HPLC Method for the Determination of Bezafibrate in Human Plasma and Application to a Pharmacokinetic Study of Bezafibrate Dispersible Tablet

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Abstract

A sensitive and selective high-performance liquid chromatographic–UV (HPLC–UV) method for the determination of bezafibrate in human plasma has been developed. Sample treatment was based on protein precipitation with a perchloric acid–methanol solution 10:90 (v/v). Analytical determination was carried out by HPLC with UV detection at 235 nm. Chromatographic separation was achieved on a C18 column by isocratic elution with acetonitrile–ammonium acetate aqueous solution (10 mmol/L; pH 4.0) (44:56, v/v) at a flow rate of 1.0 mL/min. The method was linear in the concentration range of 0.1–15.0 μg/mL. The lower limit of quantitation was 0.1 μg/mL. The intra-and inter-day relative standard deviation across three validation runs over the entire concentration range was less than 6.96%. The accuracy determined at three concentrations (0.2, 2.0, and 10.0 μg/mL for bezafibrate) was within ± 10.0% in terms of accuracy. The method was successfully applied for the evaluation of pharmacokinetic profiles of bezafibrate dispersible tablet in 20 healthy volunteers. The results show that AUC, C_{max}, and $T_{1/2}$ between the testing formulation and reference formulation have no significant difference ($P > 0.05$). Relative bioavailability was 105.0 ± 15.7%.

Introduction

Bezafibrate, 2-[2-(p-[2-chlorobenzamido] ethyl] phenoxy] 2-methylpropionic acid (Figure l), is a fibric acid derivate effective at decreasing the triglyceride and very low density lipoprotein (VLDL) triglyceride plasma levels and increasing high-density lipoprotein (HDL) cholesterol levels, and it also enhances anti-coagulation and reduces plasma viscosity and fibrinogen levels (1,2). Moreover, in patients with moderately controlled non-insulin-dependent diabetes mellitus, treatment with bezafibrate improves glucose tolerance (3). It has recently been suggested that dyslipidemic renal patients might be treated with bezafibrate only if facilities to monitor blood concentrations are available (4–6).

Several high-performance liquid chromatography (HPLC) methods to assess separately bezafibrate plasma levels with a LLOQ of 0.25 μg/mL have been reported (7,8). However, those sample preparation (7,8) can be very time-consuming and need an extraction solvent that increases the cost besides probably being harmful to the environment. Recently, a liquid chromatography–mass spectrometry (LC–MS) method was developed to assay bezafibrate (9,10). But the necessity of the LC–MS system was a restriction in terms of cost and general applicability. In this paper, a more simple, selective, and highly sensitive method by using high-performance liquid chromatograpy (HPLC) with UV detector for the determination of bezafibrate in human plasma is described. The total analysis time before injecting a new sample in the presented new method was 10 min. This was convenient and critical to deal with a large amount of samples. The lower limit of quantitation (LLOQ) of the new method was 0.1 μg/mL

This is sensitive enough for application to a pharmacokinetic study under a low clinic dosage (200 mg).

Experimental

Materials

Bezafibrate (99.8% purity) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile (HPLC-grade) and methanol (HPLC-grade) were purchased from Merck Company. Acetic acid (analytical grade), perchloric acid (analytical grade), trichloroacetic acid (analytical grade), and ammonium acetate (analytical grade) were from Nanjing Chemical Co. (Nanjing, China). Heparinized blank (drug-free) human plasma was
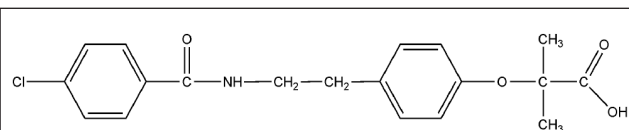

Figure 1. Structures of bezafibrate.

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obtained from Nanjing Blood Donor Service (Nanjing, China). Distilled water, doubly distilled in the laboratory, was used throughout the study.

