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SIMPLE RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF METFORMIN HYDROCHLORIDE AND FENOFIBRATE IN TABLET DOSAGE FORM

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ABSTRACT

A simple, specific, accurate and precise reverse phase high pressure liquid chromatographic method has been developed for the simultaneous determination of Metformin Hydrochloride (MET) and Fenofibrate (FENO) in tablet dosage form by using Hypersil Octadecylsilane column (250mm x4.60mm, 5µm). The samples were analyzed by using Methanol : Buffer (0.5% Ammonium dihydrogen orthophosphate adjusted to pH-3 using orthophosphoric acid)- 55 : 45v/v as a mobile phase at the flow rate of 1.0 ml/min in isocratic mode and detection wavelength is 226 nm. Both the drugs were eluted within 5 minutes and gave sharp peaks with high theoretical plate count and low tailing factor. The retention time for Metformin Hydrochloride and Fenofibrate was found to be 2.484 and 4.616 min respectively. The validation was carried according to ICH guidelines. Calibration curve was linear with correlation coefficient of 0.998 and 0.999 over a concentration range of 20-100µg/ml and 6.4-32µg/ml for Metformin Hydrochloride and Fenofibrate respectively. The percent recovery was 99.59 for Metformin Hydrochloride and 99.54 for Fenofibrate indicating accuracy and reliability of method. So the method can be used for estimation of combination of these drugs in tablet dosage form.

KEYWORDS : Metformin Hydrochloride, Fenofibrate, RP-HPLC.

INTRODUCTION

Metformin Hydrochloride (MET) is chemically 1,1-dimethyl Biguanide hydrochloride [Fig.1] Fenofibrate (FENO) is chemically 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl-propanoic acid [Fig.2]. Metformin is Oral Hypoglycaemic drug and it acts by suppressing hepatic gluconeogenesis and glucose output from liver. Fenofibrate is Hypolipidaemic drug and primarily activate lipoprotein lipase which is a key enzyme in the degradation of VLDL resulting in lowering of circulating TG's. A combination of 500mg MET and 160mg FENO is available commercially as tablets. This combination is used in the treatment of Non-insulin dependent diabetes mellitus [NIDDM]. Metformin is official in IP, BP, USP, while Fenofibrate is official in BP and USP.[1-5]

Literature survey revealed that UV spectrophotometric, HPLC methods, HPTLC and Capillary electrophoresis methods are available for estimation of MET and FENO individually and also in combination with other drugs [6-15]. Few reports are also available on the simultaneous estimation of Metformin Hydrochloride and Fenofibrate using techniques like Spectrophotometric and RP-HPLC but they are suffering from their own drawbacks like high retention time or poor resolution or use of expensive solvents etc.

The aim of present work is to develop and validate [16,17] a new, simple, better and economical method for the simultaneous estimation of Metformin Hydrochloride and Fenofibrate in tablet dosage form by RP-HPLC with improved conditions and parameters for routine use in the laboratories. The chemical structures of the assayed compounds are given below.

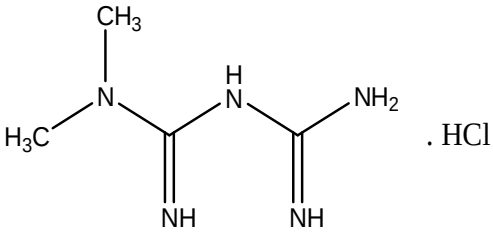

Fig.1 - Structure of Metformin Hydrochloride

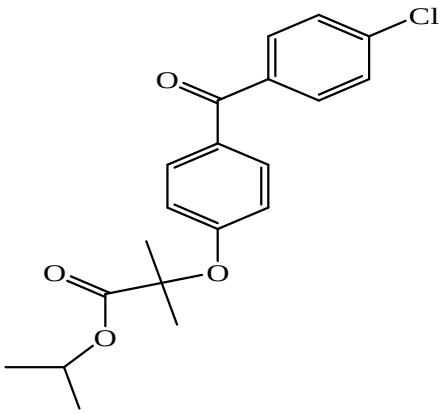

Fig.2 - Structure of Fenofibrate

1. EXPERIMENTAL WORK

1.1. Reagents and Materials

Pure drug samples of MET and FENO were obtained as gift samples from MSN Laboratories, Hyderabad, Andhra Pradesh, India. HPLC Grade Methanol, Ammonium dihydrogen orthophosphate and Orthophosphoric acid were procured from Merck. In-house Millipore water was used throughout the study. Fixed dose combination tablets (FIBMET) containing 500mg MET and 160mg FENO were procured from local market.

