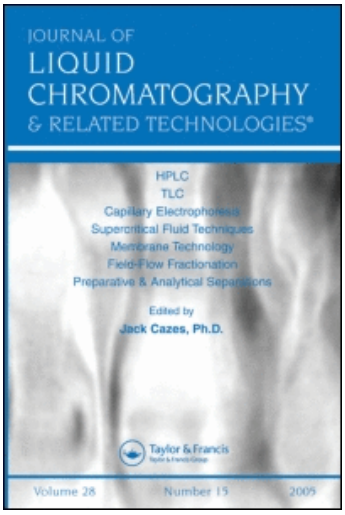

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Development of an HPLC Method for the Quantitation of Bisoprolol Enantiomers in Pharmaceutical Products using a Teicoplanin Chiral Stationary Phase and Fluorescence Detection

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Abstract: A selective high performance liquid chromatographic (HPLC) method was developed for the separation and quantification of bisoprolol enantiomers in pharmaceutical products. The method is highly specific where another co-formulated drug, hydrochlorothiazide, did not interfere. Baseline resolution was achieved by using teicoplanin macrocyclic antibiotic chiral stationary phase (CSP), known as Chirobiotic T, with fluorescence detection at excitation/emission wavelengths 275/305 nm. The polar ionic mobile phase (PIM) consisting of methanol-glacial acetic acid-triethylamine, (100:0.020:0.025), (v/v/v) has been used at a flow rate of 1.5 mL/min. All analytes with S-(−)-atenolol as the internal standard were conducted at room temperature. The stability of bisoprolol enantiomers under different degrees of temperature was also studied. The results showed that the drug is stable for at least 7 days at 70°C. The method was validated for its linearity, accuracy, precision, and robustness. An experimental design was used during validation to evaluate method robustness. The calibration curves were linear over the range of 5-250 ng/mL for each enantiomer, with a correlation coefficient of 0.999 for both enantiomers. The overall recoveries of S-(−)- and R-(+)-bisoprolol from pharmaceutical products ranged from 97.6 to 100.5% with %RSD ranging from 0.7 to 2.6%. The limit of quantification (LOQ) and limit of detection (LOD) for each

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enantiomer were 5 and 2 ng/mL, respectively. The method proved to be of chiral quality control for bisoprolol formulations by HPLC.

Keywords: Bisoprolol enantiomers, Teicoplanin chiral stationary phase, Pharmaceutical products, Fluorescence detection

INTRODUCTION

The determination of enantiomeric composition of pharmaceuticals is subject to severe attention from the clinical and toxicological point of view. Prior to the approval of a new racemic drug, the enantiomers should be analytically and unequivocally separated and the pharmacological effects, as well as the metabolic pathways, must be studied separately for each enantiomer.[1] This implies an ever increasing demand for pure enantiomeric compounds and for pertinent enantioselective technologies. Enantiomeric resolutions have acquired an important position in all stages of the drug development process. Therefore, the development of new methods for efficient chiral separations and quantitation is more than necessary.[2]

In last two decades, high performance liquid chromatography (HPLC) has become one of the most applied techniques in the chiral separation of different racemates.[3,4] Several chiral stationary phases (CSPs) have been developed and used for the chiral separation of a variety of racemates. Among these CSPs, macrocyclic glycopeptide antibiotic based CSPs are very important as they have achieved an excellent reputation in the field of chiral separation. The importance of this type of CSPs includes its ease of use, reproducible results, and a wide range of applications.[5–7]

Macrocyclic antibiotics have been introduced by Armstrong et al. as powerful chiral selectors in liquid chromatography,[8] thin-layer chromatography,[9] and capillary electrophoresis.[10] The glycopeptides macrocyclic antibiotics such as teicoplanin and vancomycin have been widely used as CSPs, and a large variety of racemic compounds have been resolved on them.[11,12] The enantioselectivity of these chiral selectors are due to several reasons: (i) they are amphoteric (i.e., contain acidic and basic ionizable groups); (ii) they have the necessary geometry and functionalities that accentuate chiral recognition in solution; and (iii) they contain both hydrophilic and hydrophobic moieties.[13]

