Enantioanalysis of Bisoprolol in Human Plasma with a Macrocyclic Antibiotic HPLC Chiral Column Using Fluorescence Detection and Solid Phase Extraction

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A sensitive, enantioselective, high-performance liquid chromatographic (HPLC) method was developed and validated to determine *S*-(−)- and *R*-(+)-bisoprolol in human plasma. Baseline resolution was achieved using the teicoplanin macrocyclic antibiotic chiral stationary phase (CSP) known as Chirobiotic T with a polar ionic mobile phase (PIM) consisting of methanol–glacial acetic acid–triethylamine (100 : 0.02 : 0.025, v/v/v) at a flow rate of 1.5 ml/min and fluorescence detection set at 275 nm for excitation and 305 nm for emission. All analyses with *S*-(−)-atenolol as the internal standard were conducted at ambient temperature. The assay involved the use of a solid-phase extraction procedure for human plasma samples prior to HPLC analysis. The C18 cartridge gave good recovery rates for both enantiomers without any interference. The method was validated over the range of 20—200 ng/ml for each enantiomer concentration. Recovery rates for *S*-(−)- and *R*-(+)-bisoprolol enantiomers were in the range of 95—102%. The method proved to be precise (within-run precision expressed as % RSD ranged from 1.0—6.2% and between-run precision ranged from 0.9—6.7%) and accurate (within-run accuracies expressed as percentage error ranged from 0.2—4.8% and between-run accuracies ranged from 0.3—1.7%). The limit of quantitation and limit of detection for each enantiomer in human plasma were 20 and 5 ng/ml, respectively.

Key words bisoprolol; teicoplanin column; solid-phase extraction; fluorescence detection; human plasma

Chirality remains an important consideration for many compounds including pharmaceuticals, biological molecules, and agrochemicals.[1,2] It has been established that frequently only one of the two enantiomers of a drug is pharmacologically active, whereas the other can be inactive or toxic.[3,4] Differences between the biological activities of enantiomers arise because of differences between protein binding and transport, mechanism of action, rate of metabolism, rate of clearance and persistence in the environment.[5—7]

In the last two decades extensive research has been performed on the resolution of enantiomers using liquid chromatography (LC) and capillary electrophoresis (CE). A search of the literature indicates that the most interesting research in this area involves the development of new chiral selectors, and different types of chiral selector have been used in chromatography for direct enantiomer resolution.[4] Macrocyclic glycopeptides, such as teicoplanin, represent a recent class of powerful chiral selectors.[8] Their success can be attributed to the diversity of their structures that have multiple stereogenic centers and a variety of functional groups known to provide multiple interactions necessary for enantioselectivity.[9,10] The glycopeptide antibiotic chiral stationary phases (CSPs) have great potential for the resolution of a variety of racemates.[11,12] Due to the strong polar groups present in macrocyclic peptides, it was possible to convert the mobile phase to 100% methanol with an acid/base added to effect selectivity. The key factor in obtaining complete resolution is still the ratio of acid to base.[12]

Bisoprolol fumarate, (±)-1-[*p*-(2-ispropoxyethoxymethyl)-phenoxy]-3-(isopropylamino)-2-propanol fumarate, is a β_1-selective adrenoceptor antagonist without membrane stabilizing activity or intrinsic sympathomimetic activity.[13,14] Bisoprolol has a chiral center in its molecule. The drug is marketed as a racemic mixture and the development of an assay method determining its individual enantiomers is important. Suzuki *et al.* determined bisoprolol enantiomers in plasma and urine using a Chiralcel OD column and fluorescence detection.[15] However, this method has the drawback of a long analysis run time (40 min) and lower percentage of recovery from biological samples. 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 CSP in a normal-mode system and UV detection.[16]

In this study, a selective HPLC method for the quantitation of *S*(+)- and *R*(+)-enantiomers of bisoprolol in human plasma is reported using a teicoplanin CSP column, commercially known as Chirobiotic T, as the chiral selector and fluorescence detection. The method is linear in the range of 20—200 ng/ml and provides the required sensitivity for monitoring the blood level of bisoprolol enantiomers (10—100 ng/ml).

