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Separation of carnitine enantiomers as the 9-anthroylnitrile derivatives and high-performance liquid chromatographic analysis on an ovomucoid-conjugated column

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Abstract

9-Anthroylnitrile was used as an achiral reagent for the derivatization of carnitine. The reagent forms UV-absorbing derivatives with the hydroxyl groups of carnitine enantiomers under very mild conditions. The derivatives were separated by high-performance liquid chromatography on an ovomucoid-conjugated column with a mobile phase of acetonitrile–20 mM KH_2PO_4 (adjusted to pH 4.5 with phosphoric acid) (17:83, v/v). The separation factor (α) and resolution (R_s) of the enantiomers were 1.44 and 5.05, respectively. The calibration plots indicated good linearity over a sample concentration ranging from 0.2 to 1.0 mg ml^{-1}, and the detection limit at 254 nm was 0.05 mg ml^{-1} for each carnitine enantiomer. The reproducibility in the analysis of 1 mg ml^{-1} of each enantiomer was within 2.0%. The method was applied successfully to the determination of carnitine enantiomers in pharmaceutical preparations.

Keywords: 9-Anthroylnitrile; Carnitine; Enantiomer separation; High-performance liquid chromatography

1. Introduction

Carnitine, β-hydroxy-γ-trimethylaminobutyric acid (Fig. 1) is one of the most active substances as a mitochondrial fatty acid acyltransferase cofactor [1,2]. The carnitine molecule has one chiral center, and its enantiomers show different pharmacological and therapeutic effects [3–5]. Consequently, the separation of enantiomers is very important for studies of their biological activities and also for quality control of the compound. Determination of achiral carnitine has been performed by high-performance liquid chromatography (HPLC) [6–11]. However, methods for the optical resolution of racemic carnitine have not been reported.

In this paper we describe a convenient procedure for the resolution of the enantiomers of carnitine in pharmaceutical preparations. The method is based on the reaction of the chiral hydroxy moiety of carnitine with 9-anthroylnitrile (9-AN) (Fig. 1) and the separation of the resulting enantiomeric derivatives by HPLC

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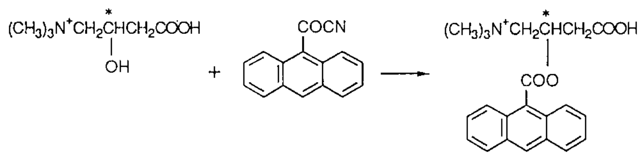

Fig. 1. Reaction of carnitine with 9-AN. The chiral center is marked with an asterisk.

on an ovomucoid-conjugated column (Ultron ES-OVM).

2. Experimental

2.1. Reagents and standards

D-, L- and DL-carnitine were obtained from Sigma (St. Louis, MO, USA). Stock solutions of 10 mg ml^{-1} D-, L- and DL-carnitine were prepared separately in water. Working standard solutions were prepared from this stock solution prior to use. 9-AN was purchased from Wako (Osaka, Japan). Approximately 100 mg of 9-AN was weighed into a 50 ml volumetric flask and dissolved in dimethyl sulfoxide (DMSO). Quinuclidine, HPLC-grade acetonitrile and methanol were obtained from Nacalai Tesque (Kyoto, Japan). Quinuclidine was dissolved in acetonitrile to a final concentration of ca. 0.1 mg ml^{-1}. Silica-gel cartridge columns (Analytichem Bond Elut, 1cc) were purchased from Varian (Harbor City, CA, USA). Water was purified in a Milli-Q II water purifier (Nihon Millipore, Tokyo, Japan). All other chemicals were of analytical-reagent grade.

2.2. Reaction and clean-up procedure

A 1 ml volume of carnitine solution (1.0 mg ml^{-1}) and 1 ml of quinuclidine solution were placed in a 5 ml vial glass tube and the solvent was evaporated to dryness at 50°C under reduced pressure in a rotary evaporator. A 1 ml volume of a DMSO solution of 9-AN (2.0 mg ml^{-1}) was then added to the residue and the mixture was kept at 80°C for 90 min. After cooling to room temperature, the mixture was made up to 5 ml with DMSO. A 100 μl aliquot of the solution was transferred into the silica-gel cartridge column. After the column had been washed with 10 ml of methanol–acetonitrile (1:9), the enantiomeric derivatives were eluted with 20 ml of water. Aliquots (10 μl) of this solution were injected into the HPLC system.

