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Ciprofibrate quantification in human plasma by high-performance liquid chromatography coupled with electrospray tandem mass spectrometry for pharmacokinetic studies

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ABSTRACT

A rapid, sensitive and specific method for quantifying ciprofibrate in human plasma using bezafibrate as the internal standard (IS) is described. The sample was acidified prior extraction with formic acid (88%). The analyte and the IS were extracted from plasma by liquid–liquid extraction using an organic solvent (diethyl ether/dichloromethane 70/30 (v/v)). The extracts were analyzed by high performance liquid chromatography coupled with electrospray tandem mass spectrometry (HPLC–MS/MS). Chromatography was performed using Genesis C18 4 μm analytical column (4.6 × 150 mm i.d.) and a mobile phase consisting of acetonitrile/water (70/30, v/v) and 1 mM acetic acid. The method had a chromatographic run time of 3.4 min and a linear calibration curve over the range 0.1–60 μg/mL ($r > 0.99$). The limit of quantification was 0.1 μg/mL. The intra- and interday accuracy and precision values of the assay were less than 13.5%. The stability tests indicated no significant degradation. The recovery of ciprofibrate was 81.2%, 73.3% and 76.2% for the 0.3, 5.0 and 48.0 ng/mL standard concentrations, respectively. For ciprofibrate, the optimized parameters of the declustering potential, collision energy and collision exit potential were −51 V, −16 eV and −5 V, respectively. The method was also validated without the use of the internal standard. This HPLC–MS/MS procedure was used to assess the bioequivalence of two ciprofibrate 100 mg tablet formulations in healthy volunteers of both sexes. The following pharmacokinetic parameters were obtained from the ciprofibrate plasma concentration vs. time curves: AUC_{last}, $AUC_{0-168\,h}$, C_{max} and T_{max}. The geometric mean with corresponding 90% confidence interval (CI) for test/reference percent ratios were 93.80% (90% CI = 88.16–99.79%) for $C_{max,}$ 98.31% (90% CI = 94.91–101.83%) for AUC_{last} and 97.67% (90% CI = 94.45–101.01%) for $AUC_{0-168\,h}$. Since the 90% CI for AUC_{last}, $AUC_{0-168\,h}$ and C_{max} ratios were within the 80–125% interval proposed by the US FDA, it was concluded that ciprofibrate (Lipless® 100 mg tablet) formulation manufactured by Biolab Sanus Farmacêutica Ltda. is bioequivalent to the Oroxadin® (100 mg tablet) formulation for both the rate and the extent of absorption.

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1. Introduction

Ciprofibrate belongs to the second generation of fibric acid derivatives [1] and is used to treat patients with dyslipidaemias of various types [2,3]. Ciprofibrate has been shown to reduce plasma triglycerides, total cholesterol and low-density lipoprotein cholesterol, and increase plasma levels of high-density lipoprotein cholesterol [1–3]. The maximum concentration time is between 1 and 2.5 h and the long half life (80–100 h) permits daily dosing of the drug [1–5]. The maximum plasma concentration is between 66 and 88 μg/mL after chronic administration of 100 mg/day of ciprofibrate [1,3,4]. Several analytical methods based on high-performance liquid chromatography (HPLC) in plasma [1,6], solution [7] and in pharmaceutical syrup [8] samples have been used for ciprofibrate quantification. Here, we describe a fast, sensitive, and specific method for quantification of ciprofibrate in human plasma using high-performance liquid chromatography

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coupled with tandem mass spectrometry (HPLC–MS/MS), using bezafibrate as an internal standard (I.S.). This HPLC–MS/MS procedure was used to assess the bioequivalence of two ciprofibrate 100 mg tablet formulations (Lipless® tablet 100 mg manufactured by Biolab Sanus Farmacêutica Ltda. and Oroxadin® tablet 100 mg manufactured by Sanofi-Aventis Farmacêutica) in healthy volunteers of both sexes.

2. Methods

2.1. Calibration standards and quality control

Stock solutions of ciprofibrate and internal standard (bezafibrate) were prepared in methanol–water (50:50, v/v) and methanol (100%) at concentrations of 1 mg/mL, respectively. Calibration curves of ciprofibrate were prepared by spiking blank plasma at concentrations of 0.1, 0.2, 0.5, 2, 10, 20, 40 and 60 μg/mL and the analysis was carried out in duplicate for each concentration. The quality control samples were prepared in blank plasma at concentrations of 0.3, 5.0 and 48.0 μg/mL (QCA, QCB, and QCC, respectively). The spiked plasma samples (standards and quality controls) were extracted from each analytical batch along with the unknown samples.

