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Enantiomeric purity determination of acetyl-L-carnitine by reversed-phase high-performance liquid chromatography using chiral derivatization

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Abstract

An indirect HPLC enantioseparation method for the determination of acetyl-D-carnitine (D-AC) in acetyl-L-carnitine (L-AC) was developed. L-AC was derivatized with a chiral amino compound which has a chromophore for UV detection. Six chiral amino compounds were examined as chiral derivatization reagents. Among them, enantiomers of acetylcarnitine derivatized with L-alanine-β-naphthylamide (L-Ala-β-NA) were successfully separated on an ODS column within 10 min with $R_s = 1.94$ and $\alpha = 1.10$. Quantitation was achieved through UV detection at 254 nm. The derivatization reaction of L-AC with L-Ala-β-NA was completed in less than 10 min at room temperature (ca. 20°C). Validation data such as linearity, detection limit, and precision are also presented. The detection limit of D-AC in L-AC in this method was below 0.05% (visual evaluation). This method was found to be applicable as a practical quality control method for the enantiomeric purity determination of L-AC. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Chiral derivatization; Derivatization, LC; Acetyl-L-carnitine; L-Alanine-β-naphthylamide

1. Introduction

Acetyl-L-carnitine (L-AC) is one of the biological substances localized in various tissues of mammals; brain, heart, liver, kidney, muscle etc., and has stimulatory actions of learned behavior [1,2]. L-AC is expected as the brain metabolic stimulant [3,4] and now under the clinical investigation in Japan. The D-enantiomer, on the other hand, does not show these effects [5,6]. Therefore, separation and determination of enantiomers of acetylcarnitine (AC) are important for the guarantee of its effectiveness and safety, as well as quality.

For chiral drugs, the minor enantiomer is regarded as one of the impurities because of its different biological activity [7,8]. Various kinds of analytical methods have been developed for the determination of enantiomeric purity. HPLC is the most generally used method and three approaches have been dis-

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cerned. First is the conversion of the enantiomers into a pair of diastereomers, which are separated on an achiral column, by chemical reaction with an enantiomerically pure chiral compound (indirect approach). Second is the addition of a chiral auxiliary to the mobile phase, and the last is the use of chiral stationary phases (direct approach).

There are some reports concerning the determination of the D-enantiomer contained in L-AC [9–12] and L-carnitine (L-C) [13–15]. Reported first by Yasuda and co-workers [9] was a method in which acetyl-D-carnitine (D-AC) in L-AC was stereoselectively converted into D-carnitine (D-C) by the enzymatic reaction with acetylcholinesterase and the D-C was determined by HPLC on an achiral column. In this method, however, trace amounts of D-AC could not be determined in the presence of carnitine as an impurity or a decomposition product from the beginning. Further, this method was not sufficient as a quality control method with respect to the cost, detection limit, and reproducibility. In 1994, Hirota and co-workers [10] reported chromatographic separation of enantiomers of AC on a commercially available chiral column (Chiralcel OD-R) after derivatization with 9-anthryldiazomethane (ADAM). Witt and co-workers [11] also reported chromatographic separation of enantiomers of AC on an ODS column after chiral derivatization. In the latter method, chiral derivatization was performed by a two step reaction. The first step is hydrolysis of AC to carnitine and the second is chiral derivatization of carnitine with (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC). That is, the enantiomeric purity of AC was measured together with that of carnitine. In 1997, Carotti and co-workers [12] reported the direct HPLC enantioseparation on a synthetic stationary phase column using teicoplanin as chiral selector. However, the theoretical plate number of this column was not good compared with that of the analytical ODS column (typically 10 000 plate number).

In this work, we describe a convenient procedure for the separation of the enantiomers of AC in pharmaceutical preparations. This method is based on the reaction of acetyl-DL-carnitine (DL-AC) with L-alanine-β-naphthylamide (L-Ala-β-NA) and the separation of the corresponding diastereomeric derivatives by reversed-phase HPLC with an ODS column. Further, the derivatives of DL-AC and DL-carnitine (DL-C) were separated simultaneously without co-elution. This method was applied for the enantiomeric purity determination of L-AC.

