Method development and validation of Gemfibrozil by RP-HPLC in bulk and Pharmaceutical dosage forms

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ABSTRACT

A simple, specific and accurate reverse phase high performance liquid chromatographic method was developed for the determination Gemfibrozil in bulk and pharmaceutical dosage form. The column used was Agilent Zorbax C8 (150 ×4.6mm, 5µ) in gradient mode, with mobile phase containing Solvent Methanol, 0.05%OPA, Water ratio 40+60 the flow rate was 0.7 mL/ min and eluents was monitored at 276 nm. The retention time Gemfibrozil was 5.158 min, respectively. The linearity for Gemfibrozil was in the range of 30-450 µg/ml respectively. The recovery of Gemfibrozil was found to be 99.5%, respectively. The proposed method was validated and successfully applied to the estimation of Gemfibrozil in capsule dosage form.

Key words: Gemfibrozil, RP-HPLC, Validation, Method Development.

INTRODUCTION

The exact mechanism of action of gemfibrozil is unknown; however, several theories exist regarding the very low density lipoprotein (VLDL) effect; it can inhibit lipolysis and decrease subsequent hepatic fatty acid uptake as well as inhibit hepatic secretion of VLDL; together these actions decrease serum VLDL levels and increase HDL-cholesterol; the mechanism behind HDL elevation is currently unknown.

Gemfibrozil increases the activity of extrahepatic lipoprotein lipase (LL), thereby increasing lipoprotein triglyceride lipolysis. It does so by activating peroxisome oil feretory-activated receptor alpha (PPARα) 'transcription factor ligand', a receptor that is involved in metabolism of carbohydrates and fats, as well as adipose tissue differentiation. This increase in the synthesis of lipoprotein lipase thereby increases the clearance of triglycerides. Chylomicrons are degraded, VLDLs are converted to LDLs, and LDLs are converted to HDL. This is accompanied by a slight increase in secretion of lipids into the bile and ultimately the intestine. Gemfibrozil also inhibits the synthesis and increases the clearance of apolipoprotein B, a carrier molecule for VLDL.

Gemfibrozil alters lipid metabolism to treat patients with hyperlipidemia. The duration of action requires twice daily dosing as the mean residence time of gemfibrozil is up to 9.6h in patients with chronic renal failure. Gemfibrozil has a wide therapeutic index as trials with twice the standard dose were not associated with severe side effects. Patients taking gemfibrozil may be at an increased risk of developing cholelithiasis and cholecystitis.

Reversed-Phase Chromatography:

Reversed-Phase Chromatography, the most widely used chromatographic mode is used to separate neutral molecules in solution on the basis of their hydrophobicity. As the name suggests, Reversed-Phase Chromatography is the reverse of Normal-Phase Chromatography in the sense that it involves the use of a non-polar stationary phase and a polar mobile phase. As a result, a decrease in the polarity of the mobile phase results in a decrease in solute retention. Modern Reversed-Phase Chromatography typically refers to the use of chemically bonded stationary phases where a functional group is bonded to silica for this reason Reversed-Phase Chromatography is often referred to in the literature as Bonded-Phase Chromatography. Occasionally, however polymeric stationary phases such as polymethacrylate or polystyrene or solid

stationary phases such as porous graphitic carbon are used. Weak acids and weak bases for which ionization can be suppressed, may be separated on reversed-phase columns by the technique known as ion suppression. In this technique a buffer of appropriate pH is added to the mobile phase to render the analyte neutral or only partially charged. Acidic buffers such as acetic acid are used for the separation of weak acids and alkaline buffers are used for the separation of weak bases.

The analysis of strong acids or strong bases using reversed-phase columns is typically accomplished by the technique known as ion-pair chromatography (also commonly called paired- ion or ion-interaction chromatography). In this technique, the pH of the eluent is adjusted in order to encourage ionization of the sample for acids pH 7.5 is used and for bases pH 3.5 is common.

Reversed-Phase Chromatography is the most popular mode for the separation of low molecular weight (<3000) neutral species that are soluble in water or other polar solvents. It is widely used in the pharmaceutical industry for separation of species such as steroids, vitamins and β-blockers. Because of the mobile phase in Reversed-Phase Chromatography is polar, Reversed-Phase Chromatography is suited to the separation of polar molecules that either are insoluble in organic solvents or bind too strongly to the polar normal-phase materials.

MATERIAL AND METHODS

Selection and Procurement of Drug

Drug sample supplier

Table 2: Drug and Drug Supplier

Name of Drug	Drug Supplier
Gemfibrozil	RSITC Jalgaon.

List of reagents & chemicals used

Table 3: List of Reagents and Chemicals used

Sr. No.	Name of chemicals	Manufacturer.
1.	Acetonitrile (HPLC grade)	Merck Ltd., India
2.	Methanol (HPLC grade)	Merck Ltd., India
3.	0.05% OPA (HPLC grade)	Merck Ltd., India
4.	water (HPLC grade)	Merck Ltd., India

Selection of formulation:

From the literature survey and market survey we selected Maxide formulation for work.

Marketed Preparation**:**

Table No.4: List of brand names of combined formulations of Gemfibrozil

The marketed preparation was obtained from local market and is referred here after in this thesis by the name as such.

Sr. No	Brand name	Formulation	Available strength	Manufacturer	Manufacturing	EXP
1	Lopid	Capsules	Gemfibrozil 600 mg	Pfizer lmt		

Selection of Analytical Technique

HPLC was selected as analytical technique for estimation of Gemfibrozil.

- **Instruments:**

The analysis of the drug was carried out on Agilent Tech. Gradient System with Auto injector, (DAD) &Gradient Detector. Equipped with Reverse Phase (Agilent) C_{18} column (4.6mm x 250mm; 5µm), and UV730D Absorbance detector and running chemstation 10.1 software.

- ❖ **Stock preparations**:
- ➢ ***Stock I : Standard Sample Preparation***

Std. GEMFIBROZIL 5 mg in 10 ml Methanol = 500 µgm/ml

- ➢ ***Stock II : Tab solution Preparation*:-**

Take 6.55 mgs in 10 ml Methanol i.e.= 1000 µgm/ml

- ❖ **For Accuracy Solution Preparations: -**
- ➢ **take 10 µgm/ml tab solution for accuracy,**

80 % = 0.1 ml tab solution and add 4 µgm/ml std gemfi

and make up vol 10 ml with mobile phase

100 % =0.1 ml tab solution and add 5 µgm/ml std gemfi

and make up vol 10 ml with mobile phase

120 % = 0.1 ml tab solution and add 6 µgm/ml std gemfi

and make up vol 10 ml with mobile phase

6.4 Instruments and Equipments

Table. 5: Instrument (HPLC) Details used during Method Development

	Name of Instrument	Company Name
1	HPLC Instrument	Agilent Tech. Gradient System with Auto injector (Chemstation software)
2	UV-Spectrophotometer	Analytical Technologies Limited
3	Column(C_{18})	Agilent C_{18} (250mmX 4.6mm,5µm)
4	pH meter	VSI pH meter(VSI 1-B)
5	Balance	WENSAR™ High Resolution Balance.
6	Sonicator	Ultrasonics electronic instrument

EXPERIMENTAL WORK

Selection of Analytical Technique

- HPLC was selected as analytical technique for estimation ofGemfibrozil
- **Instruments:**

The analysis of the drug was carried out on Agilent Tech. Gradient System with Auto injector,DAD Detector. Equiped with Reverse Phase C_{18} (Agilent) with 250mm x4.6;(5µm), UV730D Absorbance detector and running chemstation 10.1 software.

