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A SENSITIVE AND SPECIFIC BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF FENOFIBRIC ACID IN HUMAN PLASMA USING LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY

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ABSTRACT

A novel, simple, selective and rugged quantitative method for the determination of Fenofibric acid the active metabolite of fenofibrate in human plasma (Na_2EDTA) using liquid chromatography-tandem mass spectrometric (LC-MS/MS) method has been developed and validated with 200µL human plasma. Fenofibric acid-d_6 was used as an internal standard. Analyte and the internal standards were extracted from human plasma by liquid-liquid extraction using Methyl tertiary butyl ether as extraction solvent and ammonium acetate (5mM, pH 2.5) as extraction buffer. The reconstituted samples were chromatographed on a C18 column by using isocratic mobile phase. The method was validated over the concentration range of 79.89–20021.87 ng/mL. The Quattro Premier XE mass spectrometer was operated under the multiple reaction-monitoring mode (MRM) using the electro spray ionization technique for quantification of ion transitions at m/z 317.06/231.00 and 323.24/231.04 for the drug and the internal standard respectively. The method was validated for precision and accuracy, stability, matrix effect, dilution integrity, ruggedness, selectivity and extraction efficiency, and method has been proved to be simple, sensitive, selective, rugged and reproducible. A run time of 2.00 min for each sample made it possible to analyze more than 400 plasma samples per day. The proposed method can be applied for the estimation of the Fenofibric acid in real time plasma samples for pharmacokinetic, drug-drug interaction and toxicological studies.

Key words: Fenofibric acid, Validation, Human Plasma, LC-MS/MS, Electrospray ionization.

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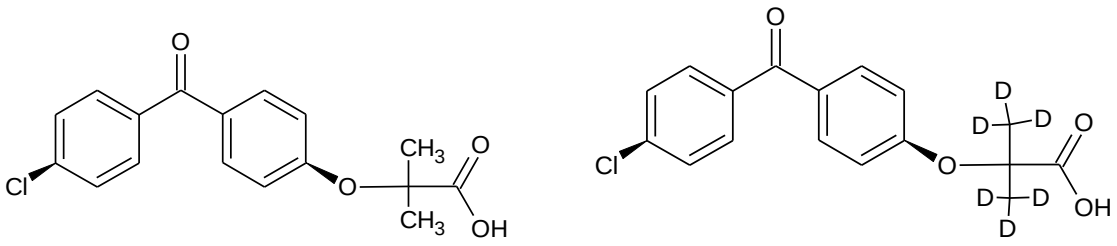
INTRODUCTION

Fenofibric acid (FA), the active moiety of fenofibrate, is responsible for the pharmacodynamic actions of the molecule. Fenofibric acid decreases triglycerides, cholesterol and very low-density lipoprotein levels, as well as increase of high-density lipoprotein-cholesterol. These effects are modulated at the transcription level through the activation of peroxisome proliferator-activated receptor-α (PPAR- α). Fenofibric acid is mainly inactivated by UDP-glucuronosyltransferases (UGTs) into FA-glucuronide (FA-G) [1]. Fenofibric acid is chemically 2-[4'-(p-chlorobenzoyl)phenoxy]-2-methylpropionic acid (Figure. 1a)[2], labled finofibric acid structure represented in figure 1b.



Fenofibric acid (1a) Fenofibric acid –d_6 (IS) (1b)

Figure1: Chemical structures of Fenofibric acid (1a) and Fenofibric acid –d_6 (1b)

The effects of fenofibric acid evaluated *in vivo* in transgenic mice and *in vitro* in human hepatocyte cultures by the activation of PPAR-α. Through this mechanism, fenofibrate increases lipolysis and elimination of triglyceride-rich particles from plasma by activating lipoprotein lipase and reducing production of Apoprotein C-III (an inhibitor of lipoprotein lipase activity) [3-5]. Fenofibric acid also has a beneficial effect on the insulin resistance feature by the metabolic syndrome. It is used alone or in conjunction with statins in the treatment of hypercholesterolemia and hypertriglyceridemia. Fenofibrate is marketed under the brand name Tricor and Trilipix by Abbott Labs, Lipofen by Kowa Pharmaceuticals America Inc, Lofibra by Teva and Supralip by Solvay Pharmaceuticals.

