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Short Communication

Direct high-performance liquid chromatographic separation of the racemates and diastereomers of nadolol

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Abstract

The direct separation of the two racemates and four diastereomers of nadolol by high-performance liquid chromatography (HPLC) is described. The racemates were well separated on non-chiral ODS columns using mixtures of phosphate buffer and methanol. The diastereomers could be separated on commercial chiral columns, using organic and aqueous mobile phases. The analytical HPLC method using an organic mobile phase was applied to semi-preparative HPLC for diastereomers.

1. Introduction

Nadolol is an important β-adrenoceptor antagonist widely used in the treatment of cardiovascular disorders and essential hypertension. Although nadolol contains three asymmetric carbons, two hydroxy groups of the tetrahydronaphthalene ring form a *cis* configuration. Therefore, it has two racemates and four diastereomers. Commercial nadolol is a mixture of approximately equal proportions of the two racemates, namely + −/− + (*RS*/*SR*) and + +/− − (*RR*/*SS*) referred to as racemates A and B, respectively [1].

Matsutera *et al.* [2] and Lacroix *et al.* [3] have reported the high-performance liquid chromatographic (HPLC) separation of the two racemates of nadolol. Schill *et al.* [4] reported that each racemate of nadolol was resolved into its diastereomers by HPLC on an α_1-acid glycoprotein (α_1-AGP) column, but they did not report the separation of all four stereoisomers in a single run. Lee *et al.* [5] studied the direct separation of the four diastereomers of nadolol using various HPLC column packings, but the separation was successful only on an α_1-AGP column. Dyas *et al.* [6] separated the four diastereomers on a Pirkle-type chiral stationary phase after derivatization with 1-naphthyl isocyanate and assigned each peak to configurations.

This paper describes HPLC methods for the direct separation of the two racemates and four diastereomers of nadolol. The method for separating the diastereomers was used also for

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semi-preparative HPLC to isolate the four diastereomers.

2. Experimental

2.1. Reagents and chemicals

Nadolol (**1**) (Fig. 1) was purchased from Sigma (St. Louis, MO, USA). Methanol, *n*-hexane and 2-propanol were obtained from Wako (Osaka, Japan). Other chemicals were of analytical reagent grade.

2.2. Preparation of racemates

Compound **1** was dissolved in acetonitrile with slight warming. The two racemates were isolated by fractional crystallization from the solution. Two courses of fractional crystallization yielded white crystalline powders. Their melting points were 140.6 and 152.8°C, in close agreement with the melting points of racemates A and B prepared by Matsutera *et al.* [2]. The IR spectra of the two racemates (KBr disc) were examined with a Model IR-700 IR spectrometer (JASCO, Tokyo, Japan), and characteristic absorption bands were observed at 1260 cm^{-1} in racemate A and at 1240 and 3580 cm^{-1} in racemate B, consistent with the literature [1].

2.3. Preparation of diastereomers

A 1-g amount of **1** was dissolved in 20 ml of ethanol, 250 μl of the solution was injected into the preparative HPLC system and fractions of various diastereomers were repeatedly collected. The fractions were pooled and white powders were obtained by evaporating the solvent at ambient temperature under reduced pressure.

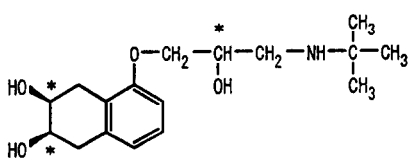

Fig. 1. Structure of nadolol. The asterisks denote the location of the chiral centres.

2.4. Apparatus and HPLC conditions

Analytical HPLC methods

The chromatograph was an LC100 system (Yokogawa, Tokyo, Japan) consisting of a pump (LC100P), an autosampler (LC100A), a column oven (LC100T) and a UV detector (LC100U). The racemates were separated on a Cosmosil $5C_{18}$ column (250 mm × 4.6 mm I.D., particle size 5 μm) (Nacalai Tesque, Kyoto, Japan) and an end-capped YMC-Pack ODS-AM-303 column (250 mm × 4.6 mm I.D., particle size 5 μm) (YMC, Kyoto, Japan). The diastereomers were resolved on an Ultron ES-OVM column (150 mm × 4.6 mm I.D., particle size 5 μm) (Shinwa, Kyoto, Japan), in which the glycoprotein ovomucoid was bound to aminopropylsilica gel, and a Chiralpak AD column (250 mm × 4.6 mm I.D., particle size 10 μm) (Daicel, Tokyo, Japan), in which a 3,5-dimethylphenylcarbamate derivative of amylose was bound to silica gel. The flow-rate was set at 0.5 or 1.0 ml/min and the detection wavelength at 220 or 230 nm. The column temperatures was maintained at 40°C for the analysis of the racemates and at 30 or 35°C for the analysis of the diastereomers. The mobile phases are specified in the figures and tables.