The possible bonding between the enantiomers and the macrocyclic glycopeptide antibiotic CSPs have been reviewed.[14] The most important bondings involved are $\pi - \pi$ complexation, hydrogen bonding, inclusion complexation, dipole interactions, steric interactions, and ionic and cationic bindings. These bondings are a result of the complex structures of this CSP, which consists of sugar moieties, phenyl, quinoline, and thiazole rings, along with several chiral centers, inclusion baskets, hydrogen donor, and acceptor sites. It has been reported that these bonding sites are responsible for the surprising chiral selectivities of these antibiotics.[6,8]

If the compound has more than one functional group capable of interacting with the stationary phase and at least one of those groups is on or near the stereogenic center, then the first mobile phase choice would be the polar ionic mobile phase (PIM). Due to the strong polar groups present in the macrocyclic peptides, it was possible to convert the original mobile phase concept to 100% methanol with an acid/base added to effect selectivity.[15] The key factor in obtaining complete resolution is still the ratio of acid to base.[5] The importance and superiority of macrocyclic antibiotics as chiral selectors, in comparison with other chiral selectors, is that they can be used in normal and reversed phases with greater stability and capacity.[16]

Bisoprolol fumarate, (±)-1-[*p*-(2-isopropoxyethoxymethyl)phenoxy] -3-(isopropylamino)-2-propanol fumarate, is a β_1-selective adrenoceptor antagonist without membrane stabilizing activity or intrinsic sympathomimetic activity.[17,18]

Bisoprolol has a chiral centre in its molecule. The drug is marketed as a racemic mixture, as are most $\beta-$adrenergic blocking agents, and the development of an assay method determining its individual enantiomerst is important.

T. Suzuki et al. determined bisoprolol enantiomers in biosamples using a Chiralcel OD column with a mobile phase of hexane-isopropanol-diethylamine (10:0.9:0.01, v/v/v) and fluorescence detection.[19] Recently, a direct liquid chromatographic separation of the enantiomers of bisoprolol has also been developed, using (R)-1-naphthylglycine and 3,5-dinitrobenzoic acid as the chiral stationary phase in a normal mode system and UV detection.[2]

The importance of the present work is the ability of the selected teicoplanin macrocyclic antibiotic CSP to separate bisoprolol enantiomers, where as the complementary chiral selector vancomycin macrocyclic antibiotic CSP failed to separate these enantiomers. Also, in this study, we used reversed mobile phase (methanol), which is considered less toxic than the mobile phases used for the reported methods.[2,19] Moreover, this method can be used as a chiral quality control for bisoprolol formulations by HPLC.

EXPERIMENTAL

Instrumentation and Chromatographic Conditions

The HPLC instrument (Jasco, Japan) equipped with a pump (model PU-980), a fluorescence detector (model FP-920), a 20 μL injector is connected to an LG computer. The CSP used in this study was the macrolide type antibiotic teicoplanin, known as Chirobiotic T (150 x 4.6 mm i.d.), purchased from Advanced Separation Technologies (Whippany, NJ, USA). The mobile phase was methanol:glacial acetic acid:triethylamine (100:0.02:0.025, v/v/v). The mobile phase was filtered through a Millipore membrane filter (0.2 μm) from Nihon, Millipore (Yonezawa, Japan) and degassed before

used. The flow rate was 1.5 mL/min and the detection wavelengths (FL) were set at 275 nm for excitation and 305 nm for emission.

Chemical and Reagents

(±)-Bisoprolol fumarate, S-(−)-bisoprolol and R-(+)-bisoprolol were purchased from RBI (Natick, MA, USA). S-(−)-Atenolol was obtained from Sigma Chemical Co. (St Louis, MO, USA). HPLC grade methanol, analytical grade triethylamine, and glacial acetic acid were purchased from BDH Chemicals (Poole, UK). Concor® 5, Concor® 10 (containing 5 and 10 mg of bisoprolol fumarate/tablet, respectively), and Concor® 5 plus (containing 5 mg bisoprolol fumarate and 12.5 mg hydrochloro-thiazide/tablet) were obtained from Merck KGaA (Germany).

