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# Simple and Accurate Simultaneous HPLC Method Development and Validation for an Anti-Diabetic Multi-Component Dosage Form

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## Abstract
The complexity of the multicomponent dosage forms is that multiple entities and excipients poses a considerable challenge to the analytical chemist during the development of an assay procedure. One of such formulation is of an anti-diabetic medicine comprising metformin hydrochloride (MET), gliclazide (GLZ) and pioglitazine (PIO). Many methods have been reported in the literature for the estimation of MET, GLZ and PIO individually and with other components by chromatographic methods. However, there is no simple reverse phase high performance liquid chromatography (RP-HPLC) method has been reported for the simultaneous estimation of MET, GLZ and PIO. Thus, we have attempted to develop and validate a simple HPLC method for MET, GLZ and PIO. The RP-HPLC method was successfully developed and validated according to ICH guidelines. The proposed method is simple, accurate, precise, and the statistical analysis proved that the method is reproducible and efficient for the simultaneous estimation of MET, GLZ and PIO in tablet dosage form without any interference from the excipients.

**Keywords**
Gliclazide, Metformin hydrochloride, Pioglitazine, RP-HPLC.

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## INTRODUCTION
The complexity of the multicomponent dosage forms is that multiple entities and excipients poses a considerable challenge to the analytical chemist during the development of an assay procedure. [1]
Earlier, colorimetric and spectrophotometric methods were employed for drug analysis as they were economical and easily available. However, the use of these methods have reduced extensively due to the lack of specificity, sensitivity and accuracy. For the simultaneous estimation of the drugs present in multicomponent dosage forms, HPLC method is considered to be most suitable since this is a powerful and rugged method. [2]
One of the most mutli-component dosage form is for diabetic disease. Metformin hydrochloride (MET), an insulin-sensitizing biguanide used to treat type-2 diabetes, has been found to be as effective as insulin

or sulfonylureas when used as monotherapy.[3-7] For many patients with type 2 diabetes, monotherapy with an oral anti-diabetic agent is not sufficient to reach target glycemic goals and multiple drugs may be necessary to achieve adequate control.[8] In such cases a combination of metformin hydrochloride and one of the sulfonylureas are used.[9] The fixed dose combination of gliclazide (80 mg) and metformin hydrochloride (500 mg) once or twice daily with meals to a maximum of 4 tablets per day (depending upon the glycemic control) showed significant efficacy in improving the glycemic control in type 2 diabetics.[10]

Gliclazide (GLZ) is a second generation sulphonylurea which that is widely used in the treatment of patients with type 2 diabetes because it has efficacy similar to other sulphonylureas but a lower risk of hypoglycemia. [11,12]

Pioglitazone hydrochloride (PIO) is an oral anti-hyperglycemic agent which acts primarily by decreasing insulin resistance. It is used in the treatment of type-II diabetes mellitus. [13]

Many methods have been reported in the literature for the estimation of MET, GLZ and PIO individually and with other components. However, there is no simple method has been reported for the simultaneous estimation of metformin hydrochloride, pioglitazine with gliclazide. Thus, we have attempted to develop and validate a simple HPLC method for MET, GLZ and PIO. [14-23]

## MATERIAL AND METHODS

### Chemicals

MET, GLZ and PIO API procured as gift sample from Ipca Laboratories Limited Mumbai. The marketed formulation (Glycinorm Total) with fixed dose combination tablets of the three drugs, (MET, GLZ, PIO) were purchased from retail pharmacy in Mumbai (Maharashtra, India). Other chemicals and reagents of analytical grade were purchased from Merck (India).

### Instrumentation

The study was carried out on Jasco Autosampler HPLC (AS-5040, PU- 2080, UV-2075). The column used was Hypersil ODS C18 (25 * 4.6 mm, 5µm).

### Development of HPLC method

### Selection of mobile phase

Various solvent systems were tried for the development of suitable HPLC methods for the analysis of MET, GLZ and PIO using API and extracted formulation sample. The suitability of the solvent system was decided on the basis of the sensitivity of the assay, retention time, tailing factor and selection of flow rate.

### Selection of flow rate

To determine the effect of flow rate, the programmed controller was set at different flow rates 0.5ml/min, 0.7 ml/min, 1.0 ml/min, 1.1 ml/min, 1.2 ml/min and 1.5 ml/min operate were performed at each flow rate.