1.2. Instrumentation

The HPLC analysis was accomplished on WATERS high pressure liquid chromatograph outfitted with 515 reciprocating dual column HPLC pump, a manually operating Rheodyne injector with 20µL sample loop, a 250mm x 4.6mm I.D. analytical column (HYPERSIL ODS) containing C-18 reversed-phase material of 5µm size and a 2489 model UV-Visible detector. All the parameters of HPLC were controlled by EMPOWER – 2 software. Other instruments used were Systronics double beam UV-Vis spectrophotometer of model 2201, Shimadzu electronic balance of model AY-220, MKVI digital pH meter and Bio-technics ultrasonic bath sonicator.

Table-1 : Optimized chromatographic conditions

Separation Variable	Optimized condition
Mobile Phase	Methanol : Buffer (0.5% Ammonium dihydrogen orthophosphate adjusted to pH 3 using orthophosphoric acid) - 55 : 45
Column	Hypersil ODS (250mm × 4.6mm, 5µm)
Flow Rate	1ml/min
Temperature	Ambient
Wavelength	226nm
Injection Volume	20µl
Retention Time	MET - 2.4min, FENO - 4.6min.

1.3. Solubility study

Table-2 : Solubility study data of Metformin Hydrochloride and Fenofibrate

SOLVENT	MET	FENO
Water	+	-
Ethanol (95%)	+	+
Acetone	-	+
Methanol	+	+
Acetonitrile	+	+

+ indicates solubility

- indicates insolubility

From solubility study data MET and FENO, both were found to be soluble in ethanol (95%), methanol and acetonitrile. For the present method methanol was selected as a suitable solvent as both drugs have excellent solubility in methanol when compared to 95% ethanol and was cheap and economical when compared to acetonitrile.

1.4. Determination of working wavelength (λ_{max})

Standard solutions of MET and FENO were scanned in the UV range (200-400nm) and the spectrums obtained were overlaid. From the overlain spectrum, 226nm was selected as the detection wavelength for the present study.

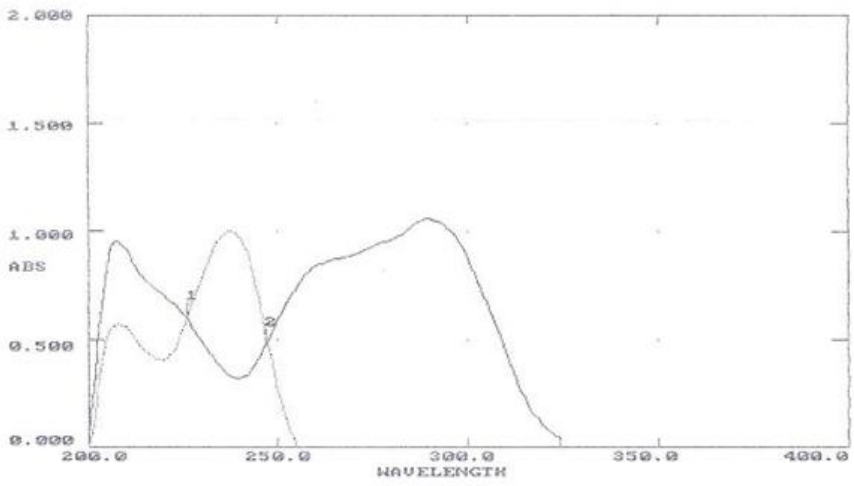

Fig.3 - Overlain UV spectra for MET and FENO

1.5. Preparation of Solutions

1.5.1. Preparation of Buffer (0.5%w/v Ammonium dihydrogen orthophosphate) : 0.5gm of ammonium dihydrogen orthophosphate was dissolved in sufficient amount of water and was made up to 100ml with the same . Then the pH of the solution was adjusted to 3 by using orthophosphoric acid.