2. Experimental

2.1. Reagents and materials

L-AC, L-C, DL-AC, DL-C, and L-Ala-β-NA were purchased from Sigma (St. Louis, MO, USA). L-AC standard was prepared through the recrystallization of purchased L-AC. L-AC formulation (60% granule) was prepared in Tanabe Seiyaku Pharmaceutics Research Laboratory (Osaka, Japan). (1*S*,2*R*)-(+)-2-Amino-1,2-diphenylethanol was from Aldrich (Milwaukee, WI, USA). (*S*)-(−)-1-Phenylethylamine and (*S*)-(−)-1-(1-naphthyl)ethylamine were from Tokyo Kasei Kogyo (Tokyo, Japan). Ethyl chloroformate was from Wako Pure Chemical Industries (Osaka, Japan). L-Phenylglycine methyl ester and L-phenylalanine methyl ester were prepared in Tanabe Seiyaku Analytical Research Laboratory. HPLC grade acetonitrile and other chemicals of analytical reagent grade were obtained from Katayama Kagaku Kogyo (Osaka, Japan). The structures of six derivatization reagents employed are shown in Table 1. The chirality of these six reagents used was selected from the preliminary investigations (data not shown) to obtain elution order D-form before L-form, which is favorable for the purity testing.

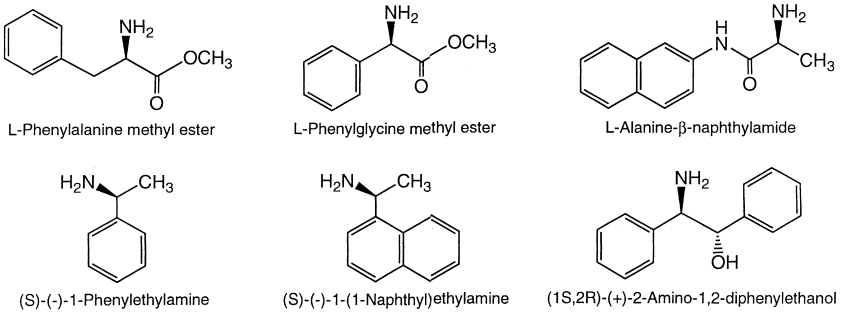
2.2. Apparatus and chromatographic conditions

The HPLC system used in this study consisted of a Shimadzu (Kyoto, Japan) LC-10AD HPLC pump, a Rheodyne Model 7725 injector with a 50 μl sample loop and a Shimadzu SPD-10A variable wavelength UV detector. Data were acquired with a Shimadzu Chromatopac C-R7A plus data processor. Chromatography was carried out at 40°C with a Shimadzu CTO-10AC column oven and flow-rate was 1.0 ml/min. The mobile phase and wavelength used for enantiomeric purity testing of L-AC were a mixture of 50 m*M* phosphate buffer solution (pH 2.5) and 23% acetonitrile, and 254 nm, respectively. The HPLC column used was Cosmosil ODS ARII (150×

Table 1
Separation of the derivatives of DL-AC with chiral amino compounds

Reagent	k_1 [a]	k_2 [a]	α	R_s	MP[b] (% CH_3CN)	Detection (nm)
L-Phenylalanine methyl ester	4.68	5.63	1.20	3.65	17	230
L-Phenylglycine methyl ester	4.03	4.46	1.11	1.84	15	230
L-Alanine-β-naphthylamide	4.59	5.06	1.10	1.94	23	254
(*S*)-(−)-1-Phenylethylamine	4.96	5.33	1.07	1.55	15	210
(*S*)-(−)-1-(1-Naphthyl)ethylamine	5.77	6.08	1.05	1.09	23	254
(1*S*,2*R*)-(+)-2-Amino-1,2-diphenylethanol	10.30	10.73	1.04	1.01	15	210

[a] k_1, k_2 (retention factors), α (separation factor)$=k_2/k_1$, R_s (resolution)$=(t_{R2}-t_{R1})/(1/2)(t_{W1}+t_{W2})$ where t_{R2} and t_{R1} are the retention times of the two peaks, t_{W1} and t_{W2} are the bandwidths at the baseline of the two peaks.
[b] MP: % acetonitrile to the 50 m*M* phosphate buffer (pH 2.5).



4.6 mm I.D., 5 μm particle size, Nacalai Tesque, Kyoto, Japan).

