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Optical resolution of imidapril hydrochloride by high-performance liquid chromatography and application to the optical purity testing of drugs

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Abstract

Imidapril hydrochloride, a newly synthesized angiotensin-converting enzyme (ACE) inhibitor, is administered as a single enantiomer (*SSS*-form) because it is the most active of the eight possible optical isomers. Three different approaches to applying high-performance liquid chromatography (HPLC) were developed for the resolution of optical isomers of imidapril hydrochloride. One is the reversed-phase HPLC method for the separation of diastereomers from the enantiomeric pairs of imidapril. The second method involves the derivatization of imidapril with a chiral reagent and separation on a silica gel column (normal-phase HPLC). The last method is the direct separation of enantiomers of imidapril hydrochloride by using chiral stationary phases (CSPs). These three methods were successfully applied to the optical purity testing of drug substances and those in tablets. The methods were also used in a stability study of imidapril hydrochloride, including its stability in aqueous solutions, and imidapril tablets.

1. Introduction

Imidapril hydrochloride is a recently developed prodrug-type angiotensin-converting enzyme (ACE) inhibitor [1,2]. Imidapril, having an ethyl ester structure, is converted into a dicarboxy-type compound (imidaprilat) in the body (Fig. 1). Imidaprilat shows about a 500 times higher activity than the ester form, imidapril.

Imidapril hydrochloride was developed as a single enantiomer (*SSS*-form) because it shows the strongest activity of the eight optical isomers [2]. It is therefore important to determine the

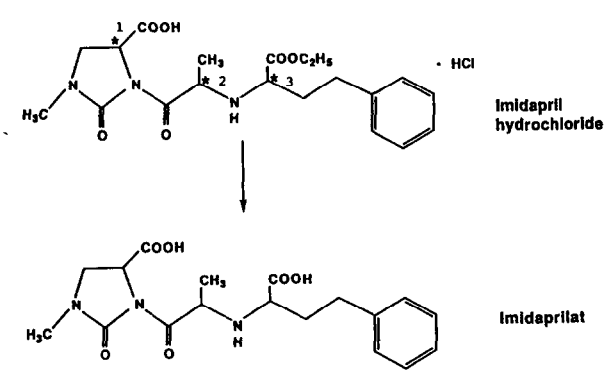

Fig. 1. Structures of imidapril hydrochloride and imidaprilat. Asymmetric carbons are indicated with asterisks. Absolute configuration of imidapril ($*^1$, $*^2$, $*^3$) is (*S*, *S*, *S*).

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optical purity of the single *SSS*-form isomer. The aim of the work was to develop a method for determining the optical purity of the drug.

Chromatographic methods, especially HPLC, have been widely accepted for the separation of optical isomers. Diastereomers can be separated by the usual HPLC modes, although the separation of enantiomers requires some special techniques such as a chiral derivatization method or chiral stationary phases (CSPs). In other words, six diastereomers of imidapril hydrochloride (*SSR*, *RRS*, *SRS*, *RSR*, *RSS* and *SRR*) can be separated from the other diastereomers, *RRR* or *SSS* (imidapril), by reversed- or normal-phase HPLC. The separation of the *RRR*- and *SSS*-forms (enantiomers of imidapril hydrochloride) can be achieved by two methods. One is a chiral derivatization method using a chiral reagent having an amino group such as L-alanine-β-naphthylamide (L-ANA), because imidapril hydrochloride has a carboxy group in the molecule. The other is a direct chiral separation method using CSPs. Among many commercially available CSPs [3,4], the ligand-exchange type may offer sufficient enantiomeric separation of imidapril hydrochloride considering that the imidapril structure is like an amino acid.

This paper describes the HPLC separation of optical isomers of imidapril hydrochloride by using three methods: the first is for the separation of diastereomers from the enantiomers of imidapril hydrochloride; the second is for the separation of enantiomers by a chiral derivatization method; and the third is a direct chiral separation method. Applications to the quality control of drug substances (single *SSS*-form) and those in tablets by these methods are reported. Stability studies of the drug substances and those in tablets and of imidapril hydrochloride in aqueous solutions were also performed by the methods developed.

