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Simultaneous Determination of Metformin and Its Related Substances in Metformin and Pioglitazone Tablets in Pharmaceutical Dosage Form by RP-HPLC Method

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ABSTRACT

A simple, fast, and precise reverse phase, gradient HPLC method was developed for the separation and quantification of metformin hydrochloride and its related compounds Cyanoguanidine impurity, Melamine impurity, 1- methylbiguanidine impurity, Monoguanylmelamine impurity, N,N Dimethylmelamine impurity in tablet formulations. Liquid chromatography with using Partisil SCX, 250 X 4.6 mm, 10µm and mobile phase is 17 gms of ammonium Dihydrogen phosphate in 1000 ml water and adjust the pH to 3.0 with phosphoric acid and degassed under sonication. The flow rate was 1.0 ml/min and the effluent was monitored at 218 nm. This new method was validated in accordance with USP requirements for new methods for assay determination, which include accuracy, precision, linearity, range and robustness. The current method demonstrates good linearity over the range of 0.01µg/mL to 10µg/mL for Impurity A and metformin HCl. Remaining all impurities with concentration from 0.02 µg/mL to 15 µg/mL for six levels. The accuracy is carried out with concentrations ranging from 50% to 200% of Target concentration the Mean % recovery for each impurity at each level should be between 85.0 % and 115.0.The precision of this method reflected by relative standard deviation of replicates all metformin Related Substances is NMT 10%. Validation of the same method was also performed according to USP requirements for quantitative determination of impurities which include robustness and limit of quantification (LOQ) and Limit of detection LOD. No significant variation in RRT of Metformin and its substances at flow rate (0.8 to 1.2mL/min.), at pH (2.8 to 3.2), column temperature (23°C to 27°C), hence the method is robust.

Key words: Metformin Hydrochloride, Metformin related substances, HPLC, method validation.

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INTRODUCTION:

Metformin is a biguanidine chemically named as N, N-Dimethylimidodicarbonimidic diamine hydrochloride .Metformin decrease the gluconeogenisis while increasing the glucose uptake by muscles and fat cells. Pioglitazone is a thiazolidine Dione derivative. No method had been reported for simultaneous estimation of Simultaneous Determination of Metformin and its Related Substances in Metformin and Pioglitazone Tablets. The present study was aimed at the Simultaneous Determination of Metformin and its Related Substances in Metformin and Pioglitazone Tablets by reverse phase HPLC method. The method was validated according to the ICH (Q2A) guidelines.

MATERIALS AND METHODS

Materials, reagents and chemicals

Metformin and Pioglitazone bulk powders were kindly gifted from Dr. Reddys Laboratories, Hyderabad and commercial fixed dose combination product mg of 850 of Metformin and 15 mg of pioglitazone procured from local pharmacy. Triethylamine, Hydrogen peroxide -30%v/v, Orthophospharic acid, Hydrochloric acid, Sodium Dodecyl Sulphate and Methanol were A.R grade from MERCK chemicals Mumbai. Acetonitrile HPLC grade from Merck chemicals, Mumbai.Cyanoguanidine (Impurity – A), Melamine Impurity (Impurity – D), Impurity B, C and E are taken from CDRL Lucknow.

Chromatographic condition: Agilent 1200 series with VWD detector was used. Ezeochrome elite software version 4.0 is used for Data acquisition. Partisil SCX, 250 X 4.6 mm column was used as a stationary phase.

Preparation mobile

Mobile phase comprised of ammonium Dihydrogen phosphate buffer and adjusted pH to 3.0±0.02 with phosphoric acid .Injection volume was 20µl and run time was 60 min and flow rate 1.0ml/min. The column was maintained at ambient temperature and the eluent was detected at 218 nm.

Solutions

Standard solutions

Metformin HCl Standard stock solution:

Weigh accurately and transfer about 50.0 mg of Metformin HCl standard into 200 mL volumetric flask and add 70mL of diluent, sonicate to dissolve and make up to mark with diluent.

Cyanoguanidine impurity stock solution:

Weigh accurately and transfer about 5.0 mg of Cyanoguanidine impurity into 25 mL volumetric flask, add about 15 mL of diluent, sonicate to dissolve the material completely, dilute to volume with diluent and mix well.

Melamine impurity stock solution:

Weigh accurately and transfer about 6.3 mg of Melamine impurity into 25 mL volumetric flask, add 3 mL Acetonitrile and add 20ml of diluent , dissolve and dilute to volume with diluent and mix well.

Standard preparation:

Pipette 4 mL of Metformin HCl standard stock solution, 4 mL of Melamine impurity stock solution and 5 mL of Cyanoguanidine impurity stock solution into a 200 mL volumetric flask, dilute to volume with diluent and mix well.

System suitability, system precision and method precision:

System suitability carried out to verify that the analytical system is working properly and could give accurate and precise results by injecting diluents as blank, standard solution into chromatographic system. System suitability and system precision.

