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Article

An HPLC Method for the Determination of Bisoprolol in Human Plasma and its Application to a Pharmacokinetic Study

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A new high-performance liquid chromatographic method is described for the determination of bisoprolol in human plasma. The proposed method was based on the derivatization of bisoprolol with 4-chloro-7-nitro-2,1,3-benzoxadiazole in borate buffer at pH 9.5 to yield a fluorescent product.

Chromatographic separation of bisoprolol was achieved by using isocratic elution at a flow rate of 1.2 mL/min on a C18 reversed-phase column (Inertsil, 4 μm, 150 × 4.6 mm) at 40°C. The mobile phase used for the analysis was methanol–water (70:30, % v/v).

Fluorescence detector was used at the excitation and emission wavelengths of 458 and 525 nm, respectively. The method was validated for linearity, limit of detection, limit of quantification, precision, accuracy, recovery and system suitability. The assay was linear over the concentration range of 10–2000 ng/mL. This method was applied in pharmacokinetic studies of bisoprolol preparations in healthy volunteers.

Introduction

Bisoprolol hemifumarate (BIS) (±)-1-{p-[(2-isopropoxyethoxy) methyl] phenoxy}-3-isopropyl-amino-2-propanol hemifumarate is indicated for the treatment of hypertension and angina pectoris (1, 2).

Several analytical methods have been studied for the determination of BIS in plasma and urine samples. Among these methods, high-performance liquid chromatography (HPLC) (3–8), liquid chromatography–tandem mass spectrometry (LC–MS-MS) (9–12), liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) (13–14) and electrophoresis (15) have been utilized for the determination of BIS.

Some methods have also been reported for the determination of BIS in pharmaceutical preparations. These articles include HPLC (16–19), voltammetry (20) and spectrophotometry (21).

In this study, a sensitive fluorimetric HPLC method has been developed. The method is based on the precolumn derivatization of BIS with 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl). The proposed method was optimized, fully validated and successfully applied to the pharmacokinetic study.

Experimental

Materials

BIS (≥98%) and ephedrine (99%) as internal standard (IS) were supplied from Sigma (Steinheim, Germany). Concor 5 mg tablet was obtained from a local pharmacy. NBD-Cl was purchased from Sigma (Steinheim, Germany). Other chemicals were provided from Merck (Darmstadt, Germany). All solvents were of analytical grade.

Apparatus and chromatographic conditions

Fluorescence spectrum of BIS-NBD derivative was recorded by a RF-1501 Model A Shimadzu (Kyoto, Japan) spectrofluorimeter.

The liquid chromatographic system was Shimadzu Liquid Chromatography (Kyoto, Japan) consisting of a Model LC 20 AT solvent delivery system with an SIL-20AHT autosampler with a 5-μL loop and a RF-10AXL fluorescence detector. The analytical column was an Inertsil C18 column (150 × 4.6 mm i.d., 5 μm) with a guard column (4 × 3 mm i.d., Inertsil) packed with the same material. The mobile phase was comprised of methanol–water (70:30 v/v). The mobile phase was prepared fresh. Analyses were run at a flow rate of 1.2 mL/min at 40°C. The fluorimetric detector was set at 458 and 525 nm for the excitation and emission wavelengths, respectively.

Preparation of solutions

Stock standard solutions of BIS was prepared in methanol at a concentration of 1,000 μg/mL and stored at +4°C. These were diluted by using methanol to give appropriate working (10 μg/mL) solutions.

A borate buffer (0.1M) was prepared by dissolving 0.620 g of boric acid and 0.750 g of potassium chloride in 100 mL of water. The pH was adjusted to 9.5 with 0.1M sodium hydroxide solution and the volume was made up to 200 mL with water. The NBD-Cl solution was prepared at 2 mg/mL in methanol.

