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Simultaneous estimation of Metformin, Pioglitazone and Glimepiride in bulk samples and in tablet dosage forms by using RP-HPLC in an Isocratic mode

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ABSTRACT

The present work was undertaken with the aim to develop and validate a rapid and consistent RP-HPLC method in which the peaks will be appear with short period of time as per ICH guidelines. The HPLC separation was achieved on an XTerra C_{18} column in an Isocratic Mode. The mobile phase was composed of phosphate buffer (25 %) [pH 3.0 adjusted with OPA] and methanol (75 %) The flow rate was monitored at 1.0mL per min. The wavelength was selected for the detection was 254 nm. The retention time found for metformin, pioglitazone and glimepiride were 1.997, 3.238 and 4.042 min respectively. The % recovery was found 98.46- 101.79 for metformin, 98.04 - 101.79 for pioglitazone and 99.60 - 100.45 for glimepiride. The linearity was established in the range of 80-120 µg/mL for metformin and 2.4-3.6 µg/mL for pioglitazone and 0.16-0.24 µg/mL for glimepiride. The LOD for metformin, pioglitazone and glimepiride were 0.07, 0.07 and 0.006 µg/mL respectively. The LOQ for metformin, pioglitazone and glimepiride were 0.23, 0.24 and 0.02 µg/mL respectively. The proposed method was adequate sensitive, reproducible, and specific for the determination of metformin, pioglitazone and glimepiride in bulk samples as well as in tablet dosage form.

Keywords: Metformin, Pioglitazone, Glimepiride, ICH Guideline, RP-HPLC, LOD, LOQ.

INTRODUCTION

Diabetes is a lifelong (chronic) disease in which there are high levels of sugar in the blood. The diabetes is classified into three major types namely, type I, II, and gestational diabetes. Type II diabetes constitutes 90% of the diabetic population. The combinational therapy for type II diabetes [1-2] is frequently prescribed when monotherapy fails. The combination of metformin (MET), pioglitazone (PIO), and glimepiride (GLIMP) is approved by FDA for treatment of type II diabetes [3]. Metformin is chemically, 1, 1-dimethyl biguanide hydrochloride. It is the first line drug of choice for the treatment of type2 diabetes. Metformin hydrochloride is a white crystalline powder. Metformin hydrochloride is freely soluble in water and is practically insoluble in acetone, ether, and chloroform. Bio-analytical, HPLC, HPTLC and UV-visible spectrophotometry methods have been reported for its individual determination of metformin and in combination with other drugs [4-8]. Glimepiride (is chemically 2-(3-ethyl-4-

methyl-2-oxo-3 pyrroline-1-carboxamido) ethyl-phenylsulfonyl-3-(trans-4-methylcyclohexyl) urea. It is a medium to long acting sulphonyl urea anti-diabetic drug. Several spectrophotometric methods, HPLC, HPTLC have been reported for estimation of glimepiride [5-7]. Glimepiride is a white to yellowish-white, crystalline, odorless to practically odorless powder and is practically insoluble in water. Pioglitazone is one of the PPAR-alpha agonist, insulin sensitizer used to reduce the insulin resistance. Pioglitazone hydrochloride is an odorless white crystalline powder. It is soluble in N, N-dimethyl formamide, slightly soluble in anhydrous ethanol, very slightly soluble in acetone and acetonitrile, practically insoluble in water, and insoluble in ether. It is a thiazolidine dione derivative and chemically (RS)-5-(4-[2-(5-ethylpyridin-2-yl) ethoxy] benzyl) thiazolidine-2, 4-dione. HPLC and UV-visible spectrophotometry methods have been reported for its individual determination of Pioglitazone and in combination with other drugs [9-15]. As per the literature, various methods are available for the estimation of these three drugs individually or in combination of two drugs in a pharmaceutical dosage form and also from biological samples. Very few methods are available for simultaneous estimation of all the three drugs together in a tablet dosage form. This paper describes a simple, precise, and accurate RP-HPLC method for simultaneous estimation of MET, PIO, and GLIMP. High performance liquid chromatography (HPLC) is a recent technique in liquid chromatography, which enables significant reductions in separation time and solvent consumption. The chemical structures for the drug are represented in fig. no. 1, 2 and 3.