For the determination of Fenofibric acid and in combination with statins in human plasma some high-performance liquid chromatographic methods, Ajay Kumar *et al.*, described method development and validation of fenofibric acid over a concentration range of 150.00 ng/mL – 20383.00 ng/mL[6]. Ravikumar trivedi *et al.* developed LC/MS/MS method for simultaneous estimation of rosuvastatin and Fenofibric acid in plasma (0.5mL) over the concentration range of 500.00 ng/mL – 2000.00 ng/mL[7]. Dasandi Bhavesh *et al.* Proposed an LC-MS/MS method for the estimation of Fenofibric acid in human plasma (0.25mL) over the concentration range of

50.00-7129.00 ng/mL[8]. B. Mertens *et al.*, proposed an LC-MS/MS method for simultaneous estimation of Pravastatin, 3-hydroxy isomeric metabolite, Pravalactone and Fenofibric acid in human plasma, Fenofibric acid the Lower limit of quantification 250ng/mL[9] have been reported.

In the present investigation, we have developed a method having a shorter run time with simple liquid-liquid extraction technique. The following are the advantages of the proposed method over those reported earlier: (1) Sample to be collected for time point from individual during the study is reduced significantly. This allows inclusion of additional time points for sample collection; (2) Employing a single step liquid-liquid extraction method simplified the sample extraction procedure , minimizing the chances of errors and saves considerable time; (3) The use of Isotope labeled internal standard, which is physically and chemically identical to the analyte thus minimizing the errors during sample preparation and mass spectrometer detection.

The above points low plasma volume, use of isotope labeled internal standard, liquid-liquid extraction and a run time of 2.0 mins. makes the method an attractive procedure in high-throughput bio-analysis of Fenofibric acid in human plasma.

MATERIALS AND METHODS

Chemicals and Reagents

The reference samples Fenofibric acid (>99.81%) and Fenofibric acid-d_6 (>99.80%) were purchased form Varda Biotech Pvt. Ltd (Mumbai, India). Water used for the LC-MS/MS analysis was prepared from Milli Q water purification system procured from Millipore (Bangalore, India). Acetonitrile, methanol and Methy tertiary butylether (MTBE) were of HPLC grade and purchased from J.T Baker (Phillipsburg, USA). Analytical grade ammonium acetate and formic acid were purchased from Qualigens (Glaxo Mumbai, India). The control human plasma (Na_2EDTA) sample was procured from Cauvery Diagnostics and Blood Bank (Secunderabad, India).

Instrumentation and Chromatographic Conditions

A HPLC system (Waters, Alliance LC, 2695 separation module, USA) consisting of a Luna, C18 column (100 X 4.6 mm, 5 µ; Phenomenex, USA), were used for the validation. Aliquots of the processed samples (10 µL) were injected onto the column, which was kept at 40 ± 5 ºC. The mobile phase, a 80:20 v/v mixture of acetonitrile and ammonium acetate (5 mM, pH 2.5) was delivered at 0.200 mL/min into the electrospray ionization chamber of the mass spectrometer. Quantitation was achieved with MRM (MS/MS data acquisition mode) in nagative ion mode for both the analyte and the internal standard using a Waters Quattro-Premier XE (LC-ESI/MS/MS,

Waters, USA). The tuning parameters were summarized in Table No: 1a &1b. Detection of the ions were carried out in the MRM, by monitoring the transition pairs of m/z 317.06/231.00 for Fenofibric acid, m/z 323.24/231.04 for Fenofibric acid-d_6. The analysis data obtained were processed by Masslynx software ™ (version 4.1).

Standard Solutions

The primary stock solution 0.5 mg/mL of the analyte was prepared in methanol and stored at 2-8 ºC. From the stock solution, appropriate dilutions were made using a 50:50 v/v mixture of methanol and water as a diluent to produce working standard solutions of 1001093.500, 800871.00, 504555.00, 250244.00, 100099.00, 50055.00, 20512.00, 10255.00, 7999.00, and 3994.50 ng/mL of Fenofibric acid. These solutions were used to prepare the relevant calibration curve (CC) standards. Another set of working solutions of Fenofibric acid were prepared in the diluent (from primary stock) at concentrations of 803442.50, 441882.50, 88381.00, 11921.20 and 4099.00 ng/mL to be used as quality control (QC) samples. The primary stock solution of Fenofibric acid-d6 (0.10 mg/mL) was prepared in methanol. A working concentration of the internal standard (50 ng/mL of Finofibric acid-d_6) solution was prepared in the diluent. These working solutions were stored at 2-8 ºC for 7 days.