Sample preparation and derivatization

Frozen human plasma samples were thawed at ambient temperature. Plasma samples were extracted employing a liquid–liquid extraction method. Calibration curves were constructed by adding various amounts (10–2,000 ng) of BIS to aliquots (1 mL) of drug-free human plasma. To a 1.0-mL aliquot of plasma samples, 10 μL of IS (50 ng) and 0.2 mL of 1M sodium hydroxide solution were added. The samples were briefly mixed and 3 mL of ethyl acetate were added. The mixture was vortex-mixed for approximately 1 min. After centrifugation at 4,500 rpm for 35 min, the organic layer was transferred to another 5-mL glass tube and evaporated to dryness under a gentle stream of air at 40°C. Onto the residue, 100 μL buffer and 100 μL NBD-Cl solutions were added and the mixture was

kept at 80°C for 20 min in a water bath. It was cooled and acidified with 100 μL of 1 N HCl. The associated compound was extracted from plasma twice with 2.5 mL chloroform and the organic layer was transferred to a tube. The organic phase was dried on anhydrous sodium sulfate. A 4.5-mL aliquot of the extract was evaporated under nitrogen at 45°C. The residue was then dissolved in 1 mL of the mobile phase and filtered through a 0.2-μm membrane filter. Typically, 5 μL aliquots of this solution are used for determination by HPLC.

Liquid–liquid extraction

A liquid–liquid extraction method was chosen. A large group of extraction solvents such as *n*-hexane, ethyl acetate, dichloromethane, chloroform and 2-propanol were tested to develop a single-step liquid–liquid extraction procedure for a good recovery. Ethyl acetate was found to be a good extracting solvent. This was found to be the most optimum condition for sample preparation because it resulted in a clean chromatogram.

Method validation

Validation was carried out following the International Conference on Harmonization (ICH) (22) guidelines determining, linearity, limits of detection, limits of quantification, precision, accuracy, recovery, stability and system suitability.

Linearity

A calibration curve of BIS-NBD derivative was constructed by the linear regression using the internal standard technique. The plots of peak area ratios ($rPN = PN_{BIS}/PN_{IS}$) versus concentrations of the associated compound were employed.

Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) of the drug by the proposed method were determined using calibration standards. LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where S is the slope of the calibration curve and σ is the standard deviation (SD) of intercept of regression equation.

Precision and accuracy

The accuracy and precision tests of BIS-NBD derivative were also tested by determining the active compound in plasma at three concentration levels on three different days. The accuracy and precision of the method was expressed by relative mean error (RME) and relative standard deviation (RSD), respectively.

Recovery

The extraction recovery for plasma at three different concentrations of BIS was determined. Known amounts of BIS were added to drug-free plasma and the IS was then added. After the derivatization and chromatography processes, the peak areas were compared to the peak areas obtained from the aqueous solutions of BIS at the same concentration.

Stability

The stability of plasma samples under different conditions was evaluated. The stability in the extraction solvent was determined at 4°C and room temperature. The stability of the derivative in mobile phase before injection into the HPLC was also tested.

Application to pharmacokinetic study

After oral administration of 5 mg of BIS to a healthy 41-year-old woman volunteer, the blood samples were collected before and at 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10, 12 and 24 h post-dosing. The plasma was rapidly separated by centrifugation and stored at −20°C until analysis. The maximum plasma concentration (C_{max}) and the time to reach C_{max} (t_{max}) were the observed values. The area under the plasma concentration–time curve (AUC_{0-t}) was calculated using the linear trapezoidal rule. The half-life ($t_{1/2}$) was calculated as 0.693/ke.

Results and Discussion

Method development and optimization

The reactions of BIS and IS with NBD-Cl in borate buffer at pH 9.5 produce a yellowish fluorescence color. Fluorescence of BIS-NBD and IS-NBD gave a spectra maximum at λ_{ex} 458 nm and λ_{em} 525 nm when spectrofluorimetry was utilized (Figure 1). Additionally, different experimental parameters affecting the intensity of BIS-NBD derivative were investigated to determine the optimum parameters.

The effect of pH on the intensity of the derivative compounds in the range of 8–10 was examined. The graphic that shows the variation of pH on the intensity is in Figure 2.

The influence of temperature and its duration on the intensity of the BIS-NBD derivative were also examined. Four different temperatures in the range of 50–80°C were investigated for NBD derivation. The best results were obtained at 80°C within 20 min.

