



Simultaneous Estimation Of Repaglinide and Metformin Hydrochloride by using RP-HPLC In Synthetic Mixture and Tablet Dosage Form

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ABSTRACT

An efficient and simple RP-HPLC method has been developed and validated for simultaneous determination of Repaglinide (REPA) and Metformin Hydrochloride (MET). The separation was carried out using mobile phase consisting of Phosphate buffer: Methanol (30:70). The column was used Luna 5u C18 (2) 100A of size 0.25m *4.6mm with flow rate of 1.0 mL/min. Drug peaks were well separated and were detected by a UV detector at 260 nm.. The described method was linear over concentration range of 10-50 ppm for assay of REPA and MET. The retention time of REPA and MET was found to be 4.31. The method has been validated according to ICH guidelines with respect to system suitability, specificity, precision, accuracy, ruggedness and robustness. Metformin limit of detection (LOD) and limit of quantification (LOQ) were 0.0386 µg/ml and 0.1169µg/ml respectively while LOD and LOQ for Repaglinide were 0.0339µg/ml and 0.1025µg/ml respectively and thus can be successfully applied for the routine analysis of REPA and MET in bulk and marketed dosage forms.

Keywords: Repaglinide, Metformin Hydrochloride, RP-HPLC, Simultaneous estimation, Validation

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INTRODUCTION

Diabetes mellitus type 2 (DMT2) is a progressive disease, and to fully control hyperglycaemia after initial monotherapy and lifestyle modification, combination treatments are usually needed to maintain glycaemic control^{1,2}

Metformin hydrochloride (MET) 3-(diaminomethylidene)-1,1-dimethylguanidine (Figure 1) is the most widely used drug for the treatment of DMT2 with a dimethylbiguanidine structure.² Metformin-based combinations are found to be better in efficacy and tolerance rather than other single hypoglycemic agent therapy.^{3,4,6} The therapeutic properties of metformin are due to its action in suppressing glucose production by liver, especially gluconeogenesis. Also, metformin increases the insulin sensitivity in peripheral tissue.

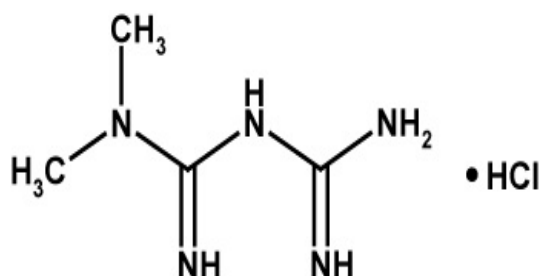


Figure 1

Repaglinide, (*S*)-(+)-2-ethoxy-4-[2-(3-methyl-1-[2-(piperidin-1-yl)phenyl]butylamine)-2-oxoethyl] benzoic acid (Figure 2), is a non-sulfonylurea insulin secretagogue that produces a hypoglycaemic effect by stimulating insulin secretion from the pancreatic β -cells, but, in contrast to the sulfonylureas, it acts via different binding sites on the β -cells.^{3,4,5}

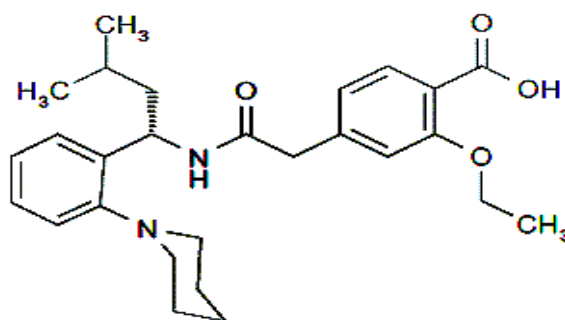


Figure 2

Literature survey revealed that various analytical methods like HPLC and UV have been reported for the determination of REPA and MET either individually or combination with some other drugs. The review of literature prompted us to develop an accurate, selective and precise simultaneous method for the estimation of REPA and MET in combined dosage forms.

MATERIALS AND METHOD

Instrumentation

Quantitative HPLC was performed on a gradient LC – 20AD SPD SHIMADZU High- Pressure Liquid Chromatographic instrument. The instrument is provided with solvent delivery module with UV-visible detector SHIMADZU SPD-M20A and Prominence Luna Reverse phase column (250 X 4.6mm X 5 μ). A 20 μ l Hamilton injecting syringe and windows based LC Solutions software was used for its semi-automatic operation, recording and analysis. A Shimadzu electronic balance was used for weighing the materials. An Elico pH meter and Sonica ultrasonic cleaner was also used for the analysis.