Table 1a: Tuning parameters.

ES - Source Parameter	Settings	Analyzer Parameter	Settings
Capillary (kV)	3.00	LM Resolution 1	10.0
Cone (V)	30	HM Resolution 1	10.0
Extractor (V)	2	Ion Energy 1	1.3
RF Lens (V)	0.0	Entrance	2
Source Temp (°C)	120	Exit	2
Desolvation Temp (°C)	400	LM Resolution 2	10.0
Cone Flow (L/h)	50	HM Resolution 2	10.0
Desolvation Flow (L/h)	800	Ion Energy 2	2.0
Collision cell Pressure (mbar)	$3.5e^{-3}$ – $4.5e^{-3}$	Multiplier	650

Table 1b: Tuning parameters.

Parameter	Setting (Analyte)	Setting (ISTD)
MS Function	MRM 317.06/231.00	MRM 323.24/231.04
Dwell (Secs)	0.2	0.2
Cone voltage (V)	15	15
Collision Energy(eV)	15	15

The calibration curve and quality control samples were prepared by spiking 20 µL of the working solution into 980 µL of control plasma. Calibration samples for Fenofibric acid were made at concentrations of 79.89, 159.78, 205.10, 410.24, 1001.10, 2001.98, 5004.88, 10091.10, 16017.42 and 20021.87 ng/mL. Quality control samples for Finofibric acid were prepared at

concentrations of 16068.85 (higher quality control, HQC), 8837.65 (middle quality control, MQC1), 1767.62 (middle quality control 2, MQC2), 238.42, (lower quality control, LQC) and 81.98 (lower limit quality control, LLOQ QC) ng/mL.

Sample processing

A 200-µL volume of the plasma sample was transferred to a 15-mL glass test tube, and 50.0 µL of 15.0 µg/mL internal standard solution was added, vortexed for 30 sec. Added 300 µL of extraction buffer, ammonium acetate (5 mM, pH 2.5), vortex for another 30 sec, and added 2 mL of extraction solvent (MTBE) using Dispensette Organic (Brand GmbH, Wertheim, Germany). The sample was shaken for 10 min using a multiplus vortexer and centrifuged all the test tubes at 4500 rpm, at 4 °C for 5 mins, using a Heraeus Megafuse 3SR (Japan centrifuge). The organic layer (1.8 mL) was transferred to a 5-mL glass test tube and evaporated at 50ºC under a stream of nitrogen (Turbo Vap LV, Zymark, Hopkinton, MA, USA). Added 200.0 µL of reconstitution solution to all the tubes and vortexed for about 2 min. Transferred 100.0 µL of the reconstituted solution into pre-labeled auto sampler vials and injected 10.0 µL onto LC-MS/MS.

Method validation

The method validation of Fenofibric acid was carried out as per the US FDA guidelines (FDA, 2001). The method was validated for selectivity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, dilution integrity, ruggedness and stability. Selectivity of the method was assessed by analyzing six blank human plasma matrix samples. The responses of the interfering substances or background noise at the retention time of the Fenofibric acid and its internal standard were acceptable if they are less than 20% of the response of the lowest standard curve point, and less than 5% of the response of the internal standard respectively.

The Sensitivity of the method was evaluated by analyzing 6 LLOQ samples. At least 67% (4 out of 6) of LLOQ samples should be within 80-120%. Matrix effect was investigated to ensure that precision, selectivity and sensitivity are not compromised by the matrix. Matrix effect was checked with six different lots of Na_2EDTA plasma. Three replicate samples each of quality control (low and high) were prepared from different lots of plasma. The QCs should be within acceptance limit 85.00 - 115.00 % (36 QC samples in total).