Linearity:

The linearity study is carried out from the range of LOQ (approximately) to 200% of impurity level of Metformin HCl, Cyanoguanidine impurity, Melamine impurity, 1- methylbiguanidine impurity, Monoguanylmelamine impurity, N,N Dimethylmelamine impurity.

Linearity solution preparation:

Linearity solutions prepared with concentration ranging from 0.01µg/mL to 10µg/mL for Impurity and metformin HCl . Remaining all impurities with concentration fron 0.02 µg/mL to 15 µg/mL for six levels, injected into the Chromatographic system. Recorded the area at each level and calculate slope, intercept, correlation coefficient and regression coefficient (R^2) and evaluate the intercept for statistical equivalence to zero.

Limit of detection / limit of quantification:

Limit of Quantitation is the lowest amount of analyte in a sample that can be quantified with acceptable accuracy and precision, under the stated experimental conditions. Limit of detection (LOD) and limit of Quantification (LOQ) were calculated from slope and the Residual standard deviation of linearity curve.

Precision at LOQ:

Carried out the precision of test method on six samples at LOQ concentration, calculated % Relative Standard Deviation of % impurity.

Accuracy at LOQ

Recovery study is carried out for Metformin impurities at LOQ level in presence of Placebo by spiking individual impurities to prove whether the impurities are recovered to the closeness to the theoretical results.

Accuracy:

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value (Standard value).

Procedure:

Carried out Recovery of Metformin impurities in presence of Excipients with concentrations ranging from 50% to 200% of Target concentration by spiking individual impurities in Homogenous blend.

For Known Impurities:

Spiked known quantity of Cyanoguanidine, Melamine Impurity, 1-Methylbiguanidine Impurity, Monoguanylmelamine Impurity, N,N-Dimethylmelamine Impurity solutions at LOQ, 50%, 100%,150% and 200% of specification level into the sample (API+Placebo), analyzed these samples in triplicate at each level and calculated % recovery at each level.

Range:

The range of analytical method is the interval between the upper and lower levels of analyte that has been demonstrated to be determined with a suitable accuracy, precision and linearity.

Robustness:

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

RESULTS AND DISCUSSION

The proposed HPLC method required fewer reagents and materials, and it is simple and less time consuming. This method could be used in quality control test in pharmaceutical industries. The chromatograms of Metformin and its related compounds were shown in (Figure 1-5). There was clear resolution between metformin and its related compounds Cyanoguanidine impurity, Melamine impurity,1- methylbiguanidine impurity, Monoguanylmelamine impurity, N,N Dimethylmelamine impurity and Metformin HCl .The RRTs are mentioned in Table 1