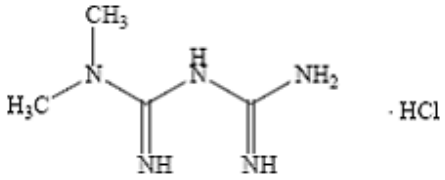

Fig. No. 1 Chemical structure of metformin HCL

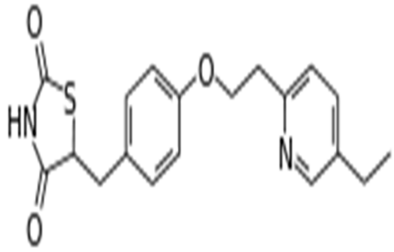

Fig. No. 2 Chemical structure of pioglitazone

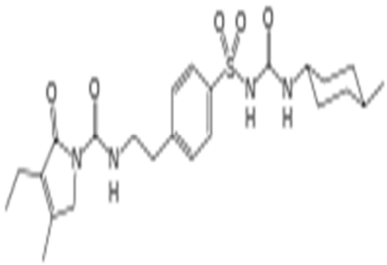

Fig. No. 3 Chemical structure of glimepiride

EXPERIMENTAL SECTION [16-20]

Chemicals and Reagents Used:

The following chemicals were procured for the process: Water [HPLC Grade], Methanol [HPLC Grade], Metformin, Pioglitazone and Glimepiride [Working standards], Orthophosphoric Acid and Potassium Dihydrogen Ortho

Phosphate all the chemicals were procured from Standard Solutions, Andhra Pradesh, India and the tablets were collected from the Local market.

Apparatus and Chromatographic Conditions:
Equipment: High performance liquid chromatography equipped with Auto Sampler and DAD or UV detector.

UV/VIS spectrophotometer: LAB INDIA UV 3000^{+}

pH meter: Adwa – AD 1020
Weighing machine: Afcoset ER-200A
Temperature: Ambient
Column: XTerra C_{18} (4.6 X 150 mm, 5 µm, Make: Thermosil) or equivalent
Phosphate Buffer: 7.0 grams of potassium dihydrogen ortho phosphate in 1000 mL water [HPLC Grade] pH adjusted with orthophosphoric acid.
pH: 3.0
Mobile phase: Phosphate Buffer: Methanol (25: 75v/v)
Flow rate: 1.0 mL per min
Wavelength: 254 nm
Injection volume: 20µl
Run time: 7 min.

Preparation of Phosphate buffer: The buffer solution was prepared by dissolving accurately weighed 7.0 grams of potassium dihydrogen ortho phosphate and transferred into a clean and dry 1000 mL volumetric flask, dissolved and diluted with 1000 mL water [HPLC Grade]. The final pH of the buffer was adjusted to 3.0 by using ortho phosphoric acid.

Preparation of mobile phase and diluent: The mobile phase was prepared by mixing 250 mL (25 %) of the above buffer and 750 mL of methanol [HPLC Grade] (75 %) and degassed in an ultrasonic water bath for 10 minutes. Then the resultant solution was filtered through 0.45 µ filter under vacuum filtration. The mobile phase was used as diluent.

Preparation of the metformin, pioglitazone and glimepiride standard and sample Solution:
Preparation of stock solution: The stock solution was prepared by weighing accurately 10 mg metformin, pioglitazone and glimepiride and transferred into clean and dry 10 mL, 100 mL and 100 mL volumetric flask respectively. About 7 mL, 70 mL and 70mL of diluent was added to the flask respectively and sonicated. The volume was made upto the mark with the same diluent. From the above prepared Stock solution pipette out 1.0 mL of metformin, 0.3 mL of pioglitazone and 1.0 mL of glimepiride solution and transferred into a clean and dry 10 mL volumetric flask, the diluent was added upto the mark to get final concentration.

