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Determination of nadolol diastereomers in dog plasma using chiral derivatization and reversed-phase high-performance liquid chromatography with fluorescence detection

Masanori Hoshino*, Koichi Yajima, Yasutaka Suzuki, Akira Okahira

Central Research Laboratories, Zeria Pharmaceutical Co., 2512-1 Oshikiri, Konan-machi, Osato-gun, Saitama 360-01, Japan

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Abstract

A stereospecific high-performance liquid chromatographic method has been developed for the determination of four diastereomers of nadolol in plasma. After the nadolol diastereomers were extracted from plasma using an Extrelut-1 solid-phase extraction cartridge, they were derivatized with (*R*)-(−)-1-(1-naphthyl)ethylisocyanate to form urea derivatives. These derivatives were then separated on a YMC-AM-303 ODS column using water–acetonitrile (60:40, v/v). The calibration curves of (*SR*)-, (*RS*)-, (*SS*)- and (*RR*)-nadolol were linear over the range 2.5–200 ng/ml, and the correlation coefficient (*r*) of the curves were higher than 0.9991 for each diastereomer. The limit of quantification was 2.5 ng/ml for each diastereomer in plasma. This method was used for a pharmacokinetic study in four dogs after oral administration of nadolol (1 mg/kg). The plasma concentrations of nadolol diastereomers showed no significant differences in C_{max}, T_{max} or AUC values. The assay appears to be readily applicable to the study of diastereoselective nadolol pharmacokinetics in animals and humans.

1. Introduction

Nadolol is a long-acting β-adrenoceptor blocking agent currently used in the treatment of angina pectoris and hypertension in many countries. Nadolol has three asymmetric carbon atoms in its chemical structure, and two hydroxyl groups in the tetrahydronaphthalene ring form a *cis* configuration. Therefore, it has two racemates and four diastereomers. One of the racemates has both (*RS*)- and (*SR*)-configurations, and the diastereomers of the other racemate are (*RR*)- and (*SS*)-configurations [1].

Since commercially available nadolol is a mixture of approximately equal proportions of the two racemates, the pharmacology, metabolism and pharmacokinetics of the four diastereomers of nadolol have important clinical implications. As a prerequisite for such studies, the concentrations of the individual stereoisomers of the drug should be determined with regard to differences in the activities of its diastereomers. Numerous analytical methods have been reported for the assay of nadolol [2–6] and the racemates [7] in biological fluids. However, to our knowledge, no pharmacokinetic studies of the effects of nadolol diastereomers in humans or animal systems have yet been reported. A sensitive analytical method is required for the

* Corresponding author.

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determination of the diastereomers of nadolol at levels of a few nanograms in plasma.

In the present report, we describe a sensitive high-performance liquid chromatographic (HPLC) method for the stereoselective determination of the four diastereomers of nadolol in dog plasma. The method involves derivatization with an optically pure fluorescent reagent, (*R*)-(−)-1-(1-naphthyl)ethylisocyanate, to enhance detection efficiency and to allow quantification of each diastereomer in the nanogram range. The method is suitable for pharmacokinetic studies of nadolol diastereomers.

2. Experimental

2.1. Reagents and chemicals

Racemic nadolol (Fig. 1) was purchased from Sigma (St. Louis, MO, USA), and *RS*(+ −), *SR*(− +), *RR*(+ +) and *SS*(− −)-nadolol were prepared in our laboratory as previously described [8]. (*R*)-(−)-1-(1-Naphthyl)ethylisocyanate [*R*(−)-NEI] was obtained from Aldrich (Milwaukee, WI, USA). The optical purity of this derivatizing reagent was higher than 99%. NEI solution was made by dissolving 50 mg of *R*(−)-NEI in 10 ml of acetonitrile. The Extrelut-1 solid-phase extraction cartridges (Merck, Darmstadt, Germany) were obtained from Kanto Chemical (Tokyo, Japan). Acetonitrile, ethanol, methanol and *n*-hexane were of HPLC grade (Wako, Osaka, Japan). All other reagents were of analytical grade and used without further purification. Water was double-distilled and filtered using a Millipore Milli-Q filtration system (Bedford, MA, USA).