Chemicals and Materials Used:

- Ortho Phosphoric Acid (AR Grade)-Prowess Chemicals, Palakkad
- Acetonitrile (HPLC Grade)-S D Fine chemicals, Mumbai
- Methanol (HPLC Grade)- Prowess Chemicals, Palakkad
- Potassium Dihydrogen Phosphate (AR Grade)-Nice Chemicals, Cochin
- Water (HPLC Grade)-Fischer Scientific
- Sodium hydroxide (AR Grade)- Prowess Chemicals, Palakkad
- Repaglinide (99.57% assay) reference standard - Sun Pharma, Chennai
- Metformin hydrochloride (99.32% assay) reference standard - Sterling QC labs, Cochin
- Prandimet (2mg REPA & 500mg MET)-Novo Nordisk Pharmaceuticals Ltd. Gurgaon

Method Development ⁷⁻¹¹

Optimized Chromatographic Method :(Table: 1)

Table: 1 Optimized Chromatographic Conditions

Parameter	Description
Column	Phenomenax ODS 3v (4.6mmX250nm, 5 μ m i.d)
Flow rate	1.5ml/min
Wavelength	239nm
Column temperature	Ambient
Injection volume	20 μ l
Mobile phase	80 ml of ACN & 20ml of phosphate buffer pH 3.0
Run time	10 mins.

Preparation of Phosphate buffer pH 3.0:

Weighed accurately 27.218gm of Potassium di-hydrogen orthophosphate into a 1000mL volumetric flask and make up the volume with HPLC water and add 0.2M Sodium Hydroxide (7.2ml for 100ml buffer) and adjusted the pH to 3.0 (\pm 0.05) with 10% v/v ortho phosphoric acid. The buffer was filtered through 0.45 μ m filter to remove all fine particles and gases.

Preparation of Mobile Phase:

About 80ml of ACN and 20 ml of pH 3.0 phosphate buffer were mixed. Filtered through 0.45 μ m filter paper & degassed.

Diluents: Methanol and ACN

Preparation of standard stock solution of REPA

Weighed accurately 10mg of REPA working standard and transferred in to a 100ml volumetric flask. About 25ml of methanol was added and ultrasonicated for 5 mins and make up was done with the ACN. The solution was filtered through 0.45 μ m filter.

Preparation of Standard stock solution of MET

Weighed accurately about 10mg of MET working standard and transferred in to a 100ml volumetric flask. About 25ml of ACN was added and ultrasonicated for 5 mins and make up was done with the same solvent. The solution was filtered through 0.45 μ m filter.

Preparation of Sample solution:

20 tablets were weighed and powdered. The powder equivalent to 500mg of MET and 2mg of REPA was transferred to into a 100ml volumetric flask. About 50ml of diluents was added & sonicated for 20 mins with occasional swirling to dissolve. It was cooled and made up to the volume with diluent. About 5ml of the above supernatant solution was transferred into 20ml volumetric flask. Diluted to volume with diluents and mixed well. The solution was filtered through 0.45 μ m filter.

Method Validation^{12,13}**System Suitability**

Stock solutions of standards were injected six times into HPLC system as per test procedure. The system suitability parameters were evaluated from standard chromatograms obtained, by calculating the % RSD of retention times, tailing factor, theoretical plates and peak areas from six replicate injections.

Acceptance criteria

- The % RSD for the retention times of principal peak from 6 replicate injections of each Standard solution should be NMT 2.0 %
- The number of theoretical plates (N) for both MET and REPA peaks should be not less than 2500.
- The Tailing factor (T) for both MET and REPA peaks should be NMT 2.0.

System-suitability conditions

Under the optimum chromatographic conditions, the retention times obtained for REPA and MET were 4.821 and 2.511 min respectively. The resolution (Rs) between REPA and MET was 2.31.

The result of capacity factor, tailing factor, theoretical plate number are reported in Table 2. The values obtained for these properties shows ($1 < k < 10$, $R_s > 2$) this chromatographic conditions are appropriate for separation and quantification of both the compounds. The number of plates (N) is a measure of column efficiency; which shows the good separation efficiency of the column used. The results were expressed in Table: 3 and Chromatogram was shown in Figure 3.

