

HPLC Determination of Bezafibrate in Human Plasma and its Application to Pharmacokinetics Studies

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Abstract

An isocratic high-performance liquid chromatographic (HPLC) method was developed and validated for the determination of bezafibrate in biological fluids. Bezafibrate was separated on a C₁₈ analytical column (150 x 4.6 mm i.d., 5 μm particle size) with 0.01 M phosphate buffer (pH 3.5)–acetonitrile–methanol (50:40:10) as mobile phase at a flow rate of 1.0 mL/min. The UV detector was set to 230 nm. Bezafibrate was extracted from human plasma using a simple liquid–liquid extraction with tert-butyl methyl ether. Parameters such as linearity, precision, accuracy, recovery, specificity, and stability were evaluated by method validation studies. All the parameters remained within acceptable limits. The validated procedure was linear in the concentration range of 0.2–50 μg/mL. The proposed method used for individual drug determinations is applicable for therapeutic monitoring purposes as well as for use in pharmacokinetic investigations. As an example, the practical quantification limit for bezafibrate in plasma was about 0.05 μg/mL with precision of 10.2% and accuracy of 112.6%. The method was applied in a study of the pharmacokinetics of bezafibrate in six healthy volunteers, who ingested a single oral dose of 200 mg.

Introduction

Epidemiological studies demonstrate that dyslipidemia is closely associated with a high incidence of atherosclerosis and cardiovascular diseases (1). Bezafibrate (BEZ), 2-[4-[2-[(4-chlorobenzoyl)amino]ethyl]phenoxy]-2-methylpropanoic acid (Figure 1) is a representative fibrate that powerfully decreases plasma triglyceride levels and increases HDL-C levels, reducing significantly the incidence of cardiovascular diseases (2).

Some analytical procedures have been reported to evaluate this fibrate in pharmaceutical products by capillary electrophoresis (3), colorimetric spectrophotometry (4), UV spectrophotometry (5), and high-performance liquid chromatography (HPLC) (6–7). Analytical techniques using HPLC have been presented for the determination of bezafibrate in human plasma (8) and urine (9). Pharmacokinetic studies of

immediate and modified-release formulations of bezafibrate have been carried out (10–13).

There has been no validated method for the determination of bezafibrate both in pharmaceutical formulations and biological fluids so far. However, our research group published a recent study reporting a chromatographic method validated initially for the evaluation of bezafibrate in pharmaceuticals (7), which was developed to be extended for the determination of the drug in biological fluids. The present paper describes the achievement of this aim and reports a validated procedure that can be applied in both formulations and biological fluids.

For analytical methods applied to biological fluids, sample preparation is a difficult and arduous step and can become a limiting stage of the procedure. Therefore, a simple sample pretreatment is advisable because pharmacokinetic and bioequivalence studies require numerous samples for analysis. Important factors in the development of the procedure include processing sample time, solvent cost, equipments needs, and sample stability. These aspects were not fully observed by the authors of the validated method for the determination of bezafibrate in human plasma (8), which employed a double extraction using 5 mL of diethyl ether followed by centrifugation and evaporation under nitrogen flow.

The method application is important to ensure the reproduction of the validated procedure when plasma samples obtained from healthy volunteers treated with a single dose of bezafibrate tablets were used in the assay. The validated procedure reported in literature (8) shows no application using volunteers.

The present work reports the development and validation of a method that can be applied for the determination of bezafibrate in human plasma from healthy volunteers and the evaluation of the drug in pharmaceutical products (tablets and compounded capsules).

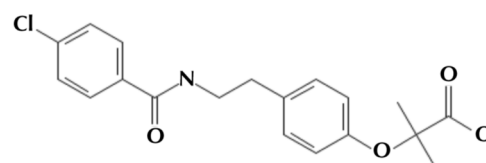


Figure 1. Chemical structure of bezafibrate.

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Experimental

Chemicals

Bezafibrate reference standard was supplied by Roche Diagnostics (Mannheim, Germany) and certified to contain 99.4%. Cedur tablets (Roche, São Paulo, Brazil) containing 200 mg of bezafibrate were obtained from commercial sources within their shelf life period.

