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METHOD DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ANALYSIS OF THREE COMPONENT TABLET DOSAGE FORM CONTAINING METFORMIN HYDROCHLORIDE, PIOGLITAZONE HYDROCHLORIDE AND GLIBENCLAMIDE

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ABSTRACT

Reverse phase High performance liquid chromatography method has developed and validated for the simultaneous analysis of glibenclamide, metformin hydrochloride and pioglitazone hydrochloride in tablet dosage form. The chromatographic separation of drugs was achieved at ambient temperature with isocratic mode by using C_{18} Hypersil BDS (150 x 4.6 mm, 5 µm) column with mobile phase containing acetonitrile: 0.1M potassium dihydrogen orthophosphate buffer (pH 4.5 adjusted with 10% sodium hydroxide solution) in the ratio 55: 45 v/v at flow rate 0.7 mL/min. The run time was 11 min and injection volume 20 µL. The eluent was monitored at 255 nm by using PDA detector. The method was validated as per ICH guidelines. The selected chromatographic conditions effectively separated metformin, pioglitazone and glibenclamide with retention time 2.1, 3.0 and 7.1 min respectively. The mean percentage recoveries were found in the range of 98-102 % which shows accuracy of the developed method. The linearity range for metformin, Pioglitazone and glibenclamide is found in the range of 50-400 µg/mL, 1-12 µg/mL and 0.5-4 µg/mL respectively. Limit of detection were 5, 0.1, 0.05 µg/mL and limit of quantification were 15, 0.3, 0.15 µg/mL for metformin, Pioglitazone and glibenclamide, respectively. The developed method was found to be specific, accurate, precise and economic.

Keywords: Metformin Hydrochloride, Pioglitazone Hydrochloride, Glibenclamide, RP-HPLC and Validation

1. INTRODUCTION

Metformin Hydrchloide:

Metformin Hydrochloride chemically known as 3-(diaminomethylidene)-1, 1-dimethylguanidene; hydrochloride. Its molecular formula is $C_4H_{12}ClN_5$ and molecular weight is 165.62 g/mol **[1]**. Metformin Hydrochloride has a melting point of 223-226°C **[4]**, it was soluble in water and has pKa 12.4 **[1]**. Metformin Hydrochloride sold under brand names Glucophage, Fortamet among others. It was used for the management of type II diabetes. Metformin was first approved in Canada in 1972, followed by 1995 in the USA. It was considered as an anti-hyperglycemic drug because it lowers glucose concentrations in type II diabetes without causing hypoglycemia. It is described as an *insulin sensitizer* leading to decrease in insulin resistance and a clinically significant reduction of plasma fasting insulin levels **[4]**.

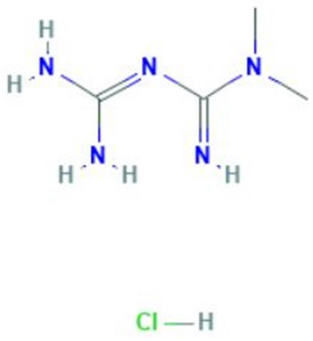


Figure 1: Metformin Hydrochloride

Pioglitazone Hydrchloride:

Pioglitazone Hydrochloride chemically known as 5-[[4-[2-(5-ethylpyridin-2-yl) ethyoxyl] phenyl] methyl]-1, 3-thiazolidine-2, 4-dione; hydrochloride. Its molecular formula is $C_{19}H_{21}ClN_2O_3S$ and molecular weight is 392.9 g/mol **[2]**. Pioglitazone Hydrochloride has a melting point of 193-194° C, it was insoluble in water and soluble in methanol. Pioglitazone Hydrochloride sold under brand name Pioglitazone-30 among others. It is an anti hyperglycemic used as an adjunct to diet, exercise, and other anti diabetic medications to manage type II diabetes **[5]**. It selectively stimulates the peroxisome proliferator activated receptor gamma (PPAR-γ) and to a lesser extent PPAR-α. Pioglitazone modulates the transcription of the insulin-sensitive genes involved in the control of glucose and lipid metabolism in the muscle, adipose tissue, and in the liver **[7]**.