1.5.2. Preparation of mobile phase: 450ml (45%) of the above buffer was mixed with 550ml of methanol (55%), this solution was filtered through 0.45µ membrane filter under vaccum filtration and degassed in ultrasonic water bath for 5minutes.

1.5.3. Diluent : Mobile phase was used as diluent.

1.5.4. Preparation of standard stock solutions: Standard stock solution of Metformin Hydrochloride and Fenofibrate were prepared by dissolving 25mg of drug in 15ml of diluent separately and it was sonicated for 10min, then the volume was made up to 25ml (1000µg/ml). This solutions were labelled as standard stock solution-I. 2.5ml of the above solutions was pipetted in to a two separate 25ml volumetric flasks and the volume was made up to the mark with the diluent to obtain a solutions of concentration 100µg/ml. This solutions were labelled as standard stock solution-II.

1.5.5. Preparation of mixed standard solution : From 100µg/ml standard solutions of Metformin Hydrochloride and Fenofibrate, 3ml of Metformin Hydrochloride and 0.96ml of Fenofibrate were pipetted in to a 10ml volumetric flask and was made up to the mark with the diluent.

1.5.6. Preparation of test solution: Twenty tablets of Fibmet were weighed, ground in to a fine powder and mixed thoroughly. A quantity of powder equivalent to 10mg of Metformin Hydrochloride (3.2mg of Fenofibrate) was weighed and transferred in to a 100ml volumetric flask and was dissolved in the diluent. The volume was made up to the mark with the same and the resulting solution was labelled as test stock solution. A solution containing 30µg/ml of Metformin Hydrochloride (9.6µg/ml of Fenofibrate) was prepared by appropriate dilution of the test stock solution. The solutions thus prepared were filtered through 0.45µ membrane filter and the filtrate was sonicated for 10min.

1.6. Assay

Procedure: 20µl of test solution (i.e., solution containing 30µg/ml of MET and 9.6µg/ml of FENO) was injected three times at the optimized method conditions and the chromatograms of three injections were recorded and the % Assay was calculated by using the formula

$$\%ASSAY = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{AW}{LC} \times 100$$

AT - Average area counts of sample preparation

AS - Average area counts of standard preparation

WS - Weight of working standard taken in mg

DS - Dilution of standard preparation

DT - Dilution of sample preparation

WT - Weight of sample taken in mg

P - Percentage purity of working standard.

LC - Label claim

AW - Average weight of tablets

1.7. Method Validation

The developed analytical method was validated as per ICH guidelines with respect to parameters such as system suitability, precision, linearity, accuracy, limit of detection, limit of quantitation, robustness and specificity.

1.7.1. System suitability

To verify that the analytical system is working properly and can give accurate and precise results, was evaluated by injecting 20µl of mixed standard solution i.e. solution containing 30µg/ml of Metformin Hydrochloride and 9.6µg/ml of Fenofibrate was injected for five times and the chromatograms were recorded for the same and the results were summarized in the Table-4.

1.7.2. Precision

The precision was studied at the levels of repeatability (intra-day) and intermediate precision (inter-day, analyst-analyst). The intra-day precision was determined by injecting sample solution containing 30µg/ml of MET and 9.6µg/ml of FENO on the same day whereas the inter-day precision was determined by injecting sample solution containing 30µg/ml of MET and 9.6µg/ml of FENO for five times, were prepared separately on different days and by different analysts. The percentage assays for repeatability and intermediate precisions were

calculated and tabulated.

1.7.3. Linearity

To establish linearity of this method, a series of dilutions ranging from 20-100µg/ml for Metformin Hydrochloride and 6.4-32µg/ml for Fenofibrate were prepared and injected in to HPLC system, the chromatograms were recorded and calibration curves were constructed by plotting peak area against concentration and the regression equations were computed.

1.7.4. Accuracy

The accuracy of the proposed method was evaluated by recovery studies which were carried out by standard addition method, where a known amount of sample solution was spiked with mixed standard solution at three levels of 50%, 100% and 150%. At each level recovery studies were carried out in triplicate and expressed as percent recoveries.