2.3. HPLC

The HPLC apparatus consisted of a JASCO Model 880-PU pump (Japan Spectroscopic, Tokyo, Japan), a Rheodyne Model 7125 injector equipped with a 10 μl loop (Rheodyne, Berkeley, CA, USA), a JASCO Model 860-CO column oven, a JASCO Model 870 UV detector set at a wave-

Table 1
Separation of enantiomeric derivatives of carnitine by HPLC with an Ultron ES-OVM column

Reagent	k'_1	k'_2	α	R_s	Mobile phase
9-Anthroylnitrile	6.8	9.8	1.44	5.05	A
1-Anthronyl cyanide	46.2	-	1.00		B
Pyrene-1-carbonyl cyanide	37.5	44.6	1.19	0.88	B

Mobile phase: (A) acetonitrile–20 mM KH_2PO_4 (pH 4.5) (17:83, v/v); (B) acetonitrile–20 mM KH_2PO_4 (pH 4.5) (8:92, v/v). Flow-rate, 1 ml min^{-1}; column temperature, 35°C; detection wavelength, 254 nm, k'_1 and k'_2 = capacity factors of L- and D-isomers, respectively; α = separation factor; R_s = resolution factor.

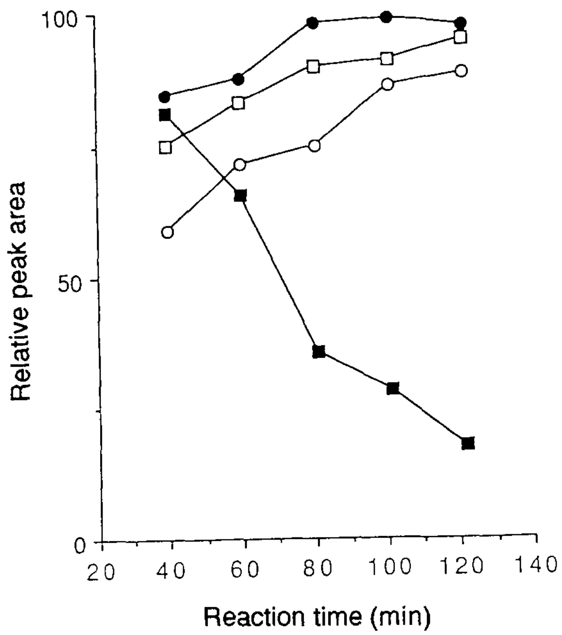

Fig. 2. Effect of reaction time and reaction temperature on the yield of carnitine-9-AN derivatives. (○) 40°C; (□) 60°C; (●) 80°C; (■) 100°C.

length of 254 nm and a Chromatopac CR-6A digital integrator (Shimadzu, Kyoto, Japan). Ovomucoid-conjugated columns (Ultron ES-OVM, 15 cm × 4.6 mm i.d.) were purchased from Shinwa Chemical Industries (Kyoto, Japan). The mobile phase was acetonitrile–20 mM KH_2PO_4 (adjusted to pH 4.5 with phosphoric acid) (17:83, v/v) at a flow rate of 1.0 ml min^{-1} and a column oven temperature of 35°C.

2.4. Sample preparation

Samples corresponding to ca. 50 mg of carnitine were placed in 50 ml volumetric flasks and ca. 40 ml of water were added. If the dosage form was tablet composites, a representative number of tablets (usually 20) were accurately weighed and ground into fine powder. After sonication for 20 min, the flasks were cooled and the solution made up to 50 ml with water. The mixtures were centrifuged at 2000 rpm for 10 min. Aliquots of 1 ml of these solutions were subjected to the reaction and then HPLC analysis was performed as above.

3. Results and discussion

The initial efforts were directed towards the direct HPLC separation of enantiomers of carnitine with chiral stationary phases such as Ultron ES-OVM, Sumichiral OA-4100, Sumichiral OA-5000, MCl Gel CRS 10W, Chiralpak OP(+) and Crownpak CR(+), but these approaches were unsuccessful. Chiral separation following pre-column derivatization was then attempted.

It was found that the carbonyl nitrile group of 9-AN, 1-anthronyl cyanide and pyrene-1-carbonyl cyanide react selectively with the chiral hydroxy moiety of carnitine under mild conditions to form the corresponding carnitine derivatives. Three derivatives of carnitine were preliminarily subjected to HPLC on ovomucoid-conjugated columns and the results are summarized in Table 1. The enantiomer separations of 9-AN derivatives showed the most significant separation of the three reagents.

