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# "DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF GLIMEPIRIDE AND LINAGLIPTIN FROM THEIR SYNTHETIC MIXTURE"

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**ABSTRACT:** The RP-HPLC was used to develop the method and for validation of glimepiride and linagliptin from synthetic mixture. For chromatographic condition the mobile phase used was Acetonitrile: Methanol: Water (35:35:30 v/v) on the stationary phase of Hibar ODS C18 column (250 mm*4.6 mm), the detection observed was at 245nm. The response of the calibration curve was found to be linear in range by Glimepiride and Linagliptin 0.9996 and 0.9998 respectively. The retention time observed is 3.198 min for Glimepiride and 7.401 min for Linagliptin at the run time of 15 minutes. The limit of detection for Glimepiride is 0.035 µg/mL and Linagliptin is 0.08 µg/mL. However, the limit of quatification for Glimepiride is 0.10 µg/mL and Linagliptin is 0.08 µg/mL. The accuracy for the Glimepiride is 99.98% and Linagliptin is 99.78 % as per method performed. The limit for precision is RSD< 2 therefore, for Glimepiride is 0.59-1.14 and Linagliptin is 0.46-1.21. The experiment was carried out as per International Conference on Harmonization (ICH) guidelines and the statistical analysis of the data is linear, precise, accurate and robust. Therefore, the method can be successfully employed for the synthetic mixture of glimepiride and linagliptin in pharmaceutical formulation.

**KEYWORDS:** glimepiride, linagliptin, RP-HPLC, validation parameter, method development and validation

## ➢ INTRODUCTION

Diabetes, is a group of metabolic disorders in which, blood sugar levels are high for a longer period of time. Diabetes occurs when the body is unable to take up sugar (glucose) into cells and use it for energy. This causes extra sugar to make up in the blood. which also called diabetes mellitus 2 that mostly occurs old age persons.

Glimepiride is an oral hyperglycemic agent (anti-diabetic drug) of the Sulfonylurea class of drugs. it was approved in FDA-1995, CDSCO -1999. Linagliptin is an oral hyperglycemic agent (anti-diabetic drug) of the DPP-4 inhibitors class of drug. It was approved in FDA-2011, CDSCO -2012.

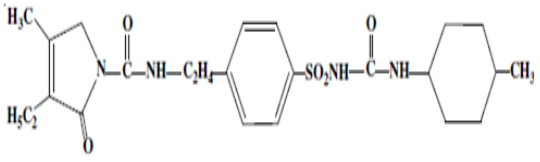

**Figure 1. Structure of Glimepiride**

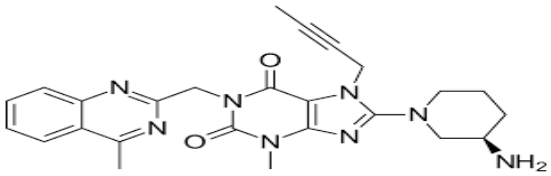

**Figure 2. Structure of Linagliptin**

Glimepiride and Linagliptin in combination are under Clinical trial Phase - 3. Dose and ratio of drug combination: Glimepiride 50 mg and Linagliptin 10 mg (5:1). Glimepiride and Linagliptin significantly improved glycemic variability, with similar glucose-lowering efficacy and safety profiles in patients with type-II diabetes.

High-performance liquid chromatography (HPLC, also known as High-pressure liquid chromatography) is the most widely used analytical technique for the qualitative and quantitative analysis of pharmaceuticals, biomolecules, polymers and other organic

compounds. HPLC is a physical separation technique performed in the liquid phase, where the sample is separated into its constituent components (or analytes) by distribution between the mobile phase (a flowing liquid) and a stationary phase (absorbent is packed inside the column).

According to the ICH (Q2R1) guideline, method validation can be defined as "Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications". Method validation is the procedure used to confirm that the analytical method used for a particular test is suitable for its intended purpose. Method validation results can be used to assess the quality, reliability and consistency of analytical results. It is an integral part of any good analytical procedure

Validation Parameters according to the ICH Q2(R1) guideline:

- Linearity and Range
- Accuracy
- Precision
- LOD (Limit of Detection)
- LOQ (Limit of Quantification)
- Robustness

## ➢ MATERIAL AND METHODS

Table 1. Instrument Specification for High Performance Liquid Chromatography

| | |
|---|---|
| **Make** | Agilent |
| **Model** | Infinity 1200 |
| **Type** | Quaternary Gradient |
| **Detector** | UV VIS |
| **Software** | Open Lab |
| **Column** | Hibar ODS C18 (250*4.6 mm. 5 um) |
| **Pump** | High Pressure Gradient |

Table 2. Instrument specification for FTIR

| | |
|---|---|
| **Make** | Agilent |
| **Model** | Carry 360 |
| **Scanning Range** | 400-4000 cm 1 |

Table 3. Instrument specification for UV double bean Spectrophotometer

| | |
|---|---|
| **Make** | Shimadzu |
| **Model** | UV 1900 |
| **Scanning Range** | 190 - 1100 |

Table 4 Instrument specification for melting point apparatus

| | |
|---|---|
| **Make** | Gallenkamp |

## ➢ IDENTIFICATION OF API

### 1. Melting Point Determination

The melting points of the Glimepiride and Linagliptin were also taken by the open capillary method by melting point apparatus.

Table 5. Identification by Melting Point

| Sr No. | Drug | Reported Melting Point | Observed Melting Point |
|---|---|---|---|
| 1 | Glimepiride | 207°C | >200°C |
| 2 | Linagliptin | 190-196°C | 191-196°C |

**2. Solubility Analysis:**

Solubility of glimepiride and linagliptin was performed using various solvents like water, methanol, acetonitrile etc.

