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LC–ESI-MS method for the determination of bisoprolol in human plasma

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Abstract

A sensitive liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS) method has been developed and validated for the determination of bisoprolol in human plasma, using metoprolol as internal standard (I.S.). After alkalization with sodium hydroxide, the samples were extracted with ethyl acetate and separated by HPLC on a ZORBAX SB-C_{18} column with a mobile phase of 10 mM ammonium acetate buffer containing 0.1% formic acid–methanol (32:68, v/v) at a flow rate of 1 ml/min. The chromatographic separation was achieved in less than 5 min. The linearity was established over the concentration range of 0.05–120 ng/ml. The intra- and inter-run standard deviation was less than 3.8 and 7.5%, respectively. The method had been successfully applied to study the relative bioavailability of bisoprolol fumarate tablets in healthy Chinese volunteers. The pharmacokinetic parameters of the reference and test tablets have been compared.

Keywords: Bisoprolol; LC–ESI-MS; Pharmacokinetics

1. Introduction

Bisoprolol, 1-(4-((2-(1-methylethoxy)ethoxy)methyl)phenoxy)-3-((1-methylethyl)amino)-2-propanol (see Fig. 1(A)), is a synthetic beta 1-selective (cardioselective) adrenoceptor blocking agent. Bisoprolol reduces the heart rate and is useful in treating abnormally rapid heart rhythms. It also reduces the force of contraction of the heart and lowers blood pressure [1,2]. Several HPLC–FL [3,4], HPLC–DAD [5] and LC–MS/MS [6] methods have been reported for the determination of bisoprolol in plasma, in which the most sensitive assay was the LC–MS/MS assay with an LLOQ of 2 ng/ml. The high sensitivity of the analytical method is very important for the investigation of the pharmacokinetics of bisoprolol in humans. So far, limited to the sensitivities of the analytical methods, the dose of 10 mg bisoprolol was chosen for the investigation in all of the pharmacokinetic studies reported in literatures. However, the usual daily dose of the drug for the patients is 5 mg. Even more, for some patients, the appropriate starting dose may be 2.5 mg bisoprolol. Therefore, the most suitable dose for the pharmacokinetic study of the drug is the dose of 5 mg bisoprolol. The results of the pilot pharmacokinetic study of the dose of 5 mg bisoprolol in our laboratory showed that many of the human plasma concentration levels of the drug on the terminal elimination phase were below 1 ng/ml; even some were below 0.2 ng/ml. The LC–MS/MS method reported in the Ref. [6], is developed for the routine therapeutic drug monitoring of 10 antiarrhythic drugs including bisoprolol, not for the pharmacokinetic study of bisoprolol. Though, it reaches an LLOQ as low as 2 ng/ml and is the most sensitive analytical method reported in literatures, it still could not meet the requirements of the pharmacokinetic studies of bisoprolol. Besides, the long chromatographic run time of this method (7 min) and its complex gradient program of the mobile phase may prove to be the other limitations for routine sample analysis. So, to evaluate the pharmacokinetics of bisoprolol in humans, a more sensitive, rapid and simple method is required. This paper described the development and validation of a sensitive and rapid LC–ESI-MS method with an LLOQ as low as 0.05 ng/ml for the quantification of bisoprolol in human plasma, when 1 ml of human plasma sample was used for sample preparation. The method was successfully applied to study the pharmacokinetics of bisoprolol in healthy Chinese volunteers.