2.2. Chemicals and reagents

Ciprofibrate was provided by Heartwell International, British Virgin Island, lot number 100170-08005. Bezafibrate was obtained from Sigma, Switzerland, lot number 1412729. Acetonitrile (HPLC grade), methanol (HPLC grade), diethyl ether (Analytic Grade), dichloromethane (Analysis Grade), formic acid (88%; Analytic Grade) and acetic acid (88%; Analytic Grade) were purchased from Mallinckrodt (Paris, ST, USA). Ultra pure water was obtained from an Elga UHQ system (Elga, UK). Blank human blood was collected from healthy, drug-free volunteers. Plasma was obtained by centrifugation of blood treated with the anticoagulant sodium heparin. Pooled plasma was prepared and stored at −20 °C until needed.

2.3. Drug analysis

Blood samples (7 mL) from a suitable antecubital vein were collected at 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.33, 2.67, 3, 3.5, 4, 5, 6, 8, 10, 12, 16, 24, 72, 120, 168 min post-dosing of reference or test ciprofibrate formulation. The blood samples were centrifuged at approximately 2000 × *g* for 1 min at 4 °C, and the decanted plasma stored at −20 °C until ciprofibrate analysis.

The extraction was performed by vortex-mixing 100 μL of each plasma sample, placed in glass tubes followed by the I.S. (50 μL of 5 μg/mL) and the samples vortex-mixed for 5 s. Formic acid (88%) was added (50 μL) to all tubes and the samples were vortex-mixed for 10 s. Diethyl ether/dichloromethane 70/30 (v/v) was then added (4 mL) to all tubes and performed the extraction by vortex-mixing for 40 s. The samples were centrifuged at 2000 × *g* for 2 min. The upper organic phase was transferred to another set of clean glass tubes and evaporated until dry under N_2 at 30 °C. The dry residues were dissolved with 1 mL of acetonitrile/water (50/50, v/v) by vortex-mixing for 40 s. The samples were centrifuged at 2000 × *g* for 5 min. The residues were transferred to 96-well plates using automatic pipettes with disposable plastic tips.

2.4. Chromatographic conditions

An aliquot (10 μL) of each plasma extract was injected into a Genesis C18 4 μm analytical column (4.6 × 150 mm i.d.). The compounds were eluted by with a mobile phase consisting of acetonitrile/water (70/30, v/v) and 1 mM acetic acid at a flow rate of 1.2 mL/min. Under these conditions, typical standard retention times were 2.00 min for bezafibrate and 2.70 min for ciprofibrate, and back-pressure values of approximately 90–100 bar. The temperature of the auto-sampler was maintained at 15 ± 2 °C and the run-time was 3.0 min.

2.5. Mass-spectrometric conditions

The mass spectrometer (API 3000) equipped with an electrospray source using a cross-flow counter electrode in a negative mode (ES-), was set up in Multiple Reaction Monitoring (MRM) for the transitions of 287.00 > 85.00 and 360.10 > 274.00, for ciprofibrate and the I.S., respectively. Fig. 1 shows a full scan mass spectra of ciprofibrate in panel "A", product ion spectra of ciprofibrate in panel "B", full scan mass spectra of bezafibrate in panel "C", and product ion spectra of bezafibrate in panel "D". The source block temperature was set at 400 °C and the turboionspray capillary voltage to −2.5 kV. For both ciprofibrate and the I.S., the optimized parameters of the declustering potential, collision energy and collision exit potential were −51 V, −16 eV and −5 V, respectively. The corresponding values for bezafibrate were −51 V, −22 eV and −13 V, respectively. Data were acquired by Analyst software (1.4.1, Applied Biosystems, Foster City, CA, USA).

2.6. Ion supression

A procedure to assess the effect of ion supression on the MS/MS was performed. The experimental set-up consisted of an infusion pump connected to the system by a "zero volume tee" before the split and the HPLC system pumping the mobile phase, which was the same as that used in the routine analysis of ciprofibrate, i.e. acetonitrile/water (50/50, v/v) by at 1.2 mL/min. The infusion pump was set to transfer (50 μL/min) of a mixture of analyte and internal standard in mobile phase (both 50 μg/mL). A sample of human pooled blank plasma was extracted by the extraction procedure. The reconstituted extract was injected into the HPLC system while the standard mixture was being infused. In this system any ion suppression would be observed as a depression of the MS signal.

2.7. Method development

Linearity was determined to assess the performance of the method. A linear least-squares regression with a weighting index of $1/x$ was performed on the peak area ratios of ciprofibrate and the I.S. vs. ciprofibrate concentrations of the eight plasma standards (0.1, 0.2, 0.5, 2, 10, 20, 40 and 60 μg/mL) in duplicate to generate the calibration curve.

The recovery was evaluated by dividing the extracted sample mean by the unextracted (spiked blank plasma extract) sample mean of the corresponding concentration. The matrix effect experiments were performed using the ratio between spiked mobile phase solutions and unextracted samples, spiked on plasma residues.