Table 6. Solubility Analysis

| Solvent | Glimepiride | Linagliptin |
|---|---|---|
| **Water** | Slightly soluble | Very slightly soluble |
| **Methanol** | Soluble | Soluble |
| **Acetonitrile** | Soluble | Soluble |
| **DMSO** | Soluble | Soluble |

**3. Identification by IR**

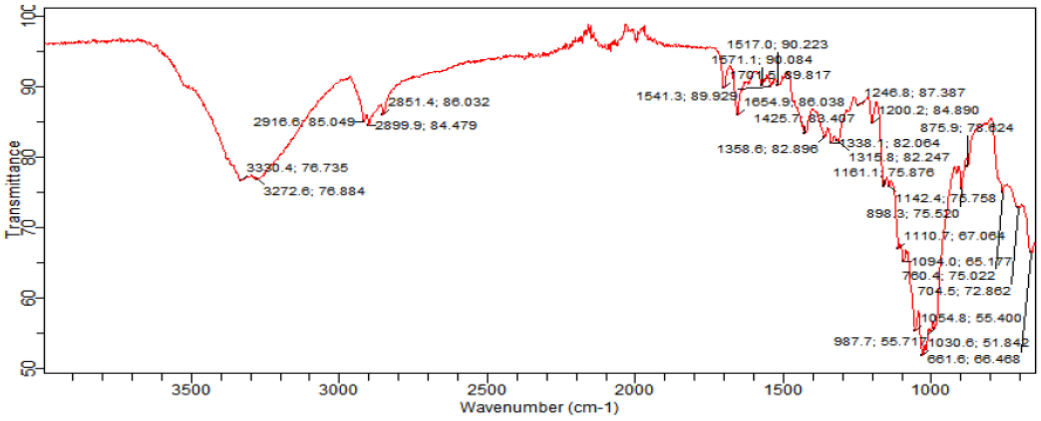


**Figure 3. IR spectrum of Glimepiride**

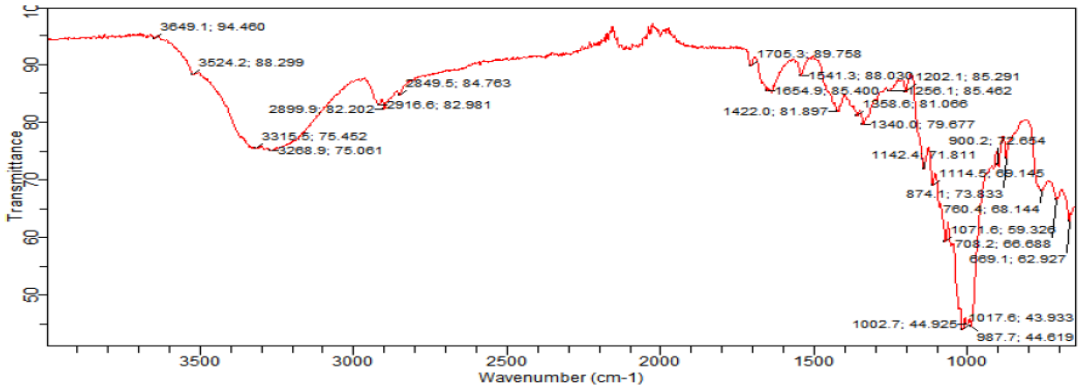


**Figure 4. IR spectrum of Linagliptin**

Table 7. Interpretation of IR spectrum of Glimepiride

| Major Functional group/ Bond present | Standard frequency $cm^{-1}$ | Observed frequency $cm^{-1}$ |
|---|---|---|
| N-H | 3400-3300 | 3315.5 |
| C=O | 1705-1725 | 1654.9 |
| C-N | 1345-1340 | 1340.0 |
| S=O | 1160-1120 | 1142.2 |
| C=C | 1650-1675 | 1654.9 |

Table 8. Interpretation of IR spectrum of Linagliptin

| Major Functional group/ Bond present | Standard frequency cm-$^{1}$ | Observed frequency cm-$^{1}$ |
|---|---|---|
| N-H | 3300-3400 | 3330.4 |
| C=O | 1605-1710 | 1701.5 |
| C=C | 1670-1600 | 1654.9 |
| C=N | 1640-1690 | 1654.9 |
| C-N | 1250-1020 | 1200.2 |

➢ **RP-HPLC METHOD FOR ESTIMATION OF GLM AND LIN**

- Ratio of Synthetic Mixture

Proposed FDC contains Glimepiride + Linagliptin (2 + 5 mg) in the form of film coated tablet. According to this dose, the ratio of synthetic mixture will be Glimepiride: Linagliptin = 2:5.

➢ Selection of Analytical Wavelength

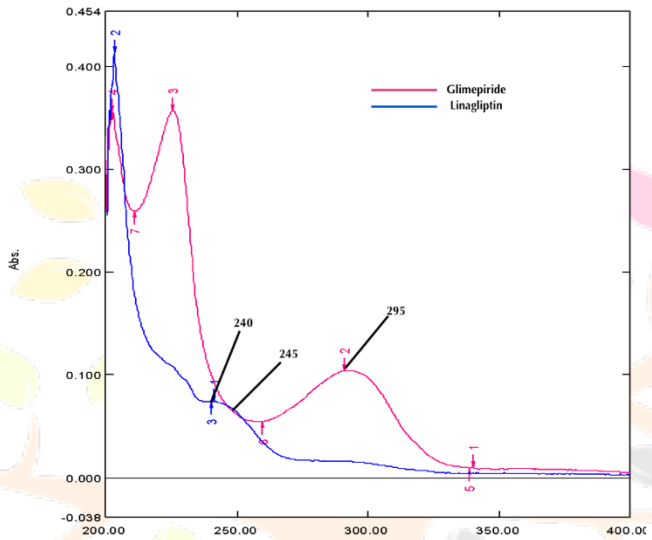


**Figure 5. Selection of analytical wavelength for HPLC**

- A Working standard of GLM (10 µg.ml$^{-1}$) and LIN (20 µg.ml$^{-1}$) using methanol as a solvent, were scanned in UV 200-400 nm region and overlapped.
- When both UV spectrum were overlapped, it was observed that at **245 nm** was **iso-absorptive point**, and both the drugs showed adequate absorbance, so it was selected as detection wavelength.