All solvents were HPLC-grade, and all reagents were of analytical-grade. Acetonitrile and methanol were obtained from Tedia (Fairfield, OH). Potassium dihydrogen phosphate and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Phosphoric acid was purchased from Quimex (São Paulo, Brazil) and *tert*-butyl methyl ether from Vetec (Rio de Janeiro, Brazil). Water was purified with Milli-Q Plus (Millipore, Billerica, MA). All solvents and solutions were filtered through a membrane filter or filtration units (Millipore Millex-HV filter units, 0.22- μ m pore size) and degassed before use.

Instrumentation and analytical conditions

The HPLC method for the determination of bezafibrate in biological fluids was performed on a Shimadzu LC-10AD HPLC system (Kyoto, Japan) equipped with diode-array detector model SPD-M10Avp. Data integration was performed using Shimadzu Class-VP software. The analytical column was a reversed phase Rexchrom Regis ODS (150 \times 4.60 mm i.d, 5 μ m particle size) from Regis (Morton Grove, IL). All analyses were done at room temperature (24 \pm 2°C) under isocratic conditions. The mobile phase consisted of a mixture of 0.01 M phosphate buffer (pH 3.5, adjusted with phosphoric acid)–acetonitrile–methanol (50:40:10, v/v/v). The flow rate was 1.0 mL/min, and the volume of injection was 50 μ L for fluids. The UV detection was made at 230 nm.

Solution preparations

Reference standard

A 20 mg portion of bezafibrate reference standard (99.4%) accurately weighed was transferred to a 20-mL volumetric flask and dissolved in methanol (final concentration 1 mg/mL).

Biological fluids

20 μ L of ezetimibe (internal standard, initial concentration of 250 μ g/mL) and 200 μ L of 0.1 N hydrochloric acid were added into eppendorf tubes containing 200 μ L of plasma-spiked aliquots. The samples were agitated for 1 min, and then 1.5 mL of *tert*-butyl methyl ether was added into the tubes. After this addition, the samples were vortex-mixed for 1 min and centrifuged at 10,000 rpm for 10 min. 1.2 mL of the supernatant was separated and evaporated to dryness under a nitrogen stream at 37°C. The residue was reconstituted with 300 μ L of a mixture of acetonitrile–water (50:50, v/v).

Method validation

The proposed bioanalytical method was validated according to the Food and Drug Administration guidelines (14). Analysis of variance (ANOVA) was used to verify the validity of the assays.

Linearity

The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method, and the curves were prepared in three different days. For biological fluids, the calibration curve was obtained with six concentrations of plasma (0.2, 0.5, 1, 10, 25, 50 μ g/mL) spiked with reference standard solution.

Precision

The precision of the procedures was determined by repeatability (intra-day) and intermediate precision (inter-day). Intra-day precision was evaluated by assaying samples at the same concentration and during the same day. The intermediate precision was analyzed by comparing the assays in three different days.

Five samples of each control concentration (0.25, 20, and 40 μ g/mL) were prepared and assayed for the evaluation of the precision in biological fluids.

Accuracy and recovery

The accuracy was determined by assaying three concentrations (0.25, 20, and 40 μ g/mL) in triplicate. The recovery was calculated for the procedure applied to biological fluids by comparing the peak areas obtained from plasma samples with detector responses achieved from suitable amounts of stock standard solutions (0.25, 20, and 40 μ g/mL).

Specificity and selectivity

Plasma samples from six different sources including fatty and hemolytic human plasma were evaluated for the determination of selectivity in fluids.

Stability

Bezafibrate stability in human plasma was evaluated after three freeze (20°C)–thaw (room temperature) cycles. Post-preparative stability was determined by assaying the processed samples, which remained for 6 h in the autosampler. The stability of stock standard solutions of bezafibrate and ezetimibe was evaluated at room temperature for 6 h. Short-term temperature stability was analyzed by thawing the plasma samples at room temperature and keeping the samples at this temperature for 4 h. Finally, the long-term stability was determined by assaying the stored samples at freezing temperature (–20°C) during 1, 2, and 3 months. The stability of bezafibrate in human plasma was evaluated by comparing to fresh samples. The samples were considered stable if the deviation (expressed as percentage bias) from the freshly samples stayed within + 15%.

