Journal of Chromatography, 445 (1988) 175–182
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 466

DETERMINATION OF CARNITINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING 9-ANTHRYLDIAZOMETHANE

TSUGUCHIKA YOSHIDA, ATSUHIRO AETAKE and HIROSHI YAMAGUCHI
Department of Analytical Chemistry, Research Center, Taisho Pharmaceutical Co. Ltd., 1-403 Yoshino-cho, Ohmiya-shi, Saitama 330 (Japan)
and
NORIYUKI NIMURA* and TOSHIO KINOSHITA
School of Pharmaceutical Sciences, Kitasato University, 9-1 Shirokane-5, Minato-ku, Tokyo 108 (Japan)
(First received December 30th, 1987; revised manuscript received March 9th, 1988)

SUMMARY

The high-performance liquid chromatographic determination of carnitine chloride was investigated by using 9-anthryldiazomethane (ADAM) as a pre-column derivatization reagent. Carnitine chloride and the internal standard *N,N*-dimethylglycine reacted with ADAM to give a stable ester derivative in the presence of sodium dodecyl sulphate (SDS) used to mask the basic function. The ADAM derivative of carnitine was separated from decomposition products of the reagent and related compounds such as amino acid derivatives on a silica gel column eluted with methanol–5% aqueous SDS–phosphoric acid (990:10:1). The calibration plot was linear over a sample concentration range from 0.02 to 100 ng per injection. The detection limit for carnitine chloride was about 1 pg per injection (signal to noise ratio = 4), by fluorometric detection.

INTRODUCTION

Carnitione [4-(*N,N,N*-trimethylammonio)-3-hydroxybutanoate] has been the object of various determination schemes because it has remarkable biological activities. It has been determined by various spectrophotometric methods based upon ion-pair formation between its quaternary ammonium functionality and anionic chromophores such as periodide[1] and bromophenol blue[2,3]. However, the ion-pair formation is liable to interference from a variety of other compounds. An enzymatic method was also devised employing carnitine acetyltransferase[4–6]. This method was further modified by the use of radioactively acetate-labelled acetyl coenzyme A followed by the measurement of radioactive acetylcarnitine[7,8], but the use of a radioisotope is not suitable for routine assay.

The direct determination of carnitine has recently been developed using labelling reagents and high-performance liquid chromatography (HPLC). In these methods, carnitine was converted into 4′-bromophenacyl esters[9–18], but the methods required tedious pre-treatment and the sensitivity was poor.

0021-9673/88/$03.50

We have previously developed 9-anthryldiazomethane (ADAM) as a reagent for the fluorescent labelling of carboxylic acids in HPLC[19]. ADAM seemed promising for improvement of the carnitine analysis because it reacts with carboxylic groups rapidly without a catalyst at room temperature, and the product is highly lipophilic and readily separable from other biological substances. In addition, we have previously established a facile procedure for the labelling of amino acids with ADAM by masking the amino groups with sodium dodecyl sulphate (SDS)[20].

In the present study, a similar procedure was examined for the derivatization of carnitine with ADAM using SDS. SDS was also effective as the counter ion for the separation of carnitine ester for normal-phase ion-pair HPLC.

EXPERIMENTAL

Materials

DL-Carnitine chloride was obtained from Kongo Kagaku (Toyama, Japan), ADAM from Funakoshi Yakuhin (Tokyo, Japan), acetonitrile and methanol from Wako Pure Chemical Industries (Osaka, Japan). Other organic solvents were from Kokysan Kagaku (Tokyo, Japan). All reagents and solvents were of analytical grade. Water was purified with a Milli-Q water purification unit (Millipore, Bedford, MA, U.S.A.) before use.

The fluorescence reagent was prepared by dissolving 10 mg of ADAM per ml of ethyl acetate. This solution was stable for 2 days in a refrigerator.

Derivatization procedure

To 10 μl of a sample solution were added 10 μl of 20% aqueous SDS solution, 10 μl of an aqueous solution of *N,N*-dimethylglycine (100 μg/ml) as the internal standard, 370 μl of isopropanol and 100 μl of ADAM solution. The mixture was allowed to stand at 50°C for 30 min and then cooled to room temperature. An aliquot of 55 μl of the resulting mixture was directly injected into the chromatograph.