➢ **PREPARATION OF STANDARD STOCK SOLUTION**

1) GLM

Table 9. Preparation of standard stock solution of GLM

| | **Preparation of solution** |
|---|---|
| **Master Stock Solution:** | Accurately weighed 20 mg GLM dissolved in 100 ml methyl alcohol (200 µg.ml$^{-1}$).<br>Dilute 1 ml of this solution 10 ml diluent, (20 µg.ml$^{-1}$) |
| **Standard Solution:** | Withdraw 1.0 ml from Master Stock Solution and make up to 10 ml with methyl alcohol (2 µg.ml$^{-1}$) |

2) LIN

Table 10. Preparation of standard stock solution of LIN

| | **Preparation of solution** |
|---|---|
| **Master Stock Solution:** | Accurately weighed 50 mg LIN dissolved in 100 ml methyl alcohol (500 µg.ml$^{-1}$).<br>Dilute 1 ml of this solution 10 ml diluent, (50 µg.ml$^{-1}$) |
| **Standard Solution:** | Withdraw 1.0 ml from Master Stock Solution and make up to 10 ml with methyl alcohol (5 µg.ml$^{-1}$) |

3) GLM + LIN

Table 11 . Preparation of standard stock solution of mixture

| | **Preparation of solution** |
|---|---|
| **Master Stock Solution:** | Weigh accurately about 20 mg of GLM and 50 mg of LIN and transfer into a 100 ml volumetric flask. Make up the volume of the flask to the mark with Methanol. (200 µg/ml GLM + 500 µg/ml of LIN). Dilute 1 ml of previous solution to 10 ml with diluent to give stock solution (20 µg/ml GLM + 50 µg/ml LIN). |
| **Standard Solution** | Withdraw 1.0 ml from Master Stock Solution and make up to 10 ml with methyl alcohol GLM+LIN (2+5 $\mu g.ml^{-1}$) |

➢ **OPTIMIZATION CHROMATOGRAPHIC CONDITION**

| | | |
|---|---|---|
| Trial 1 | Column: Hibar ODS C18 (250*4.6 mm, 5µm)<br>Mobile Phase: Methanol: Water (50:50 v/v),<br>Detection**:** 245 nm<br>Flow rate: 1 ml/min<br>Run Time: 15 minutes | Only one peak detected. |

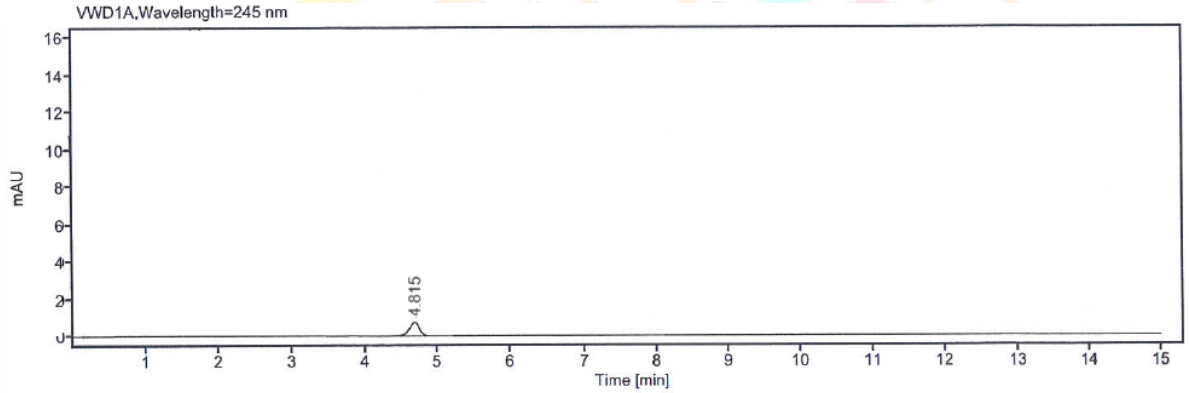


**Figure 6. Trial 1: Chromatogram of GLM+LIN (2+5 µg.ml$^{-1}$)**

| | | |
|---|---|---|
| Trial 2 | Column: Hibar ODS C18 (250*4.6 mm, 5µm)<br>Mobile Phase: Acetonitrile: Water (50:50 v/v),<br>Detection: 245 nm<br>Flow rate: 1 ml/min<br>Run Time: 15 minutes | Two peaks observed with inadequate resolution. |

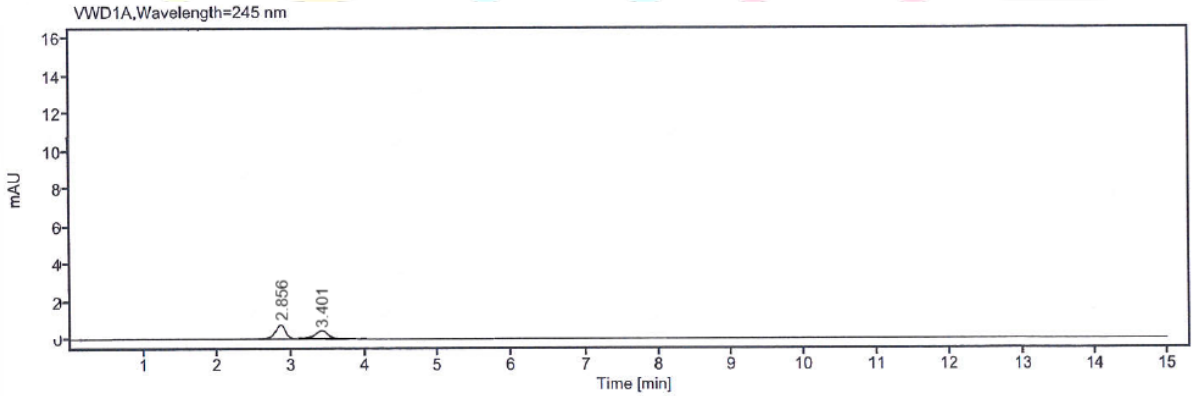


**Figure 7. Trial 2: Chromatogram of GLM+LIN (2+5 µg.ml$^{-1}$)**

| | | |
|---|---|---|
| Trial 3 | Column: Hibar ODS C18 (250*4.6 mm, 5µm)<br>Mobile Phase: Acetonitrile: Methanol (50:50 v/v),<br>Detection: 245 nm<br>Flow rate: 1 ml/min<br>Run Time: 15 minutes | Separation achieved but resolution was inadequate. |