2. Experimental

2.1. Apparatus

The HPLC system consisted of an LC-6A high-pressure pump, a CTO-6A column oven and an SPD-6A variable-wavelength UV detector (Shimadzu, Kyoto, Japan). Samples were applied to the column with a Rheodyne Model 7125 loop injector (20 μl) or a Shimadzu SIL-6B autoinjector. Peak integration was carried out with a Shimadzu Chromatopac C-R5A data processor. An M-80A mass spectrometer (Hitachi, Ibaragi, Japan) was operated in the beam electron impact mode at 70 eV.

2.2. Chemicals

Imidapril hydrochloride ($C_{20}H_{27}N_3O_6 \cdot HCl$), (4*S*)-3-[(2*S*)-2-[N-((1*S*)-1-ethoxycarbonyl-3-phenylpropyl)amino] propionyl]-1-methyl-2-oxoimidazolidine-4-carboxylic acid hydrochloride, with molecular mass (M_r) 441.91 (M_r of base = 405), and its seven optical isomers were obtained from Tanabe Seiyaku (Osaka, Japan). Potassium dihydrogenphosphate, phosphoric acid, copper(II) sulphate pentahydrate, diethylamine, pyridine, hydrochloric acid and ethanol were of analytical-reagent grade from Katayama Kagaku Kogyo (Osaka, Japan). Analytical-reagent grade N,N′-dicyclohexylcarbodiimide (DCC) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). HPLC-grade chloroform, acetonitrile and methanol were obtained from Katayama Kagaku Kogyo. Water was purified with a Millipore RO-60 water system (Millipore Japan, Tokyo, Japan). Ethyl *p*-hydroxybenzoate of analytical-reagent grade from Nacalai Tesque (Kyoto, Japan) was used as an internal standard in the assays. The chiral derivatization reagent, L-alanine-β-naphthylamide hydrobromide (L-ANA) (M_r of base = 214), was obtained from United States Biochemical (Cleveland, OH, USA). The structure of L-ANA is shown in Fig. 2. A Sep-Pak C_{18} cartridge column from Millipore was used for sample pretreatment.

2.3. HPLC columns

A Nucleosil 5C_8 (5 μm) reversed-phase col-

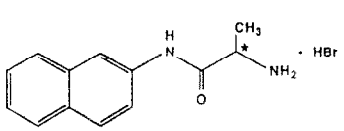

Fig. 2. Structure of L-alanine-β-naphthylamide hydrobromide (L-ANA).

umn (150 mm × 4.6 mm I.D.) from Macherey–Nagel (Düren, Germany) and a Zorbax Sil (5 μm) normal-phase column (150 mm × 4.6 mm I.D.) from DuPont (Wilmington, DE, USA) were used. A Chiralpak WH column (250 mm × 4.6 mm I.D.) from Daicel Chemical Industries (Osaka, Japan) was used for the direct separation of enantiomers of imidapril hydrochloride.

2.4. Sample preparation for reversed-phase HPLC

About 5 mg of imidapril hydrochloride were weighed and dissolved in 10 ml of the mobile phase for the Nucleosil $5C_8$ column and 20 μl of this solution were injected into the HPLC system. For the tablets, more than 20 tablets were weighed and ground. The resulting powder, equivalent to 25 mg of the drug substance according to the labelled amount, was weighed into a 50-ml volumetric flask and 40 ml of 40% methanol were added for extraction. The flask was shaken vigorously for 10 min and finally the contents were diluted to volume with 40% methanol. The solution was filtered through a membrane filter (0.45 μm) and 20 μl of the filtrate were injected into the HPLC system.

