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Development and Validation of Sensitive RP- HPLC Method for Determination of Gemfibrozil in Human plasma

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ABSTRACT

A sensitive, accurate and rapid high-performance liquid chromatography with UV-visible detection (HPLC-UV) method for the determination of Gemfibrozil in human plasma has been developed. Gemfibrozil and internal standard (Naproxen) were extracted by using solid-phase extraction technique with direct elution. The resolution of peaks was achieved with 10 mM potassium dihydrogen phosphate buffer (pH 4.0 $\pm$ 0.1) and acetonitrile in a ratio of 95:5 v/v on a reversed-phase X-Terra C_{18} column (4.6 mm Internal Diameter X 150 mm Length, 5µ particle size). The eluents were quantified by UV detection at 222 nm. The analytical run time was less than 12.5 min. The method was proved to accurate and precise at linearity range of 0.202–50.070 µg/mL. The method was applied to a pharmacokinetic study and bioequivalence study of gemfibrozil.

Key words: HPLC, Gemfibrozil, Human plasma, Validation.

INTRODUCTION

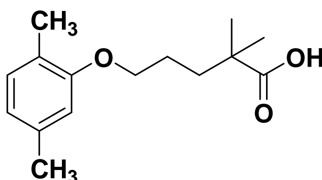

Fig-1. Structure of Gemfibrozil

Gemfibrozil [1-3] a fibric acid antilipemic agent similar to clofibrate, is used to treat hyperlipoproteinemia and as a second-line therapy for type IIb hypercholesterolemia. Gemfibrozil increases the activity of extrahepatic lipoprotein lipase (LL), thereby increasing lipoprotein triglyceride lipolysis. It does so by activating Peroxisome proliferator-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 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. Well absorbed from gastrointestinal tract (within 1-2 hours). Gemfibrozil mainly undergoes oxidation of a ring methyl group to successively form a hydroxymethyl and a carboxyl metabolite. Approximately seventy percent of the administered human dose is

excreted in the urine, mostly as the glucuronide conjugate, with less than 2% excreted as unchanged Gemfibrozil. Half life of the drug was 1.5 hrs and more than 95% of the drug was bound to plasma.

Earlier Jamshid et al reported [4] the Gemfibrozil method was developing in pharmaceutical preparation by using spectrofluorometric and micelle-enhanced-spectrofluorimetric technology. The Gemfibrozil was determined in the presence of dog plasma by using a sensitive LC–MS/MS [6] and LC/MS method for the determination of Gemfibrozil in human plasma reported by Jospeh et al [5]. In this report we are going to report the determination of Gemfibrozil in the human plasma by using RP-HPLC method.

MATERIALS AND METHODS

Chemicals and reagents

HPLC grade acetonitrile and methanol were used in this study obtained from Qualigens, INDIA. All aqueous solutions, including the buffer for the mobile phase, were prepared with Milli Q grade water (milli-Q) from Millipore (USA) equipment. Analytical grade Potassium dihydrogen phosphate salt and orthophosphoric acid purchased from Qualigens, INDIA. The working standard samples of Gemfibrozil and Ibuprofen were procured by Aurobindo Pharma Limited (Hyderabad, INDIA).

Chromatographic conditions

An Shimadzu HPLC LC-2010HT is comprised of a degassing unit, quaternary low-pressure gradient unit, pump unit, mixer, ultra fast auto sampler, column oven, and a UV-VIS detector was used for chromatographic separation, a X-Terra C_{18} (4.6 ID X 150 mm, 5μ particle size) column and the detection of the compounds was monitored at 222 nm. A mobile phase consisting of 10mM Potassium dihydrogen phosphate buffer (pH 4.0 $\pm$ 0.1) and acetonitrile in a ratio of 95:5 v/v was used at a rate of 1.2mL/min. Data was acquired and processed with LC Solution Software.