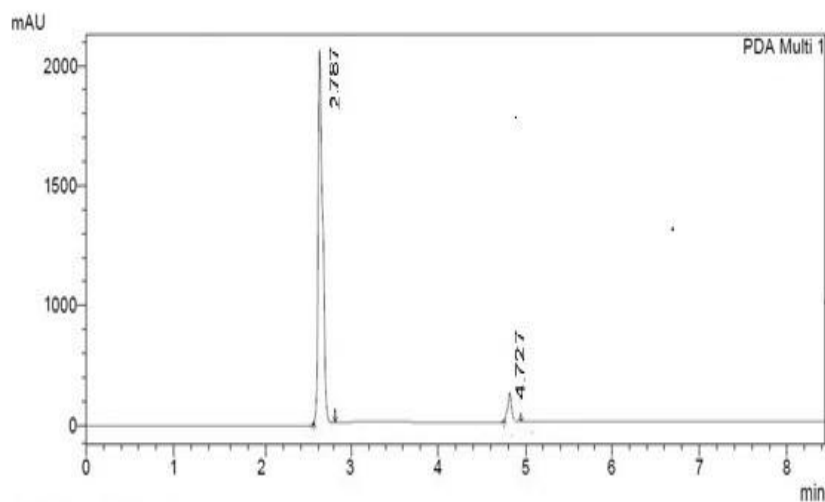


Figure 3: Chromatograph of formulation (PRANDIMET)

Table 2: Result from system suitability study

Property (n=6)	MET	REPA
Rt	2.511	4.721
T _f	1.22	1.37
K	1.51	1.23
N	5696	7992
R _s	-	2.31

Table 3: Result analysis and statistical validation of formulation

S.No.	REPA			MET		
	Amount Present (µg/ml)	Peak area	Amount Found (µg/ml) (%)	Amount Present (µg/ml)	Peak area	Amount Found (µg/ml) (%)
1	2	134666	2.01 100.5	500	22495561	498.66 99.73
2	2	135967	2.03 101.63	500	22510565	500.04 100.01
3	2	138865	1.98 99.04	500	22522284	498.99 99.79
4	2	132424	1.97 99.01	500	22533817	499.25 99.85
5	2	131862	2.03 101.63	500	22557763	499.51 99.91
Mean		134756.8	2.01 100.36		22523998	499.29 99.86
S.D.			1.36			1.81

Linearity

The linearity of the method was demonstrated over the concentration range of 125-750 µg / ml of MET and 1.5-4 µg / ml of REPA and labeled as solution 1, 2, 3, 4, 5 and 6 respectively. The solutions

were injected in to HPLC system as per test procedure. A calibration curve was plotted for concentration v/s peak area and was given in the Figure: 4,5. The results were discussed in Table 4, 5

Acceptance criteria

- Correlation Coefficient should be not less than 0.9990.
- % RSD of peak areas for Solution 1, 2, 3, 4 and 5 should be NMT 2.0 %.

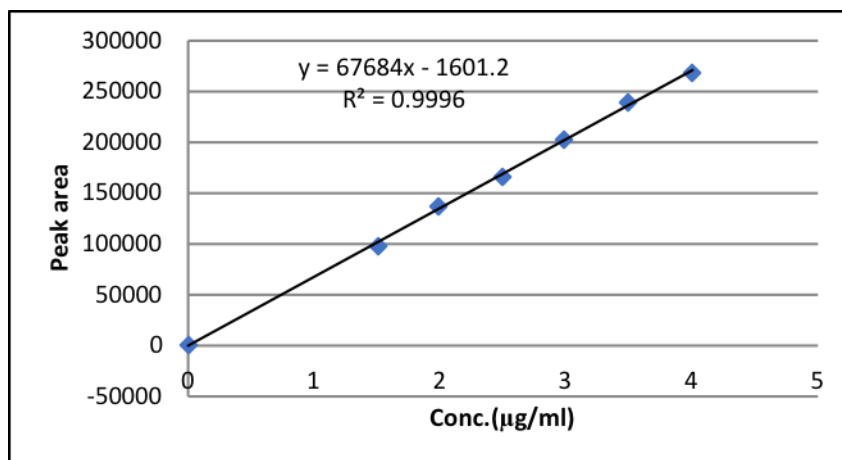


Figure 4: Calibration curve of REPA

Table 4: Linearity study of REPA

Sl no	Concentration (µg/ml)	Peak area
1	1.5	97200
2	2	134666
3	2.5	165333
4	3	202396
5	3.5	237462
6	4	268528

Table 5: Linearity study of MET

Sl no	Concentration (µg/ml)	Peak area
1	125	5623890
2	250	11515561
3	375	16893670
4	500	22495561
5	625	28159450
6	750	33823340