2.5. Chiral derivatization procedure

About 5 mg of imidapril hydrochloride and 15 mg of L-ANA were weighed into a glass-stoppered test-tube and 100 μl of chloroform, 50 μl of pyridine and 2 ml of a chloroform solution of DCC (0.45 g of DCC in 100 ml of chloroform) were added. The mixture was shaken vigorously and allowed to stand at room temperature for 1 h. After reaction, the mixture was washed with 2 ml of 1 *M* hydrochloric acid and continuously with 2 ml of water twice. A 1-ml volume of the chloroform layer was pipetted into a test-tube and diluted to 5 ml with chloroform. A 20-μl volume of the solution was injected into the HPLC system.

A blank solution was prepared by using the same procedure as described above but in the absence of the drug substance.

2.6. Sample preparation for direct chiral separation

About 20 mg of imidapril hydrochloride were weighed and dissolved in 10 ml of the mobile phase for the Chiralpak WH column and 2 μl of this solution were injected into the HPLC system. For imidapril tablets, more than 20 tablets were weighed and ground. The resulting powder, equivalent to 20 mg of the drug substance according to the labelled amount, was weighed and 20 ml of water was added for extraction. The solution was sonicated for 5 min and filtered through a membrane filter (0.45 μm). A 2-ml volume of the filtrate was charged to a previously activated Sep-Pak C_{18} column. The column was washed with 2 ml of water twice and dried with a stream of nitrogen. The sample adsorbed on the column was then eluted with 1 ml of methanol and the eluate was dried *in vacuo*. Finally, 1 ml of the mobile phase for the Chiralpak WH column was added to the residue (if necessary, the solution was filtered with a 0.45-μm membrane filter) and used as the sample solution of tablets. A 2-ml volume of each pH solution (containing 2 mg of the drug substance) for the stability testing of the drug substance in aqueous solutions (see below) was charged to a previously activated Sep-Pak C_{18} column. The column was treated continuously with the same procedure as for tablets described above. A 2-μl volume of the sample solution was injected into the HPLC system.

2.7. Stability study of drug substance in aqueous solutions

About 0.1 g of imidapril hydrochloride was weighed and dissolved in 100 ml of each buffer solution (pH range 2–11, ionic strength *ca.* 0.1). The solution was warmed on a water-bath at 90°C for 1 h. After cooling the solution with ice–water, 5 ml of the solution were diluted to 10 ml with 80% methanol and used as the sample solution for purity testing in reversed-phase HPLC. For assay, 2 ml of the internal standard solution (0.1 g of ethyl *p*-hydroxybenzoate in 200 ml of the mobile phase for the Nucleosil $5C_8$ column) and 2 ml of the buffer solution prepared above (0.1 g of drug substance in 100 ml of each

buffer solution) were pipetted into a 10-ml volumetric flask and diluted to volume with 80% methanol. A 20-μl volume of the sample solution was injected into the reversed-phase HPLC system.

3. Results and discussion

3.1. Separation of diastereomers by reversed-phase HPLC

There are eight optical isomers of imidapril hydrochloride because it has three asymmetric carbons in the molecule (Fig. 1). Six of them are diastereomers to imidapril, and these were successfully separated from imidapril (*SSS*-form) by reversed-phase HPLC employing a C_8 column, as shown in Fig. 3, although each enantiomeric pair of the diastereomer (*SRR* and *RSS*, *SSR* and *RRS*, *SRS* and *RSR*) was not resolved. Good linearity for each diastereomer over the concentration range 0.03–5% (w/w) was obtained. The limit of detection of each diastereomer at a signal-to-noise ratio of 3 was *ca.* 0.05%.

