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Bioanalytical method validation of fenofibrate by hplc using human plasma

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Abstract

The bioanalytical method validation of fenofibrate was performed with all the basic parameter by HPLC using human plasma. This research paper describes a specific procedure for validation of Fenofibrate in human plasma [1-2]. The mobile phase used was simple and column friendly and was sufficiently sensitive to quantify fenofibrate in amounts as low as 0.095µg/ml. This limit of quantitation (LOQ) was determined as the lowest concentration with a coefficient of variation lower than 20% and the total accuracy of the method ranged from 101.99 to 107.41%. The LOQ of this method is better than those of other reported methods. The calibration curve plotted concentration *versus* area and was linear from 0.095 µg/ml to 19.924 µg/ml, having r^2 greater than 0.98 during the course of validation. The above method is valid for the analysis of drug in human plasma. The method with slight modification could be used for the drug analysis in various dosage forms [2-4].

Keywords: Fenofibrate, LOQ, Bioanalytical method validation

1. Introduction

After developed an analytical methods or bio-analytical method for the quantitative determination of drugs and their metabolites in biological samples play a important role for the study and interpretation of bioavailability, bioequivalence and pharmacokinetic data. Chromatographic analytical methods are commonly used in regulatory laboratories for the qualitative and quantitative analysis of drug substances, drug products, raw materials and biological samples through all phases of drug development and from research to quality control. Throughout the drug development process, method validation is carried out to ensure that an analytical method is accurate, specific, reproducible and rugged over the specific range in which the analyte will be analysed. All the stability of a drug in a biological matrix is also important such as bench top stability, freeze-thaw stability, stability in the auto sampler and long term stability [3-4].

2. Experimental

2.1 Sample Preparation

Samples of 700 µl of plasma were taken. To this 50 µl (75 µg/ml) of Diazepam as internal standard (IS) was added. The samples were vortex mixed for about 2 min and 100 µl of 0.1 N hydrochloric acid and 5.0 ml ethyl acetate were also added. The contents were transferred to a shaker for 15 min at 100 rpm and centrifuged for 3 min at 5°C. Then 5.0 ml of the upper organic layer were transferred and evaporated under N_2 gas at100psi and 50° C. The sample was finally reconstituted with 400 µl of mobile phase, transferred into the chromatography vials and 100 µl injected [5-7].

2.2 Chromatographic Conditions

The following parameters were used for the developed method and chromatograms were obtained for the drug and IS aqueous mixtures, extracted blank plasma sample, extracted blank plasma with IS sample and extracted blank plasma with drug.(Table-I)

2.2 Validation

2.2.1 System Suitability

This parameter was performed by running six injections of drug dilution. Coefficient of variation in percentage (%CV) of areas and retention times were calculated. %CV of area was less than 2% and that of the retention times was less than 5%, which is within the acceptable range.

2.2.2 Precision:

The precision of the developed method based on within-day repeatability was determined by replicate analysis of six sets each of high, middle and low quality control samples. The reproducibility (day-to-day variation) of the method was validated using similar six sets of high, middle and low quality control samples on different days.

2.2.3 Accuracy:

Accuracy of the developed method was determined by replicate analysis of six sets of sample at high, middle and low quality control concentrations and comparing the difference between the spiked value (nominal) and that actually found. Accuracy was expressed as percentage of the nominal concentration.

2.2.4 Recovery:

In Bio-analytical method development the analytical recovery of sample preparation procedure of Fenofibrate and the internal standard (Diazepam) was estimated by comparing the peak areas obtained from plasma samples (extracted) at three concentration levels (LQC, MQC, HQS), with equivalent amounts of and IS Diazepam in aqueous solution.

2.2.5 Selectivity:

In Bio-analytical method development the selectivity of the method was verified by checking the interference of endogenous compounds in human plasma at the retention time of the drug and IS by evaluating six lots of plasma [9-12].

2.2.6 Stability

Stability parameter was evaluated by determining the following five parameters:

A. Stock solutions stability-

The drug stock solution was evaluated by injecting six replicate samples of old stock solutions and comparing the response with freshly prepared stock solution. The stock solution stability of the internal standard stock was evaluated by the same process.