### Selection of analytical wavelength

An appropriate dilution of standard stock solution with mobile phase, various concentrations of MET, GLZ and PIO were accurately prepared. The solutions were scanned between the wavelength range 400-200 nm using the UV spectrophotometer.

### Preparation of stock solution of API

MET, GLZ and PIO were weighed in ratio 33:4:1 and transferred in single vial. The stock solution of was prepared by dissolving the Api in methanol. Further dilutions were made to obtain 66, 8 and 2 µg/ml solutions of MET, GLZ and PIO respectively.

### Preparation of sample solution

Twenty tablets were weighed and crushed. The powdered drug equivalent to 500 mg of MET, GLZ of 60mg, PIO of 15mg and transferred in a single vial. 1ml of methanol was used to dissolve the powder completely.

### Method validation: [24, 25]

### Linearity and range

Under the optimized conditions, a calibration curve was prepared for MET, GLZ and PIO. Standard mixtures of different concentration were prepared for determining the working range. The corrected peaks were used to construct the calibration graph. Linearity range, regression equation, correlation coefficient, were evaluated.

### Accuracy

In order to examine the accuracy of the method standard addition method was carried out. In this method, three different concentration of MET, GLZ and PIO were added to a constant known concentration of the composite solution. Each solution was injected and the amounts determined were compared to theoretical.

### Precision

The precision of the method was tested by performing intra-day and inter-day studies. For intra-day studies, a triplicate of prepared samples was analyzed on the same day. For inter-day validation, concentrations were determined on a separate day. The % RSD values obtained from peak area for MET, GLZ and PIO were observed.

### Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were separately determined based on the signal to noise ratio. For LOD the S/N ratio is 3:1 and for LOQ the ratio is 10:1.

**System suitability parameter**

The system suitability test was carried out on freshly prepared standard stock solution of MET, GLZ and PIO. Parameters such as resolution, peak tailing HETP were studied to evaluate the suitability of the system.

**Robustness**

Robustness of the method was tested by small but deliberate variations of flow rate, mobile phase composition and temperature. Effects of variation in the flow rate and wavelength were studied at two different concentrations.

**Estimation of content of drugs in tablet formulation**

The content of all three drugs was estimated in tablet formulation (Glycinorm Total) by the developed and validated HPLC method.

## RESULTS AND DISCUSSION

**Method development**

To achieve the desired separation (resolution: Rs ≥ 1.5) and retention time value (Rt: 0.2 - 0.8) different mobile phases of various combinations were used. The mobile phase containing methanol: water (70:30 v/v) with the flow rate of 1 ml/min was selected as it gave well resolved peaks of standard MET, GLZ and PIO. The optimum wavelength 229 nm selected for detection and quantitation. The Rt for standard MET, GLZ and PIO was found to be 10.025, 3.133 and 5.817 respectively. (**Figure- 1**).

**Method validation**

**Linearity**

The calibration curves were found be linear for the concentration range of 66-330 µg/ml for MET, 8-40 µg/ml for GLZ and 2-10 µg/ml for PIO. The standard working curve equation for MET was found to be y = 89838x -101064 with correlation coefficient value $r^2$ = 0.9953, for GLZ standard working curve equation was found to be y = 5320.7x +1E+06 with correlation coefficient value $r^2$ = 0.9975 and for PIO it was y=23093x+128750 and $r^2$=0.9987. The results of linearity are given in **Table-1** and **Figure- 2, 3, and 4**.

**Recovery studies**

The mean % recovery at 80, 100, 120 % of the test concentration along with its statistical validation for MET, GLZ and PIO are given in **Table-2.1, 2.2, 2.3**. It was found that the method was accurate as the percent recovery was in the range of 99 % for MET, 98% for GLZ and 100% for PIO.

**Precision**

The repeatability of sample application and measurement of peak area were expressed in terms of % RSD and was found to be less than 2.0%. The % RSD of intra-day precision was found to be 0.97 and 1.56, 1.76 and 0.42, 0.05 and 1.29 for MET, GLZ and PIO respectively and % RSD of intermediate (inter-day) precision was found to be 0.66 and 1.17, 0.81 and 1.28, 1.93 and 0.98 for MET, GLZ and PIO respectively. The results of precision studies are shown in **Table-3.1, 3.2, 3.3 and 4.1, 4.2, 4.3**.