Linearity was tested for Fenofibric acid in the concentration range of 79.89–20021.87 ng/mL. Linearity was determined by using a 1/x2 weighted least square regression analysis of standard plots associated with a ten-point standard curve. To confirm blank interference in each of the standard curves, blank plasma samples were also analyzed. The acceptance limit of accuracy for

each of the back-calculated concentrations is ±15% except LLOQ, where it is ±20%. For a calibration run to be accepted at least 75% of the standards, including the LLOQ and ULOQ are required to meet the acceptance criterion otherwise, the calibration curve is rejected. The samples were run from low to high concentration.

Intra-assay precision and accuracy were determined by analyzing six replicates at five different QC levels on two different days. Inter-assay precision and accuracy were determined by analyzing six replicates at five different QC levels. The acceptance criteria included accuracy within ±15% deviation (SD) from the nominal values, except LLOQ QC, where it should be ±20% and a precision of ≤15% relative standard deviation (RSD), except for LLOQ QC, where it should be ≤20%.

Recovery of the analyte was determined by comparing the peak areas of the analyte in spiked plasma samples (six each of low, medium2, and high QCs) with the those of the analyte in samples prepared by spiking the extracted drug-free plasma samples with the same amounts of the analyte at the step immediately prior to chromatography. Similarly, recovery of the internal standard was determined by comparing the mean peak areas of the extracted QC samples (n=6) with those of the internal standard prepared by spiking the extracted drug-free plasma samples with the same amounts of internal standards at the step immediately prior to chromatography.

The dilution integrity was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations above the ULOQ during real time analysis. This experiment was carried out at 2.5 times the ULOQ concentration for the analyte. Six replicates each of dilution factor (DF) 5&10 concentrations were prepared and their concentrations were calculated by applying the DF 5 and 10.

Ruggedness of the method was evaluated by using a different lot of the same column and a different analyst. The precision and accuracy for the quality control samples at HQC, MQC1, MQC2, LQC and LLOQ QC concentration levels were found to be within the acceptance limit.

Stability experiments were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2-8 °C) was performed by comparing the area response of the analyte (stability samples) with the response of the sample prepared from fresh stock solution. Bench top stability [06 hr(s)], processed samples stability [Autosampler stability for 58 hr(s) 30 min(s), wet extract stability at room temperature 5 hr(s), wet extract stability at refrigerator temperature 57 hr(s), dry extracted stability (-28±50C) 59 hr(s) 25 min(s) and reinjection reproducibility 60 hr(s)

15 min(s), freeze and thaw stability (Four cycles)] were performed at low and high QC levels using six replicates at each level and stability of analyte in blood has been proven at room temperature [3 hr(s) 10 min(s)] and refrigerator temperature [3 hr(s) 15 min (s)].

RESULTS AND DISCUSSION

Method development

Mass Spectrometer parameters were tuned in nagative ionization mode using electrospray ionization for the analyte and the internal standard. For the data acquisition MRM mode was used to get better selectivity.

Chromatography was optimized using various combinations of acetonitrile and buffer with varying contents of each component on different columns like C8 and C18 of different makes like Zorbax, Xbridge, Jupiter, Hypersil, and ACE etc. A mobile phase consisting of acetonitrile and 5 mM ammonium acetate (80:20 v/v) was found suitable, as the analyte was protonated and well separated in this phase. Luna C18 column (100 X 4.6 mm, 5 µ; phenomenex, USA), column gave a good peak shape for both analyte and internal standard and at LLOQ level signal to noise ratio was found to be good. The mobile phase was operated at a flow rate of 1.0 mL/min with 80% split (0.200 mL into MS). The retention time of analyte and the internal standard were 1.15 and 1.14 respectively.

Liquid-liquid extraction (LLE) technique was employed for the extraction of drug and internal standard. LLE is helpful in producing a spectroscopically clean sample when compared to protein precipitation and avoiding the introduction of plasma components and non-volatile materials onto the LC and MS system. Clean samples are essential for minimizing the matrix effect in LC-MS/MS. Among the different solvents checked MTBE was found to be optimal, which produced a clean chromatogram for a blank sample and yielded the highest recovery for the analyte from the plasma.