B. Bench top stability-

The bench top was stability was determined at lower and higher quality control samples by evaluating 6 replicate samples at each level. The samples were processed after keeping them at bench top (room

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temperature) for about 2 hours and then analyzed against freshly spiked calibration curve standards.

C. Freeze-thaw stability-

The freeze-thaw stability in matrix was studied by assaying six replicates of QC samples at low and high concentrations previously frozen and thawed over three cycles against freshly spiked calibration standards. The samples were first frozen at -27^{0}C for at least 40 hours followed by unassisted thawing at room temperature. The samples were again frozen for at least 12 hours under the same conditions. This freeze-thaw cycle was repeated two more times and the samples were then processed after the third cycle and analyzed.

D. Auto sampler stability- Stability in the auto sampler was assessed by extracting six replicates of QC samples at low and high concentration and putting the processed samples in the atmosphere. The samples were injected after 120 hours along with freshly spiked calibration standards.

E. Long-term stability- The long-term evaluation was performed following a storage period of about 60 days. Six replicates of the stored low and high concentration QC samples were removed from the freezer/cold room and allowed to thaw. The samples were processed and analyzed against freshly spiked calibration standards [10-12].

2.3 Validation Study

After optimization of the analytical conditions, the evaluation of parameters such as selectivity, accuracy, precision, linearity, recovery (drug and internal standard), stability (freeze-thaw, bench top, auto sampler, long term, stock solution stability), ruggedness and dilution integrity were performed for the validation of the method.

2.4 Selectivity

Six lots of blank plasma were evaluated and an interfering peak was observed at the retention time of the drug, but the area of the interfering peak was small and insignificant. There were no interfering peaks at the retention time of the internal standard (Table II).

2.5 Precision and Accuracy

Precision and accuracy of the method was characterized by running five analytical batches; each batch contained the following samples:

(a) A reference standard solution (one sample, mixture with internal standard)
(b) Blank matrix (in duplicate)
(c) Blank matrix with internal standard (in duplicate)
(d) Spiked calibration standards (1set of 8 non-zero concentrations)
(e) Limit of quantitative quality control (LOQQC) (6 samples)
(f) Lower quality control (LOQ) (6 samples)
(g) Middle quality control (MQC) (6 samples)
(h) Higher quality control (HQC) (6 samples)

2.5.1 Precision: The precision of the method was measured by the present coefficient of variation (%CV) over the concentration range of high, middle and low quality control samples, respectively, of the drug during the course of validation. Within batch/interbatch precision of the method ranged from 1.14 to 6.90%.

2.5.2 Accuracy:

The accuracy of the assay is defined as the peak area ratio response of the drug and internal standard versus concentration of the quality control sample to their respective nominal values, expressed as percentage. Within batch accuracy: Within batch accuracy of the method for the drug was found in the range of 90.30 to 110.60%. Between batch/inter batch or total accuracy: The total accuracy of the method ranged from 101.99 to 107.41%.

2.6 Linearity:

The linearity of the method was determined by a weighted least square regression analysis of standard plot associated with an eight point standard curve. The calibration curve plotted concentration versus area (Table III) and was shown to be linear from 0.095µg/ml to 19.924 µg/ml as shown in fit calibration lines of peak are response of drug and internal standard versus concentration of calibration standards were determined by weighted least square regression analysis with a weighting factor $1/X^2$. The regression coefficient (r^2) were consistently greater than 0.98 during the course of validation [12-14].

Statistical parameters for the calibration curve

- Y = 0.4734 X
- r^2 = 0.9927
- r = 0.9885

2.7 Recovery

The percentage recoveries for the drug and the internal standard were determined by comparing the peak areas of the response of drug extracted from plasma quality control samples with that of the peak areas of un extracted aqueous standard samples containing the same concentration of the drug and the internal standard. The present recoveries were calculated at each QC concentration by the following equation.