Within- and between-run precision was determined as the relative standard deviation, RSD (%) = 100 (SD/*M*), and the accuracy as the percentage relative error, RE (%) = $(E - T)(100/T)$, where *M* is the mean, SD is the standard deviation of *M*, *E* is the experimentally determined concentration and *T* is the theoretical concentration.

2.8. Stability

Stability quality control plasma samples (0.3 and 48.0 μg/mL for ciprofibrate) were subjected to short-term (8 h 50 min) room temperature, four freeze/thaw (−20–25 °C) cycles, 52 h auto-sampler stability (15 °C) and long-term stability (140 days, −20 °C) tests.

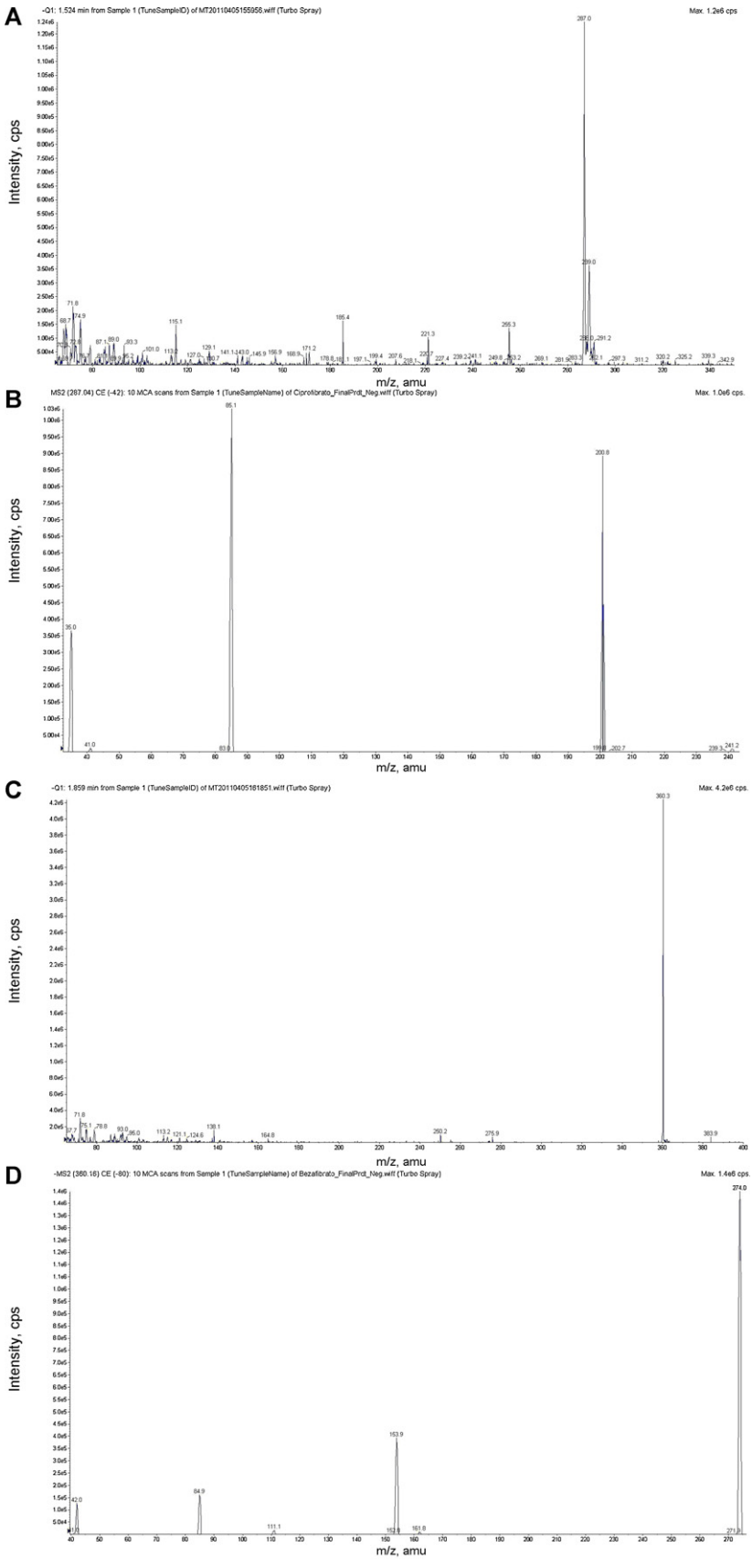

Fig. 1. Full scan mass spectra of ciprofibrate in panel "A", product ion spectra of ciprofibrate in panel "B", full scan mass spectra of bezafibrate in panel "C", product ion spectra of bezafibrate in panel "D".