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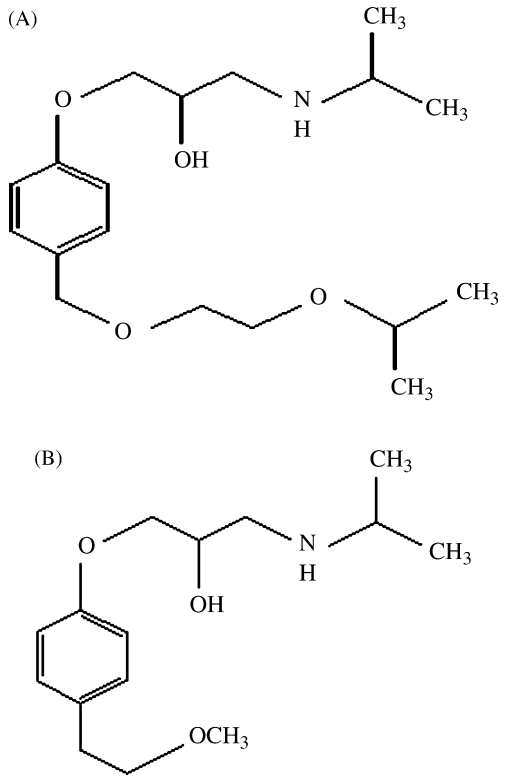

Fig. 1. Chemical structures of bisoprolol (A) and metoprolol (I.S.) (B).

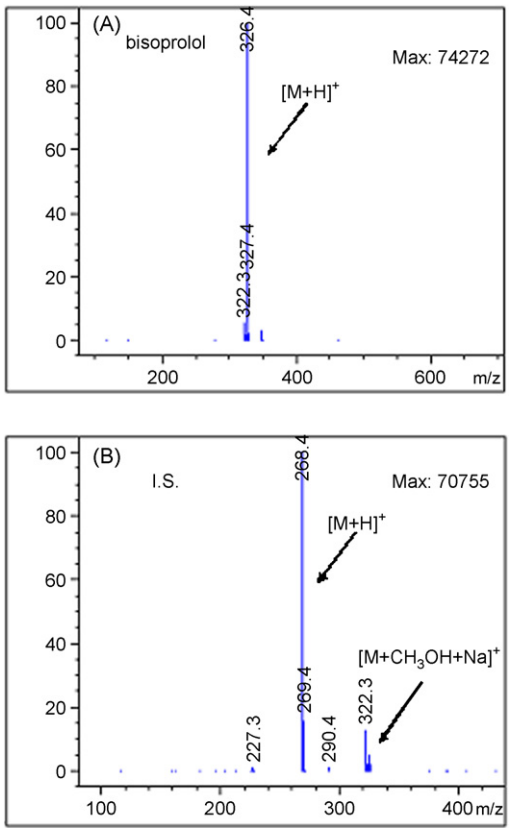

Fig. 2. Mass spectra of the positive ion of bisoprolol (A) and I.S. (B) at 70 V fragmentor voltage.

2. Experimental

2.1. Materials and reagents

Bisoprolol fumarate (99.7% purity) was obtained from Beijing Gao-Bo Pharm-Chemicals Tech. Co., Ltd. (Beijing, China). Metoprolol was supplied by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The test formulation was bisoprolol fumarate tablets (each tablet containing 5 mg bisoprolol fumarate) provided by BeiJing Sihuan Pharmaceutiacal Co., Ltd. (Beijing, China). The reference tablet containing 5 mg bisoprolol fumarate per tablet was obtained from Merck KGaA. Methanol of HPLC grade was purchased from Merck KGaA. All other reagents were of analytical grade and purchased from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China). Distilled water, prepared from demineralized water, was used throughout the study.

2.2. Instrument and conditions

LC–ESI-MS analyses were performed using an Agilent Technologies Series 1100 LC/MSD VL system (Agilent Technologies, Palo Alto, CA) with a ZORBAX SB-C_{18} column, 5 μm, 250 mm × 4.6 mm i.d. (Agilent Technology). The mobile phase was 10 mM ammonium acetate buffer solution containing 0.1% formic acid–methanol (32:68, v/v) at a flow rate of 1 ml/min. The column temperature was maintained at 25 °C. LC–ESI-MS was carried out using nitrogen to assist nebulization. A quadrupole mass spectrometer equipped with an electro-spray ionization source was set with a drying gas (N_2) flow of 10 l/min, nebulizer pressure of 40 psi, drying gas temperature of 350 °C, capillary voltage of 4 kV and the positive ion mode. The fragmentor voltage was 70 V. LC–ESI-MS was performed in selected ion-monitoring mode selecting the protonated molecules, $[M+H]^+$ at *m/z* 326.4, as the target ion for bisoprolol and $[M+H]^+$ *m/z* at 268.4, as the target ion for the I.S., respectively. Fig. 2 shows a typical full-scan ESI mass spectrum of bisoprolol and the I.S. The MS data acquisition started at 2 min after sample injection, and the stream selection valve was set to waste until data acquisition started.