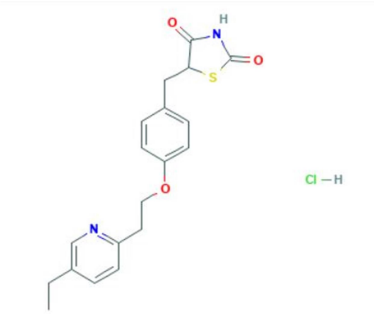


Figure 2: Pioglitazone Hydrochloride

Glibenclamide:

Glibenclamide chemically known as 5-chloro-N-[2-[4-(cyclohexyl carbamoyl

sulfamoyl) phenyl] ethyl]-2-methoxybenzamide. Its molecular formula is $C_{23}H_{28}ClN_3O_5S$ **[3]** and molecular weight is 494 g/mol **[6]**. Glibenclamide has a melting point of 169°C **[3]**, it was insoluble in water and soluble in methanol and aetonitrile mixture. Its pKa value is 6.8. Glibenclamide sold under brand name Afdiex among others. Glibenclamide shows its action on functional beta cells of pancreas and stimulate release of insulin thereby decreasing blood glucose level. After extended administration, hypoglycemic effects appear connected to extra pancreatic effects like increase peripheral sensitivity to the insulin, depletion in production of basal hepatic glucose, then later may result in changes in the events following to insulin binding or increase in insulin receptor number **[8]**.

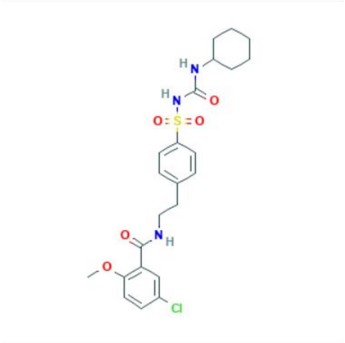


Figure 3: Glibenclamide

Literature review **[7, 10, 11, 12, 13]** reveals that there are only few methods available for the simultaneous analysis of Metformin, Pioglitazone and Glibenclamide. So my aim is to develop a specific, accurate, precise, economic and robust method for the simultaneous analysis of Metformin Hydrochloride, Pioglitazone Hydrochloride and Glibenclamide in tablet formulation and validate the developed as per International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines Q_2 (R1).

2. METHODOLOGY

2.1. Materials: Metformin, Pioglitazone and Glibenclamide were obtained from sigma Aldrich. NEW TRIGLUCORED FORTE containing Glibenclamide 5 mg, Metformin Hydrochloride 500 mg, Pioglitazone Hydrochloride 15 mg was procured from local market. Reagents and solvents such as Potassium dihydrogen orthophosphate, Methanol, Acetonitrile and HPLC Water of analytical grade were used.

2.2. Instrumentation and chromatographic conditions: The chromatographic separation was carried out on HPLC Shimadzu 2030C 3D plus with photo diode array detector, C_{18} Hypersil BDS [150×4.6, 5 µm] column with ambient temperature. The mobile phase consisting of Acetonitrile: 0.1 M Potassium dihydrogen orthophosphate (pH 4.5 was adjusted by using 10% Sodium Hydroxide Solution) in the ratio [55: 45] at flow rate 0.7 mL/min. Buffer was filtered through 0.45 µ membrane filter by using vaccum filtration apparatus. Mobile phase was

purged for 5 min for removal of any gas or air bubbled if present. Detection wavelength was set at 255 nm. The injection volume was set to be 20 µl.

2.3. Solvent or Diluent: Methanol and Acetonitrile were used in the ratio 50:50 v/v.

2.4. Preparation of Metformin(stock), Pioglitazone and Glibenclamide standard Solutions: Accurately weighed quantity of 10 mg of Metformin was transferred into a 10mL clean dry volumetric flask and added 3/4th volume of diluent, sonicated for 5 min and made up to the final volume with diluent. From the stock solution of Pioglitazone 1 mL of solution was pipette out and transferred into another 10 mL volumetric flask and made up to the final volume with diluent. From the stock solution of Glibenclamide 1 mL of solution was pipette out and transferred into another 10 mL volumetric flask and made up to the final volume with diluent. Methanol: Acetonitrile (50:50) used as diluent.

2.5. Preparation of Buffer: Weigh accurately 13.609 gm of potassium dihydrogen orthophosphate and transfer into a clean and dried 1000ml volumetric flask and pour 300 ml of HPLC water dissolve and sonicate for 15 mins. Make up to volume with HPLC water. Adjust the pH to 4.5 with 10% sodium hydroxide solution. Filter the buffer with 0.45µ membrane filter in order to remove the particle if present, to prevent blockage of pump and pressure drop.

