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Sensitive determination of bisoprolol enantiomers in plasma and urine by high-performance liquid chromatography using fluorescence detection, and application to preliminary study in humans

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ABSTRACT

A sensitive, stereoselective high-performance liquid chromatographic method with fluorescence detection for the measurement of bisoprolol enantiomers in human plasma and urine has been developed. Bisoprolol was extracted at alkaline pH with chloroform, followed by solid-phase extraction. The effluent was evaporated, and the reconstituted residue was chromatographed on a Chiralcel OD column with a mobile phase of hexane–2-propanol (10:0.9, v/v) containing 0.01% (v/v) diethylamine. Within the plasma and urine enantiomeric concentration ranges of 5–100 ng/ml and 25–1250 ng/ml, respectively, a linear relationship was obtained between the peak-height ratios and the corresponding concentrations. The limit of quantitation, defined as three times the baseline noise, was 2 ng/ml for each enantiomer in plasma. A preliminary pharmacokinetic study was undertaken in three healthy male volunteers following an oral dose of 5 mg of racemic bisoprolol. The results confirm that this assay is suitable for pharmacokinetic studies of bisoprolol enantiomers in humans following oral administration of the therapeutic dose.

INTRODUCTION

Bisoprolol hemifumarate, [±]-1-[[*p*-(2-isopropoxyethoxy)methyl]phenoxy]-3-isopropylamino-2-propanol hemifumarate, is a highly β1-selective adrenoceptor antagonist without membrane stabilizing activity or intrinsic sympathomimetic activity [1,2]. Bisoprolol has a chiral centre in its molecule and thus has two optical isomers. The drug is marketed as a racemic mixture, as are most β-adrenergic blocking agents, but there is little information on the pharmacokinetic properties of the individual enantiomers of bisoprolol [3]. A number of reports have described the analysis [4,5] and pharmacokinetics [6–9] of the racemic form of bisoprolol.

Recently, it has been shown that enantiomers of some drugs often exhibit different pharmacokinetic and/or pharmacological properties [10–13]. (−)-Propranolol, which is almost 100 times more potent as a β-blocker than (+)-propranolol, is known to be cleared more slowly from blood than (+)-propranolol after oral doses of racemic propranolol to humans [10]. For optically active drugs, therefore, their efficiency or safety in clinical use cannot be evaluated precisely without a stereoselective analysis. This also holds true for bisoprolol. Therefore, the establishment of an assay method measuring the individual enantiomers of bisoprolol and the elucidation of their pharmacokinetics after administration of its racemic mixture is important.

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For the stereoselective assay of optically active drugs, gas chromatography (GC) has generally been used. However, it has recently become less popular because its applicability is restricted to volatile drugs or drug derivatives. High-performance liquid chromatography (HPLC) has become the method of choice for the separation of enantiomers [14,15], because of its wide applicability, assay speed and separation efficiency. For chiral separation by HPLC, there are several approaches, such as derivatization with chiral reagents, the addition of chiral reagents to the HPLC mobile phase and chiral stationary phases. However, some chiral additives are expensive and not always commercially available. In addition, the use of chiral additives to the mobile phase is usually complex and often results in inaccuracy.

The first HPLC determination of the enantiomers of bisoprolol was based on the reaction of the racemic mixture with N-trifluoroacetyl-(−)prolyl chloride to form a pair of diastereoisomers, and the separation on a non-chiral column [16]. N-Trifluoroacetyl-(−)prolyl chloride has been widely used for the formation of diastereoisomers. However, Silber and Riegelman [17] reported that, in the reaction of propranolol with N-trifluoroacetyl-(−)prolyl chloride, this reagent rapidly underwent racemization during storage, and a very low reaction temperature (−78°C) was necessary to avoid racemization during the formation of the diastereoisomers. Besides, enantiomeric purity and stereochemical stability of the chiral reagents should be ensured during storage. An additional problem was that the diastereomeric derivatization method suffered from a lengthy sample preparation. These problems can be avoided by resolving the enantiomers by using enantioselective chromatography. The separation of a number of β-blocking agents has been reported on chiral stationary phases [18,19], which are becoming increasingly popular for direct separation.

Therefore, we have chosen a chiral stationary phase column and established a sufficiently sensitive method to allow quantitation of the individual enantiomers after therapeutic doses of racemic bisoprolol. This is the first paper to describe a stereospecific HPLC method for the measurement of bisoprolol enantiomers in human plasma and urine. The procedure involves a solvent and solid-phase extraction of bisoprolol from plasma and urine, followed by direct separation of the enantiomers of bisoprolol on a chiral column. Detection of the enantiomers was accomplished with a fluorimetric detector.