Preparation of stock and spiking solutions

A primary stock solution of Gemfibrozil was prepared separate weighing for calibration curve and quality control samples. The primary stock solutions of the analytes and IS were prepared in methanol (5.0 mg/mL for analyte and 1.0 mg/mL for IS) and stored at below 10 ^{0}C in refrigerator and the stock solutions were stable for 16.9 days. The spiking concentration of calibration curve standards and quality control samples LLOQQC, LowQC, Midium QC, HighQC approximately 0.202, 0.404, 2.019, 4.038, 10.094, 25.235, 35.049, 50.070 and 0.203, 0.597, 20.586, 38.123 μg/mL respectively volumes were 300 μL aliquoted into different tubes and were stored at-70 ^{0}C.

Sample extraction procedure

To an aliquot two hundred fifty micro liters of spiked plasma calibration curve standards and quality control samples were transferred to pre-labeled poly propylene tubes, was added 50 μL of internal standard (Naproxen) dilution (5 μg/mL). The tubes were vortexed on a cyclomixer (Remi Instruments, Mumbai, India) for 20 seconds. This sample mixture loaded on pre conditioned with 1 mL methanol followed by 1 mL water on HLB cartridges (1CC, 30 mg) and washed with 1 mL water and followed by 1 mL 10% methanol in water (washing solution) and the cartridges were dried for about one minute and eluted with 1mL of elution solution. The contents of the tubes were vortexed and transferred into auto-sampler vials and then analyzed by HPLC. An aliquot of 30 μL of the sample was drawn each time from the vials in the auto sampler.

RESULTS AND DISCUSSION

Method Development, Liquid Chromatography

In order to remove the interfering peaks from the bio matrices and to increase the selectivity and sensitivity of the analytical method different methods of sample pre-treatment were investigated. Protein precipitation with various organic solvents and their mixtures resulted in reproducible recoveries not obtained properly. But in the presence of solid phase extraction method, the results were obtained accurately. In this technique, there is no interference from the sample matrix with the chromatography of the analyte and IS. In pursuit of symmetric peak shape and shorter run time, feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, sodium dihydrogen phosphate, potassium dihydrogen phosphate and formic acid with variable pH range of 3.0-6.0 and along with altered flow-rates (in the range of 0.5–1.5 mL/min) were tested for complete chromatographic resolution of Gemfibrozil and IS. The resolution of peaks was achieved with 10 mM of potassium dihydrogen phosphate (pH 4.0 ± 0.1) and acetonitrile (95:5, v/v) with a flow rate of 1.2 mL/min, on a X-

Terra C18 column (4.6 mm Internal Diameter X 150 mm Length, 5μ particle size) column was found to be suitable for the determination of Gemfibrozil and IS. The UV absorption spectrum of the drug was taken in methanol and the λ_{max} was found to be at 222 nm. A model chromatogram showing the separation of Gemfibrozil is presented in Fig-5 under the above optimized conditions retention times of 10.7 and 2.7 min were obtained Gemfibrozil and Naproxen respectively.

Validation Procedures

A full validation was performed for the assay in human plasma according to the FDA and EMEA guidelines.

Specificity and selectivity

The specificity of the method was evaluated by analyzing human plasma samples from at least six different blank plasmas (from different donors) to investigate the potential interferences at the LC peak region for analyte and IS.

Precision and accuracy

The intra-day and inter-day assay precision and accuracy were estimated by analyzing six replicates at four different QC levels, i.e. 0.203, 0.597, 20.586, 38.123 μg/mL. The criteria for acceptability of the data included accuracy within ±15% standard deviation (SD) from the nominal values and a precision of within ±15% relative standard deviation (RSD), except for LLOQ, where it should not exceed ±20% of accuracy as well as precision (**Table-1**).