Preparation of sample solution: The sample solution was prepared by weighing equivalently 10mg of metformin, pioglitazone and glimepiride and transferred into a 10 mL clean and dry volumetric flask and about 7mL of diluent was added and sonicated to dissolve it completely and the volume made up to the mark with the same solvent. From above prepared stock solution pipette out 1.0 mL of solution and transferred into a clean and dry 10 mL volumetric flask, the diluent was added upto the mark to get final concentration. The standard and sample solutions were injected five times and the peak areas were recorded. The mean and percentage relative standard deviation were calculated from the peak areas.

System Suitability: The tailing factor for the peaks due to metformin, pioglitazone and glimepiride in standard solution should not be more than 2.0. The Theoretical plates for the metformin, pioglitazone and glimepiride peaks in standard solution should not be less than 2000. The system suitability of the method was checked by injecting five different preparations of the metformin, pioglitazone and glimepiride standard. The parameters of system suitability were checked.

Assay calculation for metformin, pioglitazone and glimepiride:

$$\textbf{Assay \%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{Avg.Wt.}{Label\ Claim} \times 100$$

Where

AT = average area counts of sample preparation.
AS = average area counts of standard preparation.
WS = Weight of working standard taken in mg.
WT =Weight of test taken in mg.
DS =Dilution of standard solution
DT =Dilution of sample solution
P = Percentage purity of working standard

System suitability results for metformin:

1) The Tailing factor obtained from the standard injection was **1.82.**
2) The Theoretical Plates obtained from the standard injection was **2178.**

Assay result for metformin:

$$\frac{1609966}{1603917} \times \frac{10}{10} \times \frac{1}{10} \times \frac{10}{17.7} \times \frac{10}{1} \times \frac{99.9}{100} \times \frac{884.1}{500} \times 100 = 100.17\ \%$$

System suitability results for pioglitazone:

1) The Tailing factor obtained from the standard injection was **1.48.**
2) The Theoretical Plates obtained from the standard injection was **2136.**

Assay result for pioglitazone:

$$\frac{59426}{59046} \times \frac{10}{100} \times \frac{0.3}{10} \times \frac{10}{17.7} \times \frac{10}{1} \times \frac{99.9}{100} \times \frac{884.1}{15} \times 100 = 100.44\ \%$$

System suitability results for glimepiride:

1) The Tailing factor obtained from the standard injection was **1.1.**
2) The Theoretical Plates obtained from the standard injection was **2120.0.**

Assay result for glimepiride:

$$\frac{46432}{46688} \times \frac{10}{100} \times \frac{1}{10} \times \frac{0.2}{10} \times \frac{10}{17.7} \times \frac{10}{1} \times \frac{99.9}{100} \times \frac{884.1}{1} \times 100 = 99.25\ \%$$

VALIDATION DEVELOPMENT [21]

1. PRECISION: It is a measure of degree of repeatability of an analytical method under normal operation and it is normally expressed as % of relative standard deviation (% RSD). The standard solution was injected for five times and measured the area for all five injections in HPLC. The % RSD for the area of five replicate injections was found within the specified limits. (Table no. 1)

Table no.1: Precision results for metformin, pioglitazone and glimepiride

Injection	Area for Metformin	Area for Pioglitazone	Area for Glimepiride
Injection-I	1597151	58685	46315
Injection-II	1598866	58640	46192
Injection-III	1598299	58675	45767
Injection-IV	1601110	58325	46219
Injection-V	1603191	58936	46193
Average	1599723	58652	46137
Standard Deviation	2415.2	217.6	212.9
% RSD	0.15	0.37	0.46

Acceptance Criteria: The % RSD for the area of all the five injections should not be more than 2 %.

2. INTERMEDIATE PRECISION/RUGGEDNESS: To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day by using different make column of same dimensions. The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found within the specified limits. (Table no. 2)

Table no.2: Ruggedness results for metformin, pioglitazone and glimepiride

Injection	Area for Metformin	Area for Pioglitazone	Area for Glimepiride
Injection-I	1614551	59734	43144
Injection-II	1614906	60322	44138
Injection-III	1619386	60157	44346
Injection-IV	1622241	60752	44375
Injection-V	1627787	60097	44152
Average	1619774	60213	44031
Standard Deviation	5509.7	370.4	507.6
% RSD	0.34	0.62	1.15

Acceptance Criteria: The % RSD for the area of all the five injections should not be more than 2 %.