Sensitivity

The limit of detection (LOD) was evaluated based on the ratio of signal-to-noise (3:1). The lower limit of quantification (LLOQ) for biological samples was determined experimentally by verifying the analyte concentration that causes a response with precision of 20% and accuracy of 80–120%.

Method application

Volunteer selection

Six healthy volunteers (males and females, mean age of 26.5 \pm 8.3 years) interested in participating in the pharmacokinetic

study were selected according to preset inclusion and exclusion criteria. Therefore, we selected volunteers who were healthy and had no history of heart, kidneys, neurological or metabolic diseases, no history of drug hypersensitivity, were not undergoing any pharmacological treatment, and female volunteers who were not pregnant. The subjects gave written informed consent to participate in the study after oral explanation about the experimental procedure. The clinical protocol was submitted to the appreciation and approval of the Research Ethics Committee of Federal University of Santa Maria.

Bezafibrate tablet administration

The tablets containing 200 mg of bezafibrate were administered to the volunteers in the morning after an overnight fast with 200 mL of water. Volunteers received standard lunch and afternoon snacks, respectively, 5 and 8 h after drug administration.

Acquisition of human blood samples from volunteers

The puncture was performed using an intravenous catheter, and the samples were separated into heparinized vacutainer tubes before tablet administration and in established times after drug administration (time zero/pre-dose, 0.5, 1.0, 1.5, 2.0, 2.5, 4.0, and 8.0 h). The blood samples were centrifuged at 6000 rpm for 10 min to separate plasma, which was kept frozen at -20°C until analyses.

Determination of pharmacokinetic parameters

A concentration time curve was plotted and the area under curve (AUC) was calculated by trapezoidal rule. The maximum plasma concentration (C_{max}) and the time to reach the max-

imum concentration (t_{max}) were obtained directly using Excel software (Microsoft, Redmond, WA). The elimination rate constant (k_{el}) was determined by linear regression of the last three data points, log transformed. Elimination half-time ($t_{1/2}$) was calculated using the formula $t_{1/2} = 0.693/k_{\text{el}}$. Calibration standards of 0.2, 0.5, 1.0, 10.0, 25.0, and 50.0 $\mu\text{g/mL}$ and quality-control samples of 0.25, 20.0, and 40.0 $\mu\text{g/mL}$ were prepared by spiking blank human plasma with standard solutions of bezafibrate. The HPLC injection sequence was as follows: calibration standards, volunteers' plasma samples (in duplicate), and quality-control samples throughout all sequence (in triplicate). The accuracy and precision of the validated method was monitored to ensure that it continued to perform satisfactorily during analysis of volunteer samples. To achieve this objective, a number of QC samples prepared in duplicate at three levels of concentration were analyzed in each assay run and reported at the calibration curve of the day.

Results and Discussion

Method development

The chromatographic conditions were adjusted in order to provide a good assay performance. Mobile phase selection was based on peak parameters (tailing, resolution) and run time. A typical chromatogram obtained from the analysis of a human plasma sample using the proposed method is shown in Figure 2. Bezafibrate is represented by a symmetrical peak, well-separated from the internal standard and plasma interference. The retention time observed in the assay (5.2 min) associated with the simple sample preparation allowed a rapid determination of the drug in fluids.

It is important to point out that the chromatographic conditions are the same settings employed in the reported method for the determination of bezafibrate in pharmaceutical formulations (7). Thus, we can observe that the present procedure can be used to evaluate bezafibrate both in formulations and fluids.

An exhaustive technique of sample preparation was previously reported for the determination of bezafibrate in human plasma (8). The samples were extracted twice with 5 mL of diethyl ether for 20 min followed by centrifugation for 15 min. Afterwards, the ether phases were combined and evaporated under nitrogen flow. Excessive time, large quantities of solvents and reagents consumed, and use of expensive equipment were negative aspects of this method.