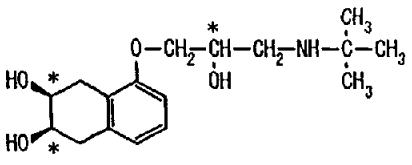


Fig. 1. Structure of nadolol. Asterisks denote the locations of chiral centres.

2.2. Apparatus and HPLC conditions

The chromatograph was an HP-1050 system consisting of a pump (HP-79851A), an autosampler (HP-79855A) (all Hewlett-Packard, Waldbronn, Germany) and a fluorescence detector (RF-550, Shimadzu, Kyoto, Japan). Separation was carried out on a YMC-AM-303 ODS column (250 × 4.6 mm I.D., particle size 5 μm) (YMC, Kyoto, Japan). ODS columns from other manufacturers were tested with this system, however, the YMC-AM-303 ODS column provided the best separation. The mobile phase used was water–acetonitrile (60:40, v/v), and the flow-rate was maintained at 1.0 ml/min. The column temperature was 40°C throughout. The excitation and emission wavelengths of the detector were set at 285 nm and 340 nm, respectively.

2.3. HPLC purities of the derivatives

R(−)-NEI derivatives of nadolol diastereomers were synthesized in our laboratory. A 5-μl volume of a 1 mg/ml methanol solution of each derivative was injected onto a YMC-AM-303 ODS column on-line in the above HPLC system, and the HPLC purity of each derivative was calculated from the areas of peaks other than those of the solvent.

The HPLC purities of *RS*(+ −)-, *SR*(− +)-, *RR*(+ +)- and *SS*(− −)-nadolol derivatives were 96.8%, 94.3%, 97.5% and 96.0%, respectively.

2.4. Stability of the derivatives

A 10-μl volume of acetonitrile was added to 50 μl of the various reaction solvents containing 100 ng of the derivatized (*SS*)-nadolol standard. After standing at 45°C for 5 min, the amount of each derivative remaining (%) was determined by HPLC.

2.5. Mass spectrometry

A mass spectrometer was used to determine the structure of the derivatized nadolol dia-

stereomers. Mass spectra were measured with a JMS-DX300 instrument (JEOL, Tokyo, Japan) in the fast-atom bombardment (FAB) mode. The samples were analyzed in an *m*-dinitrobenzyl alcohol matrix on a stainless-steel probe tip at ambient temperature at 6 keV.

2.6. Sample preparation

In a 10-ml glass tube, 0.5 ml of plasma and 0.1 ml of a 1 *M* sodium hydroxide solution were gently vortex-mixed for 1 min, after which the mixture was loaded onto an Extrelut-1 column. The tube was washed with 0.3 ml of water and the washing was also added to the column. After standing for at least 15 min, the column was eluted with 10 ml of diethyl ether. The eluate was evaporated under a gentle stream of nitrogen at 40°C, and the residue was reconstituted with 0.1 ml of water and 0.2 ml of methanol. The solution was then transferred to a 1-ml glass microtube. The empty vessel was washed with 0.2 ml of methanol, and the washing solution was also added to the microtube. The combined solution was then evaporated under a gentle stream of nitrogen at 50°C.

2.7. Derivatization

The residues were reconstituted in 50 μl of methanol, and 10 μl of NEI solution was then added. The mixtures were vortex-mixed for 30 s and heated at 45°C for 5 min in a sand bath. After derivatization, the solvent was evaporated under a gentle stream of nitrogen at room temperature, and the residue was reconstituted with 0.1 ml of methanol. Aliquots (20 μl) were injected onto the HPLC system.