Table-1 Intra- and inter-day precision of determination of Gemfibrozil in human plasma

	Intra-batch					Inter-batch			
	Theoretical concentration (μg/mL)	n	Mean concentration observed (μg/mL)	%CV	% Accuracy	n	Mean concentration observed (μg/mL)	%CV	% Accuracy
LLOQQC	0.203	6	0.1938	5.0	95.5	24	0.1973	11.4	97.2
LQC	0.597	6	0.6353	5.2	106.4	24	0.6153	3.6	103.1
MQC	20.586	6	19.0463	3.3	92.7	24	18.8803	3.1	91.5
HQC	38.123	6	41.0552	3.3	107.7	24	36.7762	6.9	96.7

Table-2 Stability samples results for Gemfibrozil

Details of Stability Experiment	n	Spiked concentration μg/mL	Mean calculated comparison sample concentration	Mean calculated stability sample concentration	%CV	% Stability
Bench top stability HQC	6	38.123	41.6862	41.5398	2.2	99.6
Bench top stability LQC	6	0.597	0.6093	0.5893	4.5	96.7
Auto-sampler stability HQC	6	38.123	38.0508	41.4962	4.1	109.1
Auto-sampler stability LQC	6	0.597	0.6342	0.6138	5.1	96.8
Freeze-thaw stability HQC	6	38.123	41.6862	40.8122	2.0	97.9
Freeze-thaw stability LQC	6	0.597	0.6093	0.6165	6.1	101.2
Dry extract stability HQC	6	38.123	38.0508	37.752	2.2	99.2
Dry extract stability LQC	6	0.597	0.6342	0.6043	4.3	95.3
Long term stability HQC	6	38.123	39.3727	40.5922	2.6	103.1
Long term stability LQC	6	0.597	0.6043	0.6182	6.1	102.3

Stabilities

The stability of the processed sample was evaluated by comparing the extracted plasma samples that were injected immediately (time 0), with the samples that were re-injected after keeping in auto-sampler at 10^{0}C for 43.20 hrs. The stability of spiked human plasma samples kept at room temperature (Bench top stability) was evaluated for 10.14 hrs and compared with that of the freshly prepared extracted samples. The freeze-thaw stability was assessed by comparing the stability of the samples that had been frozen and thawed 5 times, with that of the freshly spiked

quality control samples. The stability of spiked human plasma stored at -70^{0}C (long – term stability) was evaluated by analyzing the quality control samples that were stored at -70^{0}C for 30.12 days together with the freshly spiked calibration standards and the quality control samples (**Table-2**). All stability evaluations were based on back-calculated concentration. Analytes were considered stable if the deviation of the mean test responses were within 15% of freshly prepared or comparison samples.

Calibration curve

The calibration curve was acquired by plotting the ratio of sum of peak area of Gemfibrozil to that of IS against the nominal concentration of calibration standards. The final concentrations of calibration standards obtained for plotting the calibration curve were 0.202, 0.404, 2.019, 4.038, 10.094, 25.235, 35.049, 50.070 µg/mL. The results were fitted to linear regression analysis using$1/X^2$ as weighting factor. The calibration curve has a correlation coefficient(r) of 0.9995 (**Table-3**) (**Fig-2**). The acceptance criteria for each back-calculated standard concentration were ±15% deviation from the nominal value except at LLOQ, which was set at ±20 %.

Table-3 Summary of calibration parameters of Gemfibrozil

Calibration curve	Slope	Intercept	Correlation coefficient
1	**0.0410**	**0.0023**	**0.9989**
2	**0.0334**	**0.0209**	**0.9976**
3	**0.0310**	**-0.0061**	**0.9995**
4	**0.0316**	**0.0130**	**0.9981**