Experimental

HPLC System The HPLC instrument (Jasco, Japan) equipped with a pump (model PU-980), fluorescence detector (model FP-920), and 20-μl injector is connected to and LG computer. The CSP used in this study was the macrolide-type antibiotic teicoplanin, known as Chirobiotic T (150×4.6 mm i.d.) purchased from Advanced Separation Technologies (Whippany, NJ, U.S.A.). 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.

Chemicals and Reagents (±)-Bisoprolol fumarate, *S*-(−)-bisoprolol, and *R*-(+)-bisoprolol were purchased from RBI (Natick, MA, U.S.A.). *S*-(−)-Atenolol was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). HPLC-grade methanol and ethanol and analytical-grade triethylamine and glacial acetic acid were purchased from BDH Chemicals (Poole, U.K.). Deionized water was purified using a cartridge system (Picotech water system, RTP, NC, U.S.A.). Oasis HLB and Sep-Pak C18, C8, C2 and

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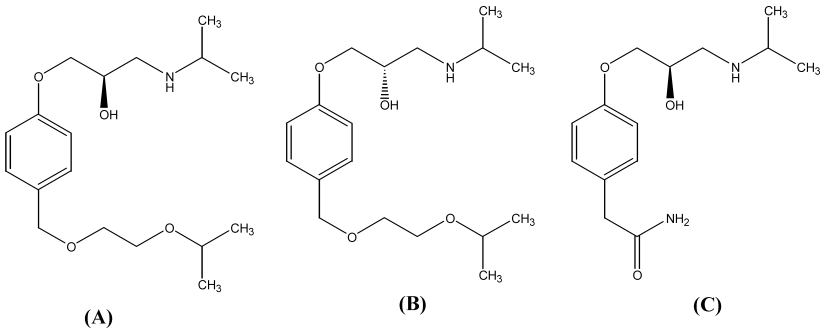

Fig. 1. Chemical Structures of (A) *S*-(−)-Bisoprolol, (B) *R*-(+)-Bisoprolol and (C) *S*-(−)-Atenolol (I—IV)

CN cartridges (1 ml) were obtained from Waters Corp. (Milford, MA, U.S.A.). Human plasma was obtained from King Khalid University Hospital (Riyadh, Saudi Arabia), and was kept frozen until use.

Preparation of Stock and Standard Solutions Stock solutions of individual *S*-(−)- and *R*-(+)-bisoprolol were prepared in methanol to give a concentration of 1 mg/ml. The internal standard *S*-(−)-atenolol was prepared in methanol to give a concentration of 1 mg/ml. Appropriate dilutions of the individual bisoprolol stock solutions were made to provide 800 ng/ml standard solutions that were used for spiking plasma. A five-point nonzero calibration standard curve, ranging from 20—200 ng/ml, was prepared by spiking the drug-free plasma with appropriate volume of *S*-(−)- and *R*-(+)-bisoprolol standard solutions. The quality control (QC) samples, at three concentration levels, *i.e.*, 40, 100, and 160 ng/ml, were prepared in a similar manner from the stock solutions. Before spiking, the drug-free plasma was tested to ensure that there was no endogenous interference at the retention times of bisoprolol enantiomers and internal standard. The QC samples were extracted with the calibration standards to verify the integrity of the method.

Preparation of Spiked Plasma Accurately measured aliquots of the individual 800-ng/ml standard *S*-(−)- and *R*-(+)-bisoprolol solutions were pipetted into individual 1.5-ml Eppendrof tubes containing 0.5 ml of human plasma. Then 100 μl of the internal standard solution was added to each tube, diluted with deionized water to 1 ml, and sonicated for 5 min to give final concentrations of 40, 100, and 160 ng/ml of each bisoprolol enantiomer. Blank human plasma samples were processed in the same manner using deionized water instead of bisoprolol ennantiomers.

Assay Method An octadecylsilane cartridge was attached to a vacuum manifold (VacElute, Harbor City, CA, U.S.A.) and conditioned with two column volumes of absolute methanol and two column volumes of deionized water before applying the plasma samples. Care was taken that the cartridge did not run dry. Blank and spiked plasma samples were transferred into the cartridge and a vacuum was applied to obtain a flow rate of 0.5 ml/min. After the entire plasma samples had been aspirated through the cartridge, the cartridge was washed with 2×500 ml deionized water. The cartridge was dried under a vacuum for 3 min. The C18 cartridge was eluted with 2× 500 μl of absolute methanol and 20 μl was injected into the HPLC system.