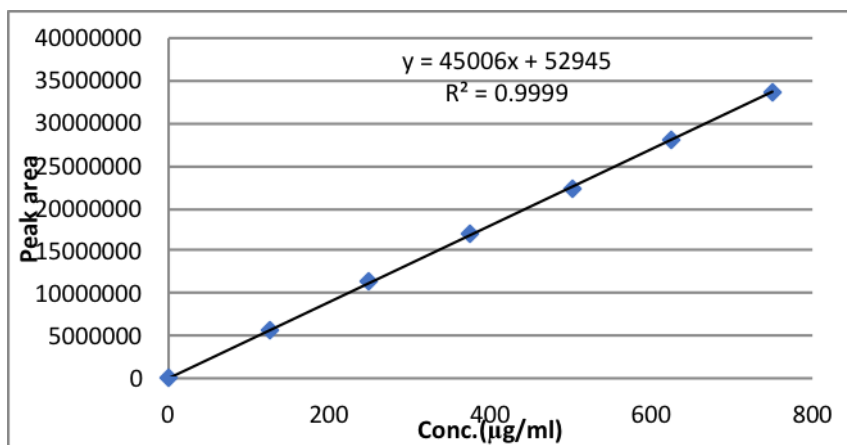


Figure 5: Calibration curve of MET

Precision

Procedure

The chromatographic condition were set as per the optimized parameters and mobile phase was allowed to equilibrate with stationary phase, five replicate injection of standard solution and each of six sample solutions were made separately and the chromatograms were recorded chromatograms of one of the sample solution is Figure 6

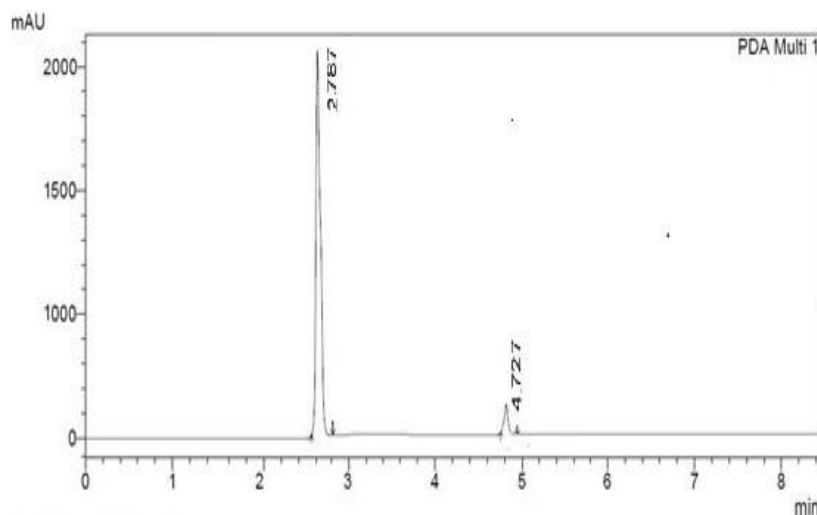


Figure 6: Chromatogram of sample solution

Acceptance criteria

- All individual assays of MET and REPA should be within 98 % - 102 %.
- RSD of % assay results should be NMT 2.0 %.

b) Intermediate precision (Analyst to Analyst variability)

Two analysts as per test method conducted the study. For Analyst-1 Refer Precision (Repeatability) results and the results for Analyst-2 were discussed in Table 6,7

Table 6: Result and statistical validation of intraday precision study

Replicate No.	Conc. found ($\mu\text{g ml}^{-1}$)					
	1 st h.		2 nd h.		3 rd h.	
	REPA	MET	REPA	MET	REPA	MET
1	2.01	495.66	1.99	501.02	2.04	496.21
2	1.99	500.04	1.97	499.04	2.03	500.94
3	1.96	496.99	2.03	495.99	1.98	497.93
4	1.97	497.85	1.98	502.15	1.99	504.75
5	2.03	504.51	2.01	499.51	1.97	496.66
Mean	1.99	499.01	1.99	499.54	2.002	499.298

Table 7: Result and statistical validation of inter-day precision study

Replicate No.	Concentration found ($\mu\text{g/ml}$)					
	1 st day		2 nd day		3 rd day	
	REPA	MET	REPA	MET	REPA	MET
1	2.01	498.66	2.07	498.01	2.02	500.08
2	2.03	500.04	2.02	499.55	2.04	498.36
3	1.98	498.99	1.99	498.19	1.96	498.89
4	1.97	499.25	1.95	499.46	1.99	500.25
5	2.03	499.51	2.01	500.51	2.05	499.12
6	2.01	498.66	2.03	500.36	1.94	498.66
Mean	2.005	499.185	2.012	499.346	2.01	499.226
S.D.	0.0251					

Accuracy

Assay was performed in triplicate for various concentrations of MET and REPA equivalent to 80, 100 and 120 % of the standard amount was injected into the HPLC system per the test procedure. The chromatograms are presented in figure: 7, 8 &9. The average % recovery of MET and REPA were calculated and the results were summarized in Table 8, 9