Selection of stationary phase:

- The column used in this method C_{18}Agilent The configuration of the column is 4.6 x 250 mm, particle size 5 µm. C_{18} column gives high non polar retentively, symmetric peak shape, highly reproducible and stable ideal for HPLC method

Solubility Studies:

- This study was carried out to find an ideal solvent in which drugs are completely soluble. Various solvents were tried for checking solubility of Gemfibrozil. From solubility studies it was concluded that of Gemfibrozil is freely soluble in Methanol andpoorly soluble in water PH adjusted 0.05%Orthophosphoric Acid, Buffer pH 3.

Chromatographic conditions :

- The following chromatographic conditions were established by trial and error and were kept constant throughout the experimentation.

Table No.6: chromatographic conditions(HPLC) details used during method Development.

1.	HPLC	Agilent Tech. Gradient System with Auto injector
2.	Software	chemstation 10.1
3.	Column	(Agilent) C18 column (4.6mm x 250mm
4.	Particle size packing	5 µm
5.	Stationary phase	C18 (Agilent)
6.	Mobile Phase	Methanol : water (0.05 % OPA) 40 : 60
7.	Detection Wavelength	272 nm
8.	Flow rate	1 ml/min
9.	Temperature	Ambient
10.	Sample size	20 µl
11.	pH	3.0
12.	Run Time	15 min
13.	Filter paper	0.45 µm

UV-VIS Spectrophotometer:

UV-VIS Spectrophotometer was selected as analytical technique for estimation of Gemfibrozil .UV absorbance range of 200-400nm.

- **Instrument** :

Analytical Technologies® Limited UV-VIS Spectrophotometer is double beam, high seed scanning spectrophotometer, The instrument needs about 1minute for initialization. Thelight source used is Deuterium lamp of spectrophotometer, a computer is attached which helps in data processing and manipulation Quartz curette with path length 1cm was used.

Study on the selection of uv spectrum use in uv-vis spectrometer of Gemfibrozil :

Accurately weigh and transfer 5 mg Gemfibrozil working standard into 100 ml volumetric flask as about dilute Methanol preparedin completely and make volume up to
the mark with the same solvent to get 100µg/ml standard (stock solution) and 15 min sonicate todissolve it and from the resulting solution 0.5ml was transferred to 10 ml volumetric flaskand the volume was made up to the mark with Methanol.(**Fig No: 19)**

Study on the chromatographic conditions of GEMFIBROZIL:

Accurately weigh and transfer 5 mg Gemfibrozil working standard into 10 ml volumetric flask as about dilute Methanol preparedin completely and make volume up to the mark with the same solvent to get 500µg/ml standard (stock solution) and 15 min sonicate to dissolve it and from the resulting solution 0.1ml was transferred to 10 ml volumetric flask and the volume was made up to the mark with mobile phase Methanol:(0.05%OPA) Water solvent. The resulting 10µg/ml of solution was subjected to chromatographic analyses using mobile phases of different strengths with chromatographic conditions mentioned below: **(Table No: 12)**

- Analytical column : AgilentC18 Column (250mm x 4.6mm), 5µm particle size.
- Injection volume : 20µl
- Flow rate : 1 ml/min
- Detection : 272nm
- Run Time : 10 min

➢ Following Mobile phase were tried:

Method development of hplc:

➢ **List of Mobile Phase :**

Table No.7: Selection of mobile Phase.

Sr.No.	Mobile Phase
1.	Methanol+ 0.05% (OPA)Water, (90+10% v/v) 20 Mcg, C_{18} (Agilent) (4.6mm x 250mm)
2.	Methanol+ 0.05% (OPA)Water, (70+30% v/v) 20 Mcg, C_{18} (Agilent) (4.6mm x 250mm)
3.	Methanol+ 0.05% (OPA)Water, (50+50% v/v) 0.7 20 Mcg, C_{18} (Agilent) (4.6mm x 250mm)
4.	Methanol+ 0.05% (OPA)Water, (40+60% v/v) 20Mcg C_{18} (Agilent) (4.6mm x 250mm)0.7
5.	Methanol+ 0.05% (OPA)Water, (20+80% v/v) 0.7 20 Mcg, C_{18} (Agilent) (4.6mm x 250mm)
6.	Methanol+ 0.05% (OPA)Water, (20+80% v/v) 20 Mcg, Flow 0.7 C_{18} (Agilent) (4.6mm x 250mm)
7.	Methanol+ 0.05% (OPA)Water, (50+50% v/v) 20 Mcg, Flow 1 C_{18} (Agilent) (4.6mm x 250mm)
8.	Methanol+ 0.05% (OPA)Water, (40+60% v/v) 20 Mcg, Flow 1 C_{18} (Agilent) (4.6mm x 250mm)

Analysis of standard drugs was done by following parameters:

- Melting point
- Solubility
- UV spectra and λ_{max}
- HPLC chromatogram and retention time

Selection of wavelength by UV-Visible Spectrophotometry:-

Preparation of standard stock solution:-

- **Gemfibrozilstandard stock solution : (Stock I)**

An accurately weighed quantity, 5 mg of Gemfibrozil (GF) was dissolved in Methanol and waterin a 100ml volumetric flask and volume made up to 10.0 ml to produce a solution of 100 µg/ml.

- **Preparation of Stock Standard Solution :(Stock II)**

Accurately weight and transfer 5mgGemfibrozil working standard into 10 ml volumetric flask as about diluent Methanol completely and make volume up to the mark with the same solvent to get 500µg/ml standard (stock solution) and 15 min sonicate to dissolve it and the resulting stock solution 0.1ml was transferred to 10 ml volumetric flask and the volume was made up to the mark with mobile phase Methanol:Water (0.05%OPA) Water, prepared in (40 mlMEOH: 60ml WATER v/v)

HPLC used for chromatographic condition apply on the Preparation of standard solution:-

- **Preparation of std. Gemfibrozilsolution: (Stock I)**

From the freshly prepared standard stock solution (500 µg/ml), 0.1-0.5ml stock solution was pipetted out in 10 ml of volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration of 5-25 µg/ml.

Selection of mobile phase:

Each mobile phase was vacuum degassed and filtered through 0.45µ membrane filter. The mobile phase was allowed to equilibrate until steady baseline was obtained. The standard solution containing of Gemfibrozil was run with individual solvents as well as combinations of solvents were tried to get a good separation and stable peak. From the various mobile phases tried, mobile phase containing Methanol and Water (0.05% OPA) with pH adjust (3.0) was selected since it gave sharp, well resolved peaks with symmetry within the limits and significant reproducible retention time for Gemfibrozil.

Studies of Calibration plot:-

7.8.1. Optimization of Chromatographic condition:

The following chromatographic conditions were established by trial and error and were kept constant throughout the analysis.

- Analytical column : Agilent C18 Column (250mm x 4.6mm)
- partical size : 5µm
- Injection volume : 20µl
- Flow rate : 1 ml/min
- Detection : 272 nm
- Run Time : 15 min
- Mobile phase : Methanol : Water(0.05% OPA) pH adjust 3.0 (40:60)

7.9. Procedure for calibration curve of Gemfibrozil:

The mobile phase was allowed to equilibrate with stationary phase until steady baseline was obtained. From the freshly prepared standard stock solution, pipette out 5 mg Gemfibrozilin 100ml of volumetric flask and diluted with mobile phase. From it 0.1, 0.2, 0.3, 0.4 and 0.5ml of solution were pipette out in 10 ml volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration 5, 10,15,20,25 µg/ml of Gemfibrozil.Sample were injected and peaks were recorded at 272 nmas the graph plotted as concentration of drug verses peak area is depicted in **(fig. no. 30, 31)** respectively.