The absolute recovery rates of each enantiomer from plasma were calculated by comparing the drug peak area of the spiked analyte samples with the unextracted analyte of stock solution that has been injected directly into the HPLC system. Calibration curves were constructed by diluting stock solutions with pooled human plasma to yield five concentrations over the range of 20—200 ng/ml for each bisoprolol enantiomer. Linear regression analysis of the normalized drug/internal standard (D/IS) peak area ratio-*versus*-concentration gave slope and intercept data for each analyte, which were used to calculate the concentration of each analyte in the human plasma sample.

The within-run and between-run precision (reported as % RSD) and accuracy (reported as % error) of the assay in plasma were determined by assaying three QC samples in triplicate over a period of 3 d. The concentrations represented the entire range of the calibration curve. The regression equations were used to determine the concentrations in the quality QC.

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.[17] The results of the statistical analysis of the experimental data, such as the slopes, intercepts, and 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 determined. The good linearity of the calibration graphs and the negligible scatter of experimental points are evident in the values of the correlation coefficient and standard deviation.

Results and Discussion

Optimization of Chromatographic Conditions The chemical structures of *S*-(−)- and *R*-(+)-bisoprolol and *S*-(−)-atenolol are shown in Fig. 1. Macrocyclic antibiotic CSPs have been widely used for enantiomer resolution because they effectively recognize the enantiomers of anionic compounds. The selectivity toward these compounds is because of the presence of amine groups in the chiral selector.[18] The polar ionic mobile phase (PIM) has been described as a method to obtain difficult enantioselective separation with macrocyclic antibiotic-based CSPs.[19] This approach uses a nonaqueous polar component (methanol) with both glacial acetic acid and triethylamine, which are necessary to achieve enantioseparation.

The HPLC method used in this study aimed at developing a chromatographic system capable of eluting and resolving bisoprolol enantiomers from human plasma. The preliminary investigations were directed toward the effect of various factors on the system. The factors assessed include the detection wavelength, type of column, and composition of the mobile phase. Bisoprolol enantiomers showed two excitation wavelength maxima at 225 and 275 nm. The 275-nm wavelength showed better sensitivity. The separation of bisoprolol enantiomers was first attempted using Chirobiotic V and Chirobiotic TAG columns. However, despite the use of a range of ratios of acetic acid and triethylamine in the mobile phase, complete separation was not achieved on column.

To improve the resolution of bisoprolol enantiomers, the Chirobiotic T 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 a mobile phase consisting of methanol : glacial acetic acid : triethylamine (100 : 0.02 : 0.025, v/v/v) (Table 1). 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 in 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 decreased the capacity factors of the studied analytes. This demonstrates that it is the concentration of

Table 1. Chromatographic Parameters of Bisoprolol Enantiomers and the Internal Standard *S*-(−)-Atenolol in Spiked Human Plasma

Analyte	R_s [a]	α [b]	K [c]	t_R (min) [c]
S-(−)-Bisoprolol	1.55	1.14	5.83±0.10	11.59±0.09
R-(+)-Bisoprolol	6.82	1.73	6.67±0.18	12.99±0.07
(*S*)-(−)-Atenolol	[d]	[d]	11.59±0.24	21.37±0.13

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_2 and w_1 are the half-peak width of the second and first peaks. *b*) Separation factor, calculated as k_2/k_1. *c*) Mean±S.D., $n=3$. *d*) Not calculated.

acetic acid and triethylamine in the mobile phase that has a substantial influence on the capacity factors and not the ionic strength of the mobile phase, which was constant.

The studied enantiomers of bisoprolol (Fig. 1) contain nitrogen and oxygen atoms, along with a benzene ring, which interact with the complementary groups on the chiral selector. The inclusion baskets and 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. In addition, the steric effect also plays an important role in the chiral resolution of the studied drug on this CSP.