Instrumentation

The chromatographic system used consisted of the LC-10AT vp HPLC (Shimadzu, Beijing, China) system with SPD-10A vp UV-vis detector. Data acquisition was performed with the N2000 chromatography data system (Zhejiang University Star Instrument Technology Co. Ltd., Hangzhou, China)

HPLC–UV conditions

The chromatographic separation was achieved on a Diamonsil C18 column (250 × 4.6 mm i.d., 5 µm, Dikma, Beijing, China) and a shim-pack GVp-ODS guard column (1.0 × 4.6 mm, Shimadzu). The mobile phase was acetonitrile–water containing 10-mmol/L ammonium acetate, pH 4.0 (adjusted with acetic acid) (44:56, v/v) at a flow rate of 1.0 mL/min. The column temperature was maintained at 30°C. The wavelength of detector was set at 235 nm.

Sample preparation

The plasma was prepared by a protein precipitation method. In a 1.5 mL centrifuge tube an aliquot of 0.2 mL of human plasma was spiked with 200 µL of a perchloric acid–methanol 10:90 (v/v) solution. The tubes were vortex mixed for 3 min. After centrifugation at 16880 g for 10 min, 20 µL of the clear supernatant fluid was injected into the HPLC–UV system for analysis.

Preparation of standard and quality control samples

Stock solution of bezafibrate was prepared in methanol at the concentration of 1000 µg/mL. Working solutions of bezafibrate were prepared daily in methanol by appropriate dilution at 100.0, 10.0, and 1.0 µg/mL. Calibration curves were prepared by spiking the appropriate standard solution to 0.2 mL of blank plasma. Effective concentrations in plasma samples were 0.1, 0.2, 0.5, 1, 2, 5, 10, 15 µg/mL for bezafibrate. The quality control (QC) samples were separately prepared in blank plasma at the concentrations of 0.2, 2.0, and 10.0 µg/mL, respectively. The spiked plasma samples (standards and quality controls) were then treated following the "Sample preparation" procedure on each analytical batch along with the unknown samples.

Method validation

Plasma samples were quantitated using the peak area of bezafibrate. As the assay parameter, peak areas were plotted against bezafibrate concentrations and standard curves were in the form of $y = A + Bx$.

To evaluate linearity, plasma calibration curves were prepared and assayed in duplicate on five separate days. The accuracy and precision were also assessed by determining QC samples at three concentration levels on three different validation days. The accuracy was expressed by (mean observed concentration)/(spiked concentration) × 100% and the precision by relative standard deviation (RSD%).

Absolute recoveries of bezafibrate at three QC levels were determined by assaying the samples as described earlier and comparing the peak areas of bezafibrate with those obtained from direct injection of the compounds dissolved in the supernatant of the processed blank plasma.