2.3. Derivatization procedure

About 0.1 mg of L-AC or 0.16 mg of 60% granule (corresponding to ca. 0.1 mg L-AC) was weighed into a glass stoppered test tube and dissolved in 0.5 ml of ethanol. A 0.5 ml volume of ethyl chloroformate solution (2 mg/ml in chloroform), 0.5 ml of triethylamine solution (5 mg/ml in chloroform), and 0.5 ml of L-Ala-β-NA (4.5 mg/ml in ethanol) were added to the test tube. Then, the mixture was vigorously shaken and allowed to stand at room temperature (ca. 20°C) for 10 min. The molar ratio of L-AC versus L-Ala-β-NA is ca. 1:20. After reaction, 1 ml of 50 m*M* sodium hydrocarbonate solution was added to the mixture. A 20 μl volume of the aqueous layer was injected into the HPLC system. The reaction scheme is shown in Fig. 1. The ^{1}H-NMR signals of derivatives were assigned as follows: L-derivative, ^{1}H-NMR ($CDCl_3$), δ 1.49 (d, 3H, J=7 Hz, CH_3), 2.11 (s, 3H, –CO–CH_3), 2.71 (dd, 1H, J=7, 15 Hz), 2.80 (dd, 1H, J=6, 15 Hz), 3.31 (s, 9H), 3.77 (d, 1H, J=14 Hz), 3.88 (dd, 1H, J=8, 14 Hz), 4.50 (m, 1H), 5.71 (m, 1H, CH), 7.41 (t, 1H, J=8 Hz), 7.46 (t, 1H, J=8 Hz), 7.60 (d, 1H, J=8 Hz), 7.77 (d, 1H, J=8 Hz), 7.81 (d, 1H, J=8 Hz), 7.82 (d, 1H, J=8 Hz), 8.20 (s, 1H). D-derivative, ^{1}H-NMR ($CDCl_3$), δ 1.50 (d, 3H, J=7 Hz, CH_3),

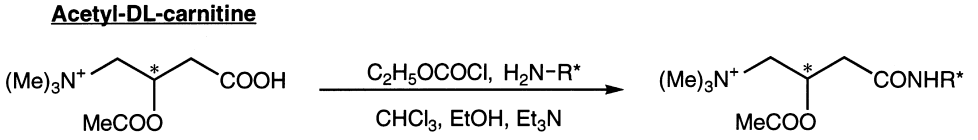


Fig. 1. Reaction of DL-AC with a chiral amino compound.

2.13 (s, 3H, –CO–CH_3), 5.64 (m, 1H, CH), the other chemical shifts are the same as in the L-derivative mentioned above.

2.4. The enantiomeric purity of L-Ala-β-NA

The enantiomeric purity testing of L-Ala-β-NA was performed by HPLC with crown ether dynamically coated chiral stationary phase (CROWNPAK CR(+)) using a mixture of 500 m*M* potassium chloride buffer solution and 15% methanol as a mobile phase [16]. As a result, D-alanine-β-naphthylamide was not detected (the detection limit is 0.05%). That is, enantiomeric purity of L-Ala-β-NA used in this work is found to be more than 99.95%.

2.5. Calculation of D-AC content

The content of the D-AC in L-AC was calculated by the following equation:

$$\text{D-AC content (\%)} = \frac{A_D}{A_D + A_L} \times 100$$

where A_D is the peak area of the derivative of D-AC and A_L is the peak area of the derivative of L-AC. Although A_D and A_L were not perfectly equal (see Figs. 3–5), a correction factor was not used in the equation mentioned above because the difference of each peak area (A_D: A_L = 1:1.04) was not so large. In other words, this difference does not give an important effect to the value of D-AC (typically 0–1%). This equation can be accepted for the quality control method.