Application to Spiked Human Plasma In the current method, solid-phase extraction is increasingly used for sample preparation instead of traditional methods such as liquid–liquid extraction with the advantages of being less time-consuming and more compatible with automization. Five solid-phase extraction cartridges (Water oasis HLB, C18, C8, C2, and CN) were investigated for plasma clean-up prior to HPLC assay. The octadecylsilane cartridge gave the best recovery rates for both bisoprolol enantiomers.

The eluting abilities of various elution solvents toward bisoprolol enantiomers were also investigated. Of these solvents, only absolute methanol was able to disrupt all types of interactions in the case of bisoprolol enantiomers and thus to elute them from the octadecylsilane sorbent. The ability of other solvents to disrupt these interactions was lower, resulting in poor elution of bisoprolol enantiomers from the C18 sorbent.

The extraction procedure used in this study afforded recovery rates ranging from 95.15 to 101.25% in the range of 20—200 ng/ml for both bisoprolol enantiomers.

Validation The method was tested for linearity, specificity, precision, and reproducibility. Using the above chromatographic procedure, linear regression equations were obtained. The linear regression equation of *S*-(−)- and *R*-(+)-bisoprolol enantiomers was constructed by plotting the peak area ratio of each enantiomer to the internal standard (y) *vs.* analyte concentration (ng/ml) in spiked plasma samples (x). The calibration curves showed good linearity in the range 20—200 ng/ml for *S*-(−)- and *R*-(+)-bisoprolol enantiomers, with correlation coefficients (r) of 0.999 and 0.998, respectively (Table 2). A typical calibration curve has the regression equation of $y=0.005x+0.011$ for *S*-(−)-bisoprolol and $y=0.005x+0.020$ for *R*-(+)-bisoprolol.

A summary of the accuracy and precision results is given in Table 3. The acceptance criteria (within-run and between-run % RSD of less than 15% and accuracy of between 85% and 115%) were met in all cases. The within-run precision and accuracy ($n=3$) expressed as % RSD and % error were 1.0—5.4% and 0.4—1.5%, respectively, for *S*-(−)-bisoprolol and 1.1—6.2% and 0.2—4.8%, respectively, for *R*-(+)-bisoprolol. The between-run precision and accuracy ($n=6$) expressed as % RSD and % error were 1.3—4.4% and 0.7—1.2% for *S*-(−)-bisoprolol, respectively, and 0.9—6.7% and 0.3—1.7% for *R*-(+)-bisoprolol, respectively.

Table 2. Validation Parameters for the Determination of Bisoprolol Enantiomers in Human Plasma Using the Proposed Method

Parameter	*S*-(−)-Bisoprolol	*R*-(+)-Bisoprolol
Concentration range (ng/ml)	20—200	20—200
Intercept (a)	0.011	0.020
Slope (b)	0.005	0.005
Correlation coefficient (r)	0.999	0.998
$S_{y/x}$	0.045	0.074
S_a	0.036	0.059
S_b	0.0003	0.0005
LOD (ng/ml) [a]	5	5
LOQ (ng/ml)	20	20

a) S/N=3.

Table 3. Accuracy and Precision Data for Bisoprolol Enantiomers in Spiked Human Plasma

Analyte	Actual concentration (ng/ml)	Experimental concentration (ng/ml)	RSD (%)	Error (%)
Within-run [a]	40	39.6±2.1	5.4	0.8
S-(−)-	100	99.5±3.0	3.0	0.4
Bisoprolol	160	157.5±1.6	1.0	1.5
Within-run [a]	40	38.0±2.3	6.2	4.8
R-(+)-	100	100.2±3.8	3.8	0.2
Bisoprolol	160	155.8±1.8	1.1	2.6
Between-run [b]	40	40.5±1.7	4.4	1.2
S-(−)-	100	99.2±2.2	2.2	0.7
Bisoprolol	160	158.7±2.1	1.3	0.8
Between-run [b]	40	39.8±2.7	6.7	0.3
R-(+)-	100	100.8±2.8	2.8	0.8
Bisoprolol	160	157.4±1.4	0.9	1.7

a) Mean±S.D. based on $n=3$. *b*) Mean±S.D. based on $n=6$.