Table 8: Accuracy results of REPA

Sl. no	Spike level	mg added	Peak area	mg recovered	% Recovery	MEAN	SD	CV
1	80%	1.6	247462	1.619	101.19	100.58	0.766	0.587
2	80%	1.6	241384.56	1.592	99.72			
3	80%	1.6	244091.92	1.632	100.83			
4	100%	2	268528	1.991	99.77	99.76	0.5001	0.250
5	100%	2	269811.84	2.011	100.25			
6	100%	2	267104.48	1.976	99.25			
7	120%	2.4	299594	2.45	101.13	100.42	0.710	0.504
8	120%	2.4	297426.91	2.418	100.41			
9	120%	2.4	295328.708	2.387	99.71			

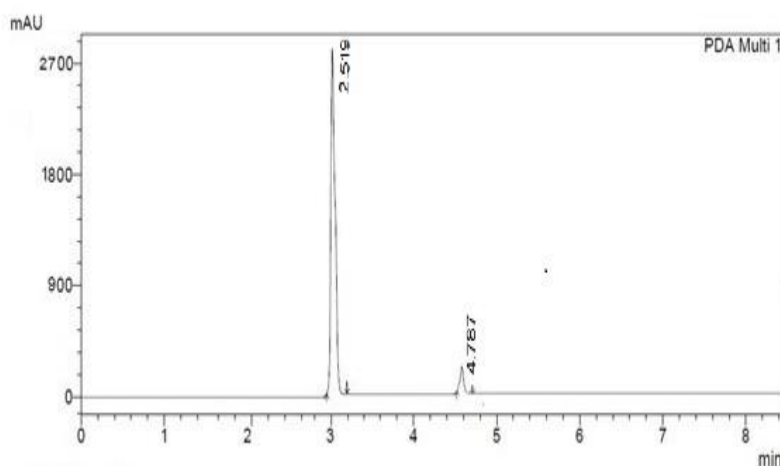
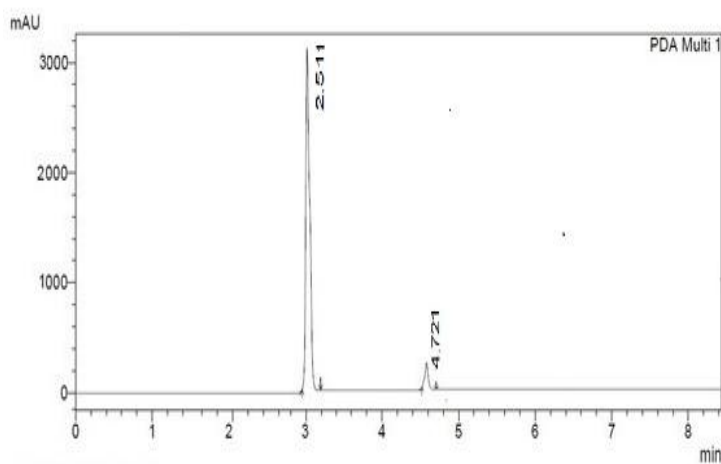
Table 9: Accuracy results of MET

Sl. no	Spike level	mg added	Peak area	mg recovered	% Recovery	MEAN	SD	CV
1	80%	400	40441708.8	397.408	99.712	99.714	0.595	0.354
2	80%	400	40614872.54	401.256	100.31			
3	80%	400	40400778.99	396.499	99.12			
4	100%	500	44935232	499.604	99.96	99.79	0.612	0.374
5	100%	500	44844446.4	495.234	100.31			
6	100%	500	45166869.39	502.398	99.12			
7	120%	600	49559545	604.443	100.40	100.16	0.404	0.163
8	120%	600	49661078.54	602.256	100.38			
9	120%	600	49477949.12	598.187	99.69			

Acceptance criteria

- The mean % recovery of MET and REPA at each spiked level should be not less than 98.0 % and NMT 102.0 %.

$$\% \text{ Recovery} = \frac{\text{Amount recovered} - \text{Amount added}}{\text{Amount present}} \times 100$$

