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Direct high-performance liquid chromatographic separation of the enantiomers of diltiazem hydrochloride and its 8-chloro derivative on a chiral ovomucoid column

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ABSTRACT

A direct enantiomeric separation of diltiazem hydrochloride and its related compounds was investigated by using an ovomucoid bonded chiral stationary phase. The enantiomers of diltiazem hydrochloride, the 8-chloro derivative of diltiazem (clentiazem maleate) and their desacetyl forms were resolved with a mobile phase of acetonitrile–0.02 *M* phosphate buffer (pH 6.0) according to the retention mechanism of reversed-phase liquid chromatography. The effects of mobile phase composition on the retention and the enantioselectivity, the effects of sample capacity on the retention time, theoretical plate number, peak height and peak area were investigated. The chromatographic conditions chosen for the separation permitted the separation of these enantiomers within 20 min. The determination of the antipode in the drug substances and those in tablets was also successfully achieved at levels down to *ca.* 0.1% by the area-percentage method.

INTRODUCTION

The desired pharmacological effect of a drug is often associated with only one of the enantiomers and the antipode may have different potencies, pharmacological activities and/or side-effects [1,2]. It is very important to characterize the pharmacological effects and the pharmacokinetics of the enantiomers of the drug in order to elucidate whether therapeutic benefits can be obtained by the use of only one enantiomer. The antipode, which has no or undesired pharmacological effects, can be regarded as one of impurities from the quality aspect. Such studies require techniques that permit the separation of the enantiomers.

High-performance liquid chromatography (HPLC) is suitable for such a purpose. Generally, there are two methods for the chromatographic separation of enantiomers: the indirect diastereomeric method using a chiral derivatization reagent, followed by separation on a non-chiral column [3], or direct chromatographic separation on a chiral column. During the last few years, interest in the direct separation of the enantiomers has increased and a number of chiral stationary phases have been prepared and some of them are now commercially available [4,5]. Especially bonded proteins such as bovine serum albumin (BSA) and α_1-acid glycoprotein (AGP) as a chiral selector have great enantioselectivity for solutes with widely different structures [6,7].

Diltiazem hydrochloride [(+)-(*S*,*S*)-form], 1,5-benzothiazepine derivative, is a representative calcium antagonist along with nifedipine and verapamil and is widely used as an antianginal and antihypertentive drug [8]. The 8-chloro derivative of diltiazem [(+)-(*S*,*S*)-form] is a new 1,5-benzothiazepine calcium antagonist that is currently being evaluated as clentiazem maleate. The calcium antagonist action of clentiazem is 2–10 times as potent as that of diltiazem is vascular smooth muscles and lasts longer [9–12].

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Enantiomers of diltiazem hydrochloride were first chromatographically resolved by the indirect method using two kinds of chiral reagents [13,14]. Recently, a direct chiral separation was successfully achieved by using a chiral stationary phase such as Chiralcel OC or OF (Daicel, Tokyo, Japan) in normal-phase HPLC [15,16]. This paper describes the direct reversed-phase HPLC separation of the enantiomers of diltiazem hydrochloride, the 8-chloro derivative of diltiazem and their desacetyl forms on recently developed ovomucoid chiral stationary phase [17–19]. The effects of pH, ionic strength of the buffer and organic solvents (species and content) on the retention and enantioselectivity of the solutes were studied. The effects of sample capacity on the retention time, theoretical plate number, peak height and peak area were also investigated. The separation of the enantiomers was achieved within 20 min according to the reversed-phase HPLC retention mechanism. The method permitted the determination of the optical purity of the drug at levels down to *ca.* 0.1% by the area-percentage method. It was found that the structure of these solutes significantly affects their enantioselectivity.

EXPERIMENTAL

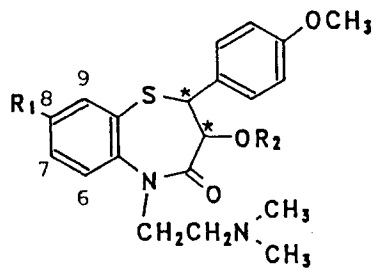
Apparatus

The chromatographic system consisted of a Shimadzu (Kyoto, Japan) LC-3A pump, a Rheodyne Model 7125 injector with a 20-μl loop, a Shimadzu CTO-2A column oven and a Shimadzu SPD-2A variable-wavelength UV detector. A Shimadzu SPD-M6A photodiode-array detector was used to monitor the UV spectra of the peaks. The chromatograms were recorded on a Shimadzu Chromatopac CR5A. An Ultron ES-OVM ovomucoid chiral column (150 mm × 4.6 mm I.D.), in which ovomucoid is chemically bonded to aminopropylsilica gel of particle size 5 μm, was purchased from Shinwakako (Kyoto, Japan).