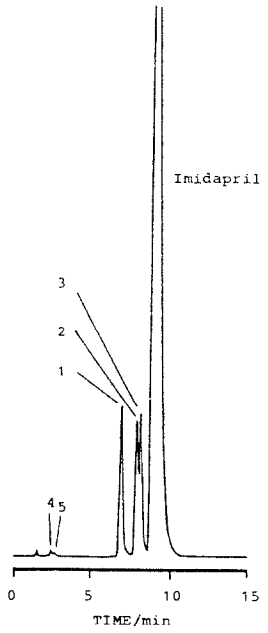

Fig. 3. Separation of imidapril, its diastereomers and the degradation products by reversed-phase HPLC. Peaks: 1 = *SRR*-form and *RSS*-form; 2 = *SSR*-form and *RRS*-form; 3 = *SRS*-form and *RSR*-form; 4 = degradation product; 5 = imidaprilat. Conditions: column, Nucleosil 5C_8 (150 mm × 4.6 mm I.D.); column temperature, 40°C; mobile phase, 0.01 *M* phosphate buffer (pH 2.7)–methanol (3:2); flow-rate, 1.0 ml/min; detection, 215 nm.

The determination of diastereomers in drug substances (single *SSS*-form) and those in tablets was performed using the proposed method. Six diastereomers were not found in six batches each of imidapril hydrochloride and imidapril tablets, which were produced for the investigation of the physico-chemical properties, testing specifications, etc. Three batches were also used in the long-term stability studying. The results of the determination of the diastereomers in the drug substances and tablets that had been stored for 3 years at room temperature are summarized in Table 1. Under these conditions, no epimerization was observed. It was found that imidapril is optically stable in the solid state.

The reversed-phase HPLC conditions mentioned above also offered a sufficient separation of related substances of imidapril hydrochloride such as metabolites and degradation products, other than the diastereomers. Fig. 3 shows the separation of imidapril and two degradation products, including imidaprilat. Imidaprilat is one of the major degradation products and also a major metabolite. The other degradation product was produced through hydrolysis of the ethyl ester and amide bond. The determination of related substances in the drug substances and in tablets was performed by the proposed method and the results are summarized in Table 1. Small amounts of the related substances (0.2–0.4%) were detected.

3.2. Separation of enantiomers by chiral derivatization method

Chiral amino compounds can be applied as derivatization reagents for imidapril hydrochloride having a carboxy group in the molecule. From the investigation of several chiral reagents, L-ANA, which is commercially available, was selected. Enantiomers of several drugs having a carboxyl group, such as naproxen (non-steroidal anti-inflammatory drug), have been successfully

Table 1
Optical purity and impurity of the drug substances and those in tablets after storage for 3 years at room temperature

Sample	Lot No.	Impurity[a] (%)	Diastereomer[a] (%)	Enantiomer (*RRR* %)		Enantiomeric purity (*SSS* %)
				Direct[b]	Derivatization[c]	
Drug substance	040	0.20	N.D.[d]	N.D.	N.D.	100.0
	050	0.20	N.D.	N.D.	N.D.	100.0
	060	0.21	N.D.	N.D.	N.D.	100.0
Tablets	040	0.37	N.D.	N.D.	–	100.0
	050	0.34	N.D.	N.D.	–	100.0
	060	0.34	N.D.	N.D.	–	100.0

[a] By reversed-phase HPLC and area-percentage method. Detection limit *ca.* 0.05%.
[b] Direct separation by using Chiralpak WH. Detection limit *c.a.* 0.2%.
[c] Derivatization method using L-ANA. Detection limit *c.a.* 0.05%.
[d] N.D. = not detected.

resolved using the chiral reagent L-ANA and normal-phase HPLC [5].

The derivatization procedure for imidapril hydrochloride was optimized by varying the amounts of the chiral reagent, DCC and pyridine by reference to the procedure for naproxen described in ref. 5. It turned out that pyridine is essential for the reaction of imidapril hydrochloride, although it was unnecessary in the derivatization of naproxen. It was also found that more than four or five times the molar amounts of both the chiral reagent and DCC with respect to the sample are required for complete reaction. The optimized conditions are described under Experimental. By-products in the reaction were efficiently removed by washing the reaction mixture with 1 *M* hydrochloride acid.