Preparative HPLC method

An HPLC system consisting of a Model LC-5A pump, FCV-100B fraction collector, SPD-2A UV detector, CR-3A Cromatopac recorder (Shimadzu, Kyoto, Japan) and Model AS-8010 autosampler (Tosoh, Tokyo, Japan) was used for preparative HPLC. A semi-preparative Chiralpak AD column (250 mm × 20 mm I.D., particle size 10 μm) (Daicel) was used. The flow-rate was 4 ml/min, the detection wavelength 230 nm and the column temperature 35°C. The mobile phase was *n*-hexane–2-propanol (80:20, v/v) containing 0.1% of diethylamine.

2.5. HPLC purities of the racemates

A 5-μl volume of a 1 mg/ml methanol solution of racemate A or B was injected into a Cosmosil $5C_{18}$ column mounted on the above HPLC

system, and the HPLC purity of each racemate was calculated from the peak areas other than those of the solvent peaks. The HPLC purities of racemates A and B were 98.1% and 90.1%, respectively. Under these conditions, the detection limits of racemates A and B were 0.05 and 0.07 μg, respectively ($S/N = 3$).

2.6. Contents of optical isomers of the isolated diastereomers

A 10-μl volume of a 2 mg/ml methanol solution of each diastereomer was injected into a Chiralpak AD column mounted on the above HPLC system and the contents of the optical isomers of each diastereomer were calculated from the peak areas other than those of the solvent peaks. Under these conditions, the detection limit of each diastereomer was 0.01, 0.014, 0.16, and 0.2 μg in the order of elution ($S/N = 3$).

2.7. Specific rotation

The specific rotation of a 5 mg/ml methanol solution of each stereoisomer was measured with a Model DIP-370 polarimeter (JASCO) using a quartz cell with a 100-mm layer length.

3. Results and discussion

3.1. Direct separation of the racemates

Tables 1 and 2 show the effect of the phosphate buffer concentration and the methanol contents in the mobile phase. The capacity factors (k') of racemates A and B decreased as the concentration of the phosphate buffer increased, but no marked changes were observed in the resolution (R_s) values. The methanol concentration in the mobile phase markedly affected the k' values. Therefore, 200 mM phosphate buffer (pH 7.5)–methanol (75:25, v/v) was used as the mobile phase in consideration of the analysis time and the R_s value. Racemate A was eluted more quickly than racemate B (Fig. 2A).

Table 1
Effect of buffer concentration on capacity factor (k') and resolution (R_s)

Buffer concentration (mM)	k'		R_s
	Racemate A	Racemate B	
10	10.01	10.92	0.70
50	6.48	7.05	0.76
100	5.53	6.01	0.76
200	4.28	4.64	0.79
300	3.75	4.05	0.80

Column, Cosmosil $5C_{18}$ (250 mm × 4.6 mm I.D.); mobile phase, methanol–phosphate buffer (pH 7.5) (3:7); flow-rate, 0.5 ml/min; column temperature, 40°C.

Similarly, the separation of the racemates was evaluated using the end-capped column. Complete separation was observed with a mobile phase of 50 mM phosphate buffer (pH 7.2)–methanol (70:30, v/v), and the elution time was markedly shorter compared with that without end-capping (Fig. 2B). This marked difference in the elution time is considered to be due to a decrease in the hydrogen bonding of residual silanol by end-capping and a resultant decrease in retention.

