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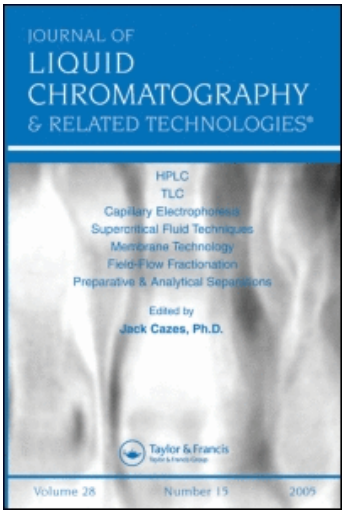

Journal of Liquid Chromatography & Related Technologies

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To cite this Article de Melo, Janine , Hurtado, Felipe Kellermann , Prado, Anelise Weich , Souza, Marinês Jost e. and Rolim, Clarice Madalena Bueno(2008) 'Validation of HPLC and UV Spectrophotometric Methods for the Determination of Bezafibrate in Pharmaceutical Formulations', Journal of Liquid Chromatography & Related Technologies, 31: 2, 269 — 280

To link to this Article: DOI: 10.1080/10826070701739108

URL: http://dx.doi.org/10.1080/10826070701739108

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Journal of Liquid Chromatography & Related Technologies®, 31: 269–280, 2008
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ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070701739108

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Validation of HPLC and UV Spectrophotometric Methods for the Determination of Bezafibrate in Pharmaceutical Formulations

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Abstract: An isocratic high performance liquid chromatographic (HPLC) and a UV spectrophotometric method were developed and validated for the determination of bezafibrate in pharmaceutical formulations. Bezafibrate was performed on a C_{18} analytical column (150 × 4.6 mm i.d., 5 μm) 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^{-1}. For both methods, detection was made at 230 nm. Method validation evaluated parameters such as linearity, precision, accuracy, and specificity, which remained within acceptable limits. Method comparison demonstrated that there is no significant difference between the procedures ($p < 0.05$).

Keywords: Liquid chromatography, Spectrophotometry, Bezafibrate, Pharmaceutical formulations, Validation

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INTRODUCTION

Cardiovascular diseases are a global problem for both the developed and developing world.[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]

The safety and efficacy of drug therapy can be ensured using a compendial or validated procedure to assess the quality of pharmaceutical products. The quality of formulations can be evaluated since analytical methods have been considered suitable for their intended purpose, like quantitation of active ingredients, and determination of performance characteristics (e.g., dissolution, drug release).[3]

There has been no compendial method for determination of bezafibrate in pharmaceutical formulations so far. Some analytical procedures have been reported to evaluate this fibrate in pharmaceutical products by capillary electrophoresis,[4] colorimeter spectrophotometry,[5] ultraviolet spectrophotometry,[6] and high performance liquid chromatography (HPLC).[7] Analytical techniques by HPLC have been presented for 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]

Since validated methods are applied routinely, some essential aspects must be observed during the technique development to avoid an excessive waste of financial resource. The mobile phase is an important factor to be considered during development of the HPLC method. Thus, solvents which have a low price and extend the column life are generally chosen. This usual choice was not fully observed in the published method for the determination of bezafibrate in pharmaceuticals by HPLC,[7] which proposed an uncommon mobile phase containing 45% propan-2-ol in 0.09 M phosphate buffer, pH 2.145.

The purpose of the procedure (e.g., identification, test for impurities, quantitation) is an important aspect that determines which parameters must be evaluated during method validation. Specificity, precision, accuracy, and linearity are some characteristics that must be assessed in procedures for the quantitation of drug substance in formulations. Since the HPLC method described in literature[7] has a quantitative intention, the specificity of the

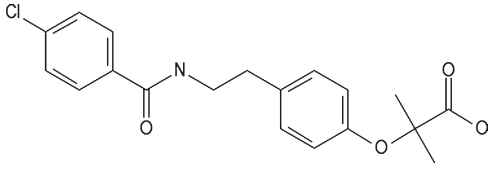

Figure 1. Chemical structure of bezafibrate.