EXPERIMENTAL

Chemicals and materials

R(+)-, *S*(−)- and racemic bisoprolol and the internal standard, 1-[*p*-(tetrahydro-3-furanyl)-phenoxy]-3-isopropylamino-2-propanol, were obtained as their hemifumarates from E. Merck (Darmstadt, Germany). The structures of bisoprolol and the internal standard are shown in Fig. 1. HPLC-grade hexane and 2-propanol and pesticide-grade chloroform were obtained from Katayama (Osaka, Japan). The remaining chemicals and solvents were of reagent grade and were used without further purification. Reagent-grade water was prepared by passage through a Milli-RO reagent-grade water system (Nihon Millipore, Tokyo, Japan). Bond Elut SI disposable cartridges were purchased from Analytichem International (Harbor City, CA, USA).

Apparatus and chromatographic conditions

The HPLC instrument consisted of a Shimadzu (Kyoto, Japan) LC-3A pump, a Hitachi (Tokyo, Japan) F-1000 fluorimetric detector, a Waters (Milford, MA, USA) 710B WISP autosampler, a Shimadzu C-R2A integrator and a tem-

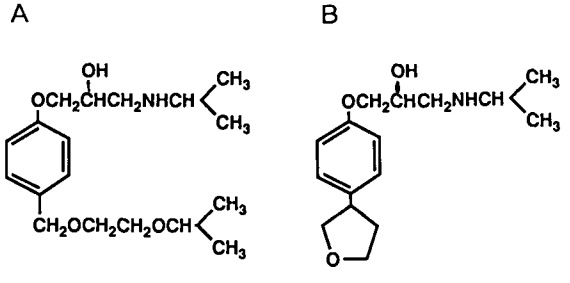

Fig. 1. Structures of (A) bisoprolol and (B) the internal standard.

perature-controlled column compartment. The analytical column (10 μm, 250 mm × 4.6 mm I.D.) and pre-column (10 μm, 50 mm × 4.6 mm I.D.) were Chiralcel OD from Daicel Chemical Industries (Tokyo, Japan). The mobile phase was hexane–2-propanol (10:0.9, v/v) containing 0.01% (v/v) diethylamine. The flow-rate of 0.5 ml/min and column temperature of 25°C were maintained throughout the analysis. An excitation wavelength of 228 nm was used, and the emission was monitored at 298 nm.

Standard solutions

A stock solution was prepared by dissolving 10 mg of bisoprolol hemifumarate in 100 ml of ethanol. When stored at 4°C, this stock solution did not show any degradation for at least two months. Every two weeks, standard solutions were prepared by dilution of this stock solution with water to the appropriate concentration. The internal standard, which has four optical isomers because it has two chiral carbons in its molecule, was separated into four peaks under the above chromatographic conditions. The third peak was chosen as the working internal standard because its retention time was suitable for analysis. A solution of the working internal standard was prepared as follows. After an injection of aliquots of the internal standard solution at a high concentration onto the analytical column, the column eluate fraction corresponding to the third peak was collected. This procedure was conducted repeatedly, and the pooled eluates were then evaporated to dryness in a nitrogen stream. The residue was reconstituted, diluted to an appropriate concentration with a mobile phase, and stored at 4°C.

Sample praparation

Plasma. To 1 ml of drug-free human plasma were added 0.2 ml of water or a racemic bisoprolol standard solution and 0.2 ml of 0.1 *M* NaOH. Then 7 ml of chloroform were added, and the resultant mixture was shaken for 10 min and centrifuged at 1800 *g* for 5 min. After the upper aqueous layer had been removed by aspiration, 5 ml of chloroform were directly applied to a separate, disposable silica extraction column (Bond Elut SI), which was previously conditioned with 3 ml of chloroform. The applied chloroform was allowed to run through, and the column was washed with 1 ml of methanol. The adsorbed bisoprolol was eluted from the column into a small test-tube with 1 ml of methanol containing 5% (v/v) triethylamine. After evaporation of the effluent under a nitrogen stream at 60°C, the residue was reconstituted with 200 μl of the working internal standard solution. Aliquots of 100 μl were injected into the HPLC column.

Urine. To 0.5 ml of urine were added 0.5 ml of water or a racemic bisoprolol standard solution and 0.3 ml of 0.1 *M* NaOH. Bisoprolol was extracted into 7 ml of chloroform. The chloroform extract was then treated in the same manner as described for plasma samples.