A typical chromatogram of enantiomers of imidapril hydrochloride after derivatization with L-ANA is shown in Fig. 4, with a chromatogram of a blank solution. The resolution (R_s) between two derivatized enantiomers was 10.5. Each of the derivatized enantiomers was collected and identified by mass spectrometry. The molecular ion peak M^+ of m/z 601, which is equal to the M_r of derivatized imidapril (405 + 214 − 18), was observed for both compounds. Both mass spectra were also identical.

For the separation of the other six diastereomers, the chiral derivatization method was also successful except for the *SSR*-form, as shown in Fig. 5. We can determine six kinds of optical isomers by the proposed method. The separation between the *SSR*- and *SSS*-forms may be successful by changing of the mobile phase, although in this case (Fig. 5) they eluted in one peak. It is not clear why the peak of the L-ANA-derivatized *RSS*-form was broad compared with the others.

The linearity of response of the *RRR*-form was investigated by derivatizing samples containing 0.05–0.5% (w/w) of the *RRR*-form in the stan-

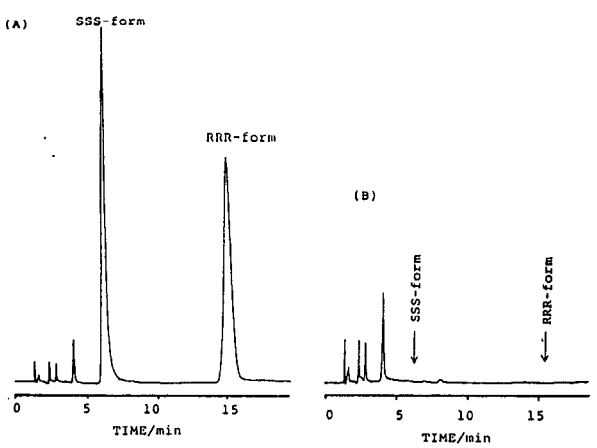

Fig. 4. (A) Separation of enantiomers of imidapril hydrochloride by the chiral derivatization method and (B) a blank chromatogram. Conditions: column, Zorbax Sil (5 μm; 150 mm × 4.6 mm I.D.); column temperature, 40°C; mobile phase, chloroform–methanol–ethanol–diethylamine (600:10:2:0.1); flow-rate, 1.0 ml/min; detection, 254 nm.

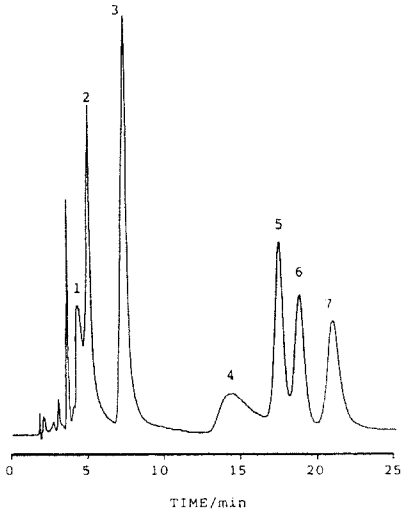

Fig. 5. Separation of eight optical isomers by the chiral derivatization method. Peaks: 1 = *SRS*-form; 2 = *SRR*-form; 3 = *SSS*-form and *SSR*-form; 4 = *RSS*-form; 5 = *RRR*-form, 6 = *RRS*-form, 7 = *RSS*-form. Conditions as in Fig. 4.

dard *SSS*-form. The results are shown in Fig. 6. The graph passed through the origin with $r = 0.999$ and the detection limit of the *RRR*-form in Fig. 7 at a signal-to-noise ratio of 3 was *ca.* 0.05%.

