ARTYKUŁ ORYGINALNY / ORIGINAL PAPER
Otrzymano/Submitted: 17.06.2025 • Zaakceptowano/Accepted: 28.07.2025

Development and validation of a UPLC MS/MS method for the determination of fenofibric acid, the active metabolite of fenofibrate

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Abstract

Introduction. Fenofibrate is a well-established drug in the treatment of hypertriglyceridemia and mixed hyperlipidemia. The drug exerts its effect through its active metabolite, fenofibric acid. This study aimed to develop and validate a simple, precise ultra-performance liquid chromatography-mass spectrometry (UPLC MS/MS) method for the determination of fenofibric acid concentration in rat plasma using isotope-labeled fenofibric acid-D_6 as an internal standard. This method can be used in preclinical and pharmacokinetic studies. ***Material and methods.*** The UPLC MS/MS method was validated in accordance with the "ICH guideline M10 on bioanalytical method validation" of the European Medicines Agency (EMA, 2022). Chromatographic separation was performed on an Acquity UPLC BEH C18 column (2.1x50 mm, 1.7 µm). The mobile phase was water with 0.1% concentrated formic acid v/v (A1) and acetonitrile with 0.1% concentrated formic acid v/v (B1). The flow rate was 0.3 mL/min. ***Results.*** The developed method met the criteria of the EMA validation guideline and showed very good linearity in the concentration range of 50-6000 ng/mL ($r^2 = 0.9984$) with limits of detection of 3.0 ng/mL, good precision (CV < 11.91%), and accuracy (97.65-111.63%). ***Conclusions.*** This method can be successfully used to analyze the drug concentration in the plasma. *(Farm Współ 2025; 18: 149-156) doi: 10.53139/FW.20251825*

Keywords: fenofibric acid, ultra-performance liquid chromatography-mass spectrometry, validation

Introduction

Fenofibric acid (FFA) is an active moiety of orally administered lipid-lowering pharmaceutical ingredients, such as fenofibrate and choline fenofibrate, that particularly targets the peroxisome proliferator-activated receptor (PPAR) alpha agonist with lower density lipid cholesterol-and triglyceride-lowering activity [1-4]. Additionally, it activates PPAR-gamma, which is involved in regulating insulin secretion [5,6]. Thereby, the administration of the drug is indicated as adjunctive therapy to diet to reduce triglycerides in patients with severe hypertriglyceridemia as well as to reduce elevated low-density lipoprotein cholesterol, total cholesterol, triglycerides, and apolipoprotein B, and to increase high-density lipoprotein cholesterol in patients with primary hypercholesterolemia or mixed dyslipidemia. Improving glycemic control in diabetic patients showing fasting chylomicronemia will usually obviate the need for pharmacological intervention [7].

It has been established through research that fenofibric acid regulates human arylacetamide deacetylase by PPARα and its significance in suppressing cellular lipid accumulation [8].

The potential for interaction with other drugs is being widely studied, primarily with hypolipidemic agents from the statin group. The combination of rosuvastatin + fenofibric acid is an effective option in patients with mixed dyslipidemia and high cardiovascular risk, providing a therapeutic alternative for those conditions that require it [9,10].

In recent years, attempts have been made to expand the indications for fenofibric acid. Experimental and clinical studies suggest a protective role of fenofibric acid on the outer blood-retina barrier in diabetic retina [11], therapeutic potential in the prevention of paclitaxel-induced peripheral neuropathy development [12], enhancement of antitumor activity and reduction of

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resistance to anticancer agents [13], as well as in the treatment of osteoporosis by increasing bone mineral density [14]. Fenofibric acid is suggested as a potential and lead-free fatty acid receptor 1 agonist, which stimulates insulin secretion without contributing to hypoglycemia, making it a favourable factor in treating type 2 diabetes [15].

Controversial results have been published regarding fibrates' effectiveness in treating COVID-19 [16,17].

The expansion of fenofibric acid and its derivatives to other fields of medicine leads to an increased number of drug classes that will be co-administered with fenofibric acid. It increases the risk of drug-drug interactions, which have the potential to enhance therapeutic efficacy, but may also contribute to the development of adverse reactions. Therefore, effective monitoring, particularly determining fenofibric acid concentration in biological fluids, remains an essential task of modern clinical pharmacy.

In previous studies, liquid chromatography with tandem mass spectrometry (MS/MS) was used to analyze fenofibric acid concentrations in rat plasma in negative mode [18], while in human plasma, liquid-liquid extraction was applied [19].