3. ACCURACY: The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and value found. The standard solution with accuracy 50, 100 and 150 % were injected into chromatographic system and calculated the amount found and amount added for metformin, pioglitazone and glimepiride and further calculated the individual recovery and mean recovery values. (Table no. 3)

4.

Table No. 3. Accuracy results for metformin, pioglitazone and glimepiride

Drug	% Concentration	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	% Mean Recovery
Metformin	50	1615331	5	5.09	101.79	99.82
	100	3148969	10	9.92	99.21	
	150	4687647	15	14.77	98.46	
Pioglitazone	50	61553	5	4.91	98.17	99.33
	100	127647	10	10.18	101.79	
	150	184429	15	14.71	98.04	
Glimepiride	50	51839	5	4.98	99.60	99.93
	100	103820	10	9.97	99.74	
	150	156844	15	15.07	100.45	

Acceptance Criteria: The % Recovery for each level should be between 98.0 to 102.0 %.

5. LINEARITY: It is the ability of the method to elicit test result that is directly proportional to analytic concentration within a given range. It is generally reported as variance of slope or regression line. It is determined by series of three to six injections of five of more standards. Different levels of solution were prepared and injected to the chromatographic system and the peak area was measured. Plotted a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient. The calibration curve is represented in fig. no. 4, 5 and 6. (Table no. 4)

Table No. 4. Linearity curve for metformin, pioglitazone and glimepiride

Linearity Level	Metformin		Pioglitazone		Glimepiride	
	Conc. (µg/mL)	Area	Conc. (µg/mL)	Area	Conc. (µg/mL)	Area
I	80	1308806	2.4	47835	0.16	37456
II	90	1441505	2.7	52947	0.18	41567
III	100	1606101	3.0	59455	0.2	45375
IV	110	1769282	3.3	64355	0.22	49579
V	120	1904680	3.6	69045	0.24	54745
Correlation Coefficient	**0.998**		**0.996**		**0.996**	

Acceptance Criteria: The correlation coefficient should not be less than 0.999.

6. LIMIT OF DETECTION: The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantities as an exact value.

Limit of detection for metformin, pioglitazone and glimepiride: The lowest concentration of the sample was prepared with respect to the base line noise and measured the signal to noise ratio. Limit of detection is the lowest concentration of the substance that can be detected, not necessarily quantified by the method. (Regression statistics) The minimum concentration at which the analyte can be detected is determined from the linearity curve by applying the following formula.

Limit of detection (LOD) = $\frac{\sigma}{S} \times 3.3$

Where S – slope of the calibration curve

σ – Residual standard deviation

Calculation of S/N Ratio for Metformin:

Average Baseline Noise obtained from Blank : 43 µV

Signal Obtained from LOD solution (0.26 % of target assay concentration) : 128 µV

S/N = 128/43 = 2.98

Calculation of S/N Ratio for Pioglitazone:

Average Baseline Noise obtained from Blank : 43 µV

Signal Obtained from LOD solution (0.62 % of target assay concentration) : 126 µV

S/N = 126/43 = 2.93

Calculation of S/N Ratio for Glimepiride:

Average Baseline Noise obtained from Blank : 43 µV

Signal Obtained from LOD solution (0.62 % of target assay concentration) : 127 µV

S/N = 127/43 = 2.95

Acceptance Criteria: The S/N Ratio value should be 3 for LOD solution.

7. LIMIT OF QUANTIFICATION: It is defined as lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy and reliability by a given method under stated experimental conditions. LOQ is expressed as a concentration at a specified signal to noise ratio.