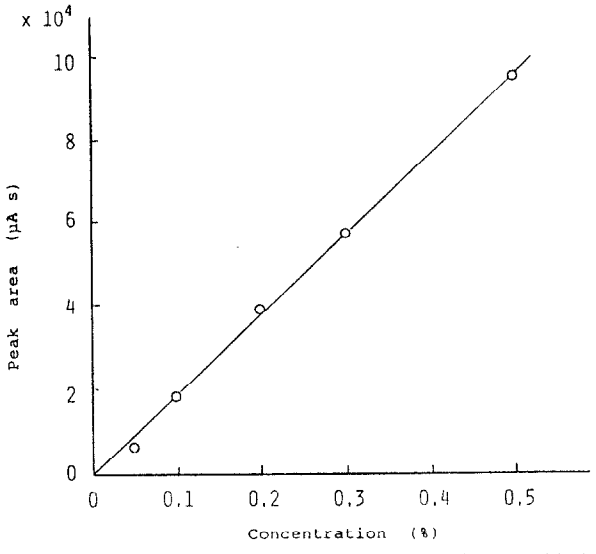

Fig. 6. Linearity of response of *RRR*-form in the chiral derivatization method.

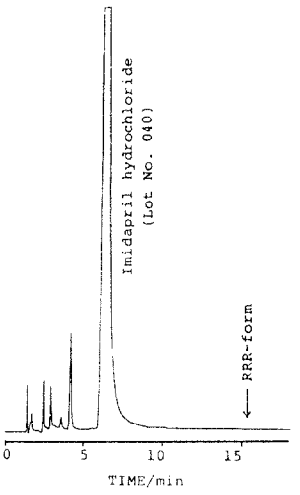

Fig. 7. Enantiomeric purity testing of imidapril hydrochloride by the chiral derivatization method. Conditions as in Fig. 4.

According to the derivatization procedure, three batches of the drug substances that had been stored at room temperature for 3 years were reacted with L-ANA and chromatographed under the conditions described in Fig. 4. The results are summarized in Table 1 and a typical chromatogram is shown in Fig. 7. The *RRR*-enantiomer was not detected in all batches.

3.3. Direct separation of enantiomers

It is well known that DL-amino acids have been successfully resolved by using ligand-exchange type CSPs [6–8], employing a solution of copper(II) sulphate as the mobile phase. Resolution of the enantiomers of imidapril hydrochloride, which can be regarded as an amino acid derivative, was achieved by employing ligand-exchange type CSPs. Among several commercially available CSPs, only Chiralpak WH offered the enantiomeric separation of imidapril. The optimization of the composition of the mobile phase was carried out by varying the concentration of copper(II) ion and the column temperature and adding some additives such as an organic solvent, by reference to ref. 8.

The effects of the concentration of copper(II) ion and the column temperature on R_s and the

Table 2
Effect of the concentration of copper(II) sulphate on the resolution of enantiomers of imidapril hydrochloride

$CuSO_4$ concentration (m*M*)	Retention time (min)		Theoretical plate number		R_s
	SSS-form	*RRR*-form	*SSS*-form	*RRR*-form	
2	12.66	14.57	1094	733	0.98
3	12.42	14.26	1084	703	0.94
4	12.18	14.15	1036	638	0.95
8	11.17	13.04	808	531	0.92

Flow-rate, 1.5 ml/min; column temperature, 40°C; detection, 230 nm.

retention times of the enantiomers are summarized in Tables 2 and 3. The higher the copper ion concentration or the column temperature, the faster the compound is eluted. The R_s values showed no noticeable change in the copper(II) concentration range 2–8 m*M*. However, it was advantageous to use a higher column temperature for improving the theoretical plate number (N), leading to a higher R_s. The effects of acetonitrile addition on R_s values and retention times are summarized in Table 4. The peak shape was markedly improved, that is, N increased with increase in the acetonitrile content, resulting in an improvement in R_s values (from 1 to 2.5).

