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High-performance liquid chromatographic determination of the enantiomers of carnitine and acetylcarnitine on a chiral stationary phase

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Abstract

A method was developed for determining the respective D-isomers in L-carnitine and acetyl-L-carnitine by high-performance liquid chromatography (HPLC). DL-Carnitine and acetyl-DL-carnitine were derivatized with 9-anthryldiazomethane to increase the interaction between the solutes and a chiral stationary phase and also the sensitivity; the simple derivatization was carried out under mild conditions of standing at 50°C for 20 min. The enantiomeric separations of the respective derivatives were achieved by HPLC on a commercially available chiral column (Chiralcel OD-R) with a mobile phase of 0.5 *M* sodium perchlorate solution–acetonitrile (3:2). It was confirmed that this method shows good specificity, reproducibility, linearity, accuracy and sensitivity (detection limit for acetyl-D-carnitine ≈0.01%).

1. Introduction

Both L-carnitine (L-C) and acetyl-L-carnitine (L-AC) are biological substances localized in various tissues such as the brain, heart, liver, kidney and muscle. L-C is essential for the transport and mitochondrial β-oxidation of long-chain fatty acids [1,2]. Another function of L-C is in the promotive excretion of accumulated short-chain fatty acids resulting from excess drug administration or metabolic disorder. Since 1960, DL-carnitine (DL-C) has been used as a drug for anorexia, dyspepsia, senile digestive hypergasia (because of its pharmacological effects of acceleration of digestive fluid secretion), enterokinesis and sugar and lipid metabolism. On the other hand, it is reported that D-C and L-C have different pharmacological activities and toxicity [3,4].

L-AC has stimulatory actions of learning behaviour from the viewpoint of ethopharmacology [5,6], so that it is expected to be useful as a cerebral metabolic enhancer [7,8]. On the other hand, the D-enantiomer does not show these effects [9,10].

It is very important to establish the enantiomeric determination and separation techniques for DL-C and DL-AC in order to ensure the quality of the drug and to investigate the phar-

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macological effects and the pharmacokinetics of the enantiomers of DL-C and DL-AC in detail.

There has been only one report concerning the determination of the D-isomer contained in L-AC [11]. This method, however, is not a direct separation technique but an indirect assay by enzymatic reaction. The D-AC contained in L-AC is stereoselectively converted into D-C by treatment with acetylcholinesterase, and the D-C produced is determined by HPLC on a non-chiral column. That is, trace amounts of D-AC would not be able to be determined in the presence of carnitine initially as an impurity or decomposition product. Further, this method has some disadvantages regarding the cost, detection limit and reproducibility.

So far, no study has been reported on the HPLC separation of the enantiomers of carnitine or acylcarnitines. It is probable that the difficulties are due to their high polarity and low UV absorption.

We tried various chromatographic techniques to establish a practical method for the enantiomeric determination of carnitine and acetylcarnitine. As the result, it was found that the enantiomeric determination of these compounds could be achieved by HPLC on a commercially available chiral column (Chiralcel OD-R) [12–14], following derivatization with (9-anthryl-diazomethane (ADAM); ADAM has been widely used for the determination of variable fatty acids, etc. [15,16] and found to be able to react with acylcarnitines under mild conditions [17,18].

This paper reports the investigation of the various derivatization and HPLC conditions in order to establish the most suitable approach. We also describe the method validation and its application to optical purity testing for L-AC drug substances.

2. Experimental

2.1. Materials

DL-Carnitine was obtained from Nacalai Tesque (Kyoto, Japan), L-carnitine and acetyl-DL-carnitine from Sigma (St. Louis, MO, USA), acetyl-L-carnitine and crotonylbetain from Sigma Tau (Rome, Italy) and ADAM from Funakoshi Yakuhin (Tokyo, Japan). Acetonitrile of HPLC grade and other organic solvents 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) and mobile phases were passed through a membrane filter of 0.45-μm pore size (Fuji Photo Film, Tokyo, Japan) prior to use. All other reagents were of analytical-reagent grade from Katayama Kagaku.