Accuracy and precision of assay

Standard samples were prepared by spiking with standard solutions of racemic bisoprolol to give final concentrations (each enantiomer) of 5, 10, 25, 50 and 100 ng/ml in plasma and 25, 50, 125, 250, 500 and 1250 ng/ml in urine. The calibration curve was constructed by plotting the ratio of the peak height of each enantiomer to that of the internal standard against the concentration of each enantiomer calculated as 50% of the concentration of racemic bisoprolol. To assess the within-day variability, three replicates were determined for each concentration on the same day in plasma and urine, respectively. The inter-day variability was determined at 25 ng/ml in plasma and at 2 μg/ml in urine on four different days. The accuracy was calculated based on the mean percentage error [(mean measured concentration − expected concentration)/expected concentration × 100%], and the precision was evaluated by calculating within-day and inter-day coefficients of variation (C.V.). Recovery values were determined by comparing extracted spiked samples with unextracted standard solutions.

Application

Three healthy male volunteers each received a 5-mg oral dose of racemic bisoprolol with *ca.* 100

ml of water. Subjects fasted overnight prior to drug administration, and up to 4 h after drug administration. Venous blood samples were collected by using heparinized plastic syringes before dosing, and 1, 2, 3, 4, 6, 8, 10 and 12 h after administration. Blood samples were centrifuged at 3000 *g* for 15 min, and plasma samples were separated and stored at −20°C until required for analysis. This study was performed under the supervision of Dr. T. Takashina, Koseikai Takaori Hospital (Kyoto, Japan).

RESULTS AND DISCUSSION

A number of efficient chiral columns for the direct separation of enantiomeric compounds have been described [14,15,18,19]. Many such chiral columns are now commercially available. Separation of *R*(+)-bisoprolol and *S*(−)-bisoprolol was first attempted using a Pirkle column with a covalently bonded α-ethylnaphthylamine phase. However, despite the use of a range of ratios of hexane to 2-propanol in the mobile phase, and the use of hexane–dichloromethane–ethanol as the mobile phase, separation was not achieved. Another column tried was a Chiralcel OD column, packed with a cellulose tris-3,5-dimethylphenylcarbamate coated on silica gel. This column showed promising resolution of the enantiomers when hexane–2-propanol was used as the mobile phase. The resolution and peak shape of the enantiomers were greatly improved when diethylamine was added to the hexane–2-propanol. Krstulovic *et al.* [20] showed the effectiveness of the addition of diethylamine in the case of betaxolol analysis. Furthermore, lowering the column temperature significantly improved the resolution. The best resolution of the enantiomers was achieved with a mobile phase of 0.01% (v/v) diethylamine in hexane–2-propanol (10:0.9, v/v) at 25°C, where the resolution coefficient was 1.5. *R*(+)-Bisoprolol and *S*(−)-bisoprolol were completely separated and eluted at *ca.* 20 and 30 min, respectively, as confirmed by chromatographing the pure enantiomers. The Chiralcel OD column was thus chosen for analysis of the plasma and urine samples.

There were only two simple extraction steps in sample preparation. One was a solvent extraction of bisoprolol from biological fluids, and the other was a solid-phase extraction, which eliminated interfering peaks present in plasma or urine. Chloroform was used as extraction solvent, and was directly applied to a disposable silica extraction column without prior solvent evaporation. The extraction efficiency of bisoprolol from aqueous solution at alkaline pH with chloroform was greater than 90%. Reagent-grade chloroform sometimes contained impurities, which gave small peaks near *R*(+)-bisoprolol on the chromatograms. This was avoided by the use of the pesticide-grade chloroform.

Washing the column with methanol before the elution of bisoprolol effectively removed interfering fluorescent compounds in plasma or urine. However, because a small amount of bisoprolol was lost when the column was washed with a large volume of methanol, the volume of washing methanol should be as small as possible. Adsorbed bisoprolol enantiomers were eluted from a Bond Elut SI column with 1 ml of methanol containing 5% (v/v) triethylamine. The presence of triethylamine enabled the volume of the effluent to be reduced, which enabled easy solvent evaporation. The recovery of bisoprolol from a Bond Elut SI column was low when diethylamine (*ca.* 60%) was used in place of triethylamine (*ca.* 100%).

Fig. 2 shows typical chromatograms of blank human plasma (A), blank human plasma spiked with racemic bisoprolol (B) and human plasma sample taken 2 h after oral administration of 5 mg of racemic bisoprolol (C). There were no interferences at the retention times of *R*(+)- and *S*(−)-bisoprolol and the internal standard. Also, the four major metabolites of bisoprolol reported in humans and animals [6,7] were found not to interfere with the assay in plasma.