% Recovery = Mean peak response of extracted samples/Mean peak response of non-extracted samples

The total recovery of the drug was 58.21 to 64.20% (mean recovery=60.86%) and that of the internal standard was 79.05%. (Tables IV)

2.8 Stability

Stock solution stability: The stability of drug stock solution was evaluated by injecting a dilution of 3.808 mg/ml solution of the stock. The results indicate the stability of the stock solution over a minimum of 15 days period when stored at or below 100 °C [12-14]. (Table V)

3. Results

Table I: Chromatographic Condition

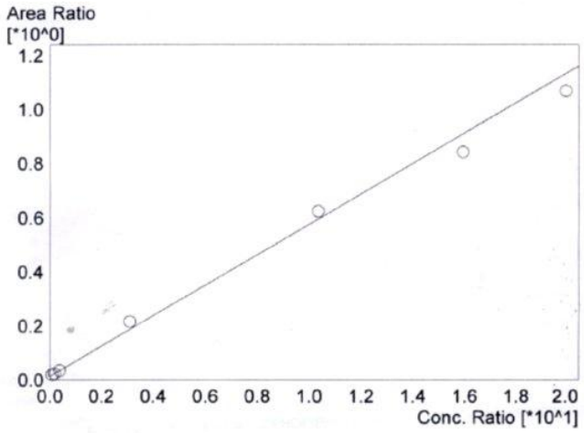
1	Column	Lithosphere 60 RP-Select B 250x4mm ; 5 µm
2	Column Temp	35°C
3	Flow Rate	0.5 to 1.5 ml/min
4	Detector	UV at 287 nm
5	Mobile phase	Phosphate buffer (pH 6.5) and Acetonitrile (75:25)
6	Injection volume:	100 µl
7	Retention Time Fenofibrate Diazepam	 6.5. to 7.5 min 5.5 to 6.5 min

Table II: Blank Plasma screening (Selectivity)

S. No.	Blanks	Interference at analyte RT (Area)	LOQ Area	% LOQ	Interference at analyte IS (Area)	IS Area	% IS
1	BLK-01	0	13138	0.0	0	354711	0.0
2	BLK-02	0	13670	0.	0	372688	0.0
3	BLK-03	56	13878	0.48	0	365678	0.0
4	BLK-04	475	14235	5.01	0	370354	0.0
5	BLK-05	0	14790	0.0	0	393184	0.0
6	BLK-06	325	14123	2.67	0	389432	0.0
	Mean		13972.33			374341.2	
	SD		509.36			13302.94	

Table III: Linearity

Concentration ratio (Drug: IS)	Area Ratio (Drug :IS)
0.095	0.0
0.190	0.1
0.381	0.2
1.088	0.5
3.108	1.6
10.361	4.4
15.940	7.6
19.924	8.4



#	Conc (Ratio)	Area Ratio	Area
1	0.095	0.0	11634
2	0.190	0.0	14967
3	0.381	0.0	21182
5	3.108	0.2	137694
6	10.361	0.6	382341
7	15.940	0.8	484081
8	19.924	1.1	607130

Table-IV (Recovery)

% Recovery			
QC	**LQC**	**MQC**	**HQC**
1	67.67	60.92	58.84
2	64.67	63.34	57..55
3	61.92	64.90	60.60
4	69.60	58.92	53.98
5	62.70	54.62	57.98
6	65.50	60.93	60.70
Mean	64.30	60.05	58.21
S.D.(+/-)	2.7	3.34	2.26
C.V.(%)	4.2	5.5	3.8
N	6	6	6

Table-V (Stability)

Stock solution stability of Fenofibrate stored at refrigerated temperature between 1-10 °C			
Stability stock		**Comparison Stock**	
Old	**Response**	**New**	**Response**
1	38680	1	38740
2	38195	2	38385
3	37985	3	38485
4	38540	4	37700
5	38720	5	38500
6	38450	6	38400
Mean	38428.5		38368.5
S.D.	262.14		320.68
C.V.%	0.6		0.8
% Stability	99.84		99.84

4. Conclusions

The method was subjected to complete validation and all the parameters showed results within the acceptance limits. A linear response between the concentration ranges of (0.095 µg/ml to 19.924 µg/ml) was obtained. The limit of quantitation using 450 µl of plasma was 0.095 µg/ mL. The accuracy and precision of the method was evaluated by peak area response of the drug and internal standardThe total precision (% CV) and accuracy (% normal) for the drug ranged from 4.35 to 8.38% and 101.99 to 107.41%, respectively. The mean recovery of the drug and IS was found to be 62.9 and 78.2% respectively.

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