In this paper, our goal was to present a precise and straightforward UPLC MS/MS method in positive mode, electrospray ionization in positive mode, which meets the criteria of the validation guidelines of the European Medicines Agency using a simple protein precipitation procedure using an organic solvent instead of the extraction method to the liquid phase.

Materials and methods

Chemicals and reagents

The LGC (Canada) supplied the fenofibric acid, while fenofibric acid-D_6 (figure 1) was procured from Merck Corp. (Germany). Acetonitrile was purchased from Merck Corp. (Germany), and ultrapure water was obtained via a direct water purification system (Milli-Q system, Millipore, Merck). Analytical reagent-grade chemicals and MS/MS-grade solvents were utilized throughout the study.

Drug-free rat plasma was obtained from the Anima Sp. z o. o. SK and ViVARI s.c. (Warszawa, Poland).

LC Instrument and Chromatographic Conditions

This novel UPLC MS/MS method was established using an LC system tandem mass spectrometer Xevo TQ-S micro coupled with the Acquity UPLC I-class Plus arrayed with MassLynx V 4.2 SCN1017 software (Waters, Milford, MA, USA). The chromatographic determination was performed using the Acquity UPLC BEH C18 column (2.1x50 mm, 1.7 µm; Waters; Milford, MA, USA) coupled with a Vanguard pre-column (2.1x5 mm, Waters; Milford, MA, USA) at a column temperature of 45°C. The mobile phase consisted of water with 0.1% concentrated formic acid v/v (A1) and acetonitrile with 0.1% concentrated formic acid v/v (B1). The following gradient was used (time: %A/%B): 0-0.3 min: 50/50 2.0 min: 95/5 2.3-3.0 min: 0/100 4.0 min: 50/50. The flow rate was 0.3 mL/min, and the injection volume for each sample was 3 µL. The total run time was 4 min. The LC eluate was directed into a tandem, quadrupole, atmospheric ionization mass spectrometer (Xevo TQ-S micro detector, Acquity, Waters, Milford, USA) equipped with an electrospray ionization source.

Mass spectrometric conditions. The mass spectrometer was run in the positive ion mode and configured in multiple reaction monitoring (MRM) mode to detect FFA and its isotope-labelled analogue. Capillary voltage, cone voltage, collision energy, and dwell time were optimized using Masslynx™ Intellistart Software (version 4.2, Waters, USA). The following settings for the Xevo TQ-S micro mass spectrophotometer were used: source temperature 150°C, desolvatation temperature 500°C, nitrogen gas flow 1000 L/h, and capillary voltage 4 kV. Transition ion pairs (parent m/z → daughter m/z) were identified using the MRM mode for the following compounds:

318.95→ 232.98 (quantitative calibration pair), dwell (s) 0.054; cone (V) 20; collision energy (V) 16,

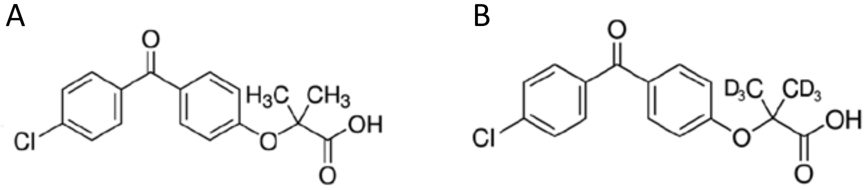

Figure 1. Chemical structure: (A) fenofibric acid, (B) fenofibric acid-D_6

318.95 → 138.88, dwell (s) 0.054; cone (V) 20; collision energy (V) 34,
318.95 → 120.85 dwell (s) 0.054; cone (V) 20; collision energy (V) 35 for FFA, and 324.93 → 110.82 dwell (s) 0.054; cone (V) 20; collision energy (V) 45 for FFA-D_6 as internal standard (IS).

Preparation of Calibration Standards and Quality Control Samples

Fenofibric acid (10 mg) was precisely weighed and completely dissolved in methanol (10 mL) to prepare the stock solution. Next, the stock solution was serially diluted with methanol to acquire the standards (fenofibric acid concentrations of 50, 100, 150, 250, 500, 750, 1000, 2000, 2500, 4000, 6000 ng/mL). Likewise, fenofibric acid-D_6 (1 mg) was dissolved in methanol (1 mL) to prepare a stock solution, then diluted to a concentration of 1 μg/mL as an internal standard solution. Then, the protein precipitation technique was used to prepare the samples. 50 μL of fenofibric acid working solution and 50 μL of FFA-D_6 internal standard solution were added to 50 μL of rat plasma. The samples were mixed, and 200 μL of acetonitrile was added to precipitate protein. Three quality control samples containing 150, 2500, and 4500 ng/mL of fenofibric acid were also selected for method validation.