3. Results and discussion

3.1. Selection of derivatization reagents

The effect of the six kinds of derivatization reagents on the enantioselectivity was investigated. The reaction was performed with DL-AC according to the derivatization procedure mentioned above, changing the chiral derivatization reagent. Obtained selectivity (α) and resolution (R_s) values are summarized in Table 1, where acetonitrile content (%) was adjusted to give *k* values around 5–10. The results indicated that the α values depended on the substituent (carboxymethyl, benzyl, methyl, phenyl, naphthyl etc.) in the α-position of the amine function of the amino compounds. L-Phenylalanine methyl ester (having a carboxymethyl and benzyl group as a substituent) gave large α value (1.20), and (1*S*, 2*R*)-(+)-2-amino-1,2-diphenylethanol (α-hydroxybenzyl and phenyl) and (*S*)-(−)-1-(1-naphthyl)ethylamine (methyl and naphthyl) gave small α values (1.04 and 1.05, respectively). The detection at 254 nm was also better to avoid the detection of the unnecessary peaks. Therefore, we selected L-Ala-β-NA as a chiral derivatization reagent for L-AC from its relative large α value and naphthyl structure having an effective UV chromophore, although the others may give larger enantioselectivity when another organic modifier is used.

3.2. Identification of diastereomeric derivatives

Both DL-AC and L-AC derivatives were prepared by the reaction (Fig. 1) and were investigated by using nuclear magnetic resonance (NMR) spectrometry. The ^{1}H-NMR spectra are shown in Fig. 2. Their spectra indicated that the target derivatives were formed from AC and L-Ala-β-NA. A further two sets of resonances were observed for almost each proton or group of equivalent protons of the diastereomeric derivatives of DL-AC.

3.3. The effects of reaction time and reagent concentration

The effect of reaction time was investigated through peak areas of diastereomeric derivatives in the range 1~10 min. DL-AC (1 m*M*) and L-Ala-β-NA (20 m*M*) concentrations and the other reaction conditions employed were the same as in the derivatization procedure described above. The result is shown in Fig. 3. The constant peak areas were obtained above 7 min reaction. Considered to be sufficient time for reaction, 10 min was adopted as the reaction time. After 10 min reaction, the absence of non-derivatized DL-AC was confirmed by HPLC with the following conditions: the mobile phase was a mixture of 50 m*M* phosphate buffer solution (pH 3.0), methanol, and sodium 1-octanesulfonate

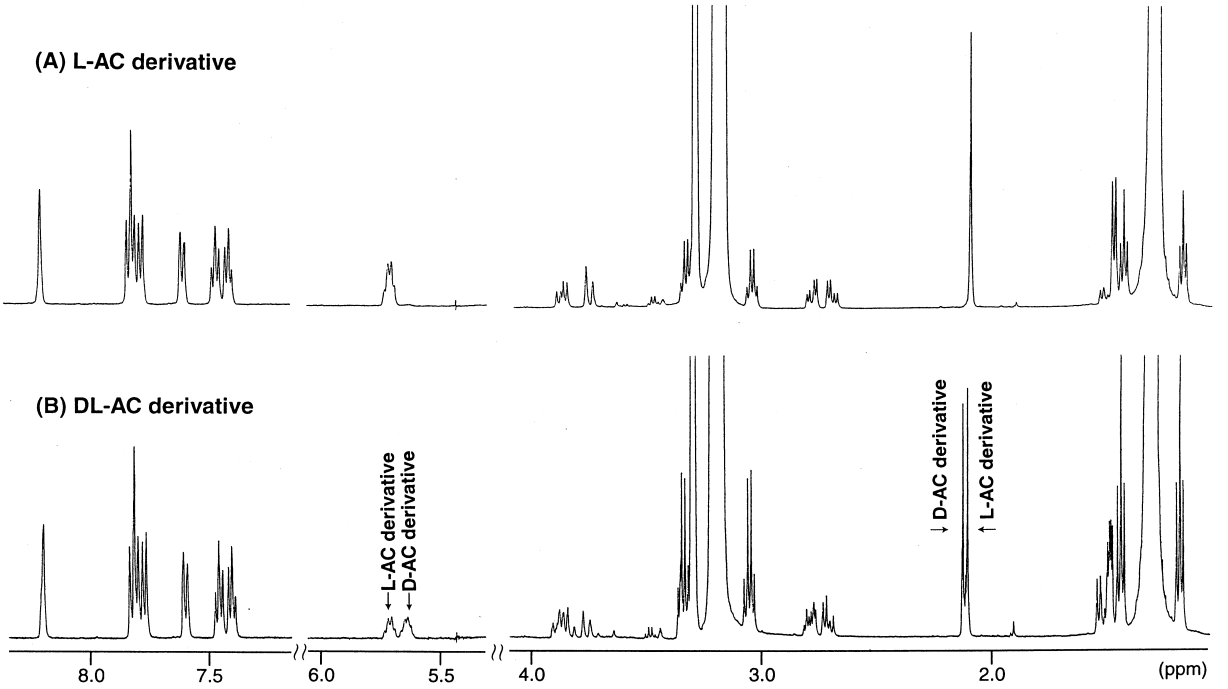


Fig. 2. NMR spectra of (A) L-AC and (B) DL-AC derivatives with L-Ala-β-NA.