Limit of quantification for metformin, pioglitazone and glimepiride: The lowest concentration of the sample was prepared with respect to the base line noise and measured the signal to noise ratio. Limit of Quantification is the lowest concentration of the substance that can be estimated quantitatively. It can be determined from linearity curve by applying the following formula

Limit of Quantification (LOQ) = $\frac{\sigma}{S} \times 10$

Where S – slope of the calibration curve

σ – Residual standard deviation

Calculation of S/N Ratio for Metformin:

Average Baseline Noise obtained from Blank : 43 µV

Signal Obtained from LOD solution (0.62 % of target assay concentration) : 428 µV

S/N = 428/43 = 9.95

Calculation of S/N Ratio for Pioglitazone:

Average Baseline Noise obtained from Blank : 43 µV

Signal Obtained from LOQ solution (2.0 % of target assay concentration) : 428µV

S/N = 428/43= 9.95

Calculation of S/N Ratio for Glimepiride:

Average Baseline Noise obtained from Blank : 43 µV

Signal Obtained from LOQ solution (2.0 % of target assay concentration) : 429µV

S/N = 429/43= 9.98

Acceptance Criteria: The S/N Ratio value should be 10 for LOQ solution.

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. The standard and samples of metformin, pioglitazone and glimepiride were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.

a. The flow rate was varied at 0.8 mL/min to 1.2 mL/min.: The standard solution of metformin, pioglitazone and glimepiride was prepared and analysed using the varied flow rates along with method developed flow rate. On evaluation of the above results, it was concluded that the variation in flow rate does not affected the method significantly. Hence it was indicated that the method was robust even by change in the flow rate. (Table No. 5).

Table No. 5 System suitability results for metformin, pioglitazone and glimepiride (change in flow rate)

Name of the drug	Flow Rate (mL/min.)	System Suitability Results	
		USP Plate Count	USP Tailing
Metformin	0.8	2109	1.91
	1.0	2178	1.82
	1.2	2158	1.71
Pioglitazone	0.8	2018	1.53
	1.0	2136	1.48
	1.2	2085	1.50
Glimepiride	0.8	2116	1.2
	1.0	2120	1.1
	1.2	3229	1.18

b. The Organic composition in the mobile phase was varied from 65 % to 85 %.: The standard solution for metformin, pioglitazone and glimepiride was prepared and analysed using the varied mobile phase composition along with the actual mobile phase composition. On evaluation of the above results, it was concluded that the variation in 10 % organic composition in the mobile phase does not affected the method significantly. Hence it was indicated that the method was robust even by change in the mobile phase ±10. (Table no. 6)

Table No. 6 System suitability results for the drugs metformin, pioglitazone and glimepiride (change % composition in organic phase)

Name of the drug	Change in Organic composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
Metformin	10 % Less	2064	1.9
	Actual	2178	1.82
	10 % More	2147	1.86
Pioglitazone	10 % Less	2055	1.25
	Actual	2136	1.48
	10 % More	2082	1.36
Glimepiride	10 % Less	2477	1.11
	Actual	2120	1. 1
	10 % More	2443	1.17

RESULTS AND DISCUSSION

The present work was undertaken with the aim to develop and validate a rapid and consistent RP-HPLC method development in which the peaks will be appear with short period of time as per ICH Guidelines. The proposed method was simple, fast, accurate and precise method for the quantification of drug in the pharmaceutical dosage form, bulk drug as well as for routine analysis in quality control. Overall the proposed method was found to be suitable and accurate for the quantitative determination of the drug in tablet dosage form. The method was simple, precise, accurate and sensitive and applicable for the simultaneous determination of metformin, pioglitazone and glimepiride in bulk samples and in combined dosage forms. The High performance liquid chromatography (HPLC) methods was developed and validated for simultaneous estimation of metformin, pioglitazone and glimepiride in bulk samples and in combined dosage forms. The HPLC separation was achieved on an XTerra C_{18} (4.6 x 150 mm, 5 µm, Make: Thermosil) or equivalent in an Isocratic Mode. The mobile phase was composed of phosphate buffer (25 %) whose pH was adjusted to 3.0 by using ortho phosphoric acid and methanol (75 %) [HPLC Grade] The flow rate was monitored at 1.0 mL per min. The wavelength was selected for the detection was 254 nm. The run time was 7 min. The retention time found for metformin, pioglitazone and glimepiride were 1.997, 3.238 and 4.042 min respectively.