LC Method Validation

Method validation was performed in accordance with the "ICH guideline M10 on bioanalytical method validation" of the European Medicines Agency (EMA, 2022) [20].

Specificity and selectivity. Six different samples were prepared to assess selectivity and specificity, with the matrix effect being defined as a drug-free blank rat plasma sample. Furthermore, the evaluation with the LC MS/MS method was performed with the plasma samples spiked with both FFA and FFA-D_6 (internal standard), the blank plasma spiked only with the FFA-D_6, and the blank plasma spiked only with FFA. No interference from endogenous compounds was observed at the analytes' retention times. Responses to interfering components were no greater than 20% of the analyte response at the lower limit of quantitation (LLOQ) and no greater than 5% of the IS response in the sample at the LLOQ.

Carry-over. Carry-over was evaluated by analysing a blank plasma sample injection after the calibration standard at the upper limit of quantification (ULOQ) containing FFA and FFA-D_6 as IS. This step was repeated five times. To meet the requirements of the EMA guidelines, carryover in the blank sample should be <20% of the LLOQ of the drug and <5% of the IS.

Matrix effect. The matrix effect was evaluated by comparing peak areas of post-extraction spiked quality control samples with corresponding standard solutions. These procedures were repeated three times at two concentrations: quality control low (LQC) and quality control high (HQC). Each concentration was prepared using a matrix from six different lots. For each matrix lot evaluated, the accuracy should be within ±15% of the nominal concentration, and the precision (per cent coefficient of variation (%CV) should not be greater than 15%.

Linearity. Linearity was demonstrated in the calibration curve, and 11 nominal concentrations ranging from 50 to 6000 ng/mL were used to plot the calibration curve of FFA. Calibration curves (n = 4) were constructed by plotting the peak area ratios of the analytes to the IS against the nominal concentration of the analytes, using a 1/x weighted linear regression. In addition, the linearity of the LC MS/MS method was demonstrated via several parameters that were produced, including correlation coefficient (r), after plotting concentration versus the mean peak area.

Accuracy and Precision. For the evaluation of the intra-day (one single day) accuracy and precision, the four nominal concentrations of FFA 50, 150, 2500, and 4500 ng/mL were utilized. The inter-day (three consecutive days) accuracy and precision were evaluated with a single concentration of the FFA sample. In this study, the %CV represented precision and recovery (%) ([calculated amount/predicted amount] × 100) characterized accuracy.

Recovery. Recovery was determined by comparing the analyte response in a biological sample that was spiked with the analyte and processed, with the response in a biological blank sample that was processed and then spiked with the analyte. The analysis was carried out by comparing the analytical results for the extracted samples in three concentrations (low, medium, and high).

Stability. The stability tests were carried out in triplicate at two different concentrations, low (150 ng/mL) and high (4500 ng/mL). The samples were kept frozen at -20°C until further testing. The stability of FFA in rat plasma was achieved under three different storage conditions. In the first storage condition, the sample was thawed at room temperature (25°C) for five hours. This was performed by leaving the quality control sample, which was stored in a refrigerator (-20°C) for five hours, on the workbench for five hours (room temperature) before the analysis.

For the second storage condition, the short-term condition, three cycles of repeated freeze and thaw were carried out before assessing the samples. Each cycle consists of 3-4 h thawing at room temperature and freezing for 24 h.

The third storage condition, long-term condition, involved keeping frozen samples (-20°C) for 1 month before the method evaluation.

Statistical analysis. The data were expressed as mean ± standard deviation (SD).

Results and discussion

Method development

The chromatographic separation of FFA and FFA-D_6 is shown in figure 2. Run time was 4 minutes.

Method validation

Method validation results are presented below (tables I-IV, figures 3, 4).

Calibration curve

System suitability is described in table I and figure 3.