2.2. Apparatus

The chromatographic system consisted of a Shimadzu (Kyoto, Japan) LC-9A pump, a Rheodyne Model 7125 injector with a 20-μl loop, a Shimadzu CTO-2A column oven, a Shimadzu SPD-6A variable-wavelength UV detector and a Shimadzu RF-535 fluorescence detector. The chromatograms were recorded on a Shimadzu Chromatopac C-R5A. A Shimadzu SPD-M6A photodiode-array detector was used to monitor the UV spectra of the peaks.

2.3. Derivatization procedure

To 0.5 ml of an aqueous solution of acetylcarnitine or carnitine (5 mg/ml) was added acetone to 100 ml. To 1 ml of this solution, 0.25 ml of a solution of ADAM in acetone (1 mg/ml) was added. The mixture was allowed to stand at 50°C for 20 min. After evaporating the solvent under a stream of nitrogen, 2 ml of dilute perchloric acid (1:1000) was added to the residue, and washed twice with 6 ml of diethyl ether to remove unchanged ADAM. To 1 ml of the aqueous phase was added 1 ml of acetonitrile, and 20 μl of the solution were injected into the HPLC system. The procedure was performed in light-resistant containers with protection from sunlight.

Fig. 1 shows the reaction of acetylcarnitine or carnitine with ADAM.

2.4. HPLC

The chromatographic separation was performed by using a Chiralcel OD-R column (250

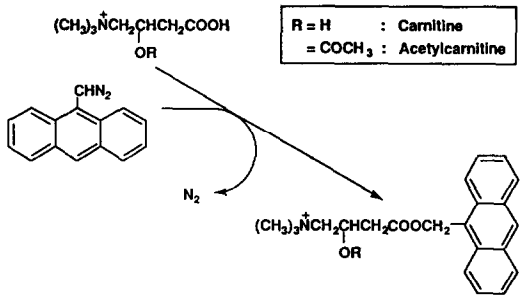

Fig. 1. Reaction of carnitine or acetylcarnitine with 9-anthryldiazomethane (ADAM).

mm × 4.6 mm I.D.) (Daicel Chemical Industries, Osaka, Japan). It was packed with silica-based cellulose tris(3,5-dimethylphenylcarbamate) as a chiral stationary phase (particle size 10 μm). The mobile phase was 0.5 *M* sodium perchlorate solution–acetonitrile (3:2) at a flow-rate of 0.8 ml/min. The column temperature was 25°C (ambient). The effluent was measured with a UV detector (254 nm) or fluorescence detector (excitation at 365 nm, emission at 412 nm); 254 nm was the UV absorption maximum of the solutes measured with a photodiode-array detector.

2.5. Calculation of D-form content

The content of the D-form in L-AC (or L-C) was calculated by the following equation:

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

where A_D = peak area of D-AC (or D-C)–ADAM and A_L = peak area of L-AC (or L-C)–ADAM.

3. Results

3.1. Preparation of the ADAM derivatives

Effect of water content in the reaction mixture

Taking into account the solubility of acetylcarnitine or carnitine and the application to a biological sample, the derivatization was carried out in acetone containing a small amount of water. The effect of the water content on the yield of the ADAM derivatives was investigated. The peak areas of L-AC–ADAM and L-C–ADAM were observed to be constant and maximum over the range of water content in the reaction mixture from 0.16 to 8.0%. The high water content causes a long evaporation time. Therefore, the water content in the reaction mixture was fixed at 0.4%, which could dissolve L-AC or L-C.

Effect of ADAM concentration

The effect of the ADAM concentration on the reaction yield was investigated and the results are shown in Fig. 2. With both L-AC and L-C the peak areas were constant and maximum when the ADAM concentration of an additional acetone solution was more than about 0.5 mg/ml. From the results, the ADAM concentration was fixed at 1.0 mg/ml, that is, about a tenfold molar excess of the reagent.