The selected mobile phase composition and a typical chromatogram of the enantiomers are shown in Fig. 8A. Other optical isomers were also successfully separated from imidapril hydrochloride (*SSS*-form), except for the *SSR*-form, as

Table 3
Effect of column temperature on the resolution of enantiomers of imidapril hydrochloride

Temperature (°C)	Retention time (min)		Theoretical plate number		R_s
	SSS-form	*RRR*-form	*SSS*-form	*RRR*-form	
40	12.42	14.26	1084	703	0.94
45	11.87	13.64	1202	792	1.00
50	11.39	13.12	1360	1019	1.12

Concentration of $CuSO_4$, 3 m*M*; flow-rate, 1.5 ml/min; detection, 230 nm.

Table 4
Effect of acetonitrile addition on the resolution of enantiomers of imidapril hydrochloride

Acetonitrile (%)	Retention time (min)		Theoretical plate number		R_s
	SSS-form	*RRR*-form	*SSS*-form	*RRR*-form	
0	12.08	13.94	1100	752	1.00
10	8.82	10.80	1558	1370	1.80
20	6.42	8.14	2158	1847	2.46
30	4.80	6.15	1980	1782	2.49

Concentration of $CuSo_4$, 3 m*M*; flow-rate, 1.5 ml/min; detection, 230 nm; column temperature, 40°C.

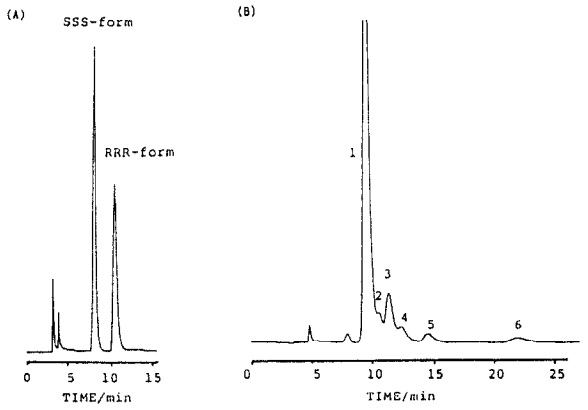

Fig. 8. Direct chiral separation of imidapril hydrochloride: (A) separation of enantiomers; (B) separation of eight optical isomers. Peaks: 1 = *SSR*-form and *SSS*-form; 2 = *RSS*-form; 3 = *SRR*-form and *RRR*-form; 4 = *RRS*-form; 5 = *RSR*-form; 6 = *SRS*-form. Conditions: column, Chiralpak WH (250 mm × 4.6 mm I.D.); column temperature, 40°C; mobile phase, 3 m*M* aqueous solution of copper(II) sulphate–acetonitrile (3:1); flow-rate, 1.0 ml/min; detection, 230 nm.

shown in Fig. 8B, where *ca.* 2% of each isomer was added to the *SSS*-isomer. Related substances of imidapril hydrochloride such as imidaprilat did not disturb the peaks of the eight optical isomers of imidapril under the conditions used in Fig. 8.

The linearity of response for the *RRR*-form was investigated over the range 0.25–3% (w/w). The graph passed through the origin with $r = 0.997$. The detection limit of the *RRR*-form at a signal-to-noise ratio of 3 was *ca.* 0.2% (see Fig. 9C). The detection limit of the antidope in the direct separation method (*ca.* 0.2%) was higher than that in the chiral derivatization method (*ca.* 0.05%). This can be interpreted by the low sample load capacity of the Chiralpak WH column and the low detection sensitivity at 230 nm.

The sample load capacity is one of the important parameters in ligand-exchange type CSPs, because it is not large compared with other common stationary phases such as an ODS column. A large sample loading affects the resolution [7]. It is necessary and recommended to decrease the sample loading in order to achieve good resolution. The results of the investigation of sample loading (1–5 μg) are

Table 5
Effect of sample load on the theoretical plate number of enantiomers of imidapril hydrochloride

Sample load (μg)	Theoretical plate number		R_s
	SSS-form	*RRR*-form	
1	3083	2450	3.05
2	2106	2860	2.99
3	3497	2471	3.00
5	3301	2846	2.81

Concentration of $CuSO_4$, 3 m*M*; concentration of acetonitrile, 30%; flow-rate, 1.0 ml/min; column temperature, 40°C; detection, 230 nm.

summarized in Table 5. There was no significant decrease in *N* values when up to 5 μg of the compound were injected.