Preparation of Standard Stock Solutions

Stock solutions of individual S-(−)-bisoprolol, R-(+)-bisoprolol, and (S)-(−)-atenolol (internal standard) (1 mg/mL) were prepared in methanol. A seven-point non-zero calibration standard curve, ranging from 5-250 ng/mL, was prepared.

Preparation of Standard Solutions from Tablets

Ten tablets were ground and powdered, an accurately weighed portion equivalent to 5 mg bisoprolol fumarate betaxolol was transferred to a 100 mL volumetric flask, and diluted to the mark with methanol. The solution was sonicated for 15 min and centrifuged at 3000 rpm for 10 min. Accurately measured aliquots of the supernatant were transferred to 5 mL volumetric flasks containing 50 μL of the internal standard, and diluted to 5 mL with methanol to give final concentration of 20, 80, and 160 ng/mL of betaxolol.

Linearity

Aliquot volumes of the final solution of S-(−)- and R-(+)-bisoprolol were transferred to a series of 10 mL volumetric flasks to produce solutions covering the concentration range of 5-250 ng/mL for each enantiomer, respectively. A volume equivalent to 10 μg/mL of S-(−)-atenolol was added to each flask and the solution was diluted to 10 mL with methanol. Calibration standards of each concentration were analyzed in triplicate. Calibration curves of bisoprolol enantiomers were constructed using

normalized drug/internal standard peak area ratio versus nominal concentrations of the analyte. Least squares linear regression analysis of the data gave slope, intercept, and correlation coefficient data. From this data, a first order polymonial model was selected for each analyte.

Recovery

A 20 μL of the selected assay solutions were injected into the HPLC system and the chromatograms recorded. The nominal contents of the drug in each solution were calculated from the linear regression equations. The percent recovery and the percent RSD were calculated.

Specificity

The specificity of the proposed method was investigated by observing any interference encountered from coformulated hydrochlorothiazide and other tablet excipients present in the formulations. The specificity of the method was checked by analyzing four independent blank tablet formulations containing coformulated hydrochlorothiazide and other tablet excipients. The chromatograms of these blank tablet samples were compared with chromatograms obtained by analyzing tablet products spiked with internal standard.

Stability of Sample Solutions

The stability of sample solutions was tested by the proposed HPLC method over a period of 7 days. The freshly prepared solutions at room temperature, and the 7 day stored samples in a thermostatic oven at 30, 50, and 70°C, were analyzed by the optimized proposed HPLC method. The concentrations of the stored samples were calculated and compared to that of the freshly prepared samples.

Limit of Detection and Limit of Quantitation

The limit of detection (LOD) and the limit of quantitation (LOQ) were determined as 3 and 10 times the baseline noise, respectively.[20] The results of the statistical analysis of the experimental data, such as the slopes, the intercepts, the correlation coefficients obtained by the linear squares treatment of the results, along with standard deviation of the slope (S_b) and intercept (S_a) on the ordinate and the standard deviation of the residuals ($S_{y/x}$), were shown.

The good linearity of the calibration graphs and the negligible scatter of experimental points are evident by the values of the correlation coefficient and standard deviation. The robustness of the method demonstrated the versatility of the experimental factors that affect the peak area.

RESULTS AND DISCUSSION

Chromatography

The chemical structures of S-(−)-bisoprolol and R-(+)-bisoprolol and (S)-(−)-atenolol (IS) are shown in Figure 1. Macrocyclic antibiotic chiral