Effects of reaction temperature and time

First the effect of the reaction temperature on the yield was investigated. The highest and constant peak area of L-AC–ADAM or L-C–ADAM was obtained over the range of the reaction temperature *ca.* 40–60°C. A high temperature leads to decomposition of ADAM and slight hydrolysis of L-AC. Next the effect of the reaction time at 50°C was examined. The results shown in Fig. 3 indicate that the derivatization reaction was completed in about 20 min at 50°C, and this condition was adopted.

Stability of ADAM derivatives

The stability of ADAM derivatives of L-AC and L-C was investigated. Fig. 4 shows the effect

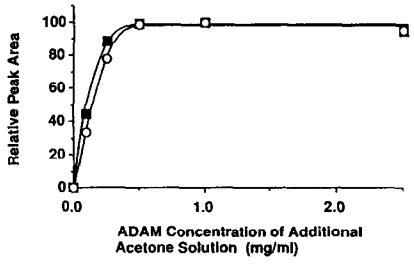

Fig. 2. Effect of ADAM concentration on the reaction yield of (○) carnitine and (■) acetylcarnitine.

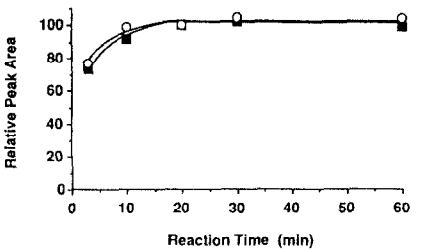

Fig. 3. Effect of reaction time on the reaction yield of (○) carnitine and (■) acetylcarnitine.

of acetonitrile addition and protection from light on the stability at room temperature. Evidently both the addition of acetonitrile and protection from light contributed to the stability of ADAM derivatives, and made them stable for 24 h at room temperature. Further, the theoretical plate numbers of the peaks increased following acetonitrile addition compared with water addition.

3.2. Conditions for HPLC

Mobile phase

In the following investigations, different aqueous solutions mixed with acetonitrile (3:2) were used for elution.

Effect of ionic species. The effect of ionic species on the solutes (D,L-AC–ADAM and D,L-C–ADAM) was investigated using perchlorate and phosphate. The retention times, theoretical plate numbers of the peaks and enantioselectivity increased with the use of perchlorate instead of phosphate.

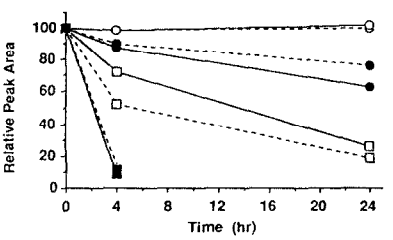

Fig. 4. Stability of carnitine–ADAM and acetylcarnitine–ADAM derivatives. ○ = Addition of acetonitrile, protection from light; □ = addition of acetonitrile, no protection from light; ● = no addition of acetonitrile, protection from light; ■ = no addition of acetonitrile, no protection from light. Dashed lines = carnitine; solid lines = acetylcarnitine.

Effect of ionic strength. The effect of ionic strength on the solutes was investigated using perchlorate over the concentration range 0.1–1.0 *M*. The retention times of the solutes increased slightly with increase in the ionic strength. However, little effect was observed on the other factors.

Effect of pH. The effect of pH on the solutes was investigated using 0.5 *M* perchlorate buffer over the pH range 2–6. The peaks were hardly affected by the pH of the buffer.

Effect of organic solvents. The difference in the effect on the solutes between acetonitrile (40%) and methanol (80%) was examined using 0.5 *M* sodium perchlorate solution. Methanol contributed to a better enantioselectivity, but resulted in decreases in the theoretical plate numbers and selectivity between carnitine and acetylcarnitine.

Column temperature

The effect of column temperature on the solutes was investigated over the range of 25°C (ambient)–50°C using 0.5 *M* sodium perchlorate solution–acetonitrile (3:2). As expected, the retention times of the solutes decreased with increase in column temperature. Further, it was found that a combination of a low column temperature and a high flow-rate gave a better enantioselectivity than that of a high column temperature and a low flow-rate.