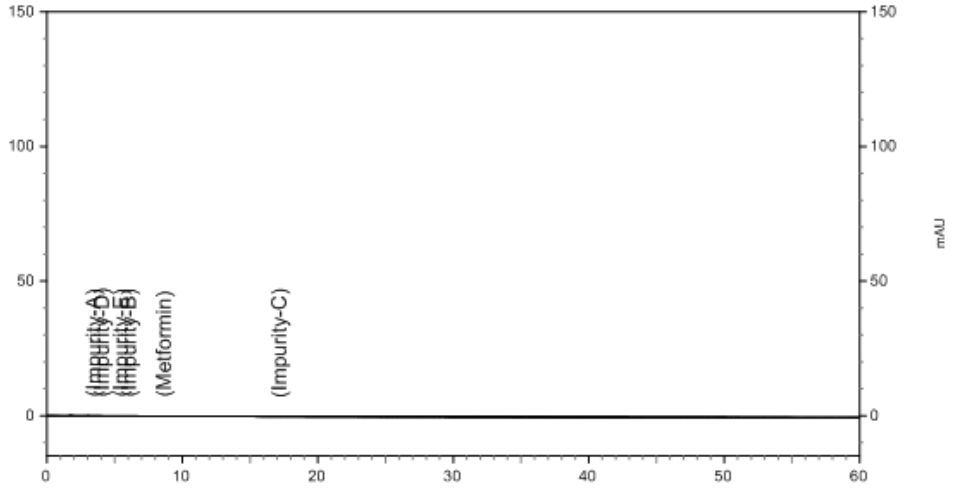

Figure 1: the Reference chromatogram of Blank

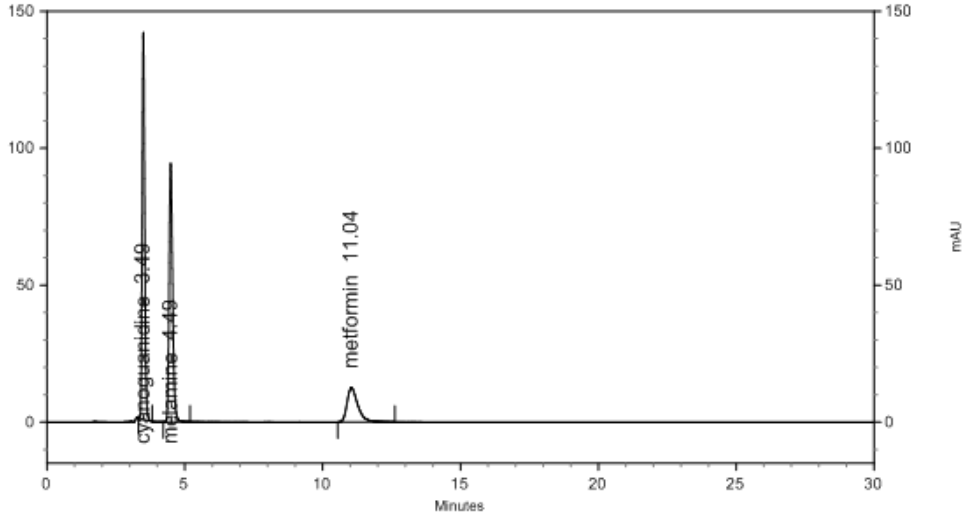

Figure 2: The Reference chromatogram of Standard

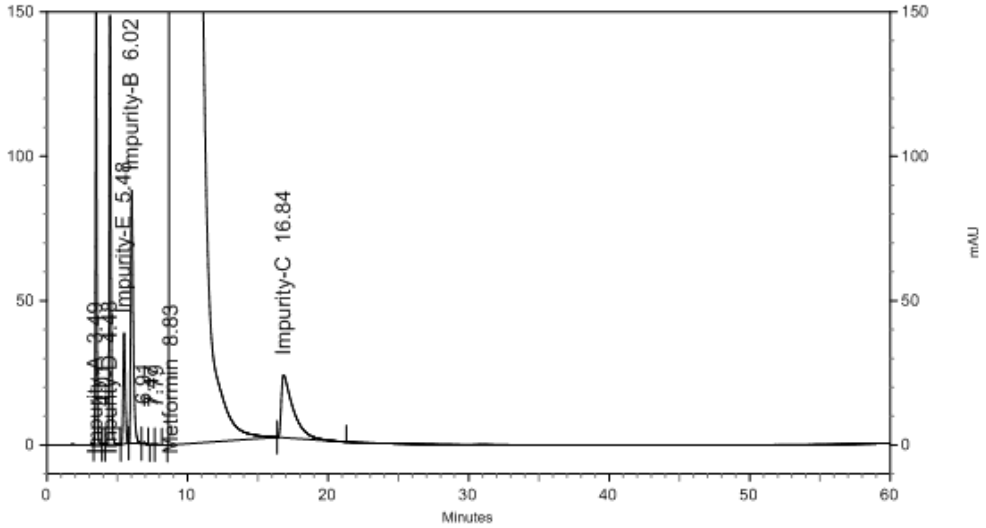

Figure 3: The Reference chromatogram of Test

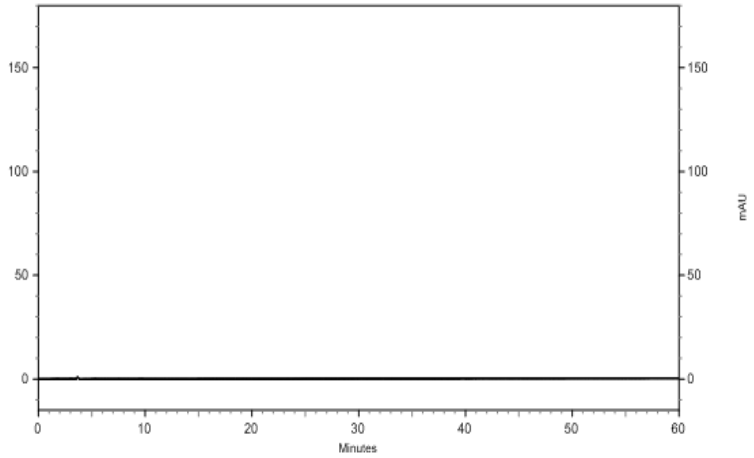

Figure 4: The Reference chromatogram of Placebo

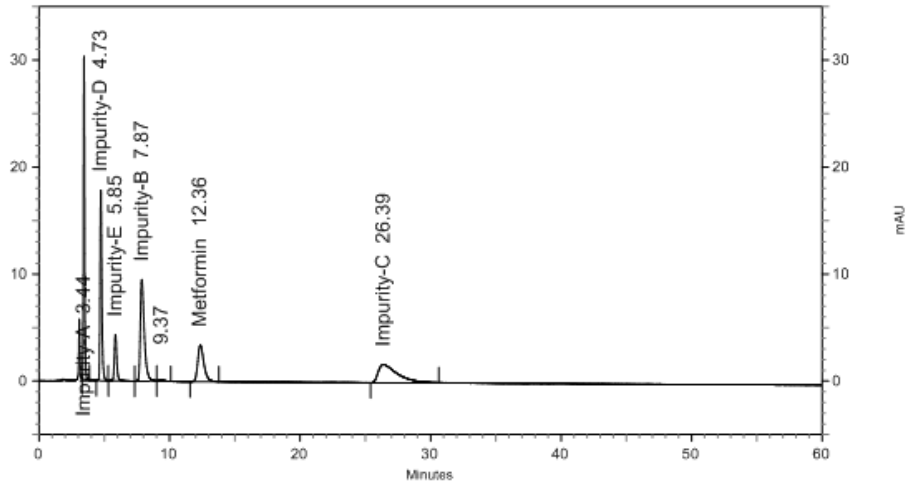

Figure 5: The Reference chromatogram of Impurity Mixture

Table-1 Metformin and its related compounds

S.No	Chemical Name of Impurity	Impurity Identification name	RRT
1	Cyanoguanidine	Impurity A	0.37
2	Melamine Impurity	Impurity D	0.49
3	1-Methylbiguanidine Impurity	Impurity E	0.58
4	Monoguanylmelamine Impurity	Impurity B	0.74
5	N,N-Dimethylmelamine Impurity	Impurity C	2.51
6	Metformin HCl	NA	1.00

System suitability

The resolution, capacity factor, theoretical plates/meter, Rt values and peak symmetry were calculated for the standard solutions. The values obtained demonstrated the suitability of the system for the analysis of the above drug combinations System suitability parameters might be fall within ± 10% standard deviation range during routine performance of the method. The

summary of the method validation results were showed in the (Table 2) and Related Impurities of metformin with Identification name in Table-3.

Table-2 Results for System Suitability

System Suitability	Observed Value	RRT	Acceptance Criteria
Asymmetry	Metformin	1.5	NMT 2.0
	Melamine	1.3	NMT 2.0
	Cyanoguanidine	1.3	NMT 2.0
Theoretical Plates for Metformin peak (1 st injection)	3426	--	NLT 2000
Resolution between Metformin and Melamine	14.09	--	NLT 10.0
% RSD for Peak area and Retention time of Metformin from six replicate injections of standard.	Area	0.7 %	NMT 10.0%
	Retention Time	**0.2 %**	**NMT 2.0 %**

Table-3 Related Impurities of metformin with Identification name