**Limit of detection and Limit of quantitation**

It was calculated by standard deviation of the response and the slope of calibration curve. LOD and LOQ of the method were calculated and found to be 0.15µg/ml and0.45µg/ml of MET, 0.12 µg/ml and 0.33 µg/ml of GLZ and 0.06µg/ml and 0.16 µg/ml of PIO.

**System Suitability**

The method developed for the simultaneous estimation of MET, GLZ and PIO was found to be suitable as the resolution factor and symmetry were within the acceptable range. The resolution (Rs) was 6.135, 1.785 and symmetry was 0.809, 0.967 and 1.023 for GLZ, PIO and MET respectively. The tailing factor was also in limits, the data is shown in **Table-5**.

**Robustness**

It was measured by multiple injections of a homogenous sample containing MET, GLZ and PIO in concentration of 132 and 264 µg/ml of MET, 16 and 32 µg/ml of GLZ and 16 and 32 µg/ml of PIO that indicates the performance of the HPLC instrument under chromatographic conditions by changing flow rate 0.8ml/min and 1.2 ml/min and by changing Wavelength i.e 226nm and 232nm nm. The method was found to be robust in the range of deliberate changes made. (**Table-6.1, 6.2, 6.3, 6.4, 6.5, 6.6**)

**Estimation of content of drugs in tablet**

The development and validation of HPLC method estimated that the content of MET, GLZ and PIO in tablet formulation was 99.68%, 100.30 % and 98.00 % respectively. The detailed data is mentioned in **Table-7**.

**Table-1: Linearity data of MET, GLZ and PIO**

| Linearity MET | | Linearity GLZ | | Linearity PIO | |
|---|---|---|---|---|---|
| Concentration (µg/ml) | Area Average | Concentration (µg/ml) | Area Average | Concentration (µg/ml) | Area Average |
| 66 | 6563341 | 8 | 1255545 | 2 | 176823 |
| 132 | 11024426 | 16 | 1304830 | 4 | 221546 |
| 198 | 17547338 | 24 | 1348685 | 6 | 263589 |
| 264 | 23154003 | 32 | 1385478 | 8 | 312088 |
| 330 | 30145042 | 40 | 1428048 | 10 | 362477 |

**Table-2.1: Recovery data of MET**

| Level (%) | Drug Conc (mg) | Amt added (mg) | Total Amt (mg) | Amt recovered (mg) | % Recovery |
|---|---|---|---|---|---|
| 80% | 500 | 400 | 900 | 898.3 | 99.7 |
| 100% | 500 | 500 | 1000 | 999.6 | 99.96 |
| 120% | 500 | 600 | 1100 | 1098 | 99.81 |

a) Conc= Concentration, Amt= Amount

**Table- 2.2: Recovery data of GLZ**

| Level (%) | Drug Conc (mg) | Amt added (mg) | Total Amt (mg) | Amt recovered (mg) | % Recovery |
|---|---|---|---|---|---|
| 80% | 60 | 48 | 108 | 106.0 | 98.14 |
| 100% | 60 | 60 | 120 | 119.2 | 99.33 |
| 120% | 60 | 72 | 132 | 130.0 | 98.48 |

a) Conc= Concentration, Amt= Amount

**Table- 2.3: Recovery data of PIO**

| Level (%) | Drug Conc (mg) | Amt added (mg) | Total Amt (mg) | Amt recovered (mg) | % Recovery |
|---|---|---|---|---|---|
| 80% | 15 | 12 | 27 | 27.65 | 102.00 |
| 100% | 15 | 15 | 30 | 30.12 | 100.40 |
| 120% | 15 | 18 | 33 | 33.04 | 100.12 |

a) Conc= Concentration, Amt= Amount

**Table- 3.1: Precision study (intra- day) of MET**

| Conc µg/ml | Area | AVG | SD | %RSD |
|---|---|---|---|---|
| 132 | 11806552 | | | |
| 132 | 11984775 | 11937935 | 115332 | 0.97 |
| 132 | 12022478 | | | |
| 264 | 24554023 | | | |
| 264 | 25302254 | 24989620 | 388976 | 1.56 |
| 264 | 25112584 | | | |

a) Conc= Concentration
b) AVG= average, SD= Standard deviation, RSD= Relative standard deviation