Fig. 5 shows a typical chromatogram for the simultaneous chiral separation of carnitine and acetylcarnitine.

3.3. Method validation

Specificity

D-AC, L-C and crotonylbetain are possible impurities or decomposition products in L-AC as a drug. Fig. 6 shows the chromatograms of the following samples obtained by this method; (A) L-AC (spiked with 1% of D-AC), (B) L-C (spiked with 1% of D-C), (C) crotonylbetain and (D) the reagent blank. The results indicate that this method has good specificity for the optical

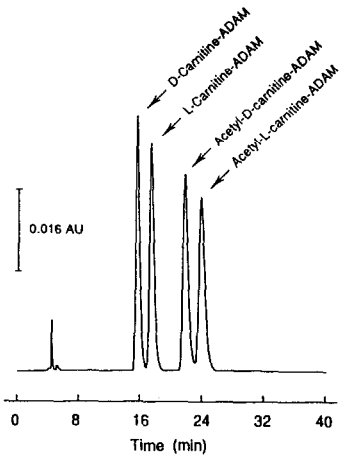

Fig. 5. Chiral separation of the enantiomers of carnitine and acetylcarnitine.

purity testing of L-AC, and permits the simultaneous determination of the enantiomers of carnitine and acetylcarnitine.

Reproducibility (precision)

The reproducibility was investigated from six determinations of the content of the D-form in a single batch of L-AC (Lot No. 3). The results are given in Table 1. The relative standard deviation (R.S.D.) was 0.84%; the good reproducibility of this method was confirmed.

Linearity and accuracy

The linearity and accuracy was investigated from determinations of the content of the D-form in L-AC standard (free from D-AC) spiked with D-AC over the range 0.2–10.0%. The relationship between the found values (y) and the theoretical values (x) was found to be a straight line that passed through the origin ($y = 0.970x - 0.053$) (correlation coefficient = 1.000); the good linearity and accuracy on this method were confirmed. In addition, similar results were obtained with respect to carnitine ($y = 0.974x - 0.059$) (correlation coefficient = 1.000).

Detection limit

The detection limit of D-AC with this method was investigated, comparing UV detection (254 nm) with fluorescence detection (excitation at 365 nm, emission at 412 nm). It was confirmed to be almost same, in both instances, *ca.* 0.01% of D-AC in L-AC, *i.e.*, *ca.* 0.05 pmol of D-AC per injection (signal-to-noise ratio = 4).

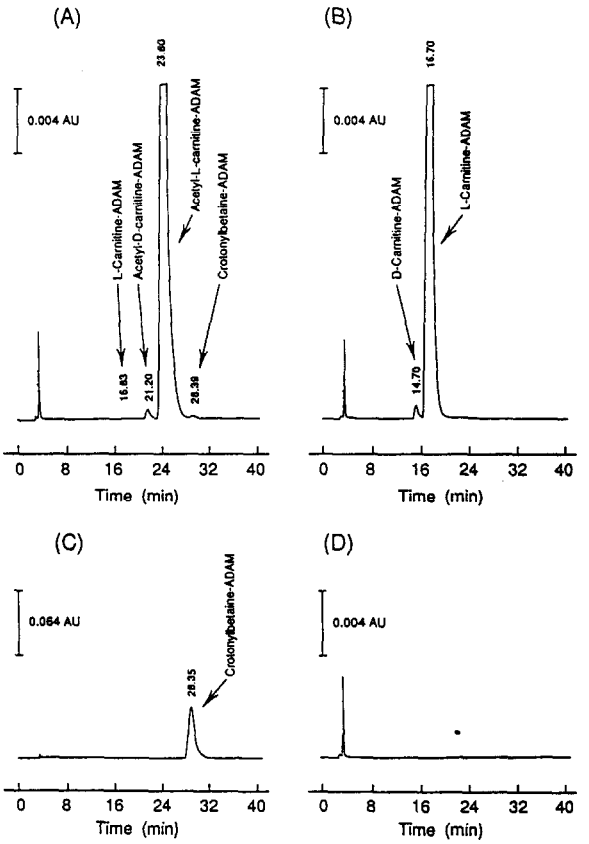

Fig. 6. Chromatograms of (A) acetyl-L-carnitine spiked with 1% of acetyl-D-carnitine, (B) L-carnitine spiked with 1% of D-carnitine, (C) crotonylbetain and (D) the reagent blank.