Table 1
Accuracy and precision data for ciprofibrate from the pre-study validation in human plasma. The calculation was performed with and without the internal standard.

Nominal concentration (μg/mL)	0.1		0.3		5		48	
Intra-batch validation with/without IS ($n = 7$)								
Arithmetic mean (μg/mL)	0.099	0.105	0.302	0.297	4.60	5.136	47.09	48.971
Precision (%)	7.90	6.98	7.44	2.55	6.34	4.12	4.47	4.24
Accuracy (%)	99.04	104.73	100.67	99.10	91.97	102.71	98.10	102.02
Inter-batch validation with/without IS ($n = 21$)								
Arithmetic mean (μg/mL)	0.102	0.11	0.305	0.30	4.97	4.95	51.95	50.30
Precision (%)	13.25	8.77	6.26	6.94	7.67	6.42	7.59	4.75
Accuracy (%)	101.54	107.60	101.73	99.84	99.42	99.00	108.23	104.78

Subsequently, the ciprofibrate concentrations were measured compared to freshly prepared samples. The significance of the results obtained was analyzed by Student's *t*-test ($p < 0.05$).

2.9. Clinical protocol

Twenty-eight healthy volunteers of both sexes, between 18 and 50 years of age and within 15% of the ideal body weight were selected for the study having their health status previously assessed by clinical evaluation (physical examination, ECG) and the following laboratory tests: blood glucose, urea, creatinine, AST, ALT, alkaline phosphatase, γ-GT, total bilirubin, albumin and total protein, triglyceride, total cholesterol, hemoglobin, hematocrit, total and differential white cell counts, and routine urinalysis. All subjects were negative for HIV, HCV and HBV (except for serological scar).

The study began with 28 volunteers and finished with 22 volunteers. Six volunteers dropped out of the study for personal reasons. The volunteers ($n = 28$) had the following clinical characteristics (according to gender and expressed as mean ± SD [range]): males: age: 28.36 ± 7.61 [20.00–45.00], height: 1.74 ± 0.08 m [1.64–1.90], body weight: 75.21 ± 12.00 [54.50–98.00]; females: age: 30.29 ± 8.80 [22.00–47.00], height: 1.63 ± 0.08 [1.51–1.75], body weight: 60.94 ± 8.45 [50.00–79.60]. All subjects provided written informed consent and the Ethical Committee of the State University of Campinas - Unicamp approved the clinical protocol. The study was conducted in accordance with the provisions of the Declaration of Helsinki (1964), Tokyo (1975), Venice (1983), Hong Kong (1989), Somerset West (1996) and Edinburgh (2000) revisions. After a screening and washout period (of at least 2 weeks), individuals who qualified were confined for 2 periods of approximately 36 h.

The study was a single dose, two-period randomized design with at least a 30-day washout period between doses. During each period, the volunteers were hospitalized at 05:00 p.m. In order to evaluate their adherence to the requirements of the clinical protocol, volunteers received specialized assistance and care during all treatment periods, which included a brief investigation of their conditions upon confinement and at time of discharge. Standard meals were administered at 07:00 p.m. (dinner) and 10:00 p.m (snack). After an overnight fast (approximately 8 h), subjects received an oral dose of ciprofibrate (100 mg of either formulation) at approximately 6:00 a.m. The following formulations were employed: Lipless® (ciprofibrate) tablet 100 mg (test formulation manufactured by Biolab Sanus Farmacêutica Ltda.; lot N° 906071, expiration: 06/2011) and Oroxadin®

Table 2
Stability tests for ciprofibrate.

Post-processing stability test (8 h 50 min; μg/mL)						
($n = 5$)	Reference values Low sample	Values after 52 h	Reference values Medium sample	Values after 52 h	Reference values High sample	Values after 52 h
Arithmetic Mean	0.276	0.291	4.49	4.7	45.6	47.8
CV (%)	4.1	4.8	1.3	2.4	1.1	3.7
Variation	5.4		4.7		4.8	

Freeze-and-thaw stability test (20–25 °C; μg/mL)						
($n = 5$)	Reference values Low sample	Values after 4 cycles	Reference values Medium sample	Values after 4 cycles	Reference values High sample	Values after 4 cycles
Arithmetic Mean	0.292	0.326	4.59	4.88	47.8	48
CV (%)	7.3	4.3	3.9	13.8	2.4	5.4
Variation	11.6		6.3		0.4	

Short-term stability test (52 h; μg/mL)						
($n = 5$)	Reference values Low sample	Values after 8 h 50 min h	Reference values Medium sample	Values after 8 h 50 min h	Reference values High sample	Values after 8 h 50 min h
Arithmetic Mean	0.292	0.331	4.59	5	47.8	46.6
CV (%)	7.3	4.2	3.9	1.1	2.4	0.7
Variation	13.4		8.9		−2.5	