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procedure should be completely evaluated, however, only matrix effects were assessed in the method.

Furthermore, the application of the method is relevant because it determines the range of linearity. The dissolution profile is an application for the determination of drug release performance and must be able to evaluate small amounts of drug substance. Thus, the HPLC validated procedure[7] could not be used in dissolution profile because the method has an inappropriate linear range (0.1–0.8 mg mL^{-1}).

Finally, the spectrophotometric method for determination of bezafibrate in pharmaceutical formulations reported in literature[6] cannot be fully understood and can be considered inaccessible because it was published in the author's language (Polish).

The present work reports the development and validation of two methods that can be applied for the determination of bezafibrate in pharmaceuticals.

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EXPERIMENTAL

Chemicals

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

All solvents were of HPLC grade and all reagents were of analytical grade. Acetonitrile and methanol were obtained from Tedia (USA). Potassium dihydrogen phosphate, sodium hydroxide, and hydrochloric acid were obtained from Merck (Germany). Phosphoric acid was purchased from Quimex (Brazil) and hydrogen peroxide was obtained from Synth (Brazil). Water was purified with Milli-Q Plus, Millipore System (USA). All solvents and solutions were filtered through a membrane filter or filtration units (Millipore Millex-HV filter units, 0.22 μm pore size) and degased before use.

Instrumentation and Analytical Conditions

The HPLC method for the determination of bezafibrate in pharmaceutical formulations was performed on a Shimadzu LC-10AD HPLC system (Japan), equipped with a diode array detector model SPD-M10Avp. Data integration was performed using Shimadzu Class-VP software. The analytical column was a reversed phase Rexchrom (150 × 4.60 mm i.d, 5 μm particle size) from Regis (USA). All analyses were carried out at room temperature (24 ± 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^{-1}, the volume of injection was 20 μL, and the detection was made at 230 nm.

The UV spectrophotometric method for quantitative assay of bezafibrate in tablets and compounded capsules was performed on a Shimadzu UV 1601 PC spectrophotometer (Japan) with detection at 230 nm, and using 1.0 cm quartz cells.

Solution and Sample Preparations

Reference Standard

Bezafibrate reference standard, 20 mg, (99.4%) accurately weighed were transfered to a 20 mL volumetric flask and dissolved in methanol for the chromatographic method (final concentration 1 mg mL^{-1}). A solution of 0.1 N sodium hydroxide (NaOH) was used to dissolve the bezafibrate reference standard for the spectrophotometric method.

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Pharmaceutical Formulations

An amount equivalent to 50 mg of bezafibrate, present in tablets and compounded capsules, was transferred to a 25 mL volumetric flask with 10 mL of methanol (for the HPLC method) or 0.1 N NaOH (for the UV method). The resulting solution was sonicated during 10 minutes and diluted with methanol or 0.1 N NaOH to obtain a final concentration of 2 mg mL^{-1}. The solution obtained from the tablets was filtered on cellulose filter discs and the solution from the capsules was centrifuged for 10 minutes at 3000 rpm before use.

Method Validation

The methods applied for the determination of bezafibrate in pharmaceuticals were validated according to the International Conference on Harmonisation guidelines for analytical procedures validation.[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. The curves were prepared on three different days.

The calibration curves were obtained with six concentrations of reference standard solution (1, 10, 50, 100, 250, and 500 $\mu g\ mL^{-1}$ for the chromatographic method, 2.5, 5, 7.5, 10, 12.5, and 15 $\mu g\ mL^{-1}$ for the spectrophotometric method).

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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 on three different days.

Six sample solutions (250 $\mu g\ mL^{-1}$ for the HPLC method and 10 $\mu g\ mL^{-1}$ for the UV method) were prepared and assayed for the determination of precision.

Accuracy

The accuracy was determined by assaying three concentrations (200, 250, and 300 $\mu g\ mL^{-1}$ for the chromatographic method, 8, 10, and 12 $\mu g\ mL^{-1}$ for the spectrophotometric method) in triplicate.