2.8. Standard solutions

Stock solutions of *RS*-, *SR*-, *RR*- and *SS*-nadolol and racemic nadolol were prepared at a concentration of 40 μg/ml in water. Working standard solutions of appropriate concentrations were made by diluting aliquots of each stock solution with water.

2.9. Extraction yields

The recovery of nadolol from plasma was examined. A 5-μl aliquot of stock standard solution of racemic nadolol and 0.1 ml of 1 *M* sodium hydroxide were added to 0.5 ml of blank control plasma. The mixture was shaken in a vortex-mixer for 1 min, then loaded onto an Extrelut-1 column and extracted with 10 ml of diethyl ether, dichloromethane or ethyl acetate. The collected eluents were evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 0.1 ml of water and 0.4 ml of methanol, and this solution was again evaporated to dryness under a stream of nitrogen. The resultant residue was then dissolved in 100 μl of methanol, and a 20-μl aliquot of this solution was injected onto the HPLC system under non-stereospecific conditions. HPLC analysis was carried out according to the method described by Noguchi et al. [6]. Absolute recovery of the drug from plasma was assessed by comparing their peak areas after the extraction procedure with those obtained by direct injection of the same amount of stock solution.

2.10. Derivatization yields

A 10-μl aliquot of NEI solution was added to 50 μl of methanol solution containing 25, 50, 100 and 400 ng of racemic nadolol ($n = 3$ for each concentration). The mixtures were derivatized according to the procedure described above. Derivatization yields were calculated by comparing peak areas of the derivatives of nadolol diastereomers to those of derivatized *RR*-nadolol standard.

2.11. Calibration curves

Calibration curves were established by analysis of 0.5-ml plasma samples spiked with different concentrations of the nadolol diastereomers. These samples were extracted and derivatized as in the assay procedure.

2.12. Precision and accuracy

The precision of the method was assessed by the intra- and inter-assay coefficients of variation (C.V.) of analyses ($n = 5$) of dog plasma samples containing individual nadolol diastereomers at three concentrations. The accuracy of the assay was expressed as: (determined concentration/spiked concentration) · 100. The inter-day C.V. was determined every day over a 5-day period. Concentrations of each diastereomer in dog plasma samples were calculated from the linear regression of the corresponding standard curve.

2.13. Data analysis

The regression line of the peak area (y-axis) versus plasma concentration (x-axis) was calculated by linear least-squares regression analysis, and the concentrations in dog plasma samples were estimated from the regression line.

The observed plasma concentration–time data for each diastereomer were used to determine the maximum plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}). The plasma concentration–time curve was fitted to a two-compartment open model by least-squares regression analysis to calculate the elimination half-lives ($T_{1/2}$). The area under the plasma concentration–time curve (AUC) up to the last sampling point was calculated by a trapezoidal rule.

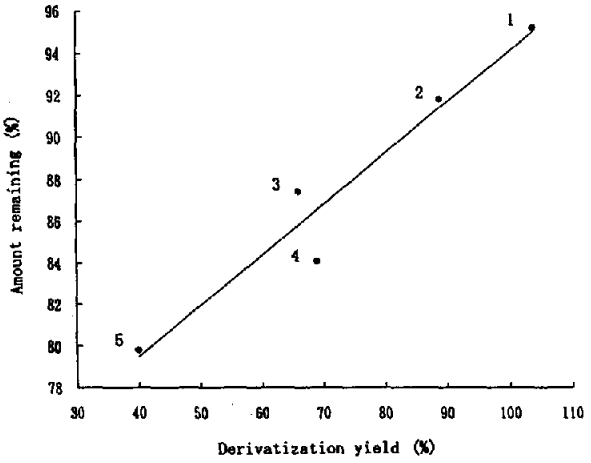


Fig. 2. Correlation between the stability of the derivatives and the derivatization yields of nadolol in reaction solvents. The derivatization yields of four diastereomers were determined in various solvents initially containing 100 ng of racemic nadolol. (1) methanol; (2) ethanol; (3) acetonitrile; (4) n-hexane; (5) dichloromethane.