Chromatography

The liquid chromatograph system consisted of a Model TRIROTAR-VI HPLC (Japan Spectroscopic, Tokyo, Japan) with an automatic sampler Model WISP 710B (Waters Assoc., Milford, MA, U.S.A.) and an ultraviolet spectrophotometer Model UVIDEC-100-IV (Japan Spectroscopic) or a Model F-1000 spectrofluoromonitor (Hitachi, Japan). The effluent was measured spectrophotometrically at 250 nm with an UV detector or fluorometrically at the excitation and fluorescence wavelengths of 365 and 412 nm.

The chromatographic separation was performed by using a stainless-steel column (150 mm × 4 mm I.D.) packed with LiChrosorb Si 100 (particle size 10 μm, Merck) kept at 50°C. The column was eluted isocratically with methanol–5% aqueous SDS–phosphoric acid (990:10:1) at a constant flow-rate of 1.0 ml/min.

Preparation of the ADAM derivative of carnitine

To 1 ml of a carnitine chloride solution (1 mg/ml) were added 100 μl of 20% aqueous SDS and 1 ml of ADAM solution and the resultant mixture was allowed to stand for 30 min at 50°C. An aliquot of 50 μl was injected for HPLC. The fraction

containing the ADAM derivative of carnitine was collected and extracted with hexane. The whole procedure was repeated several times and the hexane layers were pooled, evaporated to dryness and the residue was redissolved in methanol. The mass spectrum of the solution was measured with a mass analyzer Model JMS-DX303HF and a data system Model JMA-DA5000 (both from JEOL, Tokyo, Japan).

Application to the carnitine chloride preparations

Twenty tablets of a carnitine chloride preparation (12.5 mg per tablet) were weighed and finely powdered. A quantity of powder containing 25 mg of carnitine chloride was placed in a 50-ml volumetric flask and about 40 ml of water were added. After shaking for 10 min, water was added to a total volume of 50 ml. The mixture was then centrifuged at 3000 rpm for 5 min. The supernatant was diluted five times, and 10 μl of the solution were subjected to derivatization and HPLC as described above. Simultaneously, 10 μl of the standard solution of carnitine prepared in water (0.1 mg/ml) were also subjected to derivatization and HPLC.

The recovery of carnitine chloride from the carnitine chloride preparations was examined as follows. To the carnitine chloride preparation were added 2.5-ml of a methanolic solution of carnitine chloride (10 mg/ml) and the mixture was evaporated to dryness. To the residue were added 25 ml of water and the mixture was shaken for 10 min and centrifuged at 2000 *g*. The supernatant was diluted five times in water, and subjected to derivatization for HPLC as described.

RESULTS AND DISCUSSION

Fig. 1 shows the course of reaction of ADAM with carnitine. ADAM is highly reactive with carboxyl groups so that it esterifies most carboxylic acids at room temperature without a catalyst[19]. However, the esterification with ADAM of carboxylic acids having an amino group such as α-amino acids was found to be prevented by zwitterion formation[20]. SDS was found to increase the reactivity of amino acids with ADAM by masking the basic function. Accordingly, SDS was applied to the esterification of carnitine chloride which had previously resisted esterification.

First, several organic solvents were tested as the reaction media for the esterification. Isopropanol was found to give the largest yield of carnitine ester as in the case of α-amino acids[20]. Fig. 2 shows the yield of carnitine ester as a function of the SDS concentration. The peak area reached a plateau at a SDS concentration of more than 12%. From these results, the SDS concentration was fixed at 20% in the standard procedure.

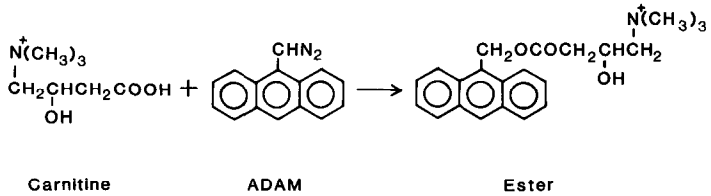

Fig. 1. The reaction course of ADAM with carnitine.