The enantiomeric purity of imidapril hydrochloride and its tablets that had both been stored for 3 years at room temperature was determined using the proposed method. Typical chromatograms are shown in Fig. 9A and B, with a chromatogram of imidapril standard spiked with

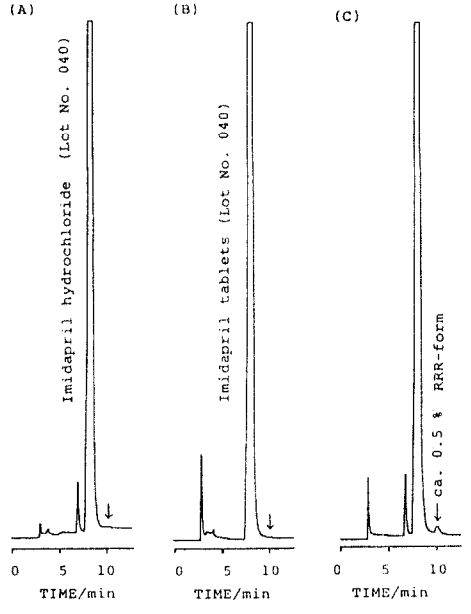

Fig. 9. Enantiomeric purity testing of (A) imidapril hydrochloride and (B) its tablet by the direct method. (C) Chromatogram of standard imidapril spiked with *ca.* 0.5% of the *RRR*-form. Conditions as in Fig. 8.

ca. 0.5% of the *RRR*-form (Fig. 9C). The *RRR*-form was not detected in all samples, as indicated in Table 1.

The method was also applied to the enantiomeric purity testing of the drug substance in aqueous solutions (pH 2–11). The results are summarized in Table 6, with the results of assay and diastereomer determination. Imidapril was found to be stable under acidic (pH 2) and neutral (pH 7) conditions. The *RRR*-form was not detected over the whole pH range investigated, although epimerization of imidapril occured at the asymmetric carbon 3 (see Fig. 1) and small amounts of *SSR*-form (*ca.* 0.5%) were detected in the pH range 5–9. The *SSR*-form was determined by using reversed-phase HPLC, considering the result in this direct separation, that is, no *RRS*-form was observed. At high pH (9–11), larger amounts of imidapril were degraded into imidaprilat and the degradation product as shown in Fig. 3.

In conclusion, the enantiomeric separation of imidapril hydrochloride was achieved by both the direct and indirect methods (derivatization method). Eight optical isomers of imidapril hydrochloride were successfully determined by the combined use of three chromatographic methods. First, reversed-phase HPLC should be performed to determine the six diastereomers because the *SSR*-isomer was not separated from the *SSS*-isomer by the succeeding enantiomeric separation method. After checking the diastereomers, enantiomeric purity testing according to the derivatization method or the direct method should be performed. It was found that no racemization of imidapril hydrochyloride occurs on long-term storage and in aqueous solutions of various pH.

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Table 6
Assay and enantiomeric purity of imidapril hydrochloride in solutions of various pH

Sample pH	Residual imidapril[a] (%)	Diastereomer[b] (%)	*RRR*-form[c] (%)
2	93.2	N.D.[d]	N.D.
3	85.9	N.D.	N.D.
5	88.7	0.48 (*SSR*)	N.D.
7	92.1	0.55 (*SSR*)	N.D.
9	75.6	0.44 (*SSR*)	N.D.
11	12.1	N.D.	N.D.

Sample solutions were heated at 90°C for 1 h.
[a] HPLC assay using internal standard method.
[b] By reversed-phase HPLC and area-percentage method. Detection limit *ca.* 0.05%.
[c] By direct chiral separation method. Detection limit *ca.* 0.2%.
[d] N.D. = not detected.