The effect of time and temperature versus intensity of BIS-NBD derivative is shown in Figure 3. We found that it was necessary to acidify the reaction mixture to pH 2 (by adding 100 μL 1N HCl) before the measurement was carried out.

Chromatographic conditions

Several parameters were examined for the optimization of HPLC analysis of the BIS-NBD associated compound. The first attempt was to find out the consistency of the mobile phase. Therefore, it was thought that a mobile phase should consist of solvent–double distilled water without any pH adjustment, and methanol was preferred as a solvent in this study.

Different mixtures of methanol and water were tried as mobile phase, including 90:10, 85:15, 80:20, 70:30 and 60:40, v/v. The most suitable peaks appeared when a 70:30, v/v solvent system was utilized.

The influence of the flow-rate of the mobile phase on the peak normalization was then studied. The optimum conditions were defined as: mobile phase consisting of 70:30, v/v methanol–water, flow-rate of 1.2 mL/min and detecting at excitation and emission wavelengths of 458 and 525 nm, respectively.

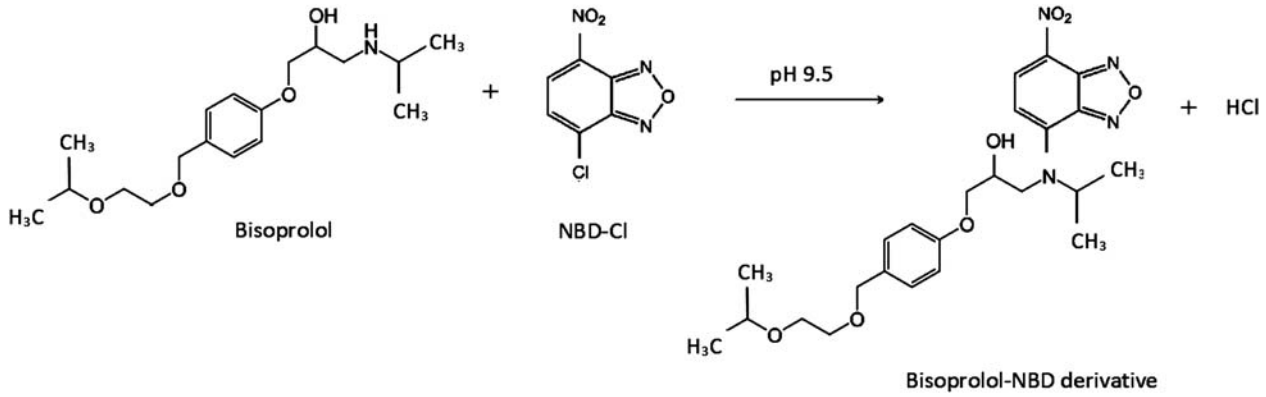

Figure 1. Reaction between BIS and NBD-Cl.

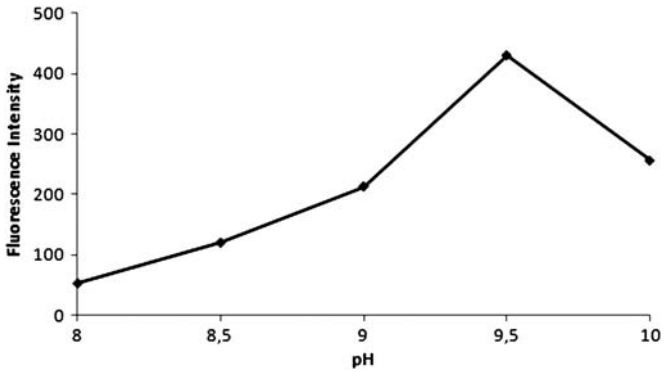

Figure 2. Effect of pH on the intensity of the reaction of BIS with NBD-Cl.