7.10. Study of system suitability parameters:

The system suitability is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis to be done. The test was performed by collecting data from five replicate injections of standard solution.

Calibration Experiment:

- **RP-HPLC Method :**

Preparation of Calibration curve standard:

The above standard stock solution (500µg/ml) of Gemfibrozilwas diluted with mobile phase to yield five calibration curve (cc) standards with concentrations of 5, 10,15,20,25µg/ml of Gemfibrozil. The calibration curve of Gemfibrozilis depicted in (**FigNo.30)**

- **UV Spectrophotometric method:**

The data obtained in the calibration experiments when subjected to linear regression analysis showed a linear relationship between peak areas and concentrations in the range 5-25 µg/mL for Gemfibrozil (**Table No:23)** The UV Spectrophotometric method respective linear equation for Gemfibrozil was y= 0.020x-0.003where x is the concentration and y is area of peak. The correlation coefficient was 1. The calibration curve of Gemfibrozil is depicted in

Selection of detection Wavelength:

Standard solutions were scanned in the range of 200-400nm ,against 10 ml Methanol and volume make with water solvent system as reference Gemfibrozil were showed absorbance maxima (lamda max) at 272 nm**(Figure No:19**).

Calibration standard drug and regression equation data :

From the standard stock solution of Gemfibrozil, different concentration were prepared respectively in the range of 5-25µg/ml for Gemfibrozil**(Figure No:30)**and measured at 272 nm. The calibration curves were plotted Regression equation data presented in **(Table No: 22)**

Calibration runs and regression analysis:

These calibration standard solutions were analyzed in three replicates using the under mentioned chromatographic conditions.

- Analytical column : C18 Column (250mm x 4.6mm, 5µm partical size).
- Injection volume : 20µl.
- Flow rate : 1 ml/min.
- Mobile phase : Methanol: Water (0.05% OPA) (40: 60 % V/V).
- Detection : 272 nm.

At the end of the calibration runs, the chromatograms of CC standards were processed to give the peak areas for Gemfibrozil. Least square linear regression analysis was used to define the functional relationship between the two variables- peak area of the drug (Y-axis) and concentration of the corresponding CC standard (X- axis) the values of which were provided by the calibration run. The peak areas of the drug were plotted against their respective concentration.

The data were subjected to linear regression in order to determine slope and intercept as:

$$\text{slope (b)} = \frac{\text{Xy} - (\text{X})(\text{y})/\text{N}}{\text{X2} - (\text{X2})/\text{N}}$$

$$\text{Intercept (a)} = \frac{\text{y}}{\text{N}} - \frac{\text{b(X)}}{\text{N}}$$

The concentrations of the calibration standards were back calculated using the calibration equation thus obtained.

Simultaneous estimation of Gemfibrozil

Determination of absorbtivity values of drugs at selected wavelengths

Suitable aliquots of standard stock solutions of Gemfibrozileach were diluted with Methanol to obtain working standard solutions of concentrations within the Beer-Lambert's range. The absorbance of each resulting solution was measured at the selected wavelengths.result as shown in formula calculation **(Chapter 8)**

The absorbtivity values for Gemfibrozilwere calculated from the following formula: as shown in chapter no :7

$$\text{Absorptivity} = \frac{\text{Absorbance}}{\text{conc. (g/lit)}}$$

Validation of method for analysis of Gemfibrozil:

- The developed method was validated as per ICH guidelines.

Linearity: Linearity of an analytical method is its ability to elicit test results that are directly or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range, The Result are shown in;**(Table No 22)**

- **Determination:**

The linearity of the analytical method is determined by mathematical treatment of test results obtained by analysis of samples with analyte concentrations across the claimed range. Area is plotted graphically as a function of analyte concentration. **(Fig No. 33-44)** Percentage curve fittings are calculated**.** The Result is shown in; **(Table No.24and Table No. 25)**

- **Acceptance Criteria:**

The plot should be linear passing through the origin.

Correlation Coefficient should not be less than 0.999. The Result are shown in;

- **Preparation of standard stock solution for linearity:**

Average weight of tablet sample (equivalent to 5 mg ofGemfibrozilwere weighed and transfered to 10 mL volumetric flask & diluent was added to make up the volume. Sonicated for 10 min with occasional swirling. 0.1 ml of this solution diluted upto 10 ml volumetric flask with diluents was added to make up the volume.

- **Preparation of linearity solution:**

A series of standard preparations of working standard of were prepared.

Table No.8: Table of linearity for **Table No.9: Tableof linearity for**

Rp-HPLC Method

Linearity of Gemfibrozil HPLC	
Sr.No.	**Concentratin (µg/mL)**
1	5
2	10
3	15
4	20
5	25

UV Method

Linearity of Gemfibrozil UV	
Sr.No.	**Concentran (µg/mL)**
1	5
2	10
3	15
4	20
5	25

Accuracy (recovery):

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Accuracy may often the expressed as percent recovery by the assay of known added amounts of analyte.The accuracy of an analytical method is determined by applying the method to analyzed samples, to which known amounts of analyte have been added. The accuracy is calculated from the test results as the percentage of analyte recovered by the assay, The RP-HPLC& UV Method Result are shown in; **(Table No:10-11)**

- **Acceptance Criteria:**

Mean recovery should be in the range of 98-102%.

The Relative Standard Deviation should not be more than 2.0%.

- **Preparation of standard stock solution:**

5 mg ofGemfibrozilworking standards were weighed and transfered to 10 mL volumetric flask & diluent was added to make up the volume 0.1 ml of this solution diluted upto 10 ml with diluent.

- **Application of proposed method for analysis of tablet formulation:**
- **Accuracy**

The accuracy was determined by Gemfibrozil(equivalent to 5 mg (80 %, 100 % and 120 % of the label claimed, respectively) to quantity equivalent to average weight of marketed tablets. This powder containing 5 mg ofGemfibrozilwere triturated and then subjected to chromatographic analysis using the described method. The resulting wasanalyzed in triplicates over three days. The % recovery of added drugwas taken as a measure of accuracy**.**

The Result are shown in; **(FigNo: 47, 48, 49)**

Table No. 10: Table of Accuracy for Rp-HPLC Method

Sample	Amount Added (mg)
	Gemfibrozil
Accuracy 80%	4
Accuracy 100%	5
Accuracy 120%	6

Table No. 11: Table of Accuracy for UV Method

Sample	Amount Added (mg)
	Gemfibrozil
Accuracy 80%	4
Accuracy 100%	5
Accuracy 120%	6

Repeatability:

Precision of the system was determined with the sample of RP-HPLC& UV Method for. three replicates of sample solution containing 20 mg of Gemfibrozilwere injected and peak areas were measured and %RSD was calculated. is was repeated for five times result are shown in; **(Table No : 28,29)& (Fig No :50,51)**

- **Application of proposed method for analysis of tablet formulation:**

Average weight of tablet sample (equivalent to 5 mg of Gemfibrozil) was weighed and transfered to 10mL volumetric flask & diluent was added to make up the volume. Sonicated for 10 min with occasional swirling. The above solution was filtered through 0.45µm membrane filter 0.1 ml of this solution diluted upto 10 ml with diluent.