Specificity

The specificity was determined by assaying placebo (tablets and capsules) and degradation components. For the UV method, the interference from the formulations matrix was evaluated. For the HPLC method, besides placebo interference, accelerated degradation studies were carried out according to the next paragraph. The possible interferences were analyzed by the peak purity, which was calculated using Shimadzu Class-VP software.

For degradation studies, reference standard solutions (1 $mg\ mL^{-1}$) were submitted to accelerated degradation. Hydrochloric acid (0.1 N) for 8 hours at 80°C, 1 N sodium hydroxide for 8 hours at 80°C, and 30% hydrogen peroxide for 24 hours at room temperature were added to the solutions; the solutions were heated at 121°C at 15 minutes and exposed to ultraviolet light ($\lambda = 254$ nm) for 22 hours (15 cm) at room temperature.

Sensibility

For both HPLC and UV methods, the limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the response and the slope by using three calibration curves.

Robustness

For the HPLC method, the robustness was determined by the analysis of the samples under a variety of conditions making small changes in the buffer pH (3.3 and 3.7), in the percentage of mobile phase compounds (phosphate buffer: acetonitrile: methanol, for 48:42:10, and 52:38:10), in the flow rate (0.9 and 1.1 $mL\ min^{-1}$), in the column manufacturer (Supelco,® USA), and

changing the wavelength (228 and 232 nm). For the UV method, the robustness was evaluated by the variation of sodium hydroxide concentration (0.01 N) and wavelength (228 and 232 nm).

Method Comparison

The chromatographic and spectrophotometric methods developed and validated for determinations of bezafibrate in formulations were compared using statistical analysis.

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. Figure 2 shows a typical chromatogram obtained from the analysis of a reference standard using the proposed method. As shown in this figure, bezafibrate is represented by a symmetrical peak. The retention time observed in the assay (5.2 minutes) associated with the simple sample preparation (for tablets and capsules), allowed a rapid determination of the drug in pharmaceutical products.

The validated procedure for the determination of bezafibrate in pharmaceuticals previously described,[7] employed a mobile phase composed of a buffer at high concentration and low pH, causing injures in the column and in the equipment. Furthermore, propan-2-ol, an expensive and uncommon solvent for non chiral separation was used in large proportions as the solvent.

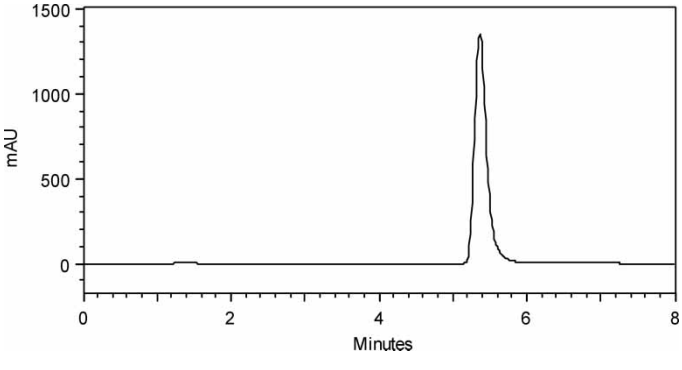

Figure 2. Chromatogram obtained from 250 μg mL^{-1} bezafibrate reference standard. Rexchrom Regis® C_{18} column (150 × 4.60 mm i.d, 5 μm), 0.01 M phosphate buffer (pH 3.5): acetonitrile: methanol (50:40:10, v/v/v), 1.0 mL min^{-1} and 230 nm.

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The spectrophotometric analyses were developed and validated using both aqueous (0.1 N sodium hydroxide) and organic solvent (methanol, data not reported). It is important to point out that the UV method using aqueous solvent was performed considering the reduction of financial resource waste. However, the UV method was easy to be carried out using both aqueous and organic solvents, and the comparison by ANOVA demonstrated that there is no difference between assays using both solvents (data not reported).