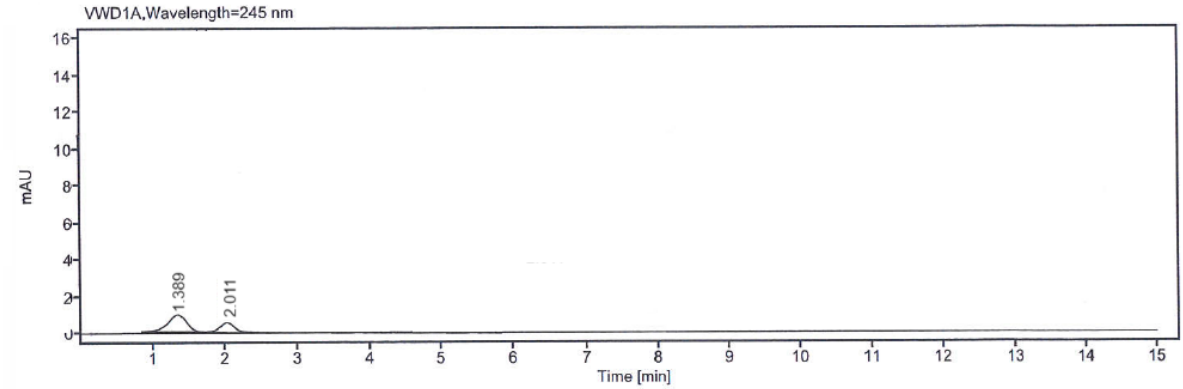


**Figure 8. Trial 3: Chromatogram of GLM+LIN (2+5 µg.ml$^{-1}$)**

| | | |
|---|---|---|
| Trial 4 | Column: Hibar ODS C18 (250*4.6 mm, 5µm)<br>Mobile Phase: Methanol: Acetonitrile: Water (35:35:30 v/v/v)<br>Detection: 245 nm<br>Flow rate: 1 ml/min<br>Run Time: 15 minutes | Separation achieved with adequate resolution |

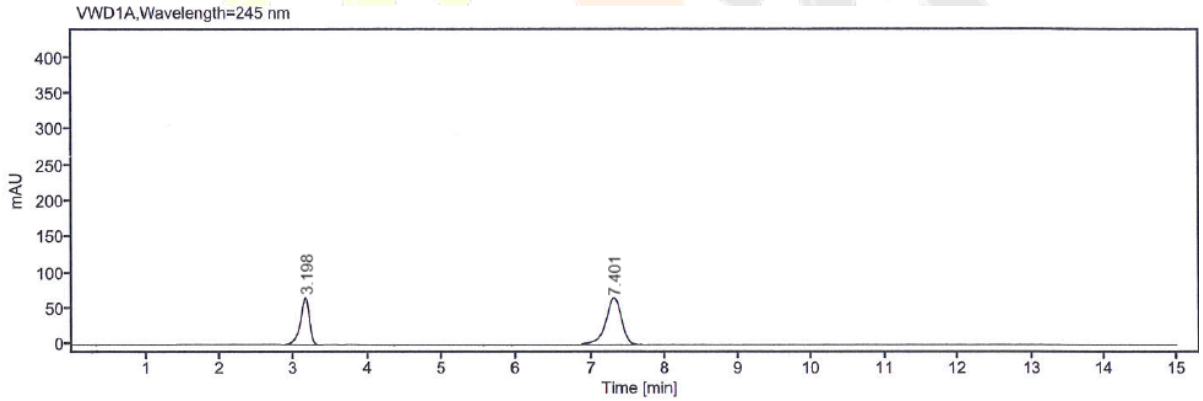


**Figure 9. Trial 4: Chromatogram of GLM+LIN (2+5 µg.ml$^{-1}$)**

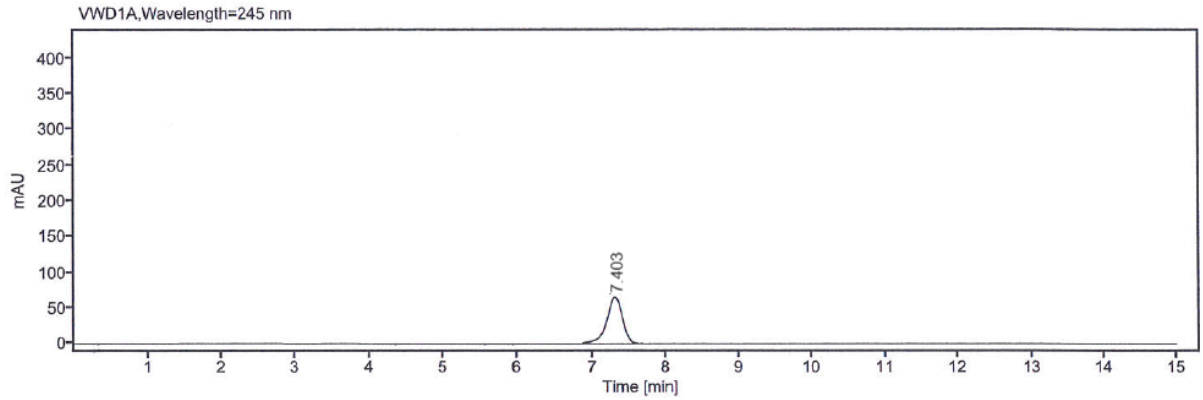


**Figure 10.** Chromatogram of LIN (5 µg.ml$^{-1}$) for peak identification

- After performing the described trials for estimation of GLM+LIN, following chromatographic conditions were optimized for development and validation of RP-HPLC method for estimation of GLM+LIN.