Chemicals and materials

Racemic diltiazem hydrochloride (DIL), the 8-

Solute	R_1	R_2
Diltiazem hydrochloride (DIL)	H	$COCH_3$
Desacetyl diltiazem (DIL-OH)	H	H
8-Chloro derivative (CHL)	Cl	$COCH_3$
Desacetyl 8-chloro derivative (CHL-OH)	Cl	H



* asymmetric carbon atom

Fig. 1. Structures of diltiazem and related compounds.

chloro derivative of diltiazem (clentiazem maleate) (CHL) and their desacetyl forms and their (+)-(*S,S*)-enantiomers were obtained from the research laboratory of Tanabe Seiyaku (Osaka, Japan). The 6-, 7- and 9-chloro derivatives of diltiazem were also used to investigate the enantioselectivity of the column. The structures are shown in Fig. 1. These solutes were dissolved in acetonitrile–water (1:1) at a concentration of *ca.* 0.2 mg/ml. The drug substances for optical purity testing were dissolved in acetonitrile–water (1:1) at 2 mg/ml. Acetonitrile of HPLC grade and ethanol and methanol of analytical-reagent grade were purchased from Katayama Kagaku (Osaka, Japan). Water was purified with a Millipore RO-60 water system (Nihon Millipore, Tokyo, Japan). All other reagents and solvents were of analytical-reagent grade from Katayama Kagaku.

Chromatographic conditions

The separation was carried out at a flow-rate of 1.0 ml/min and a column temperature of 40°C. The wavelength of detection was 240 nm, which is around the UV absorption maximum of the solutes. The mobile phase were prepared by mixing a 0.02 *M* phosphate buffer solution with an organic solvent. The pH of the buffer was adjusted to specified values by using dilute phosphoric acid (*ca.* 5%) or 0.2 *M* sodium hydroxide solution. The mobile phases were passed through a membrane filter of 0.45-μm pore size (Fuji Photo Film, Tokyo, Japan) prior to use. The injection volume was fixed at 5 μl.

RESULTS AND DISCUSSION

Effect of organic solvents

Different organic solvents such as methanol, ethanol and acetonitrile were used to regulate the retention and the enantioselectivity. The results using 0.02 *M* phosphate buffer (pH 6.0) and each organic solvent are summarized in Table I. The sample loading was 1 μg each in a 5-μl injection. Methanol was the most effective organic solvent for the enantioselectivity. With the use of methanol or ethanol, the (+)-enantiomer of the 8-chloro derivative of diltiazem did not elute within 35 min whereas its (−)-enantiomer eluted at *ca.* 6 min. This is not acceptable from the separation aspect, although these conditions are suitable for preparative purposes. The separation of the enantiomers was then optimized by using acetonitrile as the organic solvent.

The effect of the concentration of acetonitrile was investigated with a 0.02 *M* phosphate buffer (pH 6.0). The results with acetonitrile at concentrations of 10% and 20% are summarized in Table II and those with 15% acetonitrile in Table I. The retention times of the solutes increased with a decrease in the acetonitrile content. The resolution (R_s) and separation factor (α) were improved with an increase in the retention times. The same results were obtained with other organic solvents.

Effect of buffer pH

The retention of ionic solutes on protein bonded phases generally varies with changes in pH [6,7].

TABLE I

EFFECT OF ORGANIC SOLVENT ON RETENTION AND ENANTIOSELECTIVITY

Mobile phase: organic solvent–0.02 *M* phosphate buffer (pH 6.0)

Solute	Methanol (35%)				Ethanol (25%)				Acetonitrile (15%)			
	$k'(-)$	$k'(+)$	α	R_s	$k'(-)$	$k'(+)$	α	R_s	$k'(-)$	$k'(+)$	α	R_s
DIL	1.62	8.11	5.00	8.26	1.82	3.31	1.82	2.81	1.39	2.78	2.00	3.80
DIL-OH	1.85	4.48	2.42	7.27	1.99	2.69	1.35	1.98	1.41	2.01	1.43	2.35
CHL	2.68	20<[a]	–	–	2.40	20<[a]	–	–	2.29	18.59	8.10	8.79
CHL-OH	2.60	15.58	5.98	11.45	2.56	6.33	2.40	6.33	1.94	3.86	1.99	4.70

[a] Not eluted within 35 min.