The stability of the stock solution of bezafibrate was determined by placing the stock solution in the refrigerator (4°C) for a week. Bezafibrate stability in plasma was assessed by analyzing QC samples at concentrations of 0.2, 2.0, and 10.0 µg/mL, respectively, exposed to different time and temperature conditions. The long-term stability was assessed after storage of the test samples at –20°C for 14 days. The freeze-thaw stability was determined after five freeze-thaw cycles (–20 to 20°C) on consecutive days. The results were compared with those QC samples freshly prepared, and the percentage concentration deviation was calculated.
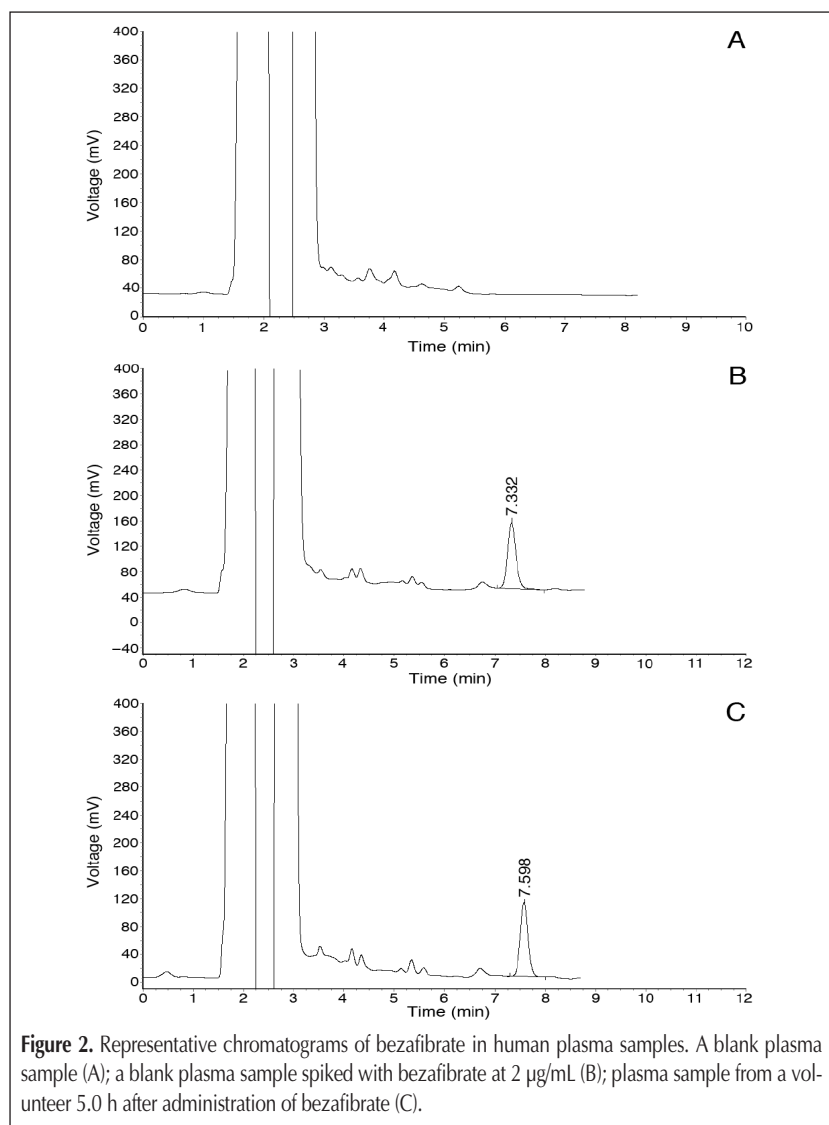

Figure 2. Representative chromatograms of bezafibrate in human plasma samples. A blank plasma sample (A); a blank plasma sample spiked with bezafibrate at 2 µg/mL (B); plasma sample from a volunteer 5.0 h after administration of bezafibrate (C).

Pharmacokinetic study

To demonstrate the reliability of this method for the study of pharmacokinetics, it was applied to determine the plasma concentrations of bezafibrate in which 20 healthy Chinese male volunteers (between 18 to 25 years old) received an oral dosage tablet (containing 200 mg bezafibrate). The pharmacokinetic study was approved by the Ethics Committee of the Institute of Dermatology, Chinese Academy of Medical Science. All volunteers gave written informed consent to participate in the study according to the principles of the Declaration of Helsinki (1964) in the revised version of 1996 (Somerset West). Serial blood samples (4 mL) from a suitable antecubital vein were collected into sodium heparin-containing tubes before and 0.33, 0.67, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, and 12 h after the administration of bezafibrate. Plasma was separated by centrifugation at $2000 \times g$ for 10 min and stored frozen at –20°C until analysis.

Pharmacokinetic parameters were determined from the plasma concentration-time data. To compare the two formulations, an analysis of variance was performed on the appropriate log-transformed and non-transformed PK parameters using BAPP2 procedures.

Results and Discussion

Chromatography

Bezafibrate is difficult to retain on the C18 column. When 0.01 mol/L ammonium acetate, pH 4.0 (adjusted with acetic acid) was added into the mobile phase, the retention time of bezafibrate was markedly delayed because the acidic solutions avoid deprotonation of the acidic compounds and, consequently increase retention of these compounds in the non-polar stationary phase. Under these optimum chromatographic conditions, the symmetry of the peak was good while the analyte was free of interference from endogenous substances.