Preparation of Mixed standard stock solution: 10mg of Metformin Hydrochloride and 3.2mg of Fenofibrate were accurately weighed and transferred in to a 100ml volumetric flask and was dissolved in the diluent. The volume was made up to the mark with the diluent. The resulting solution contains 100µg/ml of Metformin Hydrochloride and 32µg/ml of Fenofibrate.

Preparation of standard and test solutions: Mixed standard solutions containing 15µg/ml, 30µg/ml and 45µg/ml of MET (4.8µg/ml, 9.6µg/ml, 14.4µg/ml of FENO) respectively were prepared in triplicate, from the mixed standard stock solution by appropriate dilutions. Sample solution containing 30µg/ml of MET (9.6µg/ml of FENO) was prepared by appropriate dilution of the sample stock solution (containing 100µg/ml of MET and 32µg/ml of FENO).

Procedure of Spiking: Spiking at 50% level was accomplished in triplicate, by adding of 3ml of unfiltered sample stock solution (containing 100µg/ml of MET and 32µg/ml of FENO) to 1.5ml of mixed standard stock solution (containing 100µg/ml of MET and 32µg/ml of FENO) in a test tube. The contents of test tube were then shaken for some time and cautiously filtered through whatmann filter paper and the filtrate was collected in to a 10ml volumetric flask. In order to collect the remnants of the solution, the test tube and filter paper were washed with small quantities of diluent, and the washings were used to make up the

solution up to the mark. The resultant solution was filtered through 0.45µ membrane filter and sonicated for five minutes.

In the similar manner, spiking at 100% level and 150% levels was carried out by adding 3ml of unfiltered sample stock solution separately to 3ml and 4.5ml of mixed standard stock solution respectively.

Accuracy is calculated in terms of percentage recovery which is given as

$$\% \text{ Recovery} = \frac{b - a}{c} \times 100$$

Where,

a - response of test solution

b - response of spiked solution

c - response of standard solution

1.7.5. Limit of detection(LOD)

0.024µg/ml solution of MET and 0.043µg/ml solution of FENO were prepared by making appropriate dilutions from standard stock solution II of MET and FENO respectively, injected in to HPLC system at the optimized chromatographic conditions and signal to noise ratio (S/N) was calculated.

1.7.6. Limit of quantitation (LOQ)

0.06µg/ml solution of MET and 0.124µg/ml solution of FENO were prepared by making appropriate dilutions from standard stock solution II of MET and FENO respectively, injected in to HPLC system at the optimized chromatographic conditions and signal to noise ratio (S/N) was calculated.

1.7.7. ROBUSTNESS

Robustness of the method was demonstrated by making deliberate changes in the optimized conditions of the developed method. It was determined by injecting mixed standard solution of Metformin Hydrochloride (30µg/ml) and Fenofibrate (9.6µg/ml) for five times. The chromatograms were recorded and the results were summarized in the Table-33-38. % RSD of the peak areas were calculated for both the drugs at each of the following conditions :

a. At different flow rate (±0.2ml/min).

b. At different concentration of mobile phase.

c. At different wavelength (±2nm)

1.7.8. Specificity

An interference study was evaluated with placebo. Placebo in proportionate to test concentration was prepared. The solution thus prepared was filtered and sonicated for 10 minutes and then injected into HPLC system.

2. RESULTS AND DISCUSSION

To develop a precise, accurate and suitable RP-HPLC method for the simultaneous estimation of Metformin Hydrochloride and Fenofibrate, different mobile phases were tried but the proposed chromatographic conditions were found to be appropriate for the quantitative determination.