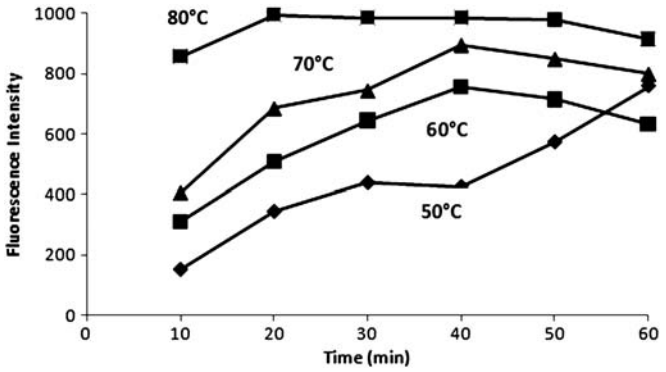

Figure 3. Effect of time and temperature on the reaction of BIS with NBD-Cl.

Typical retention times were approximately 4.79 min for BIS and 3.46 min for IS (Figure 4).

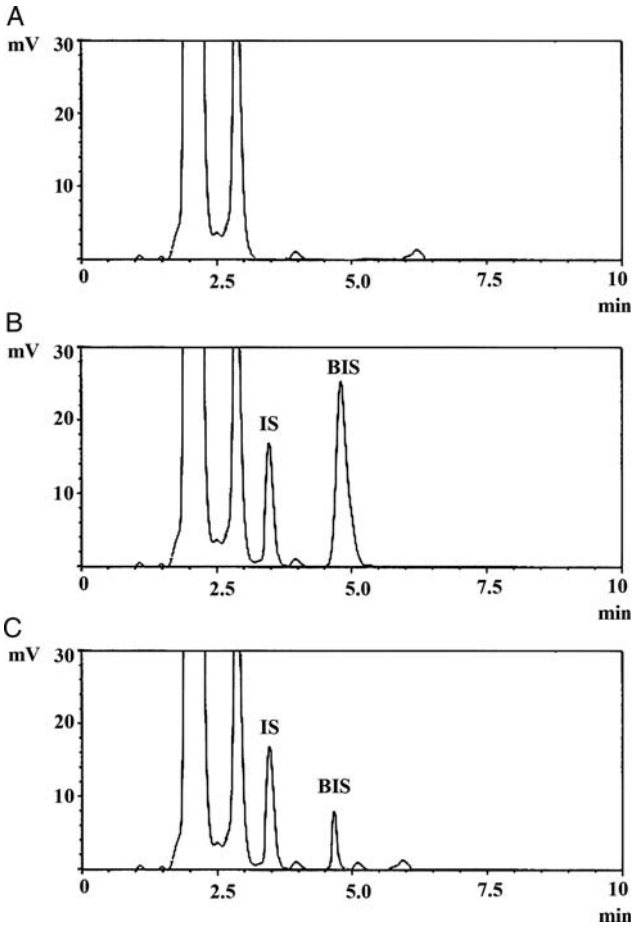

Figure 4. Chromatogram of blank human plasma with NBD-Cl (A); chromatogram of plasma spiked with 1,000 ng/mL BIS-NBD and 50 ng/mL IS-NBD (B); chromatogram of plasma sample obtained from a healthy volunteer 3 h after oral administration of 5 mg of BIS (C).

Method validation

Linearity

Calibration plots were constructed by plotting the concentration against BIS-NBD derivative to IS peak area ratio; these showed good linearity in the 10–2,000 ng/mL range for plasma. As the data show, the method is much more sensitive than most of the reported methods (16–20).

LOD and LOQ

The LOD and LOQ of BIS in plasma were 3.2 and 10 ng/mL, respectively. These values are lower than those obtained by many other reported methods (16–20).

Accuracy and precision

Precision and accuracy were tested by spiking three different concentrations (10, 500 and 2,000 ng/mL) into the plasma

Table I
Intra-Day and Inter-Day Precision and Accuracy of the Assay for BIS ($n = 3$)

Human plasma

Added concentration (ng/mL)	Found concentration (ng/mL) mean ± SD	Precision (RSD %)	Accuracy (RME %)
Intra-day			
10	10.7 ± 0.2	1.86	+7.0
500	497.6 ± 1.74	0.35	−0.48
2,000	1,899.0 ± 1.22	0.64	−5.05
Inter-day			
10	10.65 ± 0.2	1.87	+6.5
500	497.4 ± 1.15	0.23	−0.52
2,000	1,898.3 ± 1.39	0.07	−5.08

