A Validated RP-HPLC-UV Method for Identification of Reference Standards of Degradation Products of Fenofibrate

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ABSTRACT

Background: During the stress degradation studies of Fenofibrate the structures of two major degradation products, namely 2-[4-(4-chlorobenzoyl)phenoxy]-2-methyl propanoic acid and methyl 2-[4-(4-chlorobenzoyl) phenoxy]-2-methylpropanoate, were characterized using LC-MS/MS studies under alkaline and acidic hydrolytic conditions, respectively. The present communication deals with synthesis, spectral characterization and further application of those reference standards of degradation products in identification of degradation products in bulk sample of drug through retention time matching studies. **Methods:** The reference standards of the degradation products were synthesized in the laboratory and their identity were confirmed using spectral studies. These were employed for retention matching studies using HPLC studies. The chromatographic conditions were as follows: stationary phase: Waters X Bridge C_{18} column (250x4.6 mm, internal diameter, particle size 5 µm), mobile phase: acetonitrile: water in 75:25 v/v, column oven temperature: ambient. The chromatograms were monitored at 286 nm with a flowrate of 1 mL/min. The method was validated according to the ICH guidelines. **Results:** Retention time values of synthesized reference standards were found to be identical with retention time values of those degradation products in stressed samples. The calibration curves showed good linear regression (r^2>0.999) within test ranges. The method showed good reproducibility and sensitivity for quantification of both degradation products in samples. Method was precise and %recovery of both reference products was within 98-102%. **Conclusion:** This communication emphasizes use of non-compendial reference standards for quantification of degradation products by chromatographic methods. Quantitative validation parameters like Limit of detection and Limit of quantitation were established.

Key words: Degradation impurities, Fenofibrate, Non-compendial reference standards, RP-HPLC, Retention time matching.

INTRODUCTION

Fenofibrate, Propan-2-yl-2-[4-(4-chlorobenzoyl) phenoxy]-2-methylpropanoate, is used as antihyperlipidemic drug. Fenofibrate activates lipoprotein lipase, which reduces triglycerides and increases HDL cholesterol. It exerts a variable but generally modest LDL cholesterol-lowering effect. Fenofibrate is obtained by esterification of 2-[4-(4-chlorobenzoyl) phenoxy]-2-methylpropanoic acid (Fenofibric acid), which is also an active metabolite.[1]

The chromatographic methods rely on availability of pure reference standards to provide accurate quantitation. Usually two types of Reference standards are used for quantitation USP-NF and non-compendial. USP-NF reference standards are official in USP, synthesized in

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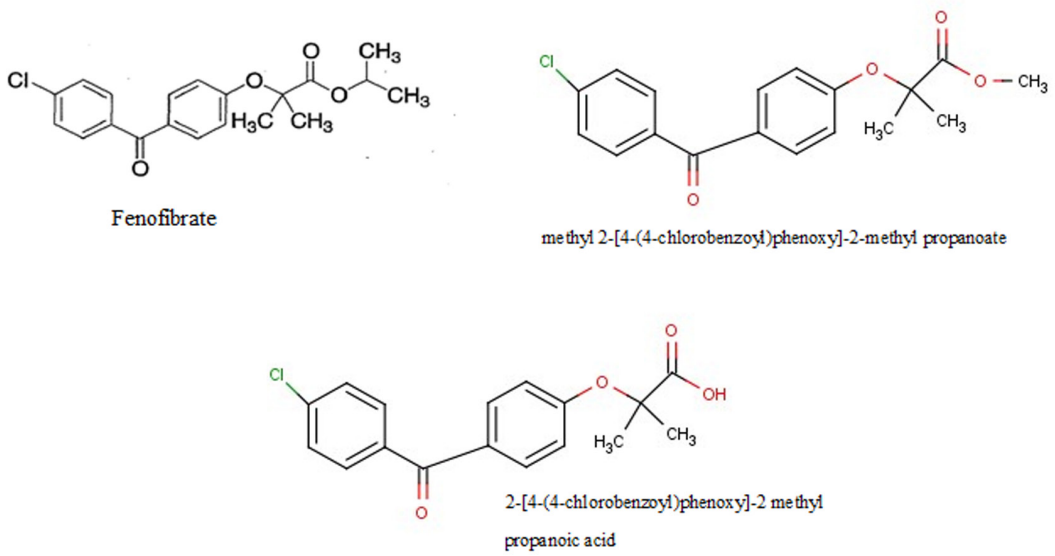