2.3. Preparation of stock and working solutions

The stock (1 mg/ml) and working solutions (10 μg/ml, 1 μg/ml, 100 ng/ml and 10 ng/ml) of bisoprolol were prepared by dissolving an accurately weighed quantity of bisoprolol in methanol and serial dilution with methanol. The stock (1 mg/ml) and working solution (0.5 μg/ml) of the I.S. were prepared in the same way. All the solutions were stored at −20 °C.

2.4. Sample preparation

A 1 ml aliquot plasma sample was transferred to a 10 ml glass tube together with 50 μl I.S. solution (0.5 μg/ml) and 100 μl 1 M sodium hydroxide solution. After vortex mixing for 10 s, 5 ml of ethyl acetate was added, the mixture was vortexed for 3 min. Following centrifugation and separation, the organic phase was

evaporated to dryness under a nitrogen stream in a waterbath of 35 °C. The residue was reconstituted with 100 μl mobile phase, and a 10 μl aliquot was injected into the LC–ESI-MS for analysis.

2.5. Assay validation

2.5.1. Selectivity, linearity, quality control samples

The selectivity of the assay was checked by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Each blank sample should be tested for interference, and no endogenous interferences were encountered.

The calibration standards of bisoprolol were constructed by spiking appropriate amount of the working solutions in 1 ml blank plasma at concentration levels of 0.05, 0.1, 0.3, 1.0, 3.0, 10.0, 30.0, 60.0 and 120.0 ng/ml. And those standard plasma samples were prepared freshly according to the procedures described in Section 2.4. The calibration curve was prepared and assayed along with quality control (QC) samples and each batch of clinical plasma samples.

The QC samples were prepared in blank plasma at the concentration levels of 0.15, 15.0 and 100.0 ng/ml, respectively. The QC samples were prepared independently of the calibration standards and analyzed with processed test samples at intervals in each run. The results of the QC samples provided the basis of accepting or rejecting the run.

2.5.2. Precision and accuracy

The validation samples were prepared and analyzed on three separate runs to evaluate the accuracy, intra- and inter-run precisions of the analytical method. The accuracy, intra- and inter-run precisions of the method were determined by analyzing five replicates at 0.15, 15.0 and 100.0 ng/ml of bisoprolol on each of the three runs. The assay precision was calculated using the relative standard deviation (R.S.D.%). The accuracy is the degree of closeness of the determined value to the nominal true value under the prescribed conditions. The accuracy is defined as the relative deviation in the calculated value (E) of a standard from that of its true value (T) expressed as a percentage (RE%). It was calculated by using the formula: $\mathrm{RE\%} = ((E - T)/T) \times 100$.

2.6. Clinical study design and pharmacokinetic analysis

The method described above was applied to the relative bioavailability study of two bisoprolol tablets. The clinical study protocol was approved by Kunming General Hospital of Chengdu Military Region. All volunteers were given written informed consent to participate in the study according to the principles of the Declaration of Helsinki. Eighteen healthy young male Chinese volunteers were involved. The mean age of the volunteers was 21.6 ± 1.3 (total range: 20–24), and the mean body weight of the volunteers was 60.3 ± 6.7 kg (total range: 51–75 kg). The volunteers were divided into two groups randomly. After an overnight fasting, each volunteer received one test or reference tablet containing 5 mg bisoprolol fumarate, which was taken with 200 ml water. Standard meals were provided at 4 and 9 h post-dose. Blood was sampled pre-dose and at 0.5, 1, 1.5, 2, 3, 4, 6, 9, 14, 24, 36 and 48 h post-dose for determination of the plasma concentrations of bisoprolol. The washout period was 1 week. The bisoprolol plasma concentrations of these samples were determined by the assay described above and the pharmacokinetics and relative bioavailability of the drugs in the volunteers was evaluated. Model-independent pharmacokinetic parameters were calculated for bisoprolol. The maximum plasma concentration (C_{max}) and the time to it (t_{max}) were noted directly. The elimination rate constant (k_{el}) was calculated by linear regression of the terminal points of the semi-log plot of plasma concentration against time. Elimination half-life ($t_{1/2}$) was calculated using the formula $t_{1/2} = 0.693/k_{el}$. The area under the plasma concentration–time curve $AUC_{0–48}$ to the last measurable plasma concentration was calculated by the linear trapezoidal rule.