Table 12. GLM+LIN Chromatogram Condition

| | |
|---|---|
| **Stationary Phase** | Hibar ODS $C1_{8,}$ 250 mm*4.6 mm |
| **Mobile Phase** | Methanol: Acetonitrile: Water (35:35:30 v/v/v) |
| **Detection wavelength** | 245 nm |
| **Flow rate** | 1 ml/minute |
| **Run Time** | 15 minutes |
| **Retention Time** | GLM: 3.198 min, LIN: 7.401 min |

## ➢ SYSTEM SUITABILITY PARAMETERS

Solution of GLM+LIN (2+5 µg.ml$^{-1}$) was injected 3 times for determination of System suitability parameters which includes Retention time ($R_t$), Tailing factor ($T_f$), Resolution ($R_s$) and number of theoretical plates.

Table 13. RETENTION TIME

| **RETENTION TIME** | | |
|---|---|---|
| | GLM | LIN |
| | 3.198 | 7.401 |
| | 3.199 | 7.403 |
| | 7.402 | 7.402 |
| Mean | 3.20 | 7.40 |
| SD | 0.00 | 0.00 |
| RSD | 0.05 | 0.01 |

Table 14. Tailing Factor

| **Tailing Factor** | | |
|---|---|---|
| | GLM | LIN |
| | 1.05 | 1.12 |
| | 1.07 | 1.15 |
| | 1.07 | 1.14 |
| Mean | 1.06 | 1.14 |
| SD | 0.01 | 0.02 |
| RSD | 1.09 | 1.34 |

Table 15. Theoretical Plates

| **Theoretical Plates** | | |
|---|---|---|
| | GLM | LIN |
| | 9857 | 12475 |
| | 9721 | 12314 |
| | 9877 | 12296 |
| Mean | 9818.33 | 12361.67 |
| SD | 84.88 | 98.56 |
| RSD | 0.86 | 0.80 |

Table 16. Resolution

| Resolution | |
|---|---|
| | 6.154 |
| | 6.155 |
| | 6.149 |
| Mean | 6.15 |
| SD | 0.00 |
| RSD | 0.05 |

Table 17. System Suitability Parameter

| | **GLM** | **LIN** |
|---|---|---|
| **Retention Time** | 3.20 ± 0.00 (0.05) | 7.40 ± 0.00 (0.01) |
| **Tailing Factor** | 1.06 ± 0.01 (1.09) | 1.14 ± 0.02 (1.34) |
| **Resolution** | 6.15 ± 0.00 (0.05) | |
| **Theoretical Plate** | 9818.33 ± 84.88 (0.86) | 12361.67 ± 98.56 (0.80) |

➢ **ANALYTICAL METHOD VALIDATION**

**1. Linearity and Range**

- Preparation of Solution for linearity studies:

For the purpose of linearity, Weigh accurately about 20 mg of GLM and 50 mg of LIN and transfer into a 100 ml volumetric flask. Make up the volume of the flask to the mark with Methanol. (200 µg/ml GLM + 500 µg/ml of LIN). Dilute 1 ml of previous solution to 10 ml with diluent to give stock solution (20 µg/ml GLM + 50 µg/ml LIN). Various aliquots from this stock solution were transferred to another 10 ml volumetric flask and volume was raised to the mark with mobile phase to give final solutions.

Table 18. Preparation of solution for linearity studies

| **Concentration of Stock solution** | **Volume taken (µl)** | **Dilution volume with methanol** | **Final concentration (GLM+LIN)** |
|---|---|---|---|
| 20 µg/ml of GLM and 50 µg/ml of LIN | 0.5 ml | Up to 10 ml | 1+2.5 µg/ml |
| | 1.0 ml | Up to 10 ml | 2+5 µg/ml |
| | 1.5 ml | Up to 10 ml | 3+7.5 µg/ml |
| | 2.0 ml | Up to 10 ml | 4+10 µg/ml |
| | 2.5 ml | Up to 10 ml | 5+12.5 µg/ml |

