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HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF *AMPHIPATHIC DERIVATIVE*-FENOFIBRATE FROM RAT PLASMA

Mohammed Omar[1], Anil Middha[2], Dr. D. Ramakrishna[3]

1. Department of Pharmacy, OPJS University, Churu, Rajasthan.
2. Department of Pharmacy, OPJS University, Churu, Rajasthan.
3. Sushrut Institute of Pharmacy, Taddanpalli, Pulkal (M), Medak Dist, Telangana

Abstract:

A rapid, sensitive, accurate HPLC analytical method development was determination of fenofibrate in rat serum. Nevirapine was used as an internal standard (IS). Absorbance UV detector at wavelength of detection was as set at 295nm. Separation was carried out on inertial C18 column (4.6x250mmx5µm) using 60:40 v/v ammonium acetate buffer, Acetonitrile as mobile phase at a flow rate of 1.0 ml/min. Analytical run time was less than 10 min. The % mean recovery for Fenofibrate in LQC, MQC and HQC was 62.7%, 64.2 % and 65.3% for 0.3 to 20.0µg/mL concentrations. The intraday and interday precision and Accuracy of the method was found to be 0.09 to 5.03% and 100.00 to 100.20% respectively. The limit of Quantification was found to be 0.3µg/ml at such concentration the inter day precision was found to be 0.07 to 0.29. The method was validated with excellent sensitivity, accuracy, precision and recovery. The assay has been applied to pharmacokinetic studies.

Key words: *HPLC, Fenofibrate, Nevirapine*

Address for Correspondence:
Dr. Anil Middha,
Department of Pharmacy, OPJS University,
Churu, Rajasthan,India.

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INTRODUCTION:

Fibric Acids (also referred to as fibrates) are amphipathic (one end is hydrophobic and one endhydrophilic) carboxylic acids characterized by the presence of a terminal carboxyl group (COOH) [1].

Fibrates are generally effective in lowering elevated plasma triglycerides and cholesterol. The magnitude of lipid changes depends, however, on the patient's pretreatment lipoprotein status [2].

The most pronounced effects of fibrates are a decrease in plasma triglyceride-rich lipoproteins (TRLs). Levels of LDL cholesterol (LDL-C) generally decrease in individuals with elevated baseline plasma concentrations, and HDL cholesterol (HDL-C) levels are usually increased when baseline plasma concentrations are low [3].

Fibrate treatment results in a reduction of the LDL fraction of atherogenic small, dense particles with an equivalent increase in the intermediate subfraction [4]. Within the triglyceride-rich apolipoprotein (apo) B-containing lipoproteins, fibrates efficiently reduce the apoC-III–containing particles [5].

The increased HDL concentrations after fibrates are generally reflected by increased plasma levels of apoA-I and apoA-II, a change that is associated with an increase in lipoprotein (Lp) A-I:A-II, and a decrease in LpA-I concentrations in patients treated with fenofibrate [6]. Fenofibrate is white off crystalline powder and stable under ordinary conditions, it has no chiral center. It is very soluble in ethanol, soluble in acetone and insoluble in water [7].

Fenofibrate belongs to a diverse group of active pharmaceutical ingredients that are used in the treatment of hyperlipidemia. They are called lipid-lowering drugs [8]. Fibrates reduce the large VLDL subfractions and attenuate the postprandial lipid response in hypertriglyceridemic subjects[9]. HDL-C levels, which are low in patients with hypertriglyceridemia, increase after treatment with fibrates [10], and increase HDL-C concentrations when used in monotherapy in patients with primary hypercholesterolemia [11]. The increase in HDL-C is related to a lower cholesteryl ester transfer protein activity, whereas LCAT activity is not affected [12]. In patients with primary hypercholesterolemia, LPL activity also increases on treatment with fibrates, resulting in a reduction of postprandial lipemia [13].

The reduction in total cholesterol is accounted for by a fall in both VLDL-C and LDL-C [14].

The aim of the present study is to develop a simple and rapid HPLC method with UV detection for the quantitative determination of fenofibrate in rat serum. The method uses HPLC-UV with Nevirapine as internal standard. This method offers the advantage of simplicity with adequate sensitivity, selectivity, precision and accuracy. This analytical method can be used for the estimation of fenofibrate in biological samples and successfully topharmacokinetic studies.