3. Results and discussion

3.1. Analysis conditions

3.1.1. Conditions for ESI-MS

The mass spectrometric conditions were optimized to obtain maximum sensitivity. Because bisoprolol is a weak basic and medium-polarity compound, the ESI in positive ion mode was adopted for the LC–MS determination of bisoprolol. The LC–ESI-MS was performed in the selected ion-monitoring (SIM) mode. In order to select the target ion for monitoring bisoprolol, the ESI mass spectra obtained by the scan monitoring at different fragmentor voltage were investigated. The test results showed that the base peak (the highest ion peak in the mass spectrum, which can be selected as the target ions of the analytes) in the mass spectra of bisoprolol obtained at different fragmentor voltage was of the same ion at m/z 326.4, which was the protonated molecule $[M + H]^+$ of bisoprolol. Therefore, the protonated molecule $[M + H]^+$ at m/z 326.4 was selected as the target ion for bisoprolol in the SIM. Fig. 2(A) shows a typical mass spectrum of the positive ions of bisoprolol at 70 V fragmentor voltage obtained by the scan monitoring. In order to achieve the highest assay sensitivity for bisoprolol, the optimal fragmentor voltage of the ESI-MS was investigated. The intensities of bisoprolol $[M + H]^+$ ion at m/z 326.4 were compared at the fragmentor voltages of 50, 60, 70, 80, 90, 100 and 110 V. The result showed that the highest sensitivity was obtained using a fragmentor voltage of 70 V. So, the fragmentor voltage was set at 70 V in the ESI-MS assay for bisoprolol. At this fragmentor voltage, the base peak in the mass spectrum of the I.S. was at $[M + H]^+$ m/z 268.4 that was the protonated molecule of the I.S., see Fig. 2(B). Therefore, the positive ion $[M + H]^+$ m/z 268.4 of the I.S. was selected as the target ion for the I.S.

3.1.2. Conditions for chromatography

Metoprolol (see Fig. 1(B)) was chosen as the internal standard, because it is structurally similar to bisoprolol and has similar retention to the bisoprolol. Because bisoprolol and the I.S. were both weak basic compounds, it would easily appear as tailing peak in their chromatograms. The different