(85:15:0.1), detection was at 220 nm, and the column temperature was 40°C.

The effect of L-Ala-β-NA concentration on the derivatization was investigated in the range from 2 to 30 m*M*. The sample concentration was fixed at 1 m*M*. The other reaction conditions were the same as in the derivatization procedure (Section 2.3). The result is shown in Fig. 4. The constant peak areas for

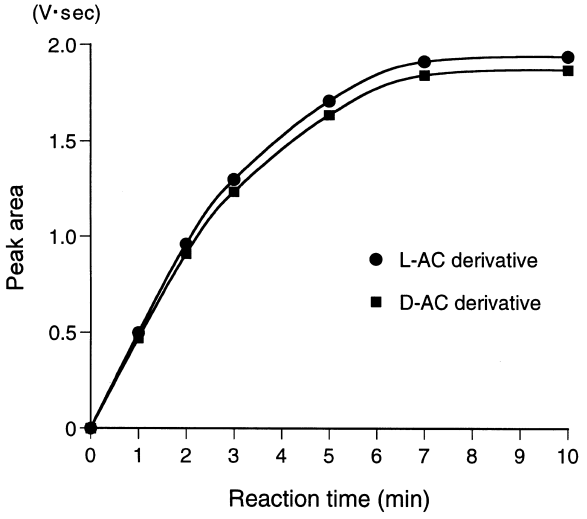


Fig. 3. Effect of reaction time on DL-AC derivatives with L-Ala-β-NA.

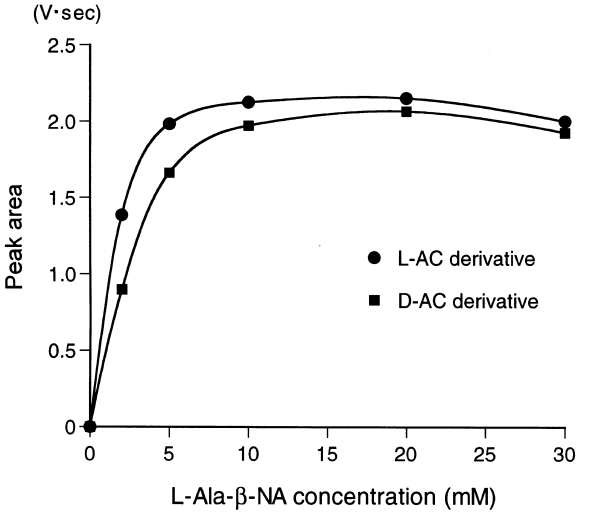


Fig. 4. Effect of L-Ala-β-NA concentration on the reaction.

L-AC and D-AC derivatives, that is, completed reactions were obtained above 20 m*M* chiral reagent concentration.

The effects of concentration of ethyl chloroformate and triethylamine on the derivatization reaction were also investigated in the range from 2 to 30 m*M* and 10 to 70 m*M*, respectively. The other reaction conditions were the same as in Section 2.3. The results are shown in Fig. 5. Concentrations of 20 m*M* for ethyl chloroformate and 50 m*M* for triethylamine were found to be sufficient for the completed reaction.

3.4. Sample solution stability

The sample solution in this method is an aqueous solution, in which AC may be hydrolyzed and turned into carnitine. Stability of the derivatized L-AC in solution was evaluated by measuring the peak areas of derivatives of L-AC and L-C over the 20 h at 5°C, which is the temperature of the refrigerator or sample cooler (Fig. 6). The result showed that about 0.5% of L-C was formed after 6 h and 1.0% after 12 h storage. The sample solution is recommended to be injected within 12 h when stored at 5°C.