MATERIALS AND METHODS

Materials: Fenofibrate and nevirapine were obtained from CIPLA Pharmaceuticals, Mumbai. HPLC grade acetonitrile, ethylacetate, methanol were purchased from SD fine chemicals, Mumbai, India. Analytical Grade ammonium acetate was purchased from SD fine chemicals, Mumbai, India.

Instrumentation:

The HPLC system consisted of Alliance Waters 2695 with dual λ Absorbance UV detector. Separation was carried out on Inertsil C_{18} column (4.6x250mmx5µm). A vortex-mixer (Remi), ultrasonic bath (Bransonic), a model C-30 centrifuge (Remi) and a model cool safe 110-4/scan speed 32 were used for sample preparation.

Chromatographic Conditions:

The HPLC system consisted of Alliance waters 2695 with dual λ Absorbance UV detector. The wavelength of detection as set at 295nm. Separation was carried out on inertsil C18 column (4.6x250mmx5µm) using 60:40 v/v ammonium acetate buffer, Acetonitrile as mobile phase at a flow rate of 1.0 ml/min.The mobile phase filtered through nylon milli pore (0.2µm) membrane filter, purchased from pall life sciences, Mumbai and degassed with Ultrasonicator prior to use. Chromatography was carried out at room temperature 25^0c and maintains the column temperature at 32^0c.

Preparation of Stock Solutions:

Stock solutions of Fenofibrate (500 µg/mL) and Nevirapine (1000 µg/mL) internal standard were prepared in Methanol. Further dilutions were carried out in 60% acetonitrile.

Preparation of Calibration Standards:

Calibration standards were prepared freshly by spiking drug free plasma with Fenofibrate stock solution to give the concentrations of 0.3, 0.6, 1.2, 2.5, 5.0, 10.0, and 20.0µg/mL. They were stored at -20^0c till the time analyzed. Detailed procedure for preparation is shown in Table-01.

Table 1: Preparation of Fenofibrate Calibration Standards in Plasma

Fenofibrate	***Concentration (µg/mL)***	***Drug stock solution (µL)***	***Blank plasma (µL)***
FFB CS1	0.3	0.6	999.4
FFB CS2	0.6	1.2	998.8
FFB CS3	1.25	2.4	997.6
FFB CS4	2.5	5.0	995.0
FFB CS5	5.0	10.0	990.0
FFB CS6	10.0	20.0	980.0
FFB CS7	20.0	40.0	960.0

Preparation of Quality Control Standards:
Quality control standards were prepared at three levels namely LQC, MQC and HQC. These standards were prepared freshly by spiking drug free plasma with Fenofibrate stock solution to give the concentrations of 1.0, 8.0 and 16.0µg/mL respectively. They were stored at -20^{0}c till the time analysed. Detailed procedure for preparation is shown in Table-2.

Table 2: Preparation of Fenofibrate Quality Control Standards in Plasma

Fenofibrate	*Concentration (µg/mL)*	*Drug stock solution (µL)*	*Blank plasma (µL)*
FFB LQC	1.0	2.0	998.0
FFB MQC	8.0	16.0	984.0
FFB HQC	16.0	32.0	968.0

Sample Preparation Method:
Drug free human plasma (500µl) is spiked with appropriate volume of drug stock. To the above prepared sample, 50µl of Nevirapine (50µg/mL) was added as an IS. The sample was vortex mixed for 4 minutes to assure complete mixing. Analytes were extracted by adding with 3ml of ethyl acetate followed by vortex mixing for 4 minutes and centrifugation at 2000 rpm/min in a cooling centrifuge for 15min at 4^0 C.
The organic phase containing analytes was separated and analytes were obtained as dried residues after drying in vacuum concentrator. The analyte residue obtained was reconstituted with 250µl of mobile phase and analysed using HPLC system according to parameters optimized.