2.6. Sample preparation: Twenty tablets were weighed and their mean weight was determined. The tablets were triturated to a fine powder. An accurately weighed quantity of powder equivalent containing 500 mg of Metformin Hydrochloride, 15 mg of Pioglitazone Hydrochloride and 5 mg of Glibenclamide was transferred to 100 ml volumetric flask and dissolved in sufficient quantity of solvent. The mixture was sonicated for 15 min. The volume was made up to the mark with same solvent. aliquots of the filtrate was diluted with methanol: acetonitrile to get final concentration 200 µg/mL, 6 µg/mL and 2 µg/mL of MET HCl, PIO HCl and GLIB respectively. The solution was filtered through PVDF filter.

2.7. Assay:

Separately blank, standard and sample solutions were injected and the areas for Metformin, Pioglitazone and Glibencl-amide were noted and % assay was calculated by using the following formula **(Refer Formula 1)**.

3. Method validation:

3.1. System suitability:

Under the optimum chromatographic conditions, the retention times obtained for Metformin, Pioglitazone and Glibencl-amide were 2.191, 3.094 and 7.175 min.

Number of theoretical plates, Tailing factor, Resolution and % RSD were reported in **Table 1**.

3.2.Specificity:

The specificity of the method was assessed by comparing blank, standard and sample (tablet solution) chromatograms. The retention times of Metformin, Pioglitazone and Glibenclamide for standard are at 2.185, 3.091 and 7.145 min, for sample the retention times are at 2.191, 3.094 and 7.175 min. No interference peak was found at the place of analyte in the blank chromatogram.

3.3. Linearity:

The linearity of the method was evaluated by analyzing different concentration of drugs. According to ICH recommendations **[9]**, at least five concentrations must be used. The linearity of Metformin was demonstrated over the concentration range of 50-400 µg/mL, the linearity of Pioglitazone was demonstrated over the concentration range of 1-12 µg/mL and the linearity of Glibenclamide was demonstrated over the concentration range of 0.5-4 µg/mL. Linear regression was applied and slope, intercept, correlation coefficient was determined and mentioned in **Table 2**. The standard calibration curve was plotted and shown in **Figure 4-6**.

3.4.Accuracy:

The accuracy was done by using weighing method. Tablet samples at three different concentrations levels 50%, 100% and 150% were taken. At each level, samples were prepared in triplicate and percentage recoveries were noted. The accuracy was determined by analyzing NEW TRIGLUCORED Tablets 50%, 100% and 150%. The values are reported in **Table 3**.

3.5. Precision:

Precision was studied for both system precision and method precision. The solution for six injections were given and the obtained areas were mentioned in table. Average area, standard deviation and %RSD were calculated. The results are reported in **Table 4 and 5**.

3.6. LOD and LOQ: The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.Series of diluted standard solutions were prepared and analyzed by both methods. The LOD and LOQ are separately determined based on standard deviation of the y-intercept and the slope of calibration curve by equations. The LOD and LOQ values are reported in **Table 6**.

$$LOD = \frac{3.3\,\delta}{S} \qquad LOQ = \frac{10\,\delta}{S}$$

Where δ = standard deviation of response

S = slope of calibration curve

3.7.Robustness: It is the capacity of analytical method to remain unaffected by small and deliberate changes of the optimized conditions. In the present research we made small changes in flow rate and mobile phase ratio of the developed method. The values are reported in **Table 7**.

3.8.Assay:

Twenty tablets (each containing Metformin Hydrochloride 500mg, Pioglitazone Hydrochloride 15mg, Glibenclamide 5mg) were taken and mean weight was determined. 750 mg of tablet powder was taken and transferred into a clean and dry 100 ml volumetric flask, pour the 3/4 th solvent to dissolve tablet powder. The solution was sonicated for 15 minutes and volume was made upto the mark (Sample solution A). From the sample solution A 4 ml was taken and transferred into 10 ml volumetric flask, volume was made up to the mark (sample solution B). From sample solution (B) 1 ml was transferred to 10 ml volumetric flask, volume was made up to 10 ml with solvent. (Sample solution C) containing Metformin Hydrochloride 200 µg/mL, Pioglitazone Hydrochloride 6 µg/mL, Glibenclamide 2 µg/mL). Filter the solution through PVDF filter. The assay values are reported in **Table 8**.

