A new rapid, selective and sensitive liquid chromatography–tandem mass spectrometric method was developed and validated for the determination of ciprofibrate, an antihyperlipidemic agent, in K$_2$EDTA human plasma. Furosemide was used as internal standard (IS). The ciprofibrate and IS were extracted using Oasis HLB 1 cc 30 mg solid-phase extraction cartridge. The chromatographic separation was performed on ACE C18, 50 × 4.6 mm, 5 μm column. The mobile phase consisted of 0.001% ammonia in methanol–acetonitrile–water (70:20:10, v/v/v). Detection and quantitation were performed by a triple quadrupole equipped with electrospray ionization and multiple reaction monitoring in negative ionization mode. The most intense [M-H]$^-$ transition for ciprofibrate at $m/z$ 287.0 → 85.0 and for IS at $m/z$ 328.90 → 204.9 were used for quantification. The method was found to linear over the range of 25–30,000 ng/mL ($r>0.998$). The lower limit of quantitation (LLOQ) was 25 ng/mL. The extraction recovery was above 90%. The accuracy was found to be 101.26–106.44%. The stability testing was also investigated and it was found that both drug and IS were quite stable. The developed method was successfully applied to the bioequivalence study of ciprofibrate 100 mg tablet after oral administration to healthy human volunteers.

Introduction

Ciprofibrate, 2-[4-(2,2-dichlorocyclopropyl)phenoxy]-2-methylpropanoic acid (Figure 1A), is an isobutyric (fibric) acid derivative and widely used for the treatment of various types of dyslipidemia (1). Ciprofibrate acts via the activation of lipoprotein lipase which is a key enzyme in the degradation of very low-density lipoprotein (VLDL) resulting in the lowering of circulating triglycerides (TGs). This effect is exerted through paroxysmalfiberactivator receptor α (PPAR α), which is the gene transcription regulating receptor expressed in the liver, fat and muscles. The activation of PPAR α enhances lipoprotein lipase synthesis and fatty acid oxidation (2). Ciprofibrate also decreases the hepatic TG synthesis and circulating free fatty acid. It is readily absorbed from the GI tract and the plasma concentration peaks within 1–4 h. Ciprofibrate is metabolized in the liver by glucuronidation conjugation pathway (3).

The literature survey revealed that several methods have been reported for the determination of ciprofibrate using tandem mass spectrometry in human plasma (4), HPLC in human plasma (5, 6), stability indicating HPLC in bulk and pharmaceutical dosage forms (7), chiral and achiral determination in urine (8) and densitometric and videodensitometric TLC (9). The reported LC–MS-MS method was developed using liquid liquid extraction (LLE) technique which is time consuming and difficult to reproduce. In the present study, attempts have been made to develop a method for the quantitation of ciprofibrate in human plasma using solid phase extraction (SPE).

Experimental

Chemicals and reagents

Ciprofibrate (Batch no. Y01L) was procured from Derivados Quimicos Ltd (NJ, USA) and Furosemide (Batch no. 8036H2R1) was supplied by IPCA Laboratories Ltd (Ratlam, India). Methanol and acetonitrile (HPLC Grade) were purchased from Merck Specialties Pvt. Ltd (Mumbai, India). Ortho-phosphoric acid, sodium hydroxide (AR Grade) and ammonia (LR Grade) were purchased from SD Fine Chemical Ltd (Mumbai, India). Drug-free control human plasmas (K$_2$EDTA) were purchased from Ashish Pathology Laboratory (Ahmedabad, India). Ultra-Pure Milli-Q-Water was prepared using Barnstead water purification system (NY, USA). The study in human plasma was carried out with institutional Ethics Committee permission (number IRB0000675). The blank human plasma used during the entire study was procured from APL Institute of Clinical Laboratory & Research Pvt. Ltd, Ahmedabad, Gujarat, India. The laboratory is ethically approved by the college of American Pathologist and National Accreditation Board for testing and calibration laboratory (NABL).

Instrumentation

The mass spectrometer API 3200 (Applied Biosystem, Foster city, CA, USA) triple quadrupole with electrospray ionization coupled with liquid chromatography LC-10ADVP (Shimadzu, Japan) and autosampler SIL-HTc were used for the study. Analytical balance (Mettler Toledo-AG285, Switzerland) was used for the weighing of chemicals.

Preparation of stock, calibration standards and quality control samples

The stock solution of ciprofibrate (5 mg/mL) was prepared by dissolving it in 1 N sodium hydroxide and then diluting with methanol–water (8:2). The stock solution of furosemide was prepared in methanol–water (8:2). The working standards were prepared by diluting stock solution with the diluent to obtain final

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concentrations of 0.5, 1, 2, 10, 20, 100, 200, 400, 600 μg/mL, and quality control (QC) samples low (LQC), medium (MQC) and high (HQC) at the concentrations of 1.5, 15 and 500 μg/mL, respectively. The methanol–water (8:2) was used as a diluent for the preparation of stock and working standards. Stock solutions and working standards were stored in the refrigerator at 2°C–8°C and the volumetric flask barb was wrapped by paraffin film to prevent methanol evaporation.