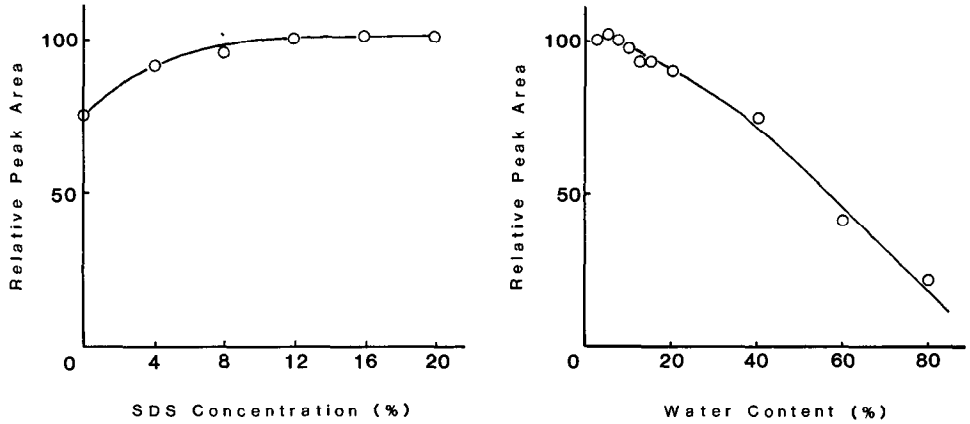

Fig. 2. The yield of carnitine ADAM ester as a function of the SDS concentration. Derivatization conditions as described in the text.

Fig. 3. The yield of carnitine ADAM ester as a function of the water content in the reaction mixture.

Fig. 3 shows the yield of carnitine ester as a function of the water content in the reaction mixture. The yield had a maximum at a water content of 7.5% and decreased thereafter presumably due to the hydrolysis of ADAM. At water contents below 7.5%, at least a 30–50 times molar excess of the reagent was required for the derivatization of the carboxyl residue.

ADAM reacts with carnitine chloride even at room temperature but the reaction requires over 3 h for completion. Accordingly, the effect of the reaction temperature on the yield was examined (Fig. 4). The highest yield was observed when the reaction was carried out 50°C for 30 min. Lower yields were obtained at 60°C presumably due to the decomposition of ADAM. The derivatization reaction required 3 h at room temperature or 30 min at 50°C for completion, and the latter condition was adopted in the standard procedure.

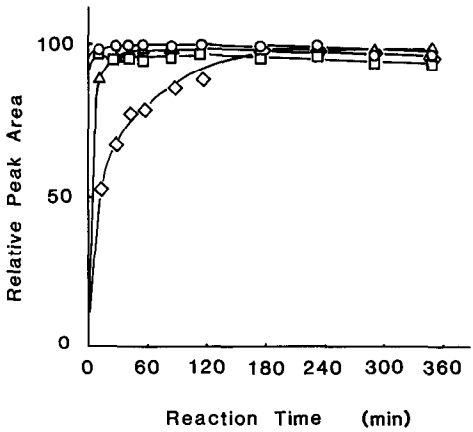

Fig. 4. The yield of the reaction as a function of the reaction time at room temperature (◇), 60°C (□), 50°C (○) and 40°C (△).

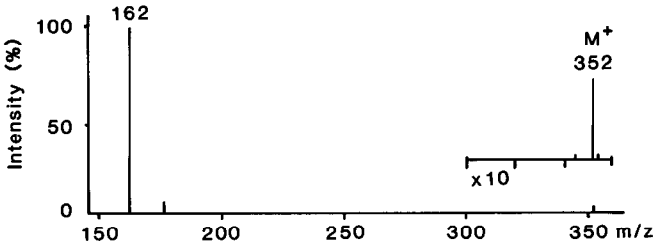

Fig. 5. Mass spectrum of the reaction product from carnitine chloride and ADAM. The proposed structure of carnitine ADAM ester is shown in Fig. 1.

In order to confirm its structure, to ADAM ester of carnitine was prepared. Fig. 5 shows the mass spectrum of the product. The base peak appeared at *m/z* 352 which should be the molecular ion peak of carnitine ester, implying the structure shown in Fig. 1.

ADAM derivatives are usually separated on reversed-phase columns. The separation of the carnitine ester was therefore first examined by reversed-phase HPLC. However, the retention time of carnitine ester was very close to those of the ADAM hydrolysates. Since SDS has been found to be an effective counter ion for the reversed-phase HPLC of ADAM derivatives of amino acids, it was also tested as the counter ion for the separation of carnitine ester. SDS prolonged the retention time of the carenitine ester, whereas those of ADAM hydrolysates were little influenced. However, the tailing of the peaks of the ADAM hydrolysates still interfered with the determination of the ester.

The separation was then examined by normal-phase HPLC on a silica gel column, SDS was again found to suppress the adsorption of carnitine ester on silica gel and decrease its retention time. Fig. 6 shows the capacity factor of the carnitine ester plotted against the SDS concentration. From the results, a mobile phase containing methanol–5% SDS–phosphoric acid (990:10:1) was found to provide the best separation.