Precision:

Precision of an analytical method is the degree of agreement among Individual test results when the procedure is applied repeatedly to multiple Samplings of a homogenous sample. Precision of an analytical method is usually expressed as standard deviation or relative standard deviation. Also, the results obtained were subjected to one way ANOVA and within-day mean square and between-day mean square was determined and compared using F-test. **(Fig No: 53)**

- **Result of Intra day and Inter day Precision studies on RP-HPLC and UV method for Gemfibrozil**

Intra-day precision:

Sample solutions containing 5 mg of Gemfibrozilthree different concentration(10µg/ml, 15µg/ml, 20µg/ml)Gemfibrozilwere analyzed three times on the same day and %R.S.D was calculated. The Result are shown in; **(Table No.30) & (Fig No: 54to56)**

Inter-day precision:

Sample solutions containing 5 mg of Gemfibrozilthree different concentrations(10µg/ml, 15µg/ml, 20µg/ml) in HPLC and three different concentrations in Gemfibrozildifferent days and % R.S.D was calculated. It is usually expressed as standard deviation or relative standard deviation. The Result are shown in; **(Table No.30) & (Fig No: 57 to 59)**

- **Acceptance criteria:**

The Relative Standard Deviation should not be more than 2% for test

- **Preparation of standard stock solution:**

5 mg of Gemfibrozilworking standards were weighed and transfered to 10 mL volumetric flask & diluent was added to make up the volume. 0.1 ml of this solution diluted upto 10 ml with diluent.

Robustness:

The mobile phase composition was changed in (±1 ml/ min^{-1}**)** proportion and the flow rate was **(Fig No:60,61)** of Methanol : Water (0.05 % OPA) in the mobile phase composition (±1ml/ min^{-1}**)** and the change in detection wavelength (±1 ml/ min^{-1}**)** and the effect of the results were examined**.(Fig No: 62,63)**and **(Fig No:64,65)** it was performed using 15µg/ml solution of Gemfibrozilin triplicate. The Result are shown in; **(Table No: 31)**

Detection Limit

Based on the S.D. of the response and the slope of calibration curve, the detection limit (DL) was calculated as,

$DL = \frac{3.3\sigma}{S}$

Where,

σ = the S.D. of the y-intercepts of regression lines.

S = the slope of the calibration curve.

The slope S may be estimated from the calibration curve and S.D. was used should be calculated from the y-intercepts of regression line in calibration curve.

The result is shown in: **(chapter: 8)**

Quantitation Limit

Based on the S.D. of the response and the slope of calibration curve, the quantitation limit (QL) was calculated as,

$QL = \frac{10\sigma}{S}$

Where,

σ = the S.D. of the y-intercepts of regression lines.

S = the slope of the calibration curve.

The slope S may be estimated from the calibration curve and S.D. was used should be calculated from the y-intercepts of regression line in calibration curve.

The results are shown in **(chapter: 8)**

Analysis of marketed formulation

To determine the content of Gemfibrozilin marketed tablets (label claim 5mg of Gemfibrozil), 20 tablets powder weighed in 15.74gms and average weight of powder was calculated in 787gms.Tablets were triturated and powder equivalent to weighed in 6.55 mg The drug was extracted from the tablet powder with 10 mL Methanol . To ensure complete extraction it was sonicated for 15 min. 0.1mL of supernatant was then diluted up

to 10 mL with mobile phase. The resulting solution was injected in HPLC and drug peak area was noted**. (Fig No: 66)**.

Regression equation was generated using peak areas of standard solutions. Using the regression equation and peak area of the sample the amount of Gemfibrozilin the sample was calculated. The amount of Gemfibrozilper tablet was obtained from the regression equation of the calibration curve as described in analysis of Tablet formulation are shown in **(Table No.32).**

RESULT AND DISCUSSION:

Preliminary studies on Gemfibrozil

Melting point

The procured reference standard of Gemfibrozil was found to melt in the range of100^0C.

Solubility

The drug was found to be

- Freely soluble in Acetonitrile, methanol.
- Practically soluble in water, but freely soluble in organic solvents.

UV Spectroscopy

Standard solutions were scanned in the range of 200-400nm ,against 10 ml methanol and volume make with water solvent system as reference Gemfibrozil in water was found to be selected wavelength is 272nm.**(Figure No:19)**

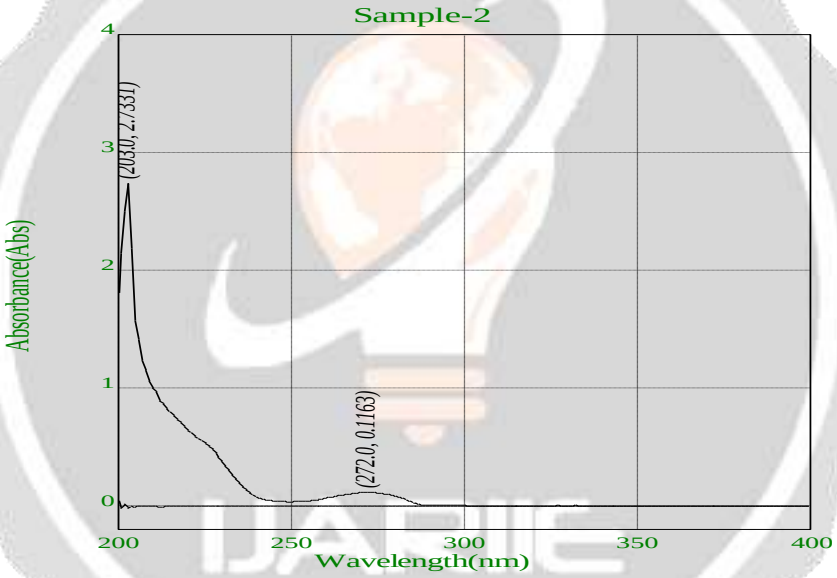

Fig No.19: UV Spectrum of Gemfibrozil

TABLE NO-12: Chromatographic behavior of Gemfibrozilmobile phase of various compositions.

Fig No	Column used	Mobile phase, Flow Rateand Wavelength	Inj. Vol.	Observation	Conclusion

1.	C_{18} (Agilent) (4.6mm x 250mm), 5.0µ)	Methanol+ 0.05% (OPA)Water, Ph3, (90+10),272nm 20 Mcg, Fl. 0.7,	20µl	Sharp Peaks were not obtained	Hence rejected
2.	C_{18} (Agilent) (4.6mm x 250mm), 5.0µ)	Methanol+ 0.05% (OPA)Water,Ph3 (70+30),272nm 20 Mcg, Fl. 0.7,	20 µl	Sharp Peaks were not obtained	Hence rejected
3.	C_{18} (Agilent) (4.6mm x 250mm), 5.0µ)	Methanol + 0.05% (OPA)Water(50+50)PH3.0, 272nm, Flow rate 0.7mL	20 µl	Sharp Peaks were not obtained	Hence rejected
4.	C_{18} (Agilent) (4.6mm x 250mm), 5.0µ)	Methanol + 0.05% (OPA)Water,(40+60)PH3.0, 272nm, Flow rate 0.7mL	20 µl	Sharp Peaks were not obtained	Hence rejected
5.	C_{18} (Agilent) (4.6mm x 250mm), 5.0µ)	Methanol + 0.05% (OPA)Water(20+80)PH3.0, 272nm, Flow rate 0.7mL	20 µl	Sharp Peaks were not obtained	Hence rejected
6.	C_{18} (agilent) (4.6mm x 250mm), 5.0µ)	Methanol + 0.05% (OPA)Water (20+80 %v/v)PH3.0, 272nm, Flow rate 1 mL	20 µl	Sharp Peaks were not obtained	Hence rejected
7.	C_{18} (agilent) (4.6mm x 250mm), 5.0µ)	Methanol + 0.05% (OPA)Water (50+50 %v/v)PH3.0, 272nm, Flow rate 1 mL	20 µl	Sharp Peaks were not obtained	Hence rejected