Method Validation

Linearity

The calibration curves of analytical methods were assessed by plotting concentration versus peak area or absorbance and showed suitable linearity in the range 1–500 $\mu g\ mL^{-1}$ for the HPLC method. The representative linear equation was $y = 59231\times + 19721$, with a highly significant correlation coefficient ($r = 0.9999$). For the UV method, the calibration curves showed appropriate linearity in the range 2.5–15 $\mu g\ mL^{-1}$ range. The representative linear equation was $y = 0.0504\times + 0.002$ ($r = 0.9999$) with sodium hydroxide as solvent. Both calibration curves were validated by ANOVA, which indicated significant linear regression and no significant deviation from linearity ($p = 0.05$).

The linear range obtained for the procedure applied to formulations by HPLC allows one to assay a dissolution profile of tablets and compounded capsules containing 200 mg of bezafibrate. The HPLC method validated and presented in literature[7] is inappropriate for dissolution profile of pharmaceutical dosage forms, since the calibration curve is linear to range 0.1–0.8 $mg\ mL^{-1}$ and cannot evaluate an amount lower than 45% when the pharmaceuticals are diluted in 900 mL of dissolution media.

Precision and accuracy

For the chromatographic method, the relative standard deviation (RSD) values for intra-day precision were 0.81 and 1.10% for tablets and capsules, respectively. The RSD for inter-day precision were 0.80% for tablets and 0.97% for capsules. Accuracy ranged from 99.76 to 100.05% for tablets and from 100.47 to 100.61% for compounded capsules (Table 1).

The RSD for intra-day precision obtained to the spectrophotometric method developed using sodium hydroxide as solvent were 0.48% for tablets and 0.76% for capsules. The RSD for inter-day precision were 0.45 and 0.83% for tablets and compounded capsules, respectively. For tablets, the accuracy ranged from 98.05 to 100.79% and for capsules ranged from 100.97 to 101.26% (Table 1).

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Table 1. Accuracy data for the methods applied to bezafibrate pharmaceutical formulations

Method	Theoretical ($\mu g\ mL^{-1}$)	Experimental[a]		
		Mean	Accuracy (%)	RSD (%)
HPLC				
Tablets	200	200.05	100.05	1.37
	250	249.40	99.76	0.76
	300	299.55	99.85	1.19
Capsules	200	201.22	100.61	0.88
	250	250.98	100.39	0.91
	300	301.41	100.47	0.52
UV (NaOH)				
Tablets	8	8.05	100.59	0.69
	10	9.88	98.80	0.55
	12	11.77	98.05	0.48
Capsules	8	8.10	101.26	0.79
	10	10.10	101.04	1.21
	12	12.12	100.97	1.74

[a]Mean of three determinations of each concentration.

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Specificity

For both HPLC and UV methods, no interference from matrix and excipients was found in placebo of tablets or capsules.

No degradation product of bezafibrate was observed after the accelerated stress caused by heating and by adding of hydrogen peroxide. However, some degradation products were found in the presence of hydrochlroric acid, sodium hydroxide, and UV light. Figures 3, 4, and 5 show that all degradation products were completely separated from the bezafibrate peak, which presented suitable purity by software analysis. Therefore, the validated procedure could be considered a stability indicative method unlike the HPLC method previously described.[7]

Sensibility

For chromatographic and spectrophotometric methods, the theoretical limits were evaluated based on three calibration curves and then the limits calculated were analyzed experimentally.