2.14. Dog study plasma samples

Blank control plasma was collected from dogs for at least two weeks before sampling. A single 1 mg/kg dose of nadolol was orally administered to each dog. Plasma samples were collected at 0.5, 1, 2, 3, 6, 8, 10, 24 and 48 h after dosing. All samples were stored at −20°C until analysis.

3. Results and discussion

3.1. Derivatization

The NEI derivatizing agent reacts with primary and secondary amines to form urea derivatives, which can be separated on a reversed-phase column. This chiral reagent has been used to separate and determine the enantiomers of a variety of β-blocker drugs as a highly sensitive and selective fluorescence derivatization reagent for primary and secondary amines [9–14].

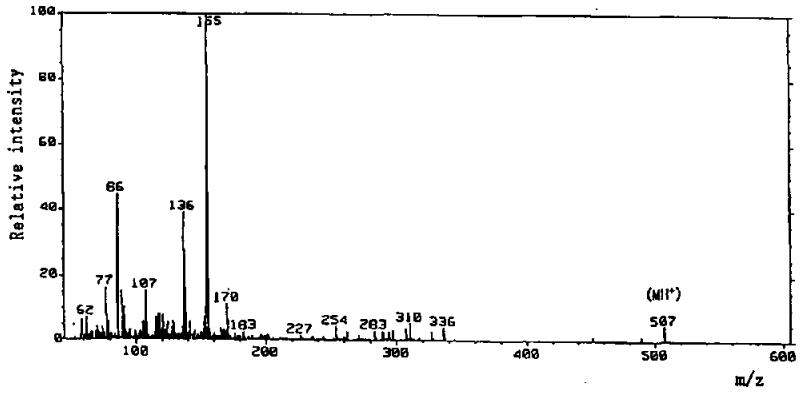
Piquette-Miller et al. [10] reported a method for the stereospecific determination of acebutolol in biological fluids using the chiral $S(+)$-NEI reagent. They described this method as applicable to a wide range of β-blocker drugs possessing a secondary amine with a hydroxyl group in the β-position such as bupranonol, nadolol and propranolol. In a similar manner, we performed nadolol derivatization with $S(+)$- and $R(-)$-NEI. However, the extent of derivatization of nadolol was less than 30%.

During the course of investigating the derivatization reaction of nadolol and $R(-)$-NEI, we found that the derivatization yield was influenced considerably by the reaction solvent. Also, the resulting derivative was found to show differences in stability in various reaction sol-

Table 1
Effect of mobile phase solvent ratio on capacity factor (k') and resolution (R_s) of the derivatives of nadolol diastereomers

Composition[a] (v/v)	k'				R_s [b]		
	(*RS*)	(*SR*)	(*SS*)	(*RR*)	(*RS*)–(*SR*)	(*SR*)–(*SS*)	(*SS*)–(*RR*)
65:35	27.7	29.1	31.9	33.9	1.3	2.3	1.5
60:40	14.3	15.1	16.1	17.2	1.3	1.5	1.5
55:45	7.4	7.9	8.2	8.8	1.0	0.8	1.3
50:50	4.4	4.7	4.7	5.1	0.9	–[c]	0.9
45:55	2.9	2.9	2.9	3.2	–	–	0.8

Column, YMC-AM-303 ODS (250 × 4.6 mm I.D.); mobile phase, water–acetonitrile; flow-rate, 1.0 ml/min; column temperature, 40°C.
[a] Composition of water–acetonitrile.
[b] R_s value between each diastereomer.
[c] Overlapping of peaks.



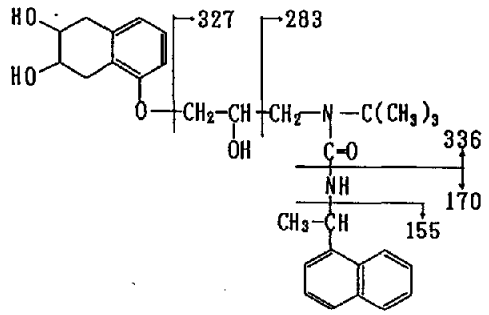


Fig. 3. Typical mass spectrum of the reaction products of (*RR*)-nadolol with *R*-(−)-1-(1-naphthyl)ethylisocyanate obtained under fast-atom bombardment.