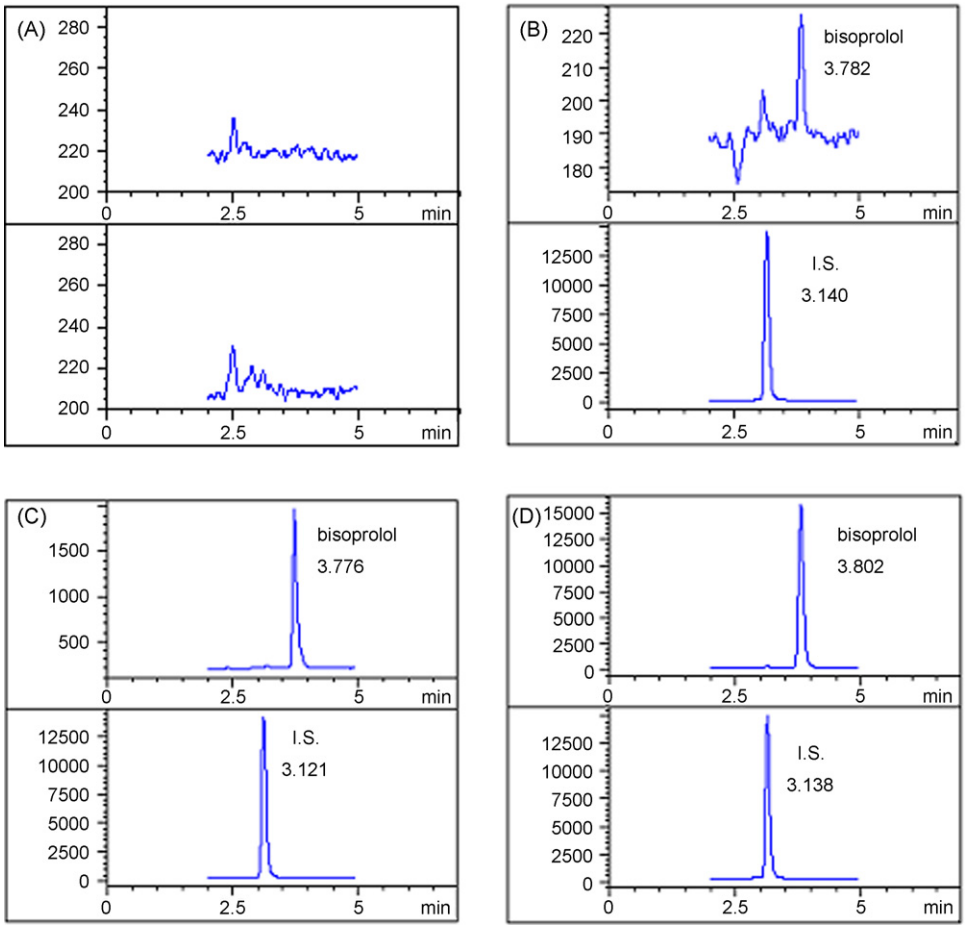

Fig. 3. Typical SIM chromatograms of blank plasma (A), LLOQ for bisoprolol in plasma (0.05 ng/ml) and I.S. (B), plasma spiked with bisoprolol (3 ng/ml) and I.S. (C) and plasma obtained from a volunteer at 1 h after oral administration of 5 mg bisoprolol fumarate, the plasma concentration of bisoprolol was estimated to be 28.42 ng/ml (D).

concentrations of ammonium acetate buffer solution at levels of 10, 20 and 30 mM were tested in the mobile phase to improve the chromatographic peak shapes of analytes [7–9]. The test results showed that the concentration of 10 mM ammonium acetate buffer was satisfying enough to improve the chromatographic peak shapes of bisoprolol and the I.S. and the symmetric chromatography peaks were obtained under this buffer condition. The weak acidic condition of the mobile phase is beneficial to the ionization efficiency of the weak basic compounds in the ESI process of the LC–ESI-MS. Finally, the good separation of target compounds was obtained with a mobile phase of 10 mM ammonium acetate buffer solution containing 0.1% acetic acid–methanol (32:68, v/v). Under the present chromatographic conditions, the run time of each sample was 5 min. The retention times were about 3.8 min for bisoprolol and 3.1 min for the I.S.

3.2. Method validation

3.2.1. Selectivity, calibration curve and lower limits of quantification

The selectivity was assessed by comparing the chromatograms of six different batches of blank plasma with the corresponding spiked plasma. Fig. 3 showed the typical chromatograms of the blank plasma, a spiked plasma sample with bisoprolol and the I.S. at the LLOQ and the plasma sample from a healthy volunteer 1 h after an oral administration. The interferences from the matrices or ion suppression at the expected retention times of the target ions were not observed. The typical retention times for bisoprolol and the I.S. were 3.8 and 3.1 min, respectively.

The bisoprolol calibration curve was constructed by plotting the peak area ratios (f) of bisoprolol to the I.S. versus the concentrations (C) of bisoprolol using weighted least squares linear regression (the weighting factor was $1/C^2$). The typical calibration curve for bisoprolol was $f = 0.0007192 + 0.04328 \times C$, and $r \geq 0.9969$ over the concentration range of 0.05–120 ng/ml.