8.	C_{18} (agilent) (4.6mm x 250mm), 5.0µ)	Methanol + 0.05% (OPA)Water (40+60 %v/v)PH3.0, 272nm, Flow rate 1 mL		Sharp and well resolved Peaks were obtained	Hence selected

Thus, from the above, it has been observed that, using mobile phase of meoh+0.05% (OPA)water,(40:60 % v/v),PH 3.0,272nm, Flow rate 1 ml gave adequate retention at 8.6333min with good peak shape (Theoretical plates: Gemfibrozil 5934.6)

Chromatogram of final Trial8:

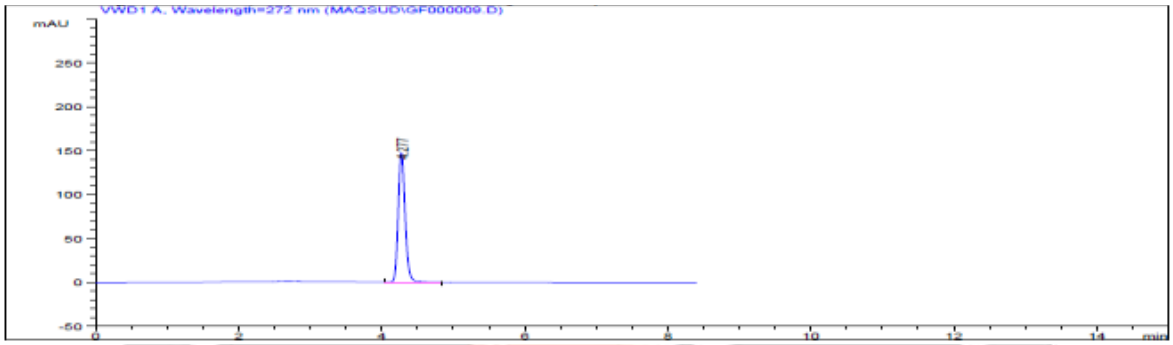

Fig No 27: Chromatogram of final Trial 8

Table.No.20. Final Trial-8 of chromatogram of Gemfibrozil

No.	Name	RT[min]	Area[mV*s]	Area%	TP	TF
1	GF	4.277	1001.1335	100.00	9164	0.84

Fig No 28: Chromatogram of blank

- **The final chromatographic conditions selected were as follow:**
  - Analytical column : Agilent C18 Column (250mm x 4.6mm, 5µm partical size).
  - Injection volume 20µl.
  - Flow rate 1 ml/min.
  - Mobile phase Methanol: water (40: 60% V/V)
  - Detection 272 nm.
  - Run Time 15 min.
- **Preparation of Standard chromatogram of Gemfibrozil**

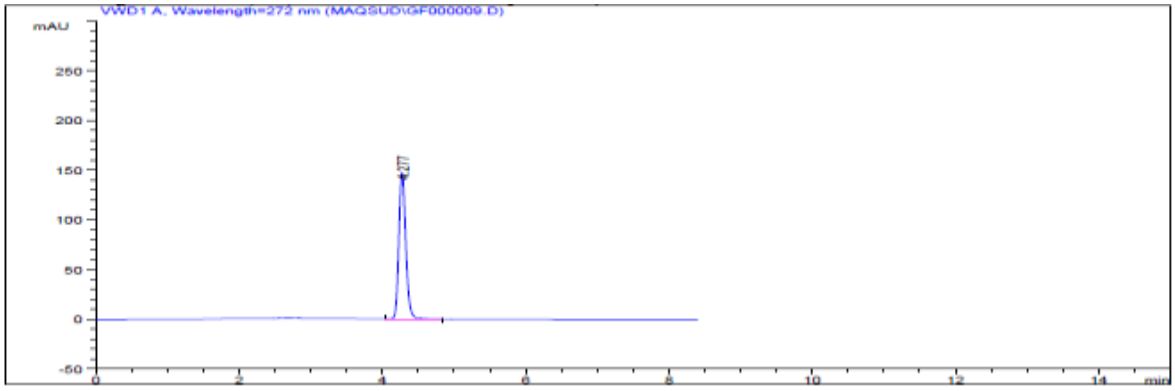

Fig No.29: Chromatogram of standard Gemfibrozil

Table.No.21. Details of chromatogram of standard Gemfibrozil

No.	Name	RT[min]	Area[mV*s]	Area%	TP	TF	Resolution
1	GF	4.277	1001.1335	100.00	6008.3	1.2273	0.0000

In the standard of Gemfibrozil theoretical plates were found above 2000 i.e. for Gemfibrozil6008.3at minimum RT 4.277.

8.1.5 Calibration experiment

- **RP-HPLC Method :**

The data obtained in the calibration experiments when subjected to linear regression analysis showed a linear relationship between peak areas and concentrations in the range 5-25µg/mL for Gemfibrozil(**Table No:22)**depict the calibration data of GemfibrozilThe respective linear equation for Gemfibrozilwas Y= 59.94x+40.13 where x is the concentration and y is area of peak. The correlation coefficient was 0.999. The calibration curve of Gemfibrozilis depicted in (**FigNo.30)**

Table No 22: Linearity data for Gemfibrozil

Method	Conc µg/ml	Peak area(µV.sec)		Average peak area (µV.sec)	S.D. of Peak Area	% RSD of Peak Area
		1	2			
RP-HPLC Method	5	344.7124	345.022	344.8674	0.22	0.06
	10	624.1492	620.969	622.5595	2.25	0.36
	15	945.3515	940.456	942.9040	3.46	0.37
	20	1251.1954	1253.53	1252.364	1.65	0.13
	25	1531.6915	1525.23	1528.465	4.56	0.30
	Equation		Y= 59.94x+40.13			
	R^2		0.999			

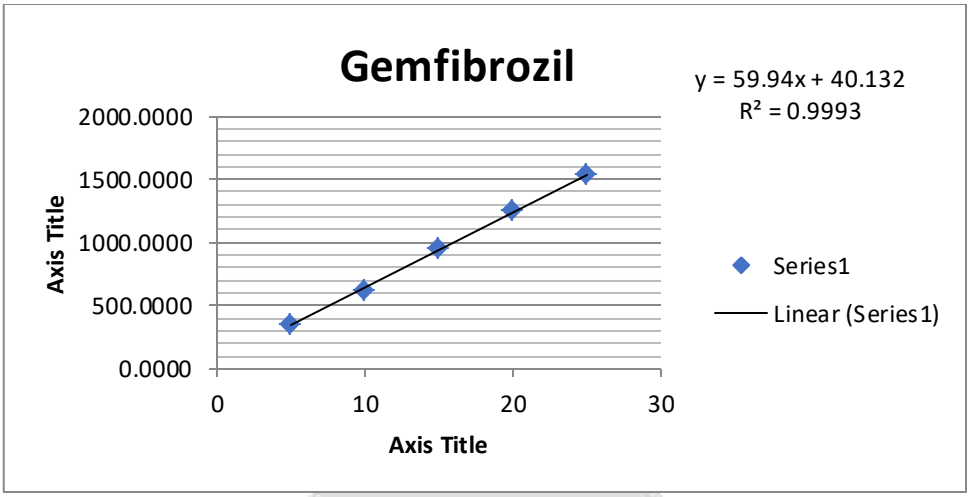

Fig.No.30: Calibration curve of Gemfibrozil (HPLC)