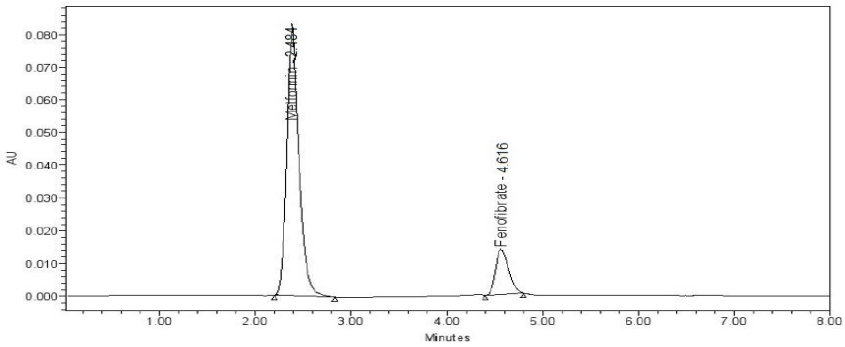

Fig.4 - Chromatogram of standard

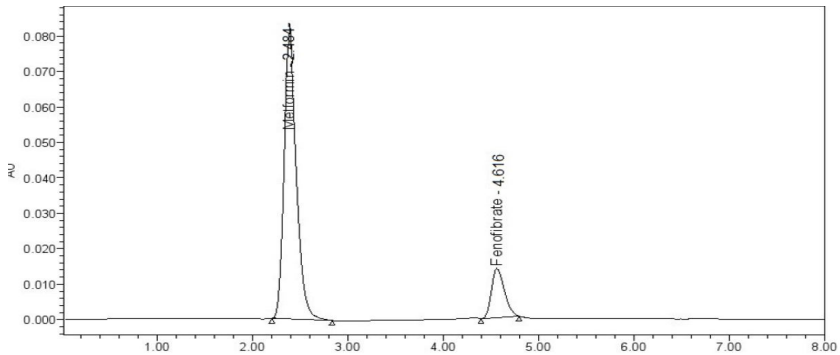

Fig.5 - Chromatogram of test

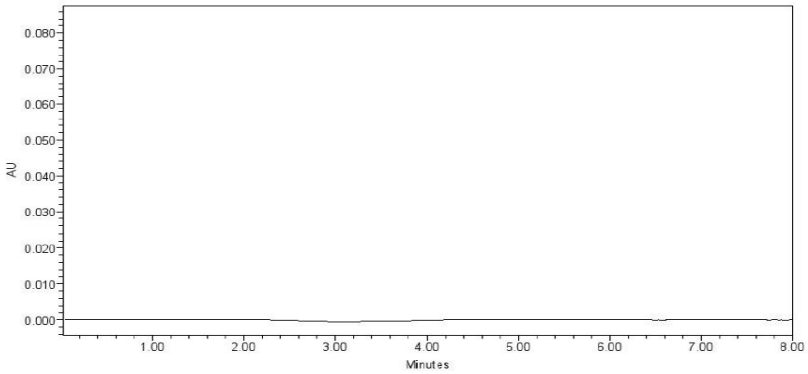

Fig-6 - Chromatogram of Placebo

The assay was performed on the tablet formulation (FIBMET, Sun Pharmaceutical Industries Ltd), and the % drug contents for Metformin Hydrochloride and Fenofibrate were found to be 99.48 and 99.68 respectively which were within the acceptance limits.

Table-3 : Results of Assay

Parameters	Metformin Hydrochloride	Fenofibrate
Standard peak area (mean)	718055	121226
Test peak area (mean)	715027	121083
Average Weight	800mg	800mg
Label claim	500mg	160mg
% Purity of Standard	99.9	99.9
Amount Obtained	497.4mg	159.4mg
% Assay	99.48	99.68

The system suitability of the proposed method was accomplished from the resolution, theoretical plate count and asymmetric factor of Metformin Hydrochloride and Fenofibrate at the optimized conditions. The parameters were recorded and tabulated (table 4) and were found to be in compliance with the acceptance specifications.

Table-4 : System Suitability Data of Metformin Hydrochloride and Fenofibrate

Drug	Retention Time	Peak Area	Theoretical Plate Count	Asymmetric Factor	Resolution
MET	2.484	716798	2718	1.29	-
FENO	4.616	121101	4065	1.13	3.78

The precision of the proposed method was established from the %RSDs of the percentage assays of the drugs at the levels of repeatability (intra-day) and intermediate precision (inter-day, analyst-analyst). The %RSDs of Metformin Hydrochloride and Fenofibrate at intra-day precision were found to be 0.15% and 0.65% respectively, at inter-day precision were found

to be 0.34 and 1.26% respectively and at the level of analyst-analyst variation, they were found to be 0.49% and 0.62% respectively. As the %RSDs were found to be within the acceptance limit (%RSD < 2%) at all the levels, the proposed method was said to be precise.