Figure 1: Chemical structures for Fenofibrate and degradation products

reputed synthetic laboratories, are of high purity (nearly 99.99% and therefore have high cost. They do not require initial characterization before their use. Non-compendial reference standards are synthesized in any laboratory with reasonable efforts but require thorough characterization to assure its identity, quality and purity so that they can be used in quantification of impurities/degradation products. Non-compendial reference standards are usually cheaper and can be used after thorough characterization. Synthesis and characterization of impurities/degradation products and clinically significant metabolites of an API utilizing optimum time and resources is one of the important areas of current pharmacoeconomic and clinical interest. The prime concerns of pharmaceutical analyst are adequate separation, selectivity and sensitivity of detection and accurate quantification in such work.[2] During the stress degradation studies of Fenofibrate, the structures of two major degradation products were proposed using LC/MS/MS studies by S. Y. Gabhe *et.al.* The names of the degradation products were proposed as 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl propanoic acid (Fenofibric acid, Degradation Product A) and methyl 2-[4-(4-chlorobenzoyl) phenoxy]-2-methylpropanoate (Degradation Product B). In order to commercialize an API, it is a mandatory requirement by regulatory authorities to identify and characterize the impurities that are present at a level of more than 0.1%.3 These impurities are required in pure form to check the HPLC method performance in areas such as linearity, range, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and system suitability testing.[2] The structures of Fenofibrate and identified degradants is given in Figure 1. While, degradation product 2-[4-(4-chlorobenzoyl)phenoxy]-2-methyl propanoic acid is identified as impurity B, degradation product methyl 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoate is identified as impurity D in European Pharmacopoeia 5.0[4] and British Pharmacopoeia 2005.[5]

Few HPLC,[6-11] and UPLC,[12,13] methods have been reported for quantitation of Fenofibrate along with its degradation products, individually and in combination with other drugs like Metformine Hydrochloride and Atorvastatin Ca. RP-HPLC14 and LC/MS[15] methods for estimation of active metabolite Fenofibric acid in biological fluids are published. In the present study, we have targeted two major degradation products of Fenofibrate. The first targeted degradation product 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl propanoic acid, was formed under alkaline hydrolytic condition. The second degradation product is methyl 2-[4-(4-chlorobenzoyl) phenoxy]-2-methylpropanoate which was formed under acidic hydrolysis. The work presented in this article is in continuation with our earlier published work. We have synthesized reference standards for those two degradation products and these were further utilized as non-compendial reference standards for retention matching studies.

EXPERIMENTAL

Reagents and Chemicals

Pure drug Fenofibrate was procured from Panacea Biotech Ltd., New Mumbai (India) as gift sample. Acetonitrile

(HPLC) was purchased from Merck Specialities Pvt. Ltd. (Mumbai, Maharashtra, India). Water (HPLC) was obtained from ELGA Purelab UHQ-II water purification unit (ELGA, Bucks, England) and filtered through 0.45 µm membrane filter before use. The degradation products, methyl 2-[4-(4-chlorobenzoyl) phenoxy]-2-methylpropanoate and 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl propanoic acid were synthesized in laboratory. All the chemicals used for synthetic procedures were of synthetic grade.

HPLC System

The separation of Fenofibrate and its degradation products during stress studies as well as retention time matching studies was carried out using Shimadzu LC-2010 CHT system. The autosampler was SIL-20 AC HT, pump was LC-20 AD and detector was SPD-20A. LC data was processed using LC solution software. 20 µL sample solutions of analytes were injected to chromatographic system and were filtered through 0.22 µ syringe filter before injection. Before delivering the mobile phase in to the system, it was degassed using ultrasonicator and filtered through 0.45 µm membrane filter.