The LLOQ was found to be 0.05 ng/ml in human plasma. The LLOQ was defined as the lowest concentration on the calibration curve at which precision was within 20% and accuracy was within ±20% [10] and it was established using five samples independent of standards. It showed that the assay was sensitive enough for bioavailability study of the bisoprolol fumarate tablets.

3.2.2. Precision and accuracy

Table 1 summarizes the intra- and inter-run precision and accuracy for bisoprolol. The precision was calculated by using

Table 1
Precision and accuracy of the assay for the determination of isoprolol in human plasma

Parameter	Concentration level (ng/ml)		
	0.15	15.0	100.0
Run 1 (mean ± S.D.)	0.1573 ± 0.0048	14.79 ± 0.27	97.36 ± 4.10
Run 2 (mean ± S.D.)	0.1682 ± 0.0074	14.61 ± 0.77	91.63 ± 3.12
Run 3 (mean ± S.D.)	0.1626 ± 0.0060	14.32 ± 0.48	93.20 ± 2.03
Overall mean	0.1627	14.57	94.06
Intra-assay R.S.D. (%)	3.8	3.8	3.4
Inter-assay R.S.D. (%)	7.5	3.6	7.0
Overall accuracy RE (%)	8.9	−2.5	−5.6

$n = 3$ runs, five replicates per run.

one-way ANOVA. The results in Table 1 demonstrate that the precision and accuracy of this assay were within the acceptable range and the method was accurate and precise.

3.2.3. Matrix effect

The matrix effect (ME) was defined as the direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in the sample [10]. The matrix effect of method was evaluated by comparing the peak area of analytes dissolved in the blank plasma sample's reconstituted solution (the final solution of blank plasma after extraction and reconstitution) (A) with that dissolved in mobile phase (B). The ME was calculated by using the formula: ME (%) = (A/B) × 100%. Three different concentration levels of bisoprolol at 0.15, 15.0 and 100.0 ng/ml were evaluated by analyzing five samples at each level. The blank plasmas used in this study were from five different batches of healthy human blank plasma. If the ratio <85% or >115%, a matrix effect was implied. The ME data at three concentration levels of bisoprolol in five different batches of human plasma were presented in Table 2. The results showed that there was no matrix effect of the analytes observed from the matrix of plasma in this study. Because the I.S. is eluted before bisoprolol from the LC column, the unforeseen matrix effect of the I.S. from the metabolites of bisoprolol in clinical samples should be considered, since the metabolites would be also eluted before bisoprolol. The matrix effect of the I.S. from the metabolites of bisoprolol was also evaluated by comparing the peak area of the I.S. dissolved in the reconstituted solutions of the clinical plasma samples obtained from the pilot study with that dissolved in mobile phase. The test results show that no matrix effect of the I.S. from the metabolites of bisoprolol was observed. Besides, the fact that the mass response of the I.S. of the clinical samples in each analytical run was very stable also demonstrated that there was no matrix effect of the I.S. from the metabolites of the drug.

3.2.4. Extraction recovery

The extraction recovery of the method was determined by comparing the peak areas obtained from the plasma samples with those of direct injected standards. The direct injected standards were dissolved in the blank plasma sample's reconstituted solution (the final solution of blank plasma after extraction and reconstitution). The extraction recovery was evaluated at three different concentration levels of 0.15, 15.0 and 100.0 ng/ml. The liquid–liquid extraction was used for the sample preparation in this work. Several solvents were tested for the extraction. Finally, ethyl acetate was chosen as the extraction solvent, because it can produce a clean chromatogram for the blank plasma samples and yield a higher recovery for bisoprolol from the plasma. The extraction recovery at three concentration levels of 0.15, 15.0 and 100.0 ng/ml were 90.8 ± 4.0, 96.9 ± 1.0 and 92.0 ± 2.3% ($n = 5$), respectively.