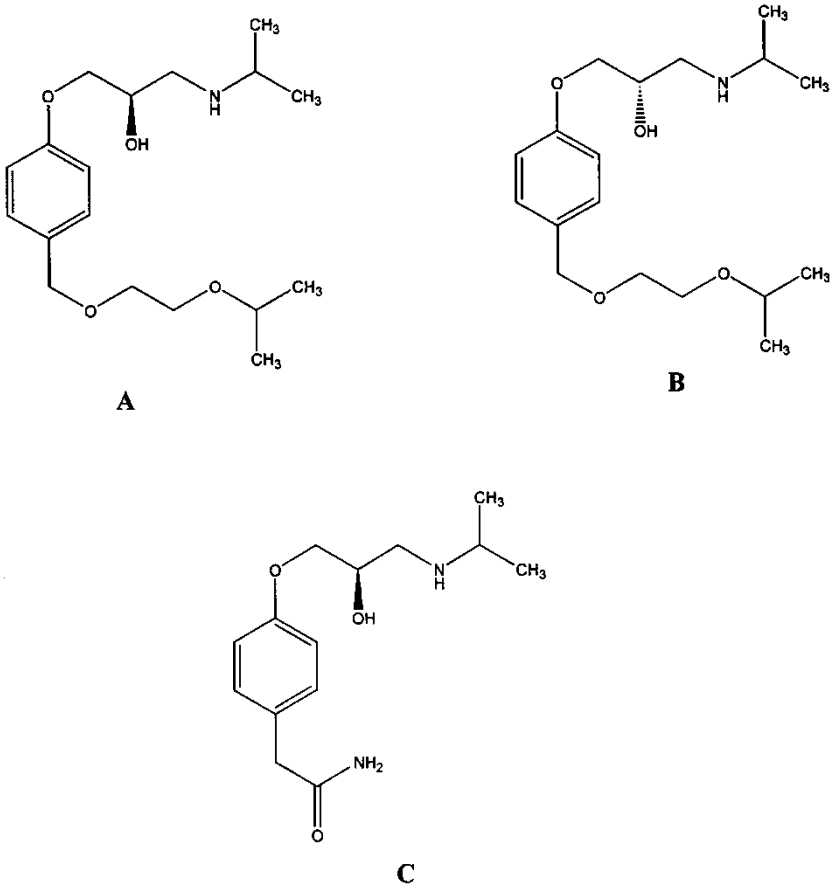

Figure 1. The chemical structure of (A) S-(−)-bisoprolol, (B) R-(+)-bisoprolol, and (C) (S)-(−)-atenolol (IS).

stationary phases have been widely used for enantiomers resolution because they very effectively recognize the enantiomers of anionic compounds. The selectivity towards these compounds is due to the presence of amine groups in the chiral selector.[15] The polar ionic mobile phase (PIM) has been described as a method developed to obtain difficult enantioselective separation with macrocyclic antibiotic based CSPs.[21] This approach uses a non-aqueous polar component (methanol) with both glacial acetic acid and triethylamine, which are necessary to achieve enantioseparation.

The HPLC method carried out in this study was aimed at developing a chromatographic system, capable of eluting and resolving bisoprolol enantiomers from pharmaceutical preparations. The preliminary investigations were directed toward the effect of various factors on the system. The factors assessed include the detection wavelength, the type of column, and the composition of mobile phase. Bisoprolol enantiomers showed two excitation wavelength maxima at 225 and 275 nm. The 275 nm wavelength showed a better resolution.

The separation of bisoprolol enantiomers was first attempted using vancomycin CSP and teicoplanin TAG CSP. However, despite the use of a range of ratios of acetic acid and triethylamine in the mobile phase, complete separation was not achieved on both columns. In order to improve the resolution of bisoprolol enantiomers, a teicoplanin column was used and several mobile phase compositions were tested. The best results in terms of resolution, analysis time, and separation factor were obtained with mobile phase consisting of methanol-glacial acetic acid-triethylamine (100:0.02:0.025 v/v/v) (Table1). No enantioseparation was observed in the absence of triethylamine when the mobile phase consisted of methanol and acetic acid. This could be explained on the basis of strong repulsive effects between the protonated amino groups of the analyte molecules and of the CSP. An increase of the triethylamine concentration in the mobile phase (to about 0.1%) decreased the retention factors of the studied analytes. Increasing the concentration of acetic acid in the mobile phase (to about 0.1%) also