TABLE II

EFFECT OF ACETONITRILE CONCENTRATION ON RETENTION AND ENANTIOSELECTIVITY

Mobile phase: acetonitrile–0.02 *M* phosphate buffer (pH 6.0).

Solute	20%				10%			
	$k'(-)$	$k'(+)$	α	R_s	$k'(-)$	$k'(+)$	α	R_s
DIL	1.05	1.40	1.33	1.57	2.80	12.23	4.37	7.99
DIL-OH	1.12	1.33	1.19	0.97	2.13	4.78	2.24	6.03
CHL	1.31	5.04	3.84	6.48	3.84	20<[a]	–	–
CHL-OH	1.34	1.85	1.38	1.98	3.52	15.42	4.35	8.51

[a] Not eluted within 35 min.

This can be interpreted by the charges generated in the protein, through the electrostatic interaction. For comparison, the retention of the solutes was investigated with 0.02 *M* phosphate buffer of pH 4.0, and the results are summarized in Table III. The sample loading was 1 μg each in a 5-μl injection. The concentration of the organic solvent required for their elution at a capacity factor of *ca.* 1 was lower than that at pH 6.0. This can be interpreted by the increase in electrostatic repulsion between the protein and the solute from the isoelectric point of the solutes of *ca.* 7 [20] and that of the ovomucoid of 3.9–4.3 [17]. The enantioselectivity at pH 4.0 was much improved by an increase in the retention of the (+)-enantiomers compared with that of the (−)-enantiomers, resulting in larger separaton factors and long analysis times. From the standpoint of analysis time and appropriate k' value ($1 < k' < 10$), a buffer of pH 6.0 was selected.

Effect of ionic strength

The effect of the ionic strength on the retention and the enantioselectivity was investigated using a mobile phase of phosphate buffer (pH 6.0)–acetonitrile (88:12) and diltiazem and its desacetyl form as test samples (1 μg each). The results are summarized in Table IV. The separation factors gradually decreased with an increase in ionic strength, owing to the decrease in retention. However, the effect is not very large and is not important for the chiral recognition and hydrophobic interaction.

The final composition of the mobile phase established through the investigation of the effects of these parameters was 0.02 *M* phosphate buffer (pH 6.0)–acetonitrile (88:12) for diltiazem hydrochloride and (82:18) for the 8-chloro derivative of diltiazem, clentiazem maleate. Typical chromatograms are shown in Figs. 2 and 3. The UV spectra of the enantiomers of diltiazem hydrochloride monitored by using the photodiode-array detector are shown in Fig. 4. Each peak gave the same spectrum and the maximum absorption at around 240 nm.

Effect of solute structure on enantioselectivity

The effect of solute structure on the enantioselec-

TABLE III

EFFECT OF pH BUFFER ON RETENTION AND ENANTIOSELECTIVITY

Mobile phase: 0.02 *M* phosphate buffer (pH 4.0)–organic solvent.

Solute	Methanol (20%)				Acetonitrile (5%)			
	$k'(-)$	$k'(+)$	α	R_s	$k'(-)$	$k'(+)$	α	R_s
DIL	0.23	2.38	10.37	3.17	0.43	3.75	8.79	6.79
CHL	0.46	17.52	38.26	7.86	1.33	20<[a]	–	–

[a] Not eluted within 35 min.

TABLE IV

EFFECT OF IONIC STRENGTH ON RETENTION AND ENANTIOSELECTIVITY

Mobile phase: phosphate buffer (pH 6.0)–acetonitrile (88:12).

Solute	Ionic strength											
	10 m*M*				20 m*M*				40 m*M*			
	$k'(-)$	$k'(+)$	α	R_s	$k'(-)$	$k'(+)$	α	R_s	$k'(-)$	$k'(+)$	α	R_s
DIL	3.37	8.77	2.60	6.01	2.96	7.63	2.58	6.41	2.59	6.63	2.56	6.18
DIL-OH	3.03	4.93	1.62	4.46	2.66	4.24	1.59	4.29	2.36	3.47	1.47	3.58

tivity was investigated using 0.02 *M* phosphate buffer (pH 6.0)–acetonitrile (82:18). The results are summarized in Table V. The retention time of the (+)-enantiomer of the 8-chloro derivative was the largest among the five solutes tested, those of the others being similar to each other. The 8-chloro derivative also eluted last among these solutes using ion-pair chromatography and an ODS column [20] and using micellar electrokinetic chromatography [21]. These results indicate that 8-substituted diltiazem acquires a very hydrophobic nature owing to the substitution at the 8-position. This hydrophobic nature probably leads to dramatic improvements in enantioselectivity through hydrophobic interaction and/or hydrogen bonding.