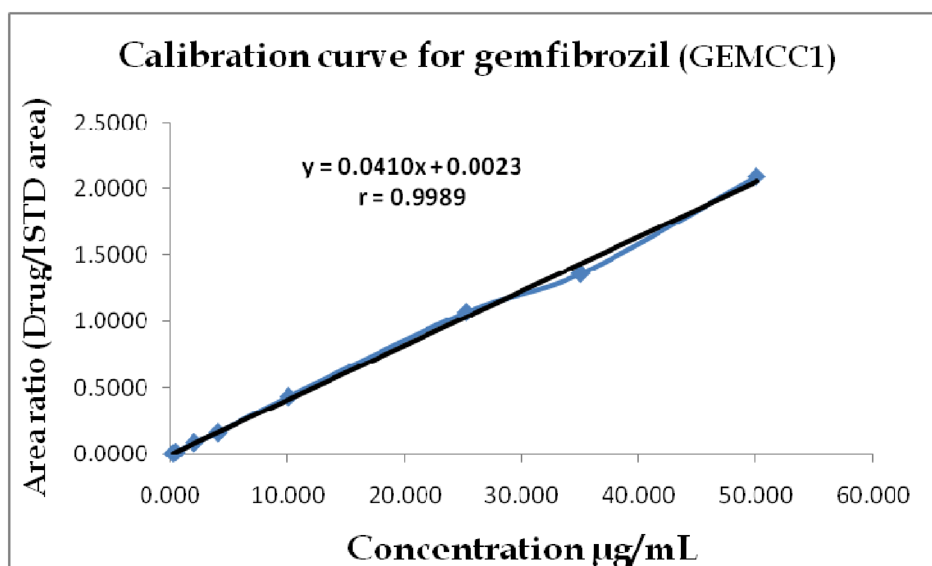

Fig-2. Calibration curve for Gemfibrozil (GEM CC1)

Recovery

The results of the comparison of extracted samples against post extracted plasma samples were estimated for Gemfibrozil at 0.597, 20.586, 38.123 µg/mL and the mean % recovery were 84.6, 85.5 and 82.3 respectively. The SPE process yielded a clean chromatogram when in the blank plasma sample was processed and further no interference was observed at the retention time of Gemfibrozil and IS. The % recovery of IS at was 71.2.

Dilution Integrity

Dilution integrity was performed to extend the upper concentration limits with acceptable precision and accuracy. Six replicates each at a concentration of double the uppermost calibration standard were diluted two fold and four fold with blank plasma. The diluted samples were processed and analyzed against calibration curve. The precision for dilution integrity of Gemfibrozil was 1.9% at 25 percent dilution and 5.3% at 50 percent dilution. The accuracy for dilution integrity of Gemfibrozil was 99.9% for 25 percent dilution and 97.1% for 50 percent dilution.