An internal standard must mimic the analyte during extraction as well as during the ionization. For LC-MS/MS analysis, use of stable isotope-labeled molecule as internal standard proved to be helpful when there is a significant matrix effect. In Fenofibric acid method development and validation, Fenofibric acid–d_6 was used as the internal standard. An isotopically labeled internal standard best compensates for sample-to-sample variability and recovery also.

Assay characteristics for method validation

A simple and novel method for determination of Fenofibric acid was developed and validated in terms of selectivity, sensitivity, linearity of response, accuracy, precision, recovery, stability,

dilution integrity as well as matrix effect according to US-FDA guidelines for validation of bioanalytical methods[10].

Selectivity and Chromatography

The specificity of the LC-MS/MS method was established by screening the standard blanks of different lots from commercially available human plasma. Ten different lots of plasma were screened for the specificity experiment. Of the ten, seven batches were intended for anticoagulant plasma (Na_2EDTA), one each of haemolytic plasma, and lipidemic plasma and one lot containing heparin as anticoagulant. All the investigated human plasma lots were found to be free of interferences at the retention time of drug and the internal standard. The peak area at the retention time of drug in standard blank samples were ≤ 20.00% of the area of the analyte in the extracted LLOQ sample; The peak area at the retention time of internal standard in standard blank samples was ≤ 5.00 % of the area of the internal standard in the extracted LLOQ sample, Representative chromatograms of Standard Blank, Standard Zero (Standard Blank with Internal Standard), ULOQ standard, LLOQ standard were mentioned in Figure: 2a, 2b, 2c and 2d respectively.

Sensitivity

The Sensitivity of the method was evaluated by analyzing 6 LLOQ samples. The LLOQ was 79.89 ng/mL for Fenofibric acid. The precision and accuracy for Fenofibric acid at LLOQ level were found to be 4.65 % and 106.25 % respectively.

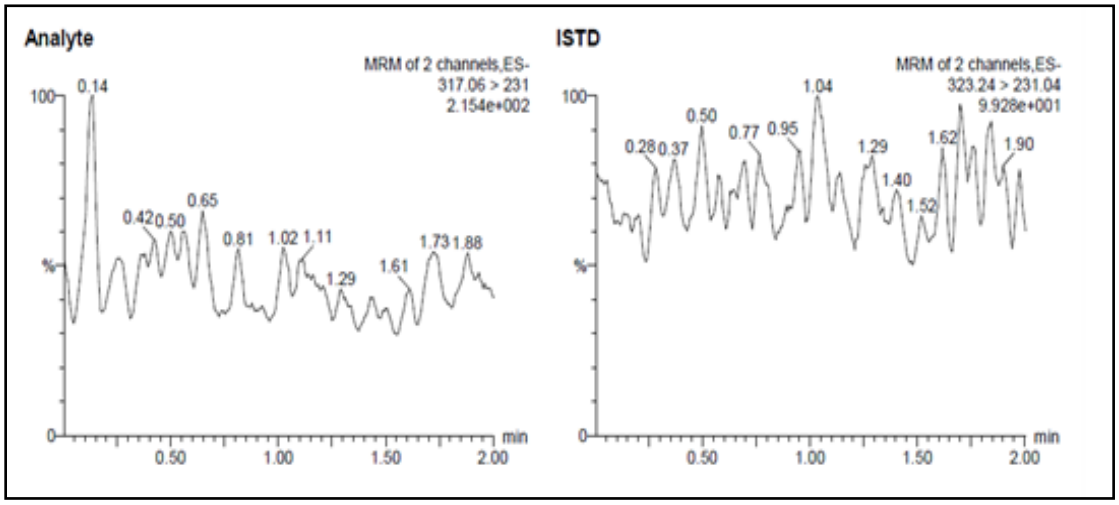


Figure 2a: Typical MRM chromatograms of Fenofibric acid (left panel) and IS (right panel) Standard Blank