Appropriate volume of working standard solutions and human plasma were added to each Eppendorf tube. The final concentrations of ciprofibrate obtained were 25, 50, 100, 500, 1000, 10,000, 20,000, 30,000 ng/mL and the QC samples LQC, MQC, HQC were 75, 750, 25,000 ng/mL, respectively.

**Sample preparation**

Plasma spiked calibration standards and QC samples of ciprofibrate were prepared by spiking 15 μL of working solution of ciprofibrate and 15 μL IS (10 μg/mL) to 285 μL drug-free control plasma in 2 mL micro-centrifuge tube. A 685 μL orthophosphoric acid (0.5%) was added to each plasma preparation and vortexed for 1 min followed by solid phase extraction.

The Oasis HLB 30 mg cartridges were conditioned with 1 mL methanol and 1 mL water followed by 1 mL sample loading (Blank plasma, Zero, Calibration Standards and QC samples). The cartridges were then washed successively with 1 mL water and then methanol (10%). Analyte and IS were eluted by 1 mL 0.001% ammonia in methanol–acetonitrile–water (70:20:10, v/v/v) and transferred in LC–MS-MS vials for injection.

**Chromatographic conditions**

The chromatographic separation was performed by injecting 20 μL sample on ACE C18 (50 × 4.6 mm, 5 μm) column at ambient temperature. The analyte and IS were eluted by mobile phase containing 0.001% ammonia in methanol–acetonitrile–water (70:20:10, v/v/v) in isocratic mode. The mobile phase flow rate was set to 1 mL/min and the total run time was 1.8 min. Under these typical conditions, the analyte and IS were eluted at retention time of 0.48 and 0.46 min, respectively. The autosampler was maintained at a temperature of 15°C ± 5°C. The mass spectrometer was set up in negative mode with multiple reactions monitoring for the transitions of m/z 287.0 → 57.0 and m/z 328.9 → 204.9 for IS. The ionization source was set at 550°C and ion spray voltage at 4500 V. The Analyst software 1.4.2 was used for instrument control and data acquisition.

**Bioanalytical method validation**

A bioanalytical method validation was performed according to USFDA and ANVISA guidelines for bioanalytical method validation (10–13). The validation assures that the method will provide accurate, precise and reproducible data during analysis. According to the existing consensus, validating quantitative analytical methods entails the evaluation of selectivity, sensitivity, carryover, calibration curves, accuracy and precision, recovery, matrix effect, stability and dilution integrity.

**Results**

**Method development**

The mobile phase used for the method development was 0.001% ammonia in methanol–acetonitrile–water (70:20:10, v/v/v). ACE C18, 50 × 4.6 mm, 5 μm column was used as the stationary phase and the flow rate was maintained at 1 mL/min. Low flow rate was selected to 1 mL/min to increase the efficiency of the column and to reduce the usage of mobile phase. Furosemide was used as internal standard (IS, Figure 1).

The LC–ESI–MS-MS with negative and multiple reactions monitoring mode was selected which provides a selective method for the determination of ciprofibrate and IS (Figure 2). The optimized parameters—Declustering potential, Collision cell entrance potential and Collision cell exit potential—for ciprofibrate and IS were −22.0, −11.0, 1.70 and −28.0, −16.0, −4.0 V, respectively.

**Method validation**

The method was validated and found to be accurate, precise, specific and selective.

System suitability studies were carried out and % CV for peak ratios was in the range of 0.61–1.25% for the analyte and IS. Assay selectivity was examined by analyzing control blank plasma samples from six different sources including lipemic and hemolized plasma. In the ion chromatograms, no interfering peaks were present at the retention times of ciprofibrate and IS (Figure 3A). Moreover, no analyte-interfering peaks were observed in all the samples collected before drug administration. The method exhibited good linear response over the concentration range of 25–30,000 ng/mL. The mean standard curve was typically described by weighted (1/x^2) linear regression equation: y = 0.000893x + 0.00504, r = 0.9980. Back calculations were made from the calibration curves to determine ciprofibrate concentrations from each calibration standard. The accuracy was within 20%.

The LLOQ was 25 ng/mL for ciprofibrate with precision 2.65% and accuracy 102.0% (Figure 3B). The intra-day precision and accuracy were in the range of 0.68–4.46 and 94.5–107.3%, respectively. Whereas the corresponding inter-day values were 4.32–7.01 and 101.3–106.4%, respectively (Table I). Specificity studies were carried out and no analyte-interfering peaks were observed in all the samples collected before drug administration, which indicates that the method is highly selective.