Table I. System suitability

Parameter	Value
CV (%) of analyte to IS peak area	± 15
Retention time (min)	4.0
Correlation coefficient (r)	0.9992
Correlation coefficient (r2)	0.9984
LOD (ng/mL)	3.0
LLOQ (ng/mL)	50.0

CV – Coefficient of Variation, LOD – Limit Of Detection, LLOQ – Lower Limit of Quantification

Linearity

Calibration standards (11 non-zero) with a 50-6000 ng/mL concentration range for plasma were prepared in blank rat plasma and analysed in 4 separate analytical runs (figure 3). Linear regression with a weighting factor of 1/x proved to result in the best fit. The calibration curves were acceptable if at least 75% of all non-zero calibration standards were within ±15% of the nominal concentrations, or ±20% for the LLOQ.

The average linear regression parameters (±SD) obtained for the quadruplicates were: $y = 0.00683557x + 0.0665919$ with $r^2 = 0.9984$. The calculated concentrations were within 15% of the nominal concentrations at all concentration levels, which is in accordance with EMA guidelines.

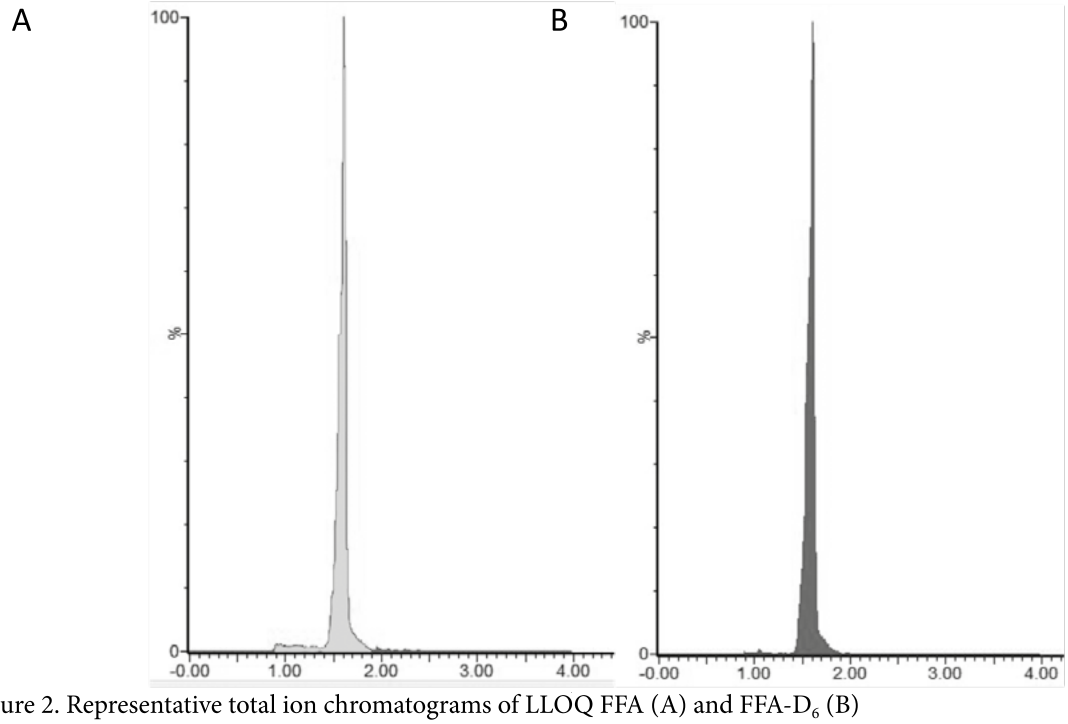

Figure 2. Representative total ion chromatograms of LLOQ FFA (A) and FFA-D_6 (B)