Method validation

Linearity

The calibration curves of the bioanalytical method were assessed by plotting concentration versus peak area and showed suitable linearity in the 0.2–50 $\mu\text{g/mL}$ range. The representative linear equation was $y = 0.073x - 0.0216$ ($r = 0.9991$). The calibration curves were validated by ANOVA, which indicated significant linear regression and no significant deviation from linearity ($p < 0.05$).

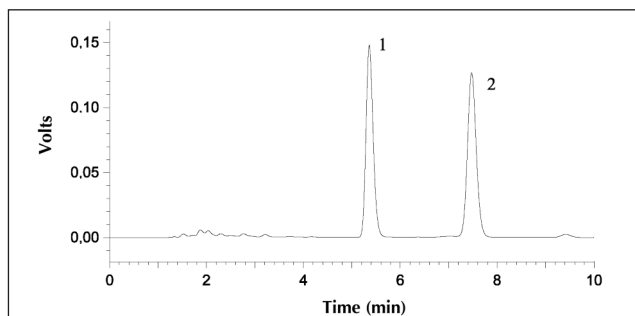


Figure 2. Chromatogram obtained from human plasma containing 20 $\mu\text{g/mL}$ bezafibrate (Peak 1) and 10 $\mu\text{g/mL}$ ezetimibe-IS (Peak 2). Conditions: Rexchrom Regis ODS column (150 \times 4.60 mm i.d, 5 μm), 0.01 M phosphate buffer (pH 3.5)–acetonitrile–methanol (50:40:10, v/v/v), flow rate 1.0 mL/min and UV detection 230 nm.

Table I. Precision and Accuracy for Determination of Bezafibrate in Biological Fluids

Theoretical ($\mu\text{g/mL}$)	Experimental				Accuracy (%)	Inter-day RSD (%)	Intra-day RSD (%)
	Day 1*	Day 2*	Day 3*	Mean			
0.25	0.27 \pm 4.1%	0.26 \pm 7.7%	0.25 \pm 5.4%	0.26	103.8	6.69	3.11
20	20.45 \pm 6.7%	19.40 \pm 5.8%	20.02 \pm 3.1%	19.96	99.80	5.48	6.68
40	42.80 \pm 4.9%	43.05 \pm 6.7%	42.35 \pm 4.4%	42.73	106.8	5.10	7.74

* Mean of five determinations of each concentration (Mean + RSD%).

Precision and accuracy

Table I shows the precision and accuracy obtained for biological fluids with quality controls (CQA, CQM, and CQB). The intra-day precision of the bioanalytical method ranged from 3.11 to 7.74% while the inter-day precision ranged from 5.10 to 6.69%. The accuracy of the method applied to the fluids ranged from 99.80 to 106.8%. The average recovery of the method was 81.61% (Table II).

Bezafibrate concentration ($\mu\text{g/mL}$)		Recovery (%)	RSD (%)
Nominal	Experimental*		
0.25	0.21	82.90	8.60
20	15.46	77.18	2.30
40	33.90	84.76	5.10
Mean		81.61	5.33

*Mean of three determinations of each concentration.

Stability	Freshly prepared sample conc. ($\mu\text{g/mL}$)*	Measured conc. ($\mu\text{g/mL}$)*	RSD (%)	Bias [†] (%)
<i>Three freeze-thaw cycles</i>				
	0.28	0.24	4.33	-14.29
	21.20	19.66	4.16	-7.26
	40.82	43.66	6.29	6.96
<i>Autosampler for 6 h</i>				
	0.28	0.29	6.79	3.57
	21.20	20.95	10.07	-1.18
	40.82	37.00	7.83	-9.36
<i>Stock solutions for 6 h</i>				
	0.28	0.25	8.45	-10.35
	21.20	18.17	9.44	-14.25
	40.82	38.68	9.99	-5.24
<i>Short-term (4 h)</i>				
	0.28	0.27	6.15	-3.57
	21.20	21.38	1.69	0.85
	40.82	43.99	7.13	7.77
<i>Long-term (Three months)</i>				
	0.28	0.27	5.94	-3.57
	21.20	20.51	8.09	-3.25
	40.82	43.64	1.30	6.91

* Mean of three determinations.
[†] Bias = (measured conc. - fresh sample conc. / fresh sample conc.) \times 100.