Calibration curves were obtained from plasma standards, which were prepared by adding racemic bisoprolol to 1 ml of blank human plasma at five concentrations. Good linearity was obtained between peak-height ratios and the corresponding plasma concentrations over the range 5–100

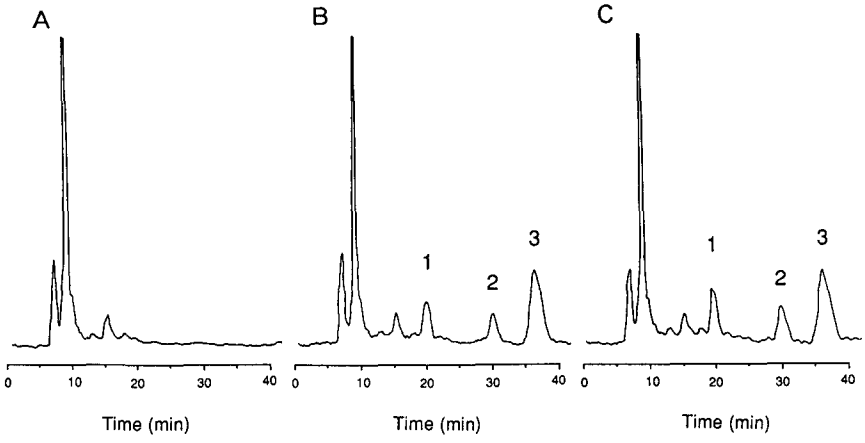

Fig. 2. Typical chromatograms of (A) blank human plasma, (B) blank human plasma spiked with 10 ng/ml of each enantiomers, and (C) a human plasma sample from a volunteer after oral administration of 5 mg of racemic bisoprolol. Peaks: 1 = *R*(+)-bisoprolol; 2 = *S*(−)-bisoprolol; 3 = internal standard.

ng/ml for each enantiomer. No interference or matrix effects occurred. Typical regression lines for *R*(+)-bisoprolol and *S*(−)-bisoprolol were $y = 0.07415x - 0.0371$ ($r > 0.999$) and $y = 0.07041x - 0.0171$ ($r > 0.999$), respectively, where y is the peak-height ratio of the enantiomer to the internal standard, and x is the enantiomer concentration.

The results in Table I describe the accuracy and precision of the assay. Within-day assay variations for each enantiomer at five concentrations are shown. At different concentrations, the associated error ranged from 0.3 to 6.0% and the C.V. was between 0.4 and 11.9%. The inter-day variability ($n = 4$) was assessed at 25 ng/ml for each enantiomer. The C.V. for inter-day analysis was less than 10%, demonstrating the good precision of the assay. The overall recovery of bisoprolol from plasma samples was 76.5 ± 3.2, 83.9 ± 0.4 and 88.1 ± 1.6% for *R*(+)-bisoprolol and 88.2 ± 5.6, 85.2 ± 4.2 and 87.0 ± 2.8% for *S*(−)-bisoprolol at 10, 25 and 100 ng/ml. The minimum detectable quantity of each enantiomer in plasma was 2 ng/ml at a signal-to-noise ratio of 3:1.

The above assay procedure was also applied to urine samples, with the slight modification of reducing the sample volume of urine and increasing the working standard solution volume to reconstitute the residue. Fig. 3 shows typical chromatograms of blank human urine (A) and blank human urine spiked with racemic bisoprolol (B).

TABLE I

ACCURACY AND PRECISION OF THE ASSAY FOR BISOPROLOL ENANTIOMERS IN PLASMA ($n = 3$)

Concentration added (ng/ml)	Measured concentration (mean ± S.D.) (ng/ml)		Mean absolute error (%)		Coefficient of variation (%)	
	R	*S*	*R*	*S*	*R*	*S*
5	4.8 ± 0.2	5.3 ± 0.3	4.0	6.0	3.8	6.1
10	9.9 ± 1.0	10.2 ± 1.2	1.0	2.0	10.2	11.9
25	24.9 ± 0.1	24.6 ± 1.2	0.4	1.6	0.4	4.9
50	49.6 ± 0.6	49.0 ± 2.1	0.8	2.0	1.2	4.3
100	100.3 ± 1.8	100.6 ± 3.2	0.3	0.6	1.8	3.2