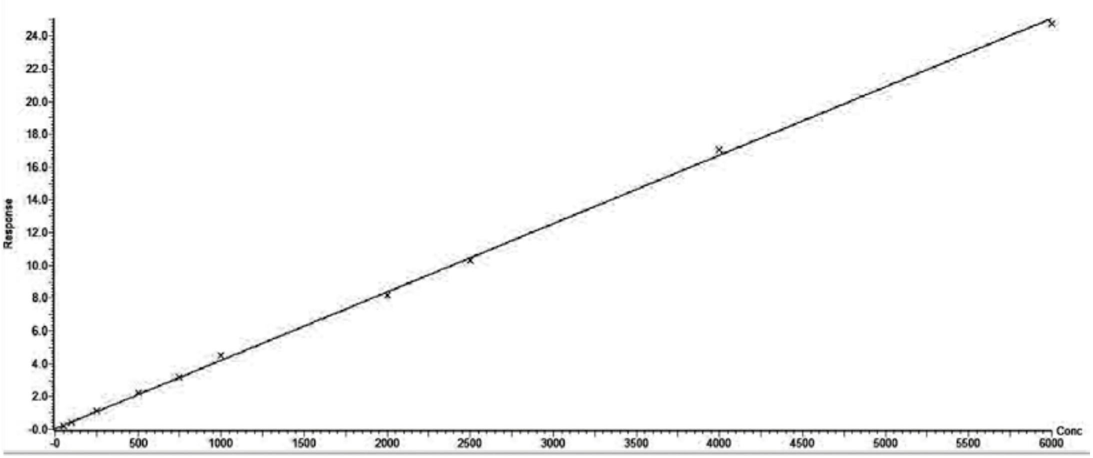

Figure 3. Calibration curve of FFA

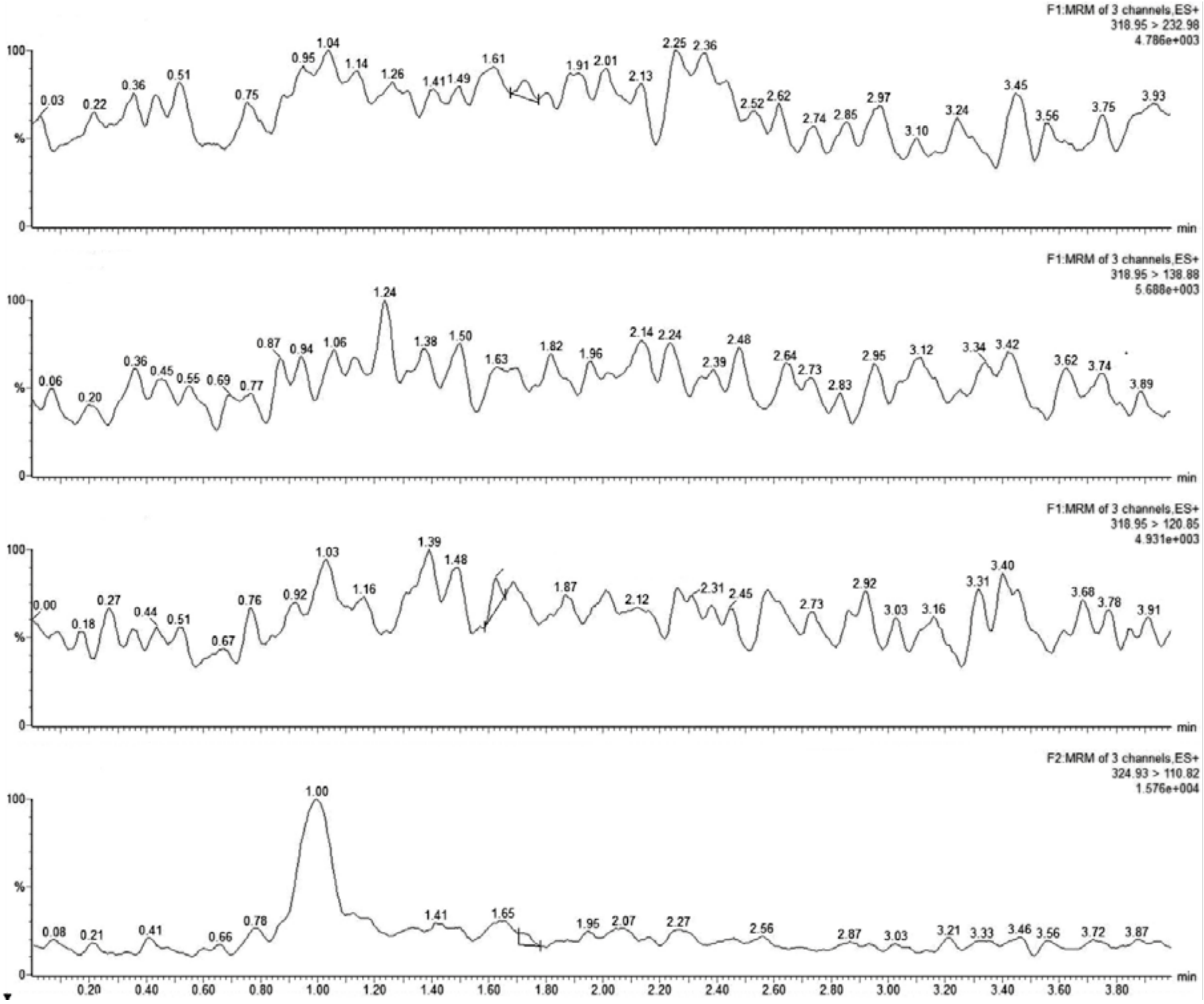

Figure 4a. Ion chromatogram of blank sample and 4b on the next page

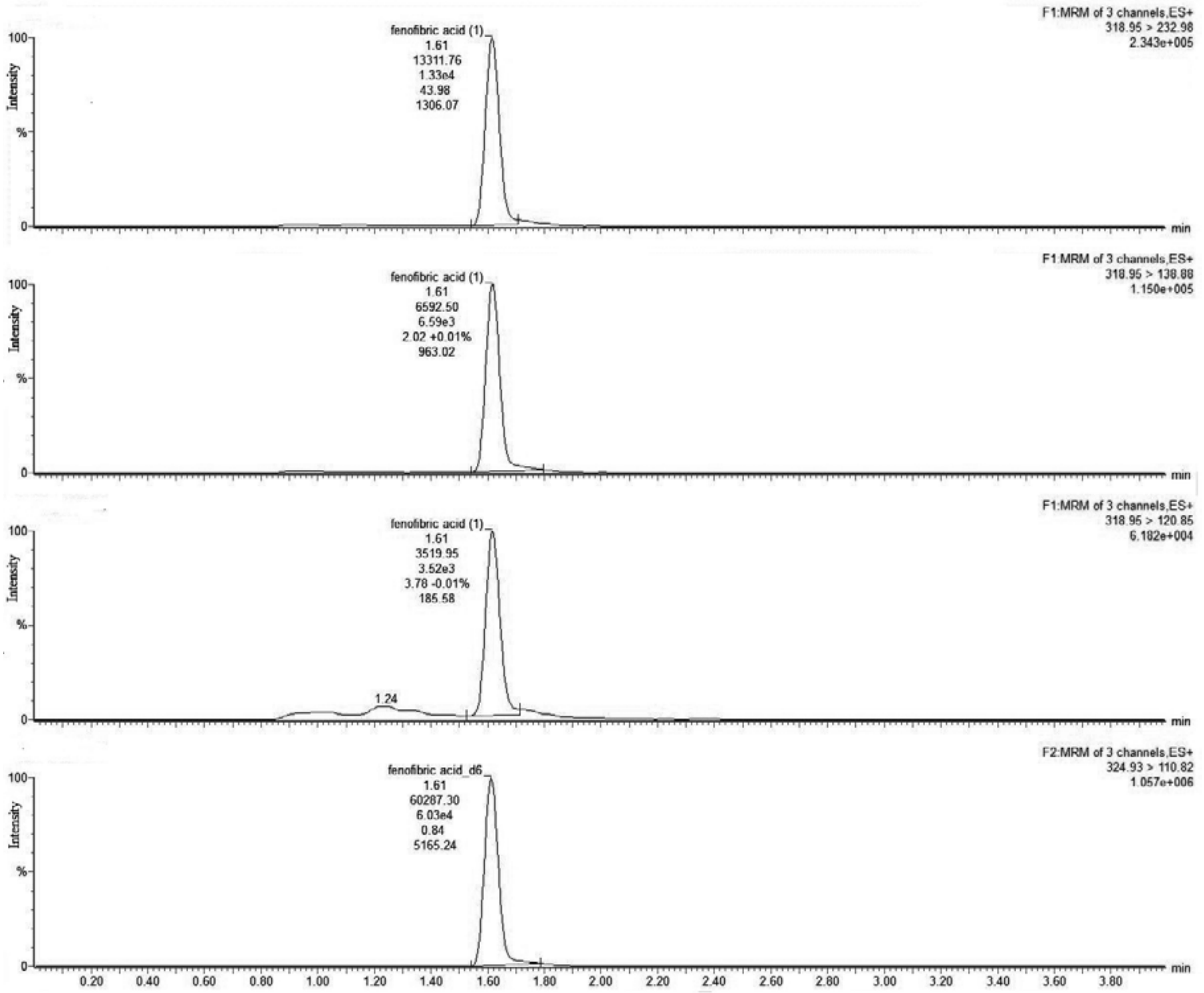

Figure 4b. LLOQ of fenofibric acid and fenofibric acid-D_6 (B)

These acceptance criteria were met, and thus the calibration curves were accepted. The calibration curve range was chosen to cover the expected clinically relevant plasma concentrations.