The RP-HPLC Method for respective linear equation for Gemfibrozilwas y = 59.94x +4.13 where x is the concentration and y is area of peak. The correlation coefficient was 0.999. The calibration curve of Gemfibrozilis depicted in **Fig30.**

- **UV Spectrophotometric method:**

The data obtained in the calibration experiments when subjected to linear regression analysis showed a linear relationship between peak areas and concentrations in the range 5-25µg/mL for Gemfibrozil(**Table No:23)**depict the calibration data of GemfibrozilThe linear equation for Gemfibrozilwasy = 0.020X+0.003where x is the concentration is area of peak. The correlation coefficient was 0.999. The calibration curve of Gemfibrozilis depicted in (**FigNo.32)**

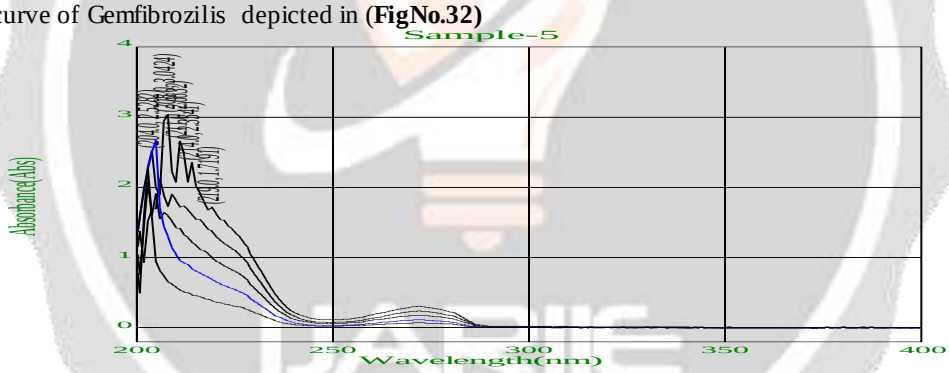

Fig.No.31 Linearity OfGemfibrozil(UV Method)

Table No 23: Linearity data for Gemfibrozil(UV Method)

Method	Conc µg/ml	Peak area(µV.sec)		Average peak area (µV.sec)	S.D. of Peak Area	% RSD of Peak Area
		1	2			
UV Method	5	0.1038	0.1044	0.104	0.0004	0.4076
	10	0.2042	0.2014	0.203	0.0020	0.9763
	15	0.3032	0.3065	0.305	0.0023	0.7654
	20	0.4001	0.4079	0.404	0.0055	1.3652
	25	0.5098	0.5009	0.505	0.0063	1.2453

	Equation	y= 0.020x+0.003
	R^2	0.999

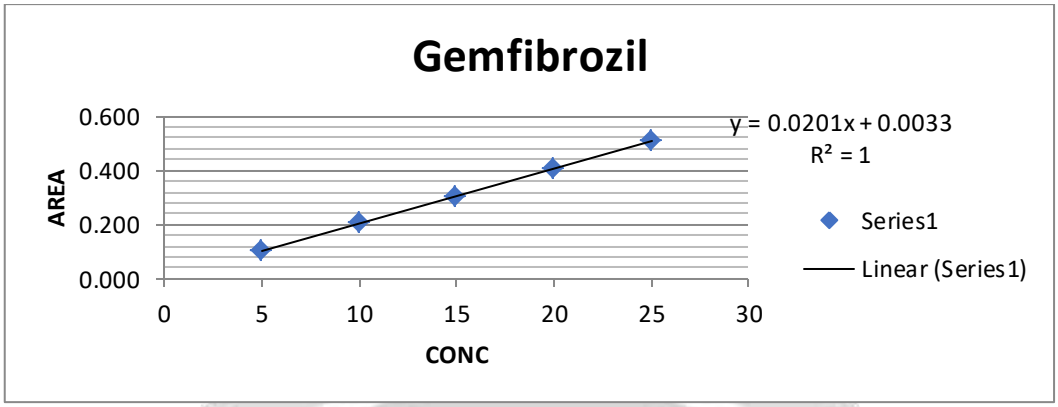

Fig.No.32: Calibration curve of Gemfibrozilfor (UV method)

The UV Spectrophotometric method respective linear equation forGemfibrozil was y= 0.020x-0.003where x is the concentration and y is area of peak. The correlationcoefficient was 0.999. The calibration curve of Gemfibrozilis depicted in **Fig32.**

a) UV spectrophotometric method for Simultaneous estimation of Gemfibrozil.

b) Determination of absorbtivity values of drugs at selected wavelengths

The absorbtivity value for Gemfibrozil was calculated from the following formula:

$$\text{Absorptivity} = \frac{\text{Absorbance}}{\text{conc.(g/lit)}}$$

GEMFIBROZIL(272)

Aborptivity = A / C

(ay2) = 0.2042

_____ = 0.02042 x 10 -6 =204200µg/ml

1 x 10 -6

(ax2) = 0.2014

_____ = 0.02014 x 10 -6 = 201400µg/ml

1x 10 -6

Aborptivity = A / C

(ay1) = 0 . 3 0 3 2

= 0.03032 x10 -6 =303200

2 x10 -6

(ax1) =0.3065

= 0.03065 x10 -6 =306500

2 x10 -6

A1 = ax1 cx + ay1,cy

A2= ax2 cx + ay2 CY

Cy= A1ax2 – A2ax1 / Ax2ay1-ax1 ay2 = (0.2042 x 201400)- (0.2014x 306500) / (201400 x 303200) - (306500x 204200)

= 0.00001038

= **1.038 µg/ml**

A1=Absorbance of GF
A2=Tablet Absorbance of GF
ax1 =Absorptivity of GF at 272 tab
ax2 =Absorptivity tab at 272 tab
ay1=Absorptivity value of GF at 272 STD
ay2=Absorptivity value of GF 272 STD
A1 absorbance of tablet at272 nm

8.2. Analytical of Method Validation:

1. Linearity:

From Gemfibrozil standard stock solution, different working standard solution (5-25µg/ml) were prepared in mobile phase 20 µl of sample solution was injected into the chromatographic system using mixed volume loop injector Chromatograms were recorded. The area for each concentration was recorded (**Table No. 24)** The Calibration curves are shown in **[Fig. No.45]**