Effect of sample capacity

The effects of the amount of sample on the retention time (t_R), theoretical plate number (N), peak height (h) and peak area (A) were investigated using diltiazem as a sample with an injection volume of 5

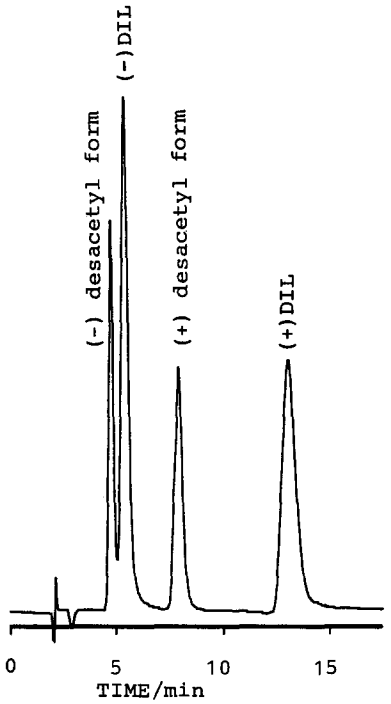


Fig. 2. Chiral separation of the enantiomers of diltiazem hydrochloride (DIL) and its desacetyl form.

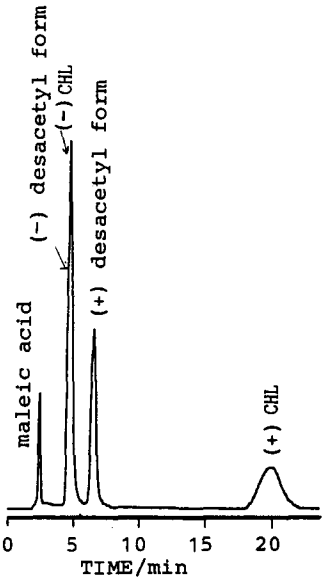


Fig. 3. Chiral separation of the enantiomers of the 8-chloro-derivative of diltiazem (clentiazem; CHL) and its desacetyl form.

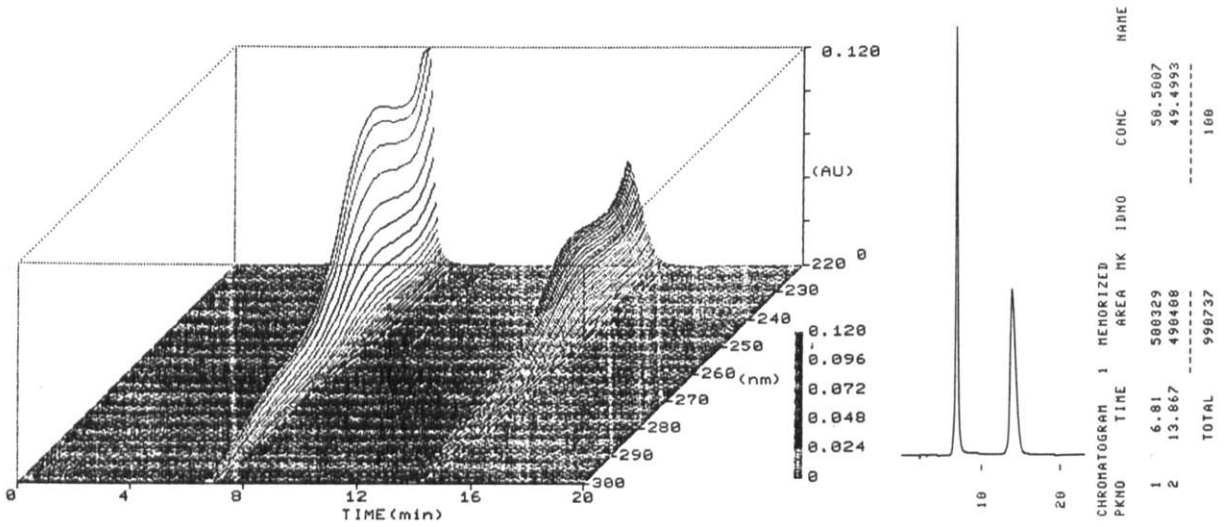


Fig. 4. UV spectra of the enantiomers of diltiazem hydrochloride obtained with the photodiode-array detector.