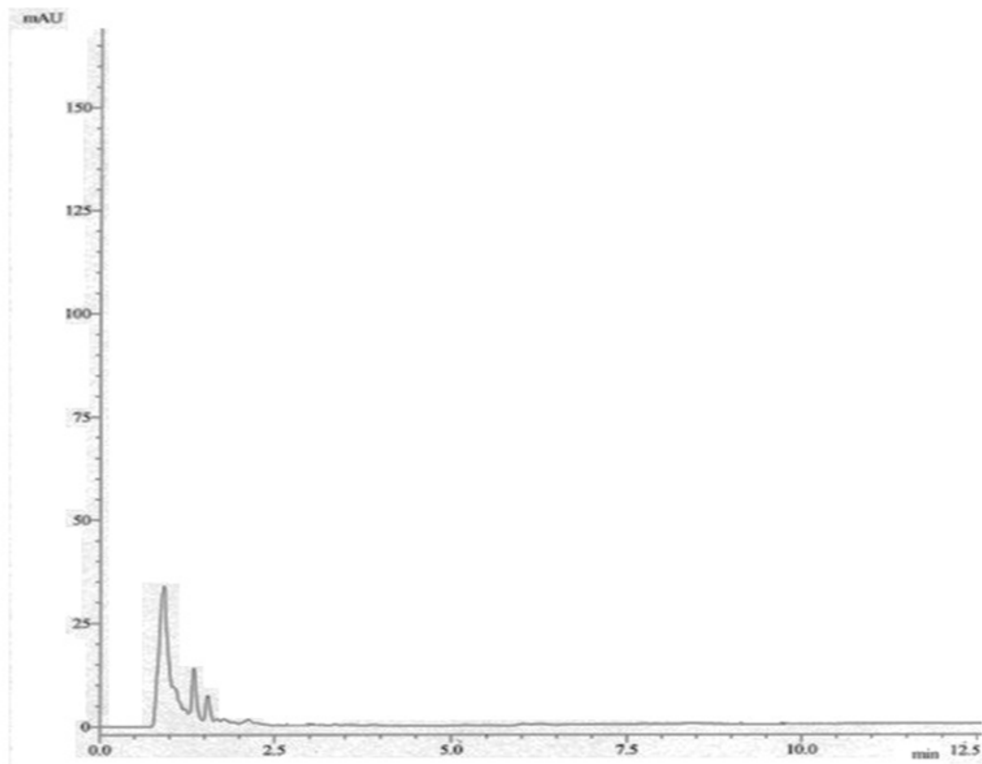

Fig-3. Representative Chromatogram of Gemfibrozil extracted blank sample

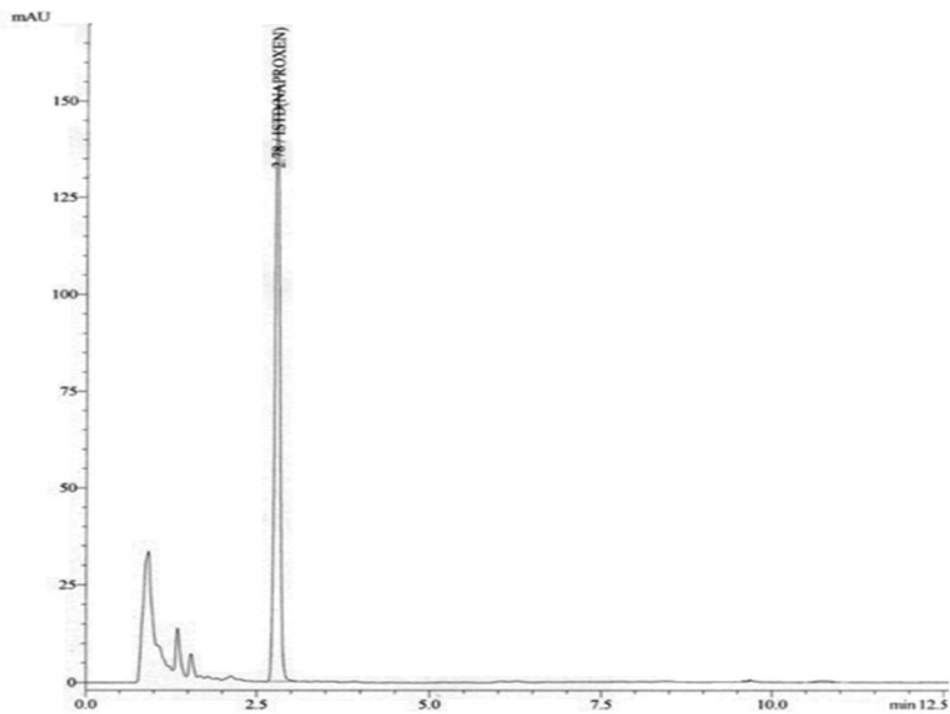

Fig-4. Representative Chromatogram of Gemfibrozil extracted blank + ISTD sample

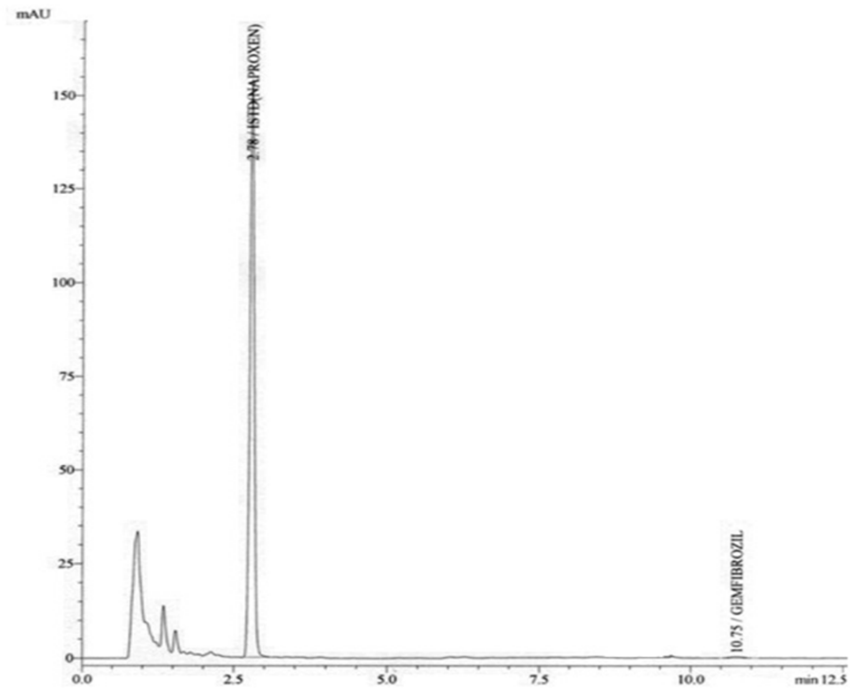

Fig-5. Representative Chromatogram of Gemfibrozil extracted STD1 (LLOQ)

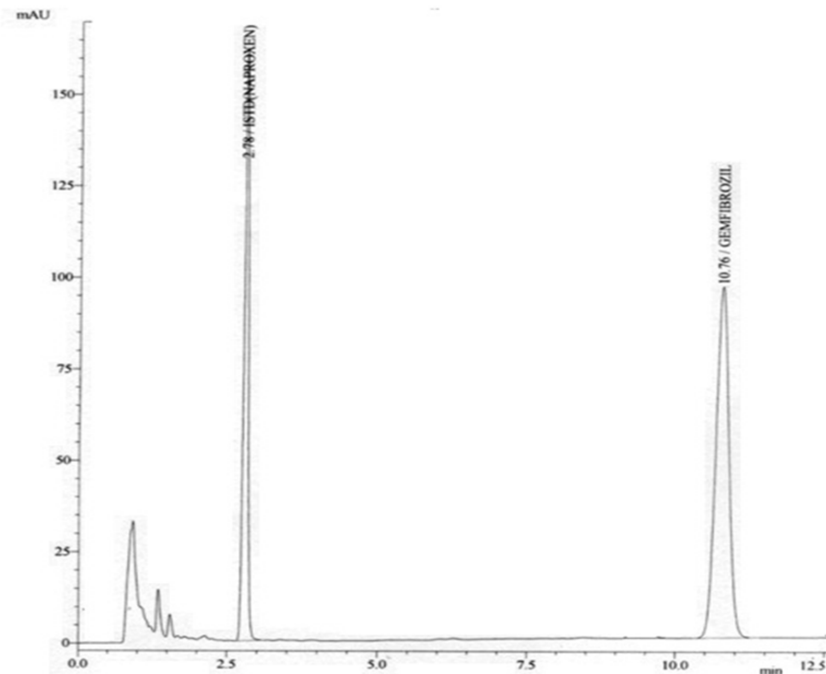

Fig-6. Representative Chromatogram of Gemfibrozil extracted STD8 (ULOQ)

According to chromatograms in **Fig-3** represented no interference at anlayte and ISTD from the extracted blank plasma sample**, Fig-4** shows there is no interference in the presence of extracted blank + ISTD plasma sample, **Fig-5** represented extracted STD-1 (LLOQ) plasma sample and **Fig-6** showed the extracted STD-8 (ULOQ) plasma sample of Gemfibrozil.

CONCLUSION

This RP-HPLC method for determination of gemfibrozil in human plasma using a simple SPE extraction procedure and commercially available IS. The method was validated in accordance with ICH guidelines like acceptance criteria with respect to selectivity, precision, accuracy, linearity, recovery and dilution integrity. The method reduces analysis time and found to be cost effective and seems to be useful further clinical pharmacokinetic studies and bioequivalence studies.

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