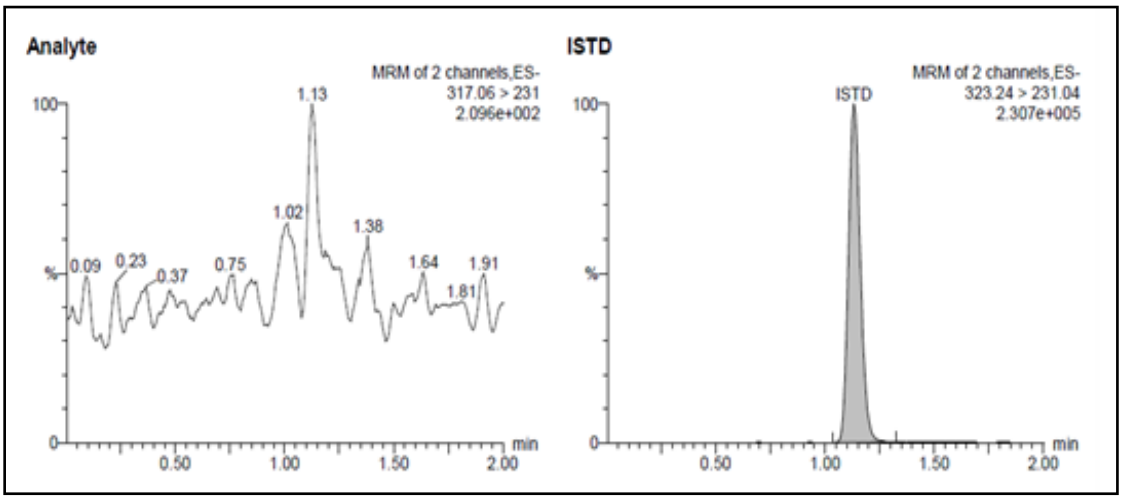


Figure 2b: Typical MRM chromatograms of Fenofibric acid (left panel) and IS (right panel) Standard Zero sample Chromatogram

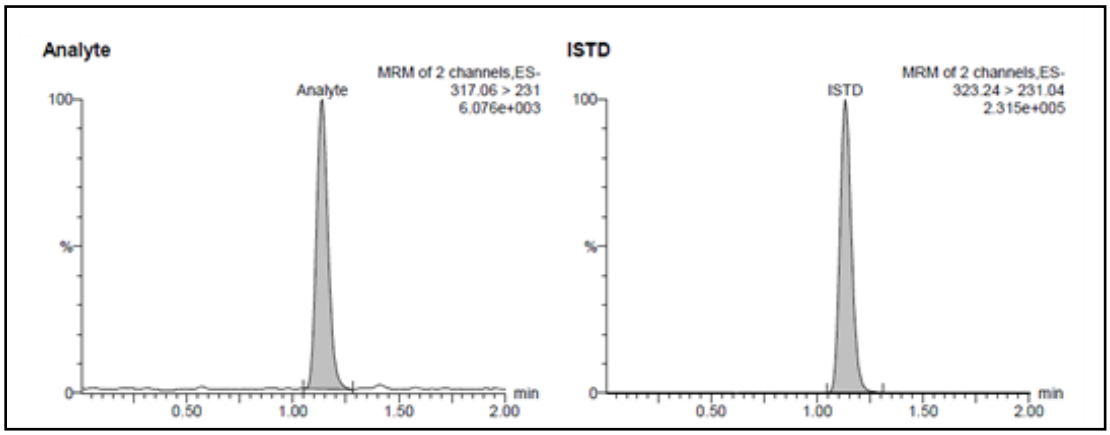


Figure 2c: Typical MRM chromatograms of Fenofibric acid (left panel) and IS (right panel) Lower Limit of Quantitation