**Table- 3.2: Precision study (intra- day) of GLZ**

| Conc µg/ml | Area | AVG | SD | %RSD |
|---|---|---|---|---|
| 16 | 1246881 | | | |
| 16 | 1250244 | 1236006 | 21814 | 1.76 |
| 16 | 1210892 | | | |
| 32 | 1361128 | | | |
| 32 | 1352411 | 1354663 | 5684 | 0.42 |
| 32 | 1350451 | | | |

a) Conc= Concentration
b) AVG= average, SD= Standard deviation, RSD= Relative standard deviation

**Table-3.3: Precision study (intra- day) of PIO**

| Conc µg/ml | Area | AVG | SD | %RSD |
|---|---|---|---|---|
| 4 | 210153 | | | |
| 4 | 209963 | 210090.7 | 111 | 0.05 |
| 4 | 210156 | | | |
| 8 | 297684 | | | |
| 8 | 305410 | 301747.0 | 3879 | 1.29 |
| 8 | 302147 | | | |

a) Conc= Concentration
b) AVG= average, SD= Standard deviation, RSD= Relative standard deviation

**Table-4.1: Precision study (inter-day) of MET**

| Conc µg/ml | Area | AVG | SD | %RSD |
|---|---|---|---|---|
| 132 | 11901552 | | | |
| 132 | 11884775 | 11938068 | 78228.5 | 0.66 |
| 132 | 12027878 | | | |
| 264 | 24714023 | | | |
| 264 | 24162254 | 24396287 | 285248.8 | 1.17 |
| 264 | 24312584 | | | |

a) Conc= Concentration
b) AVG= average, SD= Standard deviation, RSD= Relative standard deviation

**Table-4.2: Precision study (inter-day) of GLZ**

| Conc µg/ml | Area | AVG | SD | %RSD |
|---|---|---|---|---|
| 16 | 1256881 | | | |
| 16 | 1288524 | 1282099 | 22698 | 1.77 |
| 16 | 1300892 | | | |
| 32 | 1360128 | | | |
| 32 | 1362411 | 1357663 | 6350 | 0.47 |
| 32 | 1350451 | | | |

a) Conc= Concentration
b) AVG= average, SD= Standard deviation, RSD= Relative standard deviation

**Table-4.3: Precision study (inter-day) of PIO**

| Conc µg/ml | Area | AVG | SD | %RSD |
|---|---|---|---|---|
| 4 | 220153 | | | |
| 4 | 219963 | 221091 | 1791 | 0.81 |
| 4 | 223156 | | | |
| 8 | 302684 | | | |
| 8 | 295410 | 299747 | 3834 | 1.28 |
| 8 | 301147 | | | |

a) Conc= Concentration
b) AVG= average, SD= Standard deviation, RSD= Relative standard deviation

**Table-5: System suitability study**

| Std | Tailing | HETP | Resolution |
|---|---|---|---|
| MET | 0.806 | 2343 | 6.195 |
| GLZ | 1.073 | 2891 | Na |
| PIO | 0.960 | 3208 | 1.785 |

a) Std= Standard
b) HETP= Height Equivalent to the Theoretical Plate

**Table-6.1: Robustness study with change in flow rate of MET**

| Conc µg/ml | Area | AVG | SD | %RSD |
|---|---|---|---|---|
| 132 | 11756552 | | | |
| 132 | 12004775 | 11954602 | 178337 | 1.49 |
| 132 | 12102478 | | | |
| 264 | 24558023 | | | |
| 264 | 24851546 | 24841297 | 278291 | 1.12 |
| 264 | 25114321 | | | |

a) Conc= Concentration
b) AVG= average, SD= Standard deviation, RSD= Relative standard deviation

**Table- 6.2: Robustness study with change in flow rate of GLZ**

| Conc µg/ml | Area | AVG | SD | %RSD |
|---|---|---|---|---|
| 16 | 1246881 | | | |
| 16 | 1248524 | 1235432 | 21268 | 1.72 |
| 16 | 1210892 | | | |
| 32 | 1360128 | | | |
| 32 | 1352411 | 1354330 | 5116 | 0.38 |
| 32 | 1350451 | | | |

a) Conc= Concentration
b) AVG= average, SD= Standard deviation, RSD= Relative standard deviation