Chromatographic Conditions [15]:
The HPLC system consisted of Alliance Waters 2695 with dual λ Absorbance UV detector. The detection wavelength for analysing drug was set at 240 nm. Separation was carried out on Inertsil C_{18} column (4.6x250mmx5µm) using 40:60 v/v Phosphate buffer (pH-3.0) and Acetonitrile as mobile phase at a flow rate of 1.0 ml/min. The mobile phase was filtered through nylon Millipore (0.22 µm) membrane filters and degassed with ultra sonicator for 15 minutes prior to use. Chromatograms were obtained and integrated using Empower software which was provided along with the HPLC system.

Experimental Validation Parameters [16, 17]

Specificity:
A solution containing 0.3µg/ml Fenofibrate was injected on to the column under optimized chromatographic conditions to show the separation of Fenofibrate from the impurities from the plasma. The specificity of the method was checked for the interference from plasma.

Linearity and Range:
Six samples of each calibration standard were assayed. Wide range calibration was determined by solutions containing 0.3µg/ml to 20.0µg/ml. The spiked concentration and their respective peak area ratios with respective to internal standards were subjected to least squares regression. After examining the percent deviation, a proper model was chosen. The slope, Y-intercept, and coefficient of determination (r^2) were obtained from linear regression analysis performed.

Limit of Detection (LOD) and Limit of Quantification (LOQ):
The limit of quantitation refers to the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy. There are different approaches to determine the LOQ and LOD. Typically the concentration level that generates a signal-to-noise (S/N) of 10 is regarded as the LOQ and the concentration level that generates S/N = 3 is regarded as the LOD. Here LOD is calculated using the formula 3.3 times σ/s where "σ" is standard deviation of the intercept obtained for calibration curve and "s" is the slope of the calibration curve. Similarly LOQ is calculated using the formula 10 times σ/s.

Precision and Accuracy:
Intraday precision and accuracy was determined by analyzing quality control standards (1.0, 8.0 and 16.0µg/ml) and LLOQ Quality control standards (0.15µg/ml) five times a day randomly, interday precision and accuracy was determined from the analysis of each quality control stds (1.0, 8.0 and 16.0µg/ml) and LLOQC standards (0.3µg/ml) once on each of five different days.

Ruggedness:
Ruggedness of the method was determined by analysing spiked control samples of (n=6) medium concentrations i.e 8.0µg/ml using two different columns.

Recovery Studies:
The % mean recoveries were determined by measuring the responses of the extracted plasma Quality control samples at HQC, MQC and LQC against unextracted Quality control samples at HQC, MQC and LQC.
For calculation of recovery of the compound, spiked control samples were prepared at low, medium, and high concentrations i.e 1.0, 8.0 and 16.0µg/mL concentrations. The samples were processed as mentioned above and the concentration of the compound was determined from the regression of the analytical standard calibration curve. Recovery was calculated by comparing the observed concentrations in spiked samples to that of respective unextracted samples.

Stability Studies:

The stability of Fenofibrate was determined by measuring concentration change in control samples overtime under set conditions.

Freeze-thaw stability study of Fenofibrate was carried out by subjecting samples to three freeze and thaw cycles. Samples before study and after study were analysed by developed method. Results were subjected to paired two tail t-Test for means (α=0.05) to figure out any difference in initial and final set of samples. Similarly stock solution stability study of Fenofibrate (Stability after 6 Hours), bench top stability study of Fenofibrate (Stability after 10 Hours) and Inter injection stability study of Fenofibrate were carried out by subjecting samples to study conditions.

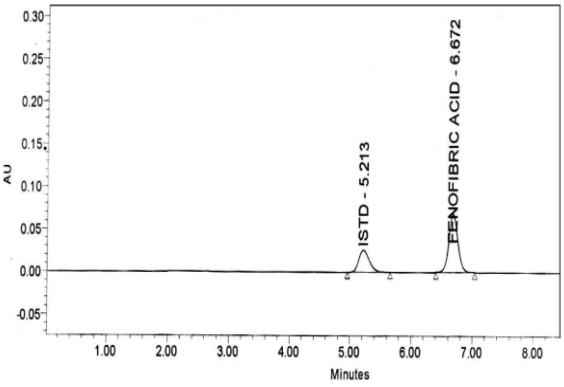

Fig 1: Chromatogram showing Retention times of Aqueous Mixture (FFB $_{aqmix}$) consist of Fenofibric acid (5µg/ml) and Nevirapine (5µg/ml).