Table 1
Reproducibility of assay of D-form in acetyl-L-carnitine (Lot No. 3)

Repetition	Assay value (%)
1	1.39
2	1.38
3	1.41
4	1.40
5	1.40
6	1.41
Mean	1.40
Standard deviation	0.0117
R.S.D. (%)	0.84

Table 2
Optical purity testing for acetyl-L-carnitine drug substance

Sample	Lot No.	D-Form content (%)
Acetyl-L-carnitine drug substances	1	3.82
	2	1.42
	3	1.40
	4	0.62
Standard sample	S1	N.D.[a]

[a] Not detected.

3.4. Application to optical purity testing

Optical purity testing for L-AC drug substances and standard samples was performed with this method. The results were given in Table 2.

Considerable differences in the content of the D-form were found between several batches of L-AC.

4. Discussion

Methods for the chromatographic separation of enantiomers may be roughly divided into indirect diastereomeric methods using a chiral derivatization reagent followed by separation on a non-chiral column, and direct methods using a chiral column or chiral mobile phase. Indirect methods have several disadvantages, *e.g.*, the cost and the steady supply of the chiral reagent, and the direct influence of the optical purity of the reagent on the analytical value.

For DL-AC and DL-C, which have weak absorption only in the low-wavelength region, direct methods using a chiral mobile phase may be inappropriate because of the background absorption of the chiral additive. Therefore, in order to develop a direct method using a chiral column for DL-AC or DL-C, various types of chiral stationary phases (CSPs) were tried under a variety of conditions; for example, the following CSPs were investigated in consideration of the chemical structure of DL-AC or DL-C (presence of β-oxycarboxyl group) and its peculiar solubility (high polarity): ligand-exchange types [Chiralpak MA (+), Chiralpak WH], crown ether type (Crownpak CR), metal complex type (Ceramospher), bonded protein type (Ultron ES-OVM) [19] and cellulose carbamate type (Chiralcel OD-R). However, all attempts at the direct separation of the enantiomers failed. The difficulties are probably due to their remarkable motility in elution and the weak interaction between DL-AC or DL-C and the CSPs.

Derivatization methods on the carboxyl site of DL-AC or DL-C were then investigated in order to increase the interaction between the solutes and the CSPs. DL-AC and DL-C methyl esters gave no effect on the enantioselectivities on these CSPs, but it was found that the ADAM derivatives achieved enantiomeric separations on a cellulose carbamate-type CSP (Chiralcel OD-R) using the reversed-phase mode. It is considered that the success depends greatly on $\pi-\pi$ interactions between the anthracene skeleton of ADAM and the CSP and the increase in hydrophobicity of the solutes. 1-Pyrenyldiazomethane (PDAM), which was developed as a reagent for the determination of carboxylic acids and is more stable than ADAM [20], was also investigated. The enantioselectivities of the PDAM derivatives on a Chiralcel OD-R column were so poor that the separation could not be achieved, probably because the bulky pyrenyl skeleton prevents effective access of the active sites for chiral recognition.

A Chiralcel OD-R column has great enantioselectivity for widely different compounds, and these results suggest that the use of ADAM makes possible a wider application to carboxylic acids, especially low-molecular-mass and highly polar compounds.

With the use of reversed-phase chromatography, the present method is probably applicable to the simultaneous determination of the enantiomers of acylcarnitines with an appropriate gradient. In addition, as the ADAM derivatization can be performed under mild and water-containing conditions and specific fluorescence detection can be used, this method can be expected to be applicable to the enantiomeric study of acylcarnitines in biological systems.

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