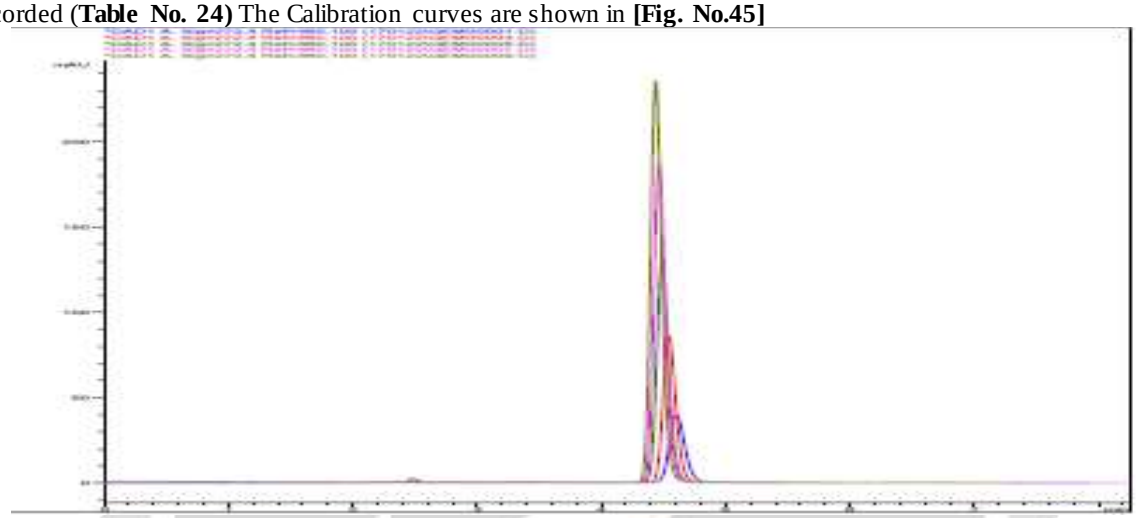

Fig.No.44.Overlay Chromatogram of linearity

Table No 24. Linearity of Gemfibrozil

Sr. No.	Concentrationµg/ml Gemfibrozil	Area Gemfibrozil
1	5	344.8674
2	10	622.5595
3	15	947.9040
4	20	1252.3643
5	25	1528.4650

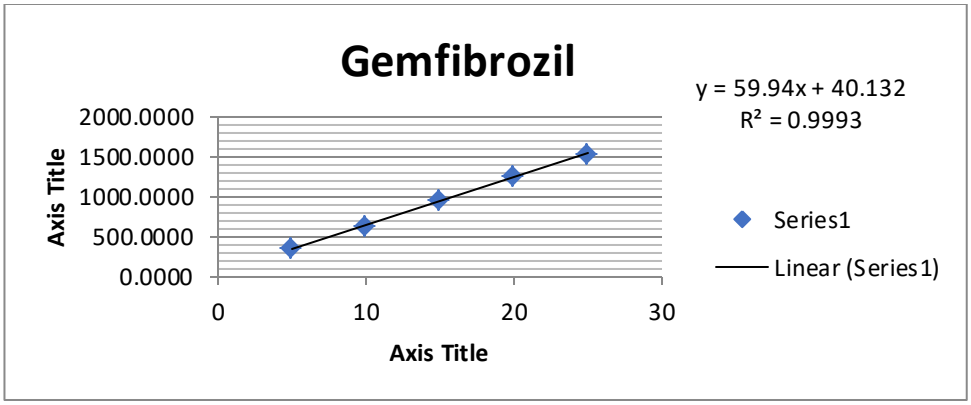

Fig.No.45. Calibration curve of Gemfibrozilfor HPLC method

Table No 25. Regression equation data for Gemfibrozil

Regression Equation Data Y=mx+c	
Slope(m)	59.94
Intercept(c)	40.13
Correlation Coefficient	0.999

Linearity of of Gemfibrozilwas observed in the range of 5-25µg/ml Detection wavelength used was 272 nm.

The plot should be linear passing through the origin; Correlation Coefficient should not be less than 0.999.that concluded. **(Table. No. 25)**

2. Accuracy:-

Recovery studies were performed to validate the accuracy of developed method. To pre analyzed tablet solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and then its recovery was analyzed **(Table No.26).** Statistical validation of recovery studies shown in **(Table No. 27)**

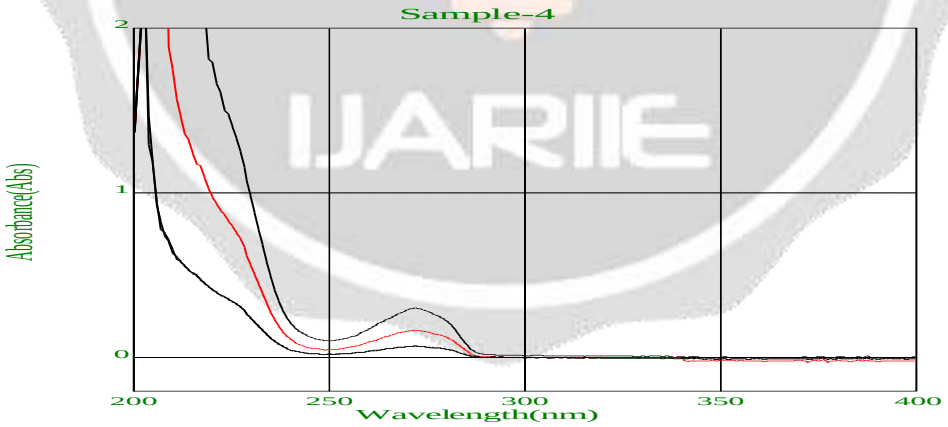

Fig.No.46Accuracy OfGemfibrozil(UV Method)

Table .26. Result of Recovery data for Gemfibrozil

METHOD	Drug	Level (%)	Amt. taken (µg/ml	Amt. Added (µg/ml	Absorbance Mean* ± S.D.	Amt. recovered Mean *±S.D.	% Recovery Mean *± S.D.
UV Method	GF	80%	10	8	17.86± 0.07	7.86± 0.07	98.22±0.93
		100%	10	10	19.89± 0.02	9.89±0.02	98.90±0.21

		120%	10	12	21.72±0.03	11.72±0.03	97.69±0.21
RP-HPLC Method	**GF**	80%	5	4	8.96±0.029	3.96±0.029	99.09±0.73
		100%	5	5	9.86±0.019	4.86±0.019	97.27±0.37
		120%	5	6	10.96±0.04	5.96±0.046	99.36±0.77

***mean of each 3 reading for RP-HPLC method and UV method**

Table.27. Statistical Validation of Recovery Studies Gemfibrozil

METHOD	Drug	Level (%)	Mean % Recovery	Standard Deviation*	% RSD
UV Method	**GF**	80%	98.22	0.93	0.94
		100%	98.90	0.21	0.21
		120%	97.69	0.21	0.21
RP-HPLC Method	**GF**	80%	99.09	0.73	0.74
		100%	97.27	0.37	0.38
		120%	98.36	0.77	0.78s

***Denotes average of three determinations for RP-HPLC and UV method**

Accuracy of RP-HPLC method and UV Spectrophotometric method is ascertained by recovery studies performed at different levels of concentrations (80%, 100% and 120%). The % recovery was found to be within 97-101% **(Table No. 26, 27)**.

3. System suitability parameters :(Repeatability)

To ascertain the resolution and reproducibility of the proposed chromatographic system for estimation of Gemfibrozilsystem suitability parameters were studied. The result shown in below **(Table No.28,29)**

Table No.28: Repeatability studies on RP-HPLC for Gemfibrozil

Sr.No.	Concentrationof Gemfibrozil (mg/ml)	Peak area	Amount found (mg)	% Amount found
1	20	1253.19	20.19	101.01
2	40	1250.324	20.21	101.07
		Mean	20.20	
		SD	2.08	
		% RSD	0.17	

Table No.29: Reapetability studies on UV method forGemfibrozil

Sr No.	Conc	Absorbance	Amt Found	% Amt Found
1	20	0.4001	19.86	99.27
2	20	0.4012	19.91	99.55

3	20	0.4011	19.90	99.53
4	20	0.4015	19.92	99.63
5	20	0.401	19.50	98.25
		Mean	19.82	99.24
		SD	0.18	0.57
		%rsd	0.90	0.58

Repeatability studies on RP-HPLC and UV method for Gemfibrozilwas found to be ,The %RSD was less than 2%, which shows high percentage amount found in between 98% to 102% indicates the analytical method that concluded .**(Table No.28,29)**

4. Precision:-

The method was established by analyzing various replicates standards ofGemfibrozil. All the solution was analyzed thrice in order to record any intra-day & inter-day variation in the result that concluded. The result obtained for intraday is shown in**(Table No. 30)** respectively.