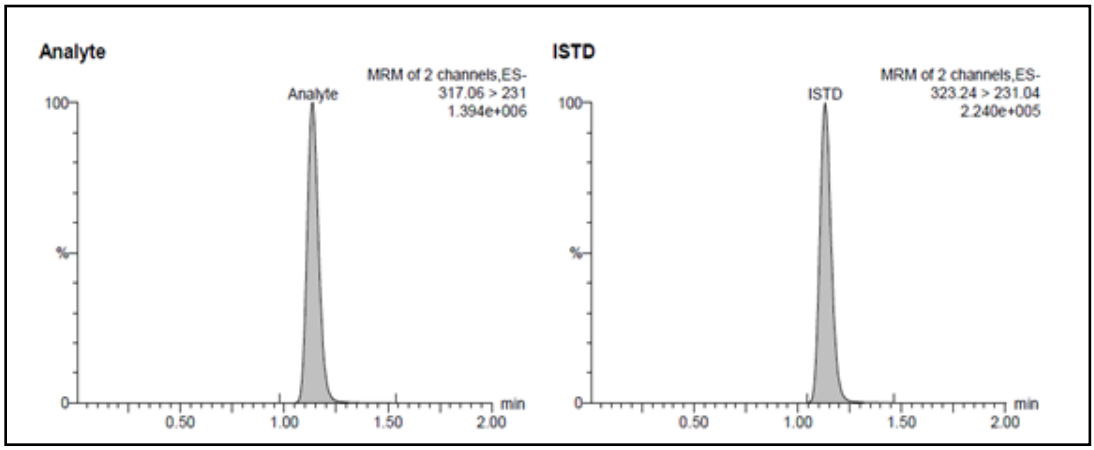


Figure 2d: Typical MRM chromatograms of Fenofibric acid (left panel) and IS (right panel) Upper Limit of Quantitation

Matrix effect

Matrix effect is due to the co-elution of phospholipids and some endogenous components present in different matrices. These components may effect the response of the analyte and in turn effects the sensitivity, accuracy and precision of the method. Thus evaluation of matrix effect is an important part of validation for quantitative LC–MS/MS method.

No significant matrix effect was observed in all the six batches of human plasma for the analyte at low and high QC concentrations. The precision and accuracy for Fenofibric acid at low QC concentration was found to be 3.31% and 96.10%, and at high QC level was 2.14 % and 100.05% respectively.

Linearity

The linearity was determined by using a 1/x2 weighted least square regression analysis of standard plots associated with a ten-point calibration curve. All the four calibration curves analyzed during the course of validation were found to be linear over the concentration ranging of 79.89–20021.87 ng/mL. The correlation coefficient (r) was observed to be ≥ 0.9994. The overall % mean accuracy for the CC standards was found to be in between 96.19–105.71 % and the overall precision was ≤ 10.44%.

Precision and Accuracy

As shown in Table 2a and 2b, The precision and accuracy of the method was evaluated by the % CV and % accuracy respectively, at different concentration levels corresponding to LLOQ QC,

Table 2a: Intra-batch precision and accuracy.

Spiked QC Concentration (ng/mL)	Concentration found (mean; ng/mL)	Precision (%)	Accuracy (%)
81.98	75.67	2.60	92.30
238.42	231.98	0.61	97.30
1767.62	1748.71	0.43	98.93
8837.65	8667.77	0.73	98.08
16068.85	16408.83	0.95	102.12

Table 2b: Inter-batch precision and accuracy

Spiked QC Concentration (ng/mL)	Concentration found (mean; ng/mL)	Precision (%)	Accuracy (%)
81.98	78.21	2.33	95.40
238.42	234.51	0.62	98.36
1767.62	1726.44	0.17	97.67
8837.65	8525.39	0.01	96.47
16068.85	15865.24	0.24	98.73

LQC, MQC2, MQC1 and HQC during the course of the validation. The precision and accuracy of analyte in the intra-batch and inter-batch runs were within ± 15% and within ± 20% at LLOQ QCs.

Extraction Efficiency

Six replicates at low, medium and high quality control concentration for Fenofibric acid was prepared for recovery determination. The percent mean recoveries were determined by measuring the responses of the extracted plasma quality control samples against unextracted quality control samples at HQC, MQC1, MQC2 and LQC levels. The mean recovery of Fenofibric acid at different levels of HQC, MQC1, MQC2 and LQC were found to be 63.39, 59.10, 61.65 and 59.85 % respectively. The mean recovery of analyte and internal standard were found to be 60.90% and 83.34% respectively.

Dilution Integrity

The upper limit of quantitation was extended to 40172.10 ng/mL for Fenofibric acid. The dilution integrity of the method was evaluated for DF 5 and 10 with screened human blank plasma. The precision for DF 5 and 10 was found to be 0.51 and 3.70 %, and the % mean accuracy for DF 5 and 10 was found to be 98.79 and 103.49 % respectively, which are within acceptance limit of 85.00 - 115.00 %. The results are summarized in the Table 3.