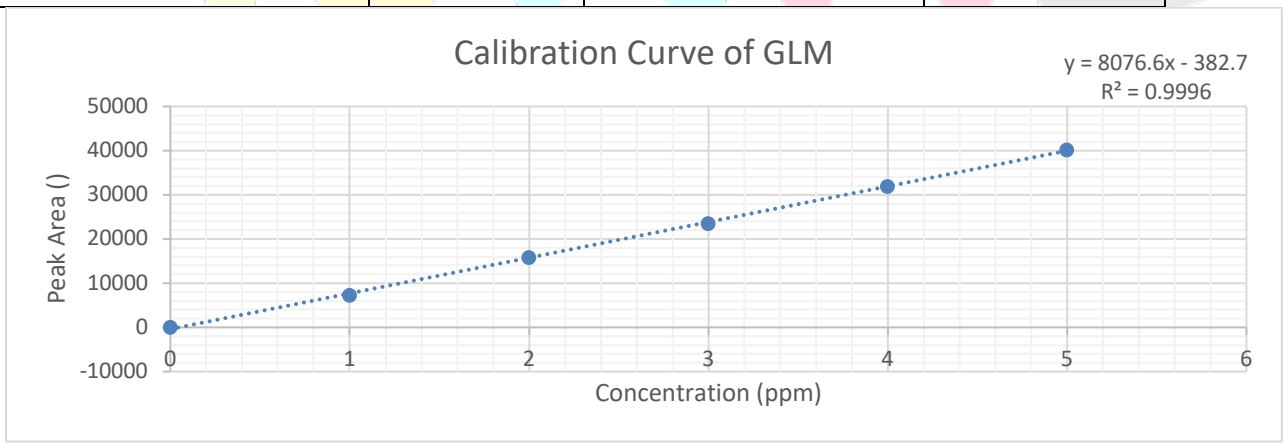


**Figure 11. Calibration Curve of GLM**

Table 19. linearity data of GLM

| Sr. No. | Concentration (µg/ml) | Mean area (µV. s) | ± SD (n=5) | RSD |
|---|---|---|---|---|
| 0 | 0 | | | |
| 1 | 1 | 7299.4 | 102.63 | 1.41 |
| 2 | 2 | 15830.6 | 180.68 | 1.14 |
| 3 | 3 | 23600 | 196.46 | 1.14 |
| 4 | 4 | 31902.4 | 252.71 | 0.79 |
| 5 | 5 | 40220.6 | 238.41 | 0.59 |

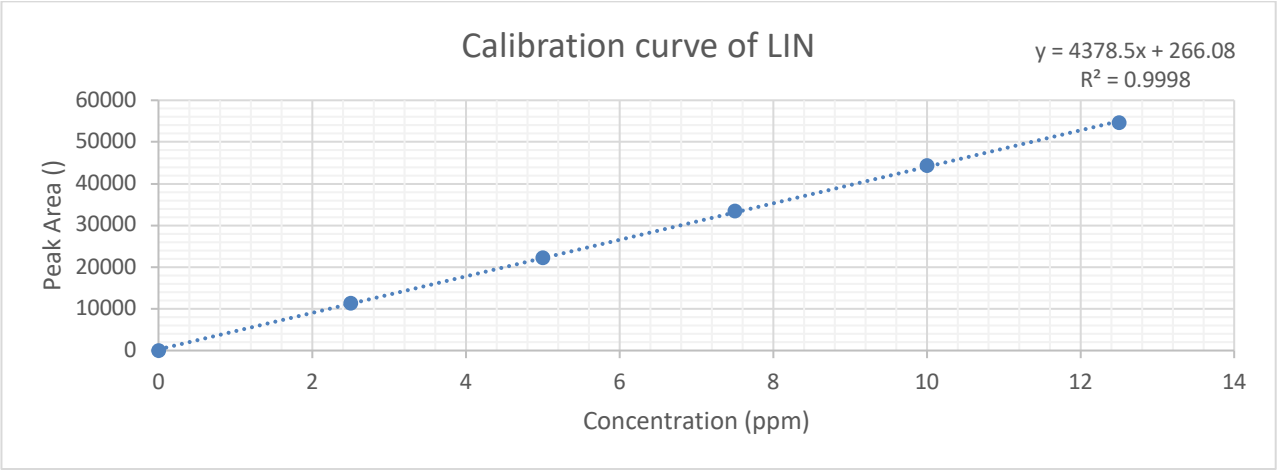


**Figure 12. Calibration Curve of LIN**

Table 20. linearity data of LIN

| Sr. No. | Concentration (µg/ml) | Mean area (µV. s) | ± SD (n=5) | RSD |
|---|---|---|---|---|
| 0 | 0 | | | |
| 1 | 2.5 | 11319.8 | 137.53 | 1.21 |
| 2 | 5 | 22173 | 234.41 | 1.06 |
| 3 | 7.5 | 33372.6 | 266.66 | 0.80 |
| 4 | 10 | 44371.4 | 303.14 | 0.68 |
| 5 | 12.5 | 54552.4 | 253.03 | 0.46 |

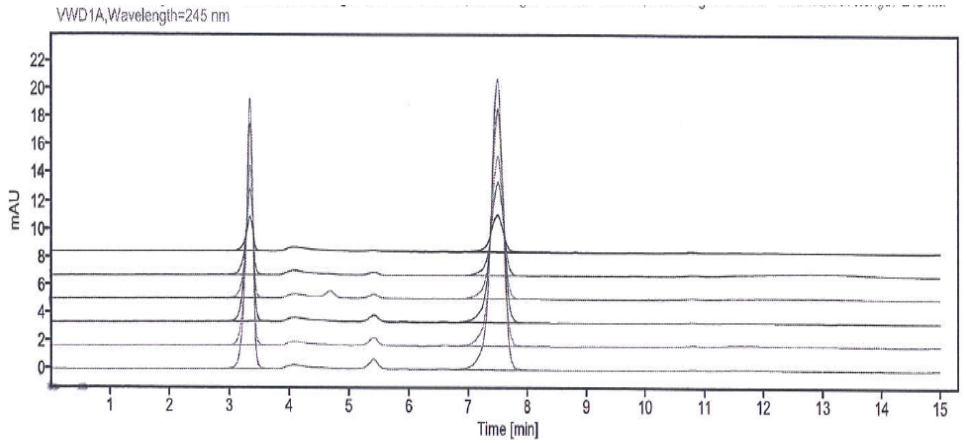


**Figure 13. Chromatogram for linearity Overlain GLM+LIN**

**2. Repeatability**

- Prepared standard working solution of mixtures having concentration of GLM (1 to 5 µg/ml) and LIN (2.5 to 12.5 µg/mL) were injected at volume of 20 µL into column by employing optimized chromatographic conditions. Each standard mixture was injected 5 time and peak area was monitored. Each concentration was monitored for repeatability by RSD.