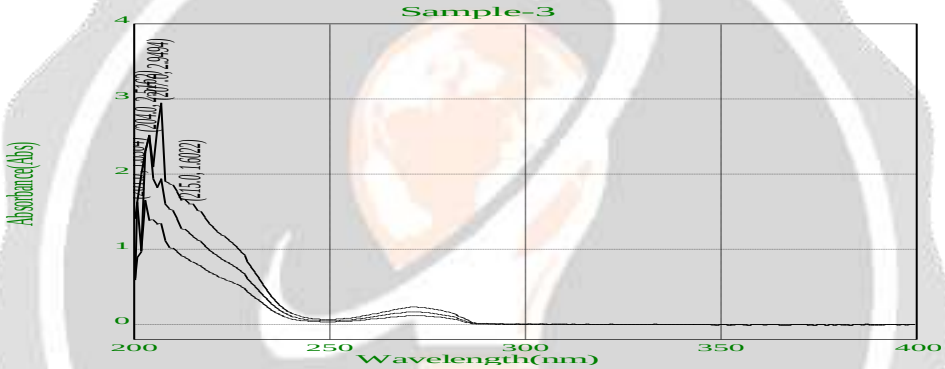

Fig.No.52Precision Of Gemfibrozil (UV Method)

Chromatogram of Precision:

Table No .30: Result of Intraday and Inter day Precision studies on RP-HPLC and UV method for Gemfibrozil

METHOD	Drug	Conc^{n} (µg/ml)	Intraday Precision		Interday Precision	
			Mean± SD	% Amt Found	Mean± SD	% Amt Found
HPLC METHOD Rp-	GF	10	621.62±1.03	97.01	627.87±1.78	98.05
		15	946.92±3.85	100.86	943.40±4.32	100.46
		20	1250.7±6.54	100.98	1251.42±1.80	101.04
UV METHOD	GF	2	0.202±0.001	99.95	0.2014±0.004	99.20
		3	0.3090±0.08	102.00	0.3063±0.003	101.07
		4	0.4006±0.002	99.40	0.4013±0.002	99.55

***Mean of each 3 reading for RP-HPLC and UV methods**

Intraday and Inter day Precision studies on RP-HPLC and UV method for Gemfibrozilwhich shows the high precision %amount in between 98% to 100% indicates to analytical method that concluded.

5. Robustness:

The Robustness of a method is its ability to remain unaffected by small deliberate changes in parameters. To evaluate the robustness of the proposed method, small but deliberate variations in the optimized method parameters were done. The effect of changes in mobile phase composition and flow rate, wavelength on retention time and tailing factor of drug peak was studied.

The mobile phase composition was changed in(±1 ml/min^{-1}**)** proportion and the flow rate was varied by(±1ml/min^{-1}**)**, and wavelength change(±1 ml/min^{-1}**)**of optimized chromatographic condition. The results of robustness studies are shown in **(Table No.31**).Robustness parameters were also found satisfactory; hence the analytical method would be concluded.

Table No.31Result of Robustness Study of Gemfibrozil

Parameters	Conc.	Amount of detected(mean ±SD)	% RSD
Mobile phase composition-(39+61)	15	943.44±3.63	0.39
Mobile phase composition-(41+59)	15	946.42±1.64	0.17
Wavelength change271nm	15	945.1±2.85	0.30
Wavelength Change 272nm	15	945.42±1.77	0.19
Flow rate change(0.9ml)	15	983.49±2.84	0.29
Flow rate change(1.1ml)	15	921.71 ± 3.42	0.37

Robustness Study of Gemfibrozil:

The changes were did flow rate (±1 ml/ min^{-1}), PH of mobile phase composition (±1 ml/ min^{-1}), and Wavelength (±1 ml/ min^{-1}). %RSD for peak area was calculated which should be less than 2%.the result shown in analytical method that concluded. (**Table No.31)**

6. Limit Detection

The LOD is the lowest limit that can be detected. Based on the S.D. deviation of the response and the slope the limit of detection (LOD) may be expressed as:

LOD= 3.3XAvd.SD/Slope
=3.3X2.43/59.94
=0.1337

Where, SD = Standard deviation of Y intercept
S = Slope

- The LOD of Gemfibrozil was found to be 0.133 (µg/mL) analytical methods that concluded.

8. Limit Quantification

The LOQ is the lowest concentration that can be quantitatively measured. Based on the S.D. deviation of the response and the slope,

The quantitation limit (LOQ) may be expressed as:

LOQ = 10 (SD)/ S
=10 X 2.43s / 59.94

= 0.4052

Where, SD = Standard deviation Y intercept

S = Slope

- The LOQ ofGemfibrozil was found to be 0.4052 (µg/mL) analytical method that concluded.

8.3 Analysis of tablet formulation:-

Procedure:

Weigh 20 GemfibrozilCapsule and calculated the average weigh 15.74 mg accurately weigh and transfer the sample equivalent to 6.55mg Gemfibrozil into 10 ml volumetric flask. Add about 10ml of diluent and sonicate to dissolve it completely and make volume up to the mark with diluent.Mix well and filter through 0.45 µm filter. Further pipette 0.3ml of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluents. (25 µg/ml). The simple chromatogram of test Gemfibrozil Shown in **(Fig No: 66)**the amounts of Gemfibrozil per Capsule were calculated by extrapolating the value of area from the calibration curve. Analysis procedure was repeated five times with tablet formulation. Tablet Assay for %Lable claim for %RSD Calculated, Result was shown in **(Table No. 32)**

Brand Name:LOPID 600 MG (PFIZER Ltd)

Total weight of 20 tab wt = 15.74gms

Avgr Weight = 0.787 gms. /Tab

Eq.wt for 5 mg= 5 X0.787 / 600 =6.55 mg

Take 6.55 mgs in 10 ml Methanol sonicate 10 min

i.e. 500 µgm/ml Gemfibrozil ----- STOCK -II

Table.32. Analysis of marketed formulation

Assay	Drug	Lable Claimed	Amt.Found	% Lable Claim	SD	% RSD
Rp-HPLC Method	**GF**	25	24.92	99.69	4.236	0.277
		25	24.82	99.29	4.213	0.284
UV Method	**GF**	15	15.10	100.70	0.35	0.9
		15	15.07	100.47	0.32	0.37

Analysis of marketed formulation were also %Lable Claim was found to be 99-101% Satisfactory are concluded*. (Table No.32)*

Conclusion:

Simple, rapid, accurate and precise RP-HPLC and UV-Spectroscopic methodhave been developed and validated for the routine analysis of Gemfibrozilin API and tablet dosage forms. Both methods are suitable for the determination of Gemfibrozilin Single-component formulations without interference of each other. The developed methods are recommended for routine and quality control analysis of the investigated drugs component pharmaceutical preparations. The amount found from the proposed methods was in good agreement with the label claim of the formulation. Also the value of standard deviation and coefficient of variation calculated were satisfactorily low, indicating the suitability of the proposed methods for the routine estimation of tablet dosage forms.

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