Pharmacokinetic parameter	Experimental (Mean \pm SD)
$\text{AUC}_{(0-t)}$ ($\mu\text{g h/mL}$)	13.76 \pm 2.47
$\text{AUC}_{(0-\infty)}$ ($\mu\text{g h/mL}$)	16.35 \pm 3.37
C_{max} ($\mu\text{g/mL}$)	4.51 \pm 1.02
t_{max} (h)	1.33 \pm 0.52
k_{el} (h^{-1})	0.50 \pm 0.10
$t_{1/2}$ (h)	1.44 \pm 0.36

Specificity, sensitivity, and stability

No significant interference from endogenous substances was detected in human plasma, including fatty and hemolytic samples.

As shown in the Table III, bezafibrate was stable in plasma for 4 h at room temperature (short-term) and after three freeze-thaw cycles, demonstrating that human plasma samples could be thawed and refrozen without interference to the analysis. Plasma samples of bezafibrate were stable for three months at -20°C (long-term) and processed samples remained stable for at least 6 h when kept in the autosampler.

Sensitivity

LOD and LLOQ for fluids were evaluated experimentally. The LOD found was $0.01 \mu\text{g/mL}$. The LLOQ was $0.05 \mu\text{g/mL}$ with precision of 10.25% and accuracy of 112.6%.

Method application

Determination of pharmacokinetics parameters

The developed method was applied to investigate the pharmacokinetics of bezafibrate. Figure 3 shows the concentration versus time curve obtained from six healthy volunteers after single oral dose of tablets containing 200 mg of bezafibrate. The parameters t_{max} , k_{el} , and $t_{1/2}$ (Table IV), generally characteristic of the drug, were similar to those reported by the method of Gandini et al. and Ali et al. (11,13). However, the values of C_{max} and AUC (Table IV), related to the formulation, were higher than reported (10,12,13). The differences between the parameters found can be explained by the use of experiments in different conditions, such as administration of two tablets each containing 200 mg (10) or tablets of 300 mg (12) and trials using multiple doses (11). Moreover, the tablets administered in the tests were produced by industries and different countries (13). The values obtained for plasma decay half-life in the present study were 1.44 h and t_{max} values were 1.33 h, which were similar to other authors' reports (10–13).

Conclusion

The proposed HPLC method enables a quantitative determination of bezafibrate in human plasma. The application of this method in routine analysis can be justified because fast sample preparation and simple reagents and solvents were used experi-

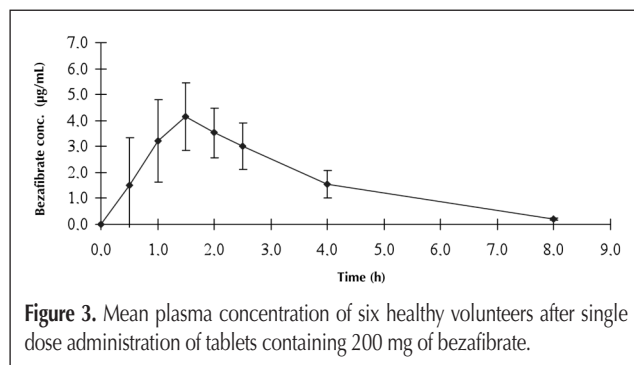


Figure 3. Mean plasma concentration of six healthy volunteers after single dose administration of tablets containing 200 mg of bezafibrate.

mentally. The validation demonstrated that this procedure is suitable for the intended purpose because the method was considered linear, precise, accurate, and specific. Besides, the method used for individual drug determinations is applicable for therapeutic monitoring purposes as well as for use in pharmacokinetic investigations. As an example, the practical quantification limit for bezafibrate in plasma was about 0.05 µg/mL with precision of 10.25% and accuracy of 112.6%, which is five times lower when compared to values previously reported, and LLOQ was 0.25 µg/mL (8). The method was applied in a study of the pharmacokinetics of bezafibrate in six healthy volunteers, who ingested a single oral dose of 200 mg.

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