Table 21. Repeatability data of GLM

| **Concentration (µg/ml)** | | | | | |
|---|---|---|---|---|---|
| **Sr. No.** | **1** | **2** | **3** | **4** | **5** |
| 1 | 7232 | 15786 | 23534 | 31824 | 40253 |
| 2 | 7194 | 15624 | 23318 | 31524 | 39863 |
| 3 | 7426 | 15942 | 23725 | 32143 | 40235 |
| 4 | 7254 | 15722 | 23589 | 31899 | 40534 |
| 5 | 7391 | 16079 | 23834 | 32122 | 40218 |
| Mean | 7299.4 | 15830.6 | 23600 | 31902.4 | 40220.6 |
| ± SD | 102.63 | 180.68 | 196.46 | 252.71 | 238.41 |
| RSD | 1.41 | 1.14 | 0.83 | 0.79 | 0.59 |

Table 22. Repeatability data of LIN

| **Concentration(µg/ml)** | | | | | |
|---|---|---|---|---|---|
| **Sr. No.** | **2.5** | **5** | **7.5** | **10** | **12.5** |
| 1 | 11253 | 22108 | 33214 | 44234 | 54746 |
| 2 | 11192 | 21813 | 33075 | 43978 | 54385 |
| 3 | 11524 | 22363 | 33532 | 44627 | 54862 |
| 4 | 11234 | 22185 | 33296 | 44296 | 54523 |
| 5 | 11396 | 22396 | 33746 | 44722 | 54246 |
| Mean | 11319.8 | 22173 | 33372.6 | 44371.4 | 54552.4 |
| ± SD | 137.53 | 234.41 | 266.66 | 303.14 | 253.03 |
| RSD | 1.21 | 1.06 | 0.80 | 0.68 | 0.46 |

### 3. Limit of detection (LOD) and Limit of quantification (LOQ)

- LOD and LOQ were determined by Statistical method by utilization of repeatability data.

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

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

Where, σ = Standard deviation of intercept

S = mean of slope

Table 23. LOD of GLM and LIN

| **GLM** | **LIN** |
|---|---|
| LOD =3.3(σ / S)<br>LOD = 3.3(86.96902454/ 8076.62)<br>=0.03553439198 µg/ml | LOD =3.3(σ / S)<br>LOD = 3.3(112.5866592/4378.46)<br>=0.08485540016 µg/ml |

Table 24. LOQ of GLM and LIN

| **GLM** | **LIN** |
|---|---|
| LOQ =10(σ / S)<br>LOQ =10(86.96902454/ 8076.62)<br>=0.1076799757 µg/ml | LOQ =10(σ / S)<br>LOQ =10(112.5866592/4378.46)<br>=0.2571375762 µg/ml |

### 4. Accuracy

- Accuracy of the analytical method has been performed by spiking of Placebo with the standard. Spiking of the sample was performed at 50, 100 and 150 % of the target concentration.

**Preparation of stock solution (Standard):** Procedure as mentioned in linearity.

Table 25. Preparation of solution for Accuracy studies

| **Amount of Placebo** | 104 mg | 104 mg | 104 mg | 104 mg |
|---|---|---|---|---|
| **Concertation of stock solution (Standard)** | 20 µg/ml of GLM and 50 µg/ml of LIN | | | |
| **Volume of Standard taken in ml from stock solution of standard** | - | 0.5 ml | 1.0 ml | 1.5 ml |
| **Diluent (Up to 10 ml)** | Mobile phase | Mobile phase | Mobile phase | Mobile phase |
| **Concertation corresponding to standard solution taken (GLM+LIN)** | - | 1+2.5 µg/ml | 2+5 µg/ml | 3+7.5 µg/ml |
| **Identification** | Unspiked | 50 % Spiked | 100 % Spiked | 150 % Spiked |

- Each solution was chromatographed for 3 time and area obtained was subjected to statistical analysis to get idea about mean % recovery.
- **Composition of placebo**: Directly Compressible Lactose (100 mg), Talc (2 mg) and Magnesium stearate (2 mg).

Table 26. Accuracy data of GLM

| **Level of spiking** | **Quantify of placebo(mg)** | **Amount of drug added (µg/ml)** | **Amount of drug recovered (µg/ml)** | **% Recovery** | **% Mean ± SD** |
|---|---|---|---|---|---|
| 50% | 104 | 1 | 1.01 | 101.00 | 99.33±1.53 |
| | | 1 | 0.98 | 98.00 | |
| | | 1 | 0.99 | 99.00 | |
| 100% | 104 | 2 | 1.98 | 99.00 | 99.67±0.76 |
| | | 2 | 2.01 | 100.50 | |
| | | 2 | 1.99 | 99.50 | |
| 150% | 104 | 3 | 2.99 | 99.67 | 100.22±0.51 |
| | | 3 | 3.02 | 100.67 | |
| | | 3 | 3.01 | 100.33 | |

Table 27. Accuracy data of LIN

| **Level of spiking** | **Quantify of placebo(mg)** | **Amount of drug added (µg/ml)** | **Amount of drug recovered (µg/ml)** | **% Recovery** | **% Mean ± SD** |
|---|---|---|---|---|---|
| 50% | 104 | 2.5 | 2.48 | 99.20 | 99.20± 1.20 |
| | | 2.5 | 2.45 | 98.00 | |
| | | 2.5 | 2.51 | 100.40 | |
| 100% | 104 | 5 | 5.02 | 100.40 | 99.47± 0.83 |
| | | 5 | 4.96 | 99.20 | |
| | | 5 | 4.94 | 98.80 | |
| 150% | 104 | 7.5 | 7.52 | 100.27 | 99.78±0.77 |
| | | 7.5 | 7.48 | 99.73 | |
| | | 7.5 | 7.45 | 99.33 | |

**5. Inter-day and Intra-day Precision**

- Method precision was determined by performing intraday and interday precision.
- Mixture that represents overall range (GLM+LIN = 1+2.5, 3+7.5 and 5+12.5 µg/mL) were analysed on same day at different time interval for intraday precision.
- Mixture that represents overall range (GLM+LIN = 1+2.5, 3+7.5 and 5+12.5 µg/mL) were analysed on different day at different time interval for inter-day precision.