Synthesis of Degradation Products A and B

1g of 4-Chloro-4'-hydroxybenzophenone dissolved in anhydrous acetone and 0.86 g of powdered NaOH were refluxed in a round bottom flask for 1-2 hrs. To this 0.51 ml of chloroform diluted with anhydrous acetone was added and mixture was further refluxed for 10 hrs. After refluxing, mixture was cooled and some quantity of water was added. Acetone was allowed to evaporate; aqueous phase was washed with ether and acidified. Organic phase was dissolved in ether and extracted into solution of bicarbonate. The bicarbonate solution was acidified to obtain desired 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl propanoic acid (Degradation Product A).[16] 2 g of Fenofibric acid was dissolved in 20 ml of methanol in a round bottom flask and 2-3 ml of sulphuric acid was added as catalyst. The mixture was refluxed for 5 hrs. Fruity smelling liquid (Degradation Product B) was formed, which followed removal of excess of methanol using distillation.[17]

Characterization of synthesized reference standards using Spectral techniques

The characterization of synthesized reference standards of degradation products were performed using FT-IR, ^{1}H-NMR and Mass spectral studies. The IR spectra were recorded in the solid state as a KBr dispersion medium using FT-IR (Perkin Elmer, Spectrum BX-II) spectrophotometer. The ^{1}HNMR spectra were recorded in DMSO using NMR Bruker (Swiss) Avance II 400 MHz spectrometer. Chemical shifts are expressed in units as parts per million, downfield from TMS (Tetramethylsilane) as an internal standard. Mass spectra were recorded on a Waters Q-TOF Micromass LC-MS system in + ESI mode using methanol as solvent. The melting points were determined by using the capillary method on a digital melting point apparatus VMP-D (Veego Pvt. Ltd., India) and were uncorrected.

RP-LC method for retention time matching studies and method validation

The chromatographic separations were achieved on Waters X Bridge C_{18} (25x4.6 mm, particle size 5 µm) column. The mobile phase was prepared by mixing water and acetonitrile in a ratio of 25:75 (v/v). The mobile phase used for diluting the samples was filtered through 0.45 µm nylon membrane filter ((Millipore, Bangalore, India). The flow rate was 1.0 mL/min and detection was at 286 nm. Column oven temperature was 25^{0}C for all determinations. The concentration of standard, assay and stressed sample was 100 µg mL^{-1}. A degassed mixture of water and acetonitrile in the ratio of 25:75% v/v was used as diluent during the standard and test sample preparations.

Preparation of Stock solutions for reference standards of degradation products

Stock solutions of reference standards of degradation products were prepared by dissolving 10 mg each in 10 ml of diluent separately, to produce solutions of 1000 µg/mL concentration.

Preparation of Mixed working solution

Mixed working solution of degradation impurities for LC analyses was prepared by spiking reference standards of degradation impurities at 1% concentration each (100 µg/mL concentration) into Fenofibrate drug solution of 1000 µg/mL concentration. Further dilutions for calibration curve were prepared by diluting appropriate aliquots from mixed working solution using diluent to obtain solutions containing 10, 20, 30, 40, 50, 60 µg/mL concentrations of each degradation product and 100, 200, 300, 400, 500, 600 µg/mL concentrations of Fenofibrate. Six different sets of spiked solutions of above concentrations were prepared separately. A 20 µl volume of each solution was injected onto column under the abovementioned chromatographic conditions. Calibration curves were constructed by plotting peak area *versus* concentrations and regression equations (n=6) were computed for degradation impurities and Fenofibrate.

Validation of RP-LC method:[18]

System suitability parameters were measured so as to verify the system, method and column performance. Mixed working solution was injected into the system for six times and system suitability parameters (tailing factor, resolution and theoretical plates) were checked. Linearity was evaluated for a set of six mixed working standard solutions

containing 10-60 µg/ml of each reference standard and 100-600 µg/ml for Fenofibrate. Linearity solutions were prepared as per the procedure explained in section 2.5.2. The relationship between peak areas *versus* concentrations of degradation products and drug in the linearity solutions was established by simple linear regression method. The regression equations were obtained as calibration curve. Intra-day and intermediate (inter-day) precision studies were performed. In repeatability, mixed working solution containing each of 20 µg/ml of reference standards of degradation products and 200 µg/ml of Fenofibrate were injected onto the column for six times. For intraday precision studies, three different mixed standard solutions were injected in triplicate across the intended range (20, 40, 60 µg/ml for reference standards) and 200, 400, 600 µg/ml for Fenofibrate and their areas were measured on the same day. These studies were repeated on three different days to determine inter-day precision. By using the obtained area values, %R. S. D. values were calculated. Accuracy studies were performed by spiking reference standards of degradation products corresponding to 75%, 100% and 125% levels into mixed working solution containing each of 20 µg/ml of reference standards and 200 µg/ml of drug). Triplicate set at each level of accuracy was prepared and solutions were injected onto column. % Recovery and % mean recovery of analyte at each level of accuracy was calculated. % Relative standard deviation (%RSD) values were computed. Specificity of the method was studied by studying the resolution factor between peaks of degradation products and Fenofibrate and amongst the peaks of degradation products.