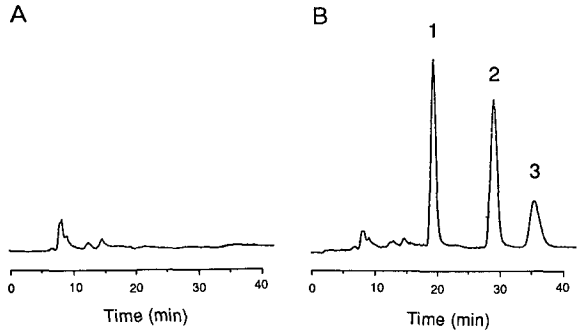

Fig. 3. Typical chromatograms of (A) blank human urine and (B) blank human urine spiked with 2 μg/ml of each enantiomer. Peaks: 1 = *R*(+)-bisoprolol; 2 = *S*(−)-bisoprolol; 3 = internal standard.

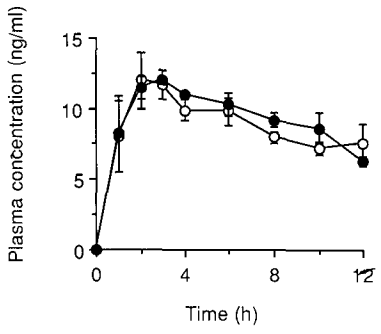

Fig. 4. Mean plasma concentration–time cruve for (○) *R*(+)-bisoprolol and (●) *S*(−)-bisoprolol after oral administration of 5 mg of racemic bisoprolol to three healthy male volunteers. Each point represents the mean ± standard error.

Urine samples assayed did not show any evidence of interference from endogenous products or from the major metabolites of bisoprolol. A linear correlation between the amounts of each enantiomer and the peak-height ratios was found over the range 25–1250 ng/ml for each enantiomer. In urine, the standard curves were best described by the equations $y = 0.01505x - 0.0076$ ($r > 0.999$) and $y = 0.01224x - 0.0118$ ($r > 0.999$) for *R*(+)- and *S*(−)-bisoprolol, respectively.

The accuracy and precision of this assay for bisoprolol enantiomers in urine are shown in Table II. At different concentrations, the associated error ranged from 0.08 to 16.4% and the C.V. was between 2.9 and 10.9%. The inter-day variability in urine over four days was assessed at 2 μg/ml for each enantiomer. The C.V. for inter-day analysis was less than 10%. The overall recovery of bisoprolol from urine samples was *ca.* 90–100%. According to the results of Leopold [6], the amount of unchanged racemic bisoprolol excreted in the urine up to 168 h is 50–60% of the dose in humans. The concentrations of each enantiomer in urine are expected to be higher than in plasma. Therefore, this assay method is adequate for the evaluation of urine samples in clinical trials.

The applicability of this assay was examined in a preliminary pharmacokinetic study in humans. An oral dose of 5 mg of racemic bisoprolol was given to three healthy male volunteers, and the concentrations of bisoprolol enantiomers in plasma were determined periodically up to 12 h. The mean plasma concentration–time curves of each bisoprolol enantiomer are shown in Fig. 4. From

TABLE II

ACCURACY AND PRECISION OF THE ASSAY FOR BISOPROLOL ENANTIOMERS IN URINE (n = 3)

Concentration added (ng/ml)	Measured concentration (mean ± S.D.) (ng/ml)		Mean absolute error (%)		Coefficient of variation (%)	
	R	*S*	*R*	*S*	*R*	*S*
25	28.4 ± 2.1	29.1 ± 1.2	13.6	16.4	7.5	4.2
50	50.3 ± 4.3	49.8 ± 5.4	0.6	0.4	8.6	10.9
125	123.3 ± 6.6	121.8 ± 6.6	1.4	2.5	5.3	5.4
250	246.8 ± 15.8	250.0 ± 18.0	1.2	0.0	6.4	7.2
500	501.6 ± 17.1	497.0 ± 25.3	0.3	0.6	3.4	5.1
1250	1251.0 ± 36.6	1251.7 ± 52.7	0.08	0.1	2.9	4.2

the results of this preliminary study, it appears that this method would be suitable for clinical trials because the oral therapeutic dose range of bisoprolol in patients has proved to be between 5 and 10–20 mg of bisoprolol once daily.

CONCLUSIONS

The described method is a rapid, sensitive and accurate procedure for the simultaneous determination of bisoprolol enantiomers in human plasma and urine. The assay is valid for the determination of bisoprolol in clinical samples, or in research applications with minimal sample preparation and a run time that is suitable for processing numerous samples on a daily basis.

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