Long-term stability test (140 days; −20 °C; μg/mL)						
($n = 5$)	Reference values Low sample	Values after 140 days	Reference values Medium sample	Values after 140 days	Reference values High sample	Values after 140 days
Arithmetic Mean	0.293	0.266	4.4	4.06	47.5	42.2
CV (%)	1.7	3.4	8.3	4.6	5.7	3.5
Variation	−9.2		−7.7		−11.2	

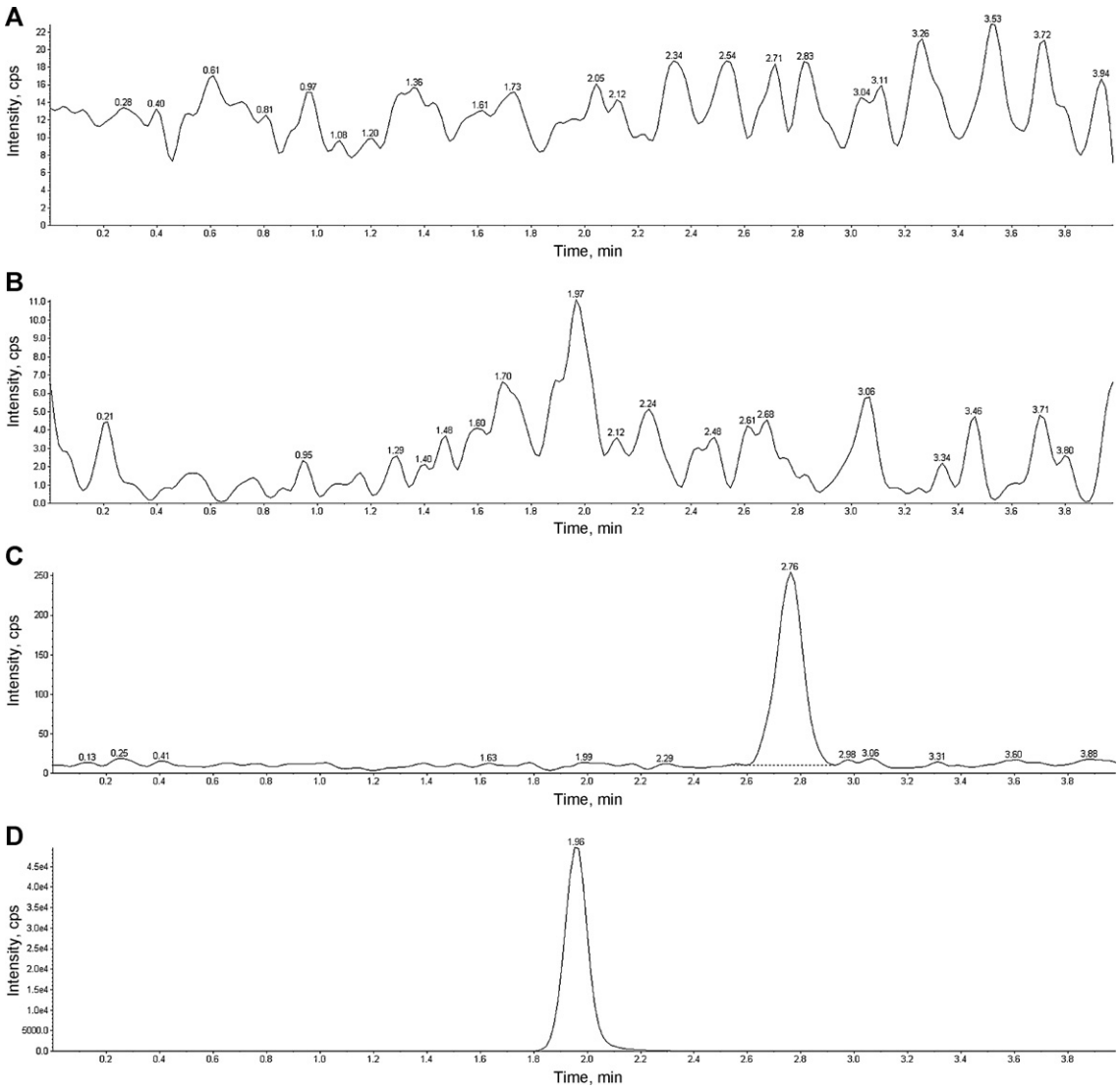

Fig. 2. MRM chromatograms of blank normal human plasma: ("A") ciprofibrate and ("B") bezafibrate; MRM chromatograms of the LOQ: ("C") ciprofibrate and ("D") bezafibrate.