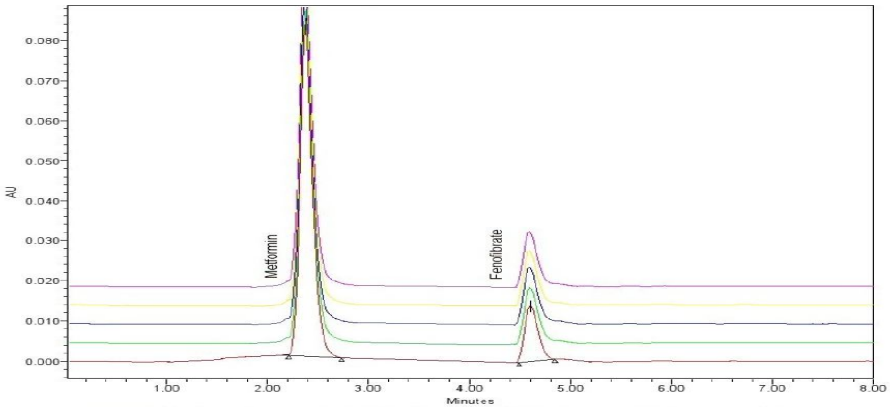

Fig.7 - Overlain Chromatogram for Intra-Day Precision

Table-5 : Results of Repeatability and Intermediate Precision

Parameters	Injection	Repeatability		Intermediate Precision			
		Day-I, Analyst-I		Day-II		Analyst-II	
		MET	FENO	MET	FENO	MET	FENO
Percentage Assay	1	99.68	100.00	99.16	101.32	99.03	99.28
	2	99.80	100.29	99.14	98.05	99.77	98.85
	3	99.42	99.87	99.66	99.14	99.87	98.79
	4	99.75	99.04	99.52	99.98	99.61	100.3
	5	99.55	98.77	99.94	100.49	98.75	99.63
Statistical Analysis	Mean	99.64	99.59	99.48	99.79	99.40	99.37
	SD	0.15	0.65	0.34	1.26	0.49	0.62
	%RSD	0.15	0.65	0.34	1.26	0.49	0.62

The linearity of the proposed method was accomplished from the correlation coefficients of the standard calibration curves of Metformin Hydrochloride and Fenofibrate which were constructed at concentration ranges of 20-100µg/ml and 6.4-32µg/ml respectively. The correlation coefficients of Metformin Hydrochloride and Fenofibrate were found to be 0.998 and 0.999 respectively which were in compliance with the acceptance criteria.

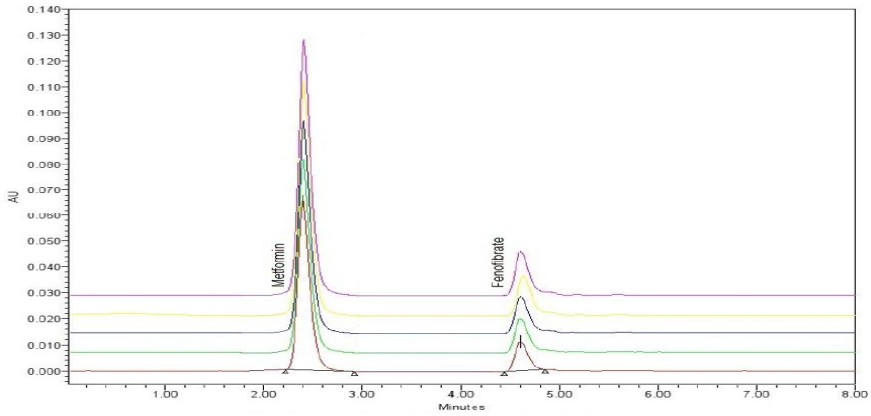

Fig.8 - Overlain Chromatogram for Linearity

Table-6 : Linearity Results for Metformin Hydrochloride and Fenofibrate

Preparations		Metformin Hydrochloride		Fenofibrate	
		Concentration (µg/ml)	Peak Area	Concentration (µg/ml)	Peak Area
I		20	465042	6.4	83217
II		40	973896	12.8	156434
III		60	1399563	19.2	249652
IV		80	1901581	25.6	332869
V		100	2296506	32	416086
Statistical Analysis	Slope	-	22953	-	13159
	Y-intercept	-	30134	-	5000.3
	Correlation Coefficient (r^2)	-	0.998	-	0.999

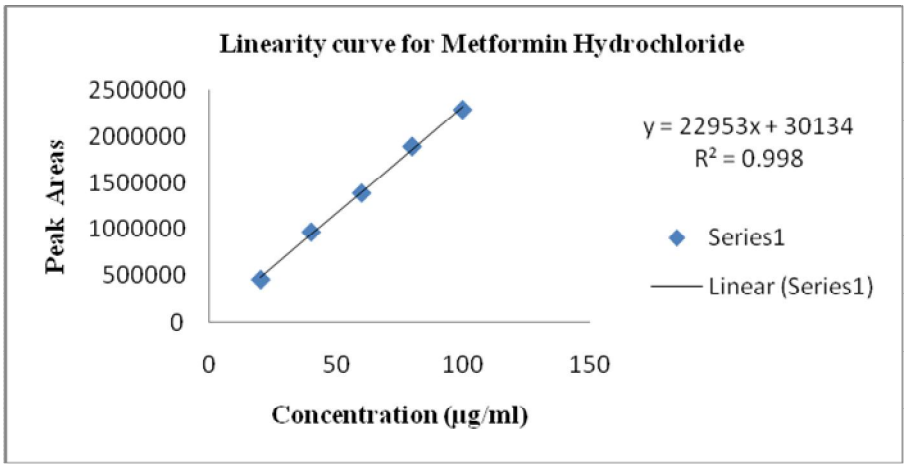

Fig.9 - Linearity curve for Metformin Hydrochloride

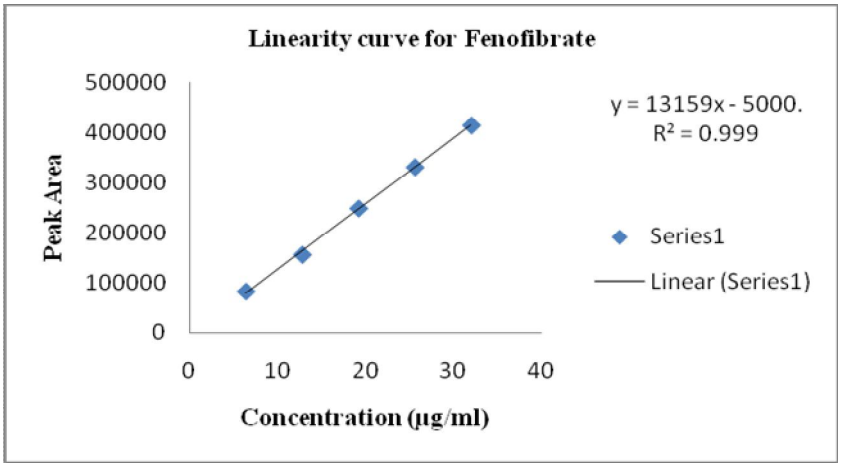

Fig.10 - Linearity curve for Fenofibrate

The accuracy of the proposed method was evaluated from the recovery studies, by standard addition method which was performed at three levels of 50%, 100% and 150%. The mean percentage recoveries at each level of Metformin Hydrochloride and Fenofibrate were found to be 99.33-99.99% and 99.35-99.78% respectively which fell within the acceptance limits. Hence the method was considered to be accurate.

Table-7 : Accuracy data for Metformin Hydrochloride

Spiked levels	Standard		Sample	Spiked		% Recovery	Mean % recovery
	Conc. (µg/ml)	Peak Area	Conc. (30 µg/ml) Peak Area	Conc. (µg/ml)	Peak Area		
50	15	352822	690868	45	1039861	99.00	99.33
		352602	690151		1041032	99.40	
		352291	690592		1041527	99.61	
100	30	690859		60	1378474	99.57	99.54
		690956			1376239	99.23	
		690486			1379853	99.83	
150	45	1037832		75	1727109	99.87	99.90
		1037972	Mean		1728150	99.97	
		1037872	690537		1727193	99.88	

Table-8 : Accuracy data for Fenofibrate

Spiked levels	Standard		Sample	Spiked		% Recovery	Mean % recovery
	Conc. (µg/ml)	Peak Area	Conc. (9.6 µg/ml) Peak Area	Conc. (µg/ml)	Peak Area		
50	4.8	63626	124360	14.4	189592	99.75	99.51
		63423	128041		189193	99.44	
		63861	125967		189579	99.36	
100	9.6	127884		19.2	253927	99.93	99.78
		125975			251869	99.81	
		126029			251683	99.62	
150	14.4	186654		24	312551	99.87	99.35
		187405	Mean		311974	99.17	
		188450	126122		312739	99.02	

LOD for Metformin Hydrochloride

Calculation of S/N ratio :

Average base line noise obtained from blank : 45 µV

Signal obtained from LOD solution : 138 µV

S/N = 138/45 =3.07

LOD for Fenofibrate

Calculation of S/N Ratio

Average base line noise obtained from blank : 45µV

Signal obtained from LOD solution : 139µV

S/N = 139/45 = 3.09

S/N ratio value should be 3 for LOD solution. The LOD for this method was found to be 0.024µg/ml for Metformin Hydrochloride and 0.043µg/ml for Fenofibrate respectively.

LOQ for Metformin Hydrochloride

Calculation of S/N Ratio

Average baseline noise obtained from blank - 45µV

Signal obtained from LOQ solution - 452µV

S/N = 452/45 = 10.04.

LOQ for Fenofibrate

Calculation of S/N Ratio

Average base line noise obtained from blank - 45µV

Signal obtained from LOQ solution - 449µV

S/N = 449/45 = 9.98

S/N Ratio value should be 10 for LOQ solution. The LOQ for this method was found to be 0.06µg/ml for Metformin Hydrochloride and 0.124µg/ml for Fenofibrate respectively.

Robustness of the proposed method was demonstrated by making deliberate changes in the flow rate, concentration of organic phase and wavelength from the optimized conditions of the developed method and computing the %RSD of the peak areas. The %RSDs for Metformin Hydrochloride and Fenofibrate at 0.8 ml/min were found to be 1.05% and 0.71% respectively and at 1.2 ml/min they were found to be 0.39% and 0.73% respectively. At a mobile phase composition of methanol and buffer in a ratio of 54:46, the %RSDs for Metformin Hydrochloride and Fenofibrate were found to be 0.41% and 1.50% respectively and at a ratio of 56:44 of methanol and buffer, they were found to be 0.22% and 0.46% respectively. At a wavelength of 224nm, the %RSDs for the drugs were found to be 0.75%, 0.38% and at 228nm they were found to be 0.22% and 0.59% respectively. As the %RSDs of peak areas and system suitability parameters were found to be within the acceptance limit, the proposed method was said to be robust.

Table-9 : Results for Robustness

Parameter		MET			FENO		
		Mean Rt	Mean Peak Area	%RSD of Peak Areas	Mean Rt	Mean Peak Area	%RSD of Peak Areas
Flow rate (ml/min)	0.8	2.511	719181	1.05	4.701	118925	0.71
	1.0	2.486	716537	0.48	4.613	119798	0.31
	1.2	2.315	718459	0.39	4.583	118252	0.73
Mobile phase (A:B)	46 : 54	2.520	725562	0.41	4.717	118558	1.50
	45 : 55	2.486	716537	0.48	4.613	119798	0.31
	44 : 56	2.323	716811	0.22	4.588	119170	0.46

Wave-length (nm)	**224**	2.485	711616	0.75	4.612	117289	0.38
	226	2.486	716537	0.48	4.613	119798	0.31
	228	2.484	714792	0.22	4.613	117879	0.59

As the peaks of analytes were well resolved and had no interference of excipients, it was concluded that the proposed method was specific to the drugs under study.

As all the validation parameters studied, complied with the acceptance criteria, the proposed method was said to be validated in accordance with ICH guidelines.

CONCLUSION

A simple and rapid reverse phase HPLC method was developed and validated according to ICH guidelines for the simultaneous determination of Metformin Hydrochloride and Fenofibrate in tablet dosage form. The developed RP-HPLC method was proved to be simple, rapid, robust, economical and reproducible. The validation data signifies good specificity, accuracy, precision, and reliability of the method. Because of its simplicity and low cost, the method can be used for routine practices in the laboratories.

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