**Figure 7: Chromatogram of accuracy (80%)****Figure 8: Chromatogram of accuracy (100%)**

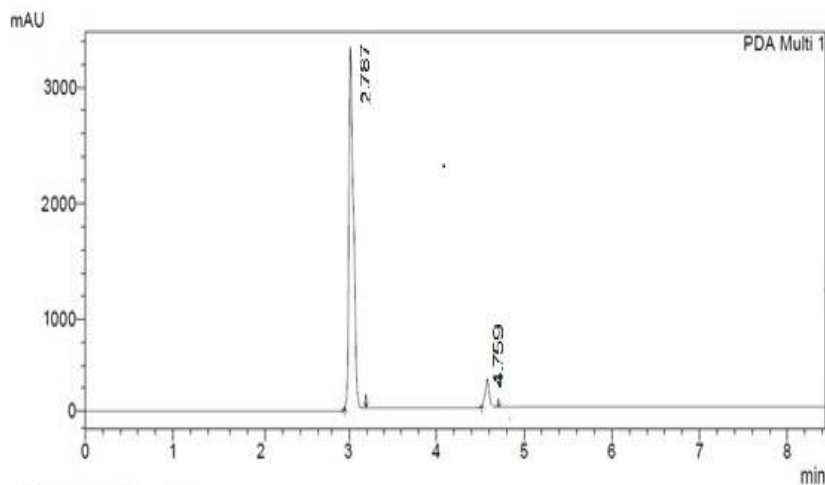


Figure 9: Chromatogram of accuracy (120%)

Specificity

a) Blank interference:

A study to establish the interference of blank was conducted. Diluent (mobile phase) was injected into HPLC system as per the test procedure. The Chromatogram of blank was shown in Figure: 10

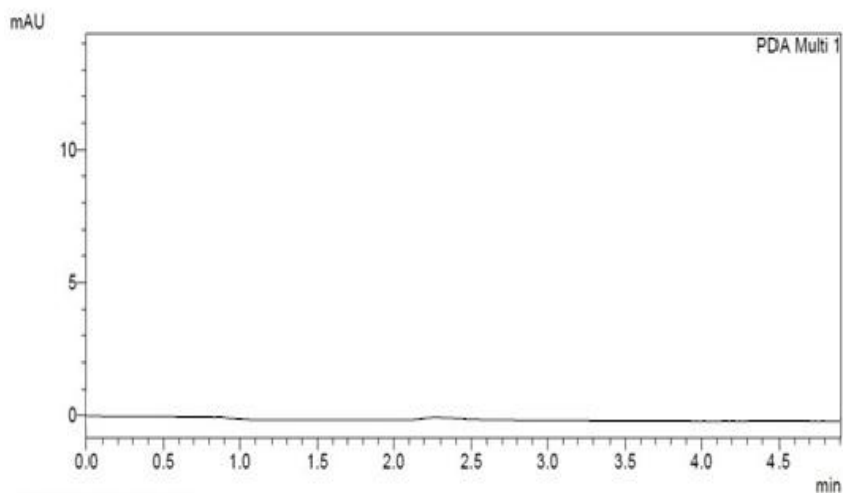


Figure 10: Chromatogram of blank

Acceptance criteria

- Chromatogram of placebo should not show any peak at the retention time of analyte peak.
- There was no interference due to placebo at the retention time of analyte Figure 11 Hence the method was specific.

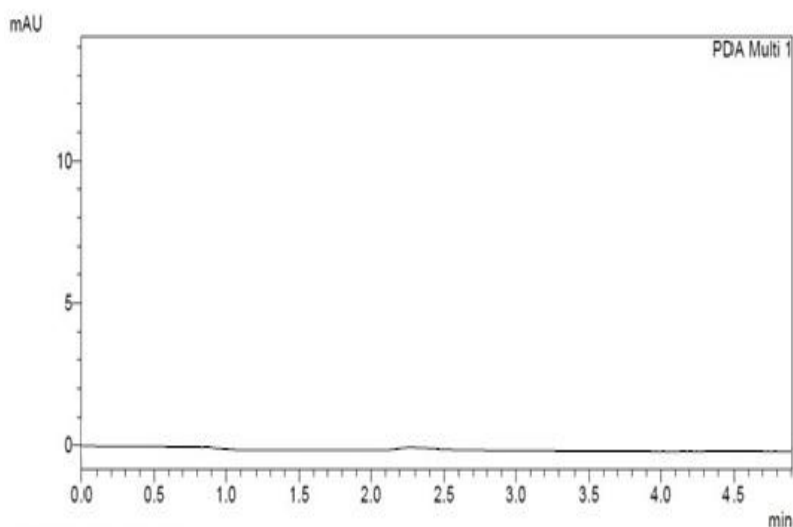


Figure 11: Chromatograph of placebo

Robustness

The robustness of the proposed method was determined by analysis of aliquots from homogenous lots by differing physical parameters like flow rate and mobile phase composition which may differ but the responses were still within the specified limits of the assay.

a) Effect of variation of flow rate:

A study was conducted to determine the effect of variation in flow rate. Standard solution was prepared and injected into the HPLC system by keeping flow rates 1.4 ml / min and 1.6 ml / min. The effect of variation of flow rate was evaluated. The results were discussed in the Table 10

Acceptance criteria

- The tailing factor of MET and REPA standard should be NMT 2.0 for variation in flow.
- The % RSD of asymmetry and retention time of MET and REPA standard should be NMT 2.0 % for variation in flow.