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**Figure 1.** Chemical structure of Ciprofibrate (A) and Furosemide (B).

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The recovery studies were carried out by extracting analyte by solid-phase extraction technique. Recoveries of analyte were measured by comparing mean peak response of analyte obtained from low- and high-QC samples \((n = 6)\) after extraction to those obtained from the reference samples which were prepared by adding the analyte to the post-extracted blank plasma samples (Relative recovery) and non-spiked samples were prepared in elution solvent (Absolute recovery) at the same concentrations. Similarly, the extraction recovery of IS was assessed at medium QC samples \((10 \text{ ng/mL})\). The absolute recovery and relative recovery of ciprofibrate were in the range of 85.8–88.3 and 90.1–96.8%, respectively, and that of IS were 88.8 and 100.0%.

**Stability**

The short-term stability of ciprofibrate at room temperature was examined by keeping four sets of low- and high-QC samples at room temperature \((25 \pm 5\, ^\circ \text{C})\) for 24 h and the results were compared with freshly prepared samples at the same concentrations (Table I).

The extracted four sets of low- and high-QC samples were kept in the auto sampler at \(15^\circ\)C for about 48 h. No analyte degradation was observed during the test period (Table I).

Analyte freeze and thaw stability was evaluated after four cycles. The four sets of low- and high-QC samples subjected to freeze and thaw cycle by freezing at \(-70 \pm 5\, ^\circ\text{C}\) followed by unassisted thaw at room temperature were extracted and analyzed (Table II).

Long-term stability is determined by storing low- and high-QC samples under the same conditions as the study samples. The four sets of low- and high-QC samples were kept in deep freezer at \(-70 \pm 5\, ^\circ\text{C}\) and in freezer at \(-15 \pm 5\, ^\circ\text{C}\) up to 11 days. The stability samples mean the back-calculated concentrations were compared with initial stability control samples of ciprofibrate (Table II).

K2EDTA interaction stability studies were carried out by preparing four sets of low- and high-QC samples in the subject sample collection vaccutainer coated with K2EDTA and kept in deep freezer at \(-70 \pm 5\, ^\circ\text{C}\) for about 48 h. After the completion of the stability period, the samples were thawed at room temperature, extracted, analyzed and the results were compared with freshly prepared samples (Table II).

The bench-top stability of ciprofibrate in whole blood was evaluated at room temperature \((25 \pm 5\, ^\circ\text{C})\) for about 4 h by keeping four sets of low- and high-QC samples. The stability samples' response was compared with the response of freshly prepared solution and it was within \(\pm 5\%\) (Table II).

The stock and working solutions stability of the analyte and the IS were evaluated at room temperature \((25 \pm 5\, ^\circ\text{C})\) for about 22 h and at refrigerated temperature \((2–8\, ^\circ\text{C})\) for 13

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**Figure 2.** MRM-negative ion spectra: (A) Ciprofibrate parent ion, (B) Ciprofibrate product ion, (C) Furosemide parent ion, (D) Furosemide product ion.
The four replicates of low- and high-QC samples were stored at the mentioned conditions (Table III).

Matrix effects were investigated using mass spectrometric method, using six lots of plasma. For each analyte and the IS, the matrix factor (MF) was calculated in each lot of matrix, by calculating the ratio of the peak area in the presence of matrix (measured by analyzing blank matrix spiked with analyte at a concentration of maximum three times the LLOQ after extraction) to the peak area in absence of matrix (pure solution of the analyte). The IS normalized MF was calculated by dividing the MF of the analyte by the MF of the IS (Table IV).

The anticoagulant effect on analyte and IS was determined by preparing four sets of low- and high-QC samples in heparinized plasma, extracted, analyzed and the results were compared against the K$_2$ EDTA anticoagulant plasma calibration. The mean % accuracy and precision at low- and high-QC samples were well within ±15% of the nominal concentration (Table IV).
For the dilution integrity study, five replicates of ULOQ were prepared in double concentration of their nominal concentration. Then the samples were diluted with drug-free control human plasma to meet their actual concentration. The samples were extracted along with precision and accuracy batch and results were compared with nominal concentration. The mean % accuracy and the precision of mean of dilution integrity samples were found to be within ±15% as per the USFDA guidelines (Table IV).