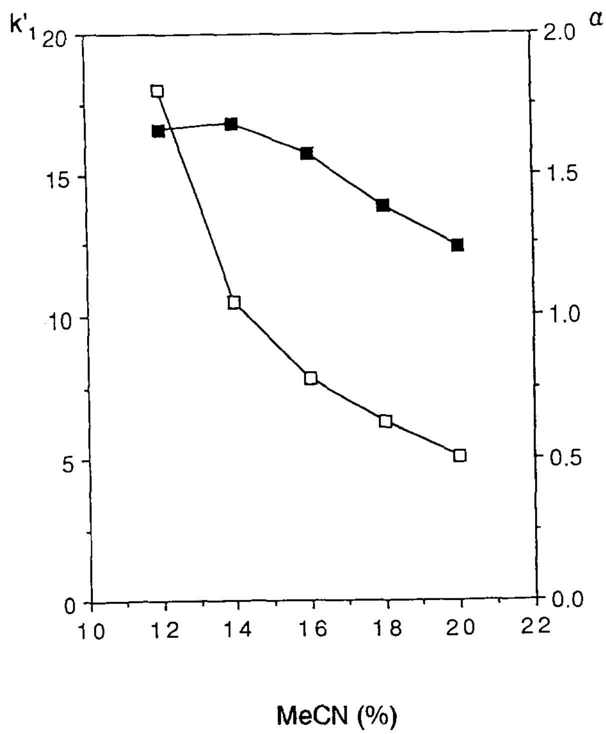

Fig. 3. Influence of acetonitrile content of the mobile phase on (□) capacity factor [k'_1 (capacity factor of the first-eluted carnitine-9-AN derivative)] and (■) separation factor (α).

Optimum conditions for the production of carnitine-9-AN derivatives were determined. An aliquot of the solution was subjected to HPLC and the yield was calculated by comparison with the peak height of the carnitine-9-AN derivatives. The effect of solvents on the reaction was examined by using DMSO, acetone, acetonitrile, tetrahydrofuran, *N*,*N*-dimethylformamide, chloroform, hexane and benzene. DMSO was chosen for subsequent studies.

Reactions with carbonyl nitrile groups are often catalyzed by bases [12,13]. To improve the reaction, catalysis by quinuclidine (bases) was examined. The relationship between the concentration of quinuclidine and the reactivity was examined by varying the amount of quinuclidine in the range 0.01–0.5 mg per 1.0 mg of racemic carnitine. The reactivity increased with increasing amount of quinuclidine and remained constant in the range 0.05–0.5 mg. The amount of quinuclidine was therefore chosen as 0.1 mg for subsequent experiments.

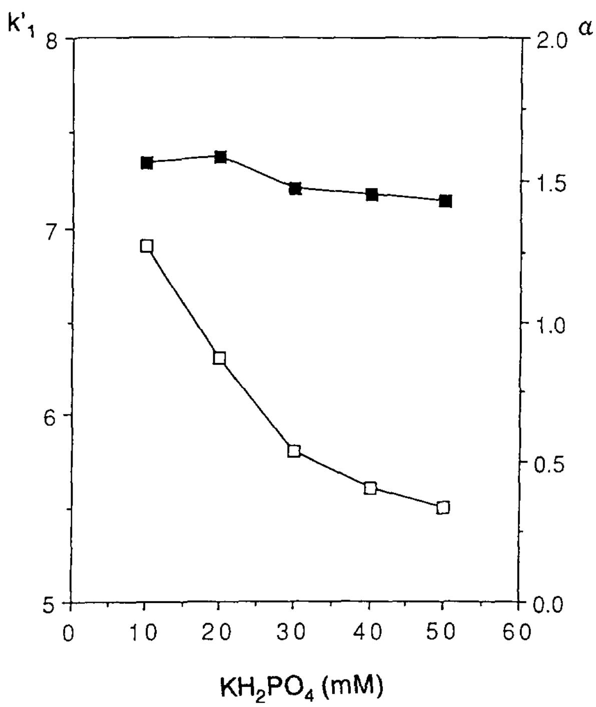

Fig. 4. Influence of ionic strength (mM) of the mobile phase on (□) capacity factor [k'_1 (capacity factor of the first-eluted carnitine-9-AN derivative)] and (■) separation factor (α).