**Table-6.3: Robustness study with change in flow rate of PIO**

| Conc µg/ml | Area | AVG | SD | %RSD |
|---|---|---|---|---|
| 4 | 211863 | | | |
| 4 | 216663 | 216227 | 4164 | 1.93 |
| 4 | 220156 | | | |
| 8 | 292684 | | | |
| 8 | 296710 | 293513 | 2873 | 0.98 |
| 8 | 291147 | | | |

a) Conc= Concentration
b) AVG= average, SD= Standard deviation, RSD= Relative standard deviation

**Table-6.4: Robustness study with change in wavelength of MET**

| Conc µg/ml | Area | AVG | SD | %RSD |
|---|---|---|---|---|
| 132 | 11806552 | | | |
| 132 | 12084775 | 11971268 | 146010 | 1.22 |
| 132 | 12022478 | | | |
| 264 | 24554023 | | | |
| 264 | 24862254 | 24842954 | 279780 | 1.13 |
| 264 | 25112584 | | | |

a) Conc= Concentration
b) AVG= average, SD= Standard deviation, RSD= Relative standard deviation

**Table-6.5: Robustness study with change in wavelength of GLZ**

| Conc µg/ml | Area | AVG | SD | %RSD |
|---|---|---|---|---|
| 16 | 1282881 | | | |
| 16 | 1298524 | 1278099 | 23189 | 1.81 |
| 16 | 1252892 | | | |
| 32 | 1360127 | | | |
| 32 | 1372411 | 1361663 | 10068 | 0.74 |
| 32 | 1352451 | | | |

a) Conc= Concentration
b) AVG= average, SD= Standard deviation, RSD= Relative standard deviation

**Table-6.6: Robustness study with change in wavelength of PIO**

| Conc (µg/ml) | Area | AVG | SD | %RSD |
|---|---|---|---|---|
| 4 | 210153 | | | |
| 4 | 209963 | 211091 | 1791 | 0.85 |
| 4 | 213156 | | | |
| 8 | 292684 | | | |
| 8 | 295410 | 296414 | 4320 | 1.46 |
| 8 | 301147 | | | |

a) Conc= Concentration
b) AVG= average, SD= Standard deviation, RSD= Relative standard deviation

**Table-7: Assay Results of Tablet Dosage Form**

| Parameter | MET | PIO | GLZ |
|---|---|---|---|
| Label claim amount (mg) | 500 | 15 | 60 |
| Amount found (mg) | 498.4 | 14.7 | 60.2 |
| % Purity | 99.68 | 98.00 | 100.30 |