μl. The results are shown in Figs. 5 and 6. N and t_R decreased with increase in the amount of sample. A tenfold increase in the amount of diltiazem (1–10 μg) resulted in a 10% decrease in t_R and a *ca.* 70% decrease in N. The peak areas, however, showed good linearity over the tested range with a correlation coefficient (r) of 0.9999. This permits the optical purity testing of the enantiomer by the area-percentage method.

Application to the optical purity testing

The linearity of the response of the (−)-enantiomer of diltiazem was investigated over the concentration range 0.1–2.0% (w/w). The total amount of diltiazem injected was 10 μg with a 5-μl injection volume. The result is shown in Fig. 7. The graph is a straight line that passes through the origin over the tested range with r = 0.9998, slope =

TABLE V

EFFECT OF SAMPLE STRUCTURE ON RETENTION AND ENANTIOSELECTIVITY

Mobile phase: 0.02 M phosphate–buffer (pH 6.0)–acetonitrile (82:18).

Solute	k' (−)	k' (+)	α
9-Chloro	2.18	2.63	1.21
8-Chloro	2.37	10.75	4.54
7-Chloro	–[a]	2.36	–
6-Chloro	1.78	2.38	1.34
Diltiazem	2.28	3.38	1.48

[a] Not examined.

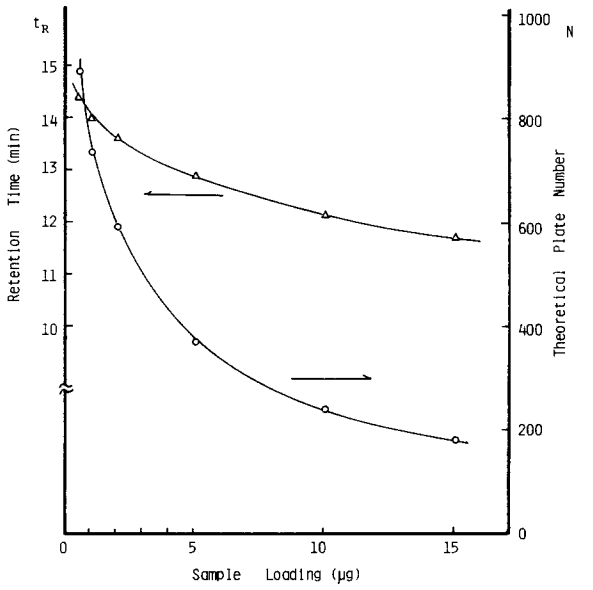


Fig. 5. Effects of sample loading on retention times and theoretical plate numbers.

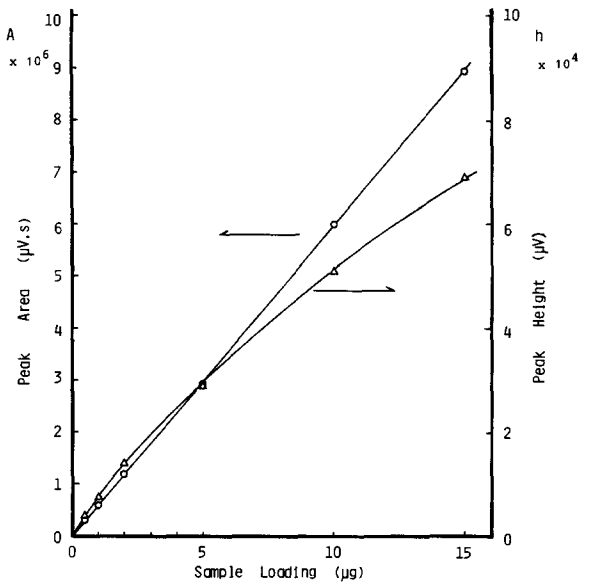


Fig. 6. Effects of sample loading on peak heights and peak areas.