For HPLC method, the theoretical LOD, 0.23 $\mu g\ mL^{-1}$, was assayed giving a RSD of 2.49% and the calculated LOQ, 0.71 $\mu g\ mL^{-1}$, was performed achieving a suitable RSD of 1.49%. For the UV method using sodium hydroxide as solvent, the assay of the calculated LOD

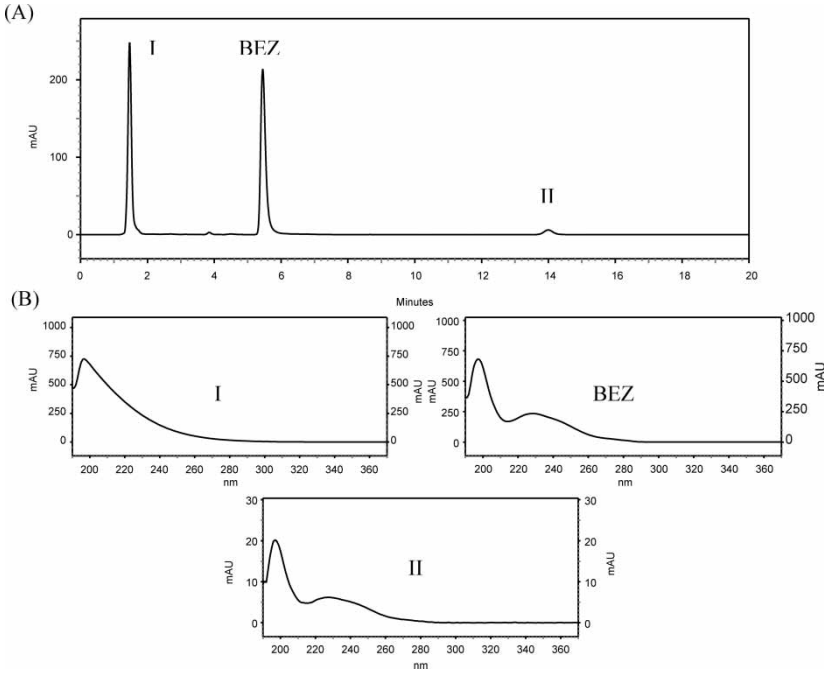

Figure 3. (A) Chromatogram of standard solution submitted to accelerated degradation using 0.1 N hydrochloric acid for 8 hours at 80°C. (B) Absorption spectrum of bezafibrate (BEZ), degradation products I and II. Rexchrom Regis® C_{18} column (150 × 4.60 mm i.d, 5 μm), 0.01 M phosphate buffer (pH 3.5): acetonitrile: methanol (50:40:10, v/v/v), 1.0 mL min^{-1} and 230 nm.

(0.44 μg mL^{-1}) gave a RSD of 14.97% and the performance of the theoretical LOQ (1.32 μg mL^{-1}) achieved a satisfactory RSD of 1.79%.

Robustness

The HPLC method demonstrated robustness for all evaluated parameters except for the flow rate of mobile phase, which caused changes in the accuracy of the procedure. The UV method remained unaffected by small changes in wavelength, but robustness was not demonstrated with the change of sodium hydroxide concentration (Table 2).

Method Comparison

In the comparison of the two methods, ANOVA demonstrated that there is no significant difference between the experimental values obtained by the

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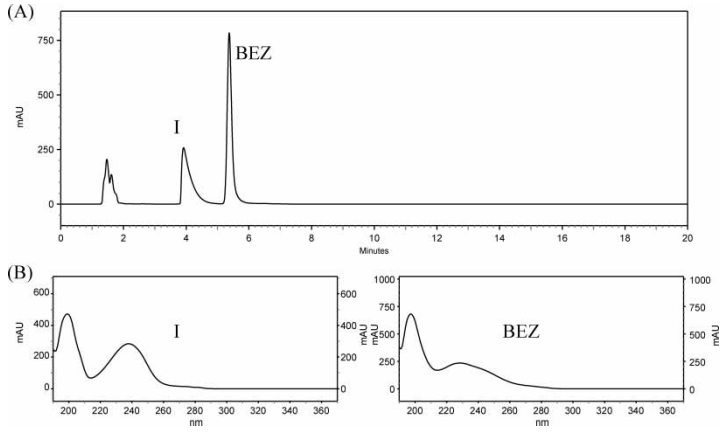

Figure 4. (A) Chromatogram of standard solution submitted to accelerated degradation using 1 N sodium hydroxide for 8 hours at 80°C. (B) Absorption spectrum of bezafibrate (BEZ) and degradation product I. Rexchrom Regis® C_{18} column (150× 4.60 mm i.d, 5 μm), 0.01 M phosphate buffer (pH 3.5): acetonitrile: methanol (50:40:10, v/v/v), 1.0 mL min^{-1} and 230 nm.