(ciprofibrate) tablet 100 mg (standard reference formulation manufactured by Sanofi-Aventis Farmacêutica Ltda., lot N° L805879, expiration date 08/2011). Water (200 mL) was given immediately after the drug administration. All volunteers were required to remain fasting for 2 h after dose administration, when a xanthine-free standard breakfast was available. A xanthine-free standard lunch was provided after five (lunch), eight (afternoon snack), twelve (lunch) and fifteen (dinner) hours after dose. After the 10 h blood withdrawal a standardized meal (breakfast) was served, and volunteers were discharged following a medical evaluation. A standard meal (lunch) consisting of rice, beans, vegetables, and fried chicken plus a fruit as dessert was consumed. A morning, afternoon and evening snack was also provided including crackers, bread, jelly, cakes and apples. No other food was per-

Table 3
Mean pharmacokinetic parameters obtained from 22 volunteers after administration of reference and test ciprofibrate (100 mg) formulations.

Variable	Unit	*N*	Mean	SD	Min	Max	CV%
Reference							
AUC_{0-168}	([μg h]/mL)	22	840.15	147.13	481.62	1105.76	17.51
AUC_{last}	([μg h]/mL)	22	838.06	154.41	424.49	1108.66	18.42
C_{last}	(μg/mL)	22	2.05	0.61	0.89	3.53	29.46
C_{max}	(μg/mL)	22	22.35	4.64	16.00	32.30	20.75
T_{last}t	(h)	22	166.02	10.31	119.88	169.03	6.21
T_{max}	(h)	22	1.67	0.76	0.50	3.50	45.21
Test							
AUC_{0-168}	([μg h]/mL)	22	818.58	164.65	504.11	1246.60	20.11
AUC_{last}	([μg h]/mL)	22	819.20	164.82	504.02	1246.26	20.12
C_{last}	(μg/mL)	22	1.92	0.66	0.91	3.35	34.58
C_{max}	(μg/mL)	22	20.93	4.33	14.20	29.40	20.69
T_{last}	(h)	22	168.27	0.57	167.27	169.80	0.34
T_{max}	(h)	22	1.74	0.67	0.75	2.67	38.73