3.2. Direct separation of the diastereomers

The separation of the four diastereomers was studied with the Chiralpak AD column using

Table 2
Effect of mobile phase solvent ratio when using methanol as organic modifier on capacity factor (k') and resolution (R_s)

Methanol–buffer (v/v)	k'		R_s
	Racemate A	Racemate B	
20:80	10.00	11.33	2.21
25:75	5.63	6.36	1.63
30:70	2.89	3.25	1.48
35:65	1.99	2.23	1.39

Column, Cosmosil $5C_{18}$ (250 mm × 4.6 mm I.D.); mobile phase, methanol–200 mM phosphate buffer (pH 7.5); flow-rate, 0.5 ml/min; column temperature, 40°C.

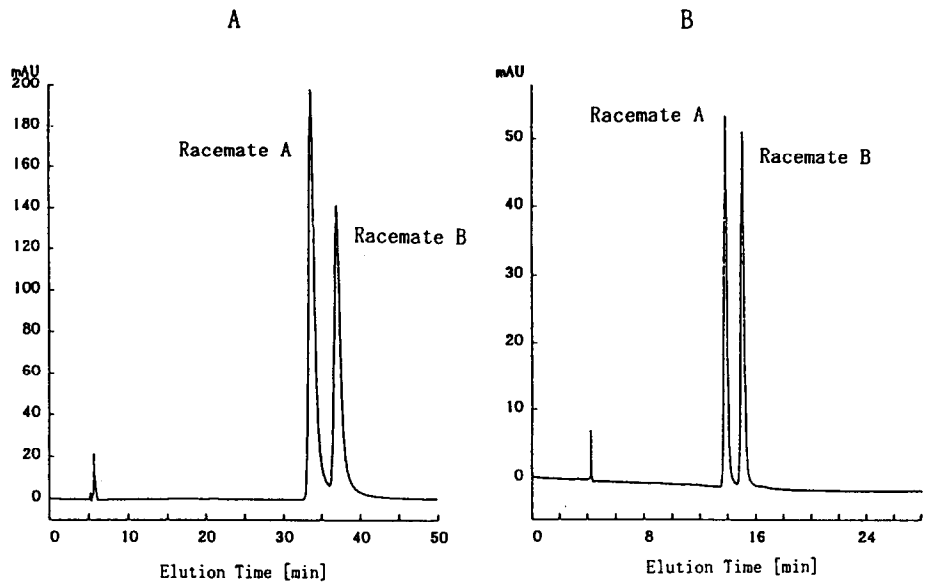

Fig. 2. Chromatograms of nadolol racemates A and B. (A) Column, Cosmosil $5C_{18}$ (250 mm × 4.6 mm I.D.); mobile phase, 200 m*M* phosphate buffer (pH 7.5)–methanol (75:25); flow-rate, 0.5 ml/min; column temperature, 40°C; volume injected, 5 μl (containing of 5 μg of nadolol). (B) Column, YMC-Pack ODS-AM-303 (250 mm × 4.6 mm I.D.); mobile phase, 50 m*M* phosphate buffer (pH 7.2)–methanol (70:30); flow-rate, 1.0 ml/min; column temperature, 40°C; volume injected, 5 μl (containing of 5 μg of nadolol).

n-hexane–2-propanol as the mobile phase and the Ultron ES-OVM column using an aqueous mobile phase of buffer solution–methanol. These mobile phases were recommended by the manufacturers of the columns to avoid their deterioration.

When the ratio of *n*-hexane and 2-propanol was changed from 60:40 (v/v) to 90:10 (v/v) with the addition of 0.1% diethylamine to improve the shape and the resolution of the peaks, the k' and R_s values improved as the proportion of *n*-hexane increased, but no changes were observed in the stereoselectivity (α). Even with the mobile phase that provided the best separation, the separation between the peak eluted first and that eluted second was insufficient, but other peaks were separated with $R_s > 2.0$ (Fig. 3A).

The elution behaviour with the Ultron ES-OVM column is reported to change with the pH of the mobile phase and the kinds and contents of organic modifiers [7,8]. From the results of a preliminary study, the composition of the mobile phase was determined as 20 m*M* phosphate buffer–methanol, and the effects of changes in the composition ratio and the pH of the phosphate buffer were evaluated.