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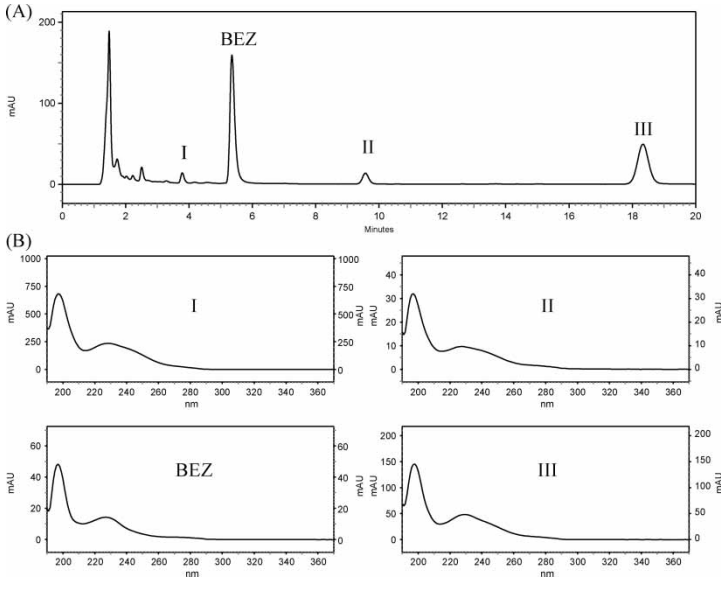

Figure 5. (A) Chromatogram of standard solution submitted to accelerated degradation using exposed to ultraviolet light ($\lambda = 254$ nm) for 22 hours at room temperature. (B) Absorption spectrum of bezafibrate (BEZ), degradation products I, II, and III. Rexchrom Regis® C_{18} column (150 × 4.60 mm i.d, 5 μm), 0.01 M phosphate buffer (pH 3.5): acetonitrile: methanol (50:40:10, v/v/v), 1.0 mL min^{-1} and 230 nm.

Table 2. Robustness of the HPLC and UV methods for determination of bezafibrate

	Normal	Variation	Bezafibrate amount[a] (%)
Chromatographic conditions			
Column C_{18} (150 × 4.6 mm, 5 μm)	Regis®	Supelco®	101.97
Mobile phase (phosphate buffer:	50:40:10	48:42:10	98.61
acetonitrile: methanol, v/v/v)		52:38:10	98.61
pH (phosphate buffer)	3.5	3.3	98.92
		3.7	100.06
Flow rate (mL min^{-1})	1.0	0.9	110.55
		1.1	91.11
Wavelength (nm)	230	228	99.24
		230	98.06
Spectrophotometric conditions			
Solvent concentration (sodium hydroxide, N)	0.1	0.01	93.67
Wavelength (nm)	230	228	101.85
		232	98.05

[a]Mean of three determination.

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procedures. The calculated F-value ($F_{calc} = 0.99$) was found to be less than the tabled F-value ($F_{tab} = 4.96$) at a 5% significance level.

CONCLUSION

The proposed HPLC and UV methods enable a quantitative determination of bezafibrate in tablets and compounded capsules. The application of these methods in routine analysis can be justified since fast sample preparation and simple reagents and solvents were used experimentally. The validation demonstrated that these procedures are suitable for the intended purpose because the methods were considered linear, precise, accurate, and specific and can be employed in control quality of pharmaceuticals containing bezafibrate. Furthermore, the HPLC method can be considered indicative of stability since degradation products were separated from bezafibrate.

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Received June 14, 2007
Accepted July 12, 2007
Manuscript 6168

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