Table 28. Intra- day Precision data for GLM and LIN

| **GLM** | | | **LIN** | | |
|---|---|---|---|---|---|
| Concentration **(µg/ml)** | **Intraday Mean± SD** | **RSD** | **Concentration (µg/ml)** | **Intraday Mean± SD** | **RSD** |
| 1 | 7222.00±98.38 | 1.36 | 2.5 | 11245.33±125.68 | 1.12 |
| 3 | 23569.00±264.24 | 1.12 | 7.5 | 33278.67±288.49 | 0.87 |
| 5 | 40227.00±342.74 | 0.85 | 12.5 | 54666.33±370.97 | 0.68 |

Table 29. Inter-day Precision data for GLM and LIN

| GLM | | | LIN | | |
|---|---|---|---|---|---|
| Concentration (µg/ml) | **Inter-day Mean± SD** | **RSD** | **Concentration (µg/ml)** | **Inter-day Mean± SD** | **RSD** |
| 1 | 7203.67±106.37 | 1.48 | 2.5 | 11239.33±150.96 | 1.34 |
| 3 | 23579.67±315.98 | 1.34 | 7.5 | 33274.67±313.43 | 0.94 |
| 5 | 40210.67±435.05 | 1.08 | 12.5 | 54681.00±477.83 | 0.87 |

- (n= 3 determination)

**6. Robustness**

- Following parameters were altered one by one for determination of robustness of the method and their effect was observed by comparing with the standard preparation.
  - I. Mobile phase flowrate (± 0.1 mL/min), optimized flowrate was 1.0 mL/min.
  - II. Mobile phase composition (± 2 mL), in optimized ratio
- 3 determinations of GLM+LIN = 2+5 µg/mL for each alteration was carried out and RSD was measured.

Table 30. Robustness for GLM and LIN

| **Condition** | | **Area** | **Mean (n=3)** | **SD (n=3)** | **%RSD** |
|---|---|---|---|---|---|
| **Glimepiride** | | | | | |
| **Flow rate (mL/min)** | **0.9** | 15632 | 15767.33 | 127.03 | **0.81** |
| | **1.0** | 15786 | | | |
| | **1.1** | 15884 | | | |
| **Mobile Phase Composition** | **37-33-30** | 15651 | 15808.25 | 118.87 | **0.75** |
| | **35-35-30** | 15786 | | | |
| | **35-37-28** | 15875 | | | |
| | **33-35-32** | 15875 | | | |
| **Linagliptin** | | | | | |
| **Flow rate (mL/min)** | **0.9** | 21874 | 22112.33 | 240.53 | **1.09** |
| | **1.0** | 22108 | | | |
| | **1.1** | 22355 | | | |
| **Mobile Phase Composition** | **37-33-30** | 21874 | 22152.75 | 217.33 | **0.98** |
| | **35-35-30** | 22108 | | | |
| | **35-37-28** | 22245 | | | |
| | **33-35-32** | 22384 | | | |

**7. Assay**

Table 31. Preparation of solution for Assay studies

| **Sample Preparation** |
|---|
| Composition of placebo: Directly Compressible Lactose (100 mg), Talc (2 mg) and Magnesium stearate (2 mg). Weigh accurately about 20 mg of GLM and 50 mg of LIN and transfer into a 100 ml volumetric flask. Make up the volume of the flask to the mark with Methanol. (200 µg/ml GLM + 500 µg/ml of LIN). |

| | |
|---|---|
| (Sonicate the solution for 10 minutes and filter the same from 0.45 µm Whatman filter paper.)<br>Dilute 1 ml of filtrate to 10 ml with diluent to give stock solution (20 µg/ml GLM + 50 µg/ml LIN). | |
| **Test Solution:** | Withdraw 1.0 ml from above filtrate in 10 mL volumetric flask; make up the volume with mobile phase, which contain GLM+LIN = 2+5 µg/ml |

Table 32. Determination of GLM and LIN from synthetic mixture

| Drug | Amount taken **(µg/ml)** | Amount found **(µg/ml)** | % Assay |
|---|---|---|---|
| GLM | 2 | 1.99±0.02 | 99.67±0.76 |
| LIN | 5 | 4.97±0.04 | 99.47±0.83 |

(n = 3 determinations)

## ➢ SUMMERY AND CONCLUSION

Table 33. Optimized Chromatographic Condition

| | |
|---|---|
| **Stationary Phase** | Hibar ODS $C_{18}$, 250 mm*4.6 mm |
| **Mobile Phase** | Methanol: Acetonitrile: Water (35:35:30 v/v/v) |
| **Detection wavelength** | 245 nm |
| **Flow rate** | 1 ml/minute |
| **Run Time** | 15 minutes |
| **Retention Time** | GLM: 3.198 min, LIN: 7.401 min |

Table 34. Summery and conclusion of developed RP-HPLC method

| **Parameter** | **limit** | **Result** | | **Conclusion** |
|---|---|---|---|---|
| | | **GLM** | **LIN** | |
| **Linearity and Range** | RSD > 0.995 | (0.996<br>1-5 µg/ml) | 0.998<br>(2.5 – 12.5 µg/ml) | Method was linear |
| **Repeatability** | RSD< 2 | 0.59-1.14 | 0.46-1.21 | Method was repeatable |
| **LOD** | - | 0.03553439198 µg/ml | 0.08485540016 µg/ml | - |
| **LOQ** | - | 0.1076799757 µg/ml | 0.08485540016 µg/ml | - |
| **Intraday Precision** | RSD < 2 | 0.85-1.36 | 0.68-1.12 | Method was precise |
| **Inter-day Precision** | RSD < 2 | 1.08-1.48 | 0.87-1.34 | Method was precise |
| **Accuracy (% Recovery)** | 98 - 102 % | 99.20-99.98% | 99.20-99.78% | Method was accurate |
| **Robustness** | RSD < 2 | 0.81-0.75 | 1.09-0.98 | Method was robust |
| **Assay** | 98 - 102 % | 99.67% | 99.48% | Pass |

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