$$\text{Assay} = \frac{\text{Sample Area}}{\text{Standard Area}} \times \frac{\text{Standard Weight}}{\text{Standard Dilution factor}} \times \frac{\text{Sample Dilution factor}}{\text{Sample Weight}} \times \frac{\text{Average Weight}}{\text{Labeled Claim}} \times \frac{\text{Potency}}{100} \times 100$$

Formula 1

Table 1: System suitability parameters of Metformin, Pioglitazone and Glibenclamide

Parameters	Metformin	Pioglitazone	Glibenclamide
Retention Time	2.191	3.094	7.175
Number of theoretical plates	4737	5307	6621
Tailing factor	1.150	1.268	1.090
Resolution	-	3.111	11.110
% RSD	0.15	0.29	0.14

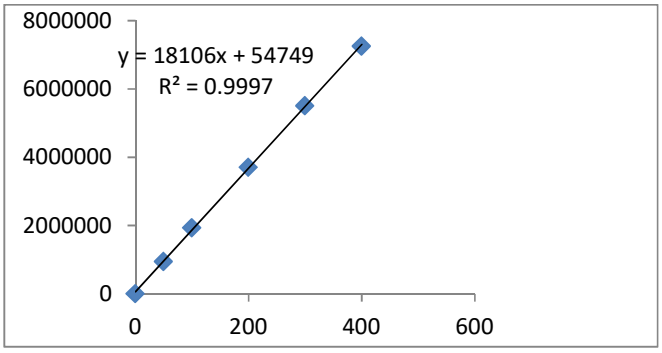


Figure 4: Linearity of Metformin

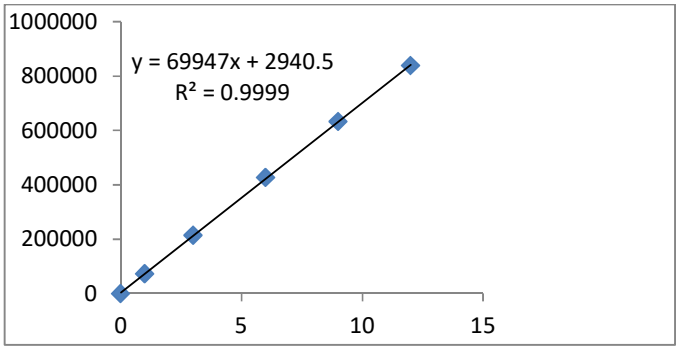


Figure 5: Linearity of Pioglitazone

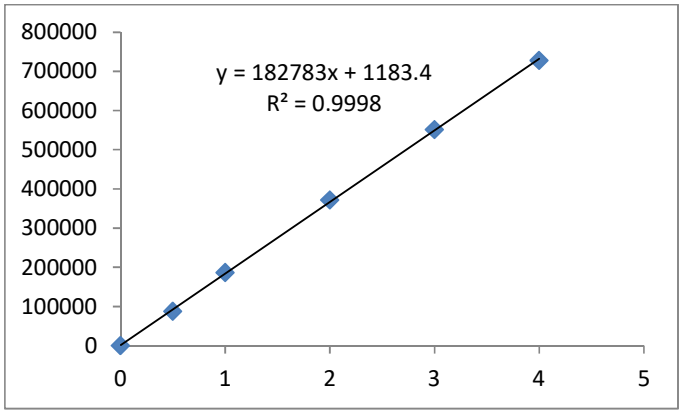


Figure 6: Linearity of Glibenclamide

Table 2: Linearity of Metformin, Pioglitazone and Glibenclamide

Metformin		Pioglitazone		Glibenclamide	
Concentrationµg/mL	Peak Area	Concentration µg/mL	Peak Area	Concentration µg/mL	Peak Area
50	944365	1	72417	0.5	87795
100	1930579	3	214354	1	187044
200	3705344	6	426576	2	372031
300	5505395	9	633313	3	551203
400	7253609	12	839328	4	728252
Correlation Coefficient (R^2) 0.999		Correlation Coefficient (R^2) 0.999		Correlation Coefficient (R^2) 0.999	

Table 3: Recovery studies

Drug	Concentration	Standard Peak Area	Sample Peak Area	%Recovery	Mean % Recovery
Metformin	50%	3705344	1874365	100.62	100.39
		3705344	1863481	100.02	
		3705344	1874365	100.54	
	100%	3705344	3705344	99.49	99.49
		3705344	3705348	99.52	
		3705344	3705353	99.48	
	150%	3705344	5553609	99.42	99.46
		3705344	5553633	99.42	
		3705344	5536601	99.56	
Pioglitazone	50%	426573	215241	100.57	99.67
		426573	212432	99.24	
		426573	212421	99.21	
	100%	426573	426576	99.69	99.96
		426573	426565	99.71	
		426573	426578	99.68	