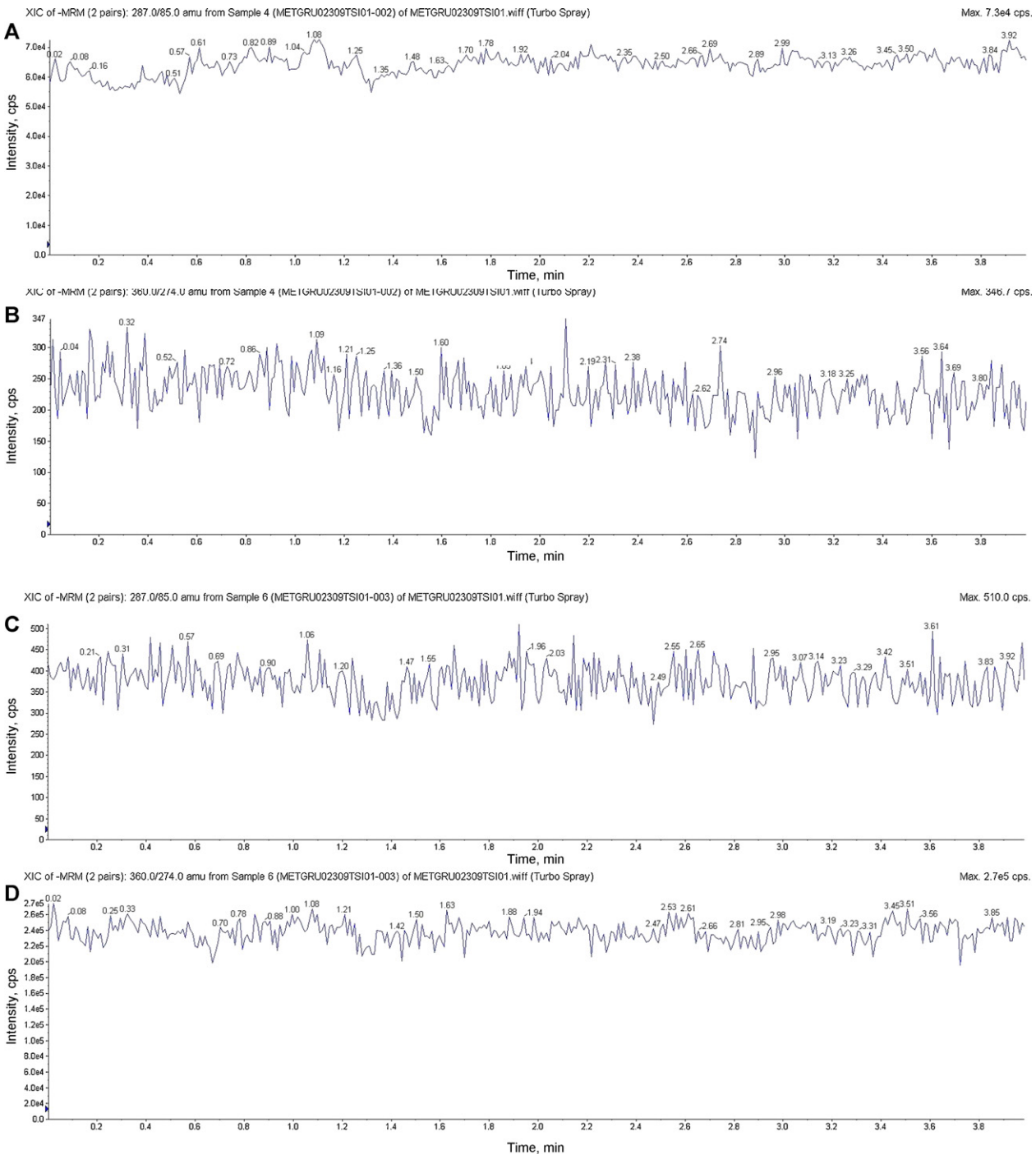

Fig. 3. Ion suppression procedure: (A) Ciprofibrate and (B) Bezafibrate sample injection.

mitted during the "in-house" period and liquid consumption was allowed *ad libitum* after lunch (with the exception of xanthine-containing drinks, including tea, coffee, and cola). At time intervals, systolic and diastolic arterial pressure (measured non-invasively with a sphygmomanometer), heart rate and temperature were recorded.

3. Pharmacokinetic and statistical analysis

Bioequivalence between the two formulations was assessed by calculating individual test/reference ratios for the peak of concentration (C_{max}), area under curve (AUC) of plasma concentration until the last concentration observed (AUC_{last}) and area under curve (AUC) of plasma concentration until 168 h ($AUC_{0-168\,h}$). The C_{max} and the time taken to achieve this concentration (T_{max}) were obtained directly from the curves. The areas under the ciprofibrate plasma concentration vs. time curves were calculated by applying the linear-log trapezoid rule. The AUC_S and C_{max} data for the two formulations were analyzed by ANOVA to establish whether the 90% CI of the ratios was within the 80–125% interval, indicating bioequivalence as proposed by the US Food and Drug Administration. The software used included WinNonlin Professional Network Edition (Pharsight v. 5.3), Microsoft Excel (v. 7.0) and GraphPad Prism (v. 3.02).

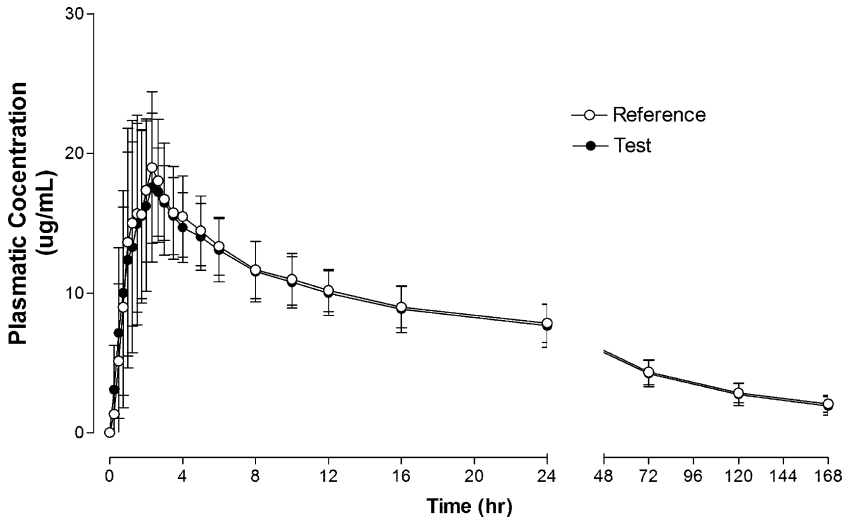

Fig. 4. Ciprofibrate plasma mean concentrations (SD) vs. time profile obtained after oral administration of 100 mg of ciprofibrate formulation.

4. Results and discussion

The calibration curve was linear over a range of 0.1–60 μg/mL, of ciprofibrate concentrations (calibration curve equation: $0.0655 \times x - 0.000448$, $r > 0.99$).

The recovery of ciprofibrate was 81.2%, 73.3% and 76.2% for the 0.3, 5.0 and 48.0 ng/mL standard concentrations, respectively. The recovery of internal standard was 94% for 5.0 μg/mL standard concentration. The limit of quantification (LOQ), defined as the lowest concentration at which both the precision and accuracy were <20%, was 0.1 μg/mL. The within- and between-run precision and accuracy (with and without internal standard) for the LOQ and QCs are summarized in Table 1. The stability tests indicated no significant degradation under the described conditions (Table 2).