Table 10: Results of robustness

Experiment(Robustness)	% RSD		USP plate count		USP Tailing/ Symmetry Factor	
	REP	MET	REP	MET	REP	MET
Control	0.1	0.1	5233	5491	1.05	1.08
Flow minus	0.4	0.4	5458	5772	1.03	1.06
Flow plus	0.6	0.5	4968	5122	1.06	1.06
Column temperature plus	0.3	0.3	5340	5162	1.04	1.06
Column temperature minus	0	1	4778	5796	1.02	1.06
P ^H minus	0.1	0.4	4442	5480	1.02	1.07
P ^H plus	0.3	0.4	4935	5273	1.01	1.08
Increased organic	0.4	0.4	4973	5192	1	1.08
Decreased organic	0.3	0.3	6232	5917	1.04	1.07

b) Effect of variation of mobile phase composition:

A study was conducted to determine the effect of variation in mobile phase ratio. Five replicate injections of standard solution and sample solution are injected at different concentration of organic phase ($\pm 10\%$) i.e. mobile phase having concentration of CAN buffer in the ratio 85:15 and 75:25 and the results were discussed in the Table 10

Acceptance criteria

- Tailing Factor of MET and REPA standard should be NMT 2.0 for variation in composition of mobile phase.
- The % RSD of tailing factor and retention times of MET and REPA standard should be not more than 2.0 for variation in composition of mobile phase.

c) Change in pH of mobile phase ($\pm 10\%$)

Five replicate injections of standard solution and sample solution were injected at different pH of buffer ($\pm 10\%$) i.e. mobile phase having buffer of pH 2.7 and 3.3. The results are shown in the Table 10

d) Change in column temperature ($\pm 5^\circ\text{C}$)

Five replicate injections of standard solution and sample solution were injected at different column temperature ($\pm 5^\circ\text{C}$) i.e. column having temperature of 35°C and 25°C . The results are shown in the Table 10

Limit of Detection (LOD)

Calibration curve was repeated for 5 times and the standard deviation (SD) of the intercepts was calculated. The LOD was determined by the formula:

$$\text{LOD} = 3.3 \sigma / S$$

Where

σ = Standard deviation of Intercepts of calibration curves

S = Mean of slopes of the calibration curves

The slope S may be estimated from the calibration curve of the analyte. Figure: 12, 13

Limit of Quantification (LOQ)

Calibration curve was repeated for 5 times and the standard deviation (SD) of the intercepts was calculated The LOQ was determined by the formula:

$$\text{LOQ} = 10 \sigma / S$$

Where

σ = Standard deviation of Intercepts of calibration curves

S = Mean of slopes of the calibration curves

The slope S may be estimated from the calibration curve of the analyte. Figure: 12,13

The LOD values were 57.12 and 12.01 ng mL⁻¹ and LOQ values were 173.15 and 36.38 ng mL⁻¹ for MET and REPA respectively. The results for LOD and LOQ are expressed in Table 11,12 for REPA and MET respectively.

Mean of slope	45342
Standard Deviation of intercept	530.382
LOD	0.0386
LOQ	0.11697