RESULTS AND DISCUSSION

Synthesis of reference standards of Degradation products A and B

After thorough characterization laboratory synthesized 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl propanoic acid (Degradation product A) and methyl 2-[4-(4-chlorobenzoyl) phenoxy]-2-methylpropanoate (Degradation product B) were used as non-compendial reference standards.

2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl propanoic acid (Fenofibric acid) was synthesized by reacting 4-chloro-4'-hydroxybenzophenone with chloroform in presence of acetone. Further reaction of 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl propanoic acid (Fenofibric acid) with methanol produced methyl 2-[4-(4-chlorobenzoyl) phenoxy]-2-methylpropanoate. The analysis of FT-IR, ^{1}HNMR and Mass spectral data confirmed the synthesis of products. Hence, these degradation products have been used as non-compendial reference standards. Identification of impurities is done by variety of chromatographic and spectroscopic techniques, either alone or in combination with other techniques. Conventional liquid chromatography, particularly, HPLC has been exploited widely in field of impurity profiling.

Characterization of synthesized reference standards using Spectral studies

2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl propanoic acid is reported as an impurity B in EP 5 and BP 2005. It was the first reference standard of degradation product synthesized as per the procedure explained. This was then converted to methyl 2-[4-(4-chlorobenzoyl) phenoxy]-2-methylpropanoate, which is reported as impurity D in EP 5 and BP 2005. The analysis of spectral data of Fenofibric acid confirmed its identity. From the FT-IR spectrum, confirmation about characteristic functional groups (values expressed in cm^{-1}) was obtained as follows: 2924.24 (C-H), 1681.80 (Ar-C=O), 1592.27, 1425.27 (Ar-C=C), 1091.89 (C-O), 760.57 (C-Cl) was obtained. The molecular ion at m/z 319 was found to be matching with the molecular mass of Fenofibric acid (318). The structure was further supported by 1H NMR data as follows: δ 1.5 (s, 3H, CH_3), δ 2.5 (s, 3H, CH_3), δ 6-8 (m, 8H, Ar-H), δ 12.9 (s, ^{1}H, COOH). Based on the above spectral data, the molecular formula of degradation product A (Fenofibric acid) was confirmed as $C_{17}H_{15}O_4Cl$.

Similarly, analysis of spectral data helped in confirmation of structure of methyl 2-[4-(4-chlorobenzoyl) phenoxy]-2-methylpropanoate. The FT-IR spectrum displayed characteristic absorptions at following wave numbers (values expressed in cm-1): 2957.68 (C-H), 1730.95 (C=O of COO), 1455.91, (Ar-C=C), 1059.88 (C-O) and 778.65 cm−1 (C-Cl). The molecular ion peak at m/z 333.1 was found to be matching with the actual molecular mass (332.5) of the compound. The structure was further supported by ^{1}H NMR data as follows: δ 0.8-2 (m, 6H, CH_3), δ 3-4.5 (m, 3H, OCH_3), δ 6.5-8.5 (m, 8H, Ar-H). Based on the above spectral data, the molecular formula of degradation product B, methyl 2-[4-(4-chlorobenzoyl) phenoxy]-2-methylpropanoate was confirmed as $C_{18}H_{17}O_4Cl$. The ^{1}HNMR and Mass spectra for the reference standards of synthesized degradation products are shown in Figures 2 and 3, respectively.

Optimisation of the chromatographic conditions and Retention time matching.

Preliminary separation studies were performed using a binary mobile phase consisting of either acetonitrile or methanol and water in a gradient elution pattern. Higher proportions of organic phases in the mobile phase resulted in early elution of drug. Use of acetonitrile as organic component improved sharpness of the peak as compared