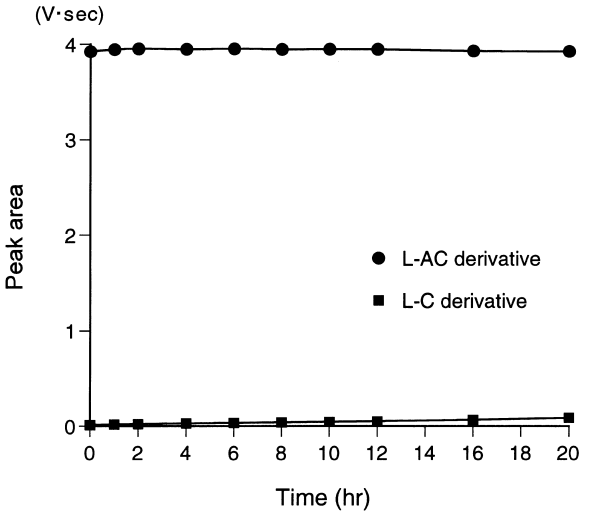


Fig. 6. Stability testing of L-AC derivative in an aqueous solution.

3.5. Separation of enantiomers of DL-AC and DL-C

Derivatives of DL-AC and DL-C were separated simultaneously by adding tetrahydrofuran to the mobile phase mentioned above, that is, by the ternary mobile phase as follows: a mixture of 50 m*M* phosphate buffer solution (pH 2.5), 9% acetonitrile, and 6% tetrahydrofuran. The separation of these four derivatives is shown in Fig. 7(A), where the last peak (derivative of L-AC) eluted at around 22 min. For the fast enantiomeric purity determination of L-AC, a mixture of 50 m*M* phosphate buffer and 23% acetonitrile was used as the mobile phase. Under

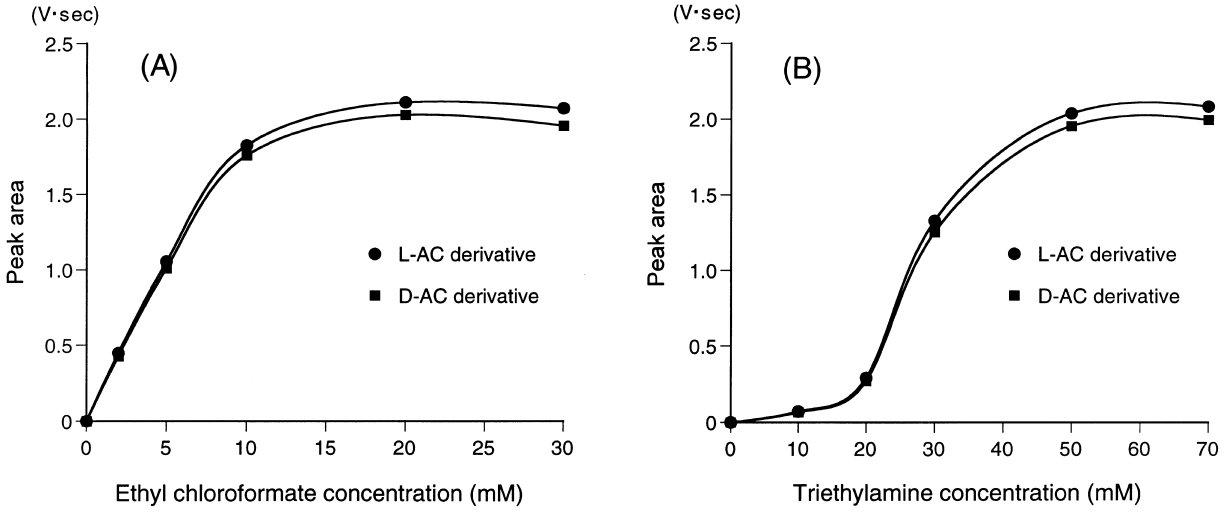


Fig. 5. Effects of (A) ethylchloroformate concentration and (B) triethylamine concentration on the reaction.