The carryover effect of the analyte and the IS was determined using LLOQ, ULOQ and solvent prepared in elution solvent, and biological matrix extracted LLOQ, ULOQ and matrix blank preparation. The carryover of the peak response of analyte in solvent run was within the set criteria.
Application of validated LC–ESI–MS–MS method

The developed and validated LC–ESI–MS–MS method was successfully applied in the bioequivalence study of ciprofibrate carried out in human volunteers (Institutional Ethics Committee number IRB0006475). The bioequivalence study involved oral administration of ciprofibrate 100 mg tablet into 36 healthy human volunteers under fasting conditions. The blood samples were drawn at different time points (0.0, 0.5, 0.75, 1.0, 1.33, 1.67, 2.0, 2.33, 2.67, 3.0, 3.50, 4.0, 4.50, 5.0, 6.0, 7.0, 8.0, 10.0, 12.0, 24.0, 36.0, 48.0 and 72.0 h) after dosing. The bioequivalence comparison was made with that of Oroxadin® 100 mg tablet M/S by Sanofi-Aventis Farmacêutica, Brazil. The plasma concentration–time profile curve is shown in Figure 4.

Discussion

The objective of the method development was to resolve chromatographic peaks for active drug ingredient and the IS. The formic acid and acetic acid were tried in the concentration range of 0.001–0.1% for the development of the mobile phase, but it was found that as the concentrations of acid increased, response of analyte decreased. The ammonium acetate and formate buffers were also tried but the response was not enough to quantify LLOQ. Hence attempts were made using various proportions of methanol–acetonitrile–water (50:40:10, v/v/v), (60:30:10, v/v/v), (70:20:10, v/v/v). Here, good chromatography was obtained but still the response was not enough. So ammonia was added from 0.001 to 0.05% and an improved response was found in the presence of ammonia at all proportions. Thus, low concentration (0.001%) ammonia was used to preserve the lifespan of the column. Finally, 0.001% ammonia in methanol–acetonitrile–water (70:20:10, v/v/v) was selected as the mobile phase. ACE C18, 50 × 4.6 mm, 5 μm column was used as the stationary phase and flow rate was maintained at 1 mL/min. Low flow rate was selected to 1 mL/min to increase the efficiency of the column and to reduce the usage of the mobile phase.

Selection of the IS was mainly based on the physicochemical property of the drug and IS. Mefenamic acid, atorvastatin and furosemide were tried as IS. Among these, furosemide was selected because of good response with symmetrical peak shape and its structural and physicochemical similarity with ciprofibrate.

The LC–ESI–MS–MS with negative and multiple reactions monitoring mode was selected which provides a selective method for the determination of ciprofibrate and IS. The negative ionization mode was chosen for ion product since there was presence of carboxylic group in the structure of ciprofibrate and IS. The ion source was set to 550 °C to enhance the sensitivity. As the [M–H]⁻ transitions at m/z 287.0 → 85.0 for ciprofibrate and m/z 328.9 → 204.9 for IS were the most intense ones, they were selected for the quantification (Figure 2). The optimized parameters—Declustering potential, Collision cell-entrance potential and Collision cell-exit potential—for ciprofibrate and the IS were −22.0, −11.0, 1.70 and −28.0, −16.0, −4.0 V, respectively.

The method was validated and found to be accurate, precise, specific and selective. System suitability studies were carried out and assay selectivity was examined by analyzing control blank plasma samples from six different sources including lipemic and hemolized plasma. In the ion chromatograms, no interfering peaks were present at the retention times of ciprofibrate and the IS. Moreover, no analyte-interfering peaks were observed in all the samples collected before drug administration. The method exhibited good linear response over the concentration range of 25–30,000 ng/mL.

The intra- and inter-day accuracy and precision studies were carried out. All the results were well within the 15% stipulated by the USFDA guidelines, which indicate that the method is accurate and precise. Specificity studies were carried out and no analyte-interfering peaks were observed in all the samples collected before drug administration, which indicate that the method is highly selective. The recovery studies were carried out by extracting the analyte by the solid phase extraction technique.

Short-term stability, post-preparative stability, freeze and thaw stability, long-term stability, K₂EDTA interaction stability, stock and working solution stability, matrix effect, dilution integrity study and the effect of anticoagulant were evaluated. The stability samples' response was compared with the response of freshly prepared solution and it was within ± 5%.

The developed and validated LC–ESI–MS–MS method was successfully applied in the bioequivalence study of ciprofibrate carried out in human volunteers.

Conclusion

The LC–ESI–MS–MS method for the quantification of ciprofibrate from K₂EDTA human plasma has been developed using the solid phase extraction technique. When compared with the reported method, the present SPE method is rapid, selective, sensitive, accurate and precise. The method exhibited good linear response over the selected concentration range 25–30,000 ng/mL. Selectivity and sensitivity were sufficient for detecting and quantifying ciprofibrate in human plasma. Accuracy and precision were well within the USFDA guidelines of <15%. These features coupled with a short run time at 1.8 min when compared with the reported methods, facilitated a high analysis throughput, with the ability to quantify a larger number of clinical samples in a shorter time frame.

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