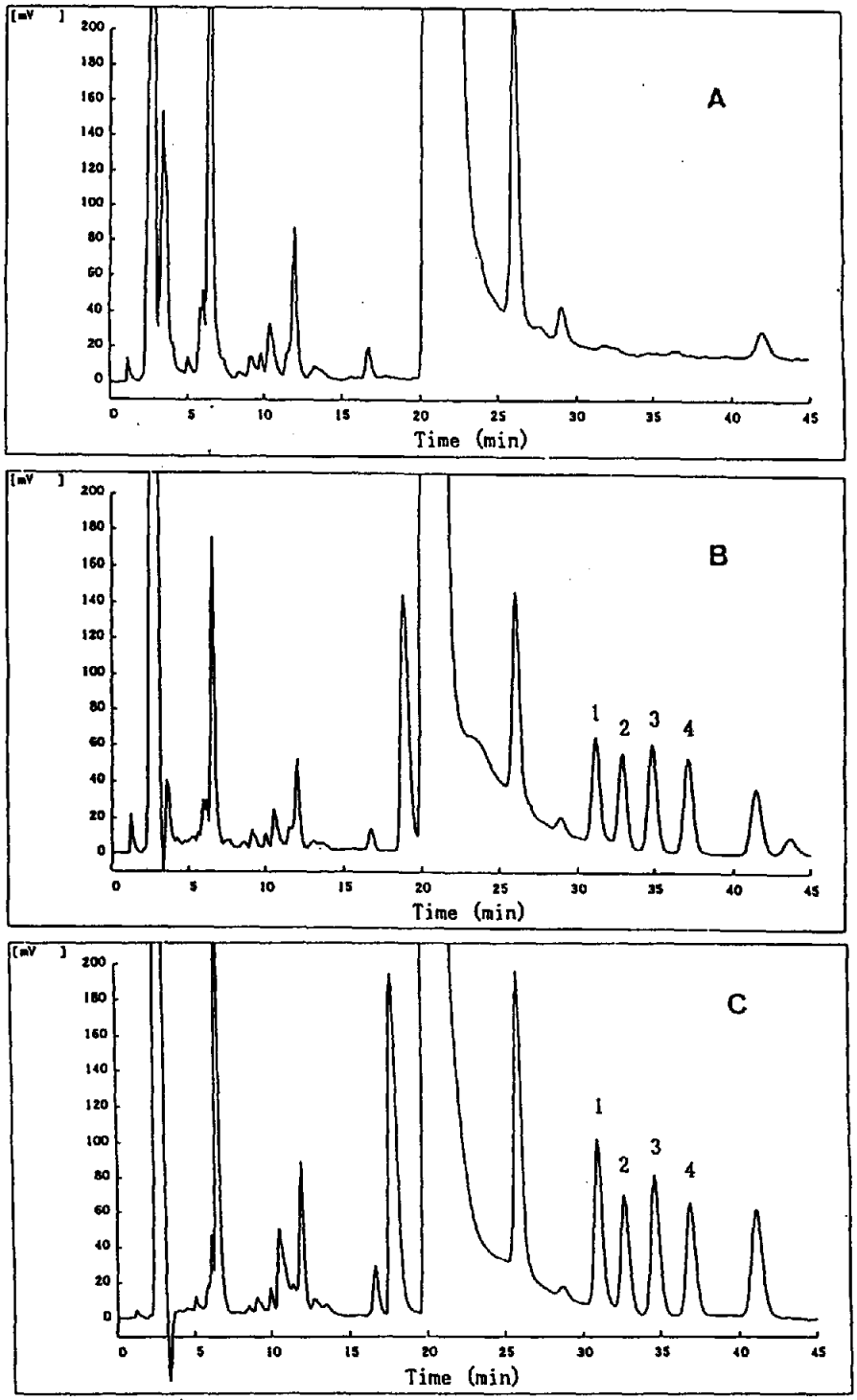


Fig. 4. Typical chromatograms of (A) blank control dog plasma, (B) plasma spiked with 50 ng/ml of each diastereomer and (C) plasma obtained 2 h after oral administration of 1 mg/kg of racemic nadolol. Peaks: 1 = (*SR*)-nadolol; 2 = (*RS*)-nadolol; 3 = (*RR*)-nadolol; 4 = (*SS*)-nadolol.

vents. As a result of a more detailed investigation, the derivatization yield and the stability of the derivative were found to show good correlations in various reaction solvents (r = 0.975) (Fig. 2). From these results, methanol was chosen as the solvent for the derivatization reaction in the present study.

The optimum conditions for NEI derivatization of nadolol were studied in terms of amounts of NEI solution (2, 6, 10 and 16 μl) and reaction time (3, 5, 10 and 20 min at 45°C). The optimal amounts of NEI solution and reaction time were 10 μl and 5 min, respectively. The derivatization yield under these optimum conditions was higher than 99% over the concentration range 25–400 ng of racemic nadolol.

The nature of the four diastereomer derivatives was confirmed by mass spectrometry. The fragmentation pattern is shown in Fig. 3. The MH^+ ion at m/z 507 corresponded to the addition of one NEI group (mol.wt. of nadolol = 309), and the fragment observed at m/z 283 indicated a reaction occurring on the secondary amine of nadolol. Thus, the selective reaction with the secondary amine group in the nadolol structure and isocyanate group of NEI was