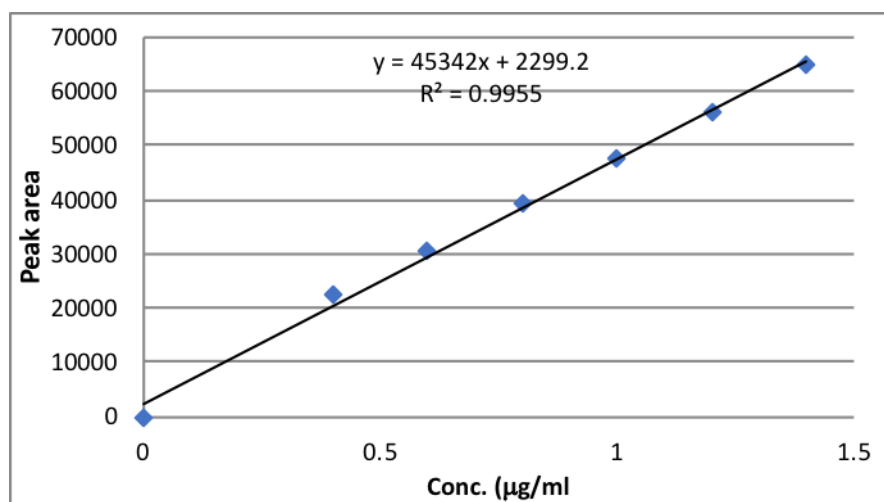


Figure 13: Calibration curve of LOD and LOQ for MET

Table 10: Results of robustness

Experiment (Robustness)	% RSD		USP plate scout		USP Tailing/ Symmetry Factor	
	REP	MET	REP	MET	REP	MET
Control	0.1	0.1	5233	5491	1.05	1.08
Flow minus	0.4	0.4	5458	5772	1.03	1.06
Flow plus	0.6	0.5	4968	5122	1.06	1.06
Column temperature plus	0.3	0.3	5340	5162	1.04	1.06
Column temperature minus	0	1	4778	5796	1.02	1.06
P ^H minus	0.1	0.4	4442	5480	1.02	1.07
P ^H plus	0.3	0.4	4935	5273	1.01	1.08
Increased organic	0.4	0.4	4973	5192	1	1.08
Decreased organic	0.3	0.3	6232	5917	1.04	1.07

RESULTS AND DISCUSSION

RP-HPLC method

In the present work method development for simultaneous estimation of REPA and MET in solid tablet dosage form in RP-HPLC were performed by using Acetonitrile and potassium dihydrogen phosphate buffer (pH 3 adjusted with orthophosphoric acid) as mobile phase in the ratio of 80:20 (v/v) at a flow rate of 1.5 ml min⁻¹.

In both the cases the developed method was validated by performing its accuracy, precision, detection limit study, and selectivity and specificity study.

Assay of REPA and MET

Content of these drugs in tablet dosage form were estimated by RP-HPLC using Acetonitrile and potassium dihydrogen phosphate buffer (pH 3 adjusted with orthophosphoric acid) as mobile phase in the ratio of 80:20 (v/v). The estimation was performed at 239nm as BOTH the analytes absorbed well at this wavelength. The flow rate was maintained at 1.5 ml/min. From the chromatogram it was found that REPA and MET eluted at retention time of 4.721 and 2.511 mins respectively with a resolution of 2.31 between REPA and MET. The total run time was within 10minutes.

The mean percentage of drug content in tablet dosage form (PRANDIMET) was performed by doing replicate study. From the result, total content of REPA and MET found to be 100.36 % and 99.86% with a standard deviation of 1.36 and 1.81 respectively.

Validation of method

The developed method was validated for its accuracy, precision, detection limit, specificity and selectivity.

Accuracy was confirmed by doing recovery study as per ICH norms, where to a preanalysed sample solution standard solutions of drugs were added equivalent to 80,100 and 120% of its drug content. The percentage of drug recoveries for REPA and MET in 80, 100 and 120% were 100.58, 99.76 and 100.42 and 99.714, 99.79 and 100.16 respectively. As all the statistical results were within the range of acceptance i.e. % CV < 2.0 and S.D. < 1.0, hence the method was accurate for simultaneous quantitative estimation of these drugs

Precision study was performed by doing intermediate precision study which includes intra-day and inter-day precision study. In intra-day study the sample solutions were analyzed on the same day at an interval of 1 hour for 3 hours and measured the total drug content in it. From the result it was observed that REPA has drug content of 1.99, 1.99 and 2.002µg/ml, MET has of 499.01,499.54 and 499.28µg/ml in first, second and third hour respectively. The accuracy was expressed as relative error and precision was expressed as the % CV.

In inter-day study the sample solutions were analyzed on 1st, 2nd and 3rd day. From the result it was found that REPA has drug content of 2.005, 2.012 and 2.01µg/ml, MET has 499.185, 499.346 and 499.226µg/ml on first, second and third day respectively. Both intra and inter day accuracy were within acceptability criteria for relative error, ± 5 % and for precision study the % CV was within acceptability criteria i.e. < 2 indicating the method was précised for quantitative estimation of both drugs.

To find the detection limit of the drugs LOD and LOQ studies were performed. The LOD values were 0.0339, 0.0386 µg/ml and LOQ values were 0.1025, 0.1169µg/ml for REPA and MET respectively.

Selectivity of the developed method was conformed as the resolution between the chromatograms of REPA and MET was 2.31 and respectively. This suggests that under the proposed chromatographic conditions REPA and MET were completely separated from each other. Which indicates the method is selective for simultaneous estimation.

Specificity was assessed by comparing the chromatograms of tablet solution with the placebo solution and also with the chromatograms obtained from standard drugs. As the retention time for both the drugs were same in standard solution as well as in tablet sample solution and also there was no extra peak co-eluted for diluents indicate the specificity of the method for quantitative estimation of both the drugs in commercially available formulation.

CONCLUSION

The proposed RP-HPLC method was found to be simple, specific, accurate, precise, robust, rapid and economical. This method gives good resolution between all the two compounds with a short analysis time. The proposed RP-HPLC method can be useful for routine analysis of Metformin hydrochloride and Repaglinide in the tablet dosage form.

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