When the pH of the phosphate buffer was below the isoelectric point of ovomucoid (p*I* ≈ 4), ovomucoid was positively charged and the analyte was scarcely retained because of the electrostatic repulsion between the stationary phase and the analyte. However, when the pH was above the isoelectric point, the k' values increased sharply. These findings were in agreement with the results of Fujima *et al.* [8] that ovomucoid becomes negatively charged and the k' value increases because of both electrostatic and hydrophobic interactions. The R_s and α values also increased with increase in pH (Table 3).

Concerning the effect of the content of methanol as an organic modifier, the k' value increased markedly and the α value increased gradually as the methanol content decreased (Table 4). This probably reflects changes in the ability to discriminate asymmetry with changes in the

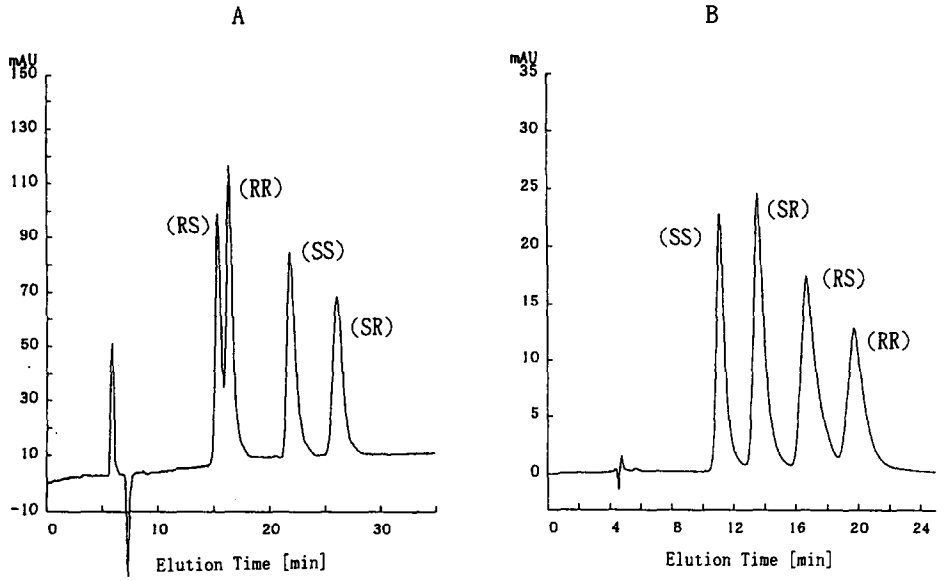

Fig. 3. Chromatogram of nadolol diastereomers. (A) Column, Chiralpak AD (250 mm × 4.6 mm I.D.); mobile phase, *n*-hexane–2-propanol (80:20) containing 0.1% of diethylamine; flow-rate, 0.5 ml/min; column temperature, 35°C; volume injected, 10 μl (containing of 10 μg of nadolol). (B) Column, Ultron ES-OVM (150 mm × 4.6 mm I.D.); mobile phase, 20 m*M* phosphate buffer (pH 6.0)–methanol (90:10); flow-rate, 0.5 ml/min; column temperature, 30°C; volume injected, 10 μl (containing of 10 μg of nadolol).

strength of the hydrophobic interaction and conformation of ovomucoid [8]. Fig. 2B shows a typical chromatogram. The peaks are mostly baseline resolved with R_s values of 1.9 or above.

3.3. Application to semi-preparative HPLC

Analytical HPLC for stereoisomers was applied to semi-preparative HPLC, and the four

Table 3
Effect of buffer pH on capacity factor (k'), resolution (R_s) and stereoselectivity (α)

pH	k'				R_s [b]			α [b]		
	Peak 1[a]	Peak 2[a]	Peak 3[a]	Peak 4[a]	Peaks 1–2	Peaks 2–3	Peaks 3–4	Peaks 1–2	Peaks 2–3	Peaks 3–4
3.0	0.00	0.00	0.00	0.00	–	–	–	1.00	1.00	1.00
3.5	0.00	0.08	0.08	0.08	0.83	–	–	1.00	1.00	1.00
4.0	0.24	0.55	0.64	0.79	2.52	0.52	0.66	2.35	1.16	1.21
4.7	0.34	0.70	0.81	0.98	2.89	0.63	0.76	2.06	1.15	1.20
5.5	0.94	1.78	2.19	2.90	3.15	1.06	1.34	1.90	1.23	1.32
6.0	3.17	5.77	7.35	9.77	4.50	1.85	2.66	1.81	1.27	1.32

Column, Ultron ES-OVM (150 mm × 4.6 mm I.D.); mobile phase, 20 m*M* phosphate buffer–methanol (97:3); flow-rate, 0.5 ml/min; column temperature, 30°C.