S.No	Chemical Name of Impurity	Impurity Identification Name
1	Cyanoguanidine	Impurity A
2	Melamine Impurity	Impurity D
3	1-Methylbiguanidine Impurity	Impurity E
4	Monoguanylmelamine Impurity	Impurity B
5	N,N-Dimethylmelamine Impurity	Impurity C
6	Metformin HCl	NA

Table-4 Results for System Precision

Injection No.	RT of Metformin HCl peak	Area of Metformin HCl
01	12.41	757970
02	12.39	760314
03	12.38	757020
04	12.37	762584
05	12.35	756890
06	12.34	746434
Mean	12.37	756869
%RSD	0.2 %	0.7 %

Validation of the method

Method Precision & system precision:

The RSD calculated for % known impurities (6 determinations) are NMT 10.0% (Table-4 &Table-5)

Table-5 Results of method precision

S.No.	RRT	% of Impurity-A	RRT	% of Impurity –D	RRT	% of Impurity –E	RRT	% of Impurity-B	RRT	% of Impurity - C
1	0.35	0.0968	0.48	0.1493	0.59	0.1524	0.78	0.1466	2.64	0.1474
2	0.36	0.0967	0.49	0.1493	0.59	0.1520	0.78	0.1464	2.58	0.1482
3	0.36	0.0971	0.49	0.1498	0.59	0.1490	0.78	0.1470	2.57	0.1484
4	0.36	0.0969	0.49	0.1503	0.59	0.1483	0.78	0.1468	2.54	0.1504
5	0.36	0.0971	0.49	0.1505	0.59	0.1486	0.78	0.1470	2.54	0.1491
6	0.36	0.0971	0.49	0.1494	0.59	0.1486	0.78	0.1468	2.53	0.1437
Mean	0.36	0.0969	0.49	0.1499	0.59	0.1498	0.78	0.1468	2.57	0.1477
%RSD		**0.0 %**		**0.0 %**		**1.0 %**		1. **%**		**2.0 %**

Linearity

The response for the detector was determined to be linear over the range of 0.01µg/mL to 10µg/mL for Impurity A and metformin HCl. Remaining all impurities with concentration fron 0.02 µg/mL to 15 µg/mL for six levels, injected into the Chromatographic system. The calibration curve was plotted as concentration of the respective drug versus the response at each level. The proposed method was evaluated by its correlation coefficient and intercept value calculated in the statistical study. They were represented by the linear regression equation **Tables-6&7**