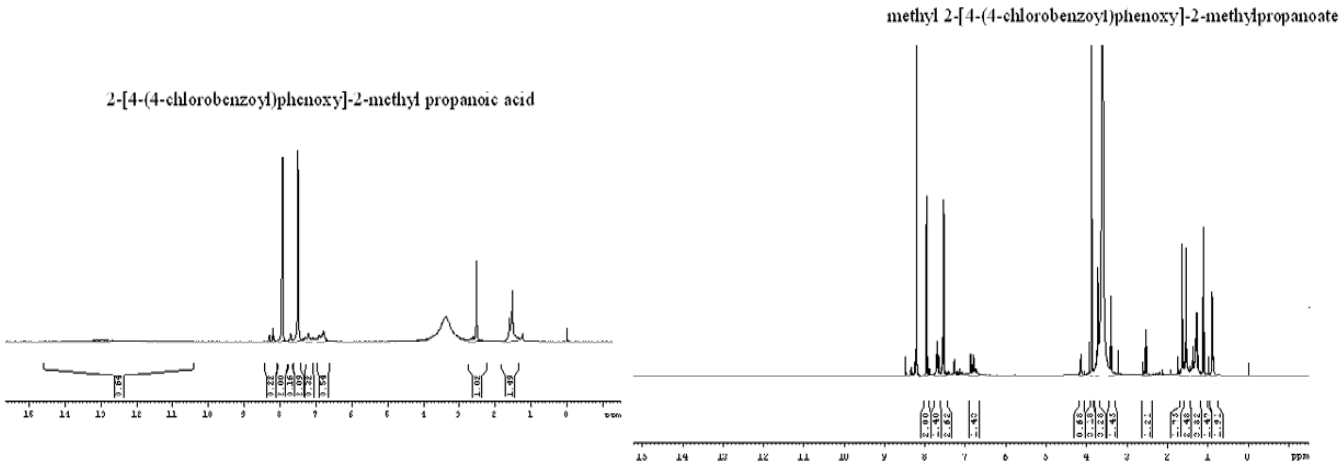

Figure 2 : ^{1}H NMR spectra for Degradation Products

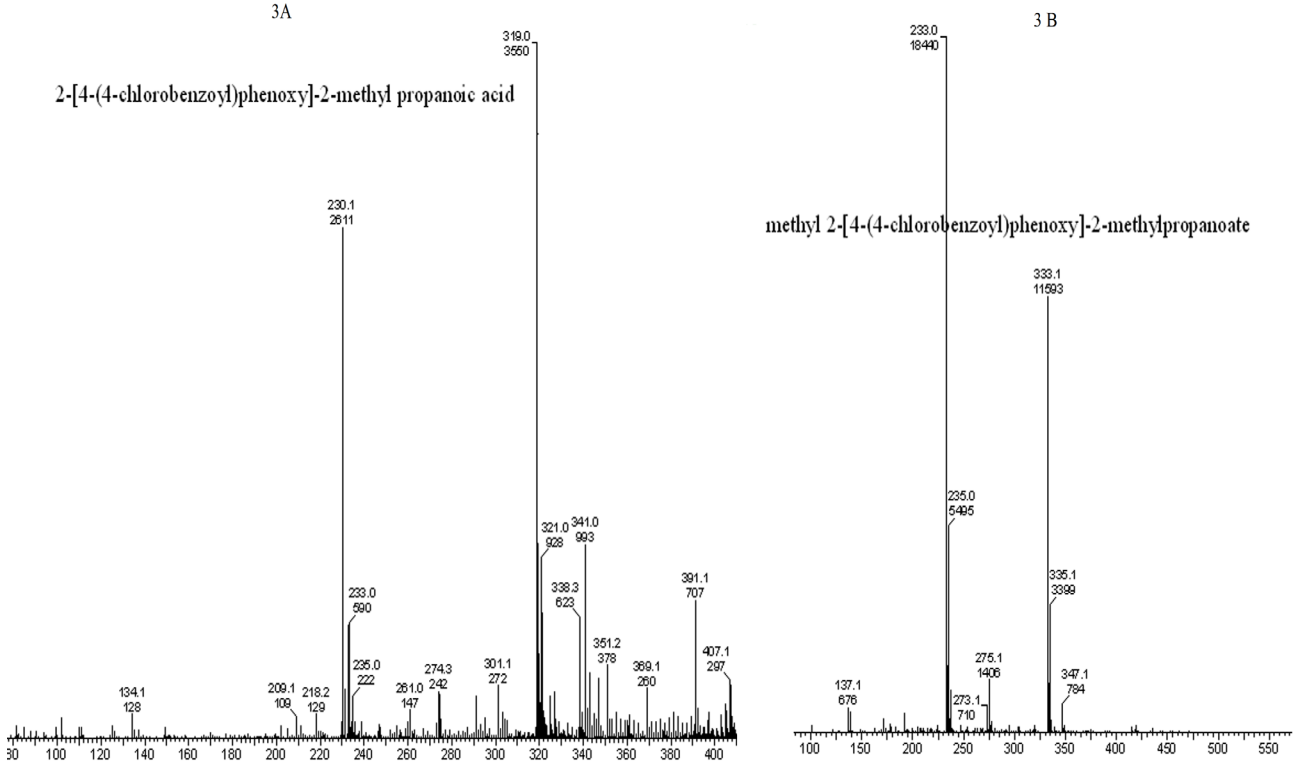

Figure 3 : Mass spectra for Degradation Products

to methanol. Satisfactory resolution was achieved using acetonitrile: water system in a 75:25 v/v proportion with a flow rate of 1 mL/min. The total run time of the method was 20 min. Acetonitrile was found to be compatible with C_{18} column.

During the stress studies, the retention time values of 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl propanoic acid formed under alkaline hydrolysis and methyl 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl propanoate formed under acidic hydrolysis were found to be 2.401 min and 6.391 min, respectively. The prepared reference standards of degradation products were spiked in the pure solution of Fenofibrate under above mentioned chromatographic conditions, in order to establish their identity. The retention time values for 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl propanoic