Evaluating the LOD, LOQ, linearity of detector response, and method precision and accuracy established the validation of the HPLC assay. The LOD was 5 ng/ml and the LOQ was 20 ng/ml for each enantiomer (Table 2). Table 2 also shows the results of the statistical analysis of the experimental data obtained by the least-squares treatment of the results.

Bisoprolol enantiomers were well separated under the HPLC conditions applied. Retention times were 11.59±0.09 min and 12.99±0.07 min for *S*-(−)- and *R*-(+)-bisoprolol enantiomers, respectively. No interference was observed in drug-free human plasma samples (Fig. 2).

Conclusion

In conclusion, a sensitive and selective HPLC method has been developed and validated for the analysis of bisoprolol enantiomers in human plasma. The enantiomers were separated with a teicoplanin. The method used an efficient solid-phase extraction procedure for sample clean-up of plasma. The total run time for this method is 25 min, which allows processing of over 55 samples per day. This method has excellent sensitivity, precision, and reproducibility.

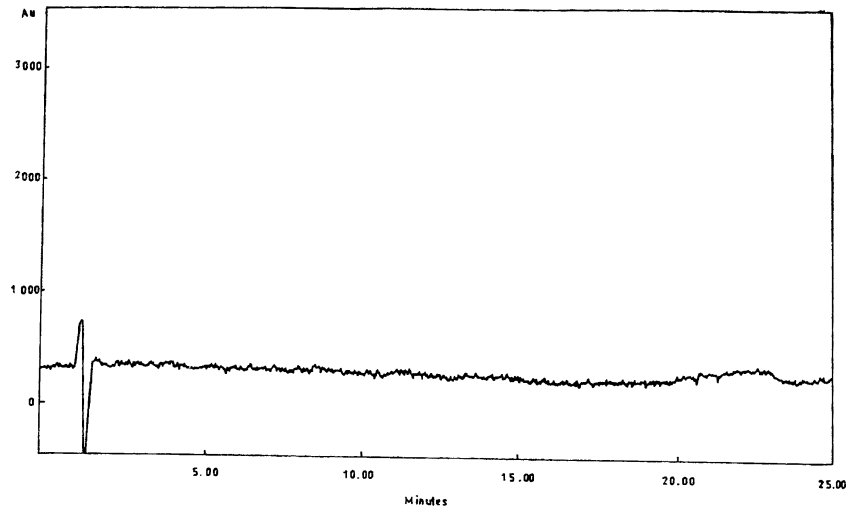

Fig. 2A. Chromatogram of Blank Human Plasma

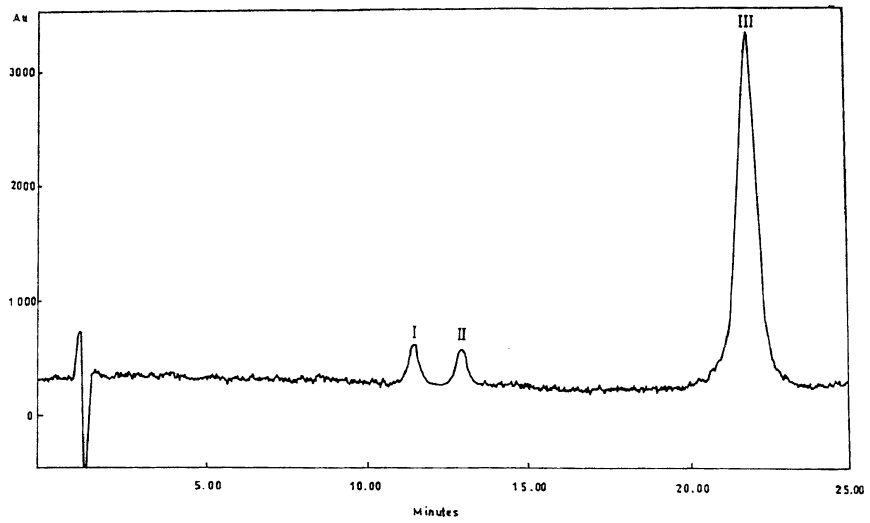

Fig. 2B. Chromatogram of Blank Human Plasma Spiked with 20 ng/ml of Each Enantiomer
Peaks: I=*S*-(−)-bisoprolol; II=*R*-(+)-bisoprolol; III=internal standard.

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