Table 1. Chromatographic parameters data for bisoprolol enantiomers and the internal standard S-(−)-atenolol

Analyte	R_s [a]	α [b]	K [c]	T_R (min) [c]
S-(−)-bisoprolol	1.46	1.12	7.02 ± 0.35	11.27 ± 0.01
R-(+)-bisoprolol	7.34	1.73	7.96 ± 0.39	12.62 ± 0.01
S-(−)-atenolol	[d]	[d]	13.88 ± 0.64	20.90 ± 0.01

[a] $R_s = (t_2 - t_1)/0.5(w_1 + w_2)$, where t_2 and t_1 are the retention of the second and first peaks and w_{b2} and w_{b1} are the half peak width of the second and first peaks.

[b] Separation factor, calculated as k_2/k_1.

[c] Mean ± SD, n = 3.

[d] Not calculated.

decreased the retention factors of the studied analytes. This demonstrates that it is the concentration of acetic acid and triethylamine in mobile phase that has a substantial influence on the retention factors, and not the ionic strength of the mobile phase that was constant.

The studied enantiomers of bisoprolol (Figure 1) contain nitrogen and oxygen atoms, along with a benzene ring, which interact with the complimentary groups on the chiral selector. The inclusion baskets and the other functional moieties provide the chiral sites in which the enantiomers fit stereogenically in a different fashion, which results in chiral discrimination between the bisoprolol enantiomers. Furthermore, the steric effect is also playing an important role for the chiral resolution of the studied drug on this CSP.

Linearity

The linear regression analysis of bisoprolol enantiomers in pure solution was constructed by plotting the peak area ratio of each enantiomer to the internal standard (y) versus analyte concentration in ng/mL (x). The calibration curves were linear in the range of 5–250 ng/mL, with a correlation coefficient (R^2) of 0.999 for both enantiomers (Table 2). A typical calibration curve has the regression equation of $y = 0.017 + 0.006\ x$ for S-(−)-bisoprolol and $y = 0.028 + 0.006\ x$ for R-(+)-bisoprolol.

Limit of Detection, Limit of Quantitation, and Accuracy

The limit of detection (LOD) and the limit of quantitation (LOQ) for each enantiomer were 2 ng/mL and 5 ng/mL, respectively (Table 2). The results

Table 2. Validation parameters for the determination of bisoprolol enantiomers using the proposed method

Parameter	S-(−)-bisoprolol	R-(+)-bisoprolol
Concentration range (ng/mL)	5–250	5–250
Intercept (a)	0.017	0.028
Slope (b)	0.006	0.006
Correlation coefficient (R^2)	0.999	0.999
$S_{y/x}$	0.050	0.026
S_a	0.033	0.018
S_b	0.0002	0.0001
LOD (ng/mL) [a]	2	2
LOQ (ng/mL)	5	5

[a] S/N = 3.

of the statistical analysis of the experimental data, such as the slopes, the intercepts, and the correlation coefficients, obtained by the least squares treatment of the results, along with standard deviation of the slopes and intercepts on the ordinate and the standard deviation of the residuals, were shown in Table 2. The accuracy of the method was tested by analyzing different concentrations of standard bisoprolol enantiomers. The results were expressed as percent recoveries of the particular components in the samples (Table 3). The overall recoveries of bisoprolol enantiomers by the proposed method were 98.6 and 99.8% for S-(−)- and R-(+)-bisoprolol, respectively, with %RSD of 0.63 and 1.67 for S-(−)- and R-(+)-bisoprolol, respectively, indicating that these values were acceptable.

Application to Pharmaceutical Products

The validity of the method developed here was applied to various concentrations taken from the pharmaceutical products (Concor® 5, Concor® 10, and Concor® 5 plus) for determining their content of bisoprolol enantiomers. The values of the overall drug percentage recoveries and the %RSD value of S(−)- and R(+)-bisoprolol are presented in Table 4, indicating that these values are acceptable and the method is accurate and precise.