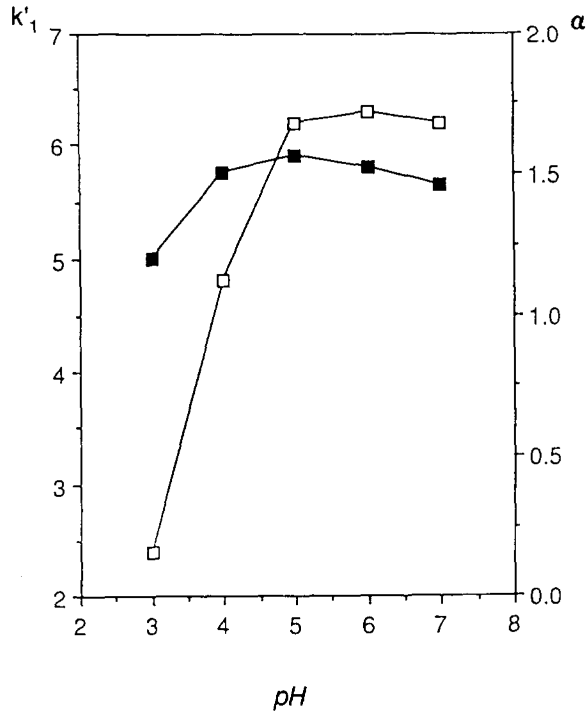

Fig. 5. Influence of pH of the mobile phase on (□) capacity factor [k'_1 (capacity factor of the first-eluted carnitine-9-AN derivative)] and (■) separation factor (α).

The relationships between the amount of 9-AN and the yield of carnitine-9-AN derivatives were examined by varying the amount of 9-AN in the range 0.1–5.0 mg per 1.0 mg of racemic carnitine. Constant yields of carnitine-9-AN derivatives were obtained above 1.0 mg of 9-AN. Consequently, the amount of 9-AN was set as 2.0 mg.

Fig. 2 shows the effects of reaction temperature and reaction time on the production of carnitine-9-AN derivatives. The reaction rate increased with increase in temperature, but the effect tended to decrease above 90°C. This suggested that carnitine-9AN derivatives may decompose to unknown products at higher temperatures (90°C). As shown in Fig. 2, the optimum reaction temperature and time were adopted as 80°C and 90 min, respectively.

The yield of the carnitine-9AN derivatives decreased in proportion to the increase in water content in the reaction. Therefore, the sample

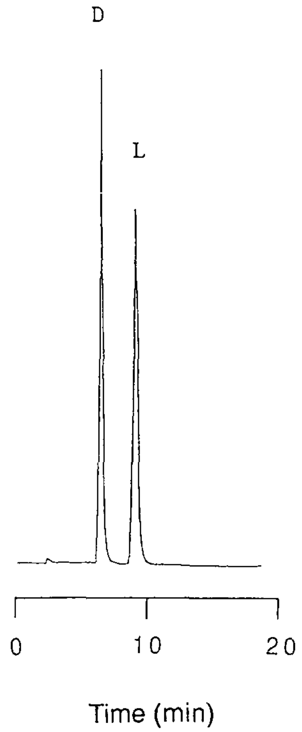

Fig. 6. Chromatogram of carnitine-9-AN derivatives. Mobile phase, acetonitrile–20 mM KH_2PO_4 (pH 4.5) (17:83, v/v); for other analytical conditions, see text.

solution was evaporated to dryness before the reaction.

Initially, the reaction mixture was injected directly into the HPLC column. However, a clear chromatogram could not be obtained owing to interference from impurities in the reagents. Therefore, it was necessary to purify the carnitine-9-AN derivatives further. Aliquots of 100 μl of carnitine-9-AN derivatives were applied to silica-gel cartridge columns after making up to 5 ml with DMSO. The columns were washed with 10 ml of methanol–acetonitrile (1:9) to remove unknown interfering compounds and carnitine-9-AN derivatives were eluted with 20 ml of water.

In this study, carnitine-9-AN derivatives were chromatographed on ovomucoid-conjugated columns with a mobile phase consisting of acetonitrile and KH_2PO_4 solution. The enantiomer separation of carnitine-9-AN derivatives was strongly dependent on the mobile phase conditions, e.g. on water content, salt concentration and pH.

The effects of acetonitrile concentration on the retention and resolution are shown in Fig. 3, where the capacity factors (k') of the first-eluted carnitine-9-AN derivative and the separation factor (α) are plotted versus acetonitrile concentration. Both k' and α decreased with increasing acetonitrile concentration. Consequently, an acetonitrile concentration of 17% was adopted.