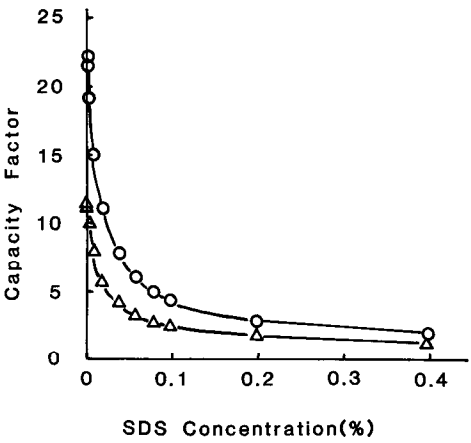

Fig. 6. Capacity factor of the carnitine (○) and *N,N*-dimethylglycine (△) ADAM esters as a function of the SDS concentration.

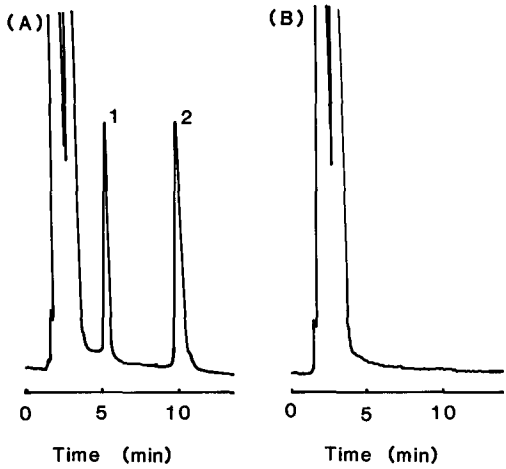

Fig. 7. Chromatogram of the ADAM derivative of carnitine (A) and a reagent blank (B). Peaks: 1 = internal standard *N,N*-dimethylglycine (1 ng), 2 = carnitine (1 ng).

Fig. 7 shows a chromatogram of carnitine and *N,N*-dimethylglycine esters. Each peak was clearly separated from decomposition products of the excess of reagent. The calibration plot for carnitine carnitine chloride showed excellent linearity in the range from 0.02 to 100 ng per injection, corresponding to 11 pmol to 55 nmol in 10 μl of the sample solution, using fluorometric detection ($y = 1.068x - 0.037$, $r = 0.99$). The reproducibility of this procedure was also sufficient, the coefficient of variation for 10 ng of carnitine chloride being 1.6% ($n = 8$). The detection limit of carnitine chloride under the conditions was about 1 pg per injection (signal-to-noise ratio, S/N = 4). The present method is about 1000 times as sensitive as the conventional HPLC method for the assay of carnitine chloride based on the precolumn derivatization with 4′-bromophenacyl triflate[17].

TABLE I

RECOVERY OF CARNITINE IN THE PRESENCE OF ANTACIDS

Antacid	*Recovery (%)*	
	50 times	*20 times*
Aluminium hydroxide	90.7	98.9
Synthetic hydrotalcite	97.1	100.3
Magnesium oxide	87.1	101.2
Magnesium carbonate	93.3	99.4
Magnesium hydroxide	89.5	100.9
Calcium carbonate	86.0	99.1
Magnesium aluminometasilicate	87.1	98.6
Dibasic calcium phosphate	97.7	99.7
Aluminium silicate	76.8	97.7

TABLER II

RECOVERY OF CARNITINE IN THE PRESENCE OF CRUDE DRUGS

Crude drug	*Recovery (%)*	
	20 times	*2 times*
Fennel	93.2	99.3
Corydalis tuber	91.1	101.5
Phellodendron bark	88.7	98.7
Glycyrrhiza	99.3	98.5
Beef bile extract powder	65.2	101.1
Ciunamon bark	96.6	99.5
Evodia fruit	87.2	100.3
Peony root	101.6	100.9
Japanese sweetflag	95.4	99.6
Dinseng extract powder	96.8	98.6
Gaijanga minor	86.1	102.1
Scopolia extract powder	88.7	99.4
Amomum seed	93.3	99.2

The established procedure was applied to the determination of carnitine chloride in a roborant preparation. As this preparation contains various antacids and crude drugs, the interferences from these substances were then examined. The recoveries of carnitine chloride in the presence of various antacids are listed in Table I. Each antacid tested had no effect on the analysis when its amount was 20 times that of carnitine chloride, but it did interfere when its amount was 50 times that of carnitine chloride.

The recoveries of carnitine chloride in the presence of crude drugs are listed in Table II. Each crude drug had no effect on the analysis when its amount was 2 times that of carnitine chloride but did so when its amount was 20 times. However,

TABLE III

RECOVERY OF CARNITINE IN THE PRESENCE OF VITAMINS AND OTHER DRUGS

When the amount of Biodiastase 1000 was 4 times that if carritine chloride the recovery was 101.1%.