acid formed under alkaline hydrolysis and methyl 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl propanoate were found to be 2.046 min and 6.359 min, respectively. The typical chromatogram of Fenofibrate

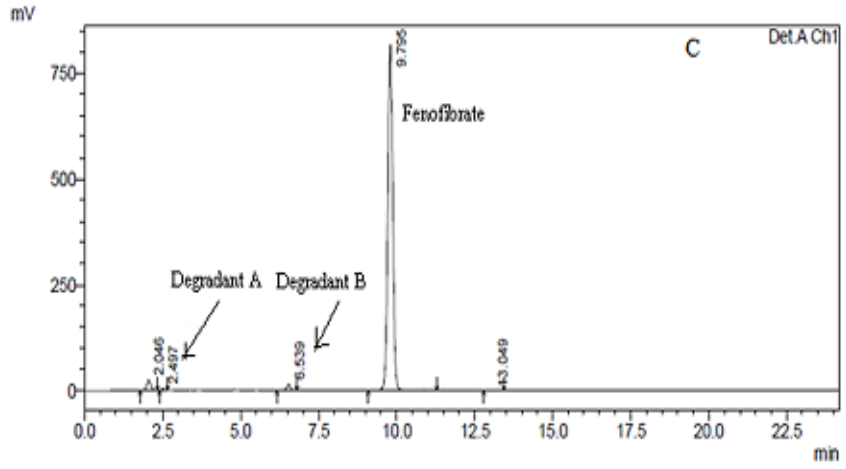

Figure 4 : HPLC chromatogram of Fenofibrate spiked with reference standards of degradation products

Table 1: Linearity, Range and SST data for Degradation Products and Fenofibrate

Parameter	Degradation Product A	Degradation Product B	Fenofibrate
Linearity range	10-60 ug/ml	10-60 ug/ml	100-600 ug/ml
Correlation coefficient (r^2) ± SD	0.9902 ± 0.0012	0.9923 ± 0.0011	0.9931 ± 0.0011
Slope ± SD*	9165.7 ± 81.02	5819.1 ± 40.66	62060.3 ± 645.78
Intercept ± SD*	92171.5 ± 1960.48	5801.1 ± 1706.86	23794.8 ± 554.98
y=mx + c	y=9236.9x + 92711	Y=5820.7x + 5537.2	Y=62177x + 23906

Degradation Product A: 2-[4-(4-chlorobenzoyl)phenoxy]-2-methyl propanoic acid, Degradation Product B: methyl 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoate, * n=6 determinations, SST=System suitability test.