[a] Elution order on Ultron ES-OVM column.

[b] R_s and α values between each peak when the first-eluted peak = 1.

Table 4
Effect of mobile phase solvent ratio on capacity factor (k'), resolution (R_s) and stereoselectivity (α)

Composition[a] (v/v)	k'				R_s[c]			α[c]		
	Peak 1[b]	Peak 2[b]	Peak 3[b]	Peak 4[b]	Peaks 1–2	Peaks 2–3	Peaks 3–4	Peaks 1–2	Peaks 2–3	Peaks 3–4
80:20	1.04	1.16	1.40	1.60	0.64	1.12	0.93	1.11	1.20	1.14
85:15	1.16	1.43	1.83	2.21	1.40	1.84	1.49	1.23	1.28	1.20
87:13	1.27	1.66	2.18	2.68	1.83	2.03	1.66	1.30	1.31	1.23
90:10	1.55	2.19	2.93	3.75	2.38	2.00	1.93	1.40	1.33	1.27
95: 5	2.42	4.14	5.48	7.15	3.90	2.05	2.03	1.70	1.32	1.30
97: 3	3.17	5.77	7.35	9.77	4.50	1.85	2.66	1.81	1.27	1.32

Column, Ultron ES-OVM (150 mm × 4.6 mm I.D.); mobile phase, 20 m*M* phosphate buffer (pH 6.0)–methanol; flow-rate, 0.5 ml/min; column temperature, 30°C.
[a] Composition of 20 m*M* phosphate buffer (pH 6.0)–methanol.
[b] Elution order on Ultron ES-OVM column.
[c] R_s and α values between each peak when the first-eluted peak = 1.

nadolol diastereomers were isolated. The semi-preparative Chiralpak AD column was used, because the use of an organic solvent system as the mobile phase facilitates the treatments of collected fractions, the injection volume is greater and the peak shapes are not altered. The composition of the mobile phase was the same as in the analytical method. Table 5 shows the contents and specific rotations of each of the resolved diastereomers.

3.4. *Assignment of separated peaks*

The configurations of the four diastereomers separated by analytical HPLC were elucidated from racemates A and B and the resolved diastereomers. Racemates A and B were analyzed using each analytical column used for the analysis of diastereomers, and + − (*RS*) or − + (*SR*) and + + (*RR*) or − − (*SS*) was established from the retention time and the specific rotatior of each diastereomer of racemates A and B Further, to confirm these assignments, racemates A and B and the resolved diastereomers were analyzed by the method of Dyas *et al.* [6] using the same column. As a result, we determined the order of elution to be (*RS*), (*RR*), (*SS*) and (*SR*) from the Chiralpak AD column and (*SS*), (*SR*), (*RS*) and (*RR*) from the Ultron ES-OVM column.

Table 5
Stereoisomer contents and optical rotations of the isolated diastereomers from nadolol

Peak No.[a]	Content (%)[b]				$[\alpha]_D^{20}$ (°)[c]	Racemate
	Peak 1	Peak 2	Peak 3	Peak 4		
1	100.0	N.D.[d]	N.D.	N.D.	+9.2	A
2	2.40	97.59	N.D.	N.D.	+20.5	B
3	0.05	0.07	98.15	1.72	−22.8	B
4	N.D.	0.19	0.75	99.05	−14.1	A

[a] Elution order on Chiralpak AD column.
[b] Area percentage of peaks on Chiralpak AD column.
[c] See Section 2.7.
[d] N.D. = not detected.

In conclusion, we established methods for the direct HPLC separation of the two racemates and four diastereomers of nadolol. In the separation of the racemates, the hydrogen bonding of residual silanols in the packing was considered to affect the retention time. The diastereomers could be separated by organic and aqueous mobile phases, and the organic mobile phase could also be applied to semi-preparative HPLC.

4. References

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