Specificity

Hydrochlorothiazide, a diuretic drug which is coformulated with bisoprolol to increase drug effectiveness, did not interfere with the determination of

Table 3. Precision of bisoprolol enantiomers in standard solutions by the proposed method

Analyte	Enantiomer	Nominal conc.(ng/mL)	Measured conc.(ng/mL)[a]	Recovery (%)
Bisoprolol	S-(−)-	30	29.83 ± 0.08	99.4
		90	88.16 ± 0.12	97.9
		180	177.16 ± 0.26	98.4
Overall recovery				98.6
RSD (%)				0.63
	R-(+)-	30	30.00 ± 0.09	100.0
		90	88.00 ± 0.31	97.7
		180	183.33 ± 0.42	101.8
Overall recovery				99.8
RSD (%)				1.67

[a]Mean ± S.D. based on n = 3.

Table 4. Precision of bisoprolol enantiomers in pharmaceutical products by the proposed method

Pharmaceutical preparation	Enantiomer	Nominal conc.(ng/mL)	Measured conc.(ng/mL) [b]	Recovery (%)
Concor® 5 tablet[a]	S-(−)-	20	19.33 ± 0.09	96.7
		80	78.66 ± 0.16	98.3
		160	156.83 ± 0.34	98.0
Overall recovery				97.7
RSD (%)				0.70
	R-(+)-	20	19.16 ± 0.11	95.8
		80	80.19 ± 0.19	100.2
		160	155.22 ± 0.42	96.9
Overall recovery				97.6
RSD (%)				1.90
Concor® 10 tablet[a]	S-(−)-	20	20.16 ± 0.07	100.8
		80	78.66 ± 0.14	98.3
		160	156.16 ± 0.29	97.6
Overall recovery				98.9
RSD (%)				1.38
	R-(+)-	20	19.66 ± 0.08	98.3
		80	82.50 ± 0.16	103.1
		160	155.33 ± 0.36	97.1
Overall recovery				99.5
RSD (%)				2.60
Concor® 5 plus tablet[a]	S-(−)-	20	19.83 ± 0.10	99.2
		80	82.16 ± 0.18	102.7
		160	159.16 ± 0.43	99.5
Overall recovery				100.5
RSD (%)				1.57
	R-(+)-	20	20.16 ± 0.09	100.8
		80	79.00 ± 0.18	98.8
		160	158.16 ± 0.39	98.9
Overall recovery				99.5
RSD (%)				0.92

[a]Obtained from Merck KGaA (Germany).
[b]Mean ± S.D. based on n = 3.

bisoprolol enantiomers, as it has no fluorescence at the selected wavelengths, indicating the high specificity of the proposed method (Figure 2). Excipients commonly coformulated with the studied drug, such as magnesium stearate, cellulose, starch, calcium hydrogen phosphate, colloidal silicon dioxide, and coloring agents, also did not interfere with the determination of bisoprolol enantiomers, indicating the high specificity of the method.

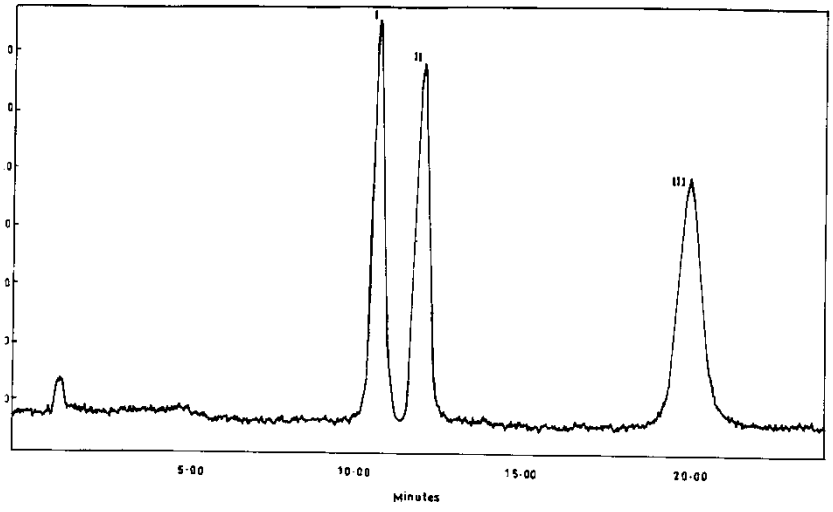

Figure 2. Chromatogram of [I] S-(−)-bisoprolol, 150 ng/mL and [II] R-(+)-Bisoprolol, 150 ng/mL, recovered from Concor® 5 plus tablet spiked with [III] 10 μg/mL (S)-(−)-atenolol (IS).