Data Analysis:

Data analysis was done using MS-Excel package for calculating mean, standard deviation, % relative standard deviation, following formulae were used in calculations.

$$\%CV = \frac{SD}{Mean} \times 100$$

$$\%Bias = \frac{\text{Observed concentration} - \text{Nominal concentration}}{\text{Nominal concentration}} \times 100$$

RESULTS AND DISCUSSIONS:

Under the chromatographic conditions employed, the sample showed sharp peaks for drug and internal standard with good resolution. The retention time for the drug was found to be 6.672±0.05 minutes and the retention time for internal standard was 5.213±0.03 minutes. The method developed was validated for specificity, accuracy, precision, linearity, range and stability as per USFDA guidelines. The results of validation parameters are given below.

Specificity of the method was proven by the absence of peaks near the retention time of the drug as well as the internal standard shown in figure-1).

The calibration function was developed for Peak area ratio Vs Concentration (in µg/ml) and it was linear over concentration range of 0.3 to 20.0µg/mL. Given in below table 3. The regression line equation for the analysis was y= 5.32e-0.05x-4.82e-0.003 with coefficient of correlation (r^2) = 0.9979 are given in below table no 3

Table 3: Mean Concentrations of Fenofibrate Calibration Standards

Fenofibrate	***Concn (µg/ml)***	***Mean of calculated concns***	***SD***	***% CV***
FFB CS1	0.3125	0.3186	0.013	3.95
FFB CS2	0.625	0.6123	0.063	10.31
FFB CS3	1.25	1.243	0.011	0.85
FFB CS4	2.5	2.558	0.010	0.41
FFB CS5	5.0	4.951	0.128	2.59
FFB CS6	10.0	10.155	0.132	1.30
EPR CS7	20.0	19.949	0.478	2.40

The LOD is calculated using the formula 3.3 times σ/s where "σ" is standard deviation of the intercept obtained for calibration curve and "s" is the slope of the calibration curve. Similarly LOQ is calculated using the formula 10 times σ/s. The calculated LOD and LOQ are shown in Table-04.

Table 4: Calibration Curve Parameters for Fenofibrate

	1	**2**	**3**	**Mean**	**SD**
Slope	0.0246	0.025	0.0247	0.0249	0.0004
Inter cept	0.0003	0.001	0.00044	0.0007	0.0006
r^2	0.999	0.997	0.998	-	-
LOD	0.084 µg/mL	-	-	-	-
LOQ	0.254 µg/mL	-	-	-	-

System suitability test was carried out by injecting six aqueous mixtures of Fenofibrate and IS. The following parameters were observed for repeated injections (n=6) of both Fenofibrate and internal standard.

The intraday and interday precision and Accuracy of the method was found to be 0.09 to 5.03% and 100.00 to 100.20% respectively for the quality control samples. This is within the acceptance limits of precision is 15% and accuracy is 85 to 115% (Tables-05 and 06).

Table 5: Intra-day Accuracy and Precision for Fenofibrate

Fenofibrate	*QC ID*	*LQC*	*MQC*	*HQC*
	conc.(ng/mL)	*1.0*	*8.0*	*16.0*
Intraday	1	1.062	8.527	17.05
	2	1.076	8.447	17.09
	3	1.068	8.975	17.075
	4	1.073	8.046	17.069
	5	1.073	9.131	17.087
	Mean	1.070	8.625	17.074
	± SD	0.0055	0.4344	0.0160
	% CV	0.5142	5.0372	0.0938
	% Accuracy	100.00	100.20	100.00

Table 6: Inter-day Accuracy and Precision for Fenofibrate

Fenofibrate	*QC ID*	*LQC*	*MQC*	*HQC*
	Actual conc.(ng/mL)	*1.0*	*8.0*	*16.0*
Interday	1	1.049	8.176	16.495
	2	1.055	8.177	16.481
	3	1.05	8.184	16.514
	4	1.055	8.181	16.493
	5	1.049	8.191	16.508
	Mean	1.051	8.181	16.498
	± SD	0.0031	0.0060	0.0130
	% CV	0.2976	0.0740	0.0789
	% Accuracy	100.00	100.00	100.00

The limit of Quantification was found to be 0.3µg/ml at such concentration the inter day precision was found to be 0.07 to 0.29 and the accuracy was 100.0% to 100.0% respectively for the quality control samples. Which are within the acceptance limits of precision is 20% and accuracy is 80 to 120% (Table-07).