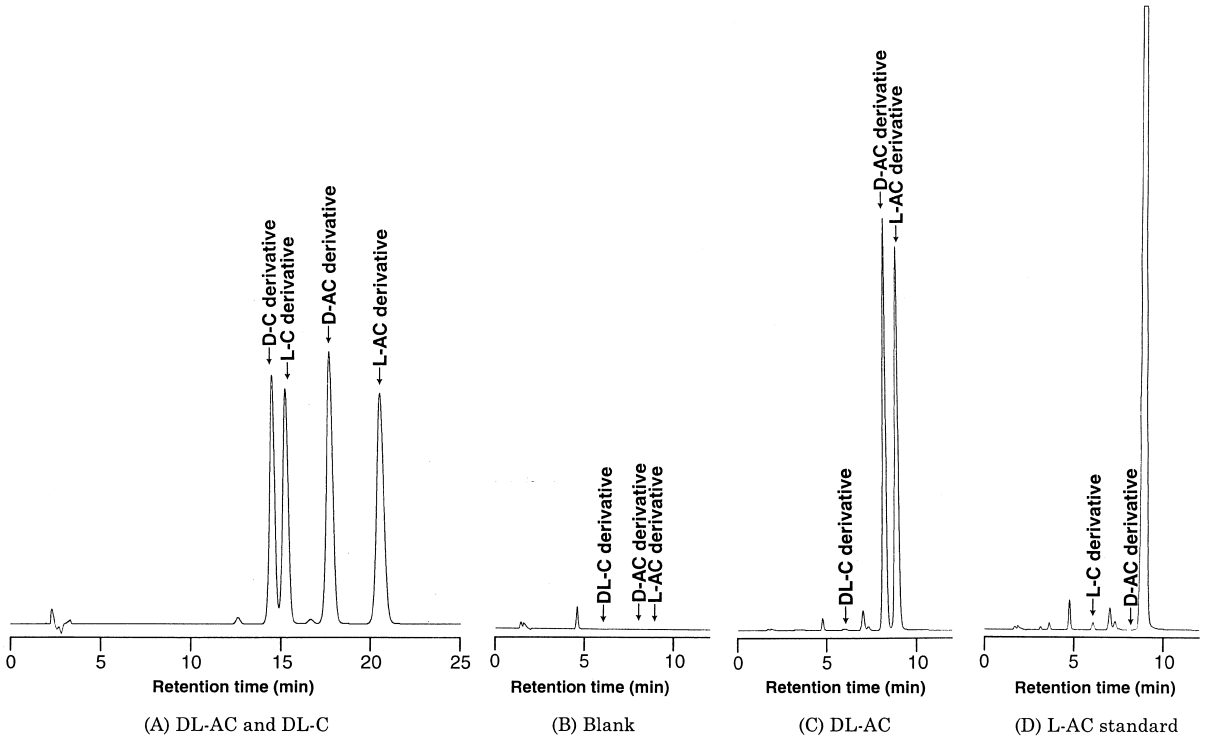


Fig. 7. Chromatogram of (A) DL-AC and DL-C, (B) blank, (C) DL-AC, and (D) L-AC standard. Mobile phase (A): 9% acetonitrile and 6% tetrahydrofuran to the 50 m*M* phosphate buffer (pH 2.5). (B–D): 23% acetonitrile to the 50 m*M* phosphate buffer (pH 2.5).

these conditions, DL-AC derivatives were separated within 10 min with $R_s = 1.94$, although DL-C derivatives were eluted as one peak before the peaks of DL-AC derivatives (see Fig. 7(C) and (D)).

3.6. Validation and application for the enantiomeric purity determination

Linearity and recovery of the D-AC in the method were evaluated using L-AC standard solutions added DL-AC correspond to 0.05, 0.1, 0.2, 0.5, 1, and 3% of D-AC. These solutions were treated according to the derivatization procedure in Section 2.3. Theoretical concentration of D-AC versus their responses obtained is shown in Fig. 8, and the recoveries are summarized in Table 2. The linear correlation coefficient $r = 0.9999$ and almost 100% recoveries were obtained in this experiment. The detection limit of D-AC was also investigated. D-AC (0.05%) was successfully detected (visual evaluation) and a limit of detection for D-AC seems to be below 0.05%.