Table-6 Linearity of Related Impurities

	Impurity-A		**Melamine Impurity**		**1-Methylbiguanidine Impurity**	
S.N	**Concentration (PPM)**	**Area**	**Concentration (PPM)**	**Area**	**Concentration (PPM)**	**Area**
01	0.0100	3955	0.0249	9206	0.0250	3455
02	2.3964	900289	3.7386	1355697	3.7432	394175
03	3.1952	1197304	4.9848	1795788	4.9910	526129
04	4.7929	1836544	7.4772	2780094	7.4865	804026
05	7.9881	2988748	12.4621	4585427	12.4775	1306170
06	9.9851	3842964	15.5777	5788695	15.5968	1664728
Coefficient of Correlation		0.9995		0.9999		0.9997
Slope		382247		371762		106132.2
Intercept		12279		23540		849.6
Residual Sum of Square		0.999		0.999		0.999
Bias for 100% response (Intercept*100/Area counts for 100%)		0.7		0.8		0.1

Table-7 Linearity of Related Impurities

	Monoguanylmelamine Impurity		N,N-Dimethylmelamine Impurity		Metformin HCl	
S.No	**Concentration (PPM)**	**Area**	**Concentration (PPM)**	**Area**	**Concentration (PPM)**	**Area**
01	0.0126	5946	0.0499	13285	0.0198	7574
02	3.7861	1458684	3.7405	1300149	2.3808	351640
03	5.0482	1952767	4.9874	1773108	3.1745	470910
04	7.5722	2978411	7.4811	2744691	4.7617	722404
05	12.6204	4988981	12.4685	4578709	7.9361	1186327
06	15.7755	6217621	15.5856	5808815	9.9202	1514447
Coefficient of Correlation		0.999		0.9997		0.9995
Slope		395782.2		374122.5		151943.5
Intercept		22428		59956		4742
Residual Sum of Square		0.999		13285		0.999
Bias for 100% response (Intercept*100/Area counts for 100%)		0.8		1300149		0.7

Y (Impurity A) = 382247X+12279, 'r' value= 0.9995

Y (Melamine Impurity) = 371762X-23540, 'r' value= 0.999

Y(1-Methylbiguanidine Impurity) = 106132.2X+849.6 'r' value= 0.9995

Y (Monoguanylmelamine Impurity) = 395782.2X-22428, 'r' value= 0.999

Y(N,N-Dimethylmelamine Impurity) = 374122.5X+59956 'r' value= 0.999

Y (Metformin HCl) = 151943.5X-4742, 'r' value= 0.999

The correlation coefficient and regression coefficient is more than 0.997for Metformin HCl Cyanoguanidine impurity, Melamine impurity, 1-Methylbiguanide impurity, Monoguanylmelamine impurity, N, N Dimethylmelamine impurity and Metformin HCl.

Slopes and intercepts were obtained by using regression equation (y=mx+c) and least square treatment of the results used to confirm linearity of the method developed.

Accuracy & Range

- The % Recoveries of Metformin HCl impurities are not less than 85.0% and not more than 115.0% for all Levels.(Table-8 to Table-15)
- Relative standard deviation obtained for 21 determinations (3 levels x 3, 2 levels x 6) found to be NMT 15.0%.(Table-8 to Table-15)

Table-8 Accuracy at LOQ

Sample	Impurity Name	"µg" added	"µg" found	%Recovery	Mean
01	Cyanoguanidine	0.01965	0.02049	104.3	104.6
02		0.01965	0.02063	105.0	
03		0.01965	0.02056	104.6	
04		0.01965	0.02055	104.6	
05		0.01965	0.02058	104.8	
06		0.01965	0.02049	104.3	
01	Melamine Impurity	0.01996	0.02035	101.9	102.0
02		0.01996	0.02037	102.1	
03		0.01996	0.02034	101.9	
04		0.01996	0.02035	101.9	
05		0.01996	0.02044	102.4	
06		0.01996	0.02029	101.6	
01	1-Methylbiguanidine	0.01977	0.02090	105.7	105.8
02	Impurity	0.01977	0.02093	105.9	
03		0.01977	0.02090	105.7	
04		0.01977	0.02091	105.8	
05		0.01977	0.02088	105.6	
06		0.01977	0.02098	106.1	
01	Monoguanylmelamine	0.02055	0.01824	91.0	91.3
02	Impurity	0.02055	0.01834	91.5	
03		0.02055	0.01824	91.0	
04		0.02055	0.01848	92.1	
05		0.02055	0.01821	90.0	
06		0.02055	0.01830	91.3	
01	N,N Dimethylmelamine	0.02013	0.01805	89.7	90.2
02	Impurity	0.02013	0.01842	91.5	
03		0.02013	0.01809	89.9	
04		0.02013	0.01819	90.4	
05		0.02013	0.01795	89.2	
06		0.02013	0.01820	90.4	
01	Metformin HCl	0.02018	0.02266	112.4	111.5
02		0.02018	0.02253	111.8	
03		0.02018	0.02236	110.9	
04		0.02018	0.02229	110.6	
05		0.02018	0.02242	111.2	
06		0.02018	0.02258	112.0	

Table-9 Accuracy for Cyanoguanidine Impurity:

Sample No.	Spike Level	"µg" added	"µg" found	%Recovery	Mean
01	50%	2.5672	2.6676	103.9	104.5
02	50%	2.5672	2.6852	104.6	
03	50%	2.5672	2.6893	104.6	
04	50%	2.5672	2.6921	104.9	
05	50%	2.5672	2.6843	104.6	
06	50%	2.5672	2.6808	104.4	
07	75 %	3.8508	4.0682	105.6	105.5

08	75 %	3.8508	4.0591	105.4	
09	75 %	3.8508	4.0682	105.6	
10	100%	5.1344	5.4474	106.1	106.1
11	100%	5.1344	5.4389	105.9	
12	100%	5.1344	5.4940	106.2	
13	150%	7.7016	8.1239	105.5	106.9
14	150%	7.7016	8.1306	105.6	
15	150%	7.7016	8.1324	109.6	
16	200%	10.2687	10.8354	105.5	105.7
17	200%	10.2687	10.8490	105.7	
18	200%	10.2687	10.8348	105.5	
19	200%	10.2687	10.8818	106.0	
20	200%	10.2687	10.8810	106.0	
21	200%	10.2687	10.8151	105.3	

Table-10 Accuracy for Melamine Impurity

Sample No.	Spike Level	"µg" added	"µg" found	%Recovery	Mean
01	50%	3.7595	3.7434	99.6	
02	50%	3.7595	3.7972	101.0	
03	50%	3.7595	3.7584	100.0	100.2
04	50%	3.7595	3.7626	100.1	
05	50%	3.7595	3.7647	100.1	
06	50%	3.7595	3.7677	100.2	
07	75 %	5.6393	5.5625	98.6	
08	75 %	5.6393	5.5423	98.3	98.5
09	75 %	5.6393	5.5618	98.6	
10	100%	7.5191	7.4414	99.0	
11	100%	7.5191	7.4447	99.0	99.0
12	100%	7.5191	7.7735	99.0	
13	150%	11.2786	11.1169	98.6	
14	150%	11.2786	11.1315	98.7	98.7
15	150%	11.2786	11.1485	98.8	
16	200%	15.0382	14.7694	98.2	
17	200%	15.0382	14.8068	98.5	
18	200%	15.0382	14.8288	98.6	98.6
19	200%	15.0382	14.8894	99.0	
20	200%	15.0382	14.9089	99.1	
21	200%	15.0382	14.8015	98.4	

Table-11 Accuracy for 1-Methylbiguanidine Impurity

Sample No.	Spike Level	"µg" added	"µg" found	%Recovery	Mean
01	50%	3.8784	4.1913	107.0	107.5
02	50%	3.8784	4.2040	108.4	
03	50%	3.8784	4.1544	107.1	
04	50%	3.8784	4.1690	107.5	
05	50%	3.8784	4.1766	107.7	

06	50%	3.8784	4.1672	107.4	
07	75 %	5.8176	6.3569	109.3	109.1
08	75 %	5.8176	6.3327	108.9	
09	75 %	5.8176	6.3515	109.2	
10	100%	7.7568	83604	107.8	107.7
11	100%	7.7568	8.3537	107.7	
12	100%	7.7568	8.3511	107.7	
13	150%	11.6351	12.4476	107.0	107.0
14	150%	11.6351	12.4576	107.1	
15	150%	11.6351	12.4576	107.0	
16	200%	15.5135	16.5252	106.5	106.8
17	200%	15.5135	16.5455	106.7	
18	200%	15.5135	16.5570	106.7	
19	200%	15.5135	16.6316	107.2	
20	200%	15.5135	16.6320	107.2	
21	200%	15.5135	16.5269	106.5	

Table-12 Accuracy for Monoguanylmelamine Impurity

Sample No.	Spike Level	“µg” added	“µg” found	%Recovery	Mean
01	50%	3.5261	3.0427	86.3	87.2
02	50%	3.5261	3.1485	89.3	
03	50%	3.5261	3.0451	86.4	
04	50%	3.5261	3.0599	86.8	
05	50%	3.5261	3.0862	87.5	
06	50%	3.5261	3.0560	86.7	
07	75 %	5.2891	4.5371	85.8	85.8
08	75 %	5.2891	4.5303	85.7	
09	75 %	5.2891	4.5380	85.8	
10	100%	7.0521	6.0888	86.3	86.2
11	100%	7.0521	6.0817	86.2	
12	100%	7.0521	6.0789	86.2	
13	150%	10.5782	9.1119	86.1	86.2
14	150%	10.5782	9.1258	86.3	
15	150%	10.5782	9.1247	86.3	
16	200%	14.1043	12.1051	85.8	86.1
17	200%	14.1043	12.1210	85.9	
18	200%	14.1043	12.1342	86.0	
19	200%	14.1043	12.1803	86.4	
20	200%	14.1043	12.1853	86.4	
21	200%	14.1043	12.1080	85.8	

Table-13 Accuracy for N, N Dimethylmelamine Impurity

Sample No.	Spike Level	“µg” added	“µg” found	%Recovery	Mean
01	50%	3.7852	4.2686	112.8	104.7
02	50%	3.7852	4.1255	109.0	
03	50%	3.7852	4.0930	108.1	
04	50%	3.7852	3.6971	97.7	
05	50%	3.7852	3.8534	101.8	
06	50%	3.7852	3.7446	98.9	