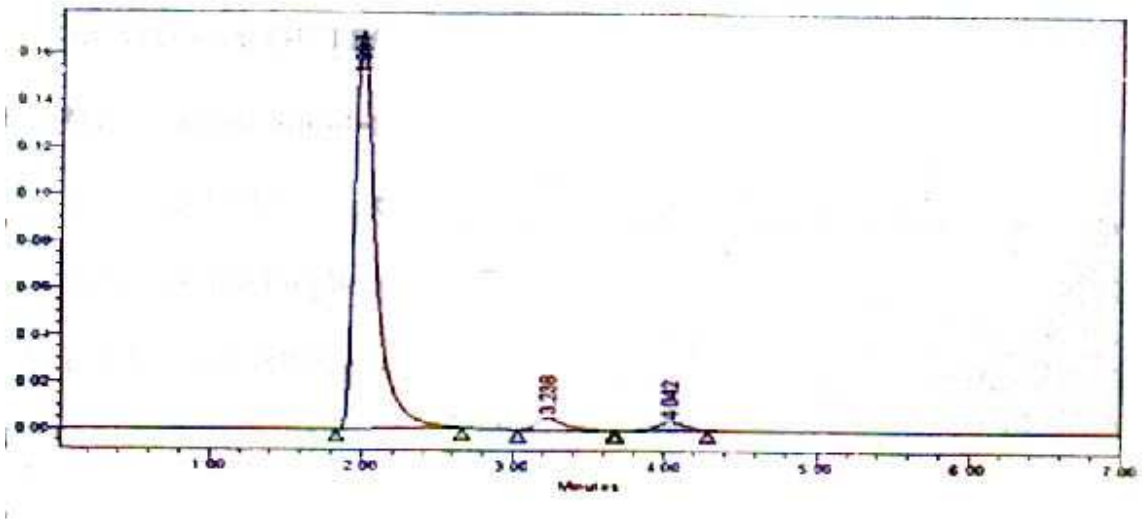

Fig. 4 Chromatogram for metformin, pioglitazone and glimepiride (optimized)

The precision data for metformin, pioglitazone and glimepiride are represented in table no. 1. The % RSD for sample should be NMT 2. The % RSD for the standard solution was found 0.15, 0.37 and 0.46 for metformin, pioglitazone and glimepiride respectively, which is within the limits hence the method, was precise. When metformin, pioglitazone and glimepiride were analyzed by the proposed method in the intra and inter-day (Ruggedness) variation, a low coefficient of variation was observed it is represented in table no. 2, which shows that the developed RP-HPLC method was highly precise. The % RSD was found 0.34, 0.62 and 1.15 for metformin, pioglitazone and glimepiride respectively, which are within the limits. The standard solution with accuracy -50, 100 and 150 % were injected into chromatographic system and calculated the amount found and amount added for metformin, pioglitazone and glimepiride and further calculated the individual recovery and mean recovery values (Table no. 3). The % recovery was found 98.46-101.79 for metformin. The % recovery was found 98.04-101.79 for pioglitazone. The % recovery was found to be 99.60-100.45 for glimepiride. In order to test the linearity of the method, five dilutions of the working standard solutions for metformin, pioglitazone and glimepiride were prepared. The linearity was established in the range of 80-120 µg/mL for metformin and 2.4-3.6 µg/mL for pioglitazone and 0.16-0.24 µg/mL for glimepiride. The data are represented in table no.4. Each of the dilution was injected into the column and the linearity curves are represented in fig. no.5, 6 and 7.