No endogenous peak was observed in the mass chromatogram of blank plasma. The mass chromatograms of a sample are shown in Fig. 2, in which the retention times of both ciprofibrate and bezafibrate were 2.70 and 2.00, respectively. In the case of ciprofibrate and its internal standard, bezafibrate, there was no significant ion suppression in the region where the analyte and internal standard are eluted as shown in Fig. 3.

This is the first HPLC–MS/MS method developed for measuring ciprofibrate in human plasma. Ciprofibrate has been determined in plasma (LLOQ 0.25 μg/mL, RT 4.9 min, run-time 17 min) [1,6] and in pharmaceutical syrup (LLOQ 0.049 μg/mL, RT 4.9 min, run-time 16 min) [7,8] by high-performance liquid chromatography. Our method has good sensitivity (LLOQ of 0.1 μg/mL) and can be carried out in a short time (run time 3 min), permitting a high throughput. Furthermore, this method involves a very simple liquid–liquid extraction.

Internal standards are routinely used in bioanalytical methods and LC–MS-MS has enough selectivity to allow the use of deuterated standards. However, these standards are expensive and not easily available. Thus, they are commonly replaced by structurally related compounds. Considering that the extraction procedures for LC–MS-MS bioanalysis are very simple (protein precipitation is

Table 4
Geometric mean of the individual AUC_{last}, AUC_{inf}, and C_{max} ratios (test/reference), the respective 90% confidence intervals (CIs) and CVs.

Ciprofibrate/Oroxadin 100 mg ($n = 22$)	Ciprofibrate			
	Parametric analysis			
	Geom. mean	90% CI	Power	Intra-subject CV
C_{max}	93.80	88.16–99.79	0.9999	11.90%
AUC_{last}	98.31	94.91–101.83	1.0000	6.75%
$AUC_{0-168\,h}$	97.67	94.45–101.01	1.0000	6.44%
Ciprofibrate/Oroxadin 100 mg ($n = 8$)	Ciprofibrate – Males			
	Parametric analysis			
	Geom. mean	90% CI	Power	Intra-subject CV
C_{max}	101.02	90.56–112.69	0.9558	10.92%
AUC_{last}	100.70	96.54–105.04	0.9999	4.21%
$AUC_{0-168\,h}$	100.68	96.56–104.97	0.9999	4.16%
Ciprofibrate/Oroxadin100 mg ($n = 14$)	Ciprofibrate – Females			
	Parametric analysis			
	Geom. mean	90% CI	Power	Intra-subject CV
C_{max}	89.79	82.85–97.32	0.9959	11.99%
AUC_{last}	96.45	91.83–101.30	0.9999	7.29%
$AUC_{0-168\,h}$	95.57	91.26–100.09	0.9999	6.86%

routinely employed), it should be asked whether the use of a structurally related internal standard is really required or advisable. The method described above validated well (if not better) without the use of the internal standard. Whether this approach applies to other bioanalytical methods should be further investigated.

The ciprofibrate was well tolerated at the administered doses and no significant adverse reactions were observed or reported. A total of four adverse events were reported during the study, three of them were considered probably related to the administration of ciprofibrate (headache). The other adverse event was low hemoglobin (10.8 g/dL) in one female. The biochemical parameters presented no clinically relevant alterations.

The mean ciprofibrate plasma concentrations vs. time profiles after oral dose (100 mg) of ciprofibrate is shown in Fig. 4. Table 3 shows the mean pharmacokinetic parameters obtained from 22 volunteers after the administration of 100 mg ciprofibrate. Table 4 shows geometric mean of the individual C_{max}, AUC_{last} and AUC_{inf} (test/reference formulation), the respective 90% confidence intervals CI power, intra-subject CV for the 22 volunteers and by gender (male and female).

After oral administration of the ciprofibrate (100 mg) tablets to the volunteers, the peak plasma concentrations (C_{max}) and area under curve (AUC) of ciprofibrate were equivalent between the formulations (Table 4). In Table 4, we demonstrated that is possible bioequivalence using 8–14 volunteers and that there is no difference between genders was observed.

Since the 90% CI for C_{max}, AUC_{last} and $AUC_{0-168\,h}$ ratios were all within the 80–125% interval proposed by the US Food and Drug Administration Agency, it was concluded that ciprofibrate formulation (Lipless® 100 mg tablet) produced by Biolab Sanus Farmacêutica Ltda. is bioequivalent to Oroxadin® formulation for both the rate and the extent of absorption [9,10].

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