Table 3: Dilution Integrity

DI Spiked Standard conc 40172.50 ng/mL				
Dilution Factor	**DIQC (spiked concentration (ng/mL)**	**Concentration found (mean; ng/mL**	**Mean Accuracy (%)**	**Precision (%)**
1/5	8034.40	7937.39	98.79	0.51
1/10	4017.21	4157.23	103.49	3.70

Ruggedness

Ruggedness was performed by using a different lot of the same column and a different analyst. The precision and % mean accuracy for the quality control samples at HQC, MQC1, MQC2 and LQC concentration levels found to be within acceptance limit 15.00 %. For all the samples of LLOQ QC was found to be within the acceptance limit of ≤ 20.00 %. The results are summarized in the Table 4.

Stability

In the different stability experiments carried out viz. bench top stability [06 hr(s)], autosampler stability [58 hr(s), 30 min(s)], repeated freeze-thaw cycles (4 cycles), reinjection reproducibility [60 hr(s), 15 min(s)], wet extract stability at room temperature [05 hr(s)], wet extract stability at

refrigerator temperature [57 hr(s)] dry extract stability [59 hr(s), 25 min(s) -28±5 0C] and stability of the analyte in blood at room temperature [03 hr(s), 10 min(s)] and at refrigerator temperature [03 hr(s), 15 min(s)] have been proved. The mean % nominal values of the analyte were found to be within ±15% of the predicted concentrations for the analyte at their low and high QC levels thus, the results were found to be within the acceptable limits during the entire validation. Long term stability at –70 °C for 40 days the mean % nominal values of the analyte was found to be within ±15% of the predicted concentrations for the analyte at their low and high QC levels. The results are summarized in Table 5.

Table 4: Ruggedness Precision and Accuracy

Experiment Name	QC (spiked concentration (ng/mL)	Concentration found (mean; ng/mL	Mean Accuracy (%)	Precision (%)
Different column	81.98	82.63	2.47	100.79
	238.42	239.19	1.10	100.32
	1767.62	1723.38	1.63	97.50
	8837.65	8590.87	0.53	97.21
	16068.85	15899.03	1.07	98.94
Different analyst	81.98	77.63	4.20	94.69
	238.42	241.26	2.07	101.19
	1767.62	1744.79	0.99	98.71
	8837.65	8679.31	0.87	98.21
	16068.85	16352.84	1.35	101.77

Table 5: Stability Samples Results for Fenofibric acid

Stability test	QC (spiked concentration (ng/mL)	Mean ± SD (ng/mL)	Accuracy/ Stability (%)	Precision (%)
Autosampler	238.42	241.366 ± 9.20	101.23	3.81
	16068.85	16414.10 ± 244.42	102.15	1.49
Wet extract (RF)	238.42	242.38 ± 5.13	101.66	2.12
	16068.85	16330.0845± 206.42	98.01	1.26
Wet extract (RT)	238.42	244.00 ± 6.21	102.34	2.55
	16068.85	16784.57 ± 212.38	104.45	1.27
Bench top	238.42	240.78 ± 4.98	100.99	2.07
	16068.85	16322.86± 238.11	101.58	1.46
FT	238.42	247.12 ± 6.10	103.65	2.47
	16068.85	16687.84 ± 383.16	103.85	2.30
Dry extract	238.42	239.80 ± 4.56	100.58	1.90
	16068.85	16633.17 ± 243.89	103.51	1.47

CONCLUSION

The use of LC-MS/MS technology can enable the performance of highly accurate analysis. This method had a suitable LLOQ of 79.89 ng/mL along with a very short retention time of 2.0 minutes and was useful for analyzing more than 400 samples in a single day. This method employs a very simple and economical liquid-liquid extraction method. The LC-MS/MS method development and validation presented in this paper is simple, selective, sensitive, fast, reliable, specific and rugged for quantification of Fenofibric acid in human plasma and the method is validated according to FDA Guidance for industry on bioanalytical method validation. The method was suitable for pharmacokinetic, drug-drug interaction and toxicokinetic studies in humans.

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