07	75 %	5.6778	5.5716	98.1	98.7
08	75 %	5.6778	5.4612	96.7	
09	75 %	5.6778	5.7528	101.3	
10	100%	7.5704	8.2850	109.4	103.1
11	100%	7.5704	7.6989	101.7	
12	100%	7.5704	7.4328	98.2	
13	150%	11.3556	11.24246	100.6	101.4
14	150%	11.3556	11.9214	105.0	
15	150%	11.3556	11.1962	98.6	
16	200%	15.1409	15.2312	100.6	100.9
17	200%	15.1409	14.9361	98.6	
18	200%	15.1409	15.4705	102.2	
19	200%	15.1409	15.6539	103.4	
20	200%	15.1409	15.4288	101.9	
21	200%	15.1409	15.0241	99.2	

Table-14 Accuracy for Metformin HCl

Sample No.	Spike Level	"µg" added	"µg" found	%Recovery	Mean
01	50%	2.5199	2.5290	100.4	99.5
02	50%	2.5199	2.4990	99.2	
03	50%	2.5199	2.5142	99.8	
04	50%	2.5199	2.4934	98.9	
05	50%	2.5199	2.5200	100.0	
06	50%	2.5199	2.4906	98.8	
07	75 %	3.7798	3.8190	101.0	100.9
08	75 %	3.7798	3.7934	100.4	
09	75 %	3.7798	3.8296	101.3	
10	100%	5.0398	5.0172	99.6	99.5
11	100%	5.0398	5.0233	99.7	
12	100%	5.0398	5.0038	99.3	
13	150%	7.5596	7.5063	99.3	99.8
14	150%	7.5596	7.5799	100.3	
15	150%	7.5596	7.5542	99.9	
16	200%	10.0795	9.9169	98.4	98.3
17	200%	10.0795	9.9225	98.4	
18	200%	10.0795	9.9386	9.6	
19	200%	10.0795	9.8946	98.2	
20	200%	10.0795	9.8897	98.1	
21	200%	10.0795	9.8753	98.0	

Table-15 Range

S.No	Impurity Name	Mean Recovery at LOQ	Mean Recovery at 200% Level
01	Cyanoguanidine impurity mean % recovery	104.6	105.7
02	Melamine impurity mean % recovery	102.0	98.6
03	1-Methylbiguanide impurity mean % recovery	105.8	106.8
04	Monoguanylmelamine impurity mean % recovery	91.3	86.1
05	N, N Dimethylmelamine impurity mean % recovery	90.2	100.9
06	Metformin HCl mean % recovery	111.5	98.3

Quantification limit

The limit of detection (LOD) and limit of quantification (LOQ) of the developed method determined by injecting progressively low concentrations of the standard solutions using the developed methods. Results of Limit of Detection / Limit of Quantification mentioned in Table 16 to Table-17)

Table-16 Limit of Detection / Limit of Quantification

S.No	Name	LOD (µg/mL)	LOQ (µg/mL)	% Impurity LOD	% Impurity LOQ
1	Cyanoguanidine	0.2994	0.9073	0.0060	0.0181
2	Melamine Impurity	0.2587	0.7839	0.0052	0.0157
3	1-Methylbiguanidine Impurity	0.3560	1.0786	0.0071	0.0216
4	Monoguanylmelamine Impurity	0.1702	0.5159	0.0034	0.0103
5	N,N-Dimethylmelamine Impurity	0.3862	1.1763	0.0078	0.0235
6	Metformin HCl	0.2372	0.7192	0.0047	0.0144

Table-17 Precision at LOQ

S.No.	% Cyano guanidine Impurity	% Melamine Impurity	% 1-Methyl biguanidine Impurity	% Monoguanyl melamine Impurity	% N,N-Dimethyl melamine Impurity
1	0.02049	0.02035	0.02090	0.01824	0.01805
2	0.02063	0.02037	0.02093	0.01834	0.01842
3	0.02056	0.02034	0.02090	0.01824	0.01809
4	0.02055	0.02035	0.02091	0.01848	0.01819
5	0.02058	0.02044	0.02088	0.01821	0.01795
6	0.02049	0.02029	0.02091	0.01830	0.01820
Mean	0.02055	0.0204	0.02092	0.01830	0.01815
%RSD	0.3	0.2	0.2	0.5	0.9

- The RSD of the % impurities obtained from 6 injections (LOQ level) found to be NMT 10.0%.
- Distinct visible peak found to be observed at LOD level concentration.
- The RSD of the % impurities obtained from 6 preparations (LOQ level) found to be NMT 10.0%.
- The RSD of % for Metformin HCl from six preparations found to be not more than 15.0%.