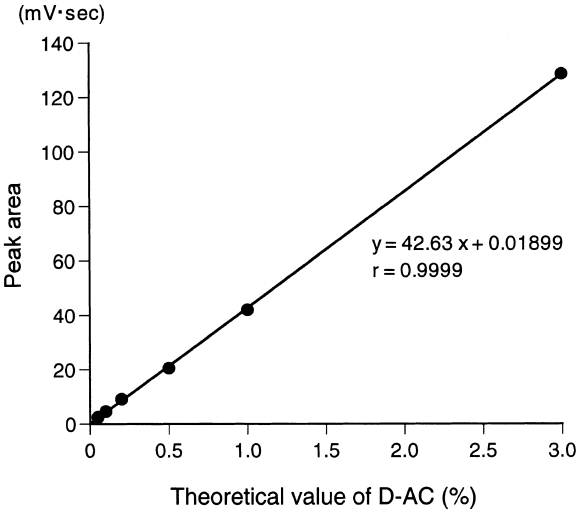


Fig. 8. Linearity of D-AC in L-AC.

Table 2
Recoveries of D-AC added to L-AC

Theoretical content (%)	Found content (%)
0.05	0.065
0.10	0.117
0.20	0.221
0.50	0.511
1.00	1.032
3.00	3.061

The HPLC chromatograms of a blank solution, derivatives of DL-AC and L-AC standard are shown in Fig. 7(B), (C), and (D). There was no peak to interfere with the peak of DL-AC, and D-AC was not detected (less than 0.05%) in the L-AC standard. This also indicated that no racemization occurred in the derivatization procedure.

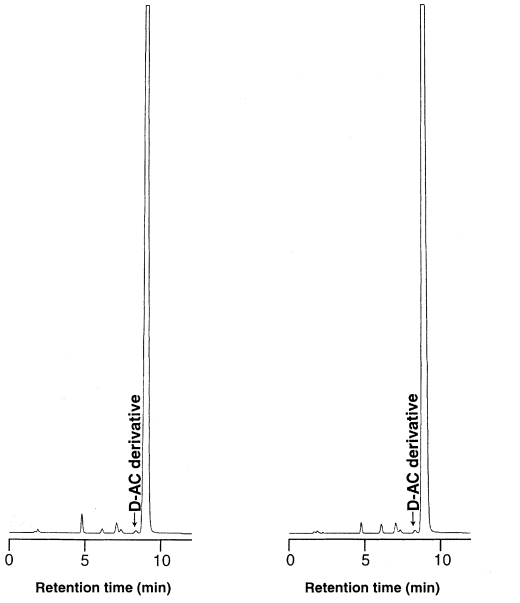
Precision was evaluated by six replicates analysis. Sample solution added 1% of the D-AC was treated according to the derivatization procedure (Section 2.3). A mean value of 1.074% of D-AC was found and the relative standard deviation (RSD) was 2.14%, indicating that this method is reliable and can be used.

Finally, the enantiomeric purity determination of L-AC drug substances and L-AC in formulations (60% granules) were performed. The results are summarized in Table 3 and the typical chromatograms are shown in Fig. 9. Almost the same values as in those obtained by the previous method [10] were obtained in this method. Further enantiomeric purity determination of stability testing was performed for the L-AC drug substance stored in a closed bottle for 6 months under the 40°C/75% relative humidity. It was found that racemization did not occur for L-AC in the solid state. This method was found to be useful for the enantiomeric purity determination of L-AC and applicable as a quality control method.

Table 3
Enantiomeric purity determination of L-AC drug substances and L-AC in formulations (60% granules)

Sample	Lot no.	D-AC content (%)	
		This method	Previous method [10]
L-AC drug substances	1	0.22	0.18
	2	0.12	0.08
	3	N.D.[a]	N.D.
L-AC in formulations (60% granules)	1	0.20	0.17
	2	0.11	0.08
	3	N.D.	N.D.

[a] N.D.: Not detected (detection limit: 0.05%).



(A) L-AC drug substance (B) L-AC in formulation (60 % granule)

Fig. 9. Enantiomeric purity determination of L-AC.

4. Conclusion

Derivatization of DL-AC and DL-C with L-Ala-β-NA leads to corresponding diastereomers which are separable by an ODS column. The derivatization was simple and completed in less than 10 min. These derivatives were simultaneously separated within 25 min with the mobile phase of a mixture of phosphate buffer, acetonitrile, and tetrahydrofuran. For a fast enantiomeric purity testing of L-AC, a mixture of phosphate buffer and acetonitrile was employed as the mobile phase. This enables the separations of derivatives of DL-AC within 10 min. The described method was successfully applied for determining the enantiomeric purity of L-AC drug substances and L-AC in formulations.

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