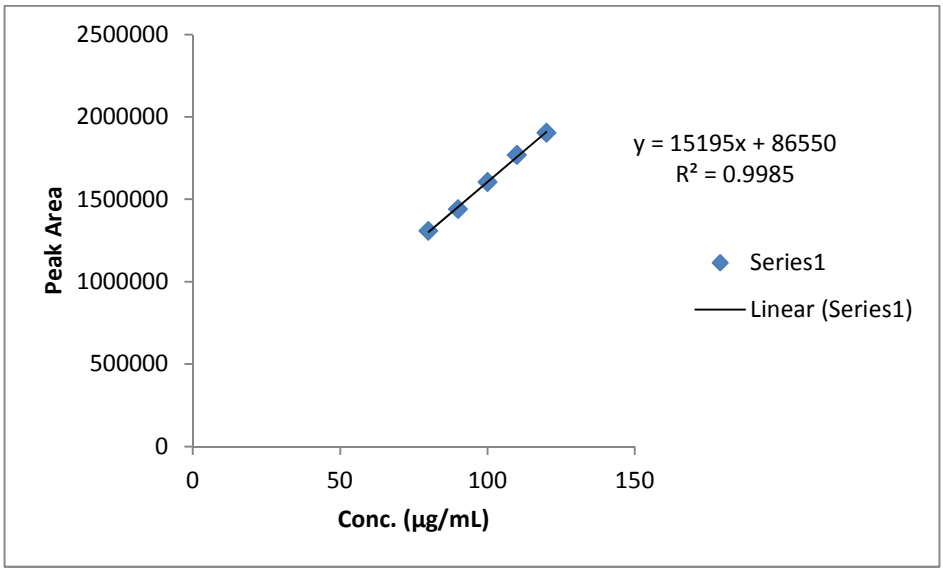

Fig. No. 5 Calibration curve for metformin

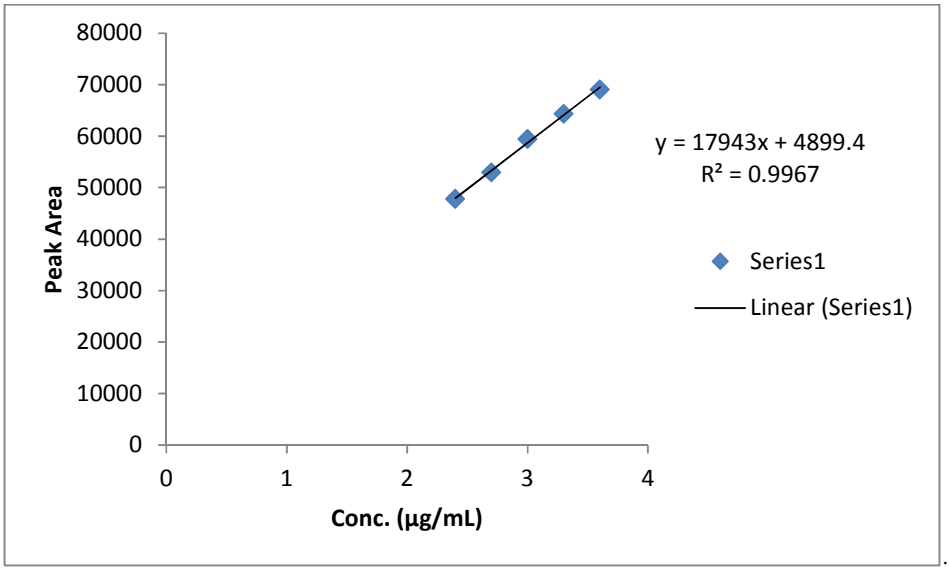

Fig. No. 6 Calibration curve for pioglitazone

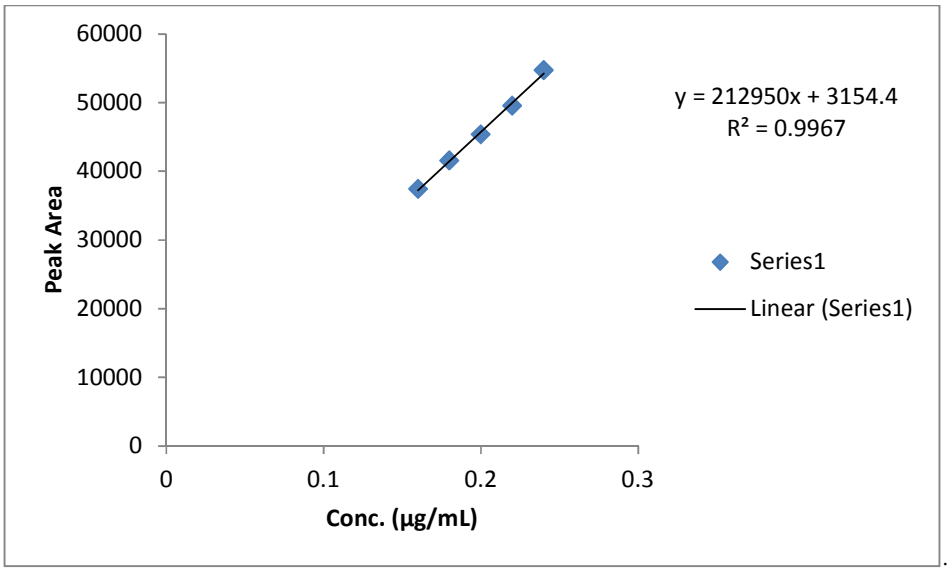

Fig. No. 7 Calibration curve for glimepiride

The correlation coefficient (R^2) should not be less than 0.999. The correlation coefficient obtained was 0.999 which was in the acceptance limit. The limit of detection and limit of quantification of the method were calculated basing on standard deviation of the response and the slope (s) of the calibration curve at approximate levels of the limit of detection and limit of quantification. The LOD for metformin, pioglitazone and glimepiride were 0.07**,** 0.07 and 0.006 µg/mL respectively. The LOQ for metformin, pioglitazone and glimepiride were 0.23**,** 0.24 and 0.02 µg/mL respectively. The signal to noise ratio should be 3 for LOD. The results obtained were within the limit. The signal to noise ratio should be 10 for LOQ solution**.** The results obtained were within the limit. The Robustness of the method was found out by testing the effect of small deliberate changes in the chromatographic conditions in the chromatographic conditions and the corresponding peak areas. The factors selected for this purpose were flow rate and percentage composition variation in phosphate buffer and methanol [HPLC Grade] in the mobile phase. The method was found to be robust enough that the peak area was not apparently affected by small variation in the chromatographic conditions. The system suitability parameters were within the limits and shown in table no. 5 and 6

CONCLUSION

Development of new analytical methods for the determination of drugs in pharmaceutical dosage is important in pharmacokinetic, toxicological biological studies. Pharmaceutical analysis occupies a pivotal role in statuary certification of drugs and their formulations either by the industry or by the regulatory authorities. In industry, the quality assurance and quality control departments play major role in bringing out a safe and effective drug or dosage form.

The current good manufacturing practices (CGMP) and the Food Drug Administration (FDA) guidelines insist for adoption of sound methods of analysis with greater sensitivity and reproducibility. Therefore, the complexity of problems encountered in pharmaceutical analysis with the importance of achieving the selectivity, speed, low cost, simplicity, sensitivity, specificity, precision and accuracy in estimation of drugs. It was concluded that the proposed new RP-HPLC method developed for the quantitative determination of metformin, pioglitazone and glimepiride in bulk as well as in its formulations was simple, selective, sensitive, accurate, precise and rapid. The method was proved to be superior to most of the reported methods. The mobile phases were simple to prepare and economical. The sample recoveries in the formulation were in good agreement with their respective label claims and they suggested non-interference of formulation excipients in the estimation. Hence the method can be easily adopted as an alternative method to report routine determination of metformin, pioglitazone and glimepiride depending upon the availability of chemicals and nature of other ingredients present in the sample. The method also finds use in clinical, biological and pharmacokinetic studies for the drug metformin, pioglitazone and glimepiride. The method was validated as per ICH guidelines, and validation acceptance criteria were met in all cases.

Future Aspect
The proposed method can be use in future for the clinical, biological and pharmacokinetic studies of metformin, pioglitazone and glimepiride.

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