Selectivity and carry-over

Multiple reaction monitoring traces of all six blank rat plasma samples showed the absence of interference as responses were <20% of the LLOQ and 5% of the IS. Chromatogram of the analyte at the LLOQ level and its respective blank sample are shown in figure 4. Carry-over in the blank sample after injection of the ULOQ sample was <20% of the LLOQ for each drug and <5% of the IS.

Matrix effect

The CV of the IS-normalized matrix effect calculated from the six rat plasma batches at both concentrations (LQC and HQC) was <6.16% for all analytes (table II).

Table II. Assay performance data of the matrix effect of FFA

Nominal concentration (ng/mL)	Matrix effect	
	mean ± SD	CV (%)
150	137.33 ± 8.46	6.16
4500	4555.45 ± 166.25	3.65

Accuracy and precision

Table III shows the results of inter-day and intra-day accuracy and precision within the calibration range [LLOQ, LQC, HQC, and quality control

Table III. Intra-day (n = 5) and inter-day (3 consecutive days) accuracy and precision data for the quantitation of fenofibric acid in rat plasma

Nominal concentration (ng/mL)	Intra-day assay (n = 5)				Inter-day assay (n = 15)			
	mean	SD	%CV	accuracy %	mean	SD	%CV	accuracy %
50	49.32	2.40	4.88	98.64	48.83	4.88	9.99	97.65
150	160.62	14.76	9.19	107.08	156.53	18.64	11.91	104.35
2500	2679.26	78.79	2.94	107.17	2732.17	226.00	8.27	109.29
4500	5191.94	210.35	4.05	103.84	5023.98	282.4	3.26	111.63

Table IV. Stability study (n = 3)

Nominal concentration (ng/mL)	Stability								
	1st day			short-term			long-term		
	mean ± SD	accuracy %	CV (%)	mean ±SD	accuracy %	CV (%)	mean ±SD	accuracy %	CV (%)
150	133.40 ±4.88	88.93	3.66	137.71 ±3.83	91.81	2.78	131.87 ±6.49	87.92	4.92
4500	4647.00 ±475.55	103.27	10.23	4273.14 ±377.73	94.96	8.84	4289.62 ±603.79	95.32	14.08

at medium (MQC) concentrations] that met the requirements of RSD <20% for LLOQ and RSD <15% for all other concentrations. The value of precision for FFA in the intra-and inter-day was under 12% in all cases. Inter-day accuracy values ranged from 97.65 to 111.63% with intra-day accuracy <7.2%. Consequently, the accuracy and precision values were within the suggested limits of the EMA guidelines.

Recovery

Recovery was analyzed by comparing the analytical results for the extracted samples in three concentrations: LQC, MQC, and HQC. For QC samples, the recovery was 93, 101, and 98%, respectively.

Stability Studies

The initial sample concentration, analyzed immediately after sample preparation, was compared with the concentration after storage under two different conditions. The stability studies indicated that FFA did not show any significant degradation, with stability ranging from 87.92% to 103.27% (table IV). The peaks of FFA had little interference with the matrix components in all three different storage conditions when the plasma was stored at -20°C. Thus, the results verified that this method is suitable for determining the stability of FFA in rat plasma.

Conclusion

This study established the novel UPLC MS/MS method for analyzing fenofibric acid in rat plasma by employing fenofibric acid-D6 as an internal standard [20].

This method validation includes all procedures required to show that determining FFA concentration in rat plasma is reliable for the intended application. The calibration range considers the expected threshold concentration of the drug metabolite (C0) and the expected C_{max}.

In addition, the stability test in three different storage conditions indicated that the method is suitable for examining the stability of the drug. Furthermore, this novel method could support pharmacological assay as a valuable tool for evaluating the amount of fenofibric acid in rat plasma.

Acknowledgments.
This work was supported by a “Phospholipids as excipients in Amorphous Solid Dispersions – an attempt to establish hot-melt-extrusion for oral formulations of poorly soluble drugs” project grant funded by the Phospholipid Research Center, Heidelberg, Germany (No. PSM-2020-085/1-1).
Part of the work was carried out with the support of the Center of Innovative Pharmaceutical Technology at Poznan University of Medical Sciences.

Conflict of interest

None

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