Robustness

The robustness of the method was studied by deliberate changes in the method like alteration in pH of the mobile phase, percentage organic content, changes in the wavelength. It was observed that there was no marked changes in the chromatograms demonstrate that the HPLC methods have developed are robust (Table-18 to Table-23)

Table-18 Variation in pH

System suitability		pH-2.8	pH-3.0	pH-3.2
Asymmetry for Metformin HCl peak from standard Injection		1.6	1.7	1.7
Asymmetry for Cyanoguanidine peak from standard injection		1.4	1.4	1.4
Asymmetry for Melamine peak from standard injection		1.4	1.4	1.4
Resolution between Melamine and Metformin		10.68	10.86	11.67
USP Plate count for Metformin peak		2929	2766	2672
%RSD for peak area and Retention time of Metformin HCl from replicate injections of standard.	Peak area	0	0.5	1
	Retention time	0	0.1	0

Table-19 Variation in pH

Sample No.	Metformin HCl Impurities RRT's									
	Cyanoguanidine		Melamine		1-Methylbiguanide		Monoguanylmelamine		N,NDimethylmelamine	
	Impurity RRT	Difference	Impurity RRT	Difference	Impurity RRT	Difference	Impurity RRT	Difference	Impurity RRT	Difference
pH-2.8	0.44	NA	0.55	NA	0.64	NA	0.73	NA	2.02	NA
pH-3.0	0.44	0.00	0.55	0.00	0.64	0.00	0.73	0.00	2.00	0.02
pH-3.2	0.44	0.00	0.55	0.00	0.64	0.00	0.73	0.00	1.99	0.03

Table-20 Change in flow rate

System suitability		0.8 mL/min	1.0 mL/min	1.2 L/min
Asymmetry for Metformin HCl peak from standard injection		1.7	1.6	1.6
Asymmetry for Cyanoguanidine peak from standard injection		1.4	1.4	1.4
Asymmetry for Melamine peak from standard injection		1.5	1.5	1.4
Resolution between Melamine and Metformin		11.77	11.05	10.81
USP Plate count for Metformin peak		3266	2856	2760
%RSD for peak area and Retention time of Metformin HCl from replicate injections of standard.	Peak area	0	1	0
	Retention time	0	0	0

Table-21 Change in flow rate

Sample No. ml/min	Metformin HCl Impurities RRT's									
	Cyanoguanidine		Melamine		1-Methylbiguanide		Monoguanylmelamine		N,NDimethylmelamine	
	Impurity RRT	Difference	Impurity RRT	Difference	Impurity RRT	Difference	Impurity RRT	Difference	Impurity RRT	Difference
1.0	0.44	NA	0.55	NA	0.64	NA	0.73	NA	2.02	NA
0.8	0.44	0.00	0.55	0.00	0.64	0.00	0.73	0.00	2.00	0.02
1.2	0.44	0.00	0.55	0.00	0.64	0.00	0.73	0.00	1.99	0.03

Table-22 Change in column Temperature:

System suitability		23 °C	25 °C	27 °C
Asymmetry for Metformin HCl peak from standard injection		1.6	1.6	1.6
Asymmetry for Cyanoguanidine peak from standard injection		1.3	1.3	1.3
Asymmetry for Melamine peak from standard injection		1.4	1.4	1.4
Resolution between Melamine and Metformin		11.32	11.33	11.40
USP Plate count for Metformin peak		2976	3062	3229
%RSD for peak area and Retention time of Metformin HCl from replicate injections of standard.	Peak area	2	1	1
	Retention time	0	0	0

Table-23 Change in column Temperature

Sample No.	Metformin HCl		Impurities RRT's							
	Cyanoguanidine		Melamine		1-Methylbiguanide		Monoguanylmelamine		N,N Dimethyl melamine	
	Impurity RRT	Difference	Impurity RRT	Difference	Impurity RRT	Difference	Impurity RRT	Difference	Impurity RRT	Difference
25 °C	0.44	NA	0.55	NA	0.65	NA	0.73	NA	1.99	NA
23 °C	0.44	0.00	0.56	0.01	0.65	0.00	0.75	0.02	2.09	0.10
27 °C	0.45	0.01	0.56	0.01	0.65	0.01	0.74	0.01	1.91	0.08

- The system suitability parameters are passed for all the conditions.
- All known and unknown impurities are separated from each other in sample spiked with impurities.
- From the results obtained it is observed that there is no significant variation in RRT of Metformin and its impurities at flow rate (0.8 to 1.2mL/min.), at pH (2.8 to 3.2), column temperature (23°C to 27°C), hence the method is robust.

CONCLUSIONS:

In conclusion, the developed method is simple, accurate, and precise. This method was validation good results for linearity, accuracy and precision of Metformin Hcl and its related substances in Metformin pioglitazone tablet dosage form. The RSD values for all parameters were found to be less than 10 for impurities, which indicates the validity of method and results obtained by this method are in fair agreement. This method enables simultaneous determination of Metformin Hydrochloride and its related substances because of good separation and resolution of the chromatographic peaks. The method was found to be accurate, precise, linear and robust.

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