Preparation of plasma samples

Protein precipitation was necessary and important because it purified the sample. Acetonitrile, methanol, 10% trichloroacetic acid, and perchloric acid solution of methanol were tested for extraction. The recoveries of bezafibrate (0.2 µg/mL) were 62.13%, 54.69%, 75.41%, and 92.22%, respectively. Finally, 10% perchloric acid solution of methanol was adopted because of its high extraction efficiency.
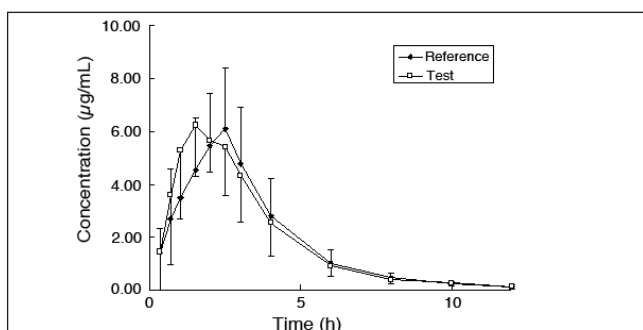

Figure 3. Mean plasma concentration-time curve of bezafibrate after a single oral dose of bezafibrate in 20 volunteers.

Method validation

Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Figure 2 shows the typical chromatograms of a blank, spiked plasma sample with bezafibrate (2.0 µg/mL), and plasma sample from a healthy volunteer 4.0 h after an oral administration. There was no significant interference from endogenous substances observed at the retention times of the analytes. Typical retention times for bezafibrate were approximately 7.46 ± 0.13 min.

Linearity of calibration curves and LLOQ

Inspection of the plotted duplicate calibration curves and correlation coefficients > 0.999 confirmed that the calibration curves were linear over the concentration ranges 0.1–15.0 µg/mL for the analyte. Typical standard curve was f = 1.79E–06Ci + 0.025. Where f represents the peak area of bezafibrate and Ci represents the plasma concentrations of bezafibrate (µg/mL).

The lower limit of quantitation was defined as the lowest concentration on the calibration curve for which an acceptable accuracy of ± 15% and a precision below 15% were obtained. The present LC–UV method offered an LLOQ of 0.1 µg/mL in 0.2 mL plasma sample. Under present LLOQ of 0.1 µg/mL, the bezafibrate concentration can be determined in plasma samples until 12 h after a single oral dose of 200 mg bezafibrate, which is sensitive enough to investigate the pharmacokinetic behaviors of bezafibrate and to establish the relationship between dose and pharmacological effect in human.

Precision and accuracy

Table I summarizes the intra-and inter-day precision and accuracy for bezafibrate evaluated by assaying the QC samples. The precision was calculated by using one-way ANOVA (11). In this assay, the intra-run precision was 6.96% or less, and the inter-run precision was 6.66% or less for each QC level of bezafibrate. The accuracy was within ± 10.0%. The results described demonstrate that the values were within the acceptable range and the method was accurate and precise.

Recovery and stability

The recovery of bezafibrate, determined at three concentrations (0.2, 2.0, and 10.0 µg/mL), were 92.22 ± 6.68%, 89.12 ± 5.42%, and 94.39 ± 3.02% (n = 5), respectively.

The results of stability experiments showed that no significant degradation occurred at –20°C for 14 days and after five freeze-

Table I. Accuracy and Precision for the Analysis of Bezafibrate in Human Plasma (in Pre-study Validation, n = 3 Days, Five Replicates per day)

Added C (µg/mL)	Found C (µg/mL)	Intra-run RSD (%)	Inter-run RSD (%)	Accuracy (%)
0.2	0.19	6.66	6.96	–7.5
2.0	2.08	5.75	6.10	4.0
10.0	9.74	4.92	5.97	–2.6

Table II. Pharmacokinetic Parameters for 20 Volunteers After Administration of a Single Dose of Bezafibrate

Parameter	Reference formulation	Test formulation
$T_{1/2/h}$	1.97 ± 0.34	2.09 ± 0.47
C_{max}/µg·mL^{-1}	7.39 ± 1.47	7.67 ± 1.19
T_{max}/h	2.0 ± 0.6	1.5 ± 0.7
$AUC_{0\rightarrow 12h}$/µg·h·mL^{-1}	22.30 ± 5.40	22.98 ± 4.78
$AUC_{0\rightarrow \infty}$/µg·h·mL^{-1}	22.74 ± 5.45	23.48 ± 4.78

thaw cycles for bezafibrate plasma samples. The accuracy values of low (0.2 µg/mL), medium (2.0 µg/mL), and high (10.0 µg/mL) concentrations of bezafibrate in human plasma were 98.71%, 97.73%, and 94.52% after five freeze-thaw cycles, and 95.16%, 96.17%, and 98.63% at –20°C for 14 days. The stock solution of bezafibrate in methanol was stable at 4°C for a week.