Table 7: Precision & Accuracy of LLOQC Standard

Fenofibrate	*LLOQC*
Actual conc.(µg/mL)	*0.3125*
1	0.307
2	0.323
3	0.319
4	0.317
5	0.332
Mean	0.3196
±SD	0.0090
%CV	2.8471
% Accuracy	100.06

Ruggedness: the data of fenofibrate ruggedness is given on below table: 08

Table 8: Ruggedness of the Method Developed for Fenofibrate (on different columns)

MQC	*Column 1 (area ratio)*	*Column 2 (area ratio)*
1	0.869	0.886
2	0.873	0.886
3	0.878	0.864
4	0.884	0.882
5	0.862	0.842
6	0.852	0.855
Mean	0.870	0.869
± SD	0.011	0.018
% CV	1.32	2.12
% Accuracy	100.06	

The % mean recovery for Fenofibrate in LQC, MQC and HQC was 62.7%, 64.2 % and 65.3% respectively (Tables-09 to 11).

Table 9: Recovery Study for Fenofibrate from rat Plasma

Fenofibrate	*LQC*		
	Unextracted (area ratio)	*Extracted (area ratio)*	*%Recovery*
1	0.099	0.062	62.63
2	0.098	0.063	64.29
3	0.098	0.064	65.31
4	0.098	0.058	59.18
5	0.098	0.061	62.24
6	0.099	0.062	62.63
Mean	0.098	0.062	62.712
±SD	0.001	0.002	2.092
%CV	0.53	3.35	3.34

Table 10: Recovery study for Fenofibrate from rat plasma

Fenofibrate	*MQC*		
	Unextracted (area ratio)	*Extracted (area ratio)*	*%Recovery*
1	0.867	0.554	63.90
2	0.867	0.556	64.13
3	0.867	0.549	63.32
4	0.866	0.569	65.70
5	0.867	0.574	66.21
6	0.867	0.542	62.51
Mean	0.867	0.557	64.296
±SD	0.000	0.012	1.410
%CV	0.05	2.17	2.19

Table-11: Recovery study for Fenofibrate from rat plasma

Feno fibrate	*HQC*		
	Unextracted (area ratio)	*Extracted (area ratio)*	*%Recovery*
1	1.573	1.048	66.62
2	1.572	1.057	67.24
3	1.574	0.993	63.09
4	1.572	0.992	63.10
5	1.569	1.082	68.96
6	1.566	0.992	63.35
Mean	1.571	1.027	65.394
±SD	0.003	0.040	2.546
%CV	0.19	3.89	3.89

Stability was assessed by comparing against the freshly thawed quality control samples. The % mean stability for HQC and LQC were 100.0 and 100.0 respectively, which is within the acceptance limits of 85 to 115%. Plasma Quality control samples of Fenofibrate were found to be stable for at least one month. Results given in the Table-12.

Table 12: Stability of Quality Control Standards

Stability after 3 freeze thaw cycles		
Fenofibrate	***LQC***	***HQC***
conc.(µg/mL)	***1.0***	***16.0***
1	0.975	17.473
2	0.983	17.516
3	0.983	17.657
4	0.956	17.48
5	0.982	17.473
6	0.98	17.628
Mean	0.976	17.537
± SD	0.0104	0.0831
% CV	1.0735	0.4740
% Accuracy	100.00	100.00

Calibration standard- fenofibrate (FFB) Chromatographs:

The following figures from 02 to 08 are the chromatographs of calibration standards taken for fenofibrate in rat plasma.