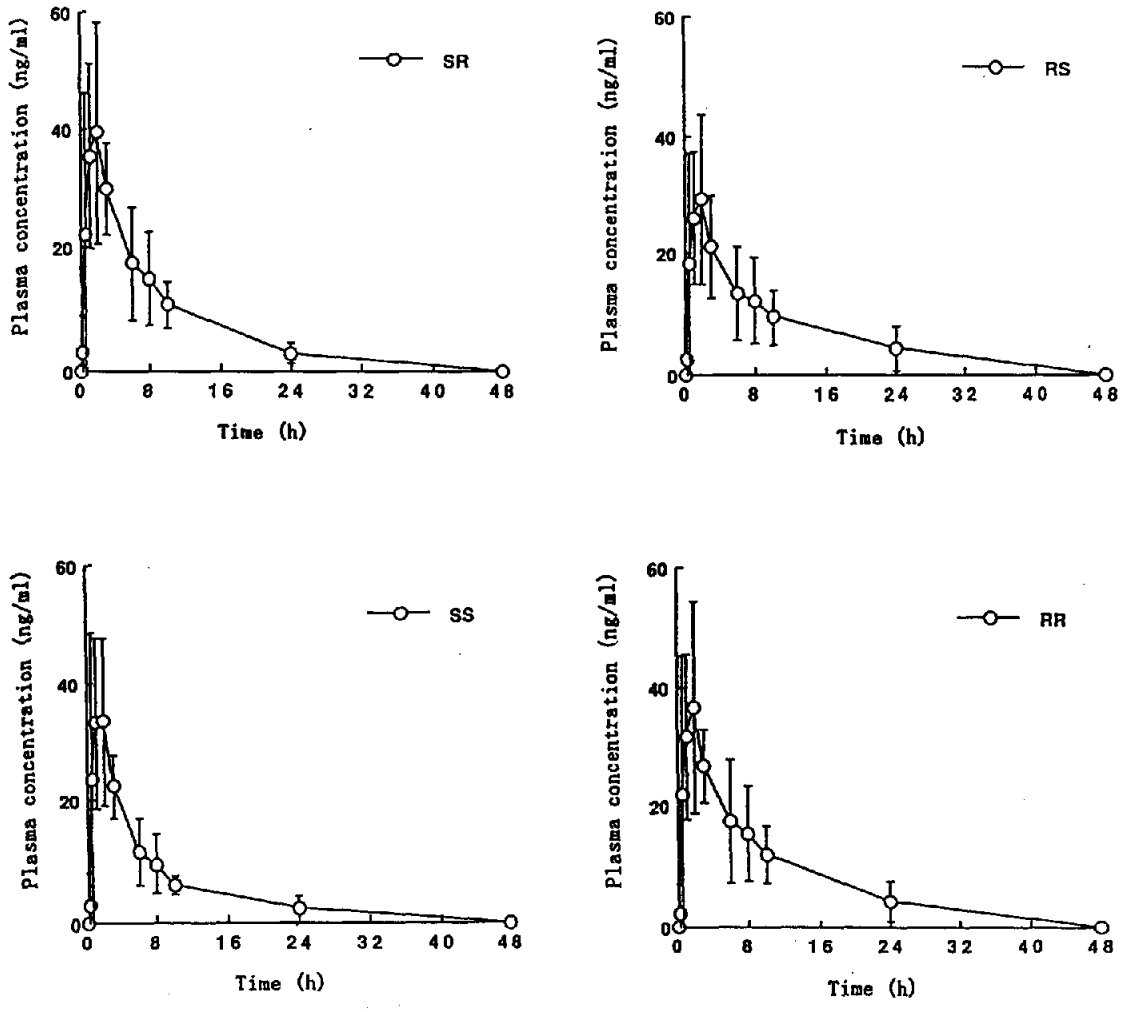


Fig. 5. Plasma concentrations of (*SR*)-, (*RS*)-, (*RR*)- and (*SS*)-nadolol versus time after a single oral administration of 1 mg/kg of racemic nadolol to four dogs. Each point represents the mean ± S.D. of four dogs.

confirmed to have occurred with the derivatives of nadolol.

3.2. Chromatographic separation

From the results of a preliminary study, the mobile phase used was acetonitrile–water, and effects of changes in the compositional ratio were evaluated on a YMC-AM-303 ODS column. The k' and R_s values improved as the proportion of acetonitrile decreased (Table 1). Therefore, acetonitrile–water (40:60, v/v) was used as the mobile phase considering the sharpness of peaks, the analysis time and the R_s value. The peaks of the diastereomers did not suffer from interference from excess reagent, reaction by-products or endogenous components from plasma. Typical chromatograms of blank control dog plasma, spiked dog plasma and dog plasma obtained 2 h after administration of an oral dose of 1 mg/kg of racemic nadolol are shown in Fig. 4. The retention times of (*SR*)-, (*RS*)-, (*RR*)- and (*SS*)-nadolol were approximately 31, 33, 35 and 37 min, respectively.

3.3. Extraction

Nadolol in plasma was extracted based on a solid-phase procedure using an Extrelut-1 column. The plasma sample was alkalized with 0.1 ml of 1 *M* sodium hydroxide prior to application onto the Extrelut-1 column. Although the eluents were examined using dichloromethane, ethyl acetate and diethyl ether, the extraction efficiency was less than 90%, except with diethyl ether which gave a yield of 96.6% (mean of $n = 6$) with a C.V. of 2.1%.

3.4. Accuracy and precision

The accuracy and precision of the present method were evaluated by analyzing plasma samples spiked with various concentrations of each diastereomer (Table 2). The C.V.s of the

Table 2
Precision and accuracy in the assay of nadolol diastereomers in plasma ($n = 5$)