Table 2: Results for Precision Studies for Degradation Products and Fenofibrate

Drug/ Degradation Product	Conc. (µg/ml)	Intraday precision		Interday precision	
		Mean area ± SD*	%RSD*	Mean area ± SD*	%RSD*
Degradation Product A	20	272347.67 ± 5205.63	0.911	272337.67 ± 5105.63	0.889
	40	448675 ± 1049.61	0.233	439675 ± 1009.61	0.214
	60	542352.33 ± 972.93	0.179	552382.33 ± 972.02	0.195
Degradation Product B	20	133850 ± 494.13	0.232	133540 ± 344.13	0.221
	40	293283 ± 619.31	0.211	285283 ± 615.31	0.189
	60	354664 ± 537.06	0.151	354544 ± 437.06	0.240
Fenofibrate	200	9534978 ± 22137.10	0.232	9414978 ± 21137.10	0.224
	400	24760423 ± 27908.50	0.112	25440423 ± 27908.5	0.178
	600	36556174 ± 147906.73	0.404	36556174 ± 135806.73	0.514

Degradation Product A: 2-[4-(4-chlorobenzoyl)phenoxy]-2-methyl propanoic acid, Degradation Product B: methyl 2-[4-(4-chlorobenzoyl) phenoxy]-2-methylpropanoate, * n=3 determinations.

Table 3: Results for recovery studies for Degradation Products and Fenofibrate

	Level (%)	Amount added in µg/ml	Mean amount found in µg/ml	Average % recovery ± SD and %RSD*
Degradation Product A	75	35	35.55	101.57 ± 0.709, 0.698
	100	40	40.11	100.28 ± 0.159, 0.159
	125	45	44.98	99.97 ± 0.338, 0.338
Degradation Product B	75	35	35.41	101.2 ± 0.377, 0.373
	100	40	40.13	100.34 ± 0.229, 0.228
	125	45	44.83	99.57 ± 0.318, 0.320
Fenofibrate	75	350	351.19	100.32 ± 0.017, 0.016
	100	400	399.40	99.85 ± 0.453, 0.453
	125	450	451.47	100.52 ± 0.391, 0.389

Degradation Product A: 2-[4-(4-chlorobenzoyl)phenoxy]-2-methyl propanoic acid; Degradation Product B: methyl 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoate.* n=3 determinations.

spiked with reference standards of degradation products is shown in Figure 4.

Method Validation

The method was validated according to ICH Q2R1 guideline[18] meant for validation of analytical methods to check accuracy, precision, linearity range, limit of detection, limit of quantitation and robustness.

Linearity studies

The response for 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl propanoic acid, methyl 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl propanoate and Fenofibrate was found to be the strictly linear in the concentration range of 10-60 µg/ml (R^2=0.9902), 10-60 µg/ml (R^2=0.9923) and 100-600 µg/ml (R^2=0.9931), respectively. The values of correlation coefficients indicate linearity of the method within above stated concentration range. The linear regression data is presented in Table 1.

Precision Studies

Intraday precision studies were performed on three different mixed standard solutions containing 20, 40, 60 µg/ml each of reference standards and 200, 400, 600 µg/ml of Fenofibrate and their areas were measured on the same day. These studies were repeated on three different days to determine inter-day precision. By using the obtained area values, %R. S. D. values were calculated. The result for precision studies is represented in Table 2.

Accuracy Studies

Reference standards of degradation products corresponding to 75%, 100% and 125% levels were spiked into mixed working solution containing 20 µg/ml of each of reference standard and 200 µg/ml of drug. Triplicate set at each level of accuracy were analysed. %Recovery at each level of accuracy was found to be between 98-102% and % relative standard deviation values (%RSD) values were found to be less than 2% at each level. Detailed results for accuracy are given in Table 3.

The Limit of detection and Limit of quantitation values were found to be 0.70, 0.96 and 0.02 µg/ml and 2.13, 2.93 and 0.08 µg/ml for 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl propanoic acid, methyl 2-[4-(4-chlorobenzoyl)phenoxy]-2-methyl propanoate and Fenofibrate, respectively by the intended method. LOD and LOQ value suggests that the method is sensitive for detection and quantitation of those degradation related impurities. The results of the robustness study indicated that the developed method is robust and is unaffected by small variations in the chromatographic conditions.

CONCLUSION

Synthesis of non-compendial reference standards was performed in the laboratory and their identity is established by spectroscopic and chromatographic techniques. A simple and convenient method was developed for estimation of degradation related impurities of Fenofibrate. In conclusion, the results of this study showed that, the degradation products 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl propanoic acid and methyl 2-[4-(4-chlorobenzoyl) phenoxy]-2-methylpropanoate can be employed as non-compendial reference standards.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ABBREVIATIONS

RP-LC	: Reverse Phase - Liquid Chromatography
FT-IR	: Fourier Treansform - Infrared Spectrophotometer
LC-MS/MS	: Liquid Chromatography-Mass Spectrometry/Mass Spectrometry

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