Application of the method to a pharmacokinetic study in healthy volunteers

The method was applied to determine the plasma concentration of bezafibrate after an oral administration of reference formulation and test formulation of bezafibrate to 20 volunteers. The mean plasma concentration-time curve of bezafibrate is shown in Figure 3. The main pharmacokinetic parameters of bezafibrate in 20 volunteers are shown in Table II. The known disintegration time of the dispersible tablet is shorter than a normal tablet. Thus the T_{max} of test formulation is short than reference formulation which is general tablet.

The relative bioavailability of the test formulation was (105.0 ± 15.7)%, and there were no remarkable differences between test formulation and reference formulation.

Conclusions

The proposed method of analysis provided a sensitive and specific assay for bezafibrate determination in human plasma. Simple protein precipitation extraction procedure and short run time can increase sample throughput that is important for large sample batches. It was shown that this method is suitable for the analysis of bezafibrate in human plasma samples collected for pharmacokinetic, bioavailability, or bioequivalence studies in humans.

References

1. J.P. Monk and P.A. Todd. Bezafibrate: a review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in hyperlipidemia. *Drugs*. **33:** 539–76 (1987)
2. M. Leschke, M. Podder, J. Jehle, and E. Strauer. Influence of long-term bezafibrate therapy on lipid parameters, fibrinogen and plasma viscosity. In: E. Ernst, W. Koening, G.D.O. Lowe, T.W. Meade TW, Eds. *Fibrinogen: A "New" Cardiovascular Risk Factor*. Vienna: Blackwell MTV, 1993, pp. 380–83.
3. I.R. Jones, A. Swai, R. Taylor, M. Miller, M.F. Laker, and K.G. Alberti. Lowering of plasma glucose concentrations with bezafibrate in patients with moderately controlled NIDDM. *Diabetes Care* **13:** 855–63 (1990)
4. T. Ushiroyama, K. Sakuma, and S. Nosaka. Effects of bezafibrate on HDL2/HDL3 ratio in postmenopausal hypertriglyceridemic women. *J. Cardiovasc. Pharmacol. Ther.* **11:** 142–48 (2006)
5. K. Ohmoto, N. Yoshioka, and S. Yamamoto. Long-term effect of bezafibrate on parameters of hepatic fibrosis in primary biliary cirrhosis. *J. Gastroenterol.* **41:** 502–503 (2006)
6. O. Ali, M.A. Khan, R. Obaid and S.W. Ahmed. Bioavailability study of tablet Bezafibrate 200 mg (Lipocor). *Pak. J. Pharm. Sci.* **15:** 35–42 (2002)
7. D. Castoldi, V. Monzani, and O. Tofanetti. Determination of bezafibrate in human plasma and urine by high-performance liquid chromatography. *J. Chromatogr.* **344:** 259–65 (1985)
8. D.M. Lucas, A.C. Luis, H.R. Ricardo, and P.W. Jose. Determination of bezafibrate, ciprofibrate and fenofibric acid in human plasma by high-performance liquid chromatography. *J. Chromatogr.* **687:** 437–42 (1996)
9. M. Farré, M. Petrovic, and D. Barceló. Recently developed GC/MS and LC/MS methods for determining NSAIDs in water samples. *Anal. Bioanal. Chem.* **387:** 1203–14 (2007).
10. M. Gros, M. Petrovic, and D. Barceló. Multi-residue analytical methods using LC-tandem MS for the determination of pharmaceuticals in environmental and wastewater samples: a review. *Anal. Bioanal. Chem.* **386:** 941–52 (2006).
11. D.G. Kleinbaum, L.L. Kupper, and A. Nizam. *Applied Regression Analysis and Other Multivariable Methods,* 3rd ed. K. E. Muller, Ed. Duxbury Press, Pacific Grove, USA, 1998, pp. 434–90.