Robustness

The optimum HPLC conditions set for this method have been slightly modified for samples of bisoprolol as a means to evaluate the method's robustness. The small changes made include the flow rate, detection wavelength, time (day), and temperature (Table 5). It was found that the percent recoveries of bisoprolol enantiomers were good under most conditions, and remained unaffected by small changes of experimental parameters. Variation in the experimental parameters, as well as carrying out the experiment at room temperature, provided an indication of its reliability during normal use and concluded that the method conditions were robust.

Stability of Sample Solutions

The stability of sample solutions was tested by HPLC over a period of 7 days. The freshly prepared solutions at room temperature and the 7 day stored samples at 30, 50, and 70°C, were analyzed by the proposed HPLC method. The concentrations of bisoprolol enantiomers in the stored samples were calculated and compared to that present in the freshly prepared samples (Figure 3). From these results, we can conclude that there were no degradation products at elevated temperatures and the drug is stable at 70°C for 7 days, indicating the °possibility of using bisoprolol samples over a period of 7 days at 70°C without °degradation.

Table 5. Effect of experimental parameters on the percent recoveries of bisoprolol enantiomers

Parameters	Modification		Recovery (%)
	S-(−)-	R-(+)-	
Flow rate (mL/min)	1.2	98.5	102.7
	1.5	99.2	101.7
	1.8	98.9	100.4
Wavelength of excitation (nm)	270	97.9	101.0
	275	99.2	102.7
	280	99.8	101.9
Wavelength of emission (nm)	300	99.6	97.5
	305	100.4	98.5
	310	99.4	98.3
Temperature[a] (°C)	30	98.6	99.4
	50	102.3	102.9
	70	101.0	100.4
Day[b]	1	102.1	99.8
	2	101.9	102.5
	3	103.1	98.8

[a]7-day stored solutions at 30, 50 and 70°C.
[b]Solutions were stored at room temperature.

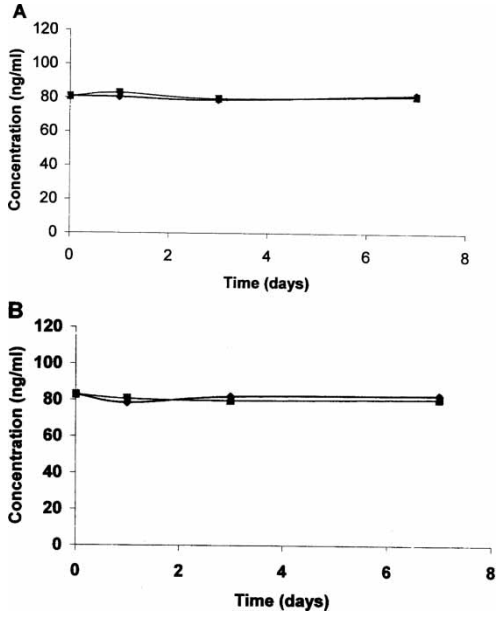

Figure 3. Unaltered concentrations vs. time of (A) S-(−)-bisoprolol and (B) R-(+)-bisoprolol at 50 (▼) and 70°C (■).

CONCLUSION

An enantioselective HPLC method that enables sensitive determination of S-(−)- and R-(+)-bisoprolol in pharmaceutical products was developed. The method is selective where coformulated hydrochlorothiazide does not interfere. With the present broad range of available CSPs and advances in column technology, the present enantioselective HPLC can be considered as the method of choice.

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