The retention time was also influenced by the ionic strength of the mobile phase; carnitine-9-AN derivatives exhibited shorter retention times with higher ionic strength. However, the separation factor (α) was almost constant (Fig. 4).

The capacity factor (k') of carnitine-9-AN derivatives increased with increase in pH in the range 3.0–7.0, whereas the separation factor (α) was affected only slightly. As shown in Fig. 5, carnitine-9-AN derivatives were mostly resolved at pH 4.50.

Table 2
Determination of carnitine enantiomers in pharmaceutical preparations

Sample	Component	Nominal amount	Found	
			Enantiomer	Amount (mg)[a]
A (syrup)	DL-Carnitine	100 mg ml^{-1}	D-Carnitine	48.5 ± 0.82
			L-Carnitine	50.3 ± 0.85
B (injection)	DL-Carnitine	100 mg ml^{-1}	D-Carnitine	50.8 ± 1.16
			L-Carnitine	52.2 ± 1.20
C (tablet)	L-Carnitine	100 mg per tablet	D-Carnitine	0 ± 0
			L-Carnitine	98.9 ± 1.30

[a] Each value is given as the mean ± SD of five measurements.

The results led to the conclusion that carnitine-9-AN derivatives could be mostly resolved using acetonitrile–20 mM KH_2PO_4 (adjusted to pH 4.5 with phosphoric acid) (17:83, v/v) as the mobile phase in HPLC with ovomucoid-conjugated columns. A typical chromatogram of carnitine-9-AN derivatives is shown in Fig. 6.

The capacity factors of the enantiomers, k'_1 and k'_2, were 6.8 and 9.8, respectively. The separation factor (α) and resolution (R_s) were determined as 1.44 and 5.05, respectively.

The relationships between the peak area (x) and the amount of each carnitine enantiomer (y) were linear over the range 0.2–1.0 mg ml^{-1}:

D-carnitine: $y = 2.015x - 0.1858$ ($r = 0.9998$)

L-carnitine; $y = 2.113x - 0.1864$ ($r = 0.9994$)

The relative standard deviations for 1.0 mg ml^{-1} of each carnitine enantiomer were below 2.0% ($n = 5$). The detection limit was 0.05 mg ml^{-1} (signal-to-noise ratio = 3) for each carnitine enantiomer.

The present method was applied to the determination of carnitine enantiomers in pharmaceutical preparations (Table 2). The chromatograms of three samples showed sharp peaks without any interference from other substances. These results indicate that this method is suitable for the determination of carnitine enantiomers in pharmaceutical preparations.

References

[1] J. Bremer, Nature, 196 (1962) 993–994.

[2] I.B. Fritz and K.T.N. Yue, Am. J. Physiol., 206 (1964) 531–535.

[3] D.J. Paulson and A.L. Shug, Life Sci., 28 (1981) 2931–2938.

[4] O. Fanelli and F. Tenuta, Pharmacology, 22 (1981) 371–377.

[5] S. Fujisawa, K. Shimatani, H. Yamada and Y. Hironaka, Nippon Yakurigaku Zasshi, 93 (1989) 305–314.

[6] P.E. Minkler, S.T. Ingalls, S. Kormos, D.E. Weir and C.L. Hoppel, J. Chromatogr., 336 (1984) 271–283.

[7] J. Gorham, J. Chromatogr., 361 (1986) 301–310.

[8] P.E. Minkler, S.T. Ingals and C.L. Hoppel, J. Chromatogr., 420 (1987) 385–393.

[9] T. Yosida, A. Aetake and H. Yamaguchi, J. Chromatogr., 445 (1988) 175–182.

[10] M. Takahashi, N. Nakayama, S. Uehara, K. Kamata, T. Hagiwara and K. Akiyama, Annu. Rep. Tokyo Metro. Res. Lab. Publ. Health, 41 (1990) 51–54.

[11] K. Kamata, M. Takahashi, K. Terashima and M. Nishijima, J. Chromatogr., 667 (1994) 113–118.

[12] J. Goto, M. Saito, T. Chikai, N. Goto and T. Nambara, J. Chromatogr., 276 (1983) 289–300.

[13] M. Kudoh, H. Ozawa, S. Fudano and K. Tsuji, J. Chromatogr., 287 (1984) 337–344.