	150%	426573	639328	99.62	99.60
		426573	639342	99.62	
		426573	639325	99.58	
Glibenclamide	50%	372031	187795	100.71	100.67
		372031	187783	100.68	
		372031	187698	100.62	
	100%	372031	372031	99.79	99.80
		372031	372133	99.84	
		372031	372035	99.78	
	150%	372031	558252	99.84	99.83
		372031	558348	99.85	
		372031	558233	99.80	

Table 4: System precision Metformin, Pioglitazone and Glibenclamide

Injection Number	Peak Area		
	Metformin	Pioglitazone	Glibenclamide
1.	3705355	426573	372033
2.	3717343	429065	372035
3.	3711355	426368	373355
4.	3702809	425478	372365
5.	3720343	425478	371953
6.	3709322	426335	372025
Average	3711088	426641	372294
SD	6773.171	1247.05	539.371
%RSD	0.18	0.29	0.14

Table 5: Method precision Metformin, Pioglitazone and Glibenclamide

Injection Number	Peak Area		
	Metformin	Pioglitazone	Glibenclamide
1.	3705346	426574	372035
2.	3717345	426066	372038
3.	3711355	426370	373358
4.	3708825	425479	372363
5.	3720345	426032	371951
6.	3709333	423635	372021
Average	3712092	425693	372294
SD	5357.62	1074.22	540.676
%RSD	0.15	0.25	0.14

Table 6: LOD and LOQ of Metformin, Pioglitazone and Glibenclamide

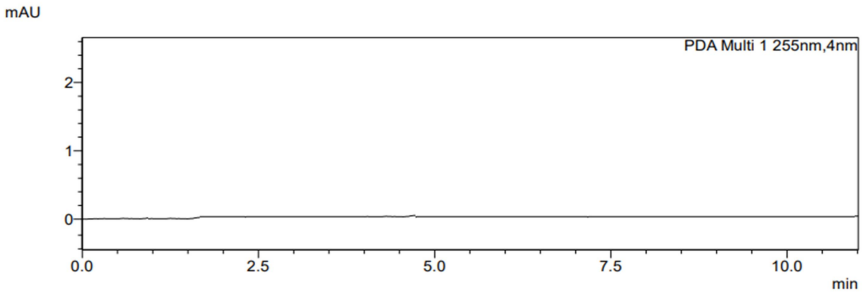
	Metformin	Pioglitazone	Glibenclamide
LOD	5 µg/mL	0.1 µg/mL	0.05 µg/mL
LOQ	15 µg/mL	0.3 µg/mL	0.15 µg/mL

Table 7: Robustness of Metformin, Pioglitazone and Glibenclamide

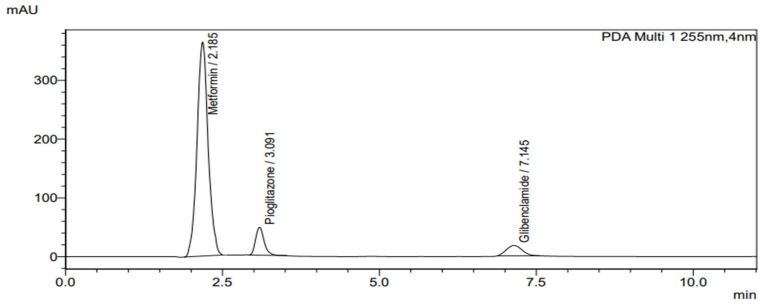
Drug	Flow rate mL/min	Retention time obtain after change in flow rate	Mobile phase ratio	Retention time after change in mobile phase ratio
Metformin	0.8	1.9 min	Acetonitrile:Buffer[50:50v/v]	2.1 min
	0.6	2.5 min	Acetonitrile:Buffer[60:40v/v]	2.2 min
Pioglitazone	0.8	2.7 min	Acetonitrile:Buffer[50:50v/v]	3.2 min
	0.6	3.6 min	Acetonitrile:Buffer[60:40v/v]	2.9 min
Glibenclamide	0.8	6.2 min	Acetonitrile:Buffer[50:50v/v]	10.0 min
	0.6	8.3 min	Acetonitrile:Buffer[60:40v/v]	5.4 min