a) GL= GLZ, PI=PIO, M= MET

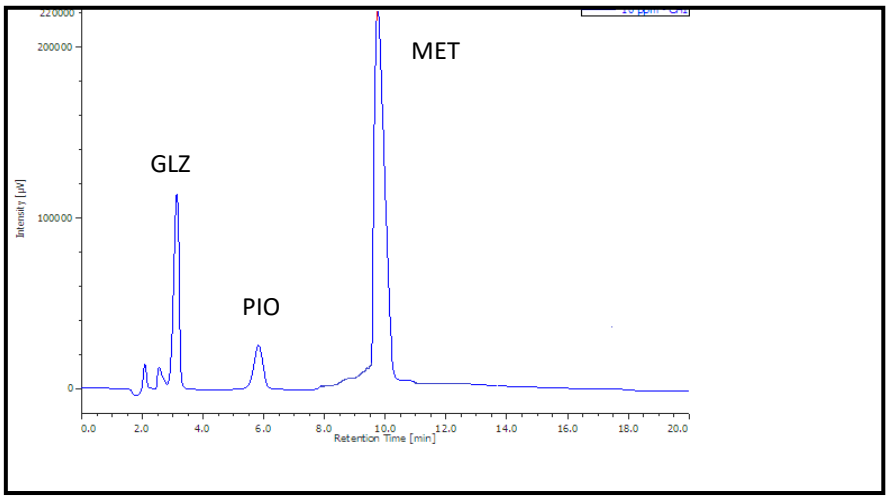



**Figure-1: HPLC Chromatogram with three resolved peaks of GLZ, PIO and MET**

**Figure-2: Linearity curve of standard MET**

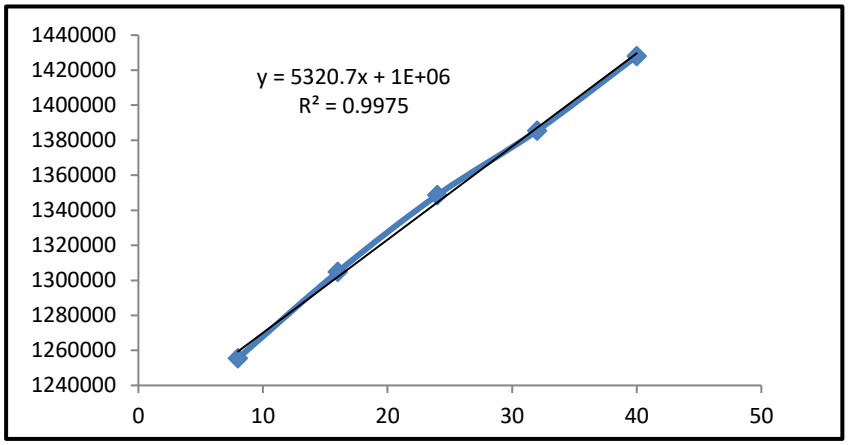
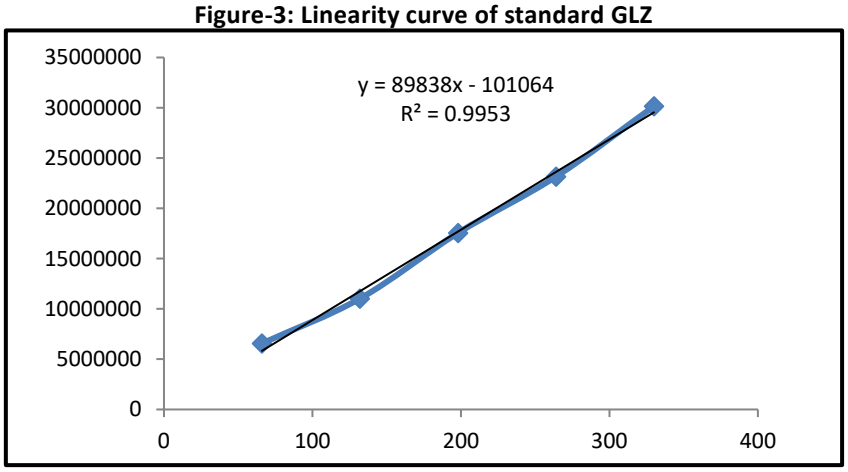



**Figure-3: Linearity curve of standard GLZ**



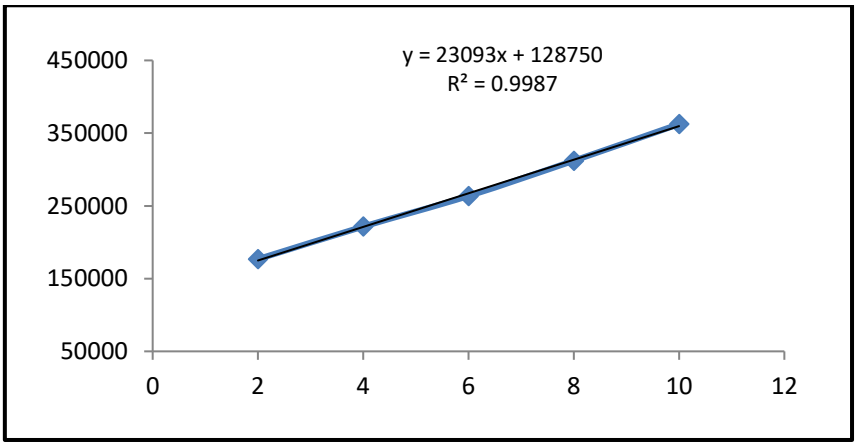




**Figure-4: Linearity curve of standard PIO**

## CONCLUSION

The RP-HPLC method was successfully developed and validated. The proposed method is simple, accurate, precise, and the statistical analysis proved that the method is reproducible and efficient for the simultaneous estimation of MET, GLZ and PIO in tablet dosage form without any interference from the excipients. From this study it was concluded that, the method can be employed for routine Quality Control analysis.

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