1.017 and intercept = −0.003. The method was then applied to the optical purity testing of diltiazem drug substances and tablets and the 8-chloro derivative drug substances. The results are summarized in Table VI. Diltiazem in tablets was extracted

TABLE VI

RESULTS OF OPTICAL PURITY TESTING

Sample	Lot	Concentration (%)	
		(−) Enantiomer	(+)-Desacetyl form
DIL drug substances	TA1	ND[a]	0.07
	TA2	ND	0.07
	TA3	ND	0.08
	FE1	ND	ND
	FA1	ND	0.16
DIL tablets (30 mg)	TA1	ND	0.19
	TO1	ND	1.98
CHL drug substances	520	ND	0.06
	530	ND	0.07
	540	ND	0.07
	810	0.68	0.05
	820	0.25	0.07

[a] Not detected.

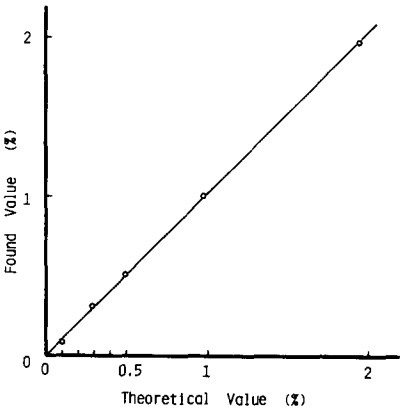


Fig. 7. Linearity of response of the (−)-enantiomer of diltiazem hydrochloride.

from the matrix using methanol–0.02 *M* hydrochloride solution (1:1). The extract solution was then filtered through the membrane filter and the filtrate was injected directly into the HPLC column. Typical chromatograms of optical purity testing are shown in Fig. 8.

CONCLUSIONS

The enantiomers of diltiazem hydrochloride, its 8-chloro derivative and their desacetyl forms were succesfully separated on an ovomucoid chiral column in the reversed-phase HPLC mode. The retention and enantioselectivity were optimized through the pH of buffer solutions and the type and concentration of organic solvent. The chromatographic conditions chosen permitted enantiomeric separation within 20 min. The good linearity of the peak area *versus* sample amount plot permitted the optical purity determination of small amounts of the antipode at levels down to *ca.* 0.1%. This column will be applied to the other pharmaceuticals owing to its wide enantioselectivity.

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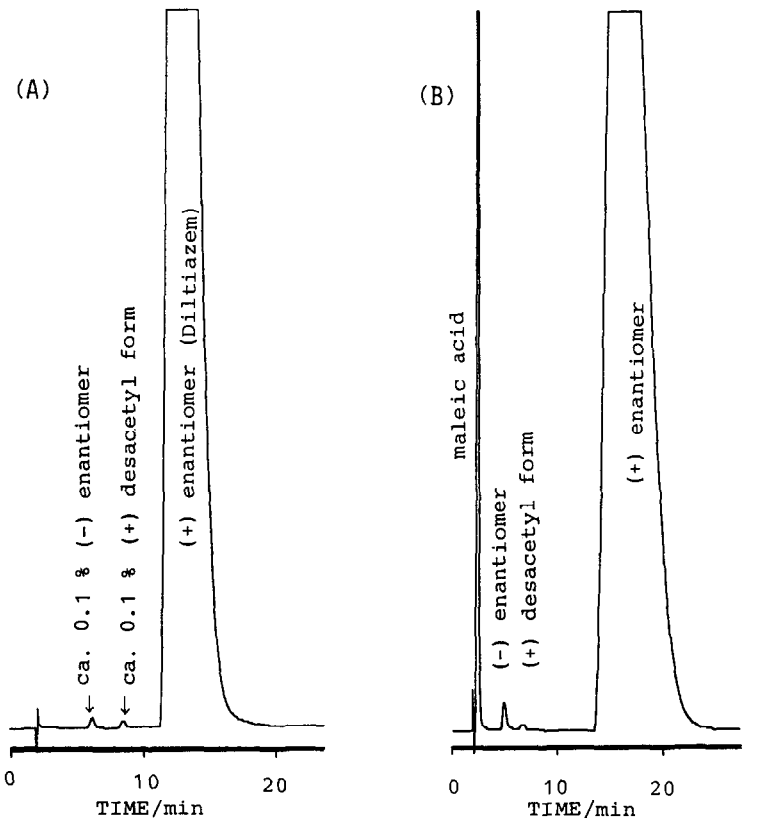

Fig. 8. Typical chromatogram fo optical purity testing of (A) drug substances of diltiazem spiked with *ca.* 0.1% (−)-enantiomer and *ca.* 0.1% of the (+)-desacetyl form and (B) drug substance of the 8-chloro derivative of diltiazem.

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