Table II
Recovery results of BIS-NBD from plasma ($n = 3$)

Concentration added (ng/mL)	Concentration found (ng/mL) mean ± SD	Recovery (%)	RSD (%)
Plasma			
10	9.76 ± 0.19	97.60	1.94
500	489.0 ± 1.73	97.70	0.35
2,000	1,951.8 ± 1.21	97.59	0.06

Table III
System Suitability Parameters ($n = 3$)

Capacity factor (k')	Tailing factor (T)	Theoretical plates (N)	HETP
9.09	1.04	1,4463	69.14

samples: these were injected concurrently for three days. The results of the precision and accuracy test of the method in human plasma are listed in Table I.

Recovery

The mean recovery results were obtained in the range of 97.59 to 97.70%. These are highly suitable values for the determination of the compounds. Data of the recoveries of BIS are presented in Table II. These values are much better than those obtained by many other methods (3, 5).

Stability

NBD derivatives of BIS in the chloroform were stable for at least four days at 4°C and approximately 3 h at room temperature. The stability in the mobile phase was also tested, and it was found that the samples were stable for at least 3 h at 4°C and 1.5 h at room temperature (in the autosampler).

System suitability

To ascertain the resolution and reproducibility of the HPLC method, system suitability tests were performed using the standard solution of BIS. The optimum HPLC conditions were examined and then resolution (Rs), RSD, theoretical plate number (N), capacity factor (k̀) and tailing factor (T) were investigated as criteria for system suitability testing. The results are all within acceptable limits (Table III).

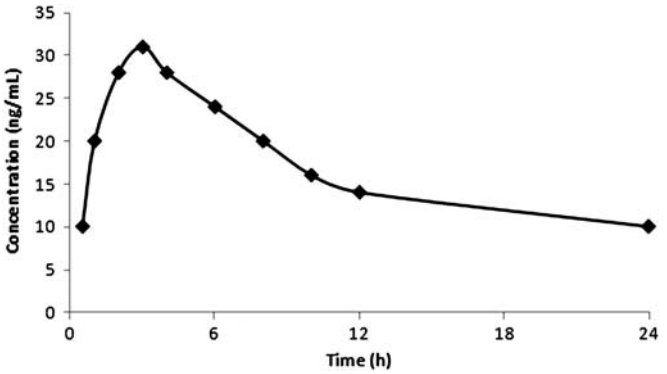

Figure 5. Plasma concentration–time profile of BIS in a healthy volunteer after a single oral administration of 5 mg.

Table IV
Pharmacokinetic Parameters for BIS in Healthy Volunteers

t_{max} (h)	$t_{1/2}$ (h)	C_{max} (ng/mL)	AUC_{0-24} (h ng/mL)
3.0	10.1	31	274.5

Application to a pharmacokinetic study

After a single oral dose administration of 5 mg of BIS to a healthy volunteer, a maximum plasma concentration of 31 ng/mL (C_{max}) was reached at 3 h (t_{max}). The elimination half-life of the drug ($t_{1/2}$) and area under the curve (AUC_{0-24}) were found to be 10.1 h and 274.5 ng x h/mL, respectively (Figure 5, Table IV). These pharmacokinetic parameter values are similar to reported values (2).

Conclusions

A sensitive fluorescence HPLC method was developed and validated for BIS after precolumn NBD-Cl derivatization in human plasma.

In this study, the purpose of the derivatization reaction is to raise the sensitivity and thus the possibility of working in low concentrations. The advantages of the liquid–liquid extraction method include good extraction recovery and simple and less time consuming procedure. In this study, the recovery percentage of BIS is high (3, 5); the derivatization and extraction processes do not take much time. Additionally, according to the other methods, the retention time is quite short (3, 5). In this study, the purpose of the derivatization reaction is to raise the sensitivity and thus the possibility of working in low concentrations.

In summary, this paper describes a sensitive and accurate HPLC method for the quantitation of BIS. The method is suitable to monitor plasma concentrations during clinical pharmacokinetic studies in humans.

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