.

[10] Krstulovic AM, Brown PR. Reversed-Phase High Performance Liquid Chromatography: Theory, Practice and Biomedical Applications, Wiley, New York, (1982).

[11] U.S. FDA, Title 21 of the U.S. Code of Federal Regulations: 21 CFR 211- Current good manufacturing practice for finished pharmaceuticals.

[12] U.S. FDA - Guidance for Industry (draft) Analytical Procedures and Methods Validation: Chemistry, Manufacturing, and Controls and Documentation, (2000).

[13] ISO/IEC 17025, General requirements for the competence of testing and calibration laboratories, (2005).

[14] International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, Validation of analytical procedures: definitions and terminology, Q2A, Geneva (1996).

[15] International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, Validation of analytical procedures: Methodology, Q2B, Geneva (1996).

[16] U.S. EPA, Guidance for methods development and methods validation for the Resource Conservation and Recovery Act (RCRA) Program, Washington, D.C. (1995).,

[17] General Chapter 1225, Validation of compendial methods, United States Pharmacopeia 30, National Formulary 25, Rockville, Md., USA, The United States Pharmacopeial Convention, Inc., (2007).