3.2.5. Stability

The stability of bisoprolol in plasma was studied under a variety of storage and handling conditions at low (0.15 ng/ml) and high (100.0 ng/ml) concentration levels. The short-term temperature stability was assessed by analyzing three aliquots of each of the low and high concentration samples that were thawed at room temperature and kept at this temperature for 8 h. Freeze–thaw stability (−20 °C in plasma) was checked through three cycles. Three aliquots at each of the low and high concentrations were stored at −20 °C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. The freeze–thaw cycles were repeated three times and then analyzed on the third cycle. The long-term stability was determined by analyzing three aliquots of each of the low and high concentrations stored at −20 °C for 4 weeks. The results in Table 3 showed that no significant degradation of bisoprolol was observed under the tested conditions.

3.3. Application

The method was successfully applied to determine the plasma concentration of bisoprolol up to 48 h after an oral administration of the reference and the test formulations of bisoprolol fumarate

Table 2
Matrix effect evaluation of bisoprolol and I.S. in human plasma ($n = 5$)

Samples	Concentration level (ng/ml)	A (mean ± S.D.)	B (mean ± S.D.)	Matrix effect (%)
Bisoprolol	0.15	530 ± 41	481 ± 23	110.1
	15.0	39618 ± 660	36517 ± 645	108.5
	100.0	262242 ± 16627	244753 ± 17659	107.1
I.S.	500.0	57391 ± 2826	52891 ± 3353	108.5

(A) The peak area of analytes dissolved in blank plasma sample's reconstituted solution (the final solution of blank plasma after extraction and reconstitution). (B) The peak area of analytes dissolved in mobile phase.

Table 3
Stability data of bisoprolol in human plasma under various storage conditions ($n = 3$)

Storage conditions	Concentration level (ng/ml)	Calculated concentration (ng/ml)	Intra-run R.S.D. (%)	RE (%)
Room temperature for 8 h	0.15	0.1590	1.3	6.4
	100.0	100.2	1.5	0.6
Three freeze–thaw cycles	0.15	0.1692	0.3	13.3
	100.0	91.47	2.6	−8.2
4 weeks at −20 °C	0.15	0.1668	1.3	11.6
	100.0	95.37	0.9	−4.2

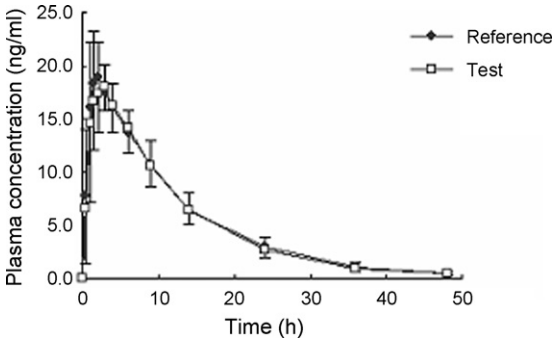

Fig. 4. Mean plasma concentration–time profile of bisoprolol after an oral administration of 5 mg bisoprolol fumarate to 18 healthy volunteers.

Table 4
Mean pharmacokinetic parameters of bisoprolol for 18 volunteers after oral administration of the test and reference bisoprolol fumarate tablets

Parameters	Test tablet	Reference tablet
$t_{1/2}$ (h)	8.4 ± 1.2	8.1 ± 1.0
C_{max} (ng/ml)	20.3 ± 3.3	20.6 ± 3.3
t_{max} (h)	1.9 ± 0.9	1.9 ± 0.7
$AUC_{0–48}$ (h ng/ml)	244.0 ± 38.5	249.0 ± 41.0

Mean ± S.D., $n = 18$.

to 18 volunteers. The mean plasma concentration–time curve of bisoprolol was shown in Fig. 4. The main pharmacokinetic parameters of bisoprolol in 18 volunteers were calculated and summarized in Table 4. The relative bioavailability of the test formulation was 98.4 ± 10.6%, based on the test–reference ratio of AUC. There were no remarkable differences between the test formulation and the reference formulation in bioavailability.

4. Conclusion

The method had a good sensitivity and specificity for the determination of bisoprolol in human plasma. No significant interferences and matrix effect caused by endogenous compounds were observed. The method is suitable for the pharmacokinetic study and bioavailability evaluation of bisoprolol formulations and can also be used as a therapeutic drug monitoring method in clinic to check the plasma concentration of bisoprolol in the patients.

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