Vitamins and other drugs	*Recovery (%)*	
	20 times	*2 times*
Ethyl aminobenzoate	93.8	98.8
Tiamine nitrate	94.5	100.4
Riboflavin sodium phosphate	93.3	101.1
Pyridoxine hydrochloride	99.7	99.2
Ascorbic acid	92.1	98.7
Nicotinamide	92.9	102.1
Calcium pantothenate	93.1	99.5
Dicycloverine hydrochloride	95.4	100.8
Papaverine hydrochloride	61.6	99.5
Methylbenactyzium bromide	94.1	99.5

TABLE IV

THE MODEL PREPARATION OF CARNITINE

Ingredient	*Content (mg per four tables)*
Carcitine hydrochloride	50
Calcium pantothenate	30
Nicotinamide	30
Riboflavin sodium phosphate	30
Pyridoxine hydrochloride	5
Thiamine nitrate	30
Calcium ascorbate	50
Retinol acetate	10
Tocopherol calcium succinate	10
Ginsing extract powder	50
Phellodendron bark	50
Beef bile extract powder	50

glycyrhiza and peony root did not interfere with the reaction even when their amounts were 20 times that of carnitine chloride.

The recoveries of carnitine chloride in the presence of vitamins, etc. are listed in Table III. Each drug had no effect on the analysis when its amount was 2 or 4 times that of carnitine chloride. This procedure was applied to the analysis of carnitine chloride in the model vitamin preparation whose contents are shown in Table IV. The recovery of carnitine chloride was 99.8%, and the coefficient of variation for the reproducibility of this procedure was 2.2% ($n = 4$).

The present method provides a sensitive, simple and selective tool for microanalysis of carnitine in various samples. Its application to the estimation of carnitine in biological fluids is now under investigation in our laboratories.

REFERENCES

1 J. S. Wall, D. D. Christianson, R. J. Dimler and F. R. Senti, *Anal. Chem.*, 32 (1960) 870.
2 S. Friedman, *Arch. Biochem. Biophys.*, 75 (1958) 24.
3 M. A. Mehlman and G. Wolf, *Arch. Biochem. Biophys.*, 98 (1962) 146.
4 N. R. Marquis and I. B. Fritz, *J. Lipid Res.*, 5 (1964) 184.
5 N. Takeyama, D. Takagi, K. Adachi and T. Tanaka, *Anal. Biochem.*, 158 (1986) 346.
6 R. E. Dugan, M. J. Schmidt, G. E. Hoganson, J. Steele, B. A. Gilles and A. L. Shug, *Anal. Biochem.*, 160 (1987) 275.
7 A. Daveluy, R. Parvin and S. V. Pande, *Anal. Biochem.*, 119 (1982) 286.
8 E. P. Brass and C. L. Hoppel, *J. Biol. Chem.*, 253 (1978) 2688.
9 J. Gorham, E. McDonnell and R. G. Wyn Jones, *Anal. Chim. Acta*, 138 (1982) 277
10 H. D. Drust, M. Milano, E. J. Kikta, Jr., S. A. Connelly and E. Grushka, *Anal. Chem.*, 47 (1975) 1797.
11 M. J. Barcelona, H. M. Liljestrand and J. J. Morgan, *Anal. Chem.*, 52 (1980) 321.
12 K. Takayama, H. C. Jordi and J. Benson, *J. Liq. Chromatogr.*, 3 (1980) 61.
13 R. L. Patience and J. D. Thomas, *J. Chromatogr.*, 234 (1982) 225.
14 R. L. Patience and J. D. Thomas, *J. Chromatogr.*, 249 (1982) 183.
15 P. E. Minkler, S. T. Ingalls, L. S. Kormos, D. E. Weir and S. L. Hoppel, *J. Chromatogr.*, 336 (1984) 271.
16 J. Gorham, *J. Chromatogr.*, 361 (1986) 301.
17 S. T. Ingalls, P. E. Minkler, C. L. Hoppel and J. E. Nordlander, *J. Chromatogr.*, 299 (1984) 365.
18 P. E. Minkler, S. T. Ingalls and C. L. Hoppel, *J. Chromatogr.*, 420 (1987) 385.
19 N. Nimura and T. Kinoshita, *Anal. Lett.*, 13 (1980) 191.
20 T. Yoshida, A. Uetake, H. Murayama, N. Ninura and T. Kinoshita, *J. Chromatogr.*, 348 (1985) 425.