Table 8: Assay of Metformin Hydrochloride, Pioglitazone Hydrochloride and Glibenclamide

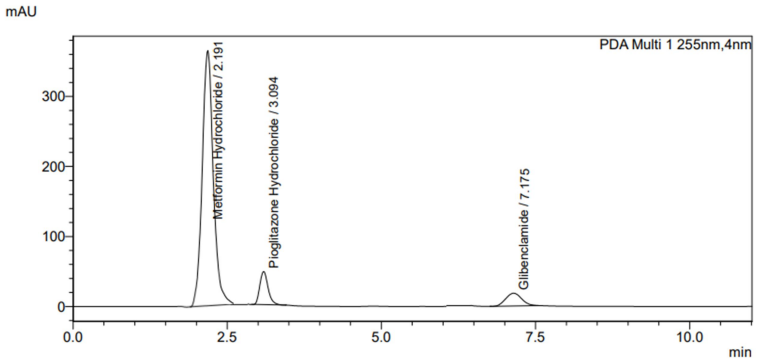
Drug	Labeled Claim	Assay
Metformin HCl	500 mg	99.49 %
Pioglitazone HCl	15 mg	99.69 %
Glibenclamide HCl	5 mg	99.78%



Blank Chromatogram



Standard Chromatogram



Sample Chromatogram

RESULTS AND DISSCUSSION

From the system suitability studies it was observed that all the parameters are within limit, hence it is concluded that the Instrument, Reagents and Column are suitable to perform Assay. The specificity of method was determined by comparing the chromatograms of blank, standard and sample. There was no interference of any peak at the retention time of the analyte the retention times of standard and sample was found to be same. Linearity of Metformin, Pioglitazone and Glibenclamide was reported in **Table 2**, the regression coefficient equation shows that the correlation coefficient was within the acceptance limit. Tablet samples at three different concentrations levels 50%, 100%

and 150% were taken and dissolved in sufficient quantity of Solvent Methanol: Acetonitrile [50:50 v/v]. At each level, samples were prepared in triplicate and the mean percentage recovery was noted. The % recovery for Metformin Hydrochloride, Pioglitazone Hydrochloride and Glibenclamide were found to be within the limit 98-102%. For precision six injections were given and the obtained areas were mentioned in table. Average area, standard deviation and %RSD were calculated. % RSD of system precision was 0.18, 0.29, 0.14 and % RSD of method precision was 0.15, 0.25, and 0.14 for Metformin, Pioglitazone and Glibenclamide. LOD and LOQ for Metformin, Pioglitazone and Glibenclamide was found to be 5, 0.1, 0.05 µg/ml and 15, 0.3, 0.15 µg/ml respectively which indicates sensitivity of the method. Various parameters such as flow rate, ratio of mobile phase was changed and all the parameters were within the acceptance limit with a tailing factor not more than 2 and plate count is more than 2000. The % assay Metformin Hydrochloride, Pioglitazone Hydrochloride and Glibenclamide was found to be 99.49%, 99.69% and 99.78%.

CONCLUSION

The developed RP-HPLC method is a simple, specific, accurate, precise and economic for simultaneous analysis of metformin hydrochloride, pioglitazone hydrochloride and glibenclamide in a tablet dosage form. The separation was achieved on C_{18} Hypersil BDS [150×4.6, 5 µm] with mobile phase at a flow rate 0.7 mL/min consisting of acetonitrile: 0.1 M potassium dihydrogen orthophosphate buffer (pH adjusted to 4.5 by using 10% Sodium Hydroxide solution) (55: 45 v/v). Detection was carried out at 255nm. The retention times of Metformin Hydrochloride, Pioglitazone Hydrochloride, Glibenclamide was found to be 2.191, 3.094 and 7.175 min, respectively. Linearity of Metformin, Pioglitazone, Glibenclamide was found in the range of 50-400 µg/mL, 1-12 µg/mL and 0.5-4.0 µg/mL Limit of detection for Metformin, Pioglitazone, Glibenclamide were, 5 µg µg/mL, 0.1 µg/mL and 0.05 µg/mL Limit of quantification for Metformin, Pioglitazone, Glibenclamide was found to be 15 µg/mL, 0.3 µg/mL, and 0.15 µg/mL respectively. Resolution and percentage RSD for robustness was within the limit. The developed method was validated as per ICH guidelines Q_2 (R1).

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