Intra-assay				Inter-assay			
Added (ng/ml)	Recovered (mean ± S.D.) (ng/ml)	C.V. (%)	Accuracy (%)	Added (ng/ml)	Recovered (mean ± S.D.) (ng/ml)	C.V. (%)	Accuracy (%)
(*SR*)							
12.0	10.5 ± 0.6	6.7	87.5	12.3	13.8 ± 1.3	10.9	112.2
96.4	99.4 ± 3.7	4.1	103.1	98.5	94.4 ± 5.4	6.4	95.8
190.6	189.1 ± 7.0	4.1	99.2	197.0	198.6 ± 9.8	5.5	100.8
(*RS*)							
11.5	9.9 ± 1.0	11.2	86.1	11.8	13.8 ± 0.9	8.0	116.9
92.3	95.7 ± 1.6	1.9	103.7	94.5	90.1 ± 2.7	3.3	95.3
182.7	181.0 ± 8.1	5.0	99.1	188.9	190.7 ± 8.3	4.7	101.0
(*RR*)							
13.6	12.7 ± 0.6	4.7	93.4	13.9	16.4 ± 1.2	7.9	118.0
108.3	110.6 ± 2.7	2.7	102.1	111.4	106.4 ± 5.4	5.7	95.5
215.4	214.4 ± 8.1	4.2	99.5	222.7	224.9 ± 10.5	5.2	101.0
(*SS*)							
13.1	12.9 ± 0.6	5.4	98.5	13.5	14.9 ± 1.0	7.4	110.4
105.2	105.6 ± 1.8	1.9	100.4	107.7	101.5 ± 4.4	4.8	94.2
208.2	208.0 ± 7.9	4.2	99.9	215.4	217.0 ± 7.3	3.8	100.7

Accuracy = (determined concentration/spiked concentration) · 100.

Table 3
Pharmacokinetic parameters of nadolol diastereomers after single oral administration of 1 mg/kg of nadolol in dogs

Parameter	Value (mean S.D.) ($n = 4$)			
	(*SR*)	(*RS*)	(*RR*)	(*SS*)
C_{max} (ng/ml)	44.95 ± 15.78	33.80 ± 12.29	43.04 ± 14.71	40.35 ± 13.95
T_{max} (h)	1.9 ± 1.0	1.9 ± 1.0	1.9 ± 1.0	1.9 ± 1.0
AUC_{0-24} (ng h/ml)	348.44 ± 126.37	350.71 ± 186.18	392.07 ± 179.24	255.36 ± 89.40
$T_{1/2\alpha}$ (h)	2.35 ± 0.82	2.36 ± 1.01	2.43 ± 1.04	1.86 ± 0.74
$T_{1/2\beta}$(h)	7.08 ± 1.54	11.33 ± 6.73	9.04 ± 2.68	7.18 ± 3.80

determined values were less than 10.9%, 11.2%, 9.2% and 9.9% for (*SR*)-, (*RS*)-, (*RR*)- and (*SS*)-nadolol, respectively, and accuracy was 94.4–111.2%. Calibration curves for each diastereomer showed good linearity in the concentration range of 2.5–200 ng/ml in plasma, and the correlation coefficient was greater than 0.999 in all cases. The limit of detection of each diastereomer in plasma was 2.5 ng/ml, representing 50 pg injected.

3.5. Application of the method

This method was used to measure plasma levels of the diasteromers of nadolol in dogs. After oral administration of racemic nadolol (1 mg/kg), the plasma concentration–time profile of (*RS*)-, (*SR*)-, (*SS*)- and (*RR*)-nadolol were obtained and are shown in Fig. 5. The plasma concentrations of (*RS*)-, (*SR*)-, (*SS*)- and (*RR*)-nadolol showed no differences, that is there were no significant differences in the C_{max}, T_{max}, AUC or MRT values between those diastereomers. Mean values of pharmacokinetic parameters are listed in Table 3.

4. Conclusions

The analytical method described in the present study is a convenient HPLC method for the simultaneous determination of the diastereomers of the nadolol, with good separation and appropriate sensitivity for detection during single-dose pharmacokinetic studies.

The extraction and derivatization procedures are rapid and simple. The method is expected to be applicable to pharmacokinetic studies in rats, mice, dogs and humans.

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