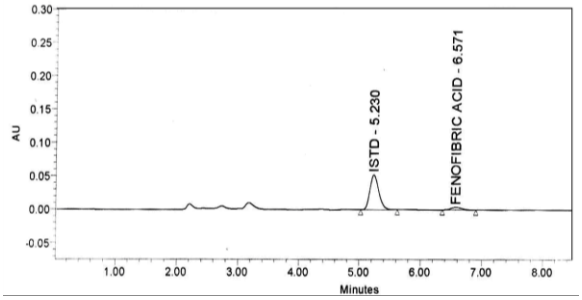

Fig 2: Chromatogram of calibration stand0ard-1(FFB CS1)

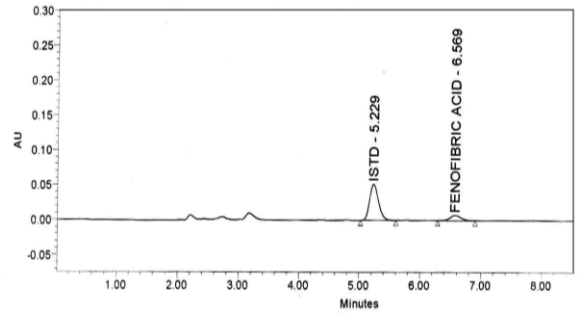

Fig 3: Chromatogram of calibration standard (FFB CS1)

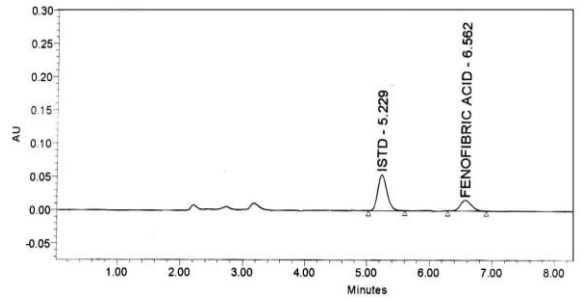

Fig 4: Chromatogram of calibration standard (FFB CS2)

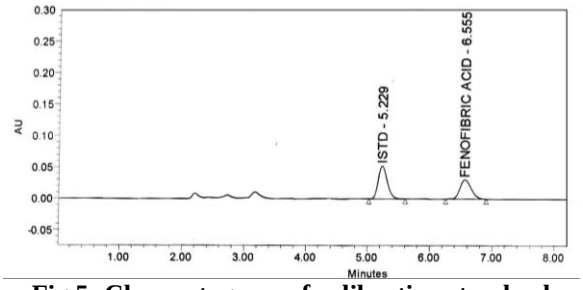

Fig 5: Chromatogram of calibration standard (FFB CS3)

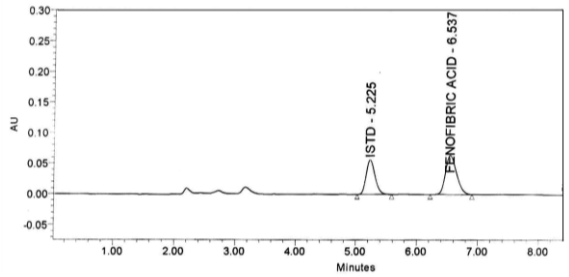

Fig 6: Chromatogram of calibration standard (FFB CS4)

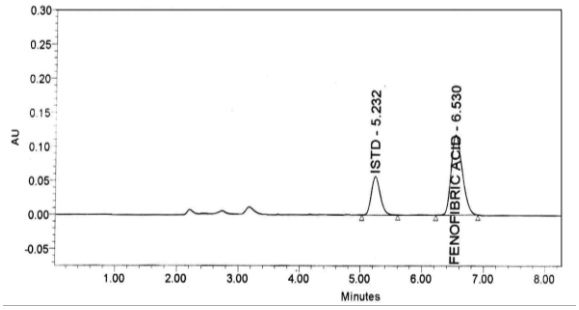

Fig 7: Chromatogram of calibration standard (FFB CS5)

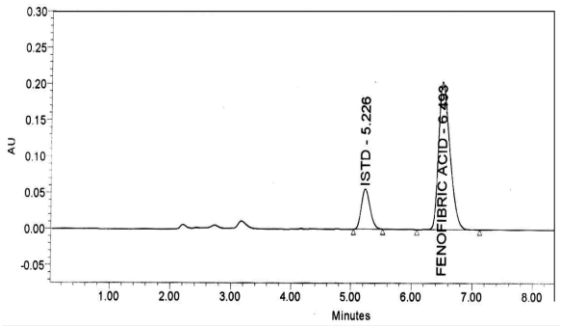

Fig 8: Chromatogram of calibration standard (FFB CS6)

Under the chromatographic conditions employed, the sample showed sharp peaks for drug and internal standard with good resolution. The retention time for the drug was found to be 6.672±0.05 minutes and the retention time for internal standard was 5.213±0.03 minutes. The method developed was validated for specificity, accuracy, precision, linearity, range and stability as per USFDA guidelines. The results of validation parameters are given below.

Specificity of the method was proven by the absence of peaks near the retention time of the drug as well as the internal standard (Figure-1).

Mean concentrations of Fenofibrate calibration standards are given in table no. 03. The calibration function was developed for Peak area ratio Vs Concentration (in µg/ml) and it was linear over concentration range of 0.3 to 20.0µg/mL. The regression line equation for the analysis was y= 5.32e-0.05x-4.82e-0.003 with coefficient of correlation (r^2) = 0.9979.

The LOD is calculated using the formula 3.3 times σ/s where "σ" is standard deviation of the intercept obtained for calibration curve and "s" is the slope of the calibration curve. Similarly LOQ is calculated using the formula 10 times σ/s. The calculated LOD and LOQ are shown in Table-04.

System suitability test was carried out by injecting six aqueous mixtures of Fenofibrate and IS. The following parameters were observed for repeated injections (n=6) of both Fenofibrate and internal standard.

The intraday and interday precision and Accuracy of the method was found to be 0.09 to 5.03% and 100.00 to 100.20% respectively for the quality control samples. This is within the acceptance limits of precision is 15% and accuracy is 85 to 115% (Tables-05 to 07). The limit of Quantification was found to be 0.3µg/ml at such concentration the inter day precision was found to be 0.07 to 0.29 and the accuracy was 98.0% to 100.0% respectively for the quality control samples. Which are within the acceptance limits of precision is 20% and accuracy is 80 to 120% (Table-07).

The Ruggedness data of fenofibrate ruggedness is given on below table: 08 on two different columns and in which where mean for two columns was found to be

0.870 And 0.869 respectively whereas accuracy for both columns was found to be

100.06

The % mean recovery for Fenofibrate in LQC, MQC and HQC was 62.7%, 64.2 % and 65.3% respectively (Tables-09 to 11).

Stability was assessed by comparing against the freshly thawed quality control samples. The % mean stability for HQC and LQC were 100.0 and 100.0 respectively, which is within the acceptance limits of 85 to 115%. Plasma Quality control samples of Fenofibrate were found to be stable for at least one month results shown in the Table-12.

SUMMARY:

A Simple, rapid, selective and sensitive HPLC method was developed and validated for the determination of fenofibrate from human plasma. The drug was extracted with ethyl acetate. Fenofibrate was measured in plasma using a validated a HPLC method with UV detector at 295nm chromatographic peaks were separated on 5µm intensil, C18 column (4.6x250mmx5µm) using 60:40 v/v 20mM ammonium acetate buffer with intial pH, Acetonitrile as mobile phase at a flow rate of 1 ml/min. The

chromatograms showed good resolution and no interference from plasma. The retention time of fenofibrate and internal standard (Nevirapine) were approximately 6.6±0.05 min and 5.2± 0.03 min respectively. The mean recovery from human plasma was found to be above 62%. The method was linear over the concentration range of 0.3 to 20.0µg/ml with coefficient of correlation (r^2) 0.9983. Both intraday and interday accuracy and precision data showed good reproducibility. This method was successfully applied to pharmacokinetics studies.

CONCLUSION:

Various methods reported in literature were studied. In the present study a simple, rapid, specific, rugged, accurate, precise and stable method is developed for estimation of Fenofibrate in human plasma. The calibration curve developed is y= 2.75e-001x -8.81e-005µg/mL with r^2=0.9974. The % mean recovery for Fenofibrate in LQC, MQC and HQC was 62.7%, 64.2 % and 65.3% respectively. The method is accurate, precise and rugged with % CV < 15% and 20% when tested at MQC, HQC and LQC levels respectively. The stability was assessed at different levels. The results of the freeze thaw stability, bench top